Biopesticides: State of the Art and Future Opportunities

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Biopesticides: State of the Art and Future Opportunities

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Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from the ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previous published papers are not accepted.

ACS Books Department

Preface

Resistance to conventional pesticides has been growing rapidly among all pests. Furthermore, there is increased public concern about the safety of conventional pesticides, and increased governmental restrictions have resulted in the need to identify new compounds that are safe and effective in controlling pests that are of concern to agriculture as well as to public and animal health. Biopesticides may aid in the control of such pests with fewer deleterious effects to the environment, people and animals. The U.S. Environmental Protection Agency (EPA) defines biopesticides as *"pesticides derived from such natural materials as animals, plants, bacteria, and certain minerals"* (www.epa.gov).

According to the U.S. EPA's website in 2014 there were more than 430 registered biopesticides along with 1320 active product registrations. Biopesticides have seen a recent growth, which is partially due to increased advances in biotechnological tools for pest control. However, the growth has been largely spurred by the growing needs for new tools to fight pesticide resistance and safer and more benign means of pest management.

This volume and the chapters contained within it resulted from the "Biopesticides: State of the Art and Future Opportunities" symposium held at the 246th ACS National Meeting in Indianapolis, Indiana, September 8–12, 2013. The symposium was comprised of 38 papers in five sessions: The Big Picture, Repellents and Attractants, Insecticides and Nematicides, Products from Genetic Improvements, and Economic, Regulatory and Future Needs.

We gratefully thank all of the authors for contributing chapters, and ACS for an Innovative Project Grant for Divisional Enhancement and the Ag Biotech Stewardship Technical Committee for generously funding this AGRO Division symposium that was the foundation for this volume. We also thank the Agrochemicals Division of ACS for its support of this symposium.

ADG dedicates this book to his family and his dear friends, Dr. Justin Bushkofsky and Liz Bushkofsky. JRC dedicates it to the excellent graduate students and postdocs that have contributed to his laboratory over the years. SOD dedicates it to the many students, postdocs, and colleagues with whom he has shared science. JNS dedicates it to staff and students in Environmental Toxicology and Environmental Sciences at the University of California, Davis, and the University of Nevada, Reno, who have been a constant source of inspiration.

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Chapter 1

Biopesticide Oversight and Registration at the U.S. Environmental Protection Agency

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The Environmental Protection Agency (EPA) is committed to encouraging the development and use of biopesticides and considers them inherently reduced-risk pesticides. Biopesticides (microbial pesticides, biochemical pesticides, and plant-incorporated protectants) are required to be evaluated by EPA. The Agency must make findings of "no unreasonable adverse effects" to man and the environment to support its registration decision to permit sale and distribution under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), as well as a "reasonable certainty of no harm" under the Federal Food, Drug, and Cosmetic Act (FFDCA) to permit residues in food and/or feed. This chapter will review areas including how EPA views the benefits of biopesticides, related laws and legal requirements, biopesticide registration, and biopesticide data requirements. EPA's commitment to low risk biological pesticides as alternatives to conventional chemical pesticides will also be emphasized.

What are Biopesticides?

Biopesticides, also known as biological pesticides, are pesticides derived from natural materials such as animals, plants, bacteria, and certain minerals. Typically, biopesticides have unique modes of action and are considered reduced risk pesticides. Biopesticides fall into three major classes:

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- Biochemical pesticides;
- Microbial pesticides; and
- Plant-incorporated protectants.

Biochemical Pesticides

Biochemical pesticides are naturally occurring substances or are synthetically derived equivalents that have a non-toxic mode of action to the target pest(s), and have a history of exposure to humans and the environment demonstrating minimal toxicity. Synthetically derived biochemical pesticides are equivalent to a naturally occurring chemical with such a history. Biochemical pesticides include, but are not limited to: semiochemicals (insect pheromones and kairomones), natural plant and insect regulators, naturally occurring repellents and attractants, induced resistance promoters, and enzymes. Biochemical pesticides typically degrade rapidly and are not persistent in the environment.

Biochemical pesticides, with the exception of pheromones, tend to have much less species-specificity and are broader spectrum pesticides than the microbials. They also may have lethal effects upon the target pest. Lethal but non-toxic biochemical pesticides include suffocating agents (e.g., soybean oil), dessicants (e.g., acetic acid), and abrasives (e.g., diatomaceous earth).

Microbial Pesticides

Microbial pesticides are microorganisms that produce a pesticidal effect. They have pesticidal modes of action that often include competition or inhibition, toxicity and even use of the target pest as a growth substrate. They may be:

- Eukaryotic microorganisms including, but not limited to, protozoa, algae, and fungi;
- Prokaryotic microorganisms, including, but not limited to, bacteria;
- Autonomous replicating microscopic elements, including, but not limited to, viruses.

Microbial pesticides can control many different kinds of pests, although each separate active ingredient is relatively specific for its target pest(s). For example, there are fungi that control certain weeds and other fungi that kill specific insects.

The most widely used microbial pesticides are subspecies and strains of *Bacillus thuringiensis*, or *Bt*. Each strain of this bacterium produces a different mix of proteins, and specifically kills one or a few related species of insect larvae. While some *Bt* strains control moth larvae feeding on plants, others are specific for larvae of flies and mosquitoes. The target insect species are determined by whether the particular *Bt* produces a protein that can bind to a larval gut receptor, thereby causing the insect larvae to starve.

Plant-Incorporated-Protectants (PIPs)

Consistent with the Coordinated Framework for Regulation of Biotechnology issued by the U.S. Office of Science and Technology Policy in 1986 (51 FR 23302) genetically modified (GM) crops with pesticidal traits fall under the oversight of EPA, the U.S. Department of Agriculture, and the U.S. Food and Drug Administration. EPA's oversight focuses on the pesticidal substance produced (e.g., *Bt Cry* proteins) and the genetic material necessary for its production in the plant (e.g., *Cry* genes). EPA calls this unique class of biotechnology-based pesticides plant-incorporated protectants (PIPs).

PIPs are pesticidal substances that plants produce and the genetic material that has been added to the plant. For example, scientists can take the gene for the *Bt* pesticidal protein and introduce the gene into the plant's own genetic material. Then the plant, instead of the *Bt* bacterium, manufactures the substance that destroys the pest. EPA regulates the protein and its genetic material, but not the plant itself.

How EPA Views Benefits of Biopesticides

In 1994, the Biopesticides and Pollution Prevention Division (BPPD) was established in EPA's Office of Pesticide Programs (OPP) to facilitate the registration of biopesticides. BPPD promotes the use of safer pesticides, including biopesticides, as components of integrated pest mangement (IPM) programs.

EPA is committed to encouraging the development and use of low risk biological pesticides as alternatives to conventional chemical pesticides (*1*). The Agency recognizes that these pesticides are often different in their mode of action and has employed numerous measures to facilitate the application process. These include distinct data requirements for microbial and biochemical biopesticides, consolidation of biological pesticide application processing to a single group within OPP, and regulatory relief activities (*2*) . EPA is committed to the efficient, effective approval of safer pesticides as well as a transparent, predictable process in decision making.

Since biopesticides tend to pose fewer risks than conventional pesticides, EPA generally requires much less data to register a biopesticide than to register a conventional pesticide, and EPA's review times are shorter for biopesticides.

While biopesticides require less data and are registered in less time than conventional pesticides, EPA always conducts rigorous reviews to ensure that pesticides will not cause unreasonable adverse effects on human health or the environment. For EPA to be sure that a pesticide is safe, the Agency requires that registrants submit a variety of data about the composition, toxicity, degradation, and other characteristics of the pesticide. These data requirements are described in more detail later in this paper.

There are several benefits to using biopesticides, including:

- Decreased risk without affecting yield. Biopesticides—when used as a component of an IPM program—can greatly decrease the use of conventional pesticides, without affecting crop yield.

- Often less toxic. Generally, biopesticides are inherently less toxic than conventional pesticides and are safer to those using them.
- Often effective in very small quantities and decompose quickly. This can result in lower exposures and avoid pesticide pollution problems.
- Targeting of specific pests. Biopesticides generally affect only the target pest and closely related organisms, in contrast to broad spectrum, conventional pesticides that may affect non-target organisms such birds, insects, and mammals.
- When used in rotation with conventional products, biopesticides can help prevent development of pest resistance problems.
- Improved residue management. Buyers and consumers are becoming increasingly selective in their purchasing habits. Illegal pesticide residues left on produce can result in loss of markets, fines, and other consumer avoidance. Biopesticides often contain natural products that are normally consumed and do not have residue concerns.

Many microbial and biochemical biopesticides are not intended to function as "stand-alone" pest control products to completely replace conventional pesticides. Instead, these biopesticides are most effective when used as a component of an IPM program because they generally affect only the target pest and closely related organisms.

Additionally, for agricultural use products, biopesticides typically qualify for a reduced restricted entry interval and have no pre-harvest interval. Restricted entry intervals are requirements that limit the time that workers can return to a field once it has been treated with a pesticide. Restricted entry intervals can delay or obstruct time-sensitive cultural practices. Many biopesticides also do not have harvest restrictions. A harvest restriction is a waiting period between when a pesticide is applied and when the treated crop can be harvested and marketed. The waiting period after treatment can often be several days. Biopesticides without harvest restrictions give a grower much greater flexibility during harvest.

Microbial and biochemical biopesticides are generally labeled for use on a wide range of crops. As a result, for some minor crops or obscure pest problems, a biopesticide may be available when no conventional product is registered for the use. In addition, for larger crops such as corn, soybean, and cotton, PIP biopesticides have reduced the use of more toxic conventional insecticides.

Overview of OPP and BPPD's Role

EPA's Office of Pesticide Programs (OPP), along with the Office of Chemical Safety and Pollution Prevention (OCSPP), works with 10 Regional Offices and other EPA program offices on a wide range of pesticide issues and topics, such as:

- Evaluating potential new pesticides and uses;
- Providing for Special Local Needs and emergency situations;
- Reviewing safety of older pesticides;
- Registering pesticide producing establishments;

- Enforcing pesticide requirements; and
- Pesticide field programs, such as the frontline implementation activities carried out by states, tribes, and EPA Regional pesticide experts.

OPP is comprised of nine divisions, three of which are divisions responsible for the registration of pesticides. The Biopesticides and Pollution Prevention Division (BPPD) is responsible for all regulatory activities associated with biologically-based pesticides. Within BPPD, the Biochemical Pesticides Branch and Microbial Pesticides branch are responsible for registering biochemical and microbial pesticides, respectively. Additionally, the Microbial Pesticides Branch registers PIPs and other biotechnology-related products.

BPPD also is working to reduce pesticide risk by promoting Integrated Pest Management (IPM) initiatives and coordinating the Pesticide Environmental Stewardship Program (PESP). BPPD's vision is to be a world leader in biopesticide regulation and pollution prevention. The mission of BPPD is to protect human health and the environment by reducing the risks of pesticides through registering biopesticides and through encouraging pollution prevention practices.

Main Statutes and Legal Requirements

EPA regulates the use of pesticides under the authority of two federal statutes: the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Federal Food, Drug, and Cosmetic Act (FFDCA) (*3*)(*4*). Additionally, the Food Quality Protection Act of 1996 (FQPA) amended FIFRA and FFDCA setting tougher safety standards for new and old pesticides and to make uniform requirements regarding processed and unprocessed foods (*5*). Finally, the Pesticide Registration Improvement Act (PRIA) establishes pesticide registration service fees for registration actions in the three registering divisions of EPA's Office of Pesticide Programs (*6*).

Other statutes that play roles in the regulation of biopesticides include:

- Endangered Species Act;
- Migratory Bird Treaty Act; and
- Clean Water Act

The following descriptions give brief overviews of the main statutes, though such descriptions are not intended to be comprehensive.

FIFRA

FIFRA provides the basis for regulation, sale, distribution and use of pesticides in the U.S. FIFRA authorizes EPA to review and register pesticides for specified uses. EPA also has the authority to suspend or cancel the registration of a pesticide if subsequent information shows that continued use would pose unreasonable risks. Some key elements of FIFRA include:

- Is a product licensing statute; pesticide products must obtain an EPA registration before manufacture, transport, and sale
- Registration based on a risk/benefit standard
- Strong authority to require data--authority to issue Data Call-ins
- Ability to regulate pesticide use through labeling, packaging, composition, and disposal
- Emergency exemption authority--permits approval of unregistered uses of registered products on a time limited basis
- Ability to suspend or cancel a product's registration: appeals process, adjudicatory functions, etc.

Microbial, biochemical, and plant-incorporated protectant biopesticides are considered pesticides under FIFRA, and generally are required to be evaluated and registered by EPA under Section 3 of FIFRA. EPA must make a finding of no unreasonable adverse effects to man and the environment from use of the pesticide in order to support its registration decision.

FFDCA and FQPA

The Federal Food, Drug, and Cosmetic Act (FFDCA) authorizes EPA to set maximum residue levels, or tolerances, for pesticides used in or on foods or animal feed. Under FFDCA and amendments to both FFDCA and FIFRA under the FQPA, EPA must make a similar finding of a reasonable certainty of no harm if the use of such agents results in residues in food or feed. If the submitted information supports this safety finding, EPA may establish a numerical tolerance or an exemption from the requirement of a tolerance regarding those residues. As of this writing, no microbial pesticides or plant-incorporated protectants registered for food use have been required to obtain a numerical tolerance. Rather, exemptions from the requirement of a tolerance have been granted based on the finding of no significant adverse effects in the supporting data.

PRIA

In 2004, Congress passed the Pesticide Registration Improvement Act (PRIA) and established a registration fee-for-service system with specific fees and decision times by type of action. PRIA 3 is the second five-year extension of the original Act and was the result of support and collaboration from a coalition of industry, grower, environmental groups, and farm worker advocates. As biopesticides are usually inherently less toxic than conventional pesticides, biopesticide registrations require a significantly reduced data set compared to conventional registrations. Additionally, biopesticides can follow truncated decision review timelines as well as reduced registration fees.

Experimental Use Permits, Emergency Exemptions, and State and Local Need Registrations

In the process of pesticide development, field testing is often necessary to evaluate the efficacy of a pesticide. Title 40 CFR Part 172 describes when it is necessary to obtain an Experimental Use Permit (EUP) under Section 5 of FIFRA for testing unregistered pesticides. Briefly, the size of the outdoor test acreage is greater than a cumulative 10 acres of land or 1 surface acre of water, an EUP is required. Any food or feed crops involved in or affected by the tests must be destroyed or consumed only by experimental animals unless a tolerance or exemption from a tolerance has been established. These acreage limitations are applicable only for outdoor terrestrial and aquatic uses. For those pesticides being tested on sites for which acreage is not relevant (e.g., tree stumps, rodent control, structural treatments or bird repellents), the determination of the need for an EUP is made on a case-by-case basis.

Other criteria to determine when an EUP must be obtained are set forth in 40 CFR Part 172.3. An EUP is of limited duration and requires that the test be carried out under controlled conditions. For small-scale field tests of genetically modified microbial pesticides or non-indigenous microbial pesticides that USDA has not previously acted upon, applicants must submit a notification to EPA for determination of whether an experimental use permit is necessary, even if the testing is on less than 10 acres

In addition to registration under Section 3 of FIFRA, there are two additional means under FIFRA whereby a pesticide product may be distributed in the absence of a Section 3 registration or an experimental use permit. One is pursuant to an emergency exemption under Section 18 of FIFRA. Under this section, Federal or State agencies may request limited approval for an unregistered use of a currently registered pesticide product or the use of an unregistered pesticide product. Such a request can only be granted when there is a potentially severe economic or human health impact and no other alternatives are available for pest control. A Section 18 exemption usually allows use of the particular pesticide product for a year; however, the duration of the exemption may be limited or expanded depending on the situation (*7*).

Cases also exist where a particular pesticide product may be registered for one or more uses, but not for a particular use which is determined by the State as being a special local need. In these cases, the State may register that use or formulation needed for the special local need under Section 24(c) of FIFRA provided that appropriate tolerances or exemptions from tolerance exist if food or feed uses are involved. The EPA has 90 days to disapprove of such State registrations.

Biopesticide Registration

Before a pesticide can be marketed and used in the United States, FIFRA requires that EPA evaluate the proposed pesticide to assure that its use will not pose unreasonable risks of harm to human health and the environment, including non-target species. This involves an extensive review of health and safety information.

Pesticide registration is also the process through which EPA examines the ingredients of a pesticide; the site or crop on which it is to be used; the amount, frequency, and timing of its use; and storage and disposal instructions. A pesticide cannot legally be used, sold, or distributed if it has not been registered with EPA's Office of Pesticide Programs. FIFRA Section 2 (u), defines the term "pesticide" as:

(1) any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest;
(2) any substance or mixture of substances intended for use as a plant regulator, defoliant, or desiccant; and
(3) any nitrogen stabilizer.

EPA makes online resources, such as the Pesticide Registration Manual (also known as the Blue Book), available to assist applicants through the registration process (*8*).

As biopesticides are usually inherently less toxic than conventional pesticides, biopesticide registrations may require a significantly reduced data set compared to conventional registrations. Additionally, there are reduced associated timelines and fees to help expedite registration processes. Timeframes to register pesticide products vary dependent on the PRIA code assigned to the submission. Based on PRIA 3 decision review timelines and fees for FY 14/15, biopesticide submissions can range from 7 months and $6,079 USD for a new non-food use (PRIA 3 code: B650) to 19 months and $48,621 USD for a new food use active ingredient with a petition to establish a tolerance (PRIA 3 code: B580). This is compared to 12 months and $12,156 USD for a conventional new non-food indoor use (PRIA 3 code: R260) and 24 months and over $590,000 USD for a new food use active ingredient (PRIA 3 code: R010).

Additionally, the Agency recommends that registrants request a pre-submission meeting with the appropriate registering branch. The pre-submission meeting is an excellent opportunity to discuss products in development and steps to take to ensure a timely registration decision. All information exchanged at these meetings is held confidential until a pesticide registration submission is made.

Pheromone Regulatory Relief

The Agency acknowledges that use of certain types of pheromone products presents lower risk than conventional pesticides, and also acknowledges the unique properties of these niche-type products regarding their inherently narrow host range (*9*). To promote the use of pheromone products, the Agency initiated a regulatory relief program that allows flexible confidential statements of formula for pheromone experimental use permits (EUPs) to allow for active ingredient adjustments during the course of experimentation. The Agency has also published generic tolerances and relaxed the acreage cut-off when an EUP is required for pheromones.

EPA established the following exemptions from the requirement of a tolerance as a result of the pheromone regulatory relief program: 1) for inert materials in polymeric matrix dispensers (40 CFR 180.1122); 2) for pheromones in retrievably-sized polymeric matrix dispensers (40 CFR 180.1124); 3) for straight-chained lepidopteran pheromones (sprayables) (40 CFR 180.1153); and 4) for inert polymers in sprayable formulations, (40 CFR 180.1162). EPA further set forth certain policies raising the acreage limit to 250 acres for experimental use permit requirements for the testing of pheromones in polymeric matrix dispensers (59 FR 3681), for testing of non-food use broadcast pheromones (59 FR 34182), and for straight-chained pheromones (sprayables) (60 FR 168).

Products Exempt from Registration

EPA has determined that pest control organisms such as insect predators, nematodes, and macroscopic parasites are exempt from the requirements of FIFRA (40 CFR 152.20(a)). In addition, pheromones (and identical or substantially similar compounds) labeled for use only in pheromone traps for monitoring and pheromone traps in which those chemicals are the sole active ingredients are not subject to regulation under FIFRA (40 CFR 152.25(b)). However, the use of pheromones in traps in conjunction with conventional pesticides, in other application methods (other than traps), or for purposes other than monitoring, is subject to regulation under FIFRA.

Minimum risk pesticides that meet certain criteria are a special class of pesticides that are not subject to federal registration requirements because their ingredients, both active and inert, are demonstrably safe for the intended use. They are exempt from federal registration under section 25(b) of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). EPA does not review or register pesticides that satisfy the 25(b) criteria (40 CFR 152.25(f)), though registration of these products is required by most states.

International Partnerships, Involvement, and Outreach

To streamline agency resources and promote international biopesticide registration, EPA and Health Canada's Pest Management Regulatory Agency (PMRA) have established a process for the joint review of biopesticide products. The procedure entails a joint pre-submission consultation to establish specific data requirements.

Joint reviews increase the efficiency of the registration process, facilitate simultaneous registration in Canada and the U.S., and increase access to new pest management tools in both countries. Efficient work-sharing requires a mutual understanding of the responsibilities of each agency, as well as common procedures and time frames (*10*).

EPA has been an active member of the Organisation for Economic Co-operation and Development's (OECD) Biopesticide Steering Group (BPSG) which meets annually to discuss harmonization of guidelines and principles of

risk assessment. Comparisons and modifications of guidelines for toxicity and pathogenicity studies are vetted within the BPSG to reach consensus on risk assessment procedures for a variety of microorganisms used in pest management. In addition, specific organisms are reviewed to ensure that the latest scientific information on their biology is considered when evaluating their safe use in pest management. Production of toxins or secondary metabolites by some microbial pest control agents (MPCA) are of concern and it is critical that risk managers understand the prevalence of these compounds in products intended for environmental release.

While the BPSG brings together a broad range of scientists from many countries, not all aspects of dossier formating, concerns over aspects of study guidelines and which studies are critical for risk assessment will be agreed upon by all members. Despite this, the BPSG provides an important forum for discussion on a wide range of topics and is the only such venue to reach such a broad range of MPCA developers and regulators. The greater the degree of harmonization of data requirements among member countries resulting from these interactions, the more likely reduced-risk biopesticides will find widespread use in agriculture.

Regarding international outreach, EPA's Office of Pesticide Programs meets periodically with representatives from several countries to discuss products of biotechnology and their impact on trade of agricultural commodities. Updates on regulatory approvals and assessment of novel traits are presented to U.S. and foreign governmental representatives for consideration and discussion. Asynchronous approval of biotechnology products by trading partners has led to occasional rejections of shipments of commodities at great expense and disruption of trade. These meetings provide a forum for direct interaction between regulators and a greater understanding of the risk assessment process as the U.S. is often seen as the lead country in the development and regulation of genetically engineered crops. The ultimate goal of these exchanges is the acceptance of risk management decisions (i.e., approvals) from one country by an importing country without the need for a separate additional review process.

Biopesticide Data Requirements

Looking at the data that is required for biopesticide registration, biochemical and microbial pesticides are subject to a different set of data requirements for registration than conventional chemicals. These Data Requirements for Registration, which are tiered, are listed in 40 CFR Part 158: Subpart U Biochemical Pesticides 158.2000 and Subpart V: Microbial Pesticides 158.2100. EPA has published guidance for developing these data in the Biochemical Pesticides Test Guidelines, OSCPP Series 880 and the Microbial Pesticides Test Guidelines, OSCPP Series 885.

The current regulations allow for flexibility in fulfilling the required data. This can be accomplished through providing a rationale as to why a specific test is not practical to perform, or by providing scientific rationale to address the particular

endpoint. In addition, the Agency has the authority to invoke additional testing requirements if a potential risk has been identified and needs to be investigated. This flexible approach ensures that potential risks presented by biopesticides will be properly assessed.

Biochemical Data Requirements

Product Analysis and Mammalian Toxicology

In general, the product characterization information required for biochemical pesticides is the same as required for conventional chemical pesticides. These include:

- Data/information on product identity and composition;
- Information on manufacturing process; and
- Discussion of the formation of impurities, enforcement analytical methods, analysis for certification of limits, and physical/chemical properties.

The Agency has adopted a tiered testing scheme to assure the safety of biochemical pest control agents toward mammalian species, similar to that used for microbial pesticides, and is comprised of three tiers. Adverse effects in a lower tier will trigger additional testing in the next higher tier (*11*) (*12*).

The mammalian toxicology studies generally required for registration in or on a terrestrial food crop include, in Tier I, acute toxicity tests (oral, dermal, and inhalation exposures, & primary dermal and primary eye irritation studies).

In addition, a battery of genotoxicity studies, 90- day oral, dermal, and inhalation studies (depending upon likely routes of repeated exposure), an immunotoxicity study, and a developmental toxicity study may be required. Hypersensitivity incidents are to be reported, if they occur. The Agency has, on a case by case basis, considered scientifically valid information or peer reviewed literature in lieu of guideline studies. In many cases, lack of significant exposure serves as a basis for not requiring active ingredient or product specific data.

Non-Target Organism Testing

The unique nature of biochemical pesticides has led to a reduction in the data requirements for these products, as compared to synthetic chemical pesticides. Maximum hazard or limit dose testing of the technical grade of the active ingredient (TGAI) is used in assessing hazard to non-target wildlife. The TGAI is the purest and highest concentration form of the biochemical pesticide active ingredient.

There are three tiers of biochemical pesticide data requirements with regards to non-target organism testing. If adverse effects are not observed in Tier I testing (short term studies on non-target birds, aquatic organisms, plants, and insects), no further testing will be required. Should adverse effects be observed in Tier

I studies, Tier II environmental fate studies will be triggered. If Tier II studies indicate that the biochemical active ingredient will persist in the environment, potentially resulting in longer exposure periods, longer term Tier III non-target wildlife studies will be required. Rarely are biochemical pesticides subjected to testing above Tier I.

Once the potential hazards to non-target wildlife have been determined via the tiered testing scheme, risk to non-target wildlife can be assessed based on expected exposure to a biochemical active ingredient via its application in an end-use product (EP) according to its proposed product label use directions.

Product Performance

Product performance data must be developed for all biochemical pesticides. However, such data is typically not required to be submitted unless it relates to a public health pest or is requested by the agency.

Microbial Data Requirements

Product Analysis and Mammalian Toxicology

Crucial to any evaluation of the hazards presented by a microbial pest control agent is correct identification. This identification allows the Agency to ascertain possible hazards associated with the proposed microbial agent and any closely related organisms, and to utilize published literature to facilitate the review. The Agency expects a registrant to provide the most accurate, current taxonomic information to verify the identity of their active microbial agent. For bacteria this information can include genetic DNA homology, morphology, biochemical tests and antibiotic sensitivity. Information for other types of microbes such as fungi, viruses and protozoa is usually less extensive, and may therefore involve other identification methodologies such as serotyping, DNA homology, restriction mapping or isozyme analysis when available. Any adverse effects known to be associated with the microbe, or closely related species, (such as toxin production and pathogenicity in species other than the target pest) should also be reported.

Additionally, the method used to manufacture microbial products is examined to determine whether adequate quality controls are in place to insure a pure product. This quality control review includes an examination for methods to verify purity and stability of the seed or stock cultures and to ensure that the final product is not contaminated with mammalian pathogens. Consideration is also given to final quality control measures for the microbial product that determine potency to insure that these tests relate to bioactivity and label claims (*13*).

The purpose of reviewing mammalian toxicology data for microbial pesticides is to ensure that the use of these products causes no unreasonable adverse effects to human health or non-target mammals. In order to do this the Agency must verify that the microbial product is correctly identified, presents little possibility of pathogenicity or toxicity to humans or other mammals, and is manufactured in a manner to prevent contamination with human pathogens.

To assure the safety of microbial pest control agents toward mammalian species, the Agency has adopted a tiered testing scheme similar to the tiered scheme used for biochemical pesticides. Tier I is designed to expose the test animal, mice or rats, to a single acute, maximum hazard or limit dose of the live microbial pesticide. Tests involving mammalian tissue cultures are required for viral pest control agents to insure there is no possibility of mammalian infection given optimal conditions for expression of viral pathogenesis.

The major endpoints for the toxicity/pathogenicity tests are to observe any adverse effects on the test animals and to establish that the microbial test substance is being cleared from the exposed animals. The animals are observed for any unusual clinical signs during the test, and for gross abnormalities at necropsy. Specific organs are isolated from sacrificed animals during the course of the test to determine the level of microbial test substance present. This is done to assure that a high dose was administered and track the normal mammalian response which recognizes the test substance as foreign and clears it from the system. Unusual persistence of the test microbe in an organ is also considered an adverse effect. Replication of the test microbe in organs is also an adverse reaction, indicating potential for infectivity.

If any adverse effects are noted in the Tier I of the toxicity/pathogenicity tests, further testing is indicated using a tier progression to verify the observed effects and clarify the source of the effects. These Tier II tests could involve a subchronic toxicity/pathogenicity test or, if the adverse effect was believed to be due to a toxic reaction rather than pathogenicity, an acute toxicity test to establish an LD50 value for the toxin. Residue data are required if significant human health concerns arise from the toxicology testing. The majority of biopesticide products screened to date have not indicated any adverse effects to warrant testing further than Tier I.

In addition to testing the safety of the purified microbial agent, the safety of the marketed pesticide product, including inert ingredients, is ascertained. Acute oral, dermal, and inhalation toxicity as well as eye irritation, and dermal irritation testing may be required. However, rationales for no further testing may be appropriate depending on the nature of the inert ingredients and results of the initial toxicity/pathogenicity tests with the microbial agent. Any incidents of hypersensitivity in production workers, applicators or the general public must be reported to the Agency.

Genetically Modified Microbial Pesticides

Genetically modified microbial pesticides may be subject to different data or information requirements on a case-by-case basis, depending on the particular microorganism, the parent microorganism, the proposed use pattern, and the manner and extent to which the organism has been genetically modified. Additional data requirements may include:

- Information on the genetic engineering techniques used;
- The identity of the inserted or deleted gene segment (base sequence data or enzyme restriction map of the gene);

- Information on the region controlling expression of the gene in question;
- A description of the new traits or characteristics that are intended to be expressed;
- Tests to evaluate genetic stability and exchange of the new traits; and/or
- Selected Tier II environmental expression and toxicology tests.

It is important for applicants to work closely with the Agency regarding data requirements to ensure that the proper tests are done and any unique characteristics of the microbial pesticide are taken into account in specific testing procedures.

Non-Target Organism Testing

The unique nature of microbial pesticides has led to changes in the data requirements for these products as compared to synthetic chemical pesticides. This is particularly evident in assessing risk to non-target wildlife (*14*). The testing requirements have been set up to test not only toxicity but also pathogenicity. This was accomplished by increasing the length of the tests (up to 30 days), and looking for signs of infection during and after the testing period. Beneficial insect testing was added in order to ensure that potential risks from insect pathogens used as pesticides had been adequately assessed.

For microbial pesticides used to control post-harvest diseases, the non-target organism data requirements to assess potential risks would also follow this case by case procedure. For example, in many instances the use of these products would be in enclosed areas (i.e., packing houses, storage buildings, etc.) and would be considered an indoor use. If this were the case, then testing of non-target organisms would probably not be required because of a lack of exposure. However, if the proposed use was determined to be outdoor and to have potential exposure to non-target organisms, then the ecological testing requirements would need to be addressed.

Tier I short term testing utilizes maximum hazard or limit dosing of non-target organisms. If no adverse results are observed in Tier I, then further testing is not warranted and environmental fate data are not required. In the first tier of non-target organism testing, avian oral, freshwater fish, freshwater aquatic invertebrate, and honeybee testing are required. In addition, tests to evaluate microbial pesticide effects on wild mammals, plants, and beneficial insects are required depending on the proposed use site, target organism, and degree of anticipated exposure. If adverse effects are observed in the first tier, then potential exposure to non-target organisms is evaluated in Tier II studies.

Product Performance

Product performance data must be developed for all microbial pesticides. However, such data is typically not required to be submitted unless it relates to a public health pest or is requested by the agency (*15*).

Plant-Incorporated Protectant Data Requirements

In general, the data requirements for PIPs are based on those for microbial pesticides (*16*). The reason for this situation is that PIP traits registered to date have been developed from genes found in microorganisms. The exact data requirements for each product have been developed on a case by case basis. The majority of products EPA has seen have been proteins, either related to plant viruses or based on proteins from the common soil bacteria *Bacillus thuringiensis* (*Bt*). The general data requirements include product characterization, mammalian toxicity, allergenicity potential, effects on non-target organisms, and environmental fate. For the *Bt* products, insect resistance management is included to prevent the loss of benefits of both the microbial sprays and the *Bt* PIPs from overuse and selection for resistant pest populations.

Conclusion

EPA is committed to encouraging the development and use of low risk biological pesticides as alternatives to conventional chemical pesticides. This commitment is shown by having a division dedicated to the registration of biopesticides, as well as distinct review timelines, fees, and required data. The efficient, effective approval of safer pesticides as well as a transparent, predictable process in decision making are top priorities for EPA, OPP, and BPPD.

Every day, the management and staff of BPPD focus on protecting human health and the environment by reducing the risks of pesticides through regulating biopesticides and encouraging pollution prevention practices. These safer options maximize the benefits of pesticides while helping to protect the air we breathe and the water we drink for generations to come. EPA looks forward to a continued role in helping to bring a broad array of safer pesticide options to market.

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Chapter 2

Botanical Insecticides: A Global Perspective

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According to the CAB Direct database of scientific publications, there has been enormous growth in research on botanical insecticides over the past 30 years. In 1980 less than 2% of all journal papers on insecticides dealt with botanicals whereas that proportion exceeded 21% in 2011. In particular there has been explosive growth in studies on insecticidal properties of plant essential oils; over half of the 2,200 papers on essential oils as insecticides have been published since 2006. In contrast, commercialization of botanical insecticides has continued to proceed at a relative snail's pace, indicating a big disconnect between theory and practice. This is certainly the case in the jurisdictions with the most rigorous regulatory standards – the EU, USA and Japan. Using California as an example, use data for botanical insecticides also suggests a very modest market presence. According to Cal DPR data from 2011, botanicals constituted only 5.6% of all biopesticides used, and less than 0.05% of all pesticide use. However some recently introduced products have seen modest success. On the other hand, there appears to be increasing commercialization of botanical insecticides in China, Latin America and Africa, regions where socio-economic conditions have led to some of the worst examples of human poisonings and environmental contamination. Arguably, botanicals should be of greater value in developing countries where the useful plant species are often locally abundant, accessible and inexpensive. In many tropical countries semi-refined plant preparations are likely to be

In Biopesticides: State of the Art and Future Opportunities; Coats, et al.;

relatively safe for users and more cost effective than imported conventional crop protection products. In G20 countries botanical insecticides will probably remain niche products for use in public health, urban pest control and in organic food production, but with considerable market opportunities.

Introduction

Insecticides continue to be the cornerstone of insect pest management, both in agriculture and in non-agricultural situations. And while regulatory requirements for approval of new insecticides have grown increasingly stringent in industrialized countries, a wide range of insecticidal products have been developed in the past twenty years that pose far fewer risks to human health and the environment. Among the types of insecticides that meet "reduced risk" criteria are microbials and microbial products, insect growth regulators, recent generations of synthetic insecticides, and botanicals – insecticides derived from plants.

Plants produce a bewildering array of "secondary metabolites" – substances not involved in primary metabolism, but instead thought to play an ecological role in relationships between plants and other organisms. Perhaps a thousand or more of these substances have some demonstrated biological activity in insects, at least in laboratory tests. These include compounds with behavioral actions – those causing repellence, feeding deterrence or oviposition deterrence – and those with physiological actions – those causing acute toxicity, developmental disruption or growth inhibition. It is not uncommon for a plant secondary compound to have both behavioral and physiological effects in one insect species, or different effects in different species.

Increasing academic interest in botanical insecticides was recently documented in a bibliometric analysis of scientific literature on this topic (*1*). Between 1980 and 2012, the proportion of published papers on botanical insecticides among all papers published on insecticides increased from 1.43% to over 21%. In absolute terms, the numbers of papers on botanical insecticides has grown over the same period from less than 100 per year to over 1100 per year. Much of that growth can be attributed to the voluminous literature (>5000 papers) on neem-based insecticides (based on the Indian neem tree, *Azadirachta indica*) starting in the mid-1980s, and since 2000, to papers on plant essential oils as insecticides. Of the approximately 2200 papers published through 2012 on essential oils as insecticides, over half were published since 2006.

But commercialization of botanical insecticides has not kept pace with this explosive growth in academic studies that should provide the scientific foundation for the development of such products. In this chapter I discuss the current status of botanical insecticides on a global scale, as well as divergent regulatory environments that are impeding or facilitating the use of such products in different jurisdictions.

Current Status of Botanical Insecticides in the U.S.A.

Following the Second World War and the remarkably successful commercialization and implementation of synthetic organochlorine, organophosphate and carbamate insecticides, only a handful of botanical insecticides remained in use for the subsequent four decades. These included pyrethrum (from flowers of *Tanacetum cinerariaefolium*), rotenone (from rhizomes of *Derris elliptica*), nicotine (from foliage of *Nicotiana tabacum*), sabadilla (from seeds of *Schoenocaulon officinale*) and ryania (from stemwood of *Ryania speciosa*) (*2*). In recent years, the use of rotenone as an insecticide has declined and it is principally used at present as a commercial piscicide. Nicotine has fallen out of favor owing to its acute toxicity and high risk to humans. Sabadilla and ryania are little used at present. Among these original five botanicals, pyrethrum remains the only product in considerable use in the U.S.A. Some previous issues of uncertainty in supply of pyrethrum oleoresin, formerly sourced primarily from East Africa, have been mitigated by more recent large-scale production of pyrethrum in Tasmania, Australia.

Two "new" botanical insecticides entered the U.S. marketplace in the past two decades, namely, neem-based insecticides and plant essential oils. Neem insecticides (herein defined as organic extracts of de-oiled neem seed cake, rich in azadirachtin) were first registered by the EPA in 1990 and are now represented by at least half a dozen products. These have, however, not enjoyed a great deal of commercial success in the U.S.A., in part owing to their relatively high cost, and in part to their relatively slow action against target pests. On the other hand, neem insecticides are produced and used on every continent. Plant essential oils were first commercialized as botanical insecticides in the late 1990s, facilitated enormously by the inclusion of specific common oils (*viz.* rosemary, cloves, cinnamon, lemongrass, thyme, mint) on the EPA's List 25B of "Exempted Active Ingredients" (*3*). This legal distinction allowed products to be commercialized without meeting the EPA's normal regulatory requirements, saving manufacturers millions of dollars and years in development. This opportunity was best exploited by EcoSMART Technologies Inc. who have become the industry leader in this area, with products for professional pest control, agriculture, animal health and consumer markets. One notable exception is an insecticide/acaricide (Requiem™) based on terpenoids from wormwood, *Chenopodium ambrosoides* originally developed by Codena Inc. and later licensed to AgraQuest Inc. This product, approved and registered by the EPA, became part of the pesticide portfolio of Bayer CropScience when that company acquired AgraQuest in 2012.

To put these products in context, it is valuable to examine actual use data. In general such data is hard to come by (or very expensive to acquire), but the California Department of Pesticide Regulations maintains scrupulous records of all pesticides used in that state and publishes the data annually in a pesticide use report that is freely accessible from their website (www.cdpr.ca.gov). This is a particularly relevant database in that (i) California produces >400 agricultural commodities, including nearly one-half of all US-grown fruits, nuts and vegetables; (ii) California is the largest producer of certified organic crops in

the U.S.A., producing >50% of organic fruit and vegetable crops in the country; (iii) California accounts for ~22% of all agricultural use of pesticides in the U.S.A.; and (iv) growers in California tend to be progressive – willing to try new products and technologies. Data for all pesticides used and selected groups of pesticides in 2006 and 2011 are shown in Table I.

Table I. Pesticide Use (Pounds Active Ingredient Applied) in California

	2006[a]	***2011***[a]
All pesticides	187,754,207	191,969,313
Biopesticides	1,180,830	1,600,636
-- Microbials	260,885	259,300
-- Oils[b]	422,444	611,800
-- Botanicals	56,130	83,100

[a] data from refs. (*4*, *5*) [b] includes clarified neem oil.

Within that five-year window, overall pesticide use (measured as pounds of active ingredient applied) increased by 1.7%, whereas the use of biopesticides increased by 35.6%. Within the biopesticide class, use of microbials remained stable (-0.6%), while oils increased by 44.8% and botanicals increased by 48.0%. Although the growth in biopesticides and botanicals appears quite impressive, their scale of use is less so. Overall, biopesticides constituted <1% of all pesticides used in California in 2011; in that year botanicals represented only 5.2% of biopesticides used, and a mere 0.04% of all pesticide use in the state. In 2011 use of botanicals was dominated by two products: 60,500 pounds of *d*-limonene (a monoterpene from *Citrus* peels) were applied, although 71% of that total was for non-agricultural uses (primarily for structural pest control), and 10,100 pounds of pyrethrins (the active principles in pyrethrum) were applied, with non-agricultural uses (structural pest control and public health) accounting for over 65% of use. In contrast, only 2,000 pounds of azadirachtin (neem) were applied, though predominantly for agricultural purposes.

These observations and data suggest either a major disconnect between research on botanical insecticides and practice (commercialization), or an extended lag period between discovery and development. In my opinion it is the former – a disconnect between theory and practice – exacerbated by regulatory regimes that emerged and evolved with the prevalence of synthetic insecticides. In a regulatory world based on single active ingredients of known purity, complex chemical mixtures (the norm in plants) of varying composition and potency are not

easily assessed. Add to this the costs of registering a new insecticide in the major industrial jurisdictions (U.S.A., E.U., Japan), and it becomes easier to understand why there are so few botanical insecticides in use in those areas. Pyrethrum is the most widely accepted botanical insecticide in the industrialized world, with other botanicals (rotenone, neem, essential oils) only sporadically approved (*6*). However, the strict regulatory regimes found in the U.S.A. and E.U. – which place strong emphases on both mammalian and non-target (environmental) toxicity and tolerance levels for pesticide residues in foods - are not necessarily emulated in other regions or jurisdictions, providing greater opportunities for manufacturers to bring botanical insecticides to the marketplace in those regions.

Newer Botanical Insecticides Outside of the U.S.A.

While costly regulatory requirements have been an impediment to the commercialization of botanical insecticides in the U.S.A., E.U. and some other regions, less well characterized botanicals have been introduced in the past five years in parts of Asia and Latin America (Table II).

Table II. Some Recently Introduced Botanical Insecticides – Asia and Latin America

Country	*Product*	*Plant source(s)*	*Active ingredient(s)*
China	No English name	*Nicotiana tabacum* *Melia azedarach*	2.5% nicotine/toosendanin
	Matrine	*Sophora flavescens*	0.3% quinolizidine alkaloids
	Veratrine	*Veratrum nigrum*	0.5% steroidal alkaloids
Korea	Mite-Kill	*Sophora species* *Melia azedarach*	quinolizodine alkaloids /toosendanin/plant oil
India	Anosom	*Annona species*	1% acetogenins (‘squamosin’)
	Biorakshak	*Annona squamosa*	acetogenins
	Torpedo	*Sophora alopecuroides* *Stemona sessilifolia*	quinolizidine alkaloids *Stemona* alkaloids
	Biocawach	*Derris indica* (syn. *Millettia = Pongamia*)	4% extract
Colombia	CapsiAlil	Liliaceae/Solanaceae	54% sulfur compounds, 43% protoalkaloids
Mexico	Akabrown	7 different plant oils	3% essential oils
	eBioluzion	5 oils, 3 plant extracts	32% oils/extracts

In China, a product containing a combination of nicotine and the limonoid triterpene, toosendanin (from the bark of the Chinaberry tree), has been used for managing fruit and vegetable pests. Toosendanin is relatively ineffective as a standalone agent, but it does serve as a synergist for other botanicals (*7*), as also seen in a miticide from Korea (Mite-Kill™) where it synergizes the quinolizidine alkaloid active principles from *Sophora* species. These alkaloids also constitute the active ingredients of a selective aphicide recently introduced in China (Matrine™), and are combined with a unique group of alkaloids from extracts of the shrub *Stemona* in an insecticide/miticide sold in India (Torpedo™). Another recently introduced botanical in China, Veratrine™, contains extracts rich in steroidal ceveratrum alkaloids – the same active principles in seeds of sabadilla obtained from South America and sold in the U.S.A. as Veratran D™.

Two insecticides based on seed extracts of *Annona* (mostly *A. squamosa* the 'sweetsop' or custard apple) have been introduced in India. These contain long chain acetogenins whose insecticidal properties were first discovered in the pawpaw tree *Asimina triloba* and patented by McLaughlin and colleagues (*8*), as were similar compounds from *Annona* species patented by Bayer AG (*9*). However the vertebrate toxicity of these compounds, having the same toxic mode-of-action as rotenone (*10*), precluded product development in the U.S.A. or the E.U. by either group. Another product used in India (Biocawach™) includes an extract of *Derris indica* (best known in the literature as *Pongamia glabra*), the active principle of which is thought to be the flavone, karanjin.

In Colombia, Ecoflora Agro produces a range of botanical insect repellents including CapsiAlil™, consisting of plant extracts rich in sulfur compounds and protoalkaloids. Green Corp Biorganics in Mexico has produced insecticides/repellents based on complex mixtures of plant extracts and oils including essential oils, neem oil, *Capsicum* oleoresin, and garlic extract to name just a few.

Some Different Models of Pesticide Regulation and Use

In a previous paper I demarcated two distinct perspectives on botanical insecticides – that in industrialized countries such as the U.S.A., and that in less developed (mostly tropical) countries (*11*).

In brief, industrialized countries are ones where:

- pesticides are highly regulated
- many conventional/competing pesticide products are available to and affordable for growers
- growers expect strong and consistent product efficacy, demanding high quality control
- there is a high premium on human safety and environmental protection
- consumers will pay premium prices for organic produce.

Developing countries are ones where:

- there is often high plant biodiversity
- pesticide regulation/enforcement is limited or absent
- conventional products are often unaffordable
- *the vast majority of human poisonings from pesticides occur, primarily due to a complete lack of safety equipment and user training*
- lower efficacy (50% control) may still be valuable (see (*12*))
- absolute food production (and protection of crops and stored products) is paramount.

Under these conditions, it is easy to understand why the industrialized countries have few botanical insecticides in use at present. Those few products (pyrethrum, neem) are based on partially to highly refined extracts with well characterized active ingredients, often in high concentration. For example, technical grade pyrethrum and neem seed extract can contain from 25 to 50% pyrethrins and azadirachtins, respectively, generating end use products with 1-5% active ingredients.

In contrast, botanicals used in developing countries often consist of crude preparations wherein the active ingredients are often uncharacterized (although active principles in the plants may be known to science) and concentrations are very low, if known at all (*13*, *14*). Some NGOs and even government agencies in tropical regions advocate the use of crude preparations of local plants for pest control (e.g., Table III), and it is not uncommon for the same plant species to appear repeatedly on such plant lists. For example, plants frequently recommended and used in Africa include:

- *Tephrosia vogelii*, a shrub or small tree containing rotenoids
- *Piper retrofractum*, an herb containing isobutylamides
- *Annona squamosa*, the seeds of which contain acetogenins
- *Tagetes minuta*, the roots of which contain thiophenes
- *Eucalyptus globulus*, the foliage from which can produce an essential oil rich in the monoterpene 1,8-cineole ('eucalyptol')

Interestingly, only the first listed species is native to Africa - the latter four listed species are introduced, but occur widely across much of the continent.

Table III. Some Plants from Which Crude Preparations Are Recommended for Insect Control in Different Tropical Regions

Brazil[a]	***Thailand***[b]	***Malawi/Zambia***[c]
Azadirachta indica[d] (Meliaceae) Neem	*Azadirachta indica* (Meliaceae) Neem	*Azadirachta indica* (Meliaceae) Neem
Curcuma longa (Zingiberaceae) Falso acafrao	*Curcuma longa* (Zingiberaceae) Tumeric	*Tephrosia vogelii* (Fabaceae) Ububa, Mtetezga, etc.
Laurus nobilis (Lauraceae) Louro	*Zingiber officinale* (Zingiberaceae) Ginger	*Vernonia amygdalina* (Asteraceae) Soyo, Mluluzga, etc.
Ruta graveolens (Rutaceae) Arruda	*Chromolaena odorata* (Asteraceae) Bitter bush	*Tithonia diversifolia* (Asteraceae) Belibeli, Heji
Allium sativum (Amaryllidaceae) Alho	*Capsicum frutescens* (Solanaceae) Chilli	*Euphorbia tirucali* (Euphorbiaceae) Nkadze, Nkhadzi, Mduzi
Tagetes erecta (Asteraceae) Cravo-de-defunto	*Gloriosa species* (Colchicaceae) Glory lily	*Mucuna pruriens* (Fabaceae) Chitedze
Melia azedarach (Meliaceae) Cinamomo	*Stemona burkillii* (Stemonaceae) Stemona	*Solanum panduriforme* (Solanaceae) Nthula, Nthuma
Mentha spicata (Lamiaceae) Hortela		*Bobgunnia madagascarensis* (Fabaceae) Mchelekete, Mulundu, etc.
Capsicum frutescens (Solanaceae) Pimento malagueta		*Sesbania sesban* (Fabaceae) Jerejere
Cymbopogon citratus (Poaceae) Capim cidreira		*Euphorbia ingens* (Euphorbiaceae) Mlangale
Eucalyptus citriodora (Myrtaceae) Eucalipto		*Terminalia sericea* (Combretaceae) Mjoyi

[a] ref. (*15*) [b] *The Bangkok Post*, 11 February 2009 [c] ref. (*16*) [d] species, plant family, local vernacular name(s).

Conclusions

Based on recently introduced botanical insecticides in India and China (Table II) there appears to be the emergence of a 'middle ground' in the pesticide regulatory spectrum that allows for products based on minimally to partially refined plant extracts with low concentrations (0.25-1.0%) of partially characterized active ingredients – typically mixtures of closely-related compounds. This may be a sensible solution for fast-growing economies largely dependent on agriculture such as India, China and Brazil, at least for pest management of domestically-consumed food crops.

In industrialized countries (*viz.*, U.S.A., E.U.) botanical insecticides will likely remain niche products, used in situations where human safety is paramount (e.g., in and around schools, restaurants and healthcare facilities) - and may even trump efficacy. In agriculture their primary use will be in organic food production, although their use in tank mixes or in rotation with conventional insecticides is possible. There are major opportunities for the development and use of less refined botanical insecticides in developing countries for domestic use. However, safety cannot be summarily ignored in these situations – consider the acute toxicity of nicotine and strychnine to humans. It will be necessary for advocates of the use of plants for insect control to assess the safety of even crude products to the end user (e.g., see (*17*)) and to assess natural intraspecific variations in phytochemistry of putative active principles as a surrogate for quality control (*13*, *14*).

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Chapter 3

The Growing Need for Biochemical Bioherbicides

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The volume of herbicides used exceeds that of other pesticides. Evolution of resistance to the currently used herbicides has greatly increased the need for herbicides with new modes of action (MOAs). However, more than 20 years have passed since a new herbicide MOA was introduced. Natural products offer a source of new herbicide chemistries with potentially new MOAs. Additionally, there are no efficacious and economical weed management chemicals (biochemical bioherbicides) available for organic agriculture. The products that are available, such as organic acids, fats, and oils, have to be used in large amounts. Current organic products do not act at enzymatic sites as synthetic herbicides do, but instead cause rapid plant tissue desiccation by direct effects on plant cuticles and membranes. Examples are given of natural compounds that act at specific targets like conventional herbicides, but with new MOAs. Thus, new biochemical biocherbicides have the potential for greatly improving weed management in organic agriculture and providing new MOAs for conventional agriculture.

In Biopesticides: State of the Art and Future Opportunities; Coats, et al.;

Introduction

According to a recent analysis in the journal *Science*, "weeds remain the largest concern of farmers" (*1*). On an annual basis, herbicides comprise about 70% of the pesticide active ingredients used in the USA, dwarfing the use of fungicides and insecticides (*2*). Furthermore, resistance is rapidly evolving to commercial herbicides (*3*, *4*), reducing the utility of many of the leading herbicides. Since glyphosate-resistant (GR) crops became available, glyphosate has become the dominant herbicide worldwide (*5*), but resistance to glyphosate is now rapidly evolving, creating costly problems for farmers that grow GR crops (*3*, *4*, *6*). Multiple resistance to herbicides with several modes of action (MOAs) is becoming common (*e.g.* (*7*),). New herbicide MOAs are greatly needed to combat evolved resistance to herbicides with existing MOAs, but the last herbicide with a new MOA was introduced more than 20 years ago (*8*). Lastly, there are no economical means of weed management in organic agriculture, with weed management costs dwarfing the costs of controlling other pests (*e.g.* (*9*),). Biochemical bioherbicides offer either direct or indirect solutions to these problems.

Current Status of Bioherbicides

The USEPA defines three categories of biopesticides: 1) microbial pesticides, which are biocontrol organisms; 2) plant incorporated protectants (PIPs) such as *Bt* toxin produced by a transgene in the crop to be protected; and 3) biochemical biopesticides which are natural products that may or may not be used in the same way as synthetic pesticides (*10*). Both microbial and insect biocontrol agents for weeds offer little hope at this time for significantly impacting weed management in crop situations. In fact, we are no further along with biocontrol technology for crops than we were decades ago. PIPs for weed management do not exist at this time and will possibly be available only decades from now (*11*, *12*).

Therefore, this chapter will focus on biochemical biopesticides, because they offer hope for dealing with the emerging weed problems in both conventional and organic agriculture that are mentioned in the introduction. The comprehensive review by Copping and Duke (*13*) lists many natural compound pesticides, including biochemical bioherbicides. Other reviews cover natural products for pest management in less detail, with very little discussion of bioherbicides (*e.g.* (*14*),). A recent analysis of biopesticides indicates rapidly growing interest in this sector of the pesticide industry, as indicated by the flurry of corporate activity in biopesticides (*15*). Nevertheless, natural product pesticides currently represent only about 4% of the total pesticide market (*16*). The lack of market penetration of biochemical biopesticides is largely due to relatively high cost and poor efficacy compared to conventional pesticides. However, a major advantage of biochemical biopesticides over conventional pesticides is that the regulatory hurdles of the USEPA are less stringent, resulting in considerably less cost to get the product approved for use.

Compared to biochemical bioinsecticides and biofungicides, there have been relatively few biochemical bioherbicides registered by EPA between 1997 and 2010 (*17*) (Figure 1). Registration of conventional herbicides has also lagged behind other pesticide classes during the same time period (*18*), but in this case, the dominance of GR crops have largely contributed to decreased activity in getting new conventional herbicides to market (*8*). The bioherbicide market does not impinge into the realm of GR crops, and have little market in agronomic crops as a whole. Bioherbicides are used primarily for weed management in organic crops and in horticultural crops that are generally high value crops for which the farmer can afford to pay more for "greener" pest management. The signficant successes with biochemical biopesticides have been with biochemical bioinsecticides and biofungicides, suggesting that finding good biochemical bioherbicides is inherently more difficult. But, the current lack of an efficacious, economical biochemical bioherbicide offers a clear opportunity for such a product.

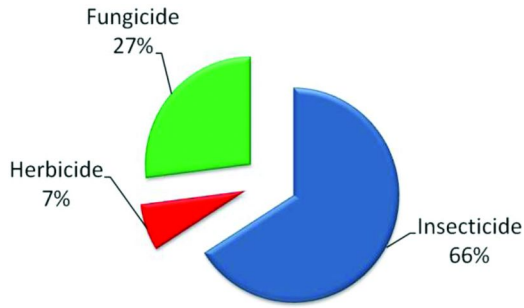

Figure 1. Biochemical biopesticides approved by the USEPA from 1997 through 2010 (18).

Biochemical Bioherbicides in Organic Agriculture

According the USEPA, certified organic farming acreage in the USA increased from 163,250 hectares in 1992 to 1,248,451 hectares in 2011, an almost eight-fold increase. The only chemicals for pest management that organic agriculture will accept are natural products, and, thus can be categorized as biochemical biopesticides. Different approving organizations for organic farmers (*e.g.*, the Organic Material Review Institute, OMRI, in the USA) have somewhat different standards regarding what they will approve. The compound must be a biosynthetic product of a non-transgenic organism. Biochemical bioherbicides that are currently approved for use in organic agriculture are expensive and have very poor efficacy compared to conventional herbicides.

The USEPA website (*10*) gives these advantages of biochemical biopesticides:

- Usually inherently less toxic
- More targeted on the pest of interest
- Effective in very small quantities and decompose quickly
- Can decrease use of conventional pesticides

These advantages are probably true for most biochemical bioinsecticides and biofungicides, but the bioherbicides that have been approved by the USEPA (*10*) are mostly not targeted on the pest of interest, generally killing or injuring almost any plant to which they are applied. Furthermore, they must usually be used in much higher doses for effective weed control than conventional herbicides. These products are mostly contact herbicides consisting of essential oils and other plant extracts, lipids, fatty acids, and organic acids (*17*, *19*) that are chemicals that cause rapid plant tissue desiccation which must be used in very high doses for effective weed management (*19*, *20*). Most conventional herbicides target a specific molecular site in the weed, and require much lower doses for good weed management than available bioherbicides. Specific examples of available bioherbicides include lemongrass oil, pine oil, acetic acid, pelargonic acid, and corn gluten (*21*). Since most of these products are herbicides that act quickly with no translocation of the active compounds, weeds can regrow from root or vegetative parts that are not in contact with the herbicide. Thus, more applications are generally required to kill a weed for available bioherbicides, compared to conventional herbicides.

Few economic comparisons of conventional herbicides and biochemical bioherbicides are available. The analysis of Young (*20*) found that bioherbicides cost from 30- to 90-fold more than the conventional herbicide glyphosate for roadside weed management in California (Table 1). Furthermore, in this study, biochemical herbicides required more applications for good weed management, increasing the use of fossil fuel. Similar results were obtained in a similar study by Barker and Prostak (*21*). In this study, the cost of organic mulches for weed management was up 215-fold that of glyphosate, not even considering the cost of application. The comparative environmental impact of large volumes of natural compounds such as acetic acid versus relatively small amounts of an environmentally benign herbicide like glyphosate (*22*) have not been studied. However in cases such as those provided in Table 1, the "natural" solution may be less friendly to the environment. There are no biochemical bioherbicides that approach the cost-effectiveness of even the more expensive conventional, synthetic herbicides. The need for better biochemical bioherbicides is great and growing yearly, as adoption of organic agriculture increases at a rapid rate. This need presents an attractive opportunity for research organizations.

Organic agriculture is in great need of a biochemical bioherbicide that kills weeds in the same way as a conventional herbicide; by targeting an enzymatic target required for an essential plant process. Such products are usually effective at much lower application rates than the biochemical bioherbicides that are now available. There are many natural compounds that act in this way (*23*, *24*). The

only commercial biochemical herbicide that we are aware of that acts in this way is bialaphos (a.ka., bilanofos), a tripeptide produced by *Streptomyces* spp. (*25*). It has a small market in Japan. It is a proherbicide that is metabolized to L-phosphinothrincin *in planta*. L-Phosphinothricin is a potent inhibitor of glutamine synthetase, and is the active molecule in the racemic mixture of synthetic L-and D-phoshinothricin sold as glufosinate, a successful conventional herbicide (*26*).

Table 1. Comparative Costs of Glyphosate and Three Bioherbicides for Control of Roadside Weeds in California at Three Different Sites. Data are from ref. (*20*).

Herbicide	*Applications Needed*	*Herbicide Cost*	*Application Cost*	*Total Cost*
		\$/ha		
Site 1				
Acetic acid	2	7000	150	7150
Glyphosate	1	40	70	110
Pine oil	2	2840	150	3010
Site 2				
Acetic acid	4	10340	300	10640
Glyphosate	2	60	150	210
Pine oil	4	10360	300	10660
Plant essentials	4	9120	300	9420
Site 3				
Acetic acid	5	17020	370	17390
Glyphosate	2	70	150	220
Pine oil	5	15690	370	16060
Plant essentials	5	11540	370	11910

Another example is that of components of the oils of several species of woody plant genera in New Zealand and Australia (*e.g.*, *Leptospermum, Eucalyptus, Xanthostemon*) (*27*, *28*). These oils contain triketone compounds that inhibit hydroxyphenylpyruvate dioxygenase (HPPD), an enzyme required for plastoquinone (PQ) synthesis (*29*). PQ is required in energy transfer of photosystem II of photosynthesis and is also a co-factor for phytoene desaturase, an enzyme required for carotenoid synthesis. Several synthetic, commercial herbicides (*e.g.*, sulcotrione, mesotrione, and tembotrione) target HPPD (*30*).

The clue to the discovery of synthetic HPPD inhibitors came from the natural triketone, leptospermone from *Calistomon citrinus*, which was observed to be an allelopathic plant species (*31*, *32*).

The oil of *Leptospermum scoparium* (manuka oil) is an effective biochemical bioherbicide, that targets HPPD with several natural triketones (*29*), of which leptospermone is the most abundant. One of the triketones in this oil, grandiflorone, is almost as active as the commercial triketone herbicide sulcotrione at the enzyme level. Manuka oil is active in soil, causing bleaching in weeds in a similar manner to that of synthetic HPPD inhbitors (Figure 2).

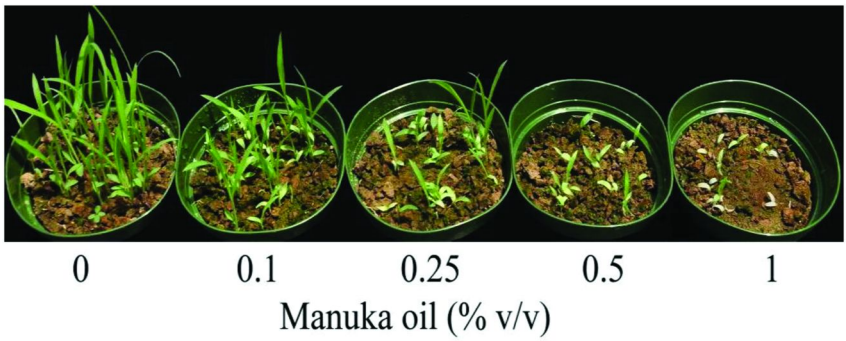

Figure 2. Effects of increasing concentrations of manuka oil applied to soil in which barnyardgrass (Echinachloa crus-galli) was planted. Solutions were sprayed onto bare soil at a rate of 360 L ha^{-1}. With permission from (33). (see color insert)

Leptospermone in soil remained at the same concentration for up to 7 days after application before starting to decline, with a half life of 18 days (*33*). Furthermore, leptospermone is not a contact herbicide, but is translocated acropetally to foliage, in much the same way as many soil-applied, commercial herbicides (*34*). However, pure leptospermone is metabolized rapidly after uptake by crabgrass. Its physicochemical properties are similar to many conventional herbicides. Pure leptospermone provides moderate herbicidal activity at 1 kg/ha (*35*), a level of activity that is comparable to some commercial herbicides, and much better than all currently available biochemical bioherbicides.

Another compound with a synthetic herbicide-like molecular target site is thaxtomin, a product of the actinobacterium that causes potato scab disease (*Streptomyces scabies*) (*36*). This plant pathogen also produces small amounts of at least a dozen 4-nitroindol-3-yl–containing 2,5-dioxopiperazines structurally related to thaxtomin A (*36*, *37*). Thaxtomin is a phytotoxic cyclic dipeptide that has been patented as a herbicide but is not yet commercialized (*38*). Nonetheless, this compound has been approved by the USEPA as a biochemical bioherbicide and has been listed by OMRI as acceptable for organic agriculture.

Plants exposed to thaxtomin have reduced growth which is accompanied by the swelling of cells and lignification of cell walls, symptoms typically associated with cellulose biosynthesis inhibitors (*39*, *40*). Inhibition of cellulose synthesis by thaxtomin was suggested in studies on *Arabidopsis thaliana*. The cell walls of seedlings treated with thaxtomin have lower cellulose content and higher proportions of pectins and hemicelluloses, relative to untreated plants. In plants, cellulose synthesis occurs in association with cortical microtubules (*41*). Thaxtomin interferes with this interaction (*42*), distinguishing it from the mechanism of action of cellulose synthesis inhibition by commercial herbicides (*i.e*. dichlobenil and isoxaben).

Sorghum species exude an oily, herbicidal substance from their root hairs that also has a synthetic herbicide-like MOA. This substance is equal parts sorgoleone, a lipid benzoquinone, and its resorcinol analog (*43*). Root hairs contain all the necessary biochemical and molecular machinery to produce these compounds (*44–47*). Sorgoleone is mineralized by microbes in soils (*48*), with the methoxy group being most readily degraded and the rest of the molecule being more recalcitrant to mineralization. However, sorgoleone is produced continuously by root hairs, which causes the level of sorgoleone to remain fairly constant in the rhizosphere layer. As such, its interaction with soil is similar to lipophilic, soil-applied synthetic herbicides such as the dinitroanilines (*43*).

Some research reported that sorgoleone has better postermergence activity than preemergence activity on a variety of weeds when applied like a conventional herbicide (*49*). However, the physicochemical properties of sorgoleone are less than ideal for foliar uptake, where its primary molecular target site (photosystem II) is located (*50*, *51*). To enhance its efficacy, sorgoleone was recently formulated as a wettable powder (*52*). This formulated sorgoleone product has a much higher herbicidal activity and broader weed spectrum than unformulated sorgoleone, making it a better potential biochemical bioherbicide.

Biochemical Bioherbicides as Sources of New Modes of Action

During the "golden age" of synthetic herbicides, a new mode of action was introduced approximately every three years from 1950 until about about 1985, when the last mode of action, HPPD, was introduced (*8*) (Figure 3). Rotating or mixing modes of action is a commonly accepted approach to combating evolution of resistance to pesticides and pharmaceuticals. Thus, an adequate number of compounds with different modes of action is needed to carry out such a strategy. For more than 20 years, evolution of resistance to herbicides has continued unabated (*3*), while no new modes of action have been introduced. Thus, we are in great need of herbicides with new modes of action. A combination of factors may explain the lack of new herbcicide modes of action introductions during the past 30 years. They may include the economic impact of the domination of the herbicide market by glyphosate and glyphosate-resistant crops, diminishing returns with traditional discovery efforts, increased regulatory costs of introducing

new herbicides, and fewer companies involved in herbicide discovery. But, the literature on molecular target sites of natural phytotoxins provides strong evidence that there are many unexploited herbicide targets (*53*).

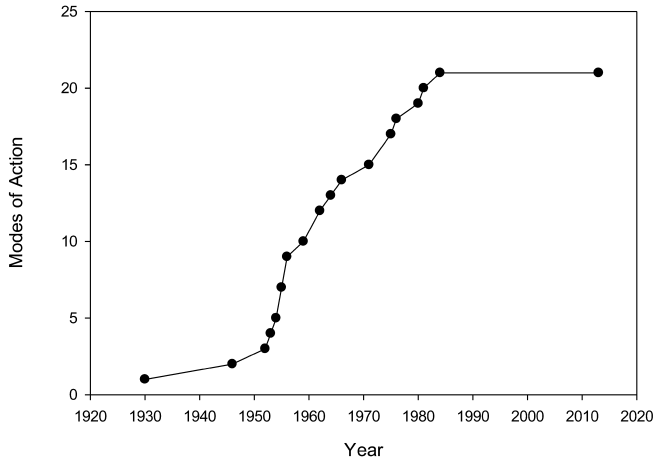

Figure 3. Cumulative introduction of new modes of action of herbicides by year.

Table 2. Examples of Natural Phytotoxins with Target Sites Not Inhibited by Commercial Herbicides

Phytotoxin	*Target Site*	*Ref.*
Actinonin	Peptide Deformylase	(*60*)
Aal- Toxin	Ceramide Synthase	(*61*)
Cyperin	Enoyl-Acp Reductase	(*62*)
Fosmidomycin	Deoxyxylulose-5-Phosphate Reductase	(*63*)
Gabaculin	Glutamate 1-Semialdehyde Aminotransferase	(*64*)
Hydantocidin	Adenylosuccinate Syntase	(*65*)
Phaseolotoxin	Ornithine Carbamyl Transferase	(*66*)
Rhizobitoxine	B-Cystathionase	(*67*)
Tentoxin	Cf1 Atpase	(*68*)

We have published several papers that detail the molecular target sites of a wide array of natural phytotoxins (*54–59*). Many of these compounds have molecular targets that differ from those included in in the *ca*. twenty targets of commercial herbicides. Table 2 provides a sampling of some of these enzyme-inhibiting phytotoxins and their modes of action. Most of these

phytotoxins kill plants at relatively low doses through disruption of these alternative targets, making it clear that, in terms of efficacy, these target sites may be viable for development as commercial herbicides.

Considering the great need for herbicides with new modes of action, why are none of these compounds being sold as herbicides? There are four main reasons. First, many of these compounds are structurally complex (*e.g.,* more than one stereogenic center), making the cost of synthetic production prohibitive. For example, the cyclic tetrapeptide tentoxin that specifically inhibits CF1 ATPase needed for photophosphorylation is expensive to synthesize. Considerable effort was made to find simpler, synthetic tentoxin analogs with the same activity without success (*e.g.* (*69*),). Second, the physicochemical properties of many natural phytotoxins are not good for a herbicide, limiting their uptake, translocation, and stability (environmental half life). For example, ferulate is an excellent selective blue-green algicide in the laboratory (*70*), but its half-life in the field is only a few hours – not long enough to be effective (*71*). Third, the target sites of some of these compounds are questionable in terms of mammalian toxicity. This is a bigger problem than some might expect, as plants and animals share many biochemical processes, and often an inhibitor of the plant enzyme is also quite active on the animal enzyme. For example, the highly potent phytotoxin AAL-toxin is an analog of the animal ceramide synthase-inhibiting fumonisin mycotoxins, and it is also quite toxic to animals by this mechanism of action (*72*). We should point out that a significant fraction of the commercial herbicide targets are also found in mammals (glutamine synthetase, protoporphyrinogen oxidase, HPPD, serine/threonine protein phosphatases, tubulin, and acetyl-CoA carboxylase) and that several pharmaceutical target sites overlap with those of herbicides (*73*). Lastly, the timing of both patents and publications may have kept some of the compounds from being developed. Quite a number of phytotoxins with new modes of action were patented at a time when there was not much value in having a new mode of action. Now, when a new mode of action is greatly needed, these patents have either expired or will soon expire, making investment in development of the compound as a herbicide economically unviable. Much of the research in the area of natural phytotoxins and their modes of action has been conducted by academic laboratories, for which publication of results is more important that protection of intellectual property. Premature publication of reports of the herbicidal effects of natural compounds can jeopardize the ability to patent the compound. Without exclusive rights afforded by a patent, industry has little incentive to spend the many millions of dollars needed for development of a new herbicide.

These hindrances have inhibited industry from using natural products-based herbicide discovery and development strategies. However, considering the extreme need for new modes of action and the clear evidence of natural phytotoxins as leads for new modes of action, greater effort may be expended in overcoming these aspects of a natural product-based strategy for herbicide discovery and development. New technologies may be helpful in overcoming the molecular complexity of some of the compounds. For example, since natural products are biosynthesized by enzymes encoded by genes of the producing organisms, modern biotechnology can be used to produce complex molecules

that would be too expensive to synthesize by traditional synthetic methods. For example, the very complex insecticidal spinosyn A and D molecules (the mixture is spinosad) are commercially produced by fermentation (*74*), and thaxtomin will be produced by fermentation (*75*). Semi synthetic modification of natural compounds can improve physicochemical properties to improve their pesticidal properties. For example improvement in the properties of spinosad has been accomplished by use of spinosyns for semisynthetic production of spinetoram (*76*). Improved technologies over the past two decades have made products viable that would have been rejected without these technologies.

Finally, the question of whether we have reached diminishing returns in discovering natural phytotoxins with new modes of action is unclear. Much of the natural product efforts of large pesticide discovery companies has been made with libraries of soil microbes, a strategy very similar to that of pharmaceutical discovery companies. From a chemical ecology perspective, one might expect that co-evolution of plant pathogens with their hosts would result in some of the most phytotoxic compounds. Indeed, some of the most potent natural phytotoxins are from plant pathogens (*e.g.*, AAL-toxin, tentoxin, thaxtomin). Only a few laboratories have taken the approach of screening toxins from plant pathogens for potential use as herbicides or herbicide leads (*77–79*). Most of what we know of phytotoxins from plant pathogens is from pathogens of crops, leaving the pathogens of weeds as an opportunity for future discovery efforts.

Conclusions

The increases in evolved herbicide resistance and adoption of organic crops have exacerbated the needs for both herbicides with new modes of action and for effective and inexpensive biochemical bioherbicides that are acceptable to organic farmers, respectively. In both cases, no new products have been introduced within the past 20 years that even partially fulfill these needs. Discovery of new, natural phytoxins with new modes of action or that can be used as effective biochemical bioherbicides at a reasonable cost can solve these problems.

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Chapter 4

G-Protein-Coupled Receptors (GPCRs) as Biopesticide Targets: A Focus on Octopamine and Tyramine Receptors

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Plants have evolved beneficial and protective mechanisms including the production of essential oils. Essential oils are the odiferous component of plant extracts, which give plants a variety of unique properties. Essential oils are composed of various terpenoid compounds, particularly monoterpenoids and related aromatic compounds, along with sesquiterpenoids. A variety of terpenoids have been shown to have a toxic effect against insects. It is thought that this toxic action occurs through a neurological mechanism of action. Octopamine receptors and tyramine receptors are G-Protein-Coupled Receptors (GPCRs) primarily found in invertebrates, including insects. GPCRs have been a popular target for pharmaceutical development but not for agrochemical development. A summary of insect octopaminergic and tyraminergic systems is discussed.

The Need for Safe and Effective Insecticides

The growing world population, which is estimated to be around 9 billion by 2050, is placing growing demands on agriculture. The agrochemical and animal health industries are trying to discover new methods to control economically devastating pests, like insects and ticks, along with the diseases these organisms are capable of vectoring. Discovery of agrochemicals and

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veterinary external-parasiticides has become difficult in a changing landscape of agricultural practices characterized by increased public and governmental scrutiny and demands. Such stipulations for agrochemicals include the discovery of compounds having characteristics of decreased toxicity to non-target vertebrate and invertebrate organisms, along with decreased environmental contamination. While significant advances have been made in reducing the use rate and environmental impact of conventional synthetic pesticides, biopesticides do not share an equal amount of the market (*1*). Additionally, biologically-based technology to aid in controlling agricultural pests still lacks public acceptance, and is not as globally accepted outside of the United States. Further restraints on agrochemical development include increased product costs and time to get a product to market (*1*). Currently, agricultural pests are controlled by over 900 types of chemistry that have over 100 mechanisms of action (*2*). However, even with this vast chemistry and mechanisms of action there is still a desideratum for new mechanisms of action. It is important to note that new mechanisms of action, along with new chemistry, are only successful with proper pesticide use and the use of integrative approaches to pest control.

New Agrochemical Targets: G-Protein-Coupled Receptors (GPCRs)

G-Protein-Coupled Receptors (GPCRs) are membrane-bound receptors, which are involved in the sensing of extracellular signals. In turn, the extracellular signal is internalized to result in some physiological or cellular response. This very nature of GPCRs allows them to be highly "druggable" targets, and they have been widely exploited by the human pharmaceutical industry. It is estimated that as much as 50% of all human pharmaceuticals target GPCRs, which indicates their vast importance to normal cellular and physiological functions and their susceptibility to pathological conditions (*3*). However, GPCRs historically have not been a dominant force in the agrochemical market. Recently, there has been growing interest in the discovery of agrochemicals targeting GPCRs (*3*–*6*).

Several ligands can activate GPCRs; here we will focus on biogenic amines as ligands for GPCRs, specifically tyramine and octopamine, and their importance to invertebrate function, particularly in relation to insects. Another significant class of ligands that are capable of activating GPCRs are neuropeptides. The physiological importance of neuropeptides in *D. melanogaster* has been recently reviewed (*7*). Since GPCRs are important to the pharmaceutical industry, there have been several systems developed to study GPCRs, which have also been previously reviewed (*3*, *4*, *6*, *8*).

Octopamine and Tyramine Synthesis

Octopamine and tyramine are biogenic monoamines that are found in the nervous system of arthropods, including ticks and insects. Octopamine and tyramine were originally identified in the salivary glands of the octopus (*9*). Octopamine and tyramine are catecholamines like dopamine, norepinephrine

(noradrenaline) and epinephrine (adrenaline). Other biogenic amines include the indolamines, such as, serotonin or 5-hydroxytryptamine (5-HT) and histamine. Catecholamines use the amino acid tyrosine as the backbone for synthesis, as shown in Figure 1. Briefly, tyramine is the rate-limiting product in the formation of octopamine. Tyramine is produced by the decarboxylation of the amino acid tyrosine via tyrosine decarboxylase (*10*). Tyramine can also be synthesized from a dopamine metabolite; however, this is not believed to be a major synthetic route (*11*). Tyramine is further acted upon by tyramine-β-hydroxylase to form octopamine (*12*).

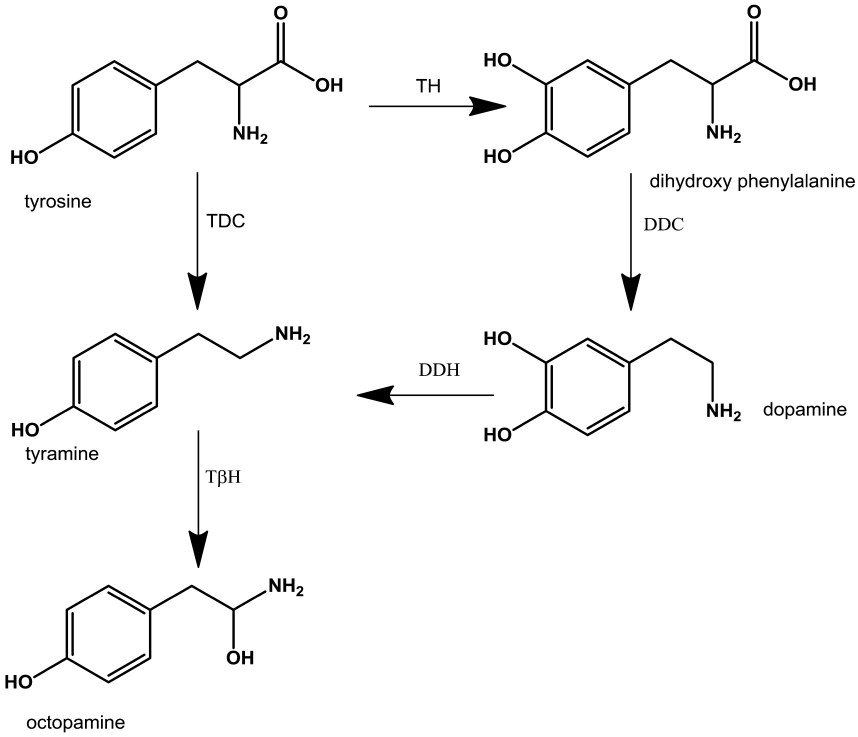

Figure 1. The amino acid, tyrosine, is vital to the synthesis of tyramine and octopamine. Tyramine is synthesized when tyrosine decarboxylase (TDC) converts tyrosine to tyramine. Octopamine is synthesized when tyramine-β-hydroxylase (TβH) converts tyramine to octopamine. It is possible that tyramine can be synthesized from a dopamine pathway when dihydroxy phenylalanine is synthesized from tyrosine via tyrosine hydroxylase (TH). Dihydroxy phenylalanine is converted to dopamine via DOPA decarboxylase. Dopamine is converted to tyramine via dopamine dehydroxylase (DDH).

Signal Transduction of Octopamine and Tyramine

Octopamine and tyramine are released from various portions of the insect's nervous system (*11*, *13*). Octopamine and tyramine's physiological functions are realized when octopamine or tyramine binds to its specific membrane-bound receptors. In turn, the receptor internalizes this original chemical message into a biochemical cascade via the production of second messenger(s), which ultimately results in a cellular response. Octopamine and tyramine primarily activate the superfamily GPCRs. Specifically, octopamine and tyramine activate rhodopsin-like metabotropic GPCRs. GPCRs are sometimes referred to as heptahelical receptors or serpentine receptors; this is because the receptor transverses the cell membrane seven times (7-TM). The seven transmembrane regions of GPCRs are connected by three extracellular loops and three intracellular loops. Residues in several octopamine receptors and several tyramine receptors have been shown to be important in ligand binding and receptor function, which has been discussed in a recent review (*14*). Receptor activation allows for the recruitment of a heterotrimeric intracellular G-protein, which are composed of an α -subunit, β-subunit and a γ-subunit.

The original classification of octopamine receptors was based on second messenger production in various invertebrate tissues. However, the advent of molecular biology has allowed for a comprehensive approach to octopamine receptor classification, now including tyramine receptors as a separate entity. The new classification system is based on sequence homology with the mammalian adrenergic receptors and signaling properties (*15*). That is, octopamine receptors are classified based on sequence similarities and the production of specific second messenger pathways realized during receptor activation. The α-adrenergic-like octopamine receptor (OctαR) shares a sequence homology with the mammalian α-adrenergic receptor(s). Activation of OctαR results in an increase of the intracellular calcium ($[Ca^{2+}]_i$) concentration, which is liberated from intracellular calcium stores, like the endoplasmic reticulum or the sarcoplasmic reticulum, via the activation of the inositol pathway. β-adrenergic-like octopamine receptors (OctβRs) share sequence homology with the mammalian β-adrenergic receptor(s). Activation of OctβRs results in the increase of the intracellular concentration of cyclic adenosine monophosphate (cAMP), via activation of the membrane-bound enzyme adenylate cyclase. It is not unusual for OctαRs and OctβRs to respond to either octopamine or tyramine at different concentrations; this is probably due to structure similarity between octopamine and tyramine. Ligand-agonist coupling or ligand-trafficking, which is peculiarized as the activation of different second-messenger pathways based on the ligand, has been reported for octopamine and tyramine at a single receptor (*16*). Ligand-agonist coupling is commonly found with the octopamine/tyramine or tyramine receptor, which were later classified as tyramine-1 receptors (TAR1). When octopamine activates these receptors, it can result in an increase of the intracellular concentration of calcium. When tyramine activates TAR1, it can result in an inhibitory effect on adenylate cyclase, decreasing the intracellular concentration of cAMP. It is now accepted that tyramine is the preferred ligand of TAR1 (*14*, *17*). Recently, tyramine-2 receptors (TAR2) have been identified, which are also specifically activated by

tyramine, versus octopamine, and have been shown to result in the release of calcium from intracellular stores (*18*, *19*). Bayliss *et al.* has proposed a third class of tyramine receptors (TAR3), which have a different pharmacological profile, and result in an increase of intracellular cAMP, when heterologously expressed in Chinese hamster ovary (CHO-K1) cells. Additionally, TAR3 seems to be specific to *Drosophila melanogaster*, where it is expressed in the crop and eye of the adult flies and the hindgut in larvae (*17*). The signal transduction pathways for these GPCRs are shown in Figure 2.

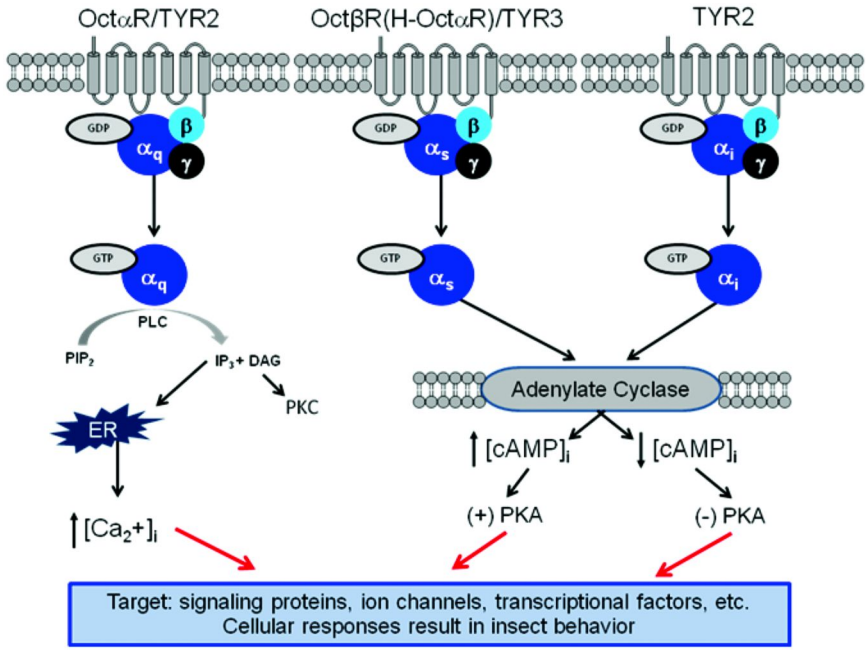

Figure 2. Signal transduction of tyramine and octopamine G-protein-coupled receptors (GPCRs). Here the cellular biochemical pathway is shown for the α-adrenergic-like octopamine receptor (OctαR), the β-adrenergic-like octopamine receptor (OctβR), the Type-1 tyramine receptor (TAR1), the Type-2 tyramine receptor (TAR2) and the Type-3 tyramine receptor (TAR3). Activation of cellular biochemical pathways results in an insect behavior or function. Abbreviations: GDP, guanosine diphosphate; GTP, guanosine triphosphate; PLC, phosphoinositide phospholipase C; PIP_2, phosphatidylinositol-4,5-bisphosphate; IP3, inositol-1,4,5-trisphosphate; DAG, diacyl glycerol; PKC, protein kinase C; cAMP, cyclic adenosine monophosphate; PKA, protein kinase

Ligand-gated ion channels, like GPCRs, are transmembrane ion channels and are involved in the flow of ions into or out of a cell upon the binding of a ligand or chemical message. Recently, ligand-gated chloride channels that are preferentially activated by tyramine have been identified in *Caenorhabditis elegans,* Cel LGC-55 (*20*), and in *Haemonchus contortus*, Hco-LGC-55 (*21*). Cel-LGC-55

appears to act on neck muscles to suppress head oscillation and promote backward movement or reversal behavior in *C. elegans* (*20*). Hco-LGC-55 has been shown to be expressed in all life stages of the parasite; expression may be reduced in the adult male (*21*).

Octopamine and Tyramine: Diverse Physiologically Active Biogenic Amines

There is a plethora of studies examining the physiological importance of octopamine and its receptors in various invertebrates; this has been the topic of several excellent reviews (*11*, *14*, *15*, *22–24*), and therefore, will not be discussed here. Tyramine and its receptors, on the other hand, have not had as much research attention. This is largely because tyramine was initially thought to only be the biosynthetic precursor to octopamine. Therefore, we will focus on the advances made in understanding the physiological role of tyramine through a brief review of the literature.

Insects undergo differential behavioral states using semiochemicals, and this is extended to the complex interactions of social insects, like the honey bee, *Apis mellifera*. Previous studies have indicated neurohormonal and neuromodulatory effects on honey bee behavior to aid in the support of social hierarchy in the bee hive (*25–29*). Previous studies have indicated that the honey bee queen uses pheromones, which are produced and released from the mandibular gland and/or the Dufur's gland, to maintain a reproductive hierarchy in the colony (*30*). The concentration of pheromones produced in the mandibular gland is high in the queen bee, but low in the worker bees (*31*, *32*). Recently, tyramine has been shown to result in reproductive dominance over the fertility of the bee. Specifically, tyramine has been shown to be involved in ovary development, and pheromone production and secretion; specifically, a pheromone that is consistent with a queen (*33*). Tyramine did not have effects opposite of octopamine, which had been thought to be a major role of tyramine in insects (*24*). Instead octopamine appears to be involved in cast differentiation and the production of specific worker pheromones (*26*, *27*, *33*).

Insects are able to respond to environmental cues via a variety of chemosensory organs. The molecular mechanism of odor reception in insects has been recently reviewed (*34*). While octopamine and tyramine may not be the original sensing signals, they are involved in the neuronal modulation of the signal. A *D. melanogaster* mutant has been identified as having an olfactory defect resulting in behavioral changes (reduced avoidance). It was determined that this reduced avoidance was a result of a p-element upstream of the type-1 tyramine receptor (TAR1); this decreased the expression of the tyramine receptor. This indicates that tyramine has a role in modulation of *D. melanogaster* sensory processing (*35*). Mutation of the tyramine-β-hydroxylase (TβH) gene results in an abnormally low concentration of octopamine with a high concentration of tyramine. The decreased level of octopamine results in a poor locomotion phenotype in *D. melanogaster*. For instance, TβH mutant larvae were described as being slow and "pausing", compared to wild-type, described as a decrease

in linear translocation; this phenotype was recovered by feeding the larvae octopamine (*36*). Tyramine was able to decrease flight, possibly via a central motor pattern generator, in honey bees; this is an opposite effect of octopamine (*37*). Tyramine has also been shown to affect egg laying, reversal movement and head oscillations in *C. elegans* (*38*). It is important to note that more sensory behavior effects, head oscillations and reversal movement, were observed via the effects at the chloride-gated tyramine receptor (LGC-55) (*20*).

Octopamine has previously been reviewed to have effects in the reproductive system of insects (*11*); however, tyramine also has such a role. It has been demonstrated that there are tyraminergic innervations in the *Locusta migratoria* oviduct muscles. Tyramine was shown to increase the amplitude of excitatory junctional potentials and hyperpolarize the oviduct muscle; this effect was seen at low concentrations of tyramine (*39*). Tyramine has also been reported to have an effect on other types of muscles, specifically, muscles involved in insect flight. In *D. melanogaster*, tyramine has been shown to inhibit flight initiation at high concentrations (*40*).

Botanical Insecticides

Botanical insecticides, such as pyrethrum, rotenone, neem and plant essential oils, have been used for over 150 years in the United States; however, some botanical insecticides have been used for thousands of years in other countries (e.g. China, Egypt, Greece and India). Essential oils can be characterized as lipophilic liquids, which when isolated from the plant, display a strong odor. Essential oils function as plant secondary metabolites, which means they are not involved in the primary metabolism of the plant but still serve a variety of functions; for instance they can deter herbivorial feeding (*41*, *42*). Essential oils are commonly obtained via steam distillation from various plant tissues/organs or plant foliage under a variety of conditions (*41–43*). Essential oils are a complex mixture of different chemistries including various types of terpenes/terpenoids and related aromatic terpenoid compounds. Here we will use "terpene" interchangeably with "terpenoid". Since botanical compounds, like essential oils, are widely found in everyday items, like cosmetics and fragrances, food additives and pharmaceuticals, they are generally believed to have minimal mammalian toxicity (*44*). Some essential oils and essential oil components are found on the United States Environmental Protection Agency's exempt lists (25b and 4a). Additionally, some essential oils are Generally Recognized As Safe (GRAS), according to the Food and Drug Administration (FDA).

Essential oil terpenoids are synthesized from isoprene units, which are the five-carbon building blocks of terpenoids. The coupling of these isoprene units can lead to structures that have 5 – 40 carbons. Here, we focus on terpenoid structures composed of two isoprene units, monoterpenoids (10-carbons), and terpenoid structures composed of three isoprene units, sesquiterpenoids (15-carbons). The carbon backbone of terpenoids is further targeted by a variety of enzymes that give terpenoids diverse characteristics. For instance, terpenoids can be cyclic or acyclic, and they can contain a variety of heteroatoms to create

alcohols, aldehydes, ketones, esters, epoxides, ethers, and acids (*45–47*). Not all terpenoids are aliphatic; some related aromatic terpenoids are synthesized from the shikimic acid pathway, which is the pathway that plants commonly use to synthesize aromatic amino acids. In particular, phenylalanine and tyrosine are responsible for the phenylpropane/phenylpropene units that are the building blocks for the aromatic compounds found in essential oils (*46*).

Terpenoid Mechanism of Action: Focus on Octopamine and Tyramine Receptors

An understanding of the mechanism of action of insecticidal activity of essential oils, and their terpenoids, will aid in the integration of these compounds into a pest control strategy. While several studies have indicated that these compounds have a neurotoxic mechanism of action (*48*, *49*), it is possible that several targets or mechanisms are involved, both inside and outside of the insect's nervous system. Several studies have been performed assessing different mechanisms of neurotoxic action. These studies included the compound's ability to inhibit the enzyme acetylcholinesterase, leading to an increased concentration of acetylcholine in the synaptic cleft (*50–55*). Another study evaluated the ability of essential oil components to affect chloride conductance by altering the γ-aminobutyric acid ($GABA_A$) receptor (*56*). Additionally, different modulations of the $GABA_A$ receptor along with physicochemical properties, to predict successful modulation of this $GABA_A$ receptor, have been described (*57*, *58*). Recently, binding at the house fly (*Musca domestica*) nicotinic acetylcholine receptor (nAChR) has been reported (*59*). In addition to GABA and nAChR receptors, essential oil terpenoids have also been reported to have an effect at other ion channels. Specifically, essential oil terpenoids have been reported to inhibit transient receptor potential (TRP) channels, which are important sensory channels in humans (*60*).

Essential oil toxicity may be attributed to the multi-functionality of octopamine, and now tyramine, to insect physiology. Application of essential oil terpenoids may result in hyperactivity, hyperextension of extremities and abdomen, knockdown, which can be followed by death. Homogenate of the American cockroach (*Periplaneta americana*) nervous system resulted in an increase of cAMP upon terpenoid application, leading the author to suggest that toxicity was mediated via the octopaminergic system in the insect's nervous system (*49*). Later studies performed in *Helicoverpa armigera* homogenate also showed an increase of cAMP (agonistic activity) from abdominal dermal tissue with the application of several essential oil terpenoids, which was blocked by the octopamine receptor antagonist, phentolamine (*61*). A cloned α-adrenergic-like octopamine receptor (Pa oa1) has been described from the American cockroach (*49*) and an α-adrenergic-like octopamine receptor (OAMB) from *D. melanogaster* (*62*). When these octopamine receptors were expressed in human embryonic kidney (HEK-293) cells they resulted in an increase of the intracellular concentration of cAMP and calcium, which is peculiar since these both are OCTαR's and should signal via the inositol pathway (increase in

intracellular calcium). This may be an artifact of the heterologous expression system or this may indicate the ability of essential oils to recruit different G-proteins, activating multiple second messenger pathways (*23*). When eugenol, a plant essential oil monoterpenoid, was applied to HEK-293 cells expressing Pa oa1 it decreased the basal level of cAMP. Application of trans-anethol to HEK-293, expressing OAMB, resulted in an increase of the cellular concentration of cAMP (*62*). However, little effect was reported on the calcium response (*62*). Essential oil activity has also been reported on a cloned tyramine receptor, from *D. melanogaster* that was expressed in *Drosophila S2* cells. Here, a strong calcium response, along with a decrease of forskolin-stimulated cAMP was observed with the addition of tyramine (*63*). In addition to *in vitro* heterologous expression studies performed for the *D. melanogaster* tyramine receptor; *in vivo* studies were also performed to determine the toxicity of essential oil terpenoids against *D. melanogaster* (*63*). The aromatic monoterpenoid, thymol, which was the only tested monoterpenoid that resulted in an increase of the basal level of cAMP, and an increase of the intracellular concentration of calcium resulted in the lowest mortality (*63*). Thymol's stereoisomer, carvacrol, which had a strong calcium response against this *D. melanogaster* tyramine receptor, had similar mortality to thymol (*63*). These results indicate a correlation between tyramine receptor activity and insect mortality.

Conclusion

GPCRs have diverse physiological functions within invertebrates, including insects, mites, ticks and nematodes; however, they are an underutilized target for agrochemical development. Since GPCRs are a widely utilized target for the pharmaceutical industry there are several screening systems available, which can be applied to insects and ticks, to study GPCRs. The use of botanically-based insecticides may allow for the development of efficacious biopesticide products or serve as the starting material for safer arthropod and nematode control programs. Hopefully GPCRs will be exploited as targets for insecticides or acaricides in the future.

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Chapter 5

RNA Interference in Insect Pest Management: Assessing Potential Benefits and Environmental Risk

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RNA interference involves the downregulation of gene expression by exogenous double stranded RNA molecules (dsRNA). RNAi has facilitated significant technological advances in diverse fields from functional genomics to agricultural biotechnology and pest management. Recent studies have demonstrated that RNAi responses in insects can be achieved by exposure to dsRNA in artificial diet or expressed in transgenic host plants, resulting in mortality of targeted species. There is clear evidence for potential applications of RNAi for the control of insect pests. However, the environmental and ecological risks of RNAi including potential toxicity to non-target organisms, environmental fate and the risk of resistance evolution have yet to be determined.

RNA Interference (RNAi)

The discovery of RNAi arose from the unexpected observations in plants, fungi and nematodes that the introduction of sense or antisense RNA resulted in reduced expression of genes with homologous sequence (*1*). Fire, Mello and others provided the explanation for these findings in experiments documenting RNA-dependent gene repression that earned them the 2006 Nobel Prize in Physiology or Medicine. Using *Caenorhabditis elegans* as a model, they demonstrated that the silencing response resulted neither from the sense nor antisense RNA but rather the mixture of the two. RNA preparations were contaminated with a small amount of the opposite strand and it was determined that the resulting double stranded

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RNA (dsRNA) caused the silencing response. When dsRNAs were deliberately prepared, they were shown to be very potent in eliminating expression of the target gene, and the phenomenon RNA interference (RNAi) was first described (*2*).

RNAi can be defined as the downregulation of expression of specific genes by dsRNA. Eukaryotic organisms have a common machinery for specific gene silencing that is triggered by the presence of dsRNA. This process is called RNAi in animals and post-transcriptional gene silencing in plants (*3*). RNAi involves the degradation of a target mRNA and is mediated by small interfering RNAs (siRNA). The siRNAs are produced from the longer strands of dsRNA that are cleaved by dsRNA specific endonucleases referred to as Dicers. The siRNAs are 21-25 bp dsRNA fragments carrying a two base extension at the 3' end of each strand (*4*, *5*). The siRNAs serve as sequence specific guides for the argonaute proteins, which contains a RNAaseH-like domain responsible for target mRNA degradation and are an essential component of the RNA-induced silencing complex (RISC) (*4*, *6*). The process is closely related to post-transcriptional gene regulation by microRNAs and the end result is the inhibition of translation initiation (*4*).

RNAi is a conserved mechanism that likely evolved as a defense against invading nucleic acids (e.g., viruses and transposable elements) (*1*, *6*, *7*). When exposed to foreign genetic material (RNA or DNA), organisms use RNAi to silence the invading nucleic-acid sequences before dsRNA sequences integrate into the host genome or affect cellular processes (*7*). In some organisms, RNAi signals are transmitted horizontally between cells and, in certain cases, vertically through the germ line from one generation to the next (*7*).

RNAi has facilitated significant technological advances in diverse fields. It has been instrumental in assigning function to genes and has been proposed as a therapeutic technique for treating human diseases (*1*, *4*, *5*). Recently, RNAi has been receiving much attention as a novel and selective method to control agricultural pests (*5*).

dsRNA Uptake by Insects and Diversity of the RNAi Pathway

RNAi uptake can be divided into cell-autonomous and non-cell autonomous RNAi (*6*). Cell-autonomous RNAi is limited to the cell in which the dsRNA is introduced resulting in reduced gene expression within that cell (*5*, *6*). In contrast, non-cell autonomous RNAi refers to the silencing effect in tissues/cells beyond the site of application of the dsRNA (*6*). Non-cell autonomous RNAi was first described in *C. elegans* when injection into the head or tail led to target gene silencing throughout the injected animal and its progeny (*2*). There are two types of non-cell autonomous RNAi: environmental RNAi and systemic RNAi. Environmental RNAi occurs when dsRNA is taken up from the environment of the cell and the effect is observed in all the cells that take up dsRNA. Systemic RNAi can only take place in multicellular organisms and involves the transference of the silencing signal from those cells exposed to dsRNA to other cells and tissues (*5*, *6*). The machinery required for uptake of environmental dsRNA or the spread of RNAi is distinct from the cell autonomous RNAi machinery (*6*).

In plants and nematodes, RNAi can have systemic effects on gene expression that involves the spread of silencing to distant parts of the organism via dsRNA movement from cell to cell (*4*, *6*). Systemic RNAi in plants and nematodes is facilitated by the presence of an RNA-dependent RNA polymerase (RdRP) that interacts with the RISC complex and generates new dsRNA using siRNAs as templates (*4*, *8*). RdRP provides the amplification of the RNAi trigger (dsRNA) allowing it to spread throughout the organism. However, based on the genome sequences for a number of species, insects apparently lack RdRP (*8*). The lack of RdRP in insects suggest that any effects of RNAi in insects should be limited to cells that have taken up dsRNA and will require continuous exposure to dsRNA for the effect to persist (*4*). However, both environmental and systemic RNAi have been observed in insects indicating that the spread of dsRNA is based on a mechanism other than RdRP-dependent amplification (*5*).

Systemic RNAi in insects through oral ingestion of dsRNA has been documented in a number of insect orders including Hymenoptera, Isoptera, Diptera and Coleoptera (*9–12*). In these instances, the dsRNA is taken up from the gut lumen, and the silencing signal spreads to other tissues (*5*). At least two pathways for environmental dsRNA uptake have been described: the transmembrane channel-mediated mechanisms based on the *systemic RNA interference deficient-1* (*SID-1*) gene described in *C. elegans* and the endocytosis mediated uptake mechanism shown in *Drosophila* S2 cells (*5*). Genetic screens in *C. elegans* have identified two *systemic RNA interference deficient* genes, *SID-1* and *SID-2*, which participate in environmental RNAi (*13*, *14*). *SID-1* encodes a widely conserved multipass transmembrane protein with homologs in almost all animals except Diptera and functions as a dsRNA channel (*13*). *SID-2* appears to be involved in the initial import of dsRNA from the gut lumen but is not required for the systemic spread of silencing signals between cells and tissues (*14*). The endocytosis mediated uptake mechanism described in *Drosophila* S2 cells involves the scavenger receptors, *SR-CI* and *Eater*. These genes possibly play a role in natural dsRNA uptake (*15*, *16*). However, the precise mechanism by which dsRNA is recognized and brought into the cell by the endocytosis mechanism is unclear (*5*).

The mechanism of uptake at the cellular level is still unknown for most insects (*12*). Additional studies are necessary to determine the mechanism(s) of uptake of dsRNA and systemic spreading of RNAi (*4*, *12*). Further understanding of insects' uptake mechanisms of dsRNA will be important to facilitate the use of RNAi as a pest management tool (*4*).

RNAi as a Pest Management Tool?

RNAi-mediated gene knockdown has been reported in several insect orders, although in most instances the response has been observed after injection of dsRNA into the hemocoel, a technique not practical for pest management (*4*, *5*). For RNAi to be effective as a pest management option, the pest insect must be able to autonomously take up the dsRNA through feeding (*4*, *5*). A number of recent reports have indicated that feeding dsRNA to insects can trigger an RNAi

response suggesting that RNAi may provide an effective and selective insect pest management tool (*4*). Although RNAi-mediated gene knockdown has been described in different insect orders, not all reported species show the same degree of sensitivity toward RNAi (*17*).

Price and Gatehouse (*4*) described that the keys to successfully achieve a systemic RNAi response are the identification of suitable target genes and the ability to deliver sufficient intact dsRNA. Targeted genes selected for insect pest management should be essential for the insect to function (*4*). In some cases the efficiency of RNAi-mediated gene knockdown appears to depend on the identity and nature of the target gene. Different genes might have dsRNA or siRNA molecules that are subject to sequence-specific degradation and/or specific stability of the targeted mRNA. Thus, genes with efficient feedback mechanisms of regulation might prevent depletion of mRNA levels with higher rates of transcription (*18*). Furthermore, the ability to deliver sufficient amounts of dsRNA is dependent on the deployment method, the stability of dsRNA in the environment and within the insect gut, and a method to cost-effectively synthesize dsRNA (*4*). Induction of RNAi via an oral route of exposure requires efficient uptake of dsRNA by midgut cells followed by suppression of the target mRNA causing significant adverse effects on growth, development and survival of the target pest species (*12*). Variation in the midgut and hemolymph environment between different species is likely to determine whether a feeding approach will be successful (*19*, *20*). For example, experiments with *Bombyx mori* have shown that dsRNA is degraded by midgut juice in less than 30 minutes, but is more stable in the hemolymph (*19*). Differences in abundance and activity of dsRNA-degrading enzymes between the gut and hemolymph may partially explain why dsRNA triggers an RNAi response after injection but not after feeding (*20*).

Different methods to achieve oral ingestion of dsRNA will be necessary for insects with different life histories, and a number of recent examples have illustrated the potential for a variety of approaches. For example, the Eastern subterranean termite, *Reticulitermes flavipes*, was exposed to a dsRNA bait consisting of filter paper treated with high concentrations of dsRNA for two termite genes: an endogenous cellulase enzyme and a caste-regulatory hexamerin storage protein (*10*, *21*). Knockdown of either gene through dsRNA feeding led to significantly reduced group fitness and increased mortality. Hexamerin silencing in combination with juvenile hormone treatments led to lethal molting and increased differentiation of presoldier caste phenotypes. Results from these studies indicate that baits containing dsRNA may offer an effective means of managing urban pests provided that the dsRNA can be stabilized (*10*, *21*).

Another method for delivery of dsRNA by ingestion involves the use of nanoparticles. Polymeric nanoparticles have been used for RNAi-therapeutics in humans to increase the stability of dsRNA and to enhance the cellular uptake (*11*). One of the most commonly used polymers is chitosan, a virtually nontoxic and biodegradable polymer that can be prepared from chitin (*22*). Chitosan nanoparticles were tested in *Anopheles gambiae* larvae targeting the chitin synthase gene (*11*). The chitosan/dsRNA nanoparticles were formed by self-assembly caused by electrostatic interaction between amino groups of chitosan phosphate groups of dsRNA (*11*). Following exposure of *A. gambie*

larvae to chitosan/dsRNA nanoparticles, larvae were exposed to diflubenzuron and calcofluor white (CF) or dithiothreitol. Larvae fed the nanoparticles showed increased susceptibilities to the pesticides suggesting that mortality resulted from the reduction of chitin biosynthesis in the midgut. Nanoparticles have the advantage of enhancing dsRNA stability and the potential to be used in aquatic environments. Such characteristics offer the potential for RNAi to be used in managing mosquito vectors of human diseases (*11*).

An alternative delivery method for exposure of pest insects to dsRNA involves transforming bacteria to express dsRNA for essential genes that could be formulated and applied as a traditional pesticide. This method involves transformation of a bacterial strain deficient in RNAase III, an enzyme that degrades dsRNA in normal bacterial cells. Zhu *et al*. (*23*) described the expression of dsRNA for five genes predicted to provide an essential biological function in the Colorado potato beetle (CPB), *Leptinotarsa decemlineata*. Exposure of CPB larvae to potato foliage treated with transformed bacteria successfully triggered the knockdown of the five tested genes, caused significant mortality and reduced body weight in the exposed insects. These results suggest that the efficient induction of RNAi using bacteria to deliver dsRNA is a possible method for management of CPB and perhaps other insects. The most significant advantage of this technique is the low cost of dsRNA synthesis relative to other *in vitro* synthesis methods. However, the effect of exposure to bacteria-expressed dsRNA was relatively slow and different target genes may be necessary to achieve a faster rate of mortality (*23*).

Perhaps the most likely RNAi technology to be commercially developed for insect pest management involves engineering crop plants to express dsRNA targeting essential genes in insect herbivores (*24*). In 2007, a paper was published demonstrating that harpin dsRNA constructs could be expressed in crop plants such as corn (*3*). In this study, corn was engineered to express vATPase-A dsRNA directed against the western corn rootworm (WCR) *Diabrotica virgifera virgifera* larvae. Transgenic plants infested with WCR larvae showed a significant reduction of corn root damage compared to controls (*3*). A second report described a system in which the cotton bollworm, *Helicoverpa armigera* were fed transgenic *Arabidopsis* or tobacco plants expressing dsRNA targeting a cytochrome P450 gene (CYP6AE14) involved in the detoxification of cotton secondary metabolite, gossypol (*25*). Insects fed with these plants exhibited reduced expression of the cytochrome P450 protein resulting in reduced larval tolerance to gossypol containing diet. Additional studies indicated that cotton plants engineered to express dsRNA for the specific cytochrome P450 exhibited enhanced resistance to bollworms (*26*). Plant-produced dsRNA has also been reported to induce RNAi silencing of two genes in the green peach aphid, *Myzus persicae*; one gene predominantly expressed in salivary glands and involved in aphid interaction with the host plant (*MpC002*), and a second gene primarily expressed in the aphid gut and involved in signal transduction cascades (*Receptor of Activated Kinase C*; *Rack-1*). Although the effects were not drastic, the knockdown of both genes resulted in reduced numbers of progeny from the exposed parental populations (*27*).

Additional target genes for RNAi in *D. v. virgifera* and *H. armigera* have recently been reported. Further studies with *H. armigera* demonstrated that when larvae were exposed to cotton plants expressing a cotton cysteine protease (GhCP1) and *H. armigera* cytochrome P450 dsRNA (CYP6AE14), the plants were better protected from bollworm than either of the single-transgene lines. Plant cysteine proteases increased the permeability of the peritrophic matrix of *H. armigera* resulting in increased uptake of dsRNA (*28*). Additional studies with *D. v. virgifera* reported the *Snf7* gene, involved in trafficking of membrane receptors. *Snf7* dsRNA was effective against *D. v. virgifera* and *D. v. howardi* larvae (*12*, *29*). Transgenic maize plants that express *Snf7* dsRNA to target *D. v. virgifera* have been developed and been shown to protect corn roots under high WCR pressure. A stacked corn event expressing two *Bt* toxins and *Snf7* dsRNA is currently in phase 3 of development, which includes regulatory data collection, trait integration and advanced field testing (*30*). This first RNAi event is planned to be released later this decade.

The use of *in planta* RNAi offers several advantages as a pest management tool. First, there are many potential target genes that have potential to produce lethality and protect plants from insect damage. For instance, 290 dsRNAs were tested against WCR and of those, 67 showed significant larval mortality and/or stunting at a low concentration (5.2 ng/cm^2) (*3*). In addition, *in planta* expression solves the problem of dsRNA stability, decreases synthesis costs, and has the potential to target sap-sucking pests such as aphids, leafhoppers and whiteflies, pests currently unaffected by *Bt* toxins (*4*, *5*, *8*). Finally, *in planta* RNAi could complement management of insect resistance to *Bt* crops (*4*, *5*, *8*).

Additional dsRNA delivery methods are being developed to make possible the use of dsRNA in products such as baits, sprays, or through irrigation systems (*24*). However, this technology will depend on the reduction of the cost associated with the production of large quantities of dsRNA and the development of strategies to increase stability of dsRNA in the environment (*5*, *24*).

RNAi Risk Assessment

The above examples offer clear evidence for the potential of RNAi to be used as an insect pest management tool. However, the environmental and ecological risks of deploying such a technology are still uncertain. The federal regulatory framework for estimating the ecological risks associated with RNAi technology is currently in development and a number of critical gaps remain. The potential toxicity to non-target organisms, environmental fate and the risk of resistance evolution in target pests are still largely undetermined (*24*).

It has been suggested that the current system used for risk assessment of *Bt* crops might provide a starting point to assess the risk of *in planta* RNAi (*31*, *32*). Existing approaches are based on a tiered assessment that begins from relatively simple and controllable lower tiers to increasingly complex higher tiers. Early tier studies performed under controlled laboratory conditions are used to test worst-case scenarios of exposure and serve to identify potential hazards (*33*).

Such experiments offer increased susceptibility to detect adverse effects because: 1) the impact of the stressor is isolated; 2) many replications are possible; 3) tests can be performed using validated protocols with arthropods reared under standardized conditions; and 4) organisms can be exposed to high concentrations that likely exceed field exposures (*32*, *34*). If no adverse effects are observed under worst-case exposures in early-tier studies, the risk can be characterized as being acceptable and further testing may not be necessary (*33*). If lower tiers detect a potential hazard, higher tier tests, which include more complex semi-field or open field tests, can serve to confirm the existence of adverse effects under more realistic conditions of exposure. The conclusion regarding risk drawn at each tier will lead to either a regulatory decision or to additional investigations (*33*, *35*). Early tier studies for *Bt* crops are preferred since higher tier studies on *Bt* crops have failed to find adverse impacts on non-target organisms that could not have been predicted from laboratory or small-scale studies (*32*, *33*, *35*).

Since it is not possible to test all species potentially present in the receiving environment and exposed to the arthropod-active protein, surrogate species representative of different ecological functions should be used (*35*). The subset of species and life-stages selected for early-tier testing should be chosen based on the potential exposure pathway, knowledge of the potential activity and mode of action of the arthropod-active molecule, the amenability of the test system and the availability of the test organism (*33*). In general, non-target species that are related taxonomically to the target pests are most likely to be affected. Selecting these taxa increases the likelihood of detecting a hazard if one exists. Species that are not exposed to the insecticidal material do not need to be tested (*35*). The current approach of species selection for genetically engineered crops provide an effective means of allocating limited regulatory resources with the effort concentrated on riskier products (*32*).

RNAi has the potential to offer high levels of specificity by carefully designing sequences of dsRNAs that are specific for the target pest (*4*, *24*, *36*) while minimizing sequence identity with non-target species. However, for identifying worst-case scenarios used in lower tier toxicity testing, it may be appropriate to select a sequence with the highest conservation between the target pest and the non-target arthropod (*24*). For *in planta* RNAi, the tested dose should be higher to that expressed in the plant and the exposure should be longer than what is expected in a field situation. This is mainly because the persistence and environmental fate of the dsRNA expressed by the plant is unknown (*24*).

Surrogate species for RNAi risk assessment should be selected based upon their phylogenetic relationship to the target organism, allowing focus on those species most likely to be susceptible due to sequence similarity (*32*). Since sequence identity is fundamental to the effectiveness of RNAi response, bioinformatic analyses may provide supplemental information to the results obtained from bioassays. However, the lack of sequence information in surrogate species may limit the usefulness of such analyses (*36*), and could place increased priority on developing genomic resources for ecologically relevant organisms (*24*). Considering that the species most likely to be affected are those phylogenetically close to the target species, it might be necessary to evaluate additional or different surrogate species from those tested for *Bt* crops. For

example, RNAi plants targeting coleopteran pests may pose increased risks to non-target Coleoptera compared with species belonging to other taxa, and therefore, it may be appropriate to include more representatives of this order (*32*). Additionally, several endpoints may need to be measured for RNAi risk assessment since limited information exists on effects other than mortality. Sublethal endpoints and effects on gene expression may provide additional useful information (*24*). Thus far, mortality has been the most common endpoint used, but few genes have been evaluated and not all relevant surrogate species have been tested. Considering that the effects of RNAi are not completely understood, further studies may be necessary to develop standardized methods involving specific endpoints (*37*). Consequently, supplementary endpoints and additional species representing related taxa might provide the most rigorous test of the risk hypothesis.

Although the risks of RNAi to non-target organisms has been predicted to be low based on requirements for sequence specificity, experimental evidence supporting this claim is only beginning to emerge (*36*). A recent study (*38*) tested specific and heterospecific vATPase dsRNA in the fruit fly *Drosophila melanogaster,* flour beetles *Tribolium castaneum,* the pea aphid *Acyrthosiphon pisum*, and tobacco hornworm M. sexta. The four species were fed each of the dsRNA at the estimated LC_{75} for the targeted species. Significant mortality was only observed when the insects were fed conspecific dsRNA, and no adverse effects were observed from heterospecific dsRNA. In a similar set of tests, four species of *Drosophila* were fed species specific vATPase dsRNA that resulted in reduced vATPase mRNA and significant mortality. However, no effects were observed when flies were treated with heterospecific dsRNA (*38*). In a different study (*3*) vATPase-A dsRNA designed to target WCR was tested against a number of other coleopterans including the Southern corn rootworm (SCR) *D. undecipunctata howardi*, Colorado potato beetle (CPB) *L. decemlineata* and cotton boll weevil *Anthonomus grandis*. WCR dsRNA caused significant mortality in both SCR and CPB, although higher concentrations of dsRNA were necessary relative to WCR. In contrast, the cotton boll weevil was insensitive to WCR dsRNA suggesting that mortality declines with decreasing sequence identity between WCR genes and their orthologs in other species (*3*). Results of the above studies demonstrate that gene silencing is potentially very selective. Likewise, the spectrum of insecticidal activity of a dsRNA targeting the *Snf7* ortholog in WCR was tested in non-target insects that were selected based on their phylogenetic relatedness to WCR (*36*). Insects representing 10 families and 4 orders were evaluated in chronic and subchronic bioassays that measured potential lethal and sublethal effects. Results demonstrated that the spectrum of activity for WCR *Snf7* is narrow and activity is only evident in a subset of beetles within a subfamily of Chrysomelidae (*36*).

Additional concerns regarding environmental risk of RNAi have been suggested but have not yet been addressed. Off-target effects due to silencing of genes other than the intended target may require further evaluation (*39*, *40*). If the original dsRNA results in sequence identity with siRNAs from other genes, unpredicted targets may be affected and partial or complete silencing of unintended genes could affect non-target organisms (*31*, *39*). Currently, little

is known about possible off-target effects of RNAi and predictions that rely on bioinformatics have yet to be fully evaluated (*39*, *40*). Additional studies are necessary to determine the relative amount of mismatch allowed between the target and non-target organisms (*23*) and the potential of off-target gene silencing (*39*). This information may enhance overall risk assessments.

An additional component of environmental risk assessment involves the stability and persistence of dsRNA in the environment (*40*). Understanding the stability, persistence and half-life of small RNAs in different ecosystems will be essential for the characterization of exposure pathways and further define the overall risk to non-target organisms (*37*). Preliminary studies indicate that *Snf7* dsRNA expressed in corn to control WCR degrades and loses its biological activity within 2 days after application to different types of soil (*41*). For exposure assessment of *in planta* RNAi, it will also be important to define the plant mediated processing mechanism of dsRNA and the form of dsRNA that is actually released into the environment (*37*). In addition, the potential for resistance evolution has not yet been addressed. Studies assessing the evolution of resistance to RNAi will be fundamental for the development of an insect resistance management plan aimed to promote sustainable use of the technology (*23*).

Conclusions

RNAi represents a potentially important and powerful pest management alternative. This approach offers a wide range of potential genes that can be targeted to manage pest populations (*4*). Current studies suggest that dsRNA can be designed to selectively manage insect pests without affecting non-target insects, even when using dsRNAs that target genes that are highly conserved across diverse taxa (*36*, *38*). However, research indicates that not all dsRNAs effectively knockdown their target and not all dsRNAs effectively kill insects (*3*, *38*). One of the greatest challenges for future use of RNAi as a pesticide will be in the identification of target genes (*38*).

The key factors for the successful use of RNAi as a pest management tool are the choice of target sequences essential for the insect and the mode of delivery (*23*). It will also be necessary to find ways to stabilize dsRNA during and after delivery, and to develop low-cost methods for large-scale production of dsRNA (*42*). RNAi efficacy is variable between genes, organisms, life stages and tissues (*18*, *19*). In addition, differences in the RNAi machinery, cellular uptake, propagation of signal and dsRNA degrading enzymes will greatly affect the success of RNAi among different species (*23*). Further understanding of insects' mechanism(s) of uptake and systemic spreading of RNAi might facilitate the use of RNAi as a pest management tool and perhaps advance our understanding of how resistance could evolve (*4*).

Regarding risk assessment there are still many unknowns including: the nature of exposure, the environmental fate and persistence, and the specific endpoints (*23*). Since susceptibility to dsRNA in insects depends on the identity

and nature of the target gene, environmental risk assessment will most likely require information on a case-by-case basis and not as a general RNAi response (*18*, *19*).

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Chapter 6

Voltage-Sensitive Potassium Kv2 Channels as New Targets for Insecticides

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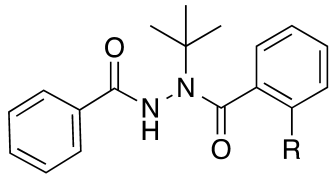
The diacylhydrazines are a class of insecticides usually thought to act by disrupting the insect endocrine system. However, at field use rates, insects affected by these compounds also show signs of neurotoxicity. Previous research found that a blocking action on Kv2 potassium channels of nerve and muscle was the cause of the observed neurotoxicity. This review will summarize the physiological role of Kv2 channels and their possible exploitation as a new target for insecticide development. Included is a description of a potassium channel homology model to visualize protein structures of mosquito and human, and to facilitate design of molecules that are safe and effective. Also included is a proposed ligand docking example with a substituted catechol as a model potassium channel blocker. Preliminary toxicity studies demonstrate that molecules of this type show greater acute toxicity to mosquitoes than the diacylhydrazines. The overall aim of this project is a new commercial insecticide for use in the fight against global malaria.

In Biopesticides: State of the Art and Future Opportunities; Coats, et al.;

Introduction

Our interest in new insecticides is focused on the pressing need for malaria vector control with compounds having contact activity against adult females, because of the growing problem of pyrethroid resistance in *Anopheles gambiae* (*1*). Accordingly, this review will discuss Kv2 potassium channels in the context of developing new insecticides for controlling this disease vector. Evidence for potassium channels as a target for insecticide action was originally documented in studies that observed neurotoxic signs following exposure to compounds thought to possess a primary mode of action as ecdysone agonists (*2*). The experimental compounds were designated RH-5849 and RH-1266, and are shown in Figure 1.



RH-5849, R = H
RH-1266, R = Br

Figure 1. Structures of neurotoxic, potassium channel-directed diacylhydrazines.

However, in addition to endocrine effects, neurotoxic symptoms (tremors and paralysis) were observed in the Colorado potato beetle and Mexican bean beetle at field use rates (*3*). Neurotoxic signs were also reported in American cockroaches exposed to RH-5849 by 50 μg/g injections, and these effects were traced to hyper-activation of central and motor nerve pathways in electrophysiological studies (*4*).

More detailed investigation in housefly larval muscle (Fig. 2) found that the most sensitive membrane current affected by RH-5849 (IC_{50} = 59 μM) and RH-1266 (IC_{50} = 40 μM) was the delayed rectifier or I_K potassium current (Fig. 2, top row), which is responsible for repolarization of the membrane potential in nerve and muscle cells in order to maintain proper function (*5*). The compounds also affected the rapidly inactivating I_A current, but to a lesser extent, and there was no blockage of these channels at a concentration of ≤ 100 μM (Fig. 2, bottom row).

A single study describes diacylhydrazine poisoning in mosquitoes, and it was focused on ecdysone agonist effects (*6*). In this paper, larval development (premature molt, the main effect of ecdysone agonists) was studied after addition of compounds to the water. Insects were evaluated for toxicity after 10 days of exposure. *Anopheles gambiae* was the most sensitive species tested, followed by *Culex quinquefasciatus,* and *Aedes aegypti* (*6*). It was also observed that the first effect of these compounds (including RH-5849) was behavioral, the larvae descending to the bottom of the cup within 2 days. Any role for neurotoxicity or potassium channel blockage was not mentioned.

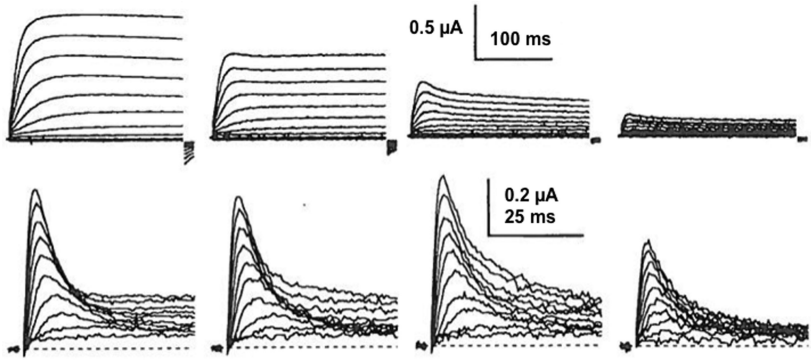


Figure 2. Effects of RH-5849 on delayed rectifier I_K (top row) and inactivating I_A (bottom row) in housefly larval muscle at 0, 31.6, 100 and 316 µM (left to right). Reproduced with permission from reference (5). Copyright 1992 Wiley.

There are a large number of different subtypes of potassium channels, and it is important to assess which subtype might form the major target site for diacylhydrazines. The available genomic sequences of both *An. gambiae* and *Drosophila melanogaster* yields clues to which channel might be involved. The potassium channel α-subunit genes of *Drosophila* and *An. gambiae* are well conserved, ranging from 42-98% with a mean of 85% amino acid sequence identity (*7*). This high degree of sequence conservation suggests that their physiological function is conserved, as well. It has been shown that the *Drosophila shab* gene (later classified as Kv2) is the primary delayed rectifier (I_K channel) of *Drosophila* nerve and muscle, and is present in all life stages (*8*). Although more Kv-type potassium channel genes were found in *An. gambiae* (eight) vs. *Drosophila* (six), only a single gene for Kv2 apparently exists in each organism, and the sequence homology is 94% (*7*).

In mammals, Kv2.1 is the primary homolog of *shab*, and is widely distributed within central and peripheral neurons, cardiac tissues, and skeletal muscle (*9*). The Kv potassium channel genes produce multiple copies of an α-subunit (Fig. 3) that associate post-translationally into a tetrameric complex to form the functional ion channel (*10*). In addition, there are smaller auxiliary subunits, Kvβ, that modulate the gating and trafficking of the channel, and impart inactivation properties to Kv1 channels (*10*). Not much is known regarding auxiliary subunits in any insect Kv channel, although one report (*11*) studied interactions of the Kv1 (I_A) channel with the β-subunit gene of the *hyperkinetic* mutant of *D. melanogaster*. Given the lack of any definitive information on Kv2 subunits interacting with β-subunits, and the non-inactivating nature of native Kv2-mediated currents in insect nerve and muscle (*5*, *6*), β-subunit influence on Kv2 channel properties is difficult to account for at present.

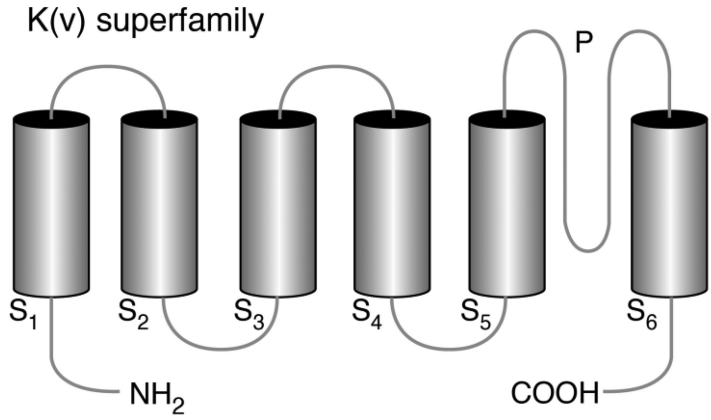


Figure 3. Membrane topology of Kv superfamily genes. Transmembrane α-helices are shown as cylinders numbered 1-6, and the conserved pore domain is "P.". Reproduced from reference (7) (2003 Genome Biology).

An important consideration in developing a potassium channel-directed insecticide is its possible interaction with the so-called hERG potassium channel (human *ether-a-go-go* related gene). This gene encodes for a fast-activating delayed rectifier potassium channel (I_{Kf}) (*12*). The channel resides in human cardiac tissue, and when blocked, mediates serious side effects (QT interval prolongation) of non-antiarrhythmic drugs (Fig. 4), sometimes resulting in sudden cardiac death (*12*). Interaction of hERG with compounds such as Sertindole requires a positively charged nitrogen in an appropriate molecular locus (Fig. 4), which the diacylhydrazines lack (Fig. 1), and which should be avoided in any insecticidal molecule. Inspection of the diacylhydrazines (Fig. 1) reveals a rather simple chemical scaffold, and with little structural similarity to known hERG channel blockers. Therefore, it would appear these compounds should have little blocking activity at hERG channels. Moreover, the potency of RH-1266 and -5849 might be improved because the essentially un-substituted nature of the phenyl rings suggests numerous possible analogs. The fact that RH-5849 has some contact activity (*4*) is another potential advantage of the existing diacylhydrazine chemistry. RH-5849 also has an oral LD_{50} in rats of 435 mg/kg, a relatively high value (*4*), suggesting that selective toxicity is achievable. Thus, the diacylhydrazines serve as potentially excellent leads for a heretofore unexploited target site of critical importance in excitable membranes; *viz*., voltage-dependent potassium channels, especially Kv2 (I_k). Action on this site will circumvent the target site (*kdr*) resistance to pyrethroids present in field populations of *Anopheles gambiae* (*1*).

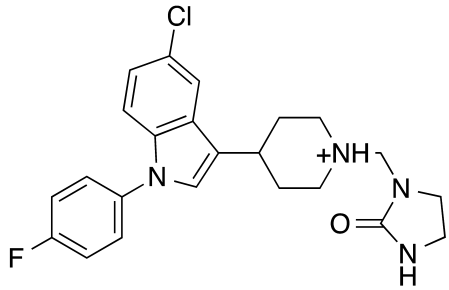


Figure 4. Example of a cationic drug (Sertindole, an antipsychotic) taken off the market due to unwanted side effects arising from hERG channel block (12).

Candidate Ligands for Kv2

Besides the diacylhydrazines, a survey of the literature revealed four additional compound classes with promising potassium channel blocking activity (Fig. 5). The catechol 48F10 is predicted to bind near the external mouth of Kv2, but is ineffective on Kv1 channels (*13*). Rutaecarpine is also a blocker of delayed rectifier potassium channels in NG-108-15 neuronal cells (*14*). Substituted biphenylpyridazines (*15*) block I_A and I_K with IC_{50}s in the low micromolar range. Flonicamid is a compound active on plant-sucking pests, and there is a single citation ascribing its mode of action to blockage of I_A potassium channels (*16*).

Among the lead compounds presented in Figures 1 and 5, three structural types stand out in terms of good drug-like properties and ability to construct diverse libraries: these are the RH compounds, 48F10, and rutaecarpine. All of these have CLogP less than 5, and tPSA less than 60 square angstroms. Those parameters should confer good ability to cross permeability barriers of the mosquito. Passage of the cuticle requires significant hydrophobicity; for reference, the carbamate insecticide propoxur has CLogP = 1.648 and tPSA = 47.56 square angstroms. We view the pyridazine compound as less favorable than the others due to carcinogenicity concerns over the terminal biphenylamine moiety. Flonicamid in turn does not appear hydrophobic enough to cross the cuticle, and the available chemical space around this commercial compound may be limited.

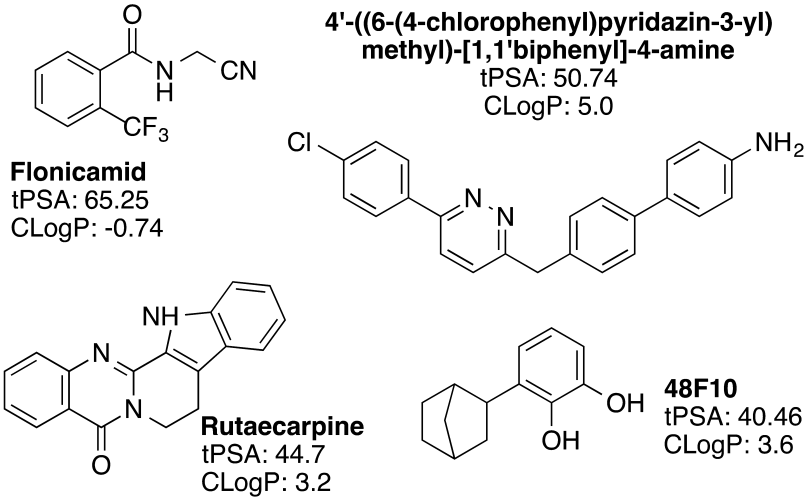


Figure 5. Other known or putative ligands for insect or mammalian Kv2 potassium channels gleaned from the literature. The abbreviations are defined as tPSA (topological polar surface area) and ClogP (calculated log P values; octanol/water partition coefficients).

Molecular Modeling of Kv2

Genomic analysis and molecular modeling evaluated Kv2 gene sequences and x-ray crystallographic information, in addition to the *in silico* computation of predicted 3-dimensional structures and ligand docking that was also performed. Sequence alignments were made of the relevant *Anopheles gambiae* and human potassium channel proteins (discussed below). Recently, the X-ray structure of a Kv1.3-Kv2.1 chimeric channel has been reported (PDB 2R9R (*17*);). The transmembrane pore domain in this structure is immersed in a lipid membrane-like environment and is in the physiologically relevant open channel conformation. This experimental structure provides a closely homologous template for comparative modeling of the target, the mosquito delayed rectifier potassium channel, *Ag*Kv2/Shab (Fig. 6). Amino acid identity across the entire transmembrane domain is 50%, and goes up to 59% if only the core channel domain is considered. Furthermore, the alignment for this domain is continuous. Lack of any insertions or deletions greatly facilitates the construction of the model (Fig. 6) and improves our confidence in its accuracy.

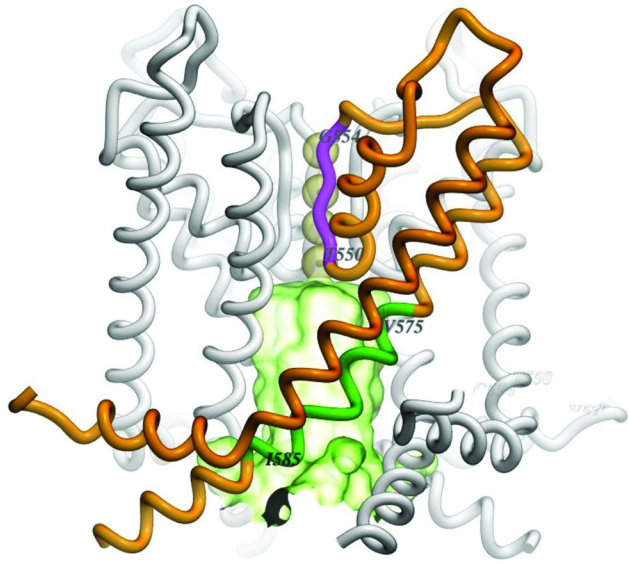


Figure 6. Model of the AgKv2 channel core. Three of the four symmetric subunits of the homo-tetrameric channel are shown in white, while regions of the fourth subunit are colored: part of helix S6 (aka, the 'inner' helix) lining the central hydrophobic cavity is in green, and the highly conserved selectivity filter in magenta. Four K^+ ions inside the selectivity filter are shown as yellow spheres. Extracellular space is at the top and cytoplasm below, while most of the channel is immersed in the membrane. Residue numbering is according to the full mosquito AgKv2 gene sequence.

The initial homology model for mosquito was built based on the X-ray structure of the Kv2.1, PDB ID 2r9r. The model of mosquito Shab was refined by global energy optimization using BPMC sampling in ICM (*18*). Molsoft's ICM software was previously applied in modeling of various drug target proteins, such as the nicotinic acetylcholine receptor (*19*). The established view is that the delayed rectifier potassium channel is a homo-tetramer (*10*). An internal variable linking method in ICM (*19*) was used to enforce identical conformations of the four subunits and thus maintain 4-fold symmetry throughout energy refinement. A fifth potassium atom at the exterior entrance of the channel was added, as seen in certain Kv structures, using PDB 2a79 as a template to place the cation. When catechol ligand 48F10 was docked into the model at the extracellular and intracellular sites, the most favorable interaction pose was observed at the extracellular opening of the channel (Fig. 7 a,b).

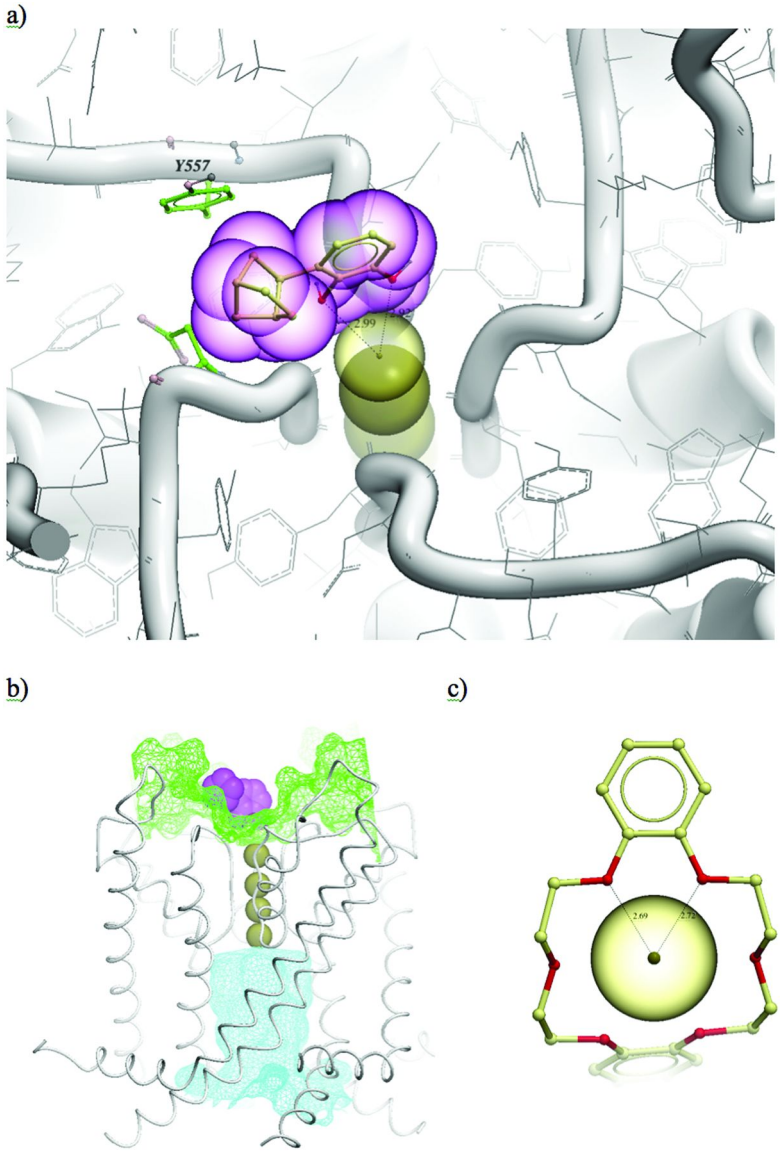


Figure 7. a) Binding pose of 48F10 (magenta); coordination of potassium ion (yellow) and hydrophobic contacts to Y557 and parts of backbone (green) can be observed. b) Location of the putative binding site of 48F10 on the overall channel structure; blue mesh is the inward facing 'vestibule', green is the outward facing surface. c) An example of experimentally observed potassium-catechol coordination in a complex with a catechol crown ether.

Two hydroxyl oxygen atoms coordinated a potassium atom at the channel entrance in a bifurcated manner, consistent with interactions seen in X-ray structures of complexes of certain catechol derivatives with potassium (Fig. 7c). The bulky lipophilic moiety extended towards a hydrophobic groove formed by the Y557 side-chain and parts of the backbone in the adjacent subunit, thus forming additional favorable interactions. This later interaction is consistent with an observation that among human channels, 48F10 is selective towards Kv2.1, which also has a tyrosine in corresponding position (Y384), over Kv1.5, which has R487 (*13*). We have also developed a preliminary model of human Kv2 for comparison with mosquito. It is quite conserved as compared to mosquito Shab; the closest substitution to the putative binding site for catechols is T559(Shab)/K386(hKv2.1), which is fairly distant. Note that this external pore model for catechols is not proposed as a hypothesis of diacylhydrazine binding.

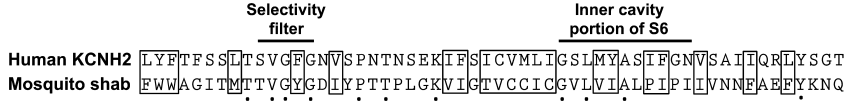


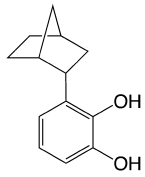
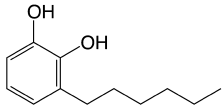
Figure 8. Gene sequence alignment of helix S6 for human KCNH2 (hERG) and mosquito Shab. Dots indicate indicate amino acid identity and boxes are conservative substitutions, respectively, in the two genes.

The most important undesirable off-target effect of K^+ channel blockers is likely to be hERG inhibition. We compared sequences of hERG and *Ag*Kv2 in the regions of the selectivity filter and inner cavity portion of S6 (Fig. 8). Homology of the helix S6 portion that lines the hydrophobic cavity is weak between these two channels. Residues adjacent to the extracellular opening are also poorly conserved, e.g., Y557(Shab)/Y384(hKv2) is a serine in hERG. This observation suggests that selective inhibition can be achieved at either site. Successful modeling of hERG and hERG interactions with a number of blocker molecules has been reported (*12*), and can be used to guide these efforts.

Preliminary Toxicity Data for Kv2 Channel Blockers

Preliminary toxicity data for diacylhydrazines and 48F10 analogs to *Anopheles gambiae* mosquitoes are shown in Table 1. Experiments were preformed on susceptible G3 and the multi-resistant Akron strain, which carries *kdr* and MACE (altered acetylcholinesterase) (*19*). The data show that diacylhydrazines are of modest topical toxicity to mosquitoes, with LD_{50}s of about 0.5 µg/female, and treated insects also displayed rapid neuroexcitatory signs of intoxication (twitching and postural collapse).

Table 1. Toxicity of K^+ Channel-Directed Compounds against Adult Females of *An. gambiae*

Chemical Class Compound #	[a]*Strain & treatment*	[b]*LD_{50} (95% CL)* [c]*RR*	[d]*LD_{50}+PBO (95% CL)* [c]*RR*	[e]*SR*
Diacylhydrazines				
RH-5849	G3 topical	735 (583-945)		
RH-1266	G3 topical	504 (393-648)		
Substituted catechols				
PRC739 	G3 topical	148 (90-262)	114 (77-142)	1.3
	Akron topical	156 (113-196) RR=1.05	123 (91-149) RR=1.08	1.27
	G3 injection	27 (20-33)		
PRC725 	G3 topical	123 (66-164)	91 (83-100)	1.35
	Akron topical	60% dead at 150 ng		

[a]Strain of *An. gambiae* is either susceptible G3 or resistant Akron strain (*20*), as indicated, and tmt = treatment mode defined as topical, WHO paper assay (*21*), or injection.

[b]LD_{50} in ng/insect (95% C.L.).

[c]RR = resistance ratio (LD_{50} in Akron/LD_{50} G3), with or without synergist, as indicated.

[d]LD_{50} (95% C.L.) when PBO treated at 200 ng/insect 4 hrs before insecticide.

[e]SR = synergist ratio (LD_{50} unsynergized/LD_{50} in the presence of PBO).

Two substituted catechols were studied in a more comprehensive series of experiments and we observed the following. The analogs tested were 4-5 fold more toxic than the diacylhydrazines. Moreover, the toxicity was not enhanced by 4 hour pretreatment with 200 ng of the monooxygenase synergist piperonyl butoxide (*22*), and there was no cross resistance in the Akron strain, as expected (Table 1). Injection increased toxicity 5-6 fold, indicating that the cuticle was not a significant barrier to penetration. For comparison, toxicity of propoxur, a WHOPES approved carbamate for mosquito control, had a topical LD_{50} of ca. 3 ng/female (*23*), which makes it about 50-fold more active than the substituted catechols reported here. Nonetheless, these compounds have significant toxicity to mosquitoes, and accordingly voltage sensitive potassium channels, in particular Kv2, appear to be new potential targets for mosquitocide development.

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Chapter 7

Mimetic Analogs of Pyrokinin Neuropeptides for Pest Insect Management

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Neuropeptides are potent regulators of critical life processes in insects, but are subjected to rapid degradation by peptidases in the hemolymph (blood), tissues and gut. This limitation can be overcome via replacement of peptidase susceptible portions of the insect neuropeptides to create analogs with enhanced biostability. The pyrokinins stimulate gut motility and regulate other functions in certain insects, but unmodified members demonstrate little or no effect when fed to pea aphids (*Acyrthosiphon pisum*). However, biostable analogs demonstrate potent oral antifeedant and aphicidal effects. The most active of the biostable analogs shows an LC_{50} value of 0.042 nmole/µl (LT_{50} = 1.0 days), approaching the potency of some commercial aphicides. The aphicidal activity of a biostable pyrokinin can be blocked with an antagonist, indicating the mechanism occurs via a neuropeptide receptor and represents a novel and selective mode of action. Biostable agonists of diapause hormone (a pyrokinin) can prevent the onset of the protective state of diapause in the corn earworm, inducing the crop pest to commit a form of 'ecological suicide'. Alternatively, an antagonist can block the activity of the native hormone. Biostable neuropeptide analogs represent important leads in the development of alternate, environmentally sound pest insect control agents.

In Biopesticides: State of the Art and Future Opportunities; Coats, et al.;

Introduction

Neuropeptides regulate numerous processes that are critical for insect survival and propagation. Due to their specificity and their high activity at extremely low doses, regulatory neuropeptides have been studied as potential leads for the development of new environmentally friendly pest control agents. However, the natural peptides are generally not useful as control agents in their native state, as they are susceptible to degradation by endogenous peptidases present in the insect digestive system, hemolymph (blood), and tissues (*1–3*). To overcome the limitations inherent in the physicochemical characteristics of peptides, the development of mimetic analogs has been used as a strategy to enhance their biological effects. It has been proposed that blocking or overstimulating the receptors of insect neuropeptides could lead to reduction of pest fitness or even increased mortality (*3*, *4*). Generally these biostable mimetics are derived by the structural modification of the lead peptide sequence to overcome a number of metabolic limitations, such as proteolytic degradation that restrict the use of peptides as agents capable of modulating aspects of insect physiology (*3*, *5*). One mimetic approach that was employed in this research is the incorporation of unnatural moieties that impart steric-hindrance such as modified Pro analogs adjacent to a peptidase hydrolysis-susceptible amide linkage within the neuropeptides (*6*, *7*). Another approach is through the appendage of polyethylene glycol (PEG) polymer or other hydrophobic moieties to the N-terminus of core sequence regions of the peptides. Both approachs are utilized in the analogs featured in this chapter.

In this chapter, we review recent research undertaken to evaluate the oral antifeedant and aphicidal activity of unmodified neuropeptides and/or core fragments along with mimetic analogs featuring enhanced biostability from the pyrokinin insect neuropeptide class. Unmodified neuropeptides and core fragments elicit little or no activity, whereas biostable analogs exhibit significant, and in some cases, potent aphicidal properties. About 250 out of a total of about 4,000 aphid species that have been identified represent serious pests to various crops around the world, causing both direct damage to plants via the process of ingestion and indirect damage by transmitting viruses that can devastate agricultural crops (*8*) Of particular significance is the pea aphid *Acyrthosiphon. pisum*, which causes hundreds of millions of dollars of crop damage every year. Many populations of this organism have already acquired resistance towards a number of traditional and modern insecticides, making a search for alternative strategies imperative (*9*). In addition, aphids are not sensitive to the toxins produced by the bacterium *Bacillus thuringiensis* (*Bt*) (*10*). However, the genome of *A. pisum* has recently been sequenced by the International Aphid Genomic Consortium providing a resource for the discovery of new targets for control (AphidBase; http://www.aphidbase.com (*11*);). The sequences of members of several neuropeptide classes native to the pea aphid have been recently reported, including two pyrokinins (SPPYSPP**F**S**PRL**-NH_2 and GGTTQSSNGIW**FG****PRL**-NH_2)., along with related PRLamide peptides QAVMAQPQV**PRL**-NH_2 and pQAVMAQPQV**PRL**-NH_2 (*12*).

In addition, we finish the chapter with a review of recent research undertaken to develop both agonist and antagonist analogs of the diapause hormone, a member of the pyrokinin super-family of neuropeptides, with the potential to disrupt this protective state of dormancy in the corn earworm (*Helicoverpa zea*), providing the basis for a novel strategy to manage populations of this important crop pest.

Pyrokinins

The pyrokinin/pheromone biosynthesis activating neuropeptides (PK/PBAN) represent a multifunctional family that plays a significant role in the physiology of insects. Leucopyrokinin (LPK), isolated from the cockroach *Leucophaea maderae* in 1986 (*13*), was the first member of the family to be discovered. Since that time, over 30 peptides have been identified. They include PKs, myotropins (MTs), PBAN, melanization and reddish coloration hormone (MRCH), diapause hormone (DH)(see next chapter sub-section), pheromonotropin (PT), all of which share the common C-terminal pentapeptide FXPRL-amide (X=S, T, G or V) (*14–17*). Functions of the PK/PBAN family include stimulation of sex pheromone biosynthesis in moths (*14*, *16–18*), and mediation of key aspects of feeding (gut muscle contractions) (*19*, *20*), development (embryonic diapause, pupal diapause and pupariation) (*21–25*) and defense (melanin biosynthesis) (*26*, *27*) in a variety of insects (cockroaches, flies, locusts and moths). All of the above functions can be stimulated by more than one peptide, and they demonstrate considerable cross-activity between various PK/PBAN assays, thereby lacking any species-specific behavior (*16*, *17*, *28–30*).

Previous work has established that a *trans* oriented Pro as an integral part of a type I β-turn structure holds broad significance for many physiological functions elicited by the PK/PBAN family of peptides, including hindgut contractile (cockroach *Leucophaea maderae*) (*19*, *31*), pheromonotropic (silk worm *Bombyx mori* and corn earworm *Helicoverpa zea*) (*4*, *32*), oviduct contractile (cockroach *Leucophaea maderae*) (*33*), egg diapause induction (silk worm *Bombyx mori*) (*4*, *32*, *34*), pupal diapause termination (corn earworm budworm *Helicoverpa zea*) (*35*), and pupariation (flesh fly *Neobellieria bullata*) (*26*) assay systems.

Due to the susceptibility of PK/PBANs to both exo- and endopeptidases in the insect hemolymph and gut, these peptides cannot be directly used as pest control agents. Members of the PK/PBAN family are hydrolyzed, and therefore inactivated, by tissue-bound peptidases of insects. The primary site for peptidase activity within the C-terminal pentapeptide (Phe^1-Xxx^2-Pro^3-Arg^4-Leu^5-NH_2) is between Pro^3 and the Arg^4 residue (*36*). To overcome the limitations inherent in the physicochemical characteristics of peptides, the development of peptidomimetic analogs has been used as a strategy to enhance their biological effects.

One peptidomimetic approach employed with the PK/PBAN class of neuropeptides is the replacement of the critical Pro residue with such sterically-hindered Pro analogs as octahydroindole-2-carboxyl (Oic) and hydroxyprolyl (Hyp) (*36*) moieties (Figure 1). This former approach has been

used to develop biostable Oic and Hyp analogs of the PK/PBAN neuropeptide family that have demonstrated markedly enhanced resistance to hydrolysis by tissue-bound peptidases.

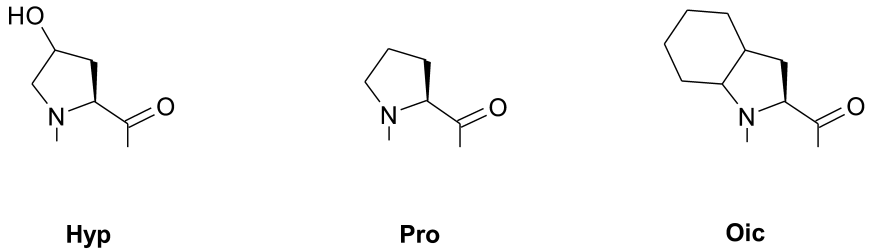

Figure 1. Hydroxyproline (Hyp; left) and octahydroindole-2-carboxylic acid (Oic; right), analogs of proline (Pro; middle) that feature greater steric bulk.

These analogs also demonstrated activity in an *in vivo* adult female *Heliothis virescens* moth pheromonotropic assay when delivered orally under conditions in which the native PBAN peptide and/or fragments do not (*36*). A Hyp-containing PK/PBAN analog proved to be more than 5-fold more potent than the native hormone DH, a member of the PK/PBAN family in terminating diapause in pupae of *Helicoverpa zea* (*35*)(see the next sub-section on DH and disruption of diapause), presumably because of a longer hemolymph residence time of the Hyp analog over the native hormone.

The two native pyrokinins (SPPYSPPFSPRL-NH_2 and GGTTQSSNGIWFGPRL-NH_2) failed to demonstrate antifeedant or aphicidal activity when fed to the pea aphid *A. pisum*. Similarly, a related native PRLamide peptide (QAVMAQPQVPRL-NH_2) proved inactive in the aphid feeding assay. Down et al. (*37*) have reported that peptides of the myosuppressin family are degraded by aphid gut enzymes. As the two PK and two PRLamide peptides native to aphids are likely inactivated by peptidases in the gut, tissues and hemolymph of the aphid (*37*), analogs containing modifications that could enhance resistance to peptidases that inactivate the native peptides were evaluated in the feeding assay. The biostable PK analogs PK-Oic-2 (Hex-FT[Oic]RL-NH_2) and PK-Hyp-1 (Hex-FT[Hyp]RL-NH_2) were shown to survive intact over at least a 2 hour period when exposed to peptidases bound to corn earworm (*H. zea*) Malpighian tubule tissue under conditions in which an unmodified, natural pyrokinin peptide was completely hydrolyzed within 30 minutes or less. Furthermore, these two analogs were shown to demonstrate *oral* pheromonotropic activity when fed to adult female *H. virescens* moths (*36*). The two biostable analogs containing either Oic and/or Hyp in the C-terminal pentapeptide core of the pyrokinins was evaluated in the aphid feeding assay. The analog PK-Hyp-1 incorporating Hyp proved to be essentially inactive, perhaps an indication that Hyp in this position is incompatible with successful interaction with the aphid PK receptor. Nonetheless, the original Oic-containing analog PK-Oic-2 and a

second analog PK-Oic-1 (FT[Oic]RL-NH_2) demonstrated potent antifeedant and aphicidal effects on the pea aphid, with LC_{50} values of 0.121 nmol/µl and 0.042 nmol/µl, respectively.

Interestingly, a PK antagonist analog containing a polyethylene glycol (PEG) polymer conjugated to the N-terminus to improve oral bioavailability (*38*, *39*), PK-dF-PEG_4 (0.5 nmol/µl), was added to the highly active agonist analog PK-Oic-1(0.1 nnmol/µl) to see if it could block its potent oral aphicidal effects. Indeed, the LC_{50} observed for the combined analog treatment was reduced by a statistically significant 55% in comparison with the LC_{50} observed for the agonist analog alone. Importantly, the results suggest that the biostable agonist PK analogs act at a PK receptor.

Two reference aphidicides that are currently used in the marketplace for selective IPM control against aphids in agriculture are pymetrozine and flonicamid. Both compounds act specifically against aphids as feeding inhibitors, although their exact mechanism(s) remain unidentified. Flonicamid [N-(cyanomethyl)-4-(trifluoromethyl)-3-pyridinecarboxamide] is a novel insecticide; its LC_{50} as determined in an experimental setup similar to that used for the pyrokinin analogs was 0.144 nmol/µl with a typical loss of honey dew formation followed by death, and its LT_{50} was 1.1 days to kill 50% of aphids feeding on diet containing 0.44 nmol/µl (*6*, *40*). For pymetrozine [1,2,4-triazin-3(2H)-one,4,5-dihydro-6-methyl-4-[(3-pyridinylmethylene)amino], Sadeghi et al. (*6*) calculated with use of a similar feeding apparatus with a diet sachet an LC_{50} of 0.01 µg/ml (*40*).

Thus, the fact that the stabilized PK analogs of this study show rapid and high activities against *A. pisum* aphids in the same order of magnitude as some commercial aphicides tested under comparable conditions in the laboratory, suggests that they represent potentially valuable leads for alternative agents in the control of aphids and in the struggle against insecticide resistance.

The aphicidal activity of the PK analogs is associated with the presence of components that enhance the resistance of the C-terminal core region to peptidases, as the unmodified PKs demonstrate no activity. The mechanism of the aphid antifeedant activity and high induction of mortality demonstrated by the biostable PK analogs cannot be clearly identified at this point, but it may be associated with disruption of the physiological processes that this important neuropeptide family regulates in insects, particularly hindgut and midgut contractile activity that may impede normal digestive processes. For this to happen, the biostable analogs with aphicidal activity would necessarily need to interact with a native aphid PK receptor(s). Evaluations of these analogs, along with related topically-active amphiphilic PK analogs (*41*) previously been shown to modify pheromonotropic activity in heliothine species, on aphids are planned in subsequent studies. The active biostable PK analogs described in this study and/or 2nd generation analogs, either in isolation or in combination with biostable analogs of other neuropeptide classes that also regulate aspects of diuretic, antidiuretic, digestive, reproductive and/or developmental processes, represent potential leads in the development of selective, environmentally friendly pest aphid control agents capable of disrupting those critical processes.

Other Insect Peptide Classes

Antifeedant effects and increased mortality have been observed as well in our laboratory for members of two other insect neuropeptide families that have been fed to the pea aphid in artificial diets. A biostable analog, Leuma-TRP-Aib-1 (*7*), of the insectatachykinin peptide family demonstrates a potent aphicidal LC_{50} of 0.0085 nmol/µl and an LT_{50} of 1.1 days (at 0.5 nmole/µl), exceeding the potency of the commercial aphicide flocnicamide. A biostable analog, K-Aib-1, of the insect kinin family demonstrated an LC_{50} of 0.063 nmol/µl (0.046 µg/µl) and an LT_{50} of about 1.68 days. In work from other laboratories, a biostable analog of the C-type allatostatin Manse-AS (Manse denotes that the peptide is native to the lepidopteran *Manduca sexta*) containing D-amino acids that was introduced in an artificial diet led to aphid mortality at an LC_{50} of 0.18 µg/µl, at least 4 times more potent than the parent unmodified allatostatin peptide (*42*), but 20-fold less potent than the biostable insectatachykinin analog Leuma-TRP-Aib-1 (*7*). A diet containing Acypi-MS or LMS (at 0.5 µg/µl), unmodified members of the myoinhibitory myosuppressins, fed to aphids also led to increased mortality over a longer 10 day period (*37*).

Pyrokinins – Diapause Hormone

Coordinating active phases of the life cycle with seasons that provide food resources and suitable environmental conditions is crucial for sustaining viable insect populations. Major portions of the year, most notably winters in temperate zones, are unsuitable for continuous development, and most insects have evolved periods of dormancy (diapause), characterized by suppressed metabolism and bolstered stress responses that enhance survival during unfavorable seasons. Short day lengths of late summer commonly trigger the onset of diapause (*43*, *44*), and these environmental signals prompt endocrine responses that directly initiate and eventually terminate the diapause state (*45*).

Desynchronizing an insect pest with its appropriate seasonal diapause could be used as a tool for disrupting pest populations (*46*). Altering the timing of diapause has the potential to evoke ecological suicide if the insect is forced to be active during a time of year when climatic conditions are adverse or food resources are absent. Blocking entry into diapause in the autumn, breaking out of an overwintering diapause prematurely, or failing to terminate diapause at the appropriate time in the spring all have potential for desynchronizing the temporal distribution of insects. We report here the development of novel agents capable of disrupting the overwintering pupal diapause of the corn earworm, *Helicoverpa zea,* a member of the *Heliothis/Helicoverpa* complex, a worldwide group of noteworthy crop pests (*47*).

One such target is diapause hormone (DH), a 24-AA (NDDKDGAASGAHSDRLGLWFGPRLamide) neuropeptide characterized from *H. virescens* and a member of the pyrokinin superfamily of peptides. The analogous DH native to *Bombyx mori* was originally known for its action in initiating embryonic diapause in the commercial silkworm (*48*), but DH native

to heliothines has also been shown to exert an opposite effect in pupae of the *Heliothis/Helicoverpa* complex: Rather than inducing diapause as it does in *B. mori,* DH actually breaks diapause in *Heliothis/Helicoverpa* (*49–52*).

The corn earworm, *Helicoverpa zea,* like other members of this pest complex, enters an overwintering diapause in the pupal stage in response to short day lengths and low temperatures of autumn (*53*). Diapause in pupae of these moths can be terminated with an injection of DH, a neuropeptide produced in the subesophageal ganglion. DH works together with another neuropeptide, prothoracicotropic hormone (PTTH), to promote synthesis of the ecdysteroids needed to break diapause and initiate adult development.

Breaking and Preventing Pupal Diapause with DH Agonists

The active core of DH in *H. zea* consists of 5-7 amino acids located at the C-terminus of the native DH. The ED_{50} for DH in terminating diapause is approximately 100 pmol per pupa. Structurally modified DH analog 2Abf-Suc-FKPRLa (DH-2Abf-K) proved to be a potent agonist with an ED_{50} of 13 pmol (*54*), almost an order of magnitude more potent than the native hormone. The higher potency exhibited by this analog likely results from greater biostability to peptidase degradation that allows it to remain for longer periods in the hemolymph.

With the highly potent, biostable analog DH-2Abf-K in hand, the question arose as to whether it could be applied to the preceding larval stage and subsequently prevent the entry into pupal diapause. Application of DH analogs to disrupt pupal diapaue could be problematic because diapausing pupae are underground and therefore remain less accessible to any sort of treatment. A much more accessible stage for applying an agent would be the larval stage that is still above ground. The hyper-potency of DH-2Abf-K and the increased half life generated by blocking the N-terminus with the hydrophobic 2Abf-Suc-complex suggested that it might be capable of being administered to larvae and retain its potency long enough to prevent diapause entry. The native hormone DH (1 nmol per larva) was injected into larvae, and although it is potent in breaking pupal diapause, it had no effect in preventing the entry into pupal diapause when injected into larvae (*55*). It is likely that DH is rapidly degraded by aminopeptidases and/or endopeptidases in the larval hemolymph, thus losing its activity before it can exert an effect on diapause. On the other hand, DH-2Abf-K apparently retained activity sufficiently long to exert an effect on pupal diapause. It reduced the incidence of pupal diapause by about 70%. A dose-response curve indicated that it displayed an ED_{50} of 7 pmol/larva (*55*). Analog DH-2Abf-K, and a couple of related analogs (*55*), represent the first peptide hormone analogs shown to be capable of preventing diapause.

The accessibility of the above-ground, prediapause larvae makes this stage a particularly good target for control efforts. Though the compound DH-2Abf-K was administrated only by injection, it is hoped that additional modifications will facilitate oral and/or topical application of future DH mimetic analogs.

Blocking Diapause Termination with a DH Antagonist

Another goal was to design DH antagonists capable of blocking the termination of diapause. Such agents could delay or completely block the natural processes of diapause termination, thus preventing the insect from exploiting favorable seasons for development and reproduction. The approach taken was to target the DH activity core and convert the agonist into an antagonist molecule by modification of the structural components important for activity. Like all members of the pyrokinin superfamily, a *trans*-oriented Pro represents an important part of the interaction between the ligand and its receptor. Strong evidence for this came from the diapause termination hyperagonist activity displayed by an analog that incorporated a mimic of the *trans*Pro called an Etzkorn moiety, in which the flexible peptide bond of the Pro is replaced with a rigid double bond that locks in the *trans* orientation (*34*). A second, novel motif that was identified as a mimic of a *trans*-oriented peptide bond was chosen to incorporate into an alternate analog of the DH C-terminal core region. The motif chosen was the dihydroimidazole moiety (or 'Jones' motif) (*54*, *55*) (Fig. 2), which was shown to serve as a surrogate for the *trans*Pro conformation (Fig. 3).

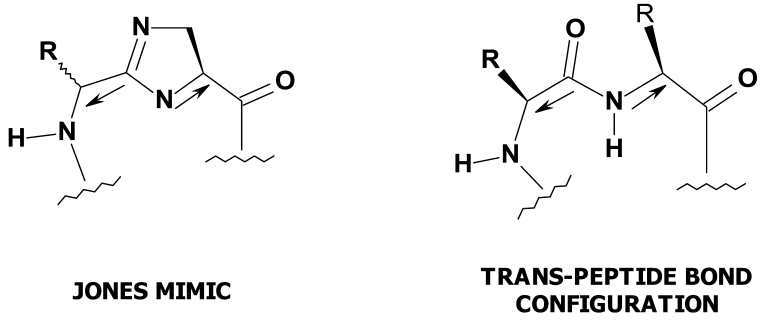

Figure 2. The dihydroimidazole moiety ('Jones' moiety)(left) can serve as a mimic of a trans-oriented peptide bond (right).

Nonetheless, the Jones motif is not as effective a mimic of a *trans* oriented peptide bond, and specifically a *trans*Pro, as is the (E)-alkene moiety in the Etzkorn motif. Possibly, a new analog containing a 'Jones' motif could mimic the *trans*Pro configuration to an extent sufficient to bind the DH receptor, and yet not closely enough to be able to elicit the downstream response. The new analog, labeled DH-Jo was designed from the C-terminal heptapeptide active core (LWFGPRLamide) of DH (*51*). The amino acids GP were replaced by the Jones motif. A further modification was introduced by replacing F with A, the rationale being that any residual agonist activity would be eliminated by removal of the aromatic side chain of the F residue that is critical for the agonist response.

Indeed, the new analog DH-Jo proved to be an effective antagonist of DH, reducing the diapause termination activity of the native DH hormone by over 50% (*55*). This new compound is the first antagonist developed for DH and represents a novel class of peptide hormone antagonists. It is the first example of an analog that incorporates a Jones *trans*-peptide bond/*trans*Pro mimic to generate an antagonistic response. This strategy may prove useful for the design of antagonists for other peptide hormones, especially those containing a *trans*Pro in the active core.

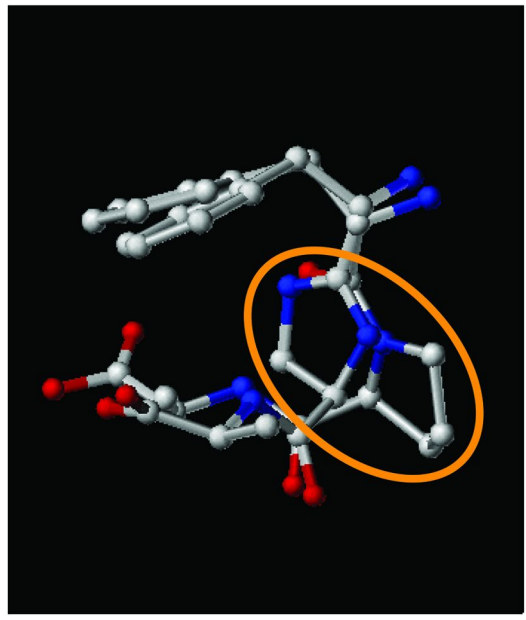

Figure 3. Within the circled portion of the computer graphics illustration is a superposition of a trans-peptide bond preceding Pro (below) with the dihydroimidazole ('Jones') motif (above) that indicates that this motif can function as a surrogate for a transPro.

Summary

While natural and unmodified neuropeptides of the pyrokinin classes demonstrate little or no activity when fed to the pea aphid *A. pisum*, biostable analogs with enhanced resistance to degradation by peptidases demonstrate potent anti-feedant and aphicidal effects that approach the potency of some commercially available aphicides. That the aphicidal effects of the biostable pyrokinin analogs can be blocked by a pyrokinin antagonist, indicates that the mechanism of action involves a neuropeptide receptor, indicative of a novel and selective mode of action. The physiological mechanism of the aphid antifeedant

activity and high induction of mortality demonstrated by these biostable insect neuropeptide analogs cannot be clearly identified at this point, but it may be associated with disruption of gut motility patterns that this neuropeptide family modulates in insects. The aphicidal activity of the biostable pyrokinin analogs may therefore result from a disruption of the digestive process by interfering with normal gut motility patterns.

Through the development of rationally-designed peptide agonists and an antagonist of diapause hormone, a member of the pyrokinin superfamily, we have prevented the entry into diapause, terminated diapause prematurely, and blocked the termination of diapause in *H. zea.* The fact that some of these peptide agonists are much more potent than the natural hormone in preventing or breaking diapause suggests that these agents or more advanced derivatives have potential as future control agents via the manipulation and/or disruption of the protective overwintering response of plant pests.

The active biostable neuropeptide analogs described in this review and/or 2nd generation analogs, either in isolation or in combination with biostable analogs of other neuropeptide classes that also regulate aspects of diuretic, antidiuretic, digestive, reproductive and/or developmental processes, represent potential leads in the development of selective, environmentally friendly pest insect control agents capable of disrupting those critical processes.

Acknowledgments

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Chapter 8

The Use of Genomics and Chemistry To Screen for Secondary Metabolites in *Bacillus* spp. Biocontrol Organisms

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Recent advances in DNA sequencing technologies have revolutionized the way we study bacterial biological control strains. These advances have provided the ability to rapidly characterize the secondary metabolite potential of these bacterial strains. A variety of bioinformatics tools have been developed to analyze sequence data, which can identify putative secondary metabolite synthetase clusters. The functionality of these clusters can then be confirmed with modern mass spectrometry techniques. The *Bacillus* biological control community is rapidly adapting these approaches to understand the bioactive compounds produced by these strains. This chapter summarizes the current state of the field as it applies to the *Bacillus* biological strains primarily focused on controlling plant diseases or are plant growth promoters, which include: *B. subtilis, B. amyloliquefaciens, B. pumilus* and *B. licheniformis* strains.

In Biopesticides: State of the Art and Future Opportunities; Coats, et al.;

Introduction

The dramatic drop in DNA sequencing costs has revolutionized how we characterize biological control organisms. Since 2007, the cost of DNA sequencing has drop 4 orders of magnitude (*1*), which has made the technology accessible for more labs. Concurrently, advances in mass spectrometry have greatly increased the sensitivity and structural analysis capabilities of modern instrumentation (*2*). Combining these two techniques has permitted the rapid characterization of some important biological control organisms. The two combine to reduce the resources needed characterize the secondary metabolite potential of an organism by using an integrated discovery workflow.

Over the past decade enormous strides have been made in understanding the secondary metabolite potential of *Bacillus* spp. biocontrol organisms. This success has been driven by technological improvements in high throughput DNA sequencing and mass spectrometry. The ability to screen and identify potentially bioactive secondary metabolites is becoming routine. *Bacillus* species represent the majority of bacterial microbial biological agents with the bioinsecticide *Bacillus thuringiensis* accounting for more than 70% of total sales (*3*). This chapter will focus on the other *Bacillus* species that have been commercialized and are primarily focused on controlling plant pathogens or are plant growth promoters. At least 20 commercial products have been developed based on these *Bacillus* species, which include: *B. subtilis, B. amyloliquefaciens, B. pumilus* and *B. licheniformis* strains (*3*). These four species are closely related and make up less than 2%of the species in the *Bacillus* genus (*4*). At the time of writing this chapter, 43 *B. subtilis,* 26 *B. amyloliquefaciens,* 10 *B. pumilus* and 13 *B. licheniformis* genomes have been sequenced and submitted to the NCBI database. This rapidly growing database will facilitate our ability to unravel the genetic strategies these species adopt and are responsible for their biological control activity.

Computational Tools and Approaches for Genomic Mining

To take advantage of the low-cost access to genome sequence data, a variety of computational tools have been developed to identify the biosynthetic clusters in bacterial or fungal genomes, for review (*5*, *6*). Most of the initial programs available were based identifying polyketide synthases (PKS) and nonribosomal peptides synthases (NRPS), which are responsible for making the majority of known bioactive secondary metabolites (*5*). These programs took advantage of the conserved backbone and modular structure of PKS/NRPS clusters to identify new and previously identified clusters. Five of the mostly commonly used programs in this class include CLUster SEquence ANalyzer (CLUSEAN) (*7*), ClustScan (*8*), Natural Product searcher (NP.searcher) (*9*), NRPSPredictor (*10*), and Structure Based Sequence Analysis of Polyketide (*11*). A newer program, antiSMASH 2.0, has taken a more comprehensive approach and added functionality to identify additional classes of secondary metabolites (*12*). Many of these programs are available as web-based programs or as stand-alone programs,

which can be run on private computing resources. It is anticipated with the rapid growth in sequencing and computational tools that the field will continue a high rate of evolution and discovery in the coming years.

Nonribosomal Peptide Synthases

Nonribosomal peptide synthases are large multidomain and modular enzyme assemblies that produce peptide based secondary metabolites. In *Bacillus* biological control strains these are responsible for some of the most important secondary metabolites associated with biological control efficacy. The most important NRPS products of these *Bacillus* biological control strains are three families of cyclic-lipopeptides: surfactins, iturins and fengycins (*13*). These compounds have antibacterial and antifungal activities and at least one can be found in most of the registered *Bacillus* products (*3*). The structures of these lipopeptides are presented in figure 1.

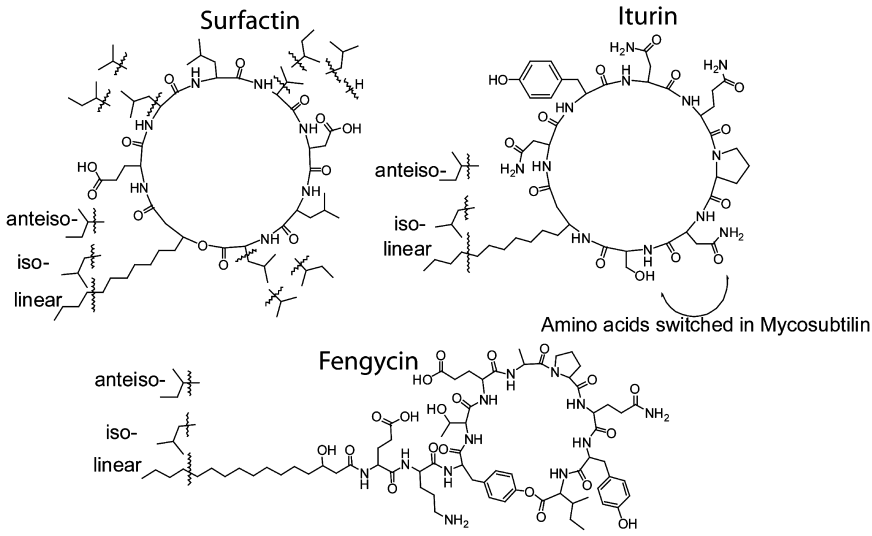

Figure 1. Structures of surfactin, iturin, and fengycin.

The widespread availability of sequence data for these NRPS clusters has allowed for comparative bioinformatics of these operons. This has led to development of degenerate primers, which allow a polymerase chain reaction (PCR) screening approach to identify each of the three major classes of lipopeptide synthetic clusters (*14*). In addition, primers for individual genes associated with these NRPS clusters have been reported (*15*, *16*). These PCR approaches provide a cost-effective method of identifying these NRPS clusters in strains were the genomic sequence data is not available. These methods are also easily scalable to screen hundreds to thousands of strains.

The Surfactin Family

Surfactins are the most common lipopeptide class found in *B. subtilis* and *B. amyloliquefaciens* strains and its production is found in most known strains (*17*). Surfactin is a cyclic heptapeptide which is formed by a β-hydroxy amino acid with a long alkyl chain forming a lactone to close the ring structure. Surfactins appear to play multiple roles in the life cycle of the species. It is required for the swarming activity of *B. subtilis* (*18*, *19*) and plays a role in the competence development pathway (*20*). The competence development pathway provides a mechanism for microorganisms to exchange DNA in response to environmental conditions. Surfactin has been reported to aid in biofilm formation in *B. amyloliquefaciens* strain FZB42 (*21*). Surfactin has been reported to have antifungal activity against the rice phytopathogen, *Magnaporthe grisea* (*22*). The activity of surfactins is related to their interactions with biological membranes, which results in a strong destabilization of the membrane and changing the physical properties of the membrane (*23*, *24*).

The surfactin lipopeptide family includes some structurally similar lipopeptides known as lichenysins, commonly produced by several *B. licheniformis* strains (*25*). Lichenysins differ from surfactins by the amino acid located in position 1 of cyclic peptide. Lichenysins have an amide amino acids (Gln or Asn), while surfactins have the acidic version (Glu) (*26*). The antibiotic activity of lichensyins is less than surfactins, but the ability to reduce surface tension is greater than that of surfactins (*25*).

Another class of lipopeptides in the surfactin family are known as pumilacidins, which were isolated from the supernatant of *B. pumilus*. Pumilacidins were shown to have antibiotic and antiviral activites (*27*). Pumilacidins are also much more hydrophobic than the other lipopeptides in the surfactin family. This property is responsible for their low solubility in water (*27*) and unique interfacial properties (*28*). A pumilacidin from a cassava endophyte, *B. pumilus* MAIIIM4a, was reported to have strong antifungal activity against *Rhizoctonia solani, Pythium aphanidermatum* and *Sclerotium rolfsii* (*29*).

In addition to surfactins, lichenysins and pumilacidins, many other closely related compounds are in the surfactin family. Bamylocin A was isolated from a *B. amyloliquefaciens* strain (*30*). Kannurin was recently isolated from a *B. cereus strain* (*31*). Another novel lipopeptide, similar to surfactin, isolated from a *B. amyloliquefaciens* strain was reported to provide strong antagonism against *Fusarium oxysporum* (*32*). These only represent a small part of the diversity of the surfactin family. Undoubtedly, additional compounds will continue to be identified at a rapid rate as the new genomic and analytical methods provide easier tools to discover new compounds.

The Iturin Family

The iturin family of lipopeptides is particularly important to the biocontrol field. The iturins have strong antifungal activity, which are effective against many plant pathogens (*13*). Iturins are cyclic heptapeptides which are formed by ring closure at a β-amino acid with a long alkyl chain. Iturins have been reported

in many *Bacillus* biocontrol strains (*33–38*) with new strains being continually reported. There are many structural and substitution variants of in the iturin family. Mycosubtilin is a variant were the final two amino acids in the chain are transposed relative to iturin A (*39*). While bacillomycin D, a common variant, varies at four amino acid positions (*40*).

The activity of the iturins has long been associated with their amphiphilic nature and their interactions with biological membranes. Early research using model membrane systems demonstrated the iturins destroyed the integrity of the membranes and allowed their contents to leak out (*41*). Additional research suggested that iturins form pores (*42*) in the membrane and modify the structural phases of the membrane (*43*). Recent research suggests the iturins may interact specifically with microdomains within the plasma membrane that have high sterol content (*44*). While a recent electron microscopy study implies that the antifungal activity is related to an interaction with intracellular targets, in addition to membrane permeabilization (*45*).

The Fengycin Family

The fengycin family is another commonly found lipopeptide in *Bacillus* biocontrol strains. Fengycins have strong anti-fungal activity against many phytopathogens and other fungi (*13*). A study in apples showed the protective effect of a *B. subtilis* strain against grey mould was associated with *in situ* production of fengycin in the infection court (*38*). The fengycins are cyclic lipodecapeptides with a β-hydroxy fatty acid chain. The mode of action of fengycin is not clearly understood. It is known to interact with the cell membrane and form aggregates at higher concentrations (*46*, *47*). These aggregates then induce curvature in the membrane (*47*) and are associated with membrane leakage (*46*).

The fengycin family also includes some structurally similar lipopeptides. Plipastatins have the same peptide sequence fengycins, but the two tyrosine are the opposite stereoisomers. Fengycin has D-Tyr at the 3 position and L-Tyr at the 9 position, while plipastatins have L-Tyr at the 3 position and D-Tyr at the 9 position (*13*). The widely used commercial strain *Bacillus subtilis* QST713, sold under the tradename Serenade®, contains a lipopeptide referred to as agrastatin. Agrastatin is the same as plipastatin, except it has a valine substituted for the isoleucine (*48*).

Other NRPS Products

In addition to the lipopeptides, NRPS clusters in *Bacillus* spp biological control strains make other metabolites with biological control activity. These products tend to fall in two classes, siderophores and antibacterials. Siderophores bind iron with high affinity and allow the microbes to compete for the limited iron available in some ecological niches. The ability to sequester iron has been recognized as an important trait in biological control antagonists (*49*).

Bacillibactin is a common NRPS derived siderophore found in these *Bacillus* spp biological control strains (*50–53*). The most common antibacterial NRPS cluster found in these strains produce bacilysin or its halogenated analogs (*54*). Bacilysin is a prodrug, that after being imported into the target cell it is hydrolyzed to L-alanine and anticapsin (*55*). Anticapsin is a strong inhibitor of glucosamine synthetase (*56*).

Polyketide Synthases

Polyketide synthases (PKS) represent a second class of biosynthetic gene clusters commonly found in *Bacillus* spp biological control strains. Similar to NRPS clusters, PKS clusters are large modular multidomain enzyme complexes that produce natural products. This modular design and wide variety of products have made them compelling targets for genomic studies (*57*). Our understanding of PKS clusters and their role in biological control systems is rapidly expanding. The widely studied PKS clusters in biological control systems produce antibiotics and are also found in *Pseudomonas* and *Streptomyces* strains (*58*, *59*). The role of PKS clusters in *Bacillus* biological control systems is not well understood, but many clusters have been identified in recently sequenced genomes (*50*).

A recent analysis of completed *B. amyloliquefaciens* genomes showed the genes unique to the biocontrol strains contained PKS clusters capable of producing the antibiotics macrolactin and difficidin (*60*). Macrolactin has been shown to be effective in controlling *Streptomyces scabiei* (potato scab) (*61*) and it was shown to inhibit the soil plant pathogenic bacteria *Ralstonia solanacearum* (*62*). While difficidin was shown to control *Erwinia amylovara,* the causal agent of fire blight (*63*). Another commonly found PKS cluster in *Bacillus* strains produces bacillaene, an antibiotic that is active against a broad spectrum of bacteria (*64*). To our knowledge, there is no knowledge on the mode of action for macrolactins. Whereas, difficidin has been shown to inhibit protein synthesis in cell-free systems (*65*). While understanding the role of antibacterials in a bacterial pathogen system is straight forward, it is unclear what impact these antibacterials have in the fungal pathogen systems where *Bacillus* biocontrol antagonists are commonly used. It most likely depends on the individual system, if altering the native bacterial community with these anitbacterials positively or negatively impacts the fungal pathogen we are attempting to control.

Other Biosynthetic Clusters

Besides NRPS and PKS clusters, *Bacillus* spp biological control strains are known to possess other important secondary metabolite clusters. A thiazole/oxazole-modified microcin (TOMM) cluster was found in *B. amyloliquefaciens* FZB42 and subsequently in multiple of other *B. amyloliquefaciens* strains (*66*). The cluster produces a compound known as plantazolicin, which was shown to have antibacterial (*66*) and nematicidal activity

(*67*). A second commonly found cluster type is lantibiotics, a class of bacteriocins (*68*). Several of these lantibiotics have been implicated to have a role in biological control systems, such as, subtilin which was shown to be effective in controlling *Gaeumannomyces graminis* in wheat (*69*). They have also been proposed as a method of biological control to limit ruminal methanogenesis (*70*). Additional lantibiotics and variants are continually being reported in *Bacillus* spp (*71–73*).

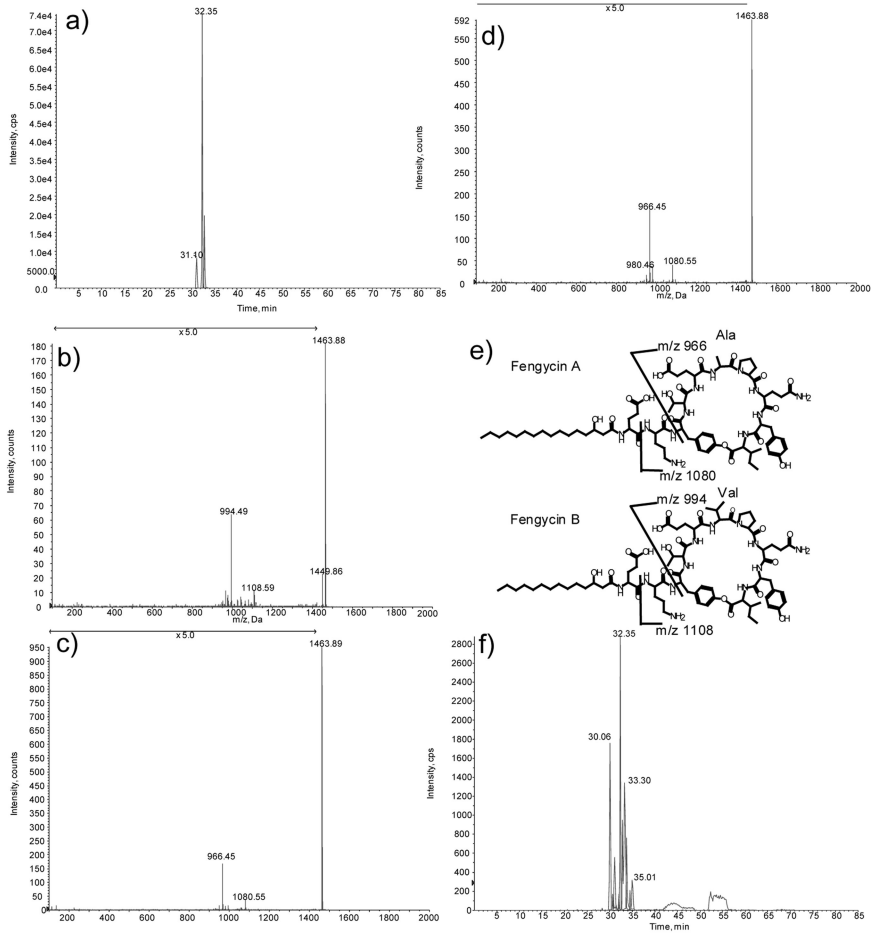

Figure 2. a) extracted ion chromatogram of m/z 1463 b) MS/MS spectrum of 31.10 min peak (fengycin B) c) MS/MS spectrum of 32.25 min peak (fengycin A) d) MS/MS spectrum of 32.8 min peak (fengycin A-tail isomer) e) Structures and diagnostic fragments of fengycin A and B. f) extracted ion chromatograms of MS/MS product ions: m/z 966 and m/z 1080 (fengycin A variants); m/z 994 and m/z 1108 (fengycin B variants).

Mass Spectrometry of Secondary Metabolites

Bacillus strains and their metabolites have been the subject of extensive study by mass spectrometry. This has been driven by advances in mass spectrometry and its use to rapidly identify microorganisms (*74*). The early advances of mass spectrometry to identify microorganisms (*75–77*), were adapted to detect *Bacillus anthracis*, when biosecurity concerns arose (*78*, *79*). Many of these studies relied upon matrix assisted laser desorption ionization mass spectrometry (MALDI-MS), because of ease of use and the ability to use whole cells (*80–82*). These mass spectrometry (MS) approaches only provide the mass of parent ions, without pre-separation of compounds. While these MS methods are useful for high-throughput initial studies, they are limited in their ability to differentiate isobaric compounds, which require pre-separation and fragmentation analysis of compounds, such as is capable with liquid chromatography tandem mass spectrometry (LC-MS/MS).

LC-MS/MS has been effectively used to identify known metabolites from different *Bacillus* strains. The lipopeptide metabolites surfactin (*60*, *83*, *84*), iturin (*60*, *83*), and fengycin (*60*, *84*, *85*) have been analyzed by LC-MS/MS using various instrument types, in the case of lichenysin quantitative LC-MS/MS data was collected (*86*). Lipopeptide analytes are conducive to informative MS/MS analysis due to the consistent peptide fragmentation patterns that provide sequence information of this portion of the molecule that can also lead to the identification of new products, as described below. In cases where new compounds are identified, a purification and characterization strategy is required to determine novel structures. For example, a novel lipopetide, kannurin, was also identified by LC-MS/MS (*31*). Additional structural characterization was performed due to the novelty of this metabolite. Some NRPS products are lower in mass and do not have the same predictable fragmentation patterns as lipopeptides; however LC-MS/MS with high mass accuracy has been used to identify bacillibactin and difficidin from a *B. amyloliquefaciens* strain. LC-MS/MS has potential to identify lantibiotics produced by *Bacillus* strains. The protein-like structures of subtilin, ericin, mersacidin, sublacin, and subtilosin have size, separation, and ionization characteristics that are conducive to LC-MS/MS; however, fragmentation information may be limited due to the crosslinking of the macrocycles of this class of molecule. These crosslinks will likely prevent the representative fragmentations across the amide bonds needed for sequence assigment.

The need for LC-MS/MS analysis of secondary metabolites of *Bacillus* strains is necessitated by the number and types of products that may be present in the extracellular matrix and the variation from strain to strain. The structural similarities between components of the lipopeptide classes reported previously stand as good examples of this diversity. The cyclic lipopeptides have been reported to have variations in the amino acids in the cyclic structures, where isomers are observed (e.g. Leu/Ile) often these involve changes to hydrophobic residues that either shorten or lengthen the side chain (e.g. Leu/Ile to Val/Ala or vice versa) (Figure 1) (*13*). The lipopeptide tail can be of varying lengths, thereby providing isobaric compounds that disguise the changes to the cyclic peptide under conditions that do not have chromatographic separation and

tandem mass spectrometry. LC-MS methods give time-resolved signals based the chromatographic matrix used with mass-to-charge (*m/z*) information to identify non-ribosomal peptide synthase gene product classes (*83*, *85–88*). Under sufficient chromatographic conditions the isomers will have peaks at discreet retention times (Figure 2a); however retention times and *m/z* may not always be sufficient for assignment. Differentiation of subclasses can be accomplished *de novo*, using fragmentation capabilities of quadrupole time-of-flight, time-of-flight/time-of-flight, triple-quadrupole, or ion-trap mass spectrometers to identify diagnostic fragment ions for surfactins, iturins, fengycins, etc (*60*, *84*, *89–91*). For example, an ion at *m/z* 1463 corresponds to fengycin, as noted in Figure 1 there are several isomers of fengycin that could be generate this ion. Under LC-MS/MS conditions, three peaks are present in the extracted ion chromatogram indicating that three isomers are present (Figure 2a). Tandem mass spectrometry involving the isolation of the desired *m/z* ion followed by fragmentation. Provides structural data for each of these three *m/z* 1463 peaks where the diagnostic fragment ions for: fengycin A with a 16 carbon tail (Figure 2c and 2d *m/z* 966 and 1080, alanine-containing macrocycle) and fengycin B with 14 carbon tail (Figure 2b *m/z* 994 and 1108, valine-containing macrocycle). The presence of the third peak (Figure 2d) indicates that a methyl branch is present on the lipophilic tail causing chromatographic separation while still fragmenting as a fengycin A macrocycle. The extracted ions from tandem mass spectrometry (*m/z* 966 and 1080 for fengycin A and *m/z* 994 and 1108 for fengycin B) can serve as markers for their presence regardless of the length of the lipid tail present on the compound (Figure 2f). As shown in the is example, the use of LC-MS/MS permits the separation of isomers and the capability of assigning structures of the analytes based on diagnostic ions, something that is lacking if the MS method used only provides parent ion information without prior fractionation.

Surfactin (*m/z* 1022, C14 tail) also serves as an example for the need for time-resolved mass spectral data with confirmatory structural information from fragment ions. In a known surfactin-producing *B. amyloliquefaciens* strain AS 43.3 (*60*, *92*) the presence of *m/z* 1022 is dominant at a retention time of 38.5 minutes (Figure 3b), additional peaks are present in the extracted ion chromatogram again indicating the presence of isomeric surfactins or similar mass compounds. In contrast, samples from a *B. subtilis* strain lacking the capability for surfactin production (data not shown), has two peaks corresponding to the same *m/z* as surfactin (*m/z* 1022). These peaks are present at much later retention times than the dominant surfactin peak of the *B. amyloliquefaciens* strain.. Under non-chromatographic conditions (e.g. MALDI-TOF) this low-intesity signal could lead to putative assignment of surfactin, when no surfactin is actually produced. The importance of all three components becomes apparent when the tandem MS data is also included. Fragmentation data from *m/z* 1022 at 38.5 min (Figure 3d) is nearly identical to 1022 at 57.4 minutes (Figure 3f), if LC was not performed on the front end of the analysis, then these two compounds would be assigned as a single component. The *m/z* 1022 peak at 45.5 minutes (Figure 3e) has altered masses from fragments in the peptide portion of the analyte suggesting that an Ile/Leu (*m/z* 685, 582, 483, 370) has been replaced with a Val (*m/z* 671, 596, 469, 356) and the hydrophobic tail is one unit longer to make up the mass

difference. As these two examples demonstrate, all three components of the LC-MS/MS system are valuable for determining the features of *Bacillus* strain secondary metabolite production, as each reinforces the information of the others leading to greater certainty in assigning products. Similarly PKS gene cluster products should also be evaluated by LC-MS/MS as they have lower masses that can be obscured by MALDI matrix effects or LC-MS ion clusters or interference. The multiple pieces of data from each peak will allow for more certainty in identifying the presence or absence of products.

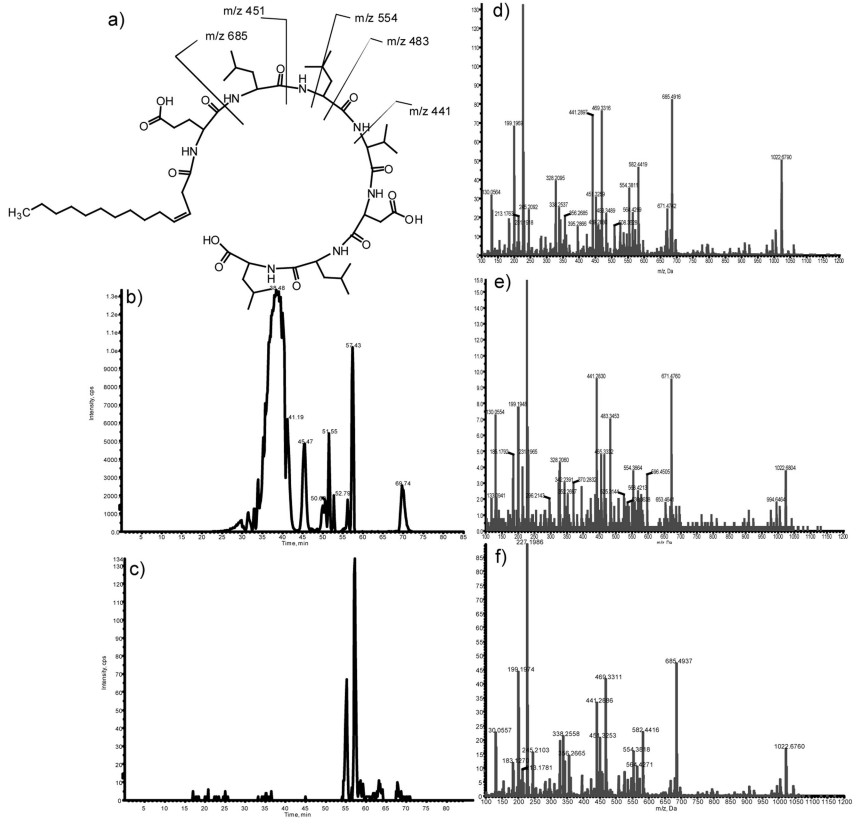

Figure 3. a) Ring-opened surfactin structure and fragmentations b) extracted ion chromatogram of m/z 1022 of B. amyloliquefaciens AS43.3. c) extracted ion chromatogram of m/z 1022 of a B. subtilis strain incapable of producing surfactin. d) MS/MS spectrum of 38.5 min peak e) MS/MS spectrum of 45.5 min peak f) MS/MS spectrum of 57.4 min peak.

Future Advances

Future advances in the field will be built up the foundation of functional genomics currently being laid. Future enhancements will see a greater integration of our knowledge of biosynthetic pathways and the ability of MS/MS techniques to identify structurally related molecules to correlate the two. An example of this type of integration was recently reported in the literature, Nguyen *et al*, reported a MS/MS networking approach to link molecular families (structurally similar molecules) to gene cluster families (*93*). Another area that will see improvement is the development of software tools for automated structure prediction based on MS/MS fragementation patterns. These enhancements will continue to reduce the resources and time needed to characterize the metabolic networks of *Bacillus* strains.

Summary

The development of genomic and LC-MS/MS techniques has greatly improved our ability evaluate the secondary metabolite potential of *Bacillus* strains. The techniques are complimentary and synergistic of eachother in that the both improve the efficiency of the other technique. Genomics can identify potential synthetic clusters that may not be active under tested conditions. Genomics can guide analytical efforts by narrowing the focus of the search and the presence of an unknown cluster could be the motivation to continue searching. Whereas, mass spectrometry can confirm the functionality of known clusters and characterize the products of new or unknown clusters. The ability of LC-MS/MS to provide accurate structural information and correlate it to the gene cluster improves the accuracy of subsequent genome mining techniques. In this manner, these techniques iteratively improve eachother through continual optimization.

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Chapter 9

Investigating the Effect of Plant Essential Oils against the American Cockroach Octopamine Receptor (Pa oa1) Expressed in Yeast

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Essential oils are produced by plants and have been shown to have repellent, attractant or insecticidal properties. It is presumed that these effects are produced through their interaction with octopamine receptors. Octopamine, an important biogenic monoamine in insects, where it functions as a neurotransmitter, neuromodulator, and a neurohormone. Octopamine activates G protein-coupled receptors (GPCRs), which, in turn, activate or inhibit various signal transduction pathways. Here the α-adrenergic-like octopamine receptor from *Periplaneta americana* (Pa oa1) was expressed in a yeast cell line (*Saccharomyces cerevisiae*). This is the first time, to our knowledge, that an insect receptor has been expressed in yeast cells. We determined that a variety of monoterpenoids and related aromatic compounds interact with this Pa oa1, and that most of these compounds act as inverse agonists to this constitutively active receptor. However, we observed that the aromatic monoterpenoid, carvacrol, acts as a positive modulator of Pa oa1.

In Biopesticides: State of the Art and Future Opportunities; Coats, et al.;

Introduction

Increased public concern about the safety of synthetic insecticides has led to increased governmental restrictions on the availability and use of some conventional synthetic insecticides and acaricides through the Food Quality Protection Act of 1996. These restrictions, along with resistance of arthropod pests, due to over use or improper use, are leaving an opportunity in the market place for new, safe, and effective insecticides to control important pests in both agriculture and public health (*1*, *2*).

Throughout evolution, plants have developed methods to resist herbivore feeding and microbial attack. A protection route often includes the plant's ability to produce volatile terpenoids, which can be extracted as an essential oil, which is characterized as a strongly odiferous lipophilic liquid. Plant essential oils can be obtained by steam distillation of plant material. Essential oils are used in everyday applications such as flavors and preservatives for food, products in the cosmetic and fragrance industries, aromatherapy, household cleaning products, and in some pharmaceutical products. Exposure to these oils, and their individual constituents, are routinely encountered in our diet and personal care products. Some oils are on the United States Environmental Protection Agency's 25b exempt list, and some are listed by the United States Food and Drug Administration as Generally Recognized As Safe (G.R.A.S.). Essential oils are composed of various terpenoids and related aromatic compounds. Terpenoids are biosynthesized from isoprene units, which are the 5-carbon building blocks. Coupling of two isoprene units can lead to 10-carbon structures known as monoterpenoids (Figure 1). These isoprene backbones are targeted by endogenous plant enzymes to create various functional groups and/or bicyclic structures (*3–5*). Aromatic compounds (Figure 2) are also found in these oils but generally to a lesser degree than terpenoids (*4*). Essential oils, and their constituents, have previously been investigated for their toxicity to insects (*6–8*), and it has been suggested that they exert this toxic effect through a neurological mechanism. Several neurological sites have been investigated including: acetylcholinesterase (*9–14*), gamma-aminobutyric acid (GABA) receptors (*15*, *16*), octopamine receptors (*17–20*), and tyramine receptors (*21*, *22*).

Octopamine, an important biogenic monoamine in invertebrates, has significant physiological actions in insects (*23–26*). Octopamine exerts these physiological actions through the superfamily of G protein-coupled receptors (GPCRs) (*27*). The original classification of these receptors was based on tissue response to agonists or antagonists and resulted in octopamine-1 and octopamine-2 receptors, the latter being subclassified into octopamine-2a, -2b, and -2c receptors. Activation of the octopamine-1 receptors produces an increase in intracellular calcium while octopamine-2 receptor activation increases intracellular cAMP. The new classification of octopamine receptors is based on sequence similarity between *Drosophila melanogaster* octopamine receptors and the mammalian adrenergic receptors. This has resulted in "octopamine-1" receptors being re-classified as α-adrenergic-like octopamine receptors, and octopamine-2 receptors being re-classified as β-adrenergic-like octopamine receptors (pharmacologically subclassified); regardless, the signal transduction

pathways remain the same. Another class of receptor was differentiated, named the tyramine/octopamine receptor that binds both ligands but displays ligand-dependent signaling activity. Therefore, when tyramine is the ligand, receptor activation has an inhibitory effect on cAMP levels, and when octopamine is present an increase in intracellular calcium concentration is observed (*28*, *29*). Octopamine receptors have generally been underutilized as a pest control target but have been identified as the site of action of the formamidine class of insecticides/acaricides (*30–35*).

GPCRs have been studied for their possible involvement in human disease and as targets for pharmaceutical intervention. It is estimated that 30-45% of current pharmaceuticals target this type of receptor (*36*). *Saccharomyces cerevisiae*, referred to as yeast hereafter, has emerged as an important organism for the study of heterologously expressed GPCRs (*37*, *38*). Functional expression of GPCRs can be achieved by linking the expressed receptor to the endogenous pheromone response pathway, which has been performed for analysis of multiple mammalian GPCRs (*38*) and some invertebrate GPCRs (*39*, *40*). Yeast has only two GPCR pathways, which makes it an attractive system for heterologous expression. Mammalian and insect cells have several GPCRs and GPCR pathways that may cause interference.

The present study aimed to (1) isolate the coding sequence of a previously identified octopamine receptor from the brain of the American cockroach, *Periplaneta americana* (Pa oa1); (2) functionally express Pa oa1 in yeast; and utilize the Pa oa1-expressing yeast cells to screen monoterpenoids and related aromatic compounds from plant essential oils to determine their ability to interact with this receptor.

Materials and Methods

Insects

American cockroaches (*P. americana*) were from an established colony maintained by the Pesticide Toxicology Laboratory in the Department of Entomology at Iowa State University, Ames IA. Insects were reared on a 14:10 light:dark photocycle at 23 ± 2°C. These insects were provided with an unlimited supply of dry cat food and water.

Chemicals

All monoterpenoids and related aromatic compounds were purchased from Sigma (St. Louis, MO) with the exception of pulegone, which was purchased from Eastman Chemical Company (Miami, FL). Eleven monoterpenoids (Figure 1) and 11 related aromatic compounds (Figure 2) were chosen to include various carbon skeletons and functional groups. For screening purposes the compounds were dissolved in certified dimethyl sulfoxide (DMSO) and serially diluted in culture medium to a screening concentration of 1×10^{-4} M. The final concentration of DMSO to which the cells were exposed was 0.1%.

Isolation of the American Cockroach Octopamine Receptor 1 (Pa oa1)

Brain tissue was obtained from adult male and female American cockroaches and homogenized with a mortar and pestle, and total RNA was extracted using TRI Reagent (Sigma). Genomic DNA was degraded using a TURBO DNA-free® kit (Applied Biosystems/Ambion; Foster City, CA). Amplification of the octopamine receptor open reading frame was prepared with gene-specific primers developed for the sense strand (5‘-ATGAGGGACGGGGTTATGGAAC-3‘) and the antisense strand (5‘CTACCTAGCCTGAGGTCCACT3‘). Primers were developed based on the sequence of a *P. americana* octopamine receptor 1 (Pa oa1) previously reported (*41*).

Cloning of the octopamine receptor’s coding sequence was performed using SuperScript® III One-step RT-PCR with Platinum® Taq DNA Polymerase (Invitrogen: Carlsbad, CA). PCR reactions were performed with a Hybaid Px2 thermal cycler (ThermoScientific, Waltham, MA). PCR conditions were 55°C for 30 min (cDNA synthesis); PCR amplification: 15 sec at 94°C, 30 sec at 53°C, and 2 min at 68°C (35 cycles); final extension: 68°C for 5 min. PCR reactions were visualized on a 1.2% agarose gel containing ethidium bromide. Amplicons were extracted from the gel using a PureLink Gel Extraction Kit (Invitrogen) and the DNA ligated into the pGEM-T Easy Vector system (Promega; Madison, WI), which was transformed into competent JM109 *Escherichia coli* cells (Promega). Plasmid DNA was isolated using Wizard® Plus SV miniprep DNA Purification System (Promega) for sequence analysis. Plasmid DNA sequences were obtained and aligned using Vector NTI v10.0 Software (Invitrogen) and compared to the previously identified octopamine receptor (*41*).

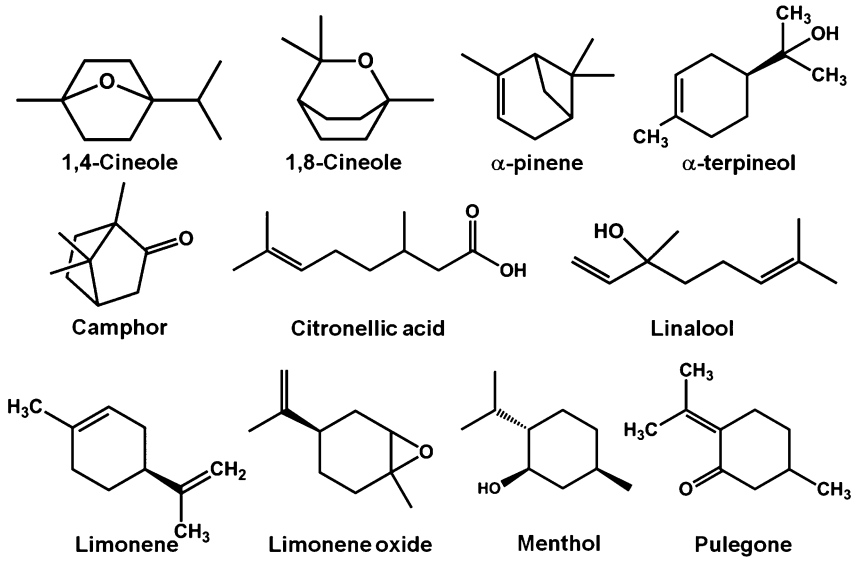

Figure 1. ***Monoterpenoid Structure.*** *Structures of monoterpenoids found in plant essential oils that were screened against the α-adrenergic-like octopamine receptor from the American cockroach (Pa oa1).*

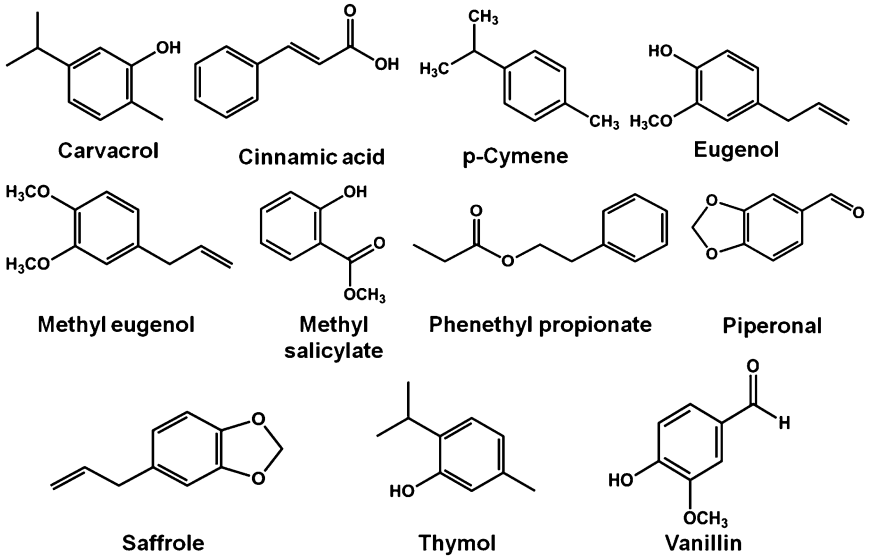

Figure 2. ***Aromatic compound structures.*** *Structures of aromatic compounds found in plant essential oils that were screened against the α-adrenergic-like octopamine receptor from the American cockroach (Pa oa1).*

Functional Expression of Pa oa1 in Yeast

The receptor coding sequence was PCR-amplified using primers that added an NcoI site (5‘GCCATA*CCATGG*GGGACGGGGTTATGAACG 3‘) and an *XbaI* site (5‘GCCATA*TCTAGA*TCACCTGGAGTCCGATCCATCGTTG3‘) at the 5‘ and 3‘ ends, respectively. PCR reactions were performed with a Hybaid Px2 thermal cycler (Thermoscientific) using High-fidelity Taq polymerase (Invitrogen). Purified amplicons were ligated into a linearized yeast expression vector, Cp4258, and then transformed into competent Jm109 *E. coli* cells. Transformation success of individual colonies was checked using PCR ,and by using gene-specific primers, for the presence of the receptor and cultured overnight in LB medium supplemented with ampicillin (30°C, 150 RPM). Plasmid DNA was isolated using the HiSpeed plasmid Midi Kit (QIAGEN; Germantown, MD) and underwent sequence analysis to confirm receptor orientation and fidelity.

A variety of genetically engineered yeast cell lines were obtained (kindly provided by J. Broach, Princeton University) that differed in their expression of the G-protein α-subunit allowing heterologously expressed proteins to interact with the endogenous pheromone-response pathway. Pa oa1 most effectively expressed in the yeast strain CY18043 (MATα PFUS1-HIS3 GPA1-Gαo(5) can1 far1Δ1442 his3 leu2 lys2 sst2Δ2 ste14::trp1::LYS2 ste18γ6-3841 Ste3Δ1156 tbt1-1 trp1 ura3). Transformation of this cell line was performed using a lithium acetate method (*42*). Briefly, yeast cells are grown in YPD medium (Sigma) to mid-log phase at 30°C and 250 RPM. Cells were pelleted (4,000 x g, 5 min, room temperature) and

washed with sterile water and resuspended in 10 mL of lithium acetate solution (1 mL of 10 X lithium acetate [10 mM], 1 mL of 10X Tris-EDTA [TE] buffer [0.01M Tris-HCl, 400μM EDTA, pH 7.5], and 8 mL of sterile water); this solution was incubated on an orbital shaker for 1 hr at 30°C and 250 RPM. Transformation was performed by adding 200 μL of yeast/lithium acetate suspension, 200 μg of UltraPure™ Salmon Sperm DNA (Invitrogen) and 1 μg of vector carrying receptor or 1 μg of empty vector (mock cells) in 1.2 mL of lithium acetate PEG solution (1 mL of 10X TE, 1 mL of 10 LiAc, 8 mL of 50% PEG4000). Transformants were selected on leucine-deficient medium (1 x Yeast Nitrogen Base (YNB) [Difco: Houston, TX], 1 x yeast synthetic dropout medium supplemented without leucine [Sigma], 10 mM ammonium sulfate [Sigma], 2% glucose [Sigma]). The presence of the octopamine receptor gene was determined by extracting yeast DNA using a "bust n' grab" method and PCR amplification with gene-specific primers (*43*). Once positive colonies were identified, the yeast cells were maintained in selective medium (-Leu) supplemented with octopamine to continually select for receptor expressions. Yeast cells were stored at -80°C in 15% glycerol stocks.

Histidine-Auxotrophic Assay

The histidine-auxotrophic assay was used to monitor the activation of the yeast's pheromone response pathway. Here, histidine synthesis is achieved with (1) the addition of histidine in the growth medium, or (2) induction of histidine synthesis. This system was chosen based on its easy end-point, yeast cell growth. (*44*) The histidine-auxotrophic assay was performed similarly to the method of Kimber et al. (*39*). Briefly, 2 mL of selective medium (-Leu), which did not contain octopamine, was inoculated with transformed yeast cells (frozen glycerol stocks) and allowed to grow overnight on an orbital shaker (30°C and 250 RPM (OD_{600} 1.0-2.0)). Cells were pelleted at 5,000 x g at room temperature and washed three times with medium deficient in leucine and histidine (1x yeast nitrogen base (Difco), 1x yeast synthetic dropout medium supplement without leucine, tryptophan, uracil, and histidine (Sigma), 170 mM uracil (Sigma), 93 mM L-tryptophan (Sigma), 10 mM ammonium sulfate (Sigma), 2% glucose (Sigma), 50 mM 4-morpholinepropanesulfonic acid (MOPS; Sigma), pH 6.8). The pellet was finally resuspended in 1 mL of leucine/histidine-deficient media supplemented with 10 mM 3-amino-1,2,4-triazole (3-AT;Sigma) to help control growth by inhibiting histidine synthesis. Cells were dispensed into a 96-well clear Costar® plate with a total volume of 200 μL at 15-20 cells/μL (OD_{600} of 0.01), which included 10 μL of DMSO vehicle or test compound solution. Cells were allowed to grow at 30°C and 98% humidity for 24 hr at which time optical density readings (absorbance of 600 nm) were taken using a Spectramax 190 (Molecular Devices, Inc. Sunnyvale, CA). Experiments were performed in quadruplicate on 96-well plates for one experiment, with a total of five experiments performed for statistical analysis. A control experiment was performed to confirm that the monoterpenoids that had a statistically significant effect on the growth of yeast cells, transformed with Pa oa1, were not interacting with an endogenous yeast pathway and were not cytotoxic to yeast cells. This control experiment was performed by incubating the candidate compound with yeast cells containing only

the vector (mock transformants)in YPD medium. Candidate ligands were placed in 8-wells of a 96-well plate, at an optical density of 0.01; these experiments were performed in triplicate. Growth of yeast was monitored over 24 hr.

Statistical analysis for yeast growth experiments was performed using SAS 9.2 (DAS Institute Inc, Cary, NC). Data from growth experiments was normalized relative to the control (vehicle only), and data was subjected to analysis of variance (ANOVA); log transformations were performed to achieve a best-fit model. Graphs were made using Prizm 5.0 GraphPad Software (San Diego, CA).

Results

Isolation and Expression of Pa oa1 in the Yeast *S. cerevisiae*

The α-adrenergic-like octopamine receptor from *P. americana* (Pa oa1) was isolated from brain tissue using gene-specific primers. Expression of the isolated octopamine receptor was performed using a lithium acetate method (*42*, *44*). The presence of the octopamine receptor transcript in the yeast was confirmed by performing RT-PCR with gene-specific primers. The size of the amplicon was approximately 2 kb (data not shown); Pa oa1 cDNA size is known to be 1.9 kb (*41*).

The isolated American cockroach octopamine receptor was expressed in a variety of genetically modified yeast cells (differed in the expression of the G-protein α-subunit). Each cell line was transformed with Pa oa1 to find a cell line suitable for use in a histidine-auxotrophic assay. In this assay, the heterologous expression of Pa oa1 is linked to the endogenous yeast pheromone response pathway. Induction of the pathway results in the activation of FUS-1 promoter, downstream of which is the *his3* reporter gene that allows production of histidine. Pa oa1 transformed yeast cells are grown in histidine-deficient medium, and cannot undergo normal protein synthesis necessary for growth unless histidine is added to the medium or histidine synthesis is induced. Histidine induction is realized upon receptor activation (*39*, *40*, *45*).

Genetically engineered yeast cells differed in their expression of a G-protein chimera. The chimera is formed with the receptor interacting domain from different G-protein signaling pathways ($G_{\alpha i}$, $G_{\alpha o}$ and $G_{\alpha s}$). The chimera is essential for successful coupling of the expressed receptor to the endogenous machinery. A functional Pa oa1 was obtained by expressing the receptor in yeast strain CY18043, a cell line containing a chimeric $G_{\alpha o}$ subunit. When Pa oa1 was expressed in CY18043 it appeared to have significant growth. This increase in cellular growth was compared to mock-transformed cells (transformed with an empty vector), and was approximately 35-fold over the growth of mock cells in the presence and absence of octopamine (Figure 3). When Pa oa1 was expressed in other engineered yeast cell lines, the degree of increased growth was generally less than 5-fold (data not shown). The increased cellular growth in the cell line CY18043 is proposed to be due to a ligand-independent (constitutive) activity (Figure 4), which is supported by the lack of cellular growth response to octopamine or tyramine. This led us to hypothesize that the receptor was constitutively active in this particular system, and is fully activated, assuming

a two-state receptor model (*36*). The two-state receptor model shows that a compound can interact with a receptor, leading to a conformation change of the receptor protein. This interaction of a compound with a receptor indicates that the receptor goes from "off" state to the "on" or activated state. Specifically, the heterotrimeric G-protein was dissociated into the G_{α}-GTP and $G_{\beta\gamma}$ dimer, allowing the cell to enter the pheromone stimulation pathway and allowing activation of the reporter gene *his3*, leading to synthesis of histidine and thereby an increase in cellular growth. Compounds were screened to determine if they could bind to the receptor and slow the growth, acting as inverse agonists Phentolamine, a previously identified antagonist of Pa oa1 (*41*), was added to constitutively active yeast cells, and it decreased yeast cell growth significantly to 63 ± 2% when compared to the vehicle control (Figure 4). Synephrine, a methylated analogue of octopamine has been shown to increase cAMP production in *P. americana* hemocytes (*48*) but has also been shown to have an inhibitory effect on forskolin-stimulated cAMP production in a heterologously expressed *D. melanogaster* octopamine/tyramine receptor; however, Pa oa1 was a different receptor subtype (*49*). Synephrine decreased yeast growth significantly to 48 ± 3% when compared to the control, as shown in Figure 4.

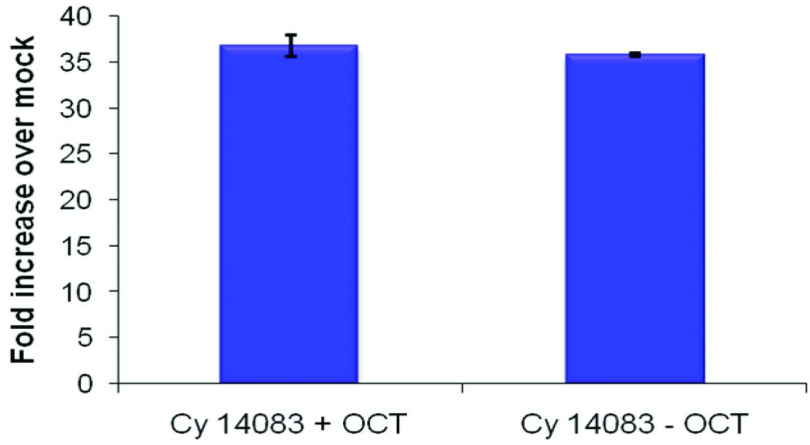

Figure 3. ***Constitutive activity of yeast cells.*** *When Pa oa1 was expressed in yeast strain CY 18043 it displaced over 35-fold increase of growth over cells transformed with an empty plasmid (mock) ± SEM. There was not an effect on the growth of the yeast cells in the presence of octopamine (+OCT) or in the absence of octopamine (-OCT).*

Finally, chlordimeform, a formamidine insecticide, was added to yeast expressing Pa oa1 and had an inhibitory effect on growth, decreasing it significantly to 73 ± 5% of the control (Figure 4). Therefore, phentolamine, synephrine, and chlordimeform all had an inhibitory effect on the expressed Pa oa1 receptor, thereby decreasing the growth of these yeast cells.

There is previous evidence that plant monoterpenoids and related aromatic compounds interact with Pa oa1 (*18*, *40*). The ability of our assay to show that some octopaminergic compounds display an inhibitory growth effect on constitutively active yeast cells expressing Pa oa1 led to the next hypothesis that this assay could be used to screen monoterpenoids and related aromatic compounds and determine their ability to bind to and alter Pa oa1, thereby indirectly affecting growth of the yeast cells. To test this hypothesis, several monoterpenoids were screened against the receptor, and their effects on yeast growth are shown in Figure 5. Several compounds did not have a statistically significant effect on growth. These compounds include α-pinene, limonene oxide, α-terpineol, linalool, 1,8-cineole, and menthol. However, several monoterpenoids did have a significant effect on the growth of the cells, when compared to the control. The effect of monoterpenoids on the constitutively active Pa oa1 expressing yeast cells can be characterized as inverse agonism when compared to the control (a decrease in growth). These compounds include1,4-cineole (77 ± 6%), citronellic acid (74 ± 4%), pulegone (73 ± 2%), limonene (68 ± 5%), and camphor (56 ± 4%) (Figure 5).

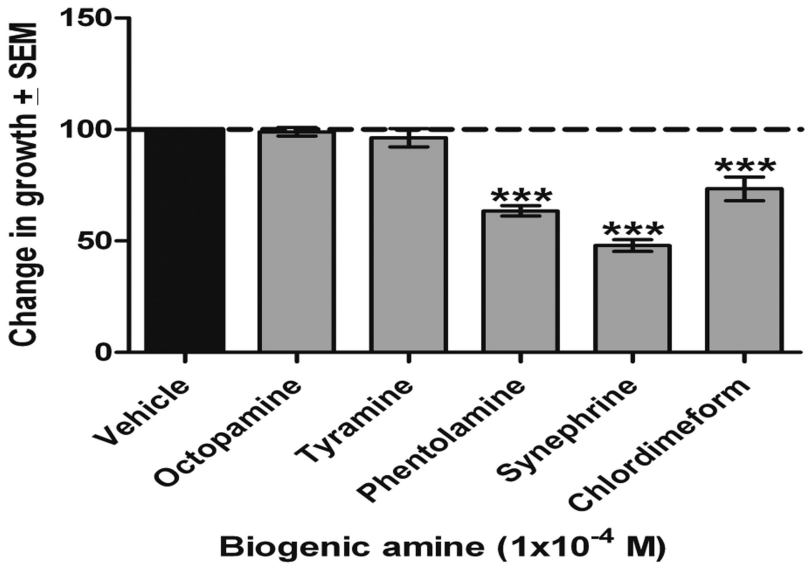

Figure 4. ***Growth responses of biogenic amines and octopaminergics.*** *The lack of a growth effect of biogenic amines, octopamine and tyramine, and other octopaminergic compounds (compounds that interact with octopamine receptors) on yeast expressing Pa oa1. Concentrations at 1x10-4 M (vehicle; *** indicates a p-value < 0.001; ANOVA).*

Aromatic compounds were also screened to see if they would bind to Pa oa1 and thereby alter the growth of the yeast cells (Figure 6). The aromatic compounds thymol and methyl salicylate had no significant activity when compared to the control (vehicle or ligand-independent activity), as shown in

Figure 6. The aromatic compound carvacrol had a stimulatory effect on growth, thereby significantly increasing growth to 130 ± 7%, when compared to the control. This was the only compound that significantly increased growth (Figure 6). Other aromatic compounds, like certain monoterpenoids, had an inhibitory effect on the Pa oa1 (as inverse agonists) thereby leading to a decrease in growth (Figure 6). The aromatic compounds that displayed this activity include safrole (80 ± 3%), p-cymene (80 ± 3%), phenethyl propionate (66 ± 4%), cinnamic acid (65 ± 4%), piperonal (54 ± 7%), vanillin (31 ± 9%), eugenol (30 ± 2%), and methyl eugenol (17 ± 10%); this data is shown in Figure 6. Comparison of the effects that monoterpenoids and related aromatic compounds have on octopamine receptors are shown in Table 1 and Table 2, respectively. These comparisons were made based on radioligand binding from a previous study using American cockroach brain homogenate (*17*) and a study on the electrophysiological activity in the nerve cord, foregut, and dorsal unpaired median neurons of the American cockroach (*20*). The most notable difference we observed is that compounds that were previously identified as antagonists have been re-classified, in our assay, as inverse agonists.

Table 1. Literature Comparison of Monoterpenoid Compounds. Comparison of the Effect of Monoterpenoids Tested in the Yeast Growth Experiments. Data Was Compared to Earlier Studies on the Same Receptor.

Monoterpenoid	*Yeast assay*	*Previous reports*
1,4-cineole	Inverse agonist	No report
1,8-cineole	No effect	No report
α-pinene	No effect	No report
α-terpineol	No effect	No report
camphor	Inverse agonist	No report
Citronellic acid	Inverse agonist	No report
linalool	No effect	No report
limonene	Inverse agonist	No effect[17]
Limonene oxide	No effect	No report
menthol	No effect	No report
pulegone	Inverse agonist	Antagonist[17]

The compounds that showed significant growth effects, presumably through an interaction with Pa oa1, which was linked to the yeast endogenous pheromone-response pathway, were tested for cytotoxicity or their ability to link with an endogenous receptor (Figure 7). The tested compounds did not have a

stimulatory or inhibitory effect on yeast cells that were not transformed with Pa oa1 (Figure 7). These results give further support that the constitutively active Pa oa1 receptor, responded to some of the tested monoterpenoids and related aromatic compounds.

Table 2. Literature Comparison of Aromatic Compounds. Comparison of Aromatic Compounds Tested in Yeast Growth Experiments. Data Was Compared to Other Studies on the Same Receptor.

Aromatic compound	*Yeast assay*	*Previous report*
carvacrol	Agonist/allosteric modulator	Agonist[17]
cinnamic acid	Inverse agonist	No effect[17]
p-cymene	Inverse agonist	Antagonist[17]
eugenol	Inverse agonist	Antagonist/ inverse agonist[17,20]
methyl eugenol	Inverse agonist	No report
methyl salicylate	No effect	Antagonist[17]
phenethyl propionate	Inverse agonist	Antagonist[17]
piperonal	Inverse agonist	Agonist[17]
saffrole	Inverse agonist	No report
thymol	No effect	No effect[17]
vanillin	Inverse agonist	Antagonist[17]

Discussion

Essential oils and their constituents have toxic effects on insects. The symptoms of their toxic action have suggested a neurological mode of action (*6–8*) resulting in numerous studies investigating different potential neurological sites (*10–21*). Octopamine receptors have emerged as a preferential target for insecticide action as they do not have a significant function in mammalian tissue, decreasing the likelihood of mammalian toxicity. Here we report that constituents of essential oils, primarily monoterpenoids and related aromatic compounds, have an effect at the octopamine receptor Pa oa1 when heterologously expressed in *S. cerevisisae*. This is the first time, to our knowledge, that a GPCR from an insect has been expressed in *S. cerevisiae*.

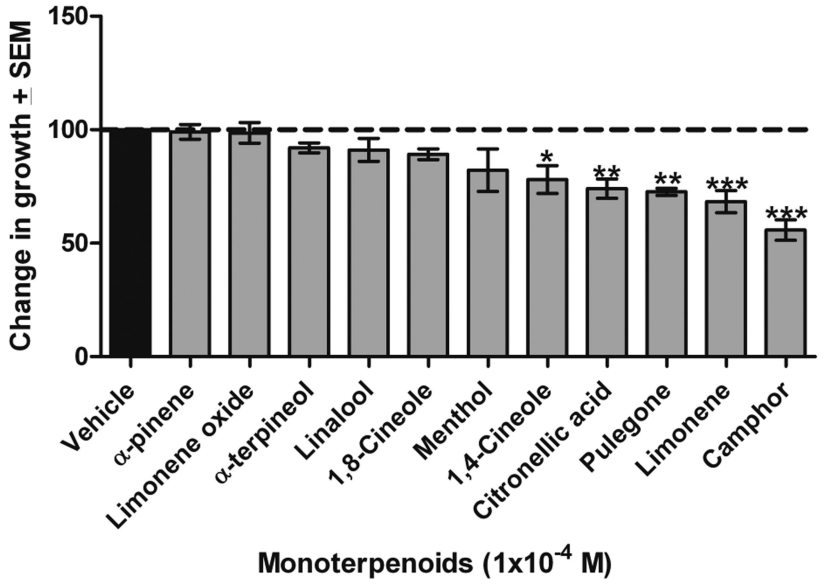

Figure 5. ***Growth responses of transformed yeast cells in response to monoterpenoids.*** *Effect of plant monoterpenoids on growth of yeast cells expressing Pa oa1 (* indicates a p-value 0.01 - 0.05; ** indicates a p-value 0.01 - 0.001; *** indicates a p-value < 0.001; ANOVA).*

In the present study we heterologously expressed Pa oa1 in an array of transgenic yeast strains that differed in their expression of chimeric G-protein alpha subunits. A receptor displaying ligand-independent activity was generated when expressed in one of these yeast strains. It is assumed that the receptor is in the activated state because it did not respond above basal levels to its previously identified ligand, octopamine at a high concentration (Figure 4), and displayed constitutive activity in the absence of the ligand (Figure 3). We believe that the screening system created here allows for the detection of molecules that are able to interact with a ligand-independent system. Previously it has been suggested that inverse agonists may block ligand-dependent activity because they prefer the inactive state of the receptor (*46*). The described screening system has the advantage of being able to identify ligands with negative intrinsic activity along with compounds that interacted with the receptor at sites which are distinct from the other steric binding site (*46*, *47*). Previously, drug discovery for mammalian targets used GPCRs heterologously expressed in *Xenopus laevis* melanophores to identify inverse agonists. These authors were able to screen for ligands that were antagonists and inverse agonists (*47*).

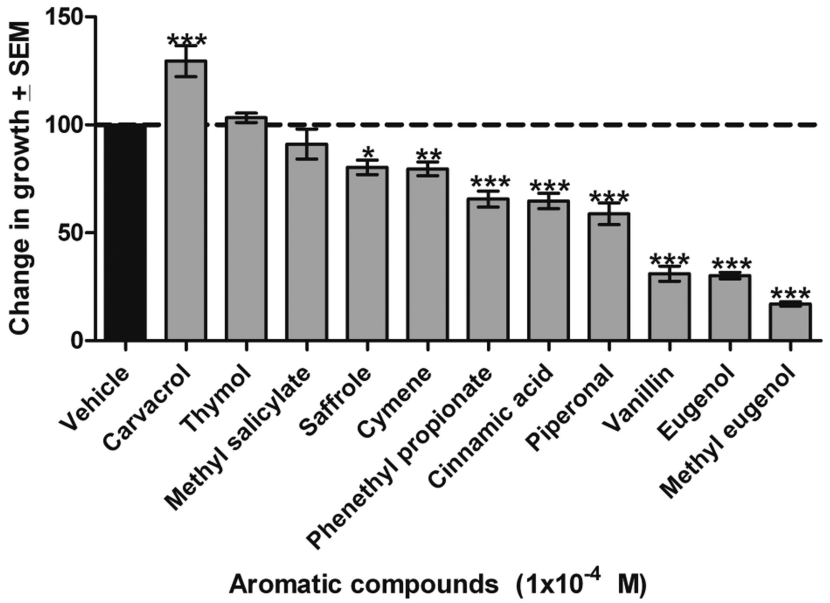

Figure 6. ***Growth responses of transformed yeast cells in response to aromatic compounds.*** *Effect of plant aromatic compounds on the growth of yeast cells expressing Pa oa1 (* indicates a p-value 0.01 – 0.05; ** indicates a p-value 0.01 - .005; *** indicates a p-value < 0.001; ANOVA).*

A search was initiated to identify compounds that would bind to the receptor and inactivate the receptor thereby changing the receptor's conformation. A two-state receptor model may be too simplified to describe the multiple conformations seen with GPCRs as we have demonstrated various degrees of efficacy. Therefore, we would expect to see differences in receptor interactions based on physicochemical differences of the ligands (partial or full inverse agonist, but allosteric modulation is also a possibility).

A variety of compounds that have been shown to interact with octopamine receptors were screened including synephrine, chlordimeform, and phentolamine (Figure 4). Synephrine, the N-methylated analog of octopamine, has high affinity for octopamine receptors (*48*, *50*). However, synephrine decreases the amount of second messenger production by some receptors, as seen with an octopamine/tyramine receptor from *D. melanogaster*. This receptor, when expressed in CHO-K1 cells, resulted in a significant increase in intracellular calcium when exposed to octopamine. However, a decrease in forskolin-stimulated cAMP occurred on exposure of the receptor to octopamine, tyramine, and synephrine (*48*). When synephrine was screened against yeast cells in our assay, it interacted with Pa oa1 to significantly decrease growth (48 ± 3%). Synephrine has not been previously investigated with Pa oa1 in a mammalian cell line to determine if it would affect second messenger production. Therefore, we cannot conclusively state that synephrine would decrease second messenger

production, but it appears to have an inhibitory action on Pa oa1 in our system. The formamidine insecticides, with chlordimeform as the lead compound, were octopaminergic compounds that have been shown to be toxic to insects; only one formamidine is available in the United States today. It is believed that the metabolite of chlordimeform (desmethyl chlordimeform) is a potent agonist to octopamine receptors (*35*). Chlordimeform has been suggested to inhibit the activity of adenylyl cyclase, acting on the enzyme instead of the octopamine receptor (*35*). Here we show that chlordimeform acts as a partial inverse agonist to a constitutively active octopamine receptor (Pa oa1), decreasing growth to 73 ± 5% when compared to the control. The results reported here do not rely on adenylyl cyclase as the output of the assay, and decrease in growth is observed with constitutively active cells. This suggests that chlordimeform is interacting with Pa oa1 and changing the receptor to an inactive state. Desmethyl-chlordimeform, a putative agonist, would be beneficial to test in this assay to see if the stimulatory effects were similar to carvacrol. Phentolamine, a nonselective α-adrenergic receptor antagonist and also an antagonist of octopamine receptors, was screened in our assay (*41*). Phentolamine interacted with Pa oa1, leading to partial inactivation of the G-protein to decrease cellular growth (Figure 4). This decrease in cellular growth may indicate that there are few true neutral antagonists, and most compounds that have effects are inverse agonists (*36*).

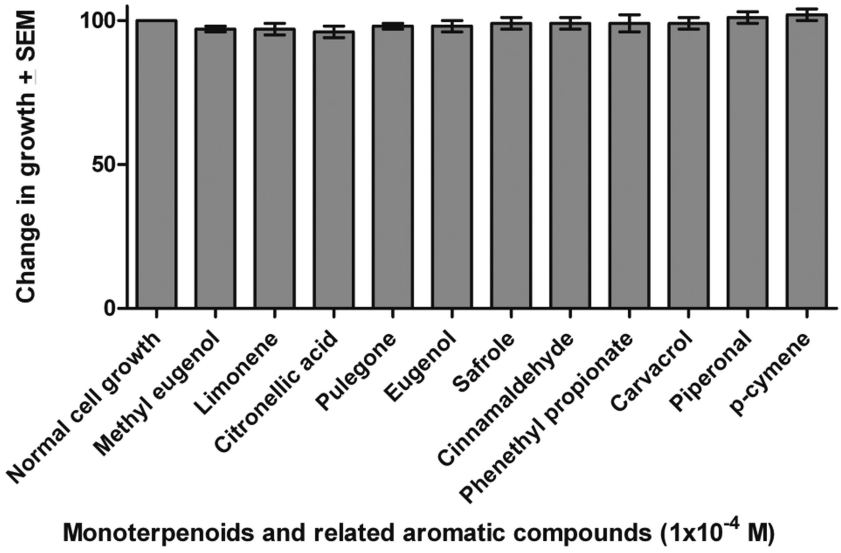

Figure 7. ***Growth responses of mock-transformed yeast cells in response to monoterpenoids and related aromatic compounds.*** *The monoterpenoids and related aromatic compounds that had a significant effect with yeast cells transformed with Pa oa1 where tested against cells that were not transformed with Pa oa1 to investigate if they had any effects in the absence of the receptor. Cells were grown in a medium that had all of the central nutrients. There were no significant results (ANOVA; α = 0.05).*

A variety of monoterpenoids and related aromatic compounds were screened in our assay (Figure 5), and several of these compounds did not significantly affect growth, and thereby did not interact with the expressed Pa oa1 receptor. However, some monoterpenoids did alter Pa oa1, leading to a significant decrease in yeast cell growth when compared to the vehicle control. Previously, pulegone was reported as an antagonist, and limonene was reported as having no effect (*17*). We are reporting both of these compounds, along with 1,4-cineol, citronellic acid, and camphor to be inverse agonists in this system. When aromatic compounds were investigated, more diverse effects on Pa oa1 were observed (Figure 6). Carvacrol is the only compound to show a stimulatory effect on Pa oa1, thereby resulting in yeast cell growth exceeding that of the control (Figure 6). This agonistic effect of carvacrol has been previously reported (*17*). However, since octopamine did not cause any significant increase in growth, presuming the receptor is fully activated, it may be possible that carvacrol is interacting with Pa oa1 at an allosteric site. It may be possible that the constitutive activity, resulting in different tertiary conformations of Pa oa1 protein, enables carvacrol to have a stimulatory effect. Eugenol is another aromatic compound that has been investigated for its octopaminergic capabilities. It has been reported as an antagonist in binding studies using American cockroach brain homogenates (*17*). However, this report was countered by electrophysiological data at the Dorsal Unpaired Median (DUM) neurons where eugenol did not behave like an agonist or antagonist. Instead eugenol decreased spontaneous nerve pulses, leading the authors to suggest that eugenol acts as a possible inverse agonist (*20*). This assumption, that eugenol exerts its actions as an inverse agonist, matches our results of eugenol decreasing yeast cell growth by interacting with Pa oa1 (Figure 6). In a separate study, when Pa oa1 was expressed in HEK-293 cells, eugenol decreased basal levels of cAMP, again consistent with what we report here. However, in the same cells, eugenol led to an increase in calcium release (*18*). Agonist-specific coupling has been suggested for some GPCRs (53). These receptors have the ability to respond differentially to the ligand thereby activating different second messenger pathways. Therefore, it is possible that eugenol would have an inhibitory effect on cAMP production but stimulate the liberation of intracellular calcium. We are only observing the inhibitory action of eugenol in our constitutively active assay. Tables 1 and 2, display a comparison of monoterpenoids and aromatic compounds and their interaction with the octopamine receptor from the American cockroach (*17*, *20*). The most significant comparison that can be concluded from Tables 1 and 2 is that compounds that were listed as antagonists were re-classified as inverse agonists with our assay.

A variety of chemical structures for related aromatic compounds with various functional groups were used in this study including: acids, aldehydes, hydrocarbons, phenols, ethers, esters, and alcohols (Figure 2). It has been suggested that a hydroxyl group increases the interaction between ligand and receptor (*18*). However, one exception was noted, with p-cymene displaying a significant decrease in growth presumably by interaction with Pa oa1. One interesting result that was obtained is the difference of the interaction of the closely related isomers thymol and carvacrol. Thymol did not interact with Pa oa1 and thereby did not alter the growth. However, carvacrol did interact with Pa oa1

and significantly increased growth of yeast cells, the only compound found to have such an effect. Safrole, which is structurally similar to piperonal (replacement of a propene group with a carboxyaldehyde), also showed significantly different interactions, and the presence of the carboxyaldehyde increased the ability of this compound to inhibit the activity of Pa oa1 (Figure 6). Significant structural differences are also present in the monoterpenoids that were screened in this study. Functional groups include acid, ether, alcohol, ketone, and epoxide (Figure 1); some of the compounds lacked a hetero atom, and some were acyclic (citronellic acid and linalool). A subtle difference, such as the connection of the cyclic ether in 1,8- and 1,4-cineole, resulted in a significant difference in interaction with Pa oa1. The presence of an epoxide group in limonene epoxide cased a decrease in its ability to interact with Pa oa1 when compared to limonene. Finally, the two ketones (camphor and pulegone) that were tested in this study show significant interaction with Pa oa1, thereby causing a decrease in growth (Figure 5).

The purpose of this study was to express Pa oa1 in the yeast, *S. cerevisiae*, to develop a high-throughput screening system to determine the efficacy of monoterpenoids and aromatic compounds from plant essential oils. This screening system does not address the second messengers produced as a result of ligand-receptor coupling. However, the screening system can be a powerful tool to the agrochemical industry to screen a large number of compounds or essential oils to determine their efficacy at an isolated receptor and identify lead compounds. While the expression resulted in a constitutively active receptor, we believe that the system still has value at identifying compounds that interact with Pa oa1. This may identify compounds that can be carried on to more robust, but more costly, assays such as mammalian systems.

There is no evidence that octopamine receptors are constitutively active in the American cockroach. Instead constitutive activity occurs as an artifact of heterologous expression in yeast (*37*). Some of the compounds have been reported to decrease basal levels of second messengers; it is possible that these compounds would inhibit the endogenous activity of octopamine. Toxicity studies are currently being performed in our laboratory to determine if there is a correlation between the activities of the tested compounds on this receptor and the whole-insect toxicity. Development of quantitative structure-activity relationship (QSAR) models will provide insight into the physicochemical properties that are important to octopaminergic activity. Future studies on a different chimeric yeast strain may provide a ligand-dependent model to screen agonist activity against this receptor.

It is possible that the monoterpenoids and related aromatic compounds have multiple mode of actions in insects and ticks. Some of the compounds that do not seem to affect octopamine have shown other neurological interactions (e.g. thymol shows effects at insect GABA receptors (*16*)). Natural product mixtures may result in more efficacious insecticides/acaracides, but this has not been fully investigated to date.

Acknowledgments

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Chapter 10

Activity of Plant-Based Compounds on Anthelmintic-Resistant *Caenorhabditis elegans*

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Anthelmintic resistance is a major global problem for livestock production and its incidence and severity have increased in recent years. With a paucity of new anthelmintics on the market, there is a need for efficacious treatments with novel modes of action. Four monoterpenoids, α-pinene, linalool, *p*-cymene, and thymol were examined for antinematodal activity in the free-living nematode, *Caenorhabditis elegans.* The order of toxicity was thymol > *p*-cymene > linalool > α-pinene, although no significant difference in mortality was observed between thymol, *p*-cymene, and linalool. Further tests were conducted using TT-7001, a blend of these monoterpenoids that exhibits 7.5-fold greater activity than expected, on three anthelmintic-resistant strains of *C. elegans*. Mortality did not differ between wild-type, benzimidazole-, levamisole-, and ivermectin-resistant *C. elegans* strains. Additionally, TT-1013, a variant of TT-7001 formulated for enteric release and to mask the flavor and fragrance of the oils, caused dose-dependent mortality after exposure to simulated small intestine conditions, while mortality did not differ from negative controls for formulated TT-1013 exposed only to simulated stomach conditions.

In Biopesticides: State of the Art and Future Opportunities; Coats, et al.;

Introduction

Gastrointestinal nematodes are a major threat to the health of humans and domestic animals worldwide. Three major drug classes exist for the treatment of these internal parasites. These are the benzimidazoles that target β-tubulin in nematodes leading to inhibition of microtubule formation, macrocyclic lactones which cause paralysis of pharyngeal pumping by increasing the opening of glutamate-gated chloride channels, and levamisole/pyrantel derivatives that act on nicotinic acetylcholine receptors in nematode muscles leading to spastic paralysis (reviewed by (*1*)). Extensive use of these anthelmintics has led to widespread resistance in parasites of livestock, including multi-drug resistance to all three classes (*2*, *3*).

Nematodes of veterinary importance exhibit a high probability of developing anthelmintic resistance due to rapid nucleotide sequence evolution, sizable populations, and host movement leading to increased gene flow (reviewed by (*2*)). Apart from the recent launches of the amino-acetonitrile derivative monepantel (*4*) and the spiroindole derquantel (*5*), no new drug class has entered the market since the introduction of ivermectin in 1981. Veterinary drug development has declined over the past two decades because of consolidation of animal health companies and the high cost associated with development of new pharmaceutical therapeutics (*6*). Plant-based compounds have demonstrated nematicidal activity against multiple helminth species (*7–10*) and may serve as an alternative to synthetic chemical anthelmintics or complement them in an integrated pest management program (IPM). Many plant compounds are commonly used in the flavor and fragrance industries and are classified as Generally Recognized as Safe (GRAS) by the United States Food and Drug Administration.

Terpenoids and related aromatic compounds are the primary constituents of many plant essential oils. In plants, terpenoids function as secondary metabolites and play important roles in plant defense against herbivory (*11*). These compounds appear to exert their activity on the nervous system of nematodes and other invertebrates by acting on various targets including the invertebrate-specific G protein-coupled receptors for octopamine and tyramine (*10*, *12–15*), acetylcholine esterase (*16–18*), and ionotropic gamma amino butyric acid (GABA) receptors (*19*, *20*). Multiple target sites of action may slow the development of resistance in nematodes. Additionally, targeting alternative modes of action to traditional anthelmintics may play a role in resistance management.

TT-7001, a synergistic blend of four monoterpenoid plant essential oil constituents: α-pinene, linalool, *p*-cymene, and thymol was developed for use as a functional food additive to control parasitic helminths in humans. One of these constituents, thymol has shown activity on the tyramine receptor, Ser2 in the free-living soil-dwelling nematode, *Caenorhabditis elegans* (*11*).

Caenorhabditis elegans has served as a model for anthelmintic screening for decades (*21*). Here we describe the activity of four monoterpenoids in *C. elegans*, and show synergy of a blend of these compounds, TT-7001 against wild-type and anthelmintic drug-resistant *C. elegans*. We also demonstrate the activity against *C. elegans* of TT-1013, a microencapsulated blend of α-pinene, linalyl acetate,

p-cymene, and thymol octanoate designed for enteric release and taste-masking of the aromatic essential oil compounds.

Materials and Methods

Caenorhabditis elegans Maintenance

Wild-type (N2 strain) and three anthelmintic-resistant *C. elegans* strains, genotypes unc-29 (e1072) I resistant to levamisole, ben-1 (e1880) III resistant to benzimidazole, and avr-14 (ad1302) I resistant to ivermectin were obtained from the *Caenorhabditis* Genetics Center (CGC, University of Minnesota, Minneapolis, MN) on nematode growth media (NGM). All nematode strains were maintained under monoxenic conditions using the OP50 strain of *Escherichia coli* received with each culture on NGM (*22*).

Caenorhabditis elegans Mortality Bioassay

Ivermectin and levamisole hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). Essential oil constituents (*a*-pinene, linalool, *p*-cymene, and thymol) were purchased from The Lebermuth Company (Mishawaka, IN). A stock solution of the essential oil blend was prepared using soybean oil (Harris Teeter, Matthews, NC) as a carrier. When examining synergy of the four constituents, TT-7001 was formulated without soybean oil. Working solutions of essential oils were made by diluting stock solutions in distilled water containing 1.2% Ryoto sugar ester S-1590 (RSE) (Mitsubishi Food Ingredients, Inc., Dublin, OH) as a surfactant. Serial dilutions of TT-7001 were tested against wild-type and anthelmintic-resistant strains of *C. elegans* to determine the LC_{50} for each strain. Levamisole hydrochloride was dissolved in distilled water. Ivermectin was first dissolved in dimethyl sulfoxide (DMSO) with further dilutions made with M9 buffer. Benzimidazole was not tested on wild-type or benzimidazole-resistant *C. elegans* because its insolubility in water made it incompatible with our testing methods. Doses are presented as ppm (µg/g) unless otherwise noted.

Caenorhabditis elegans were collected for mortality bioassays by rinsing plates with 1.5 mL M9 buffer (3.8 g Na_2PO_4, 1.5 g KH_2PO_4, 2.5 g NaCl, 0.5 mL 1M $MgSO_4$, Q.S. to 500 mL with dH2O). L4 and adult nematodes were selected by straining the M9 solution containing the worms through a 40 µm nylon cell strainer (BD Falcon, Franklin Lakes, NJ) allowing L1-L3 nematodes to pass through. L4 and adult nematodes were then rinsed from the strainer into a 50 mL conical tube (BD Falcon, Franklin Lakes, NJ) using M9 buffer and allowed to settle in the bottom of the tube before the supernatant was removed leaving 0.5-1.0 mL of worms in the tube. The nematode solution was then diluted to obtain a density of approx. 100 nematodes/750 µL.

To test compounds for mortality against *C. elegans*, 750 µL of diluted worm suspension was added to a 20 mL screw top scintillation vial (VWR, Radnor, PA). Two hundred fifty microliters of each dilution of the test compounds was then added separately to vials containing worms. Vials were swirled gently and the top was placed loosely on the vial. Vials were then placed in the dark and mortality

was assessed 16 h after treatment. Mortality was considered a lack of body and pharyngeal pump movement. Each treatment concentration and no-drug controls were replicated in duplicate or triplicate.

Average mortality was calculated as, (mean no. of dead worms / total number of worms)*100. Schneider-Orelli's formula (*23*) was used to account for mortality of *C. elegans* treated with the control vehicle (distilled water containing RSE for essential oils, DMSO + M9 buffer for ivermectin, and water for levamisole). Regression analysis was used to approximate the LC_{50} concentration for each monoterpenoid constituent and for the blend of these constituents, TT-7001, by plotting probit mortality versus log dose (*24*) using the method of least squares and inverse predictions of 95% fiducial ranges (*25*). The methods of (*26*) and (*27*) were used to estimate the variance-covariance matrix and 95% confidence intervals, respectively. Calculations were carried out in Excel spreadsheets as described previously (*28*). Average mortality caused by treatment with ivermectin or levamisole for wild-type and the respective anthelmintic-resistant strains were compared using the Student's t-test ($P < 0.05$).

To examine synergy of the essential oil components of TT-7001, α-pinene, linalool, *p*-cymene, and thymol were combined without soybean oil in their appropriate ratios on a weight:weight basis. Soybean oil aids in solubilization of the essential oil constituents, but does not contribute to the mortality of *C. elegans* (data not shown). Actual and expected mortalities for each constituent and the blend were compared to determine whether a synergistic effect was present. Expected mortality was calculated as,

$$E = (A_1Z_1) + (A_2Z_2) + (A_3Z_3) + (A_4Z_4)$$

Where, A_1 is the percentage of compound 1 in the mixture and Z_1 is the LC_{50} for compound 1 (*29*). The percentages of each essential oil are not provided because of the proprietary nature of the blend technology.

Simulated Digestion Protocol

The simulated digestion protocol was kindly provided by Kraft Foods (unpublished). Due to the light sensitivity of enzymes used in this protocol, all experiments were conducted under yellow light (CFL bug light, Sylvania, Winchester, KY). Stomach simulation solution (500 mL of 3 mg/mL pepsin, 2 mg/mL NaCl, pH 2.1) was prepared by adding 1.0 g of NaCl to 600 mL of deionized water in a beaker containing a magnetic stir bar on a stir plate set to medium speed. Upon total dissolution, stirring was reduced to minimum, and concentrated HCl was added until the pH reached 1.95-2.10 at 22.3° C. One and a half grams of pepsin from porcine gastric mucosa (Sigma-Aldrich, St. Louis, MO) was added and allowed to dissolve for 10 min. The contents of the beaker were then transferred to a plastic Nalgene bottle and refrigerated. Two concentrations (0.27 and 1.35%) of TT-1013 and empty encapsulate (negative control) (Kraft Foods, Glenview, IL) were tested for mortality to *C. elegans*. Samples were weighed into 20-mL glass screw cap headspace vials (Sigma-Aldrich, St. Louis, MO) and 7.3 g of stomach solution were added to each vial. Vials were capped

and rotated 360° on a rotator base (Glas-Col tissue culture rotator, VWR, Radnor, PA) at an angle of 60°, speed of 4 rpm at 37° C for 30 minutes.

Small intestine simulation solution (200 mL of 3.24X pancreatin, 8X concentrate NaOH, and bile salts, pH 6.8) was prepared as follows. Porcine bile extract (Sigma-Aldrich, St. Louis, MO) was preconditioned according to USP specifications by heat treating 1.98 g at 105° C for 4 h followed by 20 minutes in a desiccator at room temperature. Forty milliliters of deionized water (25 ± 1° C) were placed into a beaker containing a magnetic stir bar on a stir plate set to low. To this, 6.71 g pancreatin from porcine pancreas (Sigma-Aldrich, St. Louis, MO) was added and stirred for 15 min. In a separate beaker with a magnetic stir bar, 50 mL of deionized water (25 ± 1° C) and the heat-treated bile salts were added and mixed on low until dissolved. The pH was adjusted to 7.1 by the addition of 0.5 N NaOH. The pancreatin solution was then added to the bile salts solution by gently pouring down the side of the beaker wall. The beaker that previously contained the pancreatin was rinsed twice with 2 mL of DI H_2O. The pH was adjusted to 7.05 by the addition of 0.5 N NaOH. One hundred and fifty milliliters of DI H_2O was added to the beaker. The mixture was then transferred to 50-mL conical tubes and centrifuged at 10,000 x *g* for 20 min at 22°C. The supernatant was transferred to a plastic Nalgene container and immediately refrigerated. A premix solution was prepared in a glass beaker by combining 87.50 g of small intestine solution with 182.50 g pepsin and 6.85 g 0.5 N NaOH each per sample on an ice bath. Final pH of the premix solution was 7.74 at 7° C.

For small intestine simulation bioassays, 11.0 g of premix solution was added to each labeled vial. Vials were capped and stored on an ice bath at 7° C. Samples (0.02 and 0.10 g TT-1013 or empty encapsulate) were weighed into headspace vials containing premix solution, capped and stored at room temperature after sample addition. Samples were then rotated for 6 h on a rotator base as previously described. After incubation, samples were removed from the rotator and cooled to ambient temperature in a water bath before being tested in mortality bioassays as described above. Each concentration of treatment and control was run through the stomach and small intestine digestion simulations in duplicate (biological replicates) with three technical replicates conducted from each biological replicate. Mean percentage mortality was calculated as previously described with simulated stomach or small intestine solution serving as the negative controls for control mortality adjustment using the Schneider-Orelli formula.

Results

Of the four monoterpenoids tested for *C. elegans* mortality, thymol exhibited the lowest LC_{50}, followed by *p*-cymene and linalool. The efficacy among these three compounds, however, did not differ significantly (log probit analysis, $P < 0.05$) (Table I). No difference in mortality was found between *C. elegans* treated with TT-7001 containing soybean oil (Table I) and TT-7001 without soybean oil (Table II) based on 95% fiducial limits. α-Pinene, linalool, *p*-cymene, and thymol were 7.5-fold more active than expected when combined in the ratio in TT-7001, demonstrating synergy.

Table I. Median Lethal Concentration of Monoterpenoids and TT-7001 (without the Carrier, Soybean Oil) against Wild-Type (N2 strain) *C. elegans* Measured 16 h after Treatments Were Applied to Worms in Scintillation Vials (3 Replicates Per Treatment Dose)

Compound	n^a	LC_{50} *(ppm)*[b]	*95% FL*[c] *(ppm)*	χ^{2d}	*Slope ± SE*	r^2
α-pinene	1943	11797 C[e]	6382 - 21644	4.38	2.90 ± 0.26	0.98
linalool	2557	529 B	310 – 827	17.05	10.55 ± 1.63	0.96
p-cymene	2359	486 B	244 - 931	21.08	2.94 ± 0.38	0.97
thymol	1802	121 AB	58 – 388	170.88	6.82 ± 1.88	0.94
TT-7001 without soybean oil	1891	120 A	76 – 165	7.98	10.91 ± 1.65	0.98

[a] Number of *C. elegans* tested [b] Median lethal concentration from probit analysis. Mortality was considered the lack of motility (straightened body and lack of pharyngeal pumping). [c] Fiducial limits from probit analysis [d] Chi-square value from probit analysis [e] Values followed by a different letter indicate a statistically significant difference based on fiducial limits (log probit analysis, $P < 0.05$).

Table II. Median Lethal Concentration of TT-7001 against Wild-Type and Three Anthelmintic-Resistant Strains of *C. elegans* Measured 16 H after Treatments Were Applied to Worms in Scintillation Vials (2-3 Replicates Per Treatment Dose)

Worm strain	n^a	LC_{50} *(ppm)*[b]	*95% FL*[c] *(ppm)*	χ^{2d}	*Slope ± SE*	r^2
Wild-type (N2)	961	162 A[e]	66 - 347	19.93	6.73 ± 1.22	0.94
Benzamidazole-resistant (ben-1)	1179	172 A	30 - 690	73.93	5.43 ± 1.28	0.90
Ivermectin-resistant (avr-14)	813	202 A	123 - 326	4.23	5.74 ± 0.56	0.98
Levamisole-resistant (unc-29)	724	78 A	31 - 168	160.86	2.64 ± 0.44	0.96

[a] Number of *C. elegans* tested [b] Median lethal concentration from probit analysis. Mortality was considered the lack of motility (straightened body and lack of pharyngeal pumping). [c] Fiducial limits from probit analysis [d] Chi-square value from probit analysis [e] Values followed by a different letter indicate a statistically significant difference based on fiducial limits (log probit analysis, $P < 0.05$).

Activity of TT-7001 did not differ between wild-type, benzimidazole-, levamisole-, or ivermectin-resistant *C. elegans* strains (Table II). Ivermectin and levamisole were tested against their respective anthelmintic strains to verify phenotypic resistance. Ivermectin was 20.0-fold more active against the N2 strain compared to the avr-14 strain when tested at 1 ppm (Table III). Similarly, levamisole at 10 ppm was 27.1-fold more active against the N2 strain compared to the unc-29 strain. In both cases, mortality was significantly greater (t-test, $P < 0.0001$) for wild-type vs. anthelmintic-resistant nematodes when treated with their respective drug, demonstrating active resistance.

Table III. Mean Percentage Mortality (± 1 SEM) of Wild-Type and Anthelmintic-Resistant *C. elegans* 16 h after Treatments (Ivermectin or Levamisole) Were Applied to Worms in Scintillation Vials (2-3 Replicates Per Treatment Dose)

	Ivermectin (1 ppm)[a]	*Levamisole (10 ppm)*[b]
Wild-type (N2)	79.8 ± 2.2*	100 ± 0.0*
Ivermectin-resistant (avr-14)	4.0 ± 1.1	nt[c]
Levamisole-resistant (unc-29)	nt	3.7 ± 0.3

[a] Control mortality = 0.00% [b] Control mortality = 2.27% [c] nt = not tested * Indicates a significant difference (t-test, $P < 0.0001$) between the wild-type and anthelmintic-resistant *C. elegans* strain for a given treatment (ivermectin or levamisole).

Table IV. Mean Percentage Mortality (± 1 SEM) of *C. elegans* Exposed to Encapsulated TT-1013 and Empty Encapsulate under Simulated Stomach or Small Intestine Conditions (2 Biological with 3 Technical Replicates Per Treatment)

Sample	*Concentration (%)*	*Stomach simulation (30 min exposure)*	*Small intestine simulation (6 h exposure)*
Empty encapsulate	2.74	2.4 ± 0.7	0.0 ± 0.5*
	13.70	2.3 ± 0.6	1.3 ± 0.7*
TT-1013	2.74	5.8 ± 1.1	68.6 ± 1.7
	13.70	7.7 ± 1.4	100 ± 0.0

* Indicates a significant (t-test, $P < 0.0001$) difference between the empty encapsulate and TT-1013 at the same dose.

Microencapsulated TT-1013 and the empty encapsulate were tested against *C. elegans* after exposure to simulated stomach or small intestine conditions. Mortality of *C. elegans* did not differ between TT-1013 and the control at the low or high dose ($P = 0.45$ and 0.16, respectively) after a 30-minute exposure to the pH

and enzymatic conditions of the stomach, suggesting that the essential oils were not released under these conditions. However, under simulated small intestine conditions, *C. elegans* treated with TT-1013 demonstrated dose-dependent mortality, with the top dose achieving 100% mortality. Conversely, under the same conditions, no mortality was observed when *C. elegans* were treated with the low dose of empty encapsulate, and mortality was only 1.3% for the high dose (Table IV). These large differences in mortality of *C. elegans* under simulated small intestine conditions were significant between both doses of TT-7001 and the empty encapsulate controls (t-test, $P < 0.0001$; Table IV).

Discussion

Monoterpenoids from plant essential oils have demonstrated nematicidal activity (*8*, *11*, *30*) and are documented to act upon multiple target sites in nematodes (*11*) and other invertebrates (*16–20*). These sites are all different from the known modes of action of the three major anthelmintic drug classes. In this study, four monoterpenoids were tested, alone and in combination, for activity against wild-type *C. elegans*. Similar to the results of other studies (*8*, *11*, *30*), thymol was a potent nematicide. Evidence suggests that thymol, a major constituent of English thyme (*Thymus vulgaris* L.) essential oil (*31*), exerts its activity by binding to the tyramine receptor, Ser2 in *C. elegans*. This compound is also toxic to the parasitic pig roundworm, *Ascaris suum* Goeze (*11*), presumably by the same mechanism of action. Like thymol, linalool appears to exert its activity on GPCRs, as intracellular calcium release is observed in calcium mobilization assays in HEK293 cells stably transfected with the *C. elegans* Ser2 receptor (data not shown). Neither *p*-cymene (*11*) nor α-pinene (data not shown) elicit calcium mobilization in the transfected cell system. To our knowledge, the molecular mode of action for these compounds in nematodes has yet to be elucidated, although *p*-cymene demonstrated agonistic behavior for the American cockroach (*Periplaneta americana* (L.)) octopamine receptor in ligand binding assays (*12*), and linalool is an inhibitor of acetylcholinesterase (*32*).

Activity of TT-7001 against drug-resistant *C. elegans* strains from each of the three major anthelmintic classes was equivalent to that of the N2 wild-type strain. This verifies that the activity of monoterpenoid blend is unaffected by the genes that confer resistance in these *C. elegans*. TT-7001 with its apparent alternative mode of action versus these drugs, combined with the different mechanism of action of *α*-pinene and *p*-cymene compared to linalool and thymol, could serve as an alternative control measure to the current treatments, particularly if implemented in a multi-approach IPM program.

Essential oils are comprised of highly volatile, aromatic compounds. One difficulty with formulating these compounds for consumption by humans or domestic animals is that their flavor and odor profiles can be unpleasant. Microencapsulation is a technique used in the pharmaceutical industry to mask the flavors of unpalatable compounds (*33*). The encapsulation technique used in the formulation of TT-1013 was developed for delivery of TT-7001 as a functional food additive to reduce parasitic helminth infections of humans. When

fed daily to pigs at a dose of 1 mg/kg body weight, TT-1013 reduced the number of *A. suum* by 76.8% and fecal egg counts by 68.6% compared to placebo-treated pigs (Kaplan et al., unpublished). Although not tested, it is plausible that infection by *A. suum* could be eliminated by treatment with TT-1013 at a higher dose. Anthelmintic-resistant gastrointestinal nematodes threaten the health and well-being of all classes of livestock animals and widespread multi-drug resistance is common (*3*). TT-1013 was formulated for enteric release in the monogastric human gut. Results of the present study along with those conducted in pigs (Kaplan et al., unpublished) suggest that the active compounds survive the pig stomach and simulated conditions of the human stomach but are released under those of small intestine. Further formulation would be necessary to overcome the multiple stomach compartments and different enzymatic environment of the ruminant gut. Additionally, testing would need to be carried out against ruminant parasites of the abomasum (true stomach) and small intestine such as *Haemonchus contortus* (Rudolphi) and *Trichostrongylus colubriformis* (Giles), respectively.

Widespread and multi-drug resistance in gastrointestinal nematodes of animals necessitates the development of alternative control strategies and therapeutics. Furthermore, the development of resistance to currently used anthelmintics in human mass drug treatment programs for nematode control is a serious concern (*34*). Drug development and registration of a new active ingredient is expensive and time consuming. Alternative treatments based on plant compounds, particularly those that are GRAS-listed leading to reduced registrarion time, may play an important role in managing parasite resistance. The current study demonstrates that four individual essential oil constituents and a blend of these compounds provide nematicidal activity. Additionally, these compounds have successfully been formulated so that their flavors and fragrances are masked, and release occurs under the conditions of the small intestine of monogastrics. TT-7001 and TT-1013, with their activity upon resistant nematode strains, may provide a means to improve parasite control and help to manage the problems posed by anthelmintic-resistant nematode parasites.

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Chapter 11

Botanical Nematicides, Recent Findings

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Following the withdraw of synthetic nematicides such as organophosphates, carbamates and methyl bromide there is a great need for new compounds to control phytonematodes. The repetitive use of the few currently available commercial nematicides and the subsequent increase of microbial biodegradation in soil, led to low efficacy on nematode control under field conditions. On the other hand, high nematode population favors secondary infestations with other soil pathogens that even lead to total crop damage. Thus, new molecules with high nematicidal are needed as potential prototypes for synthesis of new nematicidal compounds by the industry. In recent years, botanical species are studied and exploited in terms of nematicidal properties in the frame of integrated crop management programs. To date, the efficacy is attributed to specific compounds that are characterized as active ingredients, and this is feasible due to the availability of analytical instruments and the familiarity with purification techniques that favor bioassay-guided fractionation. Nonetheless, the delineation of the biochemical mode of action of these compounds is a missing piece in the botanical's nematicidal efficacy puzzle. Herein we report the most recent chemical groups of botanicals compounds with nematicidal activity, together with their mode of action.

In Biopesticides: State of the Art and Future Opportunities; Coats, et al.;

Introduction

Root-knot nematode (*Meloidogyne* sp.) is the most destructive plant parasite nematode worldwide (*1*, *2*). While the global population is expanding, food needs are a challenge that is further complicated by consumer and growing regulatory concerns. In that context, biopesticides are the mainstream option for integrated crop management because they can provide with efficacy and at the same time reduce the risk of resistance management and pesticide residues, increasing production in a sustainable agriculture frame. In recent years the interest in the botanical nematicides has been continuously growing also as a result of the shortage of commercial formulates that can control phytonematodes, following arising problems such as environmental pollution, persistence and microorganisms degradation mechanisms according to the European Council Directive 91/414 and 1107/2009. Herein we present the latest data of the nematicidal activities of plant derived substances used in their pure form or as ingredients in complex plant mixtures, focusing on their modes of actions when available. Nematicidal activity is usually expressed as a paralysis effect on the second stage juveniles inducing infection or as egg hatch arrest, but the mechanisms of action are yet to be discovered in most of the natural nematicides cases.

The first barrier a nematicide must surpass to enter in the nematode body is the external cuticle. The cuticle of the infective juvenile of endoparasitic phytonematodes straddles two environments, the soil and the plant, moving between which the nematodes must adapt to both (*3*). An understanding of this biology plays a key role in developing new, novel, and environmentally benign nematode control strategies. The way nematodes adapt to and survive plant defense processes is by surface switching and increase in the lipophilicity according to chemosensation. In fact, the increase in the lipophilicity of the nematode surface may alter the permeability barrier of the nematode cuticle and may control the uptake of water, ions and lipids in the nematode, which are important for cell signalling (*3*). To date, none of the available commercial or natural nematicides are reported to damage the nematode cuticle despite the principle importance of its role in the activity of nematicides.

Isothiocyanates

Glucosinolates are secondary metabolites contained in Cruciferae. Upon plant tissue damage, they are hydrolized by myrosinase to various pungent products, among most active of which are isothiocyanates (*4*). The high number of glucosinolates is a consequence of chemical diversity of their side chain (R-group), which is derived from several amino acids. The use of Brassicaceae in nematode control is perhaps the most studied green manure technique. In general, replenishing the farm system with organic matter as either residues or amendments influences the chemical, physical and biological processes. Studies to identify which practices have the greatest positive effect on plant growth and plant disease are challenging and benefit from a multi-disciplinary approach (*5*). For example, *Ochradenus baccatus* (Resedaceae, Brassicales) as a very poor host status to *Meloidogyne javanica*, *M. incognita* and *M. hapla*, could be used

as a trap plant (*6*), whereas application of water cress (*Nasturtium officinale*) macerate, rich in mustard glycosides (glucosinolates), can reduce to zero percent *Meloidogyne* spp. infection in tomato plants (*7*).

In general, glucosinolates have little biological activity and act as precursors of derivatives acting against soilborne pests and diseases. Nonetheless, some glucosinolates like epi-progoitrin (2(S)-2-hydroxy-3-butenyl-GLS), glucoerucin (4-methylthio-butyl-GLS) and glucoiberin (3-methylsulfinylpropyl-GLS) display a significant biocidal activity (50%) regardless of their myrosinase-catalyzed hydrolysis, although addition of the enzyme to the bioassay mixture enhances activity to even 100% (*8*). Glucosinolates react with biological nucleophiles essential for the nematode, mainly thiol and amine groups of various enzymes which become irreversibly alkylated. On the other hand, isothiocyanates are of considerable activity on proteins (*9*) and contain a –N=C=S group, the electrophilic central carbon, which is an active site in the compound playing an important role to biological activity (*8*). Other nucleophilic reaction centers (–OH, –S–, –S=O) contained by glucoerucin, glucoiberin, epi-progoitrin and progoitrin and their cognate isothiocyanates can possibly induce other types of biological interactions with the nematode (*8*). Dead *M. incognita* J2s treated with degradation products from gluconasturtiin, glucotropaeolin, glucoerucin, sinigrin, and glucoraphasatin show a straight and rigid appearance and browning of internal organs, whereas after glucoconringiin and gluconapin degradation products treatment dead larvae sometimes appear to be wound on themselves. The immobile larvae are not able to penetrate the roots even if they cannot be considered as dead (*10*). Last, aliphatic isothiocyanates were found to have stronger biofumigation effects than aromatic isothiocyanates in brassicas (*11*).

A drawback in the practical use of isothiocyanates is their high volatility that makes them unstable under field conditions (*12*) and should thus be taken into consideration since it decreases their residual activity. There are though some practices that can enhance the efficacy *in situ*. The size of Brassicae plant particles incorporated into the soil affects release of isothiocyanates, and in particular coarsely chopped material reveals less isothiocyanates than thoroughly pulverized plant material. Additionally, coverage of soil with polyethylene may trap isothiocyanates to some extend, although polyethylene is permeable to methyl isothiocyanate and maybe also to larger isothiocyanates (*13*). Finally, increasing the water content of the covered soil can help to retain the bioavalaibily of isothiocyanates via partitioning to the water phase and may increase chemical exposure (*14*).

Nonetheless, glucosinolate types in *O. baccatus* and the released isothiocyanates were not toxic to *M. javanica* (*11*). In the same context, recent studies prove that in the case of glucosinolates fumigation properties its the intense mechanical disturbance, green manure and the absence of host plants for obligatory plant parasitic nematodes that exhibit the nematicidal activity rather than the release of isothiocyanates (*15*).

Interestingly, Liliaceous crops of the genus *Allium* spp.; are known to contain sulphur compounds which are hydrolyzed to form a variety of nematicidal isochiocyanates (*16*). Aqueous extracts of garlic cloves and castor seeds were found to be nematicidal against the root knot nematode *M. incognita,* infecting

tomato. When used at 20 ml of 10% extracts per pot, they reduced significantly the number of galls and egg masses on host roots as well as the number of juveniles in roots and soil, compared to Cadusafos 10G (Rugby) used at 0.02 g/pot (*17*). *Armoracia rusticana* fresh roots water-methanol extract was found nematicidal against *M. incognita* $EC_{50/3days}$ = 251 mg/L and the active ingredient was found allylisothiocyanate with an $EC_{50/3\ days}$ value of 6.6 mg/L (*12*). Raw garlic straw (2%) combined with plastic film covering and rabbit manure, inhibited by 72.4% the galling index of *M. incognita* incidence and increased tomato yield by 72.6% when compared with control treatment (*18*).

The proposed antimicrobial mechanism of action of isothiocyanates is the disruption of the major metabolic processes and the eventual cell death. In specific, benzyl isothiocyanate was found to induce on *Campylobacter jejuni* a progressive oxygen consumption impairment, shift in the energy metabolism balance, ATP content increase and lastly intracellular protein aggregation (*19*).

In fungi, bacteria and insects isothiocyanates act by inhibiting the oxygen uptake through the uncoupling oxidative phosphorylation in mitochondria. They inhibit the coupling between the electron transport and phosphorylation reactions and thus eventually hinder ATP synthesis. In bacteria isothiocyanates also act by inactivating various intracellular enzymes of the pathogen by oxidative breakdown of S–S– bridges present in the enzymes. In insects they inactivate the thiol group of essential enzymes of the pest or by alkylating the nucleophilic groups of biopolymers like DNA or as uncouplers they affect respiration, eventually leading to death (*20*). Anticancer activity studies reveal isothiocyanates interactions with topoisomerase II, an essential enzyme that regulates DNA supercoiling and removes knots and tangles from the genome. Intermediates in the catalytic cycle of the enzyme convert topoisomerase II to a cellular toxin that fragments the genome (*21*). Further studies on nematodes physiological and biochemical functions are needed for clear assignment of mechanisms of action of isothiocyanates.

Organic Acids

Acetic acid together with other organic acids produced in soil treated with wheat straw residues, due to anaerobic decomposition, and microbial degradation (*5*) is nematicidal against *Meloidogyne* spp. and the $EC_{50/1d}$ value was calculated at 38.3 mg/L (*22*). Also other acids have been found of significant nematicidal activity against *Meloidogyne* spp. like butyric acid and hexanoic acid, exchibiting an $EC_{50/1d}$ value of 40.7 and 41.1 mg/L (*22*). *Mentha piperita*, *M. pulegium* and *M. spicata* water extracts exhibit significant nematicidal activity against *M. incognita*, and the $EC_{50/72h}$ values were calculated at 1,005, 745, and 300 mg/L, respectively. The extracts yielded mainly chlorogenic acid, salvianolic acid B, luteolin-7-O-rutinoside, and rosmarinic acid (*23*). 3,4-dihydroxybenzoic acid from *Terminalia nigrovenulosa* bark provokes 94.2% J2 mortality at 1.0 mg/ml, after 12 h incubation and 85.0% hatch inhibition 3 days after incubation. Fluorescence dyeing of the eggshell surface clearly demonstrated deformation of the egg shapes and the inside layer appeared to be destroyed (*24*). This

information is very important in the context that the three-layer shell and the inner lipo-protein layer, makes the egg stage the most resistant stage in the nematode's life cycle (*25*). Caffeic and chlorogenic acids are reported as active free radical scavengers being involved in modulation of enzymatic activity and alteration of signal transduction pathway (*26*, *27*). *β*-aminobutyric acid and salicylic acid foliar applications on tomato plants reduce the development and reproduction of *Meloidogyne chitwoodi* in tomato plants, by triggering plant defence mechanisms. This chemically-induced resistance to plant parasitic nematodes has yet to be studied widely although it may be a practical alternative to current methods of root-knot nematode control (*28*). 4-hydroxyphenylacetic acid, isolated from cultures of the fungus *Oidiodendron* sp. exhibits nematicidal activity against the root-lesion nematode, *Pratylenchus penetrans*, and the pine wood nematode *Bursaphelenchus xylophilus* (*29*).

Amino Acids and Proteins

On the other hand, amino acids like DL-methionine, sodium methionate, potassium methionate, and methionine hydroxyl analog used at rates of 224 and 448 kg amino acid/ha reduced the number of *M. incognita* J2 in soil (*30*), while DL-methionine, DL-valine, DL-serine, DL-phenylalanine, L-proline and L-histidine reduced the number of galls, adult females, egg masses and juvenile stages of *M. javanica* in tomato plants, and to enhance plant growth characters (*31*). In the same frame several families of crystal proteins from *Bacillus thuringiensis*, like Cry5, Cry6, Cry12, Cry13, Cry14, Cry21, and Cry55, exhibit nematicidal activity (*32*, *33*).

The recently discovered Cry6A, sharing very low homology with Cry5B at amino acid sequence, suppress growth, decreased brood size, and even abnormal motility in *C. elegans* (*34*). Cry6Aa2 has been also found of high toxicity to *M. hapla* (*35*). The nematicidal agent in seaweed, betaine, is an amino acid that functions as an osmolyte and methyl donor. Mutating, betaine transporter SNF-3, in a sensitized background caused the worms to be hypercontracted and paralyzed, presumably as a result of excess extracellular betaine (*36*).

Terpenoids

Terpenoids constituting essential oil mixtures are nematicidal natural compounds of great importance (*37*) because they are easily extracted, are of high residue yields, they can be developed for use as nematicides themselves or can serve as model compounds for the development of derivatives with enhanced activity (*38*). In that context, carvacrol, thymol, nerolidol, *α*-terpinene, geraniol, citronellol, farnesol, limonene, pseudoionone and eugenol were found nematicidal against *C. elegans* in a descending order, on paralysis tests at concentrations varying from 3 to 30 μg/mL (*39*). Plant parasitic nematodes share many common and highly conserved biological processes with the free-living nematode *C. elegans* and for this reason the latter is often used as a laboratory model for screening nematicidal compounds. Similarly, L-carvone, pulegone,

trans-anethole, geraniol, eugenol, carvacrol, thymol, terpinen-4-ol EC_{50} values (24 h) of activity against *M. incognita* were calculated in the range of 115-392 μg/mL (*40*). The essential oil of *Agastache rugosa* flowering aerial parts was found active on *M. incognita* (LC_{50} value of 47.3 μg/mL), and some of the major components like eugenol (LC_{50} = 66.6 μg/mL) and methyl eugenol (LC_{50} = 89.4 μg/mL) exhibited significant activity (LC_{50} = 185.9 μg/mL) (*41*). Most interestingly, synergic activity has been reported for paired terpenes against *Meloidogyne* species, and the most promising ones were trans-anethole/geraniol, trans-anethole/eugenol, carvacrol/eugenol and geraniol/carvacrol (*42*). Moreover, important effects on nematodes egg hatch are attributed to the activity of terpene mixtures, like *Dittrichia viscosa* essential oil exhibiting 100% inhibition at 5 μg/mL against *M. incognita*. When plant parts containing terpenes like *Citrus sinensis* lyophilised pulp are incorporated in soil as amendments, they exhibit activity against *M. Javanica*, and the EC_{50} value was calculated at 2.0 mg/g in terms of ♀/g root (*43*).

In *Aedes aegypti*, terpenes have been found to bind to octopamine receptor and acetyl cholinesterase protein models, and most interestingly some of them were found to be selective since they did not act against the respective human protein models (*44*). Additionally, some essential oils were found to cause a significant increase in the levels of the intracellular messenger, cyclic adenosine monophosphate of abdominal epidermal tissue in *Helicoverpa armigera* (*45*).

On the other hand, terpenoids of higher molecular weight, like saponins possess significant nematicidal activity as well. Soil amendments with green or dry biomass from *Medicago* spp. applied at 40 g/kg soil exhibit significant nematicidal potential, due to the saponins contents, while plant growth and crop yield increase are also noted (*46*). The methanol extract of *Pulsatilla koreana,* rich in triterpenoid saponins, exhibiting an $LC_{50/48h}$ value of 92.8 μg/mL against *M. incognita*, disintegrates the internal structures of the nematodes body by emptying somatocysts and forming vacuoles (*47*). Saponins as insecticidal agents are known to induce anti-feedancy, disturbance of the moulting and growth regulation, and to interact with alimentary cholesterol causing a disturbance of the synthesis of moulting hormone (*48*). On fungi, saponins interact with cell membrane constituents, like sterols, phospholipids and proteins, disrupting and increasing their ion permeability. The side sugar chains attached to the saponins are very important, as monodesmoside saponins are more active than bidesmosides (*49*). An effect usually seen with saponins having sugar moieties attached to a cholesterol-like core, is leaking or disruption of the cell membrane (*50*).

Cannabinoids

Aqueous extracts of *Cannabis sativa* and *Zanthoxylum alatum*, rich in cannabinoids, were assessed on hatching, mortality and infectivity of *M. incognita* (*51*), and they were found to induce immobilization, incapacitation, mortality, poor penetration and later retardation in different activities of J2s. In fact, only few nematodes infest the roots of the hemp (*Cannabis sativa*). The activity of

9-tetrahydrocannabinol in humans is exerted via a family of G protein-coupled receptors, known as cannabinoid (CB) receptors (*52*) but these are absent in *C. elegans* (*53*).

Alkaloids

The ethanol extract of Chinese medicinal herb, *Evodia rutaecarpa* unripe fruits, of significant nematicidal activity on *M. incognita* (LC_{50} = 131.54 g/mL) was found to contain among major constituents the alkaloids evodiamine, rutaecarpine, and wuchuyuamide I (LC_{50} = 73.55, 120.85 and 147.87 g/mL) (*54*). The nematicidal extract of *Tithonia diversifolia*, inhibiting *M. incognita* egg hatch by 98% from 2 day, is attributed to alkaloids (*55*). Alkaloids of *Fumaria parviflora* are reported to possess activity against *M. incognita* (*56*), possibly by intercalating with DNA and inhibiting its synthesis (*57*). Preparations rich in cephalotaxine alkaloids (tetracyclic spirobenzazepines) and the purified component drupacine, were recently found to inhibit the protease activity in the free living nematode *Panagrellus redivivus*. The identification of specific proteases in *M. incognita* that are inhibited by alkaloids, and the physiological processes involved, can help in revealing the metabolic effects in plant-parasitic nematodes and in designing control strategies incorporating these phytochemicals (*58*). Finally, nematodes have ryanodine receptors and thus are sensitive to the plant alkaloid, ryanodine. These receptors regulate the release of stored intracellular calcium and play an important role in muscle contraction (*59*).

Lactones

Artemisia annua aqueous extract, rich on lactones, as well as its chemical components artemisinin and artesunate were found nematicidal against *M. incognita*, *Globodera rostochiensis* and *Xiphinema index*. The toxicity among different nematode species varied maybe due to differences in their anatomy and feeding behaviour (*3*, *60*). Artemisinin was more toxic than artesunate suggesting that the lactone function of the molecule plays a role in the bioactivity against nematodes, in agreement with previous findings showing a similar action towards SERCA-type enzymes of artemisinin and thapsigargin, a sesquiterpene lactone lacking the endoperoxide pharmacophore (*61*).

Polyphenols and Phenolics

Phenolic compound accumulation in the root system of tomato after treatment with *Arthrobotrys oligospora* and salicylic acid, provides efficient control against *M. javanica* (*62*). Similarly, high contents of total phenolics in cucumber roots treated with *Saccharomyces cerevisiae* and hydrogen peroxide reveals the ability of the yeast to induce plant resistance, and to thus protect it from *M. javanica* (*63*). Chalcones, which are flavonoid precursors in plants and can be easily prepared

from inexpensive reagents by aldolic condensation between benzaldehydes and acetophenones, when tested *in vitro* against *M. exigua* J2 they caused significant paralysis. In particular, chalcone LC_{50} value was 171 µg/ml while the respective value of the commercial nematicide carbofuran was 260 µg/ml. Chalcone appeared to target a putative caffeic acid 3-*O*-methyltransferase homodimer in the nematode. This enzyme, together with chalcone *O*-methyltransferase and chalcone isomerise, are used by plants as defenses against nematodes and although not found in the genome database of *M. exigua*, since amino acid sequences were identified for these three enzymes in similar nematode species, it was assumed that *M. exigua* should be able to produce caffeic acid 3-O-methyltransferase (*64*).

Tannins as constituents of *Fumaria parviflora* possess anthelmintic activity against gastrointestinal nematodes and have the capacity to bind to proteins on the surface of nematodes, thereby disturbing their metabolism (*65*) or to the cuticle of the larvae, which is high in glycoproteins, causing their death (*66*) and disturbing the integrity and growth (*67*). Tannins solutions tested at concentrations from 0. 32 to 20. 48 g/L reduced egg viability from 56 to 87% on *Globodera rostochiensis*, while in the pot experiment rates of 100, 250 and 450 g/m^2 reduced the number of cyst/100 g soil, eggs and juveniles/g soil and the reproduction rate in comparison to untreated control (*68*). A thickening of the longitudinal and transverse cuticular ridges by patches in gastrointestinal nematodes, as noticed after *in vitro* exposure to tannins, may reduce the worm's motility and possibly alter potential metabolic exchanges with the local environment (*69*). Tannins mode of action on phytonematodes is yet to be discovered, although there are some hypotheses that they can be considered as attractants or repellents for chemoreceptors causing in plant parasitic nematodes disorientation in locating root systems of host plants (*70*).

Others

Bromelain enzyme of pineapples (*Ananas comosus)* is known to damage nematode cuticles (*71*). *Verbesina encelioides*, a plant species found to be a non-host to *M. javanica, M. incognita* and *M. hapla*, was tested against *M. javanica* as extract and fresh parts powder tested *in-vitro* and in pot experiments. The aqueous extract of the flower was more nematicidal while the lipophilic compounds, did not exhibit nematicidal activity. The active nematicidal compound(s) in *V. encelioides* is not known (*72*). Tall fescue, *Schedonorus arundinaceus* cv. Jesup (Max-Q, produces root and shoot compounds that inhibit *M. incognita* hatch, and are nematostatic or nematotoxic to J2 (*73*).

Natural compounds of botanical origin can also be used in tag mixtures with nematicides to promote the activity of active ingredients. Different characteristics of emulsifiers in terms of pH specific gravity at 25°C gm/cc and critical micelle concentration (CMC) can provide the best possible nematicidal activity and minimum phytotoxicity. In addition some emulsifier exhibit nematicidal activity used by their own. For instance, canola, cotton, flax, olive, sesame and soybean oils as emulsifiable concentrates can be used as protectants against *M. incognita* infection to tomatoes since they reduce root galls and J2 in soil (*74*).

Of most interest is the suppressing effect of arbuscular mycorrhizal fungi on root-knot nematodes (*75*). Mycorrhizal symbiosis changes the chemical character of root exudation (*76*) affecting the root penetration of the nematodes. Compounds like amino acids, flavonoids, phenolic compounds, sugars and organic acids are co-involved in the chemical profile of the root exudes infested by mycorizae (*75*). In addition, root exudates from mycorrhizal plants attract bacteria like *Pseudomonas fluorescens* (*76*) and stimulated *Trichoderma*, both of which are known as biocontrol agents on plant-parasitic nematodes (*77*).

Our Experimental Results, Aldehydes

Among the most potent natural nematicides used as complex mixtures of compounds (crude extracts or/and essential oils) (*78*), tested both against the second stage juveniles provoking host infection (J2), and on biological cycle arrest in the host roots, were *Melia azedarach* (fruits methanol extract: $EC_{50/3d}$ = 429 mg/L), *Ailanthus altissima* (wood water extract: $EC_{50/3d}$ = 58.9 mg/L), *Ruta chalepensis* (aerial plant parts essential oil: $EC_{50/1d}$ = 77.5 mg/L) and *Mentha spicata* (water extract aerial parts: $EC_{50/72h}$ = 300 mg/L) (*23*, *79–81*). Chemical composition analysis of the nematicidal extracts, distinguished among constituents for activity mainly aldehydes and ketones. In specific, the most active components were furfural ($EC_{50/1d}$ = 8.5 mg/L), 2-undecanone ($EC_{50/1d}$ = 20.6 mg/L), (E,E)-2,4-decadienal ($EC_{50/1d}$ = 11.7 mg/L) and carvone ($EC_{50/1d}$ = 730 mg/L). As a first attempt to understand the mode of action, the reactivity of *α*,*β*,*γ*,*δ*-unsaturated aldehydes was studied by the means of environmental scanning electron microscopy (ESEM) on the external nematode cuticle following treatment with (E,E)-2,4-decadienal and furfural (*81*). Cuticle damage and leakage of the internal fluid material of nematode body were evident, and they were attributed to a nucleophilic addition of the cuticle amino or thiol group to the *α*,*β*-unsaturated carbonyl. Successively, in a structure-activity relationship study, twenty-six low molecular weight redox-active aldehydes were studied on *M. incognita* in terms of their chemical reaction with the nematode's cuticle peptides to better understand the toxicity. The best performing nematicidal compounds were phthalaldehyde and salicylaldehyde (EC_{50} = 11 ± 6 and 11 ± 1 mg/L, respectively) and some very interesting structure−activity relationship trends were established. In specific, the presence of a hydroxyl group in the ortho position to the formyl group provided with higher activity if compared with the para position (4-hydroxybenzaldehyde). The hydroxyl group substitution with a nitro group descreased activity and the glycation of the hydroxyl group in the ortho position led to total loss of activity. Furthermore, the treated nematodes showed evident damage on the external cuticle (*82*), thus suggesting that the potential mechanism of action of the tested aromatic aldehydes might be related to either the alteration of the external cuticle or/and that an internal biochemical target might be involved. Interestingly, a 1508.7 ± 0.3 Da molecular mass peptide was used to study the creation of adducts with the aldehydes, which can be linked to the reactivity with the peptides on the nematode's body. Finally, the toxicity of the V-ATPase inhibitor pyocyanin against *M. incognita* J2 exhibited

an EC_{50} at 24 h of 72 ± 25 mg/L, similar to those of 2-pyridinecarboxaldehyde, indole-3-carboxyaldehyde, and pyrrole-2-carboxaldehyde (3.60 ± 1.16, 301 ± 33, and 392 ± 32 mg/L) (*82*, *83*), thus suggesting the inhibition of V-ATPase enzymes as a potential target site of the aldehydes in the nematode's body (*83*).

Summary

Botanical pesticides are considered environmentally benign and less hazardous due to the public perceiving natural products to be safer than synthetic chemicals, despite evidence to the contrary (*84*, *85*). There is thus a large volume of scientific investigations and publications on botanical insecticides but still the commercialization of new botanicals is sparse (*86*). In the US, regulatory exemptions have facilitated the commercialization of some essential oil-based pesticides over the past decade, but in Europe procedures are still very slow. In any case, there are some major issues to be concerned before utilizing the knowledge on natural substances activity to produce commercial pesticides. Active ingredients identification is of course relatively easy now due to new analytical platforms of laboratories. Yet, blending and fortification does not necessarily provide a better option than integrating crude botanical products in pest management since minute amounts of compounds may have an active role in the bioactivity of the total mixture (*85*). In some cases, the use of crude or semi-refined plant extracts might be a better idea than an industrialized pesticide development. In the second case, novel formulation methods that mimic the chemical compartmentalization and storage capacity of plants are needed before commercialization, to avoid sort residual life under field conditions (*85*).

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Chapter 12

Natural and Synthetic Isothiocyanates for Pest Control in Soil

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Synthetic fumigants are widely used in agriculture to provide highly efficacious pre-plant pest control for high cash crops. However, stringent regulations aimed at controlling soil to air emissions govern fumigant use. This has led to increased interest in biofumigation using Brassica species which release volatile isothiocyanate (ITC) chemicals into the soil. These ITCs have a similar chemistry to the synthetic fumigant methyl isothiocyanate (MITC) and are, therefore, of interest in pest control. However, there are significant disadvantages to natural ITCs when compared to MITC; most notably, a relatively low release efficiency into the soil, and rapid degradation/sorption within the soil. The inconsistent pest control efficacy of biofumigation indicates a lack of robustness and suggests that non-organic growers may be reluctant to switch from traditional fumigants. MITC, despite being subject to regulations, offers efficacious pest control and its emissions to the atmosphere can be significantly reduced using plastic tarps or water sealing. Compared to other soil fumigants, MITC exhibits relatively low soil diffusion. Although this lower diffusion is advantageous in terms of limiting atmospheric emissions, it needs to be considered in relation to pest control, for example in the positioning of drip lines, emitters, or shank spacing, during application.

In Biopesticides: State of the Art and Future Opportunities; Coats, et al.;

Introduction

Both synthetic and natural isothiocyanates (ITCs) are volatile chemicals that are biocidal to a wide range of soil organisms, including nematodes, bacteria, and fungi (*1*, *2*) and are therefore of use in the pre-planting control of soil pests. Synthetic methyl isothiocyanate (MITC) has long been widely used as one of the active ingredients in pre-plant soil fumigants in agriculture. Due to similarities in chemistry between synthetic and naturally produced ITC, there is increasing interest in the use of natural ITC as a biofumigation agent for pre-plant pest control in soils. Natural ITC is produced by certain plant materials; most commonly, Brassica species. Such plants can be used for biofumigation as rotation crops, or intercrops, by incorporating fresh, chopped plant material as green manure, or by incorporating processed plant products high in glucosinolates (GSLs) such as seed meal or dried plant material (*3*). Gas phase diffusion of synthetic and natural ITCs via the soil pore space affords a degree of pest control within the root zone of agricultural soils prior to the planting of a crop. In general, fumigants are used for high cash crops such as fruits, vegetables, and nuts.

Due to their potentially toxic nature, the use of chemical pesticides is strictly controlled within the USA and a pesticide must be registered by the US Environmental Protection Agency (USEPA) and an individual state before it can be used. In the case of fumigants, where their gaseous nature can lead to a relatively high degree of off-gassing from the site of application, regulations are in place to protect air quality and human health. For example, in California, fumigant labels (produced by CA Department of Pesticide Regulations, CDPR) generally require optimal soil and weather conditions at the time of application and may require the use of plastic tarp covering the soil surface to reduce the potential for soil to air emission and its associated risks. Moreover, certain areas of the state of California have been identified as "non-attainment" areas where additional regulations to reduce emissions are in place because these areas do not meet federal air quality standards for pesticide emissions (*4*). Due to these relatively strict regulations and registration requirements, there is a perceived need for alternative approaches to pest control. Biofumigation potentially addresses this need because the release of naturally produced gas into the atmosphere is not currently subject to regulation and no pesticide registration is required.

Traditional, synthetic, fumigants are typically either shank or drip applied as a liquid formulation. Upon entering the warm soil, they are converted to gas. The physical properties of this gas (particularly its Henry's constant, vapor pressure, degradation half-life, and sorption potential) determine its fate and transport within the soil environment. Synthetic fumigants have a high Henry's constant and vapor pressure, a long half-life, and low sorption potential, and so exhibit a high degree of soil diffusion (likely resulting in effective and uniform pest control), but also potentially high emissions from soil to air. Biofumigation can be considered a distributed source of ITC since the parent plant material is generally plowed into the surface soil. The plant material then undergoes chemical transformation to release ITC into the soil. The kinetics of this transformation, together with the potential for degradation and adsorption of the ITC, may have

a significant bearing on ITC concentrations in the soil, subsequent pest control efficacy, and atmospheric emission potential.

In this chapter, the aim is to briefly review the use of natural ITC as a biofumigant and highlight some of the problems and limitations of this approach to pest control. The use of MITC will then be described, along with its environmental hazards. The concept of concentration-time index will be applied to demonstrate the relative efficacy of MITC in pre-plant pest control compared to other soil fumigants.

Natural ITC

Natural ITC is a by-product of GSL degradation. GSLs are sulfur-containing secondary plant metabolites that contain a β-thioglucose moiety, a sulfonated oxime moiety, and a side-chain derived from amino acid (*5*). They are found exclusively in dicotyledonous plants such as members of the order *Capparales*. The *Resedaceae*, *Capparidaceae* and *Brassicaceae* families have been shown to contain the greatest concentrations of GSLs (*6*). Within the plant tissue, the GSLs are separated from the endogenous enzyme, myrosinase, which catalyses their hydrolysis (*5*). GSLs themselves are of limited biological activity (*7*). However, when GSL-containing plant tissues are disrupted (for example, pulverized and plowed into soil) the constituent GSLs are hydrolyzed to a number of breakdown products such as ITCs, thiocyanates, nitriles, and oxazolidines. ITCs have been shown to be toxic to a number of soil organisms, such as nematodes, bacteria, and fungi (*1*, *2*). It is this production of ITC that has led to interest in the use of, especially Brassica, plant species as biofumigant amendments to soils. Because the production of GSL is greatest during flowering of the plant (*8*, *9*), this often represents the ideal time to incorporate plant material for optimum generation of ITC and, hence, biofumigation potential.

Production of Natural ITC in Soil

The efficacy of biofumigation using fresh Brassica leaves depends on the rate of GSL conversion to ITCs, but also on the environmental factors controlling GSL availability in the soil matrix (*10*, *11*). Omirou et al. (*11*) found that GSLs rapidly dissipated in clay loam soil with half-lives ranging from 3.2 to 15.5 h, and that increasing soil moisture increased the rate of dissipation. Similarly, other workers have found that ITCs and other GSL hydrolysis products degrade rapidly in soils, being present from as little as a few hours or days (*5*, *7*, *12*) to as much as 14 days (*13*). Hansen and Keinath (*14*) found that ITC concentrations were greatest after incorporation of mustard, intermediate with rapeseed, and lowest, or zero, with radish. Across the three crops and two experiments, these researchers found that when ITCs were detectable in the soil, mean concentrations ranged from 0.14 to 5.91 $\mu g\ g^{-1}$ dry soil. Highest concentrations were generally found after just 4 h and declined rapidly thereafter. Interestingly, they also found that ITCs were detected

at relatively low concentrations (< 0.17 μg g^{-1} dry soil) in plots under plastic film even without prior plant material incorporation. Bangarwa et al. (*15*) found that the addition of allyl ITC at a rate of around 1000 kg ha^{-1} under low density polyethylene was required to suppress weeds and produce marketable tomato yield equivalent to a standard methyl bromide application of 390 kg ha^{-1}, also under low density polyethylene. Assuming that one hectare of soil contains around 2×10^6 kg soil in the plow layer (20 cm depth), this application of allyl ITC equates to a soil concentration of around 500 μg g^{-1}; two orders of magnitude greater than the highest values observed by Hansen and Keinath (*14*) following incorporation of Brassica species.

As reported by Motisi et al. (*16*), previous field studies indicate that the GSL content of plants is not well correlated with the efficacy of biofumigation for decreasing disease expression. Gimsing and Kirkegaard (*5*) attempted to relate GSL content of plant material to ITC concentrations in the soil following incorporation. They found that total GSL contents for 'high GSL' rape were 23.2 and 28.1 μmol g^{-1} (for shoots and roots, respectively), and for 'high GSL' mustard were 31.3 and 13.7 μmol g^{-1} (for shoots and roots, respectively). They also determined that between 10.4 μmol g^{-1} (mustard roots) and 30.0 μmol g^{-1} (mustard shoots) of these GSLs were ITC-liberating. In corresponding soils, these workers found that maximum total ITC concentrations were present just 30 min after incorporation of the green manure and were approximately 80 nmol g^{-1} for mustard, and approximately 20 nmol g^{-1} for rape. The GSL to ITC release efficiency after 30 min calculated for the 'high GSL' mustard was 56%, but it was only 26% for the 'high GSL' rape. By 6h, these values decreased to 23 and 10%, respectively.

Bangarwa et al. (*10*) evaluated the biofumigation potential of seven Brassica cover crops for weed control in plasticulture tomato and bell pepper. They found that GSL concentration and composition varied between crops and between roots and shoots. Total GSLs contributed to the soil by incorporation of Brassica cover crop tissues were between 47 and 452 nmol g^{-1}. These amounts of GSL contributed to the soil were then used to estimate maximum potential ITC release to the soil; ranging from 47 nmol g^{-1} for oil seed rape (*Brassica napus* L.) to 237 nmol g^{-1} for a blend of Indian mustard (*Brassica juncea* L.) and white mustard (*Sinapis alba* L.). However, actual measured ITC concentrations ranged from 1.6 to 28.4 nmol g^{-1}, indicating that the conversion from GSLs to ITCs was lower than expected. Actual conversion efficiencies were between 1 and 39% depending on crop. Highest ITC concentrations were measured 3 h after crop incorporation and declined over the following two weeks of monitoring.

Pest Control Using Brassica Biofumigation

The incorporation of seed meal or, particularly, chopped plant material (green manuring) are considered the most effective ways to induce a concentrated release of ITCs (*3*). For example, suppression of common scab disease was enhanced using dried and ground post-harvest residues of Brassica vegetables (*17*), bacterial wilt was reduced (40-50%) by a range of Brassica amendments to potato crops, and

the agents responsible for apple replant disease were suppressed by rapeseed meal (*18*). Rapeseed green manure was shown to be effective in controlling root-knot nematodes and in increasing yield of potatoes (17-25%), due to the role of GSLs (*19*). Green manures of Indian mustard, canola, and radish have been shown to be effective in controlling *Verticillium dahlia* (*20*), and a mustard green manure has also been used to suppress common scab (*Streptomyces scabies*) (*21*). Bangarwa et al. (*10*) noted that control of yellow nutsedge weed was < 53% at two weeks and declined to < 18% later in the season following incorporation of Brassica species, and they concluded that Brassica cover crops have only marginal potential for early season weed control and cannot be used as a weed control practice in commercial tomato and bell pepper production. As an alternative to incorporation of plant material into the soil, rotation, or intercropping, has also been used for biofumigation where above-ground plant material is harvested or left to mature above ground. This approach relies on ITCs entering the soil via root exudates of growing plants, leaf washings, or root and stubble residues decomposing in the soil after crop harvest (*3*). For example, ITCs have been detected in the rhizosphere of intact plants, and intercropping of strawberries with Brassica plants has been shown to control *Verticillium* wilt (*22*).

Motisi et al. (*16*) reported that field studies have generated conflicting data concerning the efficacy of biofumigation at the field scale, limiting the use of this technique. Indeed, in a broad review of the use of biofumigation these authors cite a lack of robustness with the technique as a major limitation to its widespread use. Although the biocidal effectiveness of ITCs has been clearly demonstrated *in vitro* (*1*), the many studies carried out in agricultural conditions have not systematically shown a pathogen-suppressing effect of the ITCs released by Brassica residues (*16*).

Advantages and Disadvantages of Biofumigation

Reducing emissions of synthetic MITC is a challenge for researchers and alternative, low emission approaches are required to satisfy increasingly stringent regulations relating to atmospheric emissions. In this regard, Trott et al. (*23*) measured air concentrations of natural ITCs during and after incorporation of mustard cover crops into soil. The maximum observed concentrations of allyl, benzyl, and phenethyl ITCs were 188.6, 6.1, and 0.7 μg m^{-3}, respectively, during mustard incorporation. Based on measured concentrations, these workers concluded that airborne natural ITC concentrations did not appear to pose a human inhalation exposure concern to field operators and bystanders. Such regulatory advantages of biofumigation suggest that further research in this area, to find high GSL-yielding crops; increase rates of GSL to ITC conversion; and maintain higher concentrations of ITCs within the soil pore space over longer periods, is warranted.

In addition to the regulatory advantages of natural ITC, it also offers the advantages of improving soil texture and water holding capacity due to the addition of the plant material from which the ITCs are subsequently derived. Moreover, the addition of this material may also stimulate and improve the structure of the

existing soil microbial community, increase potentially mineralizable nitrogen in the soil, increase soil nutrient availability by mineral weathering, increase water infiltration rate, reduce soil erosion by wind, and reduce soil compaction (*3*). Thus, soils applied with plant material for the purposes of biofumigation are likely to be improved in terms of soil health and nutrition.

Conversely, a potentially major disadvantage of biofumigation with natural ITC may be limited pest control efficacy when compared to synthetic MITC. Because the production of natural ITC tends to be relatively low and subject to rapid degradation and/or sorption within the soil, a high level of soil diffusion may not be evident, resulting in a non-uniform distribution of the fumigant in soil. This is likely a primary reason for the lack of robustness of biofumigation noted by Motisi et al. (*16*). With MITC, a uniform concentration within the soil pore space can be more easily controlled and ensured, resulting in a more reliable degree of pest control. Further potential disadvantages of using natural ITC are the loss of crop production time that a grower experiences during the growing period of the biofumigation plants, and the possibility of the biofumigation crop hosting disease-causing organisms. Omirou et al. (*24*) reported that biofumigation by incorporation of Brassica plants into soil induced changes in the structure and function of the soil microbial community that were mostly related to microbial substrate availability changes derived from the soil amendment with fresh organic matter. Gilardi et al. (*25*) noted that soil amendments with Brassica products can enhance disease severity due to the increased pathogen inoculum potential when the substrate serves to sustain saprophytic growth of plant pathogens. In addition to these issues, there are also questions over the willingness of growers to accept non-traditional (non-chemical) approaches to pre-plant pest control, particularly if crop yield is adversely affected due to a lack of adequate pest control. Consequently, in California, the use of biofumigation is more likely utilized by organic growers while, in general, large scale production still relies on synthetic MITC or other fumigant chemicals.

Synthetic MITC

Methyl isothiocyanate (MITC) is a volatile organo-sulfur compound with pesticidal characteristics. In agricultural settings, MITC is generated following the degradation of the common soil fumigants metam sodium, metam potassium, and dazomet and is the active ingredient responsible for pest control via non-selective enzyme inhibition, when applying these compounds. The chemical structures of MITC, metam sodium, metam potassium, and dazomet are shown in Figure 1a-d. All three products are registered for use in the USA and are classed as non-selective fumigants for pre-plant application. In California, metam sodium and metam potassium are widely used. For example, in 2011, 4.91 and 2.58 million kg, respectively, were used to treat a total of 46.5 thousand hectares during 4300 agricultural applications (*4*). Metam sodium was ranked the fourth most used pesticide, and second most used fumigant (behind 1,3-dichloropropene) in California in 2011 while metam potassium was ranked eighth most used pesticide and fourth most used fumigant. This high level of use likely results in the release

of large quantities of volatile MITC into air. For example, California Department of Pesticide Regulations (*26*) estimated that an average of 9 million pounds per year of MITC were released into the air from agricultural applications of metam sodium in the 1995-2000 period.

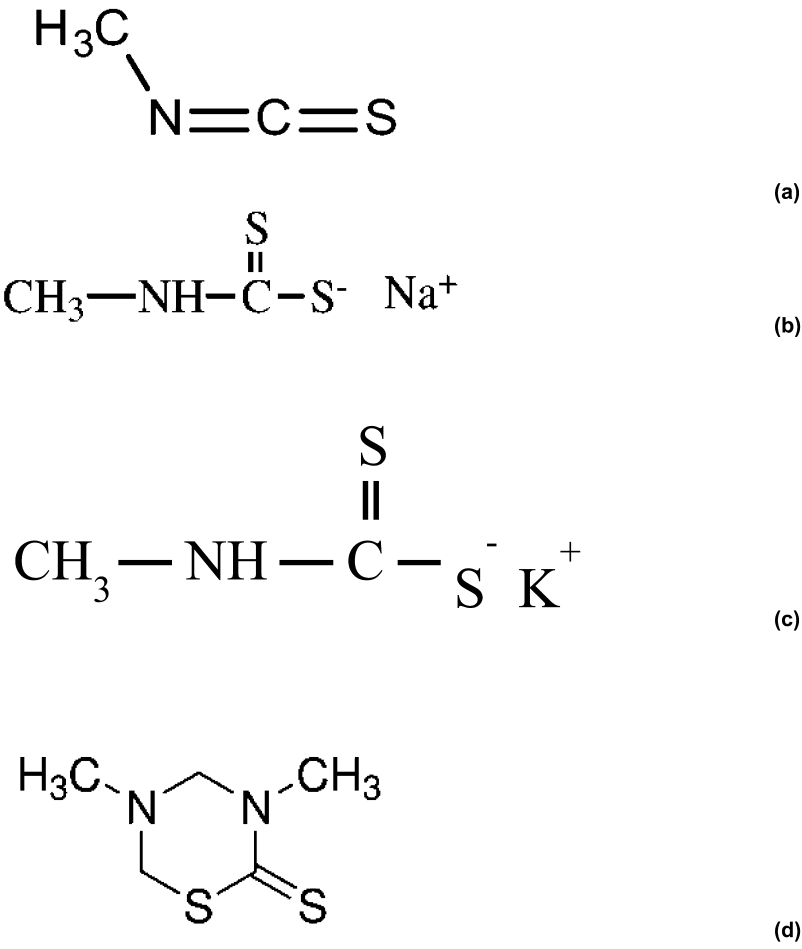

Figure 1. Chemical structures of (a) MITC, and the three MITC pre-cursors (b) metam sodium, (c) metam potassium, and (d) dazomet.

In agricultural settings, metam sodium and metam potassium are applied to pre-plant soils as a liquid and convert to volatile MITC within the soil. They both exhibit fungicidal, herbicidal, insecticidal, and nematicidal properties. As dithiocarbamate salts, they breakdown in soil to produce volatile MITC which is capable of diffusing through the soil pore space and producing highly effective

pest kill. Metam sodium converts to MITC on a mole to mole basis, which based on the molecular weights of the two compounds results in a conversion rate of approximately 60% by weight (*26*). Zheng et al. (*27*) reported that this conversion to MITC was a rapid abiotic decomposition process and complete within 30 min for a sandy loam soil. Dazomet is applied to soil as dry granules that react with water to produce MITC. Water management is therefore critical to maintain appropriate MITC gas concentrations within the soil. Dazomet is used for the control of weeds, fungi, nematodes, and rhizomes (*28*, *29*).

MITC Properties

In common with other soil fumigants, the adsorption of MITC to soil components is very low (*30*). MITC has a relatively high boiling point (119 °C), low density (1.05 g mL^{-1} at 24 °C), intermediate water solubility (8200 mg L^{-1} at 20 °C), low vapor pressure (19 mm Hg at 20 °C), and low dimensionless Henry's constant (0.01 at 20 °C). The extent of pest control of a fumigant is largely controlled by its soil diffusion and, therefore, its Henry's constant and vapor pressure for which higher values generally indicate a greater potential for diffusion through the soil pore space. Therefore, MITC is considered to have lower soil diffusion, potentially limiting the spatial extent of pest control, compared to other fumigants unless a more uniform application methodology is employed. However, these properties also indicate a relatively lower potential for soil to air emissions compared to other fumigants.

MITC Toxicity

MITC is acutely toxic by inhalation, injection, or dermal absorption (*28*). This chemical received publicity due to a spill of 19,500 gallons of 32.7% metam sodium into the Sacramento River in 1991 (*26*). Exposure of airborne MITC to local populations resulted in numerous complaints of eye and respiratory irritation, nausea, headaches, dizziness, vomiting, shortness of breath, and chemically induced asthma. Other accidental exposures have produced similar symptoms. Acute toxicity data for MITC were collated by CDPR (*26*). In humans, the no-effect air concentration for eye irritation was at 0.22 ppm (*31*). In rats, inhalation LC_{50} was determined as 180 ppm for 4 h exposure (*32*) and 633 ppm for 1 h exposure (*33*). Oral LD_{50} in rats was measured as between 50 mg kg^{-1} (*34*), and 305 mg kg^{-1} (*35*). Also in rats, dermal LD_{50} was 181-225 mg kg^{-1} (*36*), subcutaneous LD_{50} was 60 mg kg^{-1} (*37*), and intraperitoneal LD_{50} was approximately 50 mg kg^{-1} (*37*). Inhalation exposure is the principal concern with agriculturally applied MITC fumigants due to the potential for atmospheric emissions and the associated exposure risk to agricultural workers and local bystanders (*27*). However, the potential for MITC off-gassing to affect nursery seedlings in adjacent fields was also noted by Wang et al. (*38*).

MITC Degradation

The pest control efficacy of a fumigant is largely dependent upon the exposure concentration within the soil over time. Therefore, soil degradation kinetics of the chemical is a critical factor governing pest control since very rapid degradation may lead to poor efficacy. Conversely, very slow degradation may lead to relatively high concentrations of residual fumigant in the soil and the potential for atmospheric emissions over extended time periods.

In the absence of soil, the hydrolysis of MITC was investigated by Zheng et al. (*39*). In neutral aqueous solution, MITC was found to be stable with a half-life of 93 d. Hydolysis products of MITC were identified by GC-MS as gas phase methyl isocyanate and liquid phase 1,3-dimethylthiourea. Zhang et al. (*40*) studied the half-life of MITC in nursery and forest soils at three application rates (195, 390, and 785 kg ha^{-1} equivalent) and found that MITC degradation followed first order kinetics. In the nursery soils, half-lives ranged from 3.47 to 11.01 days across the three application rates, and in the forest soil from 3.14 to 11.20 days. Overall, these workers found no significant difference in MITC half-life across the three application rates or between nursery and forest soils. Dungan et al. (*41*) reported the effects of temperature and the addition of chicken manure on MITC degradation. In non-amended soil they found that half-life decreased from 5.8 days at 20°C to 1.8 days at 40°C. A similar temperature effect was observed for the chicken manure amended soils. The addition of the chicken manure also reduced half-life within each temperature treatment; for example, at 20°C, half-life decreased from 3.5 days at 1% manure addition to 2.2 days at 5% addition.

Zhang et al. (*40*) observed that enhanced degradation of MITC due to previous fumigation of the soil did not occur. Nevertheless, other workers have found that repeated fumigation of soil with MITC did lead to increased degradation rates, probably due to the increased potential for an adapted microbial community to persist (*42*, *43*). Although, in soil, MITC degradation occurs via chemical and biological processes (*28*), Chellemi et al. (*44*) reported that biological mechanisms were most important. Therefore, an adapted or supplemented soil microbial population may be significant in enhancing MITC degradation. Indeed, the addition of organic materials has been shown to enhance MITC degradation (*41*, *45*), probably due to the supplementation of the soil microbial community.

Soil-Air Emissions of MITC

Reducing soil to air emissions of agricultural fumigants such as MITC is critical to maintain air quality and to adhere to increasingly strict regulations regarding fumigant emissions. For example, in California, the Department of Pesticide Regulations places specific requirements on how fumigations must be performed, as well as prohibiting some high-emission methods, in five "non-attainment" areas of the state. The stringent regulations within these areas are due to a failure to meet federal air quality standards for pesticide volatile organic compound emissions. Scientific research in this area has focused on quantifying atmospheric emissions of fumigants and assessing methods to reduce these emissions. In addition to protecting air quality, some methods to reduce

emissions maintain fumigants within the soil environment; thereby, maximizing pest control potential. As such, a combination of field and laboratory (soil column) methods has been used to study MITC emissions.

The method by which a fumigant is applied to soil may have a strong bearing on its emission potential. Littke et al. (*46*) compared low-boom-height center pivot chemigation (surface application) with soil-incorporated shank injection for metam sodium and measured subsequent MITC emissions from the soil. They found that the estimated cumulative fumigant loss was 13% by shank injection compared with 47% by chemigation. A similar result was found by Saeed et al. (*47*). In part, the lower emissions for the shank injection were likely due to the increased path length between the site of application and the soil surface. However, Ajwa et al. (*48*) noted that emission reductions could also be associated with the improved design of shank injection systems that help to break up the soil voids after injection, and effective shank compaction of the soil. Similarly, Woodrow et al. (*49*) found that subsurface application potentially reduced MITC concentrations in air by four orders of magnitude when compared to application via surface irrigation water.

In raised-bed, plastic-mulched systems, Chellemi et al. (*50*) found that MITC emissions were < 6% under relatively impermeable films across three sites in Florida. With a more permeable film, emissions were <13% across three sites in Georgia state. In addition, peak emissions occurred much more rapidly with the more permeable film. Lower total emissions were found by Wang et al. (*38*) using high density polyethylene; 2.5-5.2%. Papiernik et al. (*51*) and Ou et al. (*52*) both found that virtually impermeable film not only reduced emissions to air but also retained MITC in the root zone longer, and at higher concentrations, compared to other plastic films. Agricultural films offer a benefit in terms of maintaining high soil gas concentrations and pest kill efficacy.

Using laboratory soil columns, Frick et al. (*53*) noted that MITC emissions were positively correlated with air-filled porosity and were suppressed by water application. Indeed, Wang et al. (*38*) found total MITC emissions of just 0.1 to 3.2% when applied with a water seal at the soil surface. Simpson et al. (*54*) compared various levels of irrigation water addition to the soil surface as an emission reduction strategy for MITC. These authors found that emissions were consistently reduced with increasing water seal application, with a 2.5-3.8 cm depth of water seal providing a 71-74% reduction in MITC emissions compared to no water seal. Zheng et al. (*27*) reported similar findings and suggested that water sealing, together with subsurface application, may be an effective and economical strategy to reduce MITC emissions while maintaining pest control efficacy. Sullivan et al. (*55*) found that intermittent water sealing significantly reduced off gassing rates of MITC for both shank injection and chemigation applications when compared to standard water sealing practices. Li et al (*56*) found that with surface drip irrigation of metam sodium under plastic tarp, MITC flux density showed a diurnal pattern with peak flux in the first 12 h after chemigation with a subsequent decline over time. During the first 60 h, 2.65% of the applied mass was lost via emissions suggesting that this drip application method (with tarp) offered relatively good control of MITC emissions from soil. In general, a water seal is considered the most cost-effective emission reduction strategy for MITC.

According to Henry's law, the more water in the soil, the less MITC would be partitioned into the gas phase. Therefore, the relatively low (compared to other fumigants) Henry's constant for MITC helps explain the observation of lower MITC emissions from irrigated soils.

MITC Soil Concentrations and Pest Control

In soil columns, Zhang and Wang (*57*) applied MITC at 20 cm soil depth at a rate of 170 kg ha^{-1} under tarp without irrigation, with a tarp after limited irrigation, and 5 days of irrigation without tarp. During the first 24 h after application, soil concentrations of MITC were greatest in the 15-25 cm depth region and were relatively low closer to the soil surface throughout the experiment; presumably due to volatilization losses at the soil-air boundary. Peak concentrations of around 5, 3, and 2 μg mL^{-1} were found for the tarp only, tarp with limited irrigation, and irrigation only treatments, respectively, soon after application (0.25 to 5 h). Peak concentrations, therefore, seemed to be inversely related to amount of irrigation water added which the authors suggested was due to MITC becoming solubilized in the water phase. This concurs with the low atmospheric emissions of MITC from irrigated soils found by these authors and others (discussed above).

In tarped (high density polyethylene) and water sealed plots, Wang et al. (*58*) measured MITC soil gas concentrations following application of dazomet (448-560 kg ha^{-1} that was surface applied and then spaded into the top 20 cm of soil) and metam sodium (686 L ha^{-1} of 0.5 kg L^{-1} metam sodium solution that was surface applied and then roto-tilled into the top 12 cm soil). They found that MITC concentrations in the soil gas tended to decrease with depth and were generally higher under tarp than with water seal. With dazomet application at two separate sites, peak MITC concentrations were found in the top 5-10 cm and measured 2.14 and 1.17 μg mL^{-1} at 1.17 and 0.3 days after application, respectively. With metam sodium application, MITC concentrations were lower, never exceeding 0.6 μg mL^{-1}. Overall, these authors concluded that, under tarp, MITC was concentrated in the upper 30 cm of the soil profile (i.e. the root zone of most crops) and the effect lasted about 3 days. With water seal, the lower concentrations were deemed to likely not provide sufficient exposure time in order to achieve desired pesticidal efficacy.

Candole et al. (*59*) studied MITC soil gas concentrations following drip application (drip lines at 2.5 cm depth) of Vapam (42% metam sodium) at 701 L ha^{-1} to raised beds under low density polyethylene. MITC concentrations decreased over time and with lateral distance from the drip emitter. Higher amounts of MITC were detected at 20 cm below the emitter than at 10 cm, with a peak concentration of around 2.2 μg mL^{-1} found at 20 cm below the emitter at 12 h after application. At 24, 48, 72, and 120 h, peak concentrations of around 1.6, 0.9, 0.6, and 0.1 μg mL^{-1}, respectively, were observed 20 cm below the emitter. These workers also determined the control of *Phytophthora capsici* Leonian, *Rhizoctonia solani* Kuhn, and *Cyperus esculentus* L. for two locations at 10 cm soil depth (both directly below the drip emitter and at a lateral distance of 20 cm away from the emitter). This was done for three rates of Vapam application (234, 468, and 701 L ha^{-1}). For each pest, survival was significantly lower at 10 cm below the emitter than at 20 cm away from the emitter. In general, organism

survival at 10 cm below the emitter was not significantly different from the survival in beds treated with a co-formulation of methyl bromide and chloropicrin (67:33) at a rate of 336 kg ha^{-1}.

Using a very similar approach but with application rates of 147 and 295 L ha^{-1}, the same workers (*60*) found that a higher rate of application resulted in higher MITC concentrations in the soil. Highest MITC concentrations were again found 20 cm directly below the emitter and lowest 30 cm laterally away from the emitter. MITC concentrations decreased with time and distance from the emitter. Peak concentrations of around 0.3 µg mL^{-1} (lower application rate at 3 h) and 0.75 µg mL^{-1} (higher application rate at 12 h) were found at 20 cm below the emitter. The authors also determined concentration-time (CT) index values (the integral of fumigant concentration in the soil over time) at each location. Lower MITC CT values at 20 and 30 cm laterally from the emitter resulted in lower mortalities of *R. solani* and *C. esculentus*. CT index values increased with time, particularly at the higher rate of application. At 240 h from application, CT values for this treatment were 62, 33, 10, and 0.8 µg-h cm^{-3} at 20 cm below the emitter, 10 cm below the emitter, 20 cm laterally away from the emitter, and 30 cm laterally away from the emitter, respectively. The results demonstrated that MITC can be delivered at lethal doses with drip applied water downward within beds. However, later diffusion of MITC from the point of application did not reach biologically active concentrations to affect the survival of *R. solani* and *C. esculentus*.

The concept of CT values is a useful one for predicting the degree of exposure of an organism to a fumigant and, consequently, for predicting pest control based on measured soil concentrations over time. The application of this concept is described further below.

Predicting MITC Pest Control Using Concentration Time (CT) Index

A 2-D chamber approach for measuring fumigant emissions, gas diffusion and pest control has been developed in this laboratory and was recently described by Luo et al (*61*, *62*). Using this approach, moist soil is pre-mixed with plant pests of interest, such as fungi (coated onto millet seeds), weed seeds, and nematode-infested roots (e.g. chopped citrus roots). The soil mix is then packed into a stainless steel 2-D soil chamber in which fumigant volatilization, spatial and temporal distribution of soil gas concentration, degradation, and organisms' survivability in the soil could be determined (*63*) (Figure 2). The soil chamber was fabricated from 0.5-cm stainless steel. The internal dimensions of the soil chamber were 60 cm x 60 cm x 6 cm so that typical agricultural management and practice methods within the root zone could be simulated. A total of 84 sampling ports extending radially from the central injection port were sampled to determine soil gas concentrations in real time after the fumigant was injected into the soil through the central port. These soil gas samples were taken at various times (e.g. 0.5, 1.5, 2.5, 3.5, 4.5, 6, 8, and 24 hr). At the end of the experiment, one side wall (60 cm x 60 cm) of the soil chamber was removed, and soil samples were taken with a stainless steel ring with a 4-cm diameter for pest survivability and residual fumigant determinations. In the case of citrus nematodes (*Tylenchulus semipenetrans* Cobb), for example, these were extracted from 50 g of soil using

the Baerman funnel method (*64*). Extracted nematodes were then enumerated using a dissecting microscope. To quantify an organism's exposure to pesticides, a concentration-time index, *CT*, the integral of concentration over time, is defined as:

$$CT(t) = \int_0^t C_T(x,z,t)dt \tag{1}$$

where: x, z are the spatial coordinates, $C_T(x,z,t)$ is the total concentration ($=C_{gas}+C_{liquid}+C_{solid}$) (μg mL^{-1}), t is time (h).

A logistic dose-response curve (Figure 3) is then used to describe the relationship between organism's survival and concentration time index:

$$SURVIVAL = \frac{100\%}{1+(CT/CT_{50})^n} \tag{2}$$

where: n is the slope at the inflection point of the logistic dose response curve.

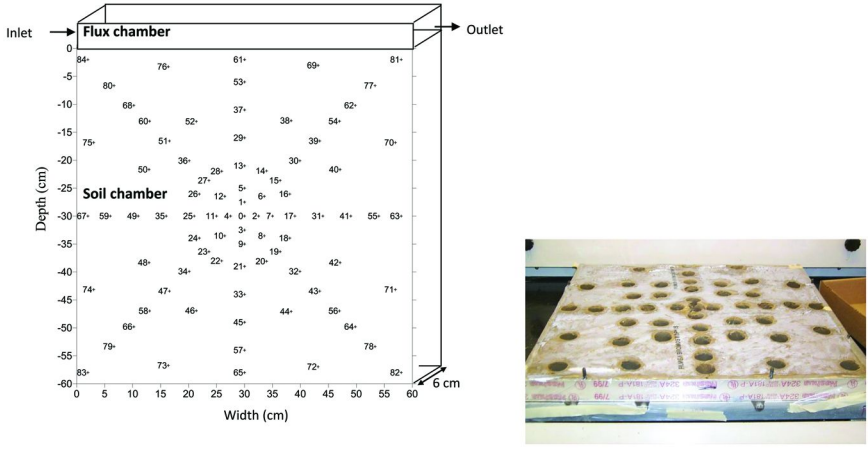

Figure 2. 2-D soil chamber system for determining fumigant gas distribution, CT index, and pest control. Fumigant is injected at Port 0 (center) and soil gas distribution determined at selected ports over time. Atmospheric emissions are determined using a dynamic flux chamber covering the soil surface. Photo shows chamber laid flat for sampling of soil and determination of pest mortality.

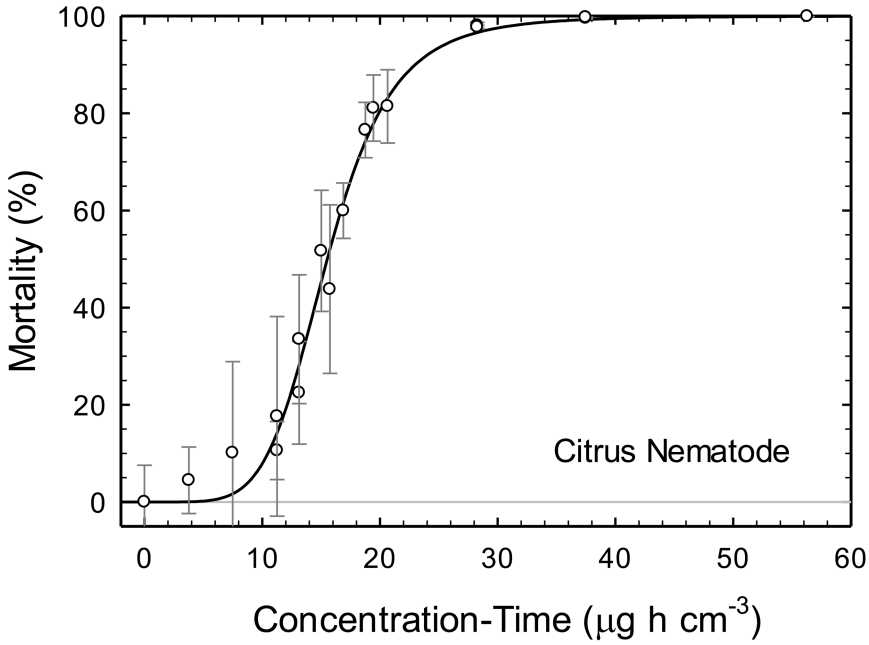

Figure 3. Typical dose response curve for effect of fumigant CT index on citrus nematode (Tylenchulus semipenetrans Cobb) pest mortality. The solid line is derived from the survival model (Equation 2), with CT_{50}, n, and the regression r^2, respectively, 15.5 μg h cm^{-3}, -5.6, and 0.99. Error bars are one standard deviation.

This approach allows for prediction of pest control at various spatial coordinates throughout the 2D chamber. Surfer 8 (Golden Software Inc., Golden, CO) is used to map the distribution of soil gas-phase concentration, and mortality of organisms. The data are interpolated using a kriging method (*65*).

A prediction of pest control using this approach for MITC and two other fumigants (methyl iodide and propargyl bromide) is shown in Figure 4 for comparison. As mentioned previously, MITC exhibits low vapor pressure (19 mm Hg) and Henry's constant (0.01) values compared to other fumigants. This is most noticeable in comparing MITC pest control with that of methyl iodide. For methyl iodide, the much higher Henry's constant (0.22) and vapor pressure (400 mm Hg) values result in a high degree of gas diffusion (note the much greater soil diffusion coefficient, D_s) and, consequently, higher CT values throughout the chamber. This, in turn, gives excellent nematode control throughout the chamber. This degree of pest control is aided by the relatively long half-life for methyl iodide (~ 20 d). In comparison, for MITC the lower potential for gas diffusion throughout the chamber results in a radial pattern in MITC concentrations which decrease with distance from the application point. Consequently, CT values

and nematode control exhibit a similar pattern. Indeed, complete nematode kill extends only within a ~5 cm radius from the application point, although fairly good control (> 60 % mortality) is seen to around a 15 cm radius. At the edges of the chamber, nematode control is relatively poor. This is likely exacerbated by the rather rapid soil degradation of MITC. For propargyl bromide, values for vapor pressure (94 mm Hg) and Henry's constant (0.04) are intermediate between methyl iodide and MITC. Therefore, nematode control again shows a radial pattern, but extends further from the point of application; complete kill being observed to around a 15 cm radius and poor control only observed towards the soil surface. In all cases, volatilization of fumigant at the soil surface is seen to reduce concentrations in this region with a consequent reduction in nematode control. The concentration-time index approach also allows for determination of CT_{50}, the effective CT required to give 50% pest mortality. Interestingly, the CT_{50} for MITC (14 µg-h cm^{-3}) is lower than for methyl iodide (19 µg-h cm^{-3}) and propargyl bromide (16 µg-h cm^{-3}) indicating its greater toxicity to nematodes in this work. Therefore, despite having a lower potential for soil diffusion, nematode control in regions close to the MITC application point is likely to be very good.

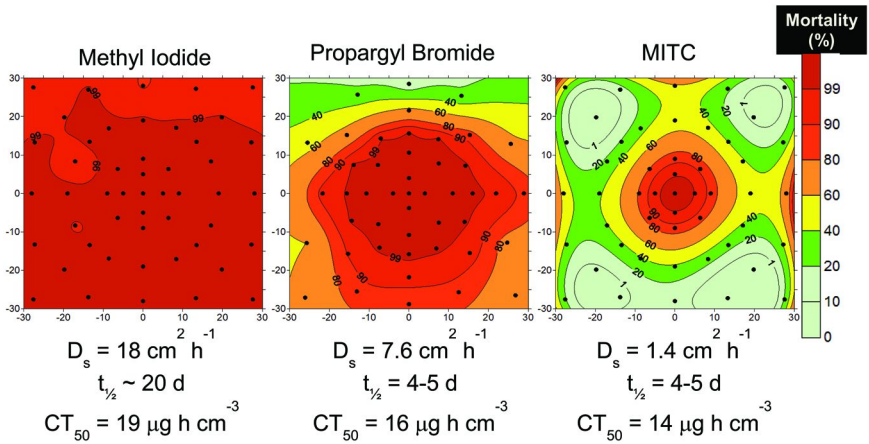

Figure 4. Citrus nematode mortality in the 2-D chamber for methyl iodide, propargyl bromide, and MITC. D_s is soil diffusion coefficient, t½ is degradation half-life of the chemical, and CT_{50} is the effective concentration-time index that results in 50% nematode mortality.

Conclusions

With increasingly stringent regulations governing the use of traditional, synthetic fumigants, there is clearly a need for non-chemical alternatives. However, such alternatives are only plausible if they can offer effective pest control and yields of economic significance. Although biofumigation using Brassica species offers a significant advantage over using synthetic fumigants

in terms of regulations and air quality protection, its conflicting pest control efficacy under field conditions indicates a lack of robustness. The promise of biofumigation may be realized with further research, particularly in identifying high GSL crops in which the GSLs are efficiently converted to ITCs. Maintaining elevated ITC concentrations in the root zone of soils also appears to be a key challenge to increasing the efficacy of pest control. With synthetic MITC, maintaining elevated concentrations in the soil gas is relatively easy to control via application rate and the use of emission reduction strategies (especially tarping and water sealing). Maintaining MITC within the soil not only increases CT values, and hence pest control, but also partially addresses the issue of emissions regulations. With MITC, high pest control efficacy can be readily achieved; as evidenced by the widespread use of its precursor fumigants (metam sodium and metam potassium), particularly in California. Compared to other common soil fumigants, MITC exhibits relatively low soil diffusion and so pest control may not be as efficacious at distance from the point of application. Although this lower diffusion is probably advantageous in terms of limiting atmospheric emissions of MITC, it needs to be considered in the application of the chemical, for example in the positioning of drip lines, emitters, or shank spacing.

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Chapter 13

Activity of Glucosinolate Aglycones and Cyanohydrin Aglycones against Nematodes, Insects, Bacteria, Fungi, and Weeds

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Use of natural botanical compounds as leads for insecticides has been fruitful. Use of botanicals as nematicides and as lead compounds for synthetic nematicides has resulted in promising data relevant to the control of the soybean cyst nematode and the root-knot nematode. Glucosinolate aglycones and cyanohydrin aglycones were tested for their efficacy against egg hatch of the nematodes. Synthetic analogs and derivatives of the aglycones were synthesized and evaluated for their potency. Soil fumigation assays included evaluating one derivative for its effectiveness against soil bacteria, soil fungi and weed seed germination.

Introduction

Plants have evolved chemical defenses to fend off numerous types of phytophagous pests. Those pests include insects, mites, mammals, birds, mollusks, fungi, bacteria, viruses, and many others, including nematodes. Some plants produce herbicidal chemical defenses that reduce competition for resources by killing or stunting other plants. Agricultural production often requires control of plant-parasitic nematodes, such as the root-knot nematode[CJR[1] [AG2](*Meloidogyne incognita*) or the soybean cyst nematode (*Heterodera*

In Biopesticides: State of the Art and Future Opportunities; Coats, et al.;

glycines). After decades of control of nematodes in strawberries, tomatoes, citrus, cut flowers and many other horticultural crops by fumigating the soil with methyl bromide, the phase out of that fumigant, along with the voluntary or involuntary deregistration of several additional nematicides, has created the need for new chemistry for nematicides.

Elucidation of plant chemistry through the past century has revealed excellent detail on the biosynthesis capabilities of plants, including their defensive chemistry. Botanical insecticides such as pyrethrins, rotenone, ryanodine and nicotine have been known for centuries, and numerous phytochemicals have been evaluated for control of harmful plant-parasitic nematodes (*1*, *2*). The current study explored the biological activity of natural botanicals as nematicides, as well as some analogs and derivatives of them. Following up on our previous studies on the insecticidal effects of cyanohydrins (Fig. 1) and glucosinolates (Fig. 2), we examined their potential as nematicidal compounds in laboratory tests. One glucosinolate of special interest was 1-cyano-2-hydroxybutene (CHB) from crambe seed meal; crambe is an oilseed crop in the Brassicaceae, related to rapeseed, canola, radish and mustards. Several other families of plants produce small defensive molecules with cyano and hydroxyl groups, but both positioned on the same carbon, in the cyanohydrin configuration. Some natural cyanogens include amygdalin (a glycoside of phenyl acetonitrile), which occurs in almonds, apricots and cherries, and dhurrin (a glycoside of 4-hydroxyphenyl acetonitrile), which is produced in sorghum species. Another common naturally occurring cyanohydrin is linamarin (a glycoside of the cyanohydrin of acetone), which has been identified in flax, cassava and bamboo.

Figure 1. Chemical structure of cyanohydrins (GA = glucosinolate aglycone).

Figure 2. Chemical structure of glucosinolates.

The candidate fumigants were tested against stored grain beetles and house flies, as well as soybean cyst nematode and root-knot nematode, in contact (Figure 3) and vapor-phase bioassays (Figure 4). Commercial fumigants utilized for comparison included chloropicrin (trichloronitromethane) and the organophosphorus insecticide dichlorvos.

Materials and Methods

CHB glucosinolate was isolated from crambe seed meal which was produced in the Center for Crops Utilization Research at Iowa State University. The meal was extracted with polar solvents (ethanol, methanol), which were then dried with anhydrous sodium sulfate, filtered and evaporated by rotary evaporation. Purification of CHB was accomplished by using silica gel gravity column chromatography with a blend of organic solvents (hexane:acetone 8:2) as the mobile phase. The purity of the CHB was estimated at 90% by silica gel thin-layer chromatography (*3*, *4*).

Mandelonitrile, 4-hydroxy-mandelonitrile, benzyl isothiocyanate, benzylisothiocyanate, chloropicrin and dichlorvos were purchased from Sigma-Aldrich, St. Louis, MO. Analogs and derivatives were synthesized using the methods described previously (*5*, *6*). The two reactions utilized a small aldehyde or ketone as the starting material, used one of two reagents to accomplish the synthesis: trimethylsilyl cyanide with zinc iodide in methylene chloride, followed by treatment with 3N HCl, or potassium cyanide in acetic acid (*5*, *6*). Esters were made from certain cyanohydrins, using one of two reactions: acetates were produced by using acetyl chloride in methylene chloride with a catalytic amount of pyridine; other esters were made by using an acid plus 4-(N,N-dimethylamino)pyridine (DMAP) and N,N′-dicyclohexylcarbodiimide (DCC) in methylene chloride. Cyanohydrins were purified by distillation, and structural confirmation was obtained by proton NMR and IR.

Insects for fumigation were reared in the Department of Entomology Insectary. Soybean cyst nematodes were hatched in the Nematology Laboratory from eggs collected at Iowa State University research farms and reared in a greenhouse. Root-knot nematodes were from a colony reared in a greenhouse. Methods for hatching the nematodes and conducting laboratory bioassays were previously described (*8*). Bioassays for anti-fungal and anti-bacterial activity in soil were previously described in a patent (*8*). A bioassay for the inhibition of weed-seed germination was carried out according to the methods previously described (*6*). A minimum of three replications was utilized for all of the bioassays.

Results

Fumigation

Our initial testing determined that several of the natural insecticides in these two classes of chemicals also showed potency against nematodes (*5–7*). Initial fumigation bioassays with the lesser grain borer (*Rhyzopertha dominica*) and the house fly (*Musca domestica*) demonstrated the potency of cyano-hydroxy-propene (CHP) (the cyanohydrin of acrolein) compared to commercial fumigants chloropicrin and dichlorvos. Table 1 shows the LC_{50} of CHP to be three times lower than that of chloropicrin but slightly higher than that of dichlorvos, against the lesser grain borer. It also reveals that CHP was less potent against the house fly than the two commercial fumigants. Three esters of CHP were evaluated in

the fumigation bioassays: the acetate, the propionate and the pivalate. Table 2 shows that the acetate and the propionate were superior to the chloropicrin against the lesser grain borer and nearly as efficacious as dichlorvos.

Table 1. The Fumigation Toxicity of CHP, Chloropicrin, and Dichlorvos against the Lesser Grain Borer and Adult House Fly after 24 hr

	Fumigation toxicity	
	Lesser grain borer	*Adult house fly*
Compound	LC_{50} *(μg/cm³)*	LC_{50} *(μg/cm³)*
CHP	0.37	0.15
Chloropicrin	1.3	0.08
Dichlorvos	2.9	0.011

Table 2. The Fumigation Toxicity of CHP Derivatives against the Lesser Grain Borer and Adult House Fly after 24 hr

	Fumigation toxicity	
	Lesser grain borer	*Adult house fly*
Compound	LC_{50} *(μg/cm³)*	LC_{50} *(μg/cm³)*
CHP-Acetate	0.37	0.26
CHP-Propio	0.70	0.66
CHP-Pival	2.4	1.37
Chloropicrin	1.3	0.08
Dichlorvos	2.9	0.011

Nematode Juvenile Contact Toxicity Assay

Incubation in CHP reduced the mobility of the soybean cyst nematode second-stage juvenile (J2) relative to four other experimental treatments[CJR[4] The average percentage of surviving mobile J2s for the four less active compounds was 27% at 1 hr and 15.7% at 24 hr; CHP was much more active against the nematodes. Incubation of J2 in CHP for 1 or 24 hours reduced mobility to 1.1% and 0.2%, respectively. After incubation in the CHP treatment, the immobile J2 appeared rigid, in contrast to the flaccid appearance of the immobile J2 for all other treatments. There was clearly a different mechanism of toxicity exerted by the CHP.

Nematode Egg Hatch

The bioassay to evaluate the effects of the fumigants on egg hatch for the soybean cyst nematode (SCN) revealed that the glucosinolate aglycone CHB, the natural methyl-ethyl ketone cyanohydrin, the synthetic cyanohydrin CHP and two esters of CHP all caused significant reduction in SCN egg hatch after the 14-day exposure compared to hatch in water (Table 3). At the 100-µg/ml concentration of the compounds in water, the CHP and CHP acetate inhibited 99% of SCN egg hatch. At the lower concentration (10 µg/ml) they caused 60-70% inhibition of egg hatch.

Table 3. Activity of Potential Natural Nematicides on Hatch of Soybean Cyst Nematode Eggs. *Difference Compared to Water Control after 14 Days (Three Replicates).

Egg hatch: Soybean cyst nematode		
Compound	*Concentration (ppm)*	*Difference in hatch**
CHB	10	-70%
	100	-73%
CHP	10	-69%
	100	-99%
CHP-Ace	10	-58%
	100	-99%
CHP-Piv	10	-25%
	100	-83%
MEK-CNOH	10	+2%
	100	-83%

The egg hatching bioassays for the root-knot nematode (RKN) were conducted with contact activity and vapor-only activity at 100 µg/ml over a 20-day period. The two best inhibitors by contact were again CHP and the CHP acetate (Table 5), while benzyl isothiocyanate and mandelonitrile had no significant effect on hatching; CHP acetate inhibited 91.6% of RKN egg hatch (Table 4). When the candidate compounds were tested for their efficacy by vapor only, the CHP and the mandelonitrile were the most potent at 20 days, suppressing 71% and 56% of RKN egg hatch, respectively.

Table 4. Root-Knot Nematode: Percentage Egg Hatch (Vapor Exposure) at 100 ppm (Three Replicates)

Egg hatch: Root-knot nematode (Vapor Exposure)					
	Days				
Compound	*0*	*4*	*8*	*12*	*20*
Benzyl isothiocyanate	0	18	37	51	59
Mandelonitrile	0	4	14	17	29
MEK CNOH	0	14	28	39	61
CHP	0	3	14	14	18
CHP-Ace	0	18	36	41	43
DH_20	0	19	41	60	66

Table 5. Root-Knot Nematode: Percentage Egg Hatch (Contact Exposure) at 100 ppm (Three Replicates)

Egg hatch: Root-knot nematode (Contact Exposure)					
	Days				
Compound	*0*	*4*	*8*	*12*	*20*
Benzyl isothiocyanate	0	19	40	53	63
Mandelonitrile	0	19	44	57	72
MEK CNOH	0	18	42	53	68
CHP	0	8	23	27	34
CHP-Ace	0	1.5	5	5	6
DH_20	0	22	46	65	72

Figure 3 and 4 show the potency of CHP and CHP-acetate at inhibiting SCN egg hatch at 100 μg/ml (100 ppm) solution in water, compared to water alone and a solution of the hatching stimulant zinc sulfate or another hatching stimulant AA, which is 2-hydroxymethylenecyclopentanone. Two exposure routes were utilized: (1) direct contact with the treatment solutions and (2) vapor-exposure only, with the treatment solution in the same closed container, but not directly contacting the nematode eggs. After 24 dyas of incubation, all eggs were transferred to and incubated in water through day 46 to determine if effects on hatch were irreversible. Through the 46-day test period, the CHP and CHP acetate both demonstrated excellent activity against SCN egg hatch, via contact and vapor exposure routes.

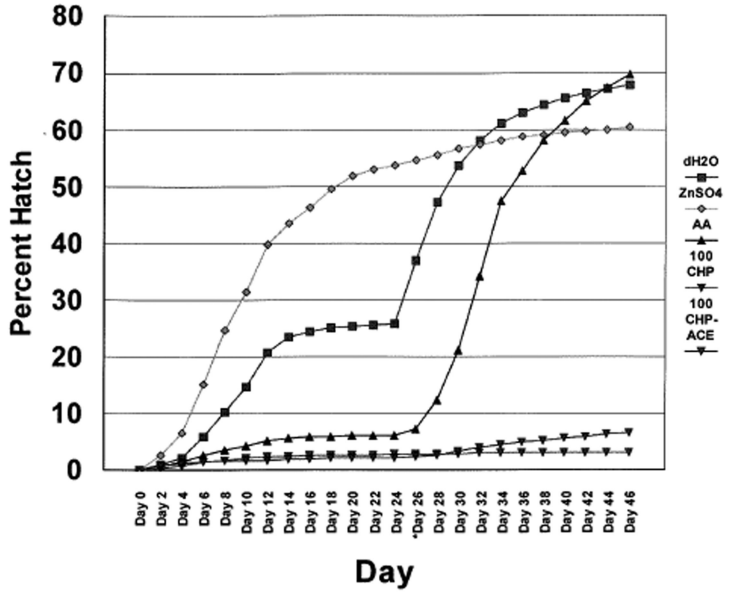

Figure 3. The effect of CHP and CHP actetate on the hatching of soybean cyst nematode eggs following contact exposure (three replicates).

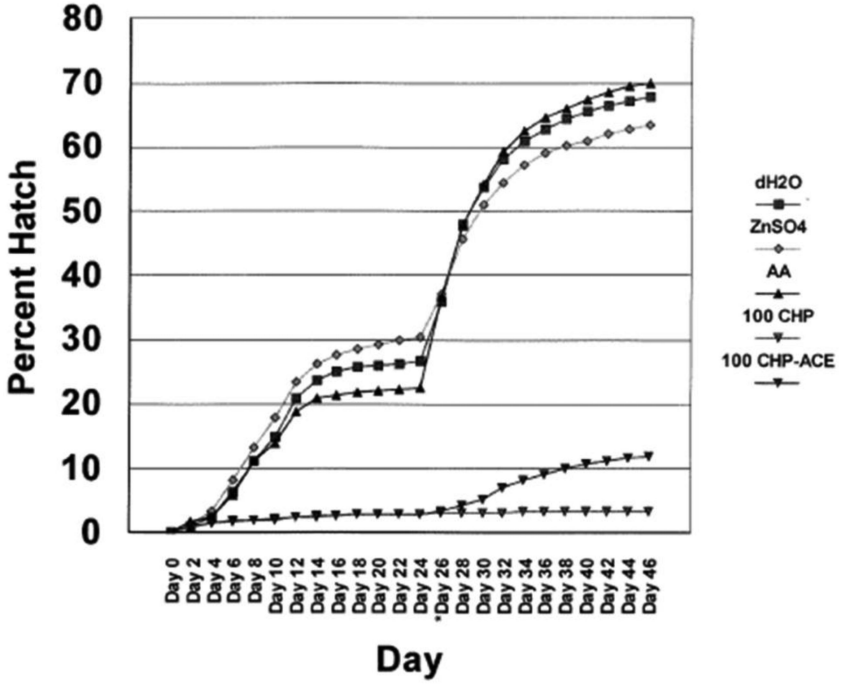

Figure 4. The effects of CHP and CHP acetate on the hatching of soybean cyst nematode eggs following volatile exposure (three replicates).

Soil Fumigation

Laboratory bioassays of soil fumigation, with CHP acetate applied at different concentrations, quantified total bacterial colony count per gram of soil (dry wt.) and total fungal colony count per gram of soil (dry wt.) Table 6 illustrates the anti-bacterial efficacy of CHP acetate applied to an agronomic soil at different concentrations. Concentrations greater than 100 μg/g suppressed bacterial colony growth on the agar plates. Table 7 shows the anti-fungal effect of treating soil with different concentrations of CHP-acetate. Weed-seed germination was also assessed, per 20 grams of soil in the greenhouse, following treatment of CHP acetate or chloropicrin (Tables 8, 9, 10).

Table 6. Total Bacteria Count in Soil after Two Days Following Application of CHP-Acetate (Three Replicates)

Soil bacteria	
Compound	*Average cell/g (dry weight) ± S.D.*
Blank (control)	$4.7 \times 10^6 \pm 1.8 \times 10^6$
Solvent (control)	$4.7 \times 10^6 \pm 0.2 \times 10^6$
10 ppm	$4.7 \times 10^6 \pm 2.6 \times 10^6$
100 ppm	$4.7 \times 10^6 \pm 3.2 \times 10^6$
1,000 ppm	$4.7 \times 10^6 \pm 0.02 \times 10^6$
10,000 ppm	0.00 ± 0.00

Table 7. Total Fungi Count in Soil after Two Days Following Application of CHP-Acetate (Three Replicates)

Soil fungi	
Compound	*Average cell/g (dry weight) ± S.D.*
Blank (control)	$1.9 \times 10^4 \pm 0.4 \times 10^4$
Solvent (control)	$1.4 \times 10^4 \pm 0.4 \times 10^4$
10 ppm	$2.0 \times 10^4 \pm 0.5 \times 10^4$
100 ppm	$0.5 \times 10^4 \pm 0.4 \times 10^4$
1,000 ppm	0.00 ± 0.00
10,000 ppm	0.00 ± 0.00

Table 8. The Number of Germinated Weed Seeds Following Application of CHP-Acetate (3 days)

	Germination of weed seeds		
	Number of germinated weed seeds (average)		
Treatment	*Replicate 1*	*Replicate 2*	*Replicate 3*
Solvent (control)	6	8	11
1,000 ppm	0	0	0
10,000 ppm	0	0	0

Table 9. The number of Germinated Weed Seeds Following Application of CHP-Acetate at 24 days

	Germination of weed seeds		
	Number of germinated weed seeds (average)		
Treatment	*Replicate 1*	*Replicate 2*	*Replicate 3*
Solvent (control)	10	20	27
1,000 ppm	0	1	0
10,000 ppm	0	0	0

Table 10. The Number of Germinated Weed Seeds Following Application of Chloropicrin at 10 days

	Germination of weed seeds		
	Number of germinated weed seeds (average)		
Treatment	*Replicate 1*	*Replicate 2*	*Replicate 3*
Solvent (control)	*0*	*12*	*3*
1,000 ppm	*0*	*3*	*0*
10,000 ppm	*0*	*0*	*0*

Discussion

Some of the natural aglycones and synthetic analogs of them show excellent toxicity as insecticides and nematicides. Against two species of soil-dwelling plant-pathogenic nematodes, some aglycones and one acetate derivative demonstrated toxicity via contact with a treatment solution and inhibition of egg hatch via both contact and vapor exposure routes.

In today's agrochemicals market, "soil fumigants" are frequently deployed for control of insects, nematodes, bacterial diseases, fungal diseases and weed seed germination. We have evaluated one of the most effective nematicides for its efficacy against soil bacteria, soil fungi and weed seed germination, in addition to its potency against insects and nematodes. When the overall results are compared against chloropicrin as a current commercial soil fumigant, the CHP acetate biorational nematicide is comparable in efficacy against those several types of soil organisms. Although the soil bacterial and soil fungal bioassays were measuring overall bacterial and fungal activity, rather than specific pathogenic bacteria or fungi, the principle of suppression of bacteria and fungi indicates that the CHP acetate could possibly be effective in the broader definition of soil fumigant.

As pressure develops for replacement of older nematicides and soil fumigants, it is possible that following the lead compounds that plants have provided us may be a valuable approach for development of new nematicides and/or soil fumigants.

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Chapter 14

Semiochemicals To Monitor Insect Pests – Future Opportunities for an Effective Host Plant Volatile Blend To Attract Navel Orangeworm in Pistachio Orchards

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The navel orangeworm (*Amyelois transitella*) has been a major insect pest of California tree nut orchards for the past five decades. In particular, almond and pistachio orchards suffer major annual economic damage due to physical and associated fungal damage caused by navel orangeworm larvae. Until recently, the only viable option, albeit inconsistent, for monitoring navel orangeworm populations within these orchards has been the use of almond meal in egg traps. Over the past several years a synthetic blend of host plant volatiles, based on various almond emissions, has demonstrated effective attractancy of both male and female navel orangeworm in field trapping studies in almond orchards. However, this attractiveness did not extend into the mid- to late-season in pistachio orchards, thus suggesting either an orchard specificity of the moth or perhaps a temporal component expressed as a change in background odors of the orchard. Using information and approaches learned during the development of the almond host plant volatile blend, research within these ARS laboratories

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has focused on the volatile emissions from various pistachio matrices to ascertain potential semiochemicals capable of attracting navel orangeworm moths in pistachio orchards. Provided herein is a perspective on the challenges and progress of developing a synthetic host plant volatile blend for an agricultural insect pest that has a diverse range of hosts and has proven difficult to monitor.

Introduction

The polyphagous navel orangeworm, *Amyelois transitella* (Lepidoptera: Pyralidae), is an insect pest of several California agricultural commodities with its host range including almond, fig, pistachio, pomegranate, and walnut (*1–4*). Literature from the 1950s and 1960s report the movement of navel orangeworm moths from southern California walnut storage areas to Central Valley fruits and tree nuts (*5*). For tree nut products, larval feeding on the nutmeat causes physical damage that lowers kernel quality and value, but more importantly also introduces mycotoxigenic fungi (*6*, *7*). Over the past five decades various methods have been used to control or monitor the navel orangeworm (*8*) with strict orchard sanitation playing a vital role in controlling moth populations (*2*). More recently, the use of a synthetic female navel orangeworm sex pheromone blend has been introduced as a means to attract male navel orangeworm for monitoring and mating disruption purposes (*8*) in almond and pistachio orchards.

In addition to the synthetic pheromone blend, a blend of host plant volatiles that attracts both male and female navel orangeworm moths in almond orchards has been identified (*8*, *9*). Because it is a generalist scavenger (*5*), it is thought the navel orangeworm may respond to numerous host plant volatile cues instead of just one or two specific semiochemicals (*10*). For attraction of navel orangeworm to host plant volatiles within almond orchards this idea has been validated by use of a synthetic five-component blend of host plant volatiles described above (*9*). In terms of insect attractancy to host plant volatiles one generally accepted theory is that phytophagous insects are attracted to specific blends of ubiquitous volatiles from host plant species (*11*). A second theory is that background volatiles, e.g., the ambient odors associated with a particular orchard, may enhance the detection of a more orchard-specific volatile or blend of volatiles (*12*). For Lepidopterans it is rare that a single host plant volatile is as effective as a pheromone; however, instances such as the pear ester for codling moth (*13*) provide an example that it is possible. Using the volatile emission profiles from two of the hosts of the navel orangeworm we present evidence that support the idea that ambient orchard-specific volatiles may provide the necessary background volatile bouquet for a host-based volatile or blend of volatile attractants (semiochemicals). We will also discuss the role of fungal spores on the host plants for attracting the navel orangeworm (*14*).

Typical Almond and Pistachio Emission Profiles

Of the numerous hosts of the navel orangeworm, two orchards are particularly negatively affected by its damage – almond and pistachio, which produce over $4 billion annually for California growers. Several studies have been performed on the volatile profiles of various matrices of almond or pistachio and include *ex situ* intact (*15*, *16*) and hulls (*17*); *in situ* (*9*, *18*); essential oil (*19*); ambient (*20*); kernels (*21*); and, mummies (*22*, *23*). Representative major volatiles from almond and pistachio orchards/fruit are provided in Table I.

Table I. Representative Major Volatiles Produced by Almond and Pistachio Orchards/Fruit (from References (*16–20*)). Volatiles Are Listed in Approximate Order of Amounts or Representative of Matrix Studied.

Almond-Produced Volatiles	*Pistachio-Produced Volatiles*
Benzaldehyde	Limonene
Acetophenone	α-Terpinolene
Nonanal	Δ^3-Carene
(Z)-Hex-3-enyl acetate	α-Pinene
Caryophyllene	Myrcene

As expected, the major volatiles produced by pistachio (Table I) are all monoterpenes; albeit, the studies performed were *ex situ* (*16*, *17*, *19*) and performed on either the kernel or intact nut. A specific study of the ambient orchard volatile emissions, similar to the one performed in almonds (*20*), would be necessary to more appropriately address the volatiles encountered by female navel orangeworm as they maneuver the orchards in search of an ovipositional site. Additionally, the phenology and the vulnerable stages of the crop need to be considered to more fully understand this phenomonen. An *in situ* analysis of a single intact pistachio is provided later in this chapter and will be discussed in greater detail.

Conversely, the major volatiles for the two almond matrices considered – ambient and *in situ* – are more diverse but do not include monoterpenes (Table I). The green leaf volatile hexenyl acetate was the major compound detected during the *in situ* study of the intact nut (*18*). Though monoterpenes were not present as a major volatile, the sesquiterpene caryophyllene was present in the *in situ* study on the cultivar Nonpareil. Interestingly, the ambient volatiles detected in collections from almond orchards (*20*) were primarily comprised of benzenoids and fatty acid breakdown products with benzaldehyde, acetophenone, and nonanal as the highest amounts. It should be noted that almond orchards typically consist of several cultivars whereas pistachios are primarily the Kerman variety; thus, volatile compositional differences due to varying cultivar emissions may be present in almond orchards.

The Role of Fungal Spores and a Navel Orangeworm Semiochemical(s)

A blend of host plant volatiles that attracts navel orangeworm in almond orchards and contains the compounds (±)-1-octen-3-ol, (±)-(*E*)-conophthorin (**1**) (Figure 1), acetophenone, ethyl benzoate, and methyl salicylate was recently discovered (*9*). The benzenoids of the blend are volatiles known to be associated with almonds or almond orchards (*9*). The compound 1-octen-3-ol has been associated with fungal emissions as well as damaged almonds (*15*).

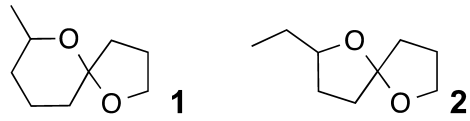

*Figure 1. The spiroketals (E)-conophthorin (**1**) and (E)-chalcogran (**2**).*

The origin of the navel orangeworm semiochemical conophthorin (**1**) was originally thought to be from naturally or mechanically damaged almond hulls (*9*, *14*, *15*). However, due to its transient detection from various almond matrices further thought was given to its actual origin (*17*). The question was if the spiroketals were produced by the plant, a fungus, or if both organisms were involved in spiroketal production.

A foundational study was performed that evaluated the volatile profiles of fungal spores – fungi that were common to tree nut orchards – placed on fatty acids that were also common to tree nuts (*24*). In this study the spiroketals conopthorin (**1**) and chalcogran (**2**) (Figure 1) were produced by the fungal spores on linoleic; however, only the chalcograns were produced by the spores when on linolenic acid. This study was important in that it definitively showed that fungal spores are capable of producing the spiroketals from a basic carbon source. It did not, however, prove that the almond (or other host tissue) does or does not play a role in the formation of conophthorin in conjunction with the fungal spores.

With the knowledge that conophthorin was detected from almonds undergoing hull split and the idea that fungal spores may be responsible for the generation of conophthorin, a study of late season almond and pistachio hulls was performed to determine if both crops generated conophthorin and to further prove hulls as the carbon source (*17*). *Ex situ* evaluation of the headspace of ground hulls and shells at varying periods during the growing season showed that almond hulls and shells were capable of producing both conophthorin (**1**) and chalcogran (**2**). The production of the spiroketals appeared to be dependent upon the water activity of the matrix in question. The water activity needed to be greater than 0.80, which is the water activity required of several xerophilic fungi common to tree nut orchards (*25*, *26*). The almond and pistachio samples were received from a commercial orchard and were assumed to have a representative fungal bouquet present. Despite the positive results of spiroketal formation from almonds, the

spiroketals were not detected from the pistachio hulls or shells. One possible hypothesis to this, which needs to be evaluated, was that the relatively large amounts of monoterpenes emitted by the fresh pistachios may inhibit fungal growth (*17*, *27*).

The consistent production of conophthorin (**1**) from *ex situ* ground almond hulls supported the results that the spiroketal was formed during almond hull split (*9*). The results from the hull split (*9*), *ex situ* (*17*), and spore emission (*24*) studies were important since they demonstrated that when fungal spores start to develop on the hull split almonds the spores are essentially calling to navel orangeworm moths. This possible relationship (Figure 2) was explored and discussed in an earlier publication (*14*) and will also be considered further for applicability to pistachio as the host plant.

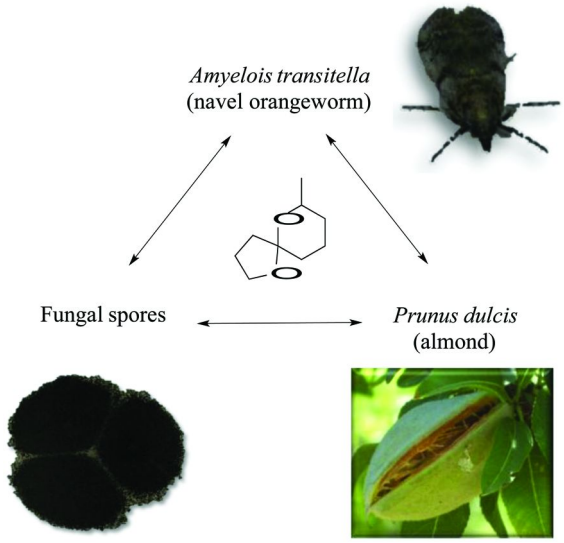

Figure 2. Postulated relationship of the navel orangeworm moth with fungal spores and its almond host plant. (Reproduced with permission from reference (14). Copyright 2013 Sociedad Química de México.)

As illustrated in Figure 2, conophthorin (**1**) is hypothesized to be the unifying chemical compound for ubiquitous orchard fungal spores – including aflatoxigenic aspergilli, the navel orangeworm moth and its larvae, and almonds undergoing hull split, which is a natural form of damage to the hull tissue. Berenbaum and co-workers (*28*) have shown that navel orangeworm larvae can neutralize the toxins produced by aflatoxigenic aspergilli, and thus are able to ingest fungi in addition to the nutmeat. There are a few ecological questions that warrant discussion and experimental exploration (*14*). For instance, what is the actual relationship between the fungi and navel orangeworm – do the fungi use navel orangeworm larvae as a transport? Do the moths use the fungal

emissions as a means to locate the host? Or both? Then there is the question of how the larvae are influenced by the surrounding volatiles that become semiochemical attractants to adults? The last question has been broadly addressed as the natal habitat preference induction hypothesis which states "phytophagous insect females should prefer to lay their eggs on the host species on which they developed as larvae" (*29–31*). This hypothesis appears to have two separate theories – the Hopkins' host-selection principle (*32*, *33*), which essentially says that adult female moths are attracted to the distinctive odors of their environment as larvae, and the chemical legacy (*34*), which says the adults are influenced by the residual odors from their life as larvae that remain until the pupal stage. Since the semiochemical conophthorin (**1**) has been proposed as the central link of the navel orangeworm, fungal spores, and almonds (*14*), the following sections will consider several experiments performed on pistachio matrices and whether there exists a different unifying semiochemical for the navel orangeworm, fungal spores, and pistachios.

Mummies and Overwintering

Because the navel orangeworm is known to overwinter in pistachio and almond nuts that remain on the tree or ground after harvest (mummies) (*2*, *35*) it could be intuited that there may be a volatile component of the mummies that attracts the navel orangeworm moths (*36*, *37*). Furthermore, since mummies can be laden with fungal growth or fungal spores (Figure 3) there exists the potential role of fungal spores or fungal emissions as the source of semiochemicals. Indeed, the idea of mummies attracting navel orangeworm (*3*, *36*, *38*) has been recently re-visited by Boyd and co-workers (*39*) who used ground almond and pistachio mummies to attract and trap navel orangeworm moths in Northern California almond and pistachio orchards. The volatile profile of both the dry (low water activity) and wet (increased water activity) almond and pistachio mummies were recently described (*22*, *23*).

Figure 3. Examples of a pistachio (left) and almond (right) mummy. An adult navel orangeworm moth is seen on the almond mummy.

The volatile profiles of the dry almond and pistachio mummy matrices had some similarities, which would corroborate the first theory discussed above that asserted phytophagous insects are attracted to specific blends of ubiquitous volatiles from host plant species (*11*). Table II shows the ten most abundant volatiles detected by GC-MS from the dry almond and pistachio mummy matrices and identifies volatiles common to both. In this instance, the listed ubiquitous compounds are marked with an asterisk and could be considered as potential attractants of navel orangeworm, but in varying ratios.

Table II. Ten Most Abundant Volatiles Produced by Dry (Low Water Activity) Almond and Pistachio Mummies (From Reference (*22*)). Volatiles Marked with * Were Common to Both Matrices in Relatively Large Abundances. Volatiles Marked with ‡ Were Also Common but in Lower Relative Abundances for the Other Matrix.

Almond Mummy Volatiles	*Pistachio Mummy Volatiles*
Acetic acid‡	Limonene*
Limonene*	α-Thujene*
2,3-Butanediol B	α-Pinene‡
Unknown	α-Terpinolene*
2,3-Butanediol A	Δ^3-Carene
γ-Pentalactone	Camphene
γ-Butryolactone	β-Pinene
α-Terpinolene*	α-Longipinene
Nonanal	Bornyl acetate
α-Thujene*	β-Myrcene

While the similarities between these two matrices support the first theory, there were also a large number of compounds unique to each matrix and could support the second theory which contends background volatiles may enhance the detection of a matrix-specific volatile or volatiles. Elucidation of these matrix-specific volatiles from the overall volatile bouquet will require extensive electrophysiological, behavioral, and field trapping studies to determine and verify any semiochemical activities. This work is ongoing with some developments reported in this chapter. As an example of the complexity of volatiles unique to each matrix, 57 volatiles were matrix-specific to dry pistachio mummies, 13 were specific to dry almond mummies, and a total seven volatiles were common to both matrices (*22*) (Figure 4).

Using the acquired knowledge that an increase in the water activity of a tree nut matrix influences the volatile profiles of the resident fungal spores (*17*), we demonstrated that by increasing the water activity of almond and pistachio

mummies the number of emitted volatiles dramatically increased (*23*) (Figure 4). Given the usual rain during the winter and spring months in the California Central Valley it is easy to assume an increase in water activity of the mummy matrices, thus providing different volatile profiles for the navel orangeworm to detect and act upon during their first flight in early spring. As illustrated in Figure 4, the wet pistachio matrix provided 22 unique volatiles compared to the dry matrix; and, the wet almond matrix provided a surprising 37 unique volatiles, a 185% increase in volatile composition, compared to the dry matrix. In the wet matrices there were 19 volatiles – diverse in their classes of compounds (monoterpenes, 2-alkanones, alkanols, and aromatics) – common to both almond and pistachio mummies.

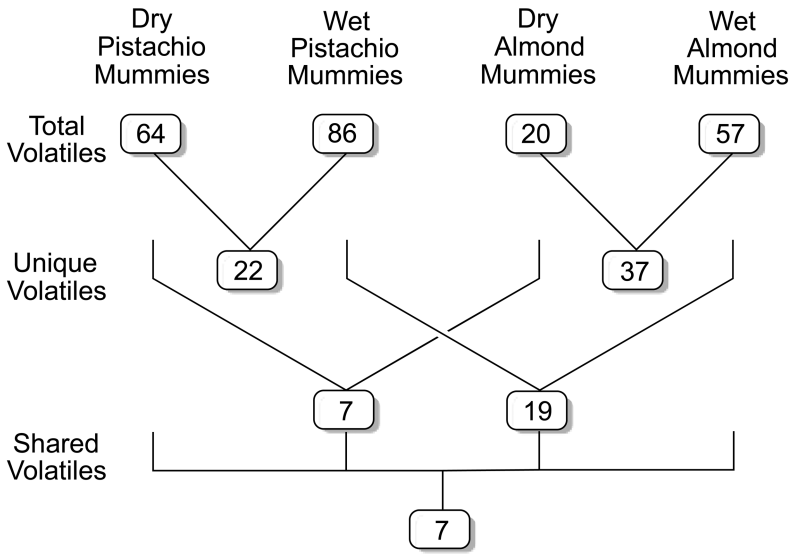

Figure 4. Tree illustrating the total number of volatiles, unique volatiles, and shared volatiles from the four mummy matrices studied. (Reproduced with permission from reference (23). Copyright 2014 Elsevier.)

Despite showing promise as a biomass-based attractant to monitor navel orangeworm populations, the use of mummies or other tree nut products may allow for undesired emission variability (*3*, *23*, *36*, *40*), and thus inconsistent trapping in the field. This volatile emission variability was recently demonstrated during normal evaluation of our treatments for laboratory-based behavioral bioassays and is described below.

Pistachio Mummy-Produced Semiochemicals?

The success of the almond tissue-based host plant volatile blend to attract navel orangeworm moths in almond orchard trapping studies provided a template for the investigative efforts toward a pistachio tissue-based attractant blend. The following series of experiments and brief discussions highlight recent efforts by ARS researchers and demonstrate the complexity of the volatile profiles from various pistachio tissues.

Table III. Volatile Differences of Different Batches of Dry Pistachio Mummies As Analyzed by HS-SPME-GCMS Analyses on a DB-Wax Column. Relative Abundances: ++ > 1 x 10^6 and + < 1 x 10^6.

Unique Volatiles…	*Relative Abundances*	*RI*	*…To What Batch*
1-Methylpyrrole	+	1134	DPME 2012
2,6-Dimethylpyrazine	+	1327	DPME 2012
Junipene	++	1565	DPME 2012
Unknown sesquiterpene	+	1571	DPME 2012
trans-p-Menta-2,8-dien-1-ol	+	1671	DPME 2012
Unknown alkene	+	1779	DPME 2012
Dehydromevalonic lactone	++	2008	DPME 2012
2-Nonanone	+	1387	DPME 2013
Nonanal	++	1391	DPME 2013
β-Thujone	+	1437	DPME 2013
Decan-2-one	+	1492	DPME 2013
Camphor	+	1510	DPME 2013

Emission Variability as a Function of Batches Collected

Dry Pistachio Mummy Emission Analysis

Pistachio mummies from a commercial orchard containing the cultivar Kerman were collected at varying intervals and shipped as different batches. The batch labeled DPME 2012 was collected in the winter months of 2011/2012, and the batch labeled DPME 2013 was collected in the winter months of 2012/2013. The samples were placed in containers identical to those used previously (*22*). The headspace volatiles of ground pistachio material (varying from 6.5 g to 12 g) were adsorbed onto a 100 μm solid-phase microextraction (SPME),

polydimethylsiloxane fiber using a fiber exposure time of 30 minutes while the sample was at room temperature (ca. 25 °C). GC-MS analyses were performed on instrumentation and methods identical to previously published conditions and on a DB-wax column (*22*). Data reported in Table III are based on the average GC-MS relative abundances.

Table III lists the volatiles that were unique to the different batches of dry ground pistachio mummies. The major volatiles, constituting greater than ca. 95% of the amounts detected, were essentially identical to the monoterpenes noted in Table II for pistachio mummies. The significance of the results in Table III was the inconsistent production of the volatiles and that one batch of mummies produced a potential semiochemical that was not produced in another. For instance, nonanal, detected in the DPME 2013 batch, but not the DPME 2012 batch, was shown to bind with navel orangeworm olfactory proteins (*41*) and was also evaluated as a semiochemical in field testing studies (*9*).

Dried Mummy Emissions as a Potential Ovipositional Attractant

Bioassay Hood

The behavioral bioassay hoods used had the following dimensions: 106.7 cm (L) x 91.4 cm (H) x 55.9 cm (W). A 10.2 cm circular hole for an in-duct fan was placed 19.1 cm from the back and 53.5 cm from the side of the hood. The front border of the hood was 6.4 cm around the front of hood, with the edge lined with Velcro® so the front cover of the hood could be easily attached and removed. The front cover was made of 0.3 cm thick plexiglass 106.7 cm (L) x 91.4 cm (H). One mm holes were drilled 5.7 cm from the edge and bottom of cover. Ten holes were drilled in 2 rows of 5, each hole was 2.54 cm apart and rows were 1.27 cm apart. A 10.2 cm diameter in-duct fan was attached to the hood in the outward direction to facilitate hood evacuation (a negative pressue inside the hood), and movement of air through the front cover holes. Evacuated bioassay hood volatiles were directed to a separate fume hood. Flow in bioassay hood was set at 0.13-0.15 m^3/min, measured in the duct approximately, 45.7 cm from the hood connection. Air flow in the fume hood was maintained at about 5.7 m^3/min. A horizontal metal wire was attached to the upper back length of the hood 17.8 cm from the top and 9.5 cm from the back. Metal wire hooks were attached to the horizontal wire 20.3 cm from each side to hang the egg traps.

Bioassay

Forty female and 10 male navel orangeworm moths, laboratory-reared from larvae on wheat bran diet, and 1-3 days old, were placed in a jar in the bioassay hood and the lid removed. Egg traps, as previously described (*9*) were filled with either dry ground pistachio mummy (DPME 2012 6.5 grams) or almond meal (AM 6.5 g). Each experiment was run for three nights, traps were removed from the hood the morning after the third night, and eggs deposited on the egg traps were counted immediately.

For the past few decades the use of egg traps baited with almond meal has been the standard tool for monitoring navel orangeworm populations in tree nut orchards (*8*, *37*, *42*). In their 2012 report Boyd and co-workers (*39*) demonstrated the ability of ground pistachio mummies to attract and capture female navel orangeworm. During their study they noted a large amount of deposited eggs in or near the trap baited with the ground pistachio mummy and suggested that the female moths considered the mummies as a viable host. Other research groups have also demonstrated the ovipositional attractancy of almond or pistachio meats (*3*, *42*). Our results from the laboratory-based behavioral bioassay (Figure 5) corroborate dried ground pistachio mummies as a possible ovipositional attractant and further advance the need to delineate what volatiles are responsible for attracting the female navel orangeworm. Our study of the volatile profile of dried ground pistachio mummies showed that ca. 95% of the profile consisted of non-oxygenated terpenoids – primarily monoterpenes and some sesquiterpenes – and only about 5% of the profile consisted of either oxygenated terpenoids (alcohols or ketones), fatty acid breakdown products, or aromatics (aryl-compounds) typically associated with fungi (*22*). Though terpenoids have not heretofore been shown to attract navel orangeworm moths the results from Figure 5 and the Boyd and co-workers study (*39*) suggest they should perhaps be considered in conjunction with other semiochemicals. To further explore the idea of pistachio and almond mummies exhibiting ovipositional activity we evaluated the dry and wet matrices of both almond and pistachio mummies and compared their ovipositional activity to that of ground pistachio kernels and almond meal (Figure 6).

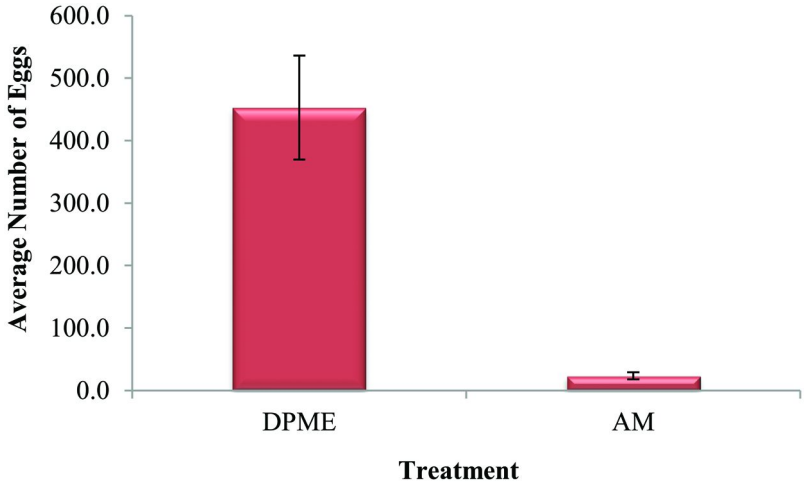

Figure 5. Average number of eggs oviposited on egg traps baited with either dry ground pistachio mummy (DPME) or almond meal (AM). Results were from a laboratory-based behavioral bioassay with n = 14. Error bars are standard error of means.

Wet and Dry Mummies as Ovipositional Attractants in the Field

Field Egg Trapping Studies

Egg traps were placed in a randomized design (*9*) in both almond and pistachio orchards and baited with each of the treatments noted in Figure 6 and in the following amounts: 12 g dry ground material, 15 g of wet ground material, which corresponded to 25% moisture w/w (*23*). Two trials were performed each with n = 10 and the results for each treatment averaged over the two collection periods to provide the data in Figure 6. The trials were performed from 5/1/2013 to 5/17/2013 and in different areas of each orchard.

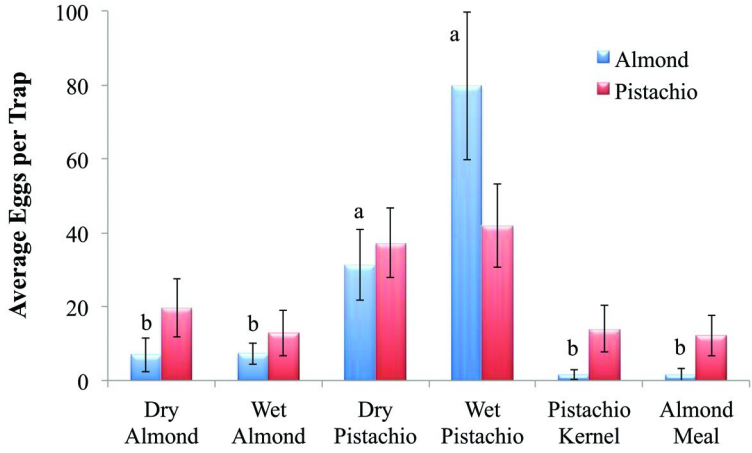

Figure 6. Average number of eggs oviposited on egg traps baited with varying mummy matrices. Results were from field-based evaluations over two one-week periods with a total n = 20 for each treatment in each orchard. Error bars are standard error of means. Bars with different letters denote significant difference (t-Test, P < 0.05) between treatments in almond orchards. The legends "Almond" and "Pistachio" refer to orchards where the treatments were placed.

The results shown in Figure 6 corroborated the laboratory-based behavioral bioassay results for the ground dried pistachio mummy treatment having some ovipositional attractancy behavior. In almond orchards the female moth oviposited significantly greater number of eggs on the traps containing the wet and dry pistachio mummy material than the other treatments, with the wet pistachio only numerically greater than the dry pistachio. There were no significant differences between treatments in pistachio orchards. When compared for egg trap efficacy between the two orchards, the treatments did not demonstrate any significant difference (t-Test, $P < 0.05$), albeit the wet pistachio mummies provided the only instance of a greater number of eggs oviposited on a treatment in the almond

orchard. An interesting result was the relatively poor performance of the almond mummy, and the control pistachio and almond kernel treatments. One possibility for the low number of eggs deposited on these treatments was the noted low amount of emissions in the almond mummy tissues relative to the pistachio mummy tissues (*22*, *23*). The low amount of volatile output has also been noted for ground almond kernels. For ovipositional activity, the data hinted that the pistachio tissues, both wet and dry, contain potential semiochemicals and warrant further investigation and delineation of activities.

To further evaluate possible sources of pistachio-based semiochemicals a series of exploratory experiments were performed on both *in situ* and *ex situ* pistachio matrices at various periods when they are vulnerable to navel orangeworm infestation.

Other Pistachio-Produced Semiochemicals?

Split and Late-Season Pistachios as Potential Hosts

Ex Situ Split Shells/Hulls versus Undamaged

Control (undamaged and intact) and split pistachios (Figure 7) were collected from the field August 2013 prior to first shake of harvest. Five nuts of the control (17.2 g), 25 shells/hulls of split (14.9 g), and 25 kernels of split (12.3 g) were placed in separate volatile collection chambers previously described (*22*, *23*). The treatments were kept whole and not ground. The containers were sealed for five minutes and the headspace volatiles were collected for 30 minutes onto 100 μm solid-phase microextraction (SPME) polydimethylsiloxane fibers. The desorbed volatiles were analyzed on instrumentation and parameters identical to previously described protocols (*17*).

Figure 7. Examples of control (left) and split (right) pistachios collected from Strain Ranches, Arbuckle, CA in August 2013.

Table IV. Survey Results Showing the Major Volatile Differences between Ex Situ Control (Intact, Undamaged, and No Fungal Growth), Shells/Hulls, and Kernels of Split Pistachios Collected in August 2013 from Arbuckle, CA and Analyzed by HS-SPME-GCMS on a DB-1 Column. Relative Abundances: nd = not detected; + = < 1 x 10^6; ++ = 1 x 10^6 to 1 x 10^8; +++ = 1 x 10^8 to 1 x 10^9; and ++++ = > 1 x 10^9.

Identity	*Control*	*Shells/Hulls*	*Kernels*
Methylpyrrole	nd	++	nd
Styrene	nd	++	++
Tricyclene	+	++	nd
α-Thujene	++	++	nd
α-Pinene	++	+++	++
Camphene	++	++	nd
β-Pinene	+	++	nd
Myrcene	nd	++	nd
Δ^3-Carene	++	+++	++
α-Terpinene	+	++	nd
p-Cymene	nd	++	nd
Limonene	++	++++	++
γ-Terpinene	nd	++	nd
Terpinolene	++	+++	++
Bornyl Acetate	++	nd	nd
Longifolene	nd	++	nd

The evaluation of volatiles from split pistachios was of interest since they have been associated with the infestation of navel orangeworm larvae as well as contamination of aflatoxin, a toxin produced by certain *Aspergillus* fungi (*43*, *44*). Similar to almond hull-split, the natural damage of an split pistachio represents a vulnerable stage for navel orangeworm to exploit as a host and thus its volatile profile may contain semiochemicals (*9*). The survey of *ex situ* volatiles illustrated the noteworthy differences between an intact pistachio and the components of its naturally damaged counterpart.

The major volatiles detected from the pistachio *ex situ* split experiment are shown in Table IV. The emissions of the control pistachios (intact) corroborate other reports of pistachio fruits at approximately similar phenological stages with limonene, terpinolene, and α-pinene among the top volatiles produced (*16*, *17*). The data also suggest that the vast majority of pistachio volatile emissions originate from the shells and hulls with substantial increases in both composition and amounts of compounds emitted. The compounds methylpyrrole and styrene were emitted in relatively large amounts and were indicative of fungal growth (*15*, *23*). In particular, the emission of methylpyrrole was shown to increase markedly in wet pistachio mummies (*23*) indicating the participation of fungi for its production.

Finally, the relatively low volatile emissions, in both content and composition, corroborate other studies performed by our laboratories on other tree nut matrices (unpublished results). More importantly, the low emissions of tree nut kernels overall may partially explain the poor results for these matrices as shown in Figure 6. However, the presence of styrene in the kernel emissions does indicate some fungal growth and thus some form of damage to the kernels of split pistachios.

Figure 8. Illustration of the single-nut in situ volatile collection chamber used to monitor the headspace of undamaged and naturally damaged post-harvest pistachios from Strain Ranches, Arbuckle, CA in October 2013.

Table V. In Situ Results Showing the Volatile Differences between the Control (Intact, Undamaged, and No Fungal Growth) and Naturally Damaged Pistachios Collected in October 2013 from Arbuckle, CA and Analyzed by HS-SPME-GCMS on a DB-Wax Column. Amounts Reported Are Averages (n = 6) of the Relative Abundances (x 10^6) and Listed in Order of Naturally Damaged Pistachio Amounts. * = not authenticated; nd = not detected.

RI	*Identity*	*Control*		*Naturally Damaged*	
		Average	*(s.e.m.)*	*Average*	*(s.e.m.)*
1197	Limonene	69.5	(68.3)	137.4	(56.2)
1281	Terpinolene	0.1	(0.1)	37.8	(17.8)
1578	Bornyl acetate	1.5	(0.6)	14.0	(6.8)
1023	α-Thujene	0.1	(0.1)	11.4	(6.4)
1496	Decanal	11.3	(3.2)	10.2	(1.3)
1391	Nonanal	3.0	(0.8)	7.5	(2.6)
1852	Geranyl acetone	2.7	(1.0)	7.3	(2.5)
1687	1,8-Menthadien-4-ol*	nd	-	6.2	(2.1)
1443	*cis*-Limonene oxide	0.4	(0.4)	4.5	(3.7)
1146	Δ^3-Carene	1.6	(1.6)	4.1	(1.2)
1019	α-pinene	0.9	(0.7)	4.1	(1.9)
1161	Myrcene	0.7	(0.7)	3.6	(1.4)
1777	Curcumene	nd	-	3.0	(2.0)
1600	Unk monoterpene	nd	-	2.6	(1.7)
1465	α-Longipinene	0.6	(0.6)	2.5	(0.6)
1119	Sabinene	nd	-	2.3	(0.7)
1847	*p*-Cymene-8-ol	nd	-	2.1	(0.7)
1060	Camphene	nd	-	2.1	(0.6)
1617	Thujopsene	nd	-	1.3	(0.8)
1106	β-pinene	nd	-	1.3	(0.9)

Single-Nut In Situ Evaluation of Post-Harvest Pistachios

In Situ Analysis of Volatiles from a Single Nut

Control (undamaged and intact) and naturally damaged pistachios were individually enclosed using thin Teflon® paper cut into a circle (ca. 8-9 cm diameter) with a small hole (4-5 mm) cut in the middle and a straight cut made from the middle to the outside of the circle. A rubber ring (cut from rubber adapter

to fit snugly on the container) was placed over the nut and onto the stem. The Teflon paper was placed at the bottom of single nut on the stem and gently folded into a cone shape to provide a snug fit to the stem, then taped on the outside. A scintillation vial with the bottom glass portion removed was placed over the nut. The cone of Teflon was secured to the vial using the rubber sleeve and a wire placed around the neck of the vial for stability. A lid with a Teflon septum was secured to the vial (Figure 8) and a SPME, secured via wire to a nearby branch, was inserted into the vial through the septum.

Once the vial was capped, the volatiles were immediately adsorbed onto a 100 μm polydimethylsiloxane SPME fiber for 60 minutes. Upon removal from the collection system the SPME cartridges were stored on ice until desorption with a total storage time of 17-18 hours. Volatiles were analyzed via GC-MS on a DB-Wax column with instrumentation and methods identical to previously published parameters (*17*). Volatile identities were verified by comparison of retention times and fragmentation patterns to authentic standards. The top 20 volatiles from the naturally damaged pistachios are listed. The natural damage of the pistachios consisted of the hull peeling off with some signs of fungal growth.

Limonene and terpinolene continued to be the most abundant of the volatiles emitted from the naturally damaged pistachios (Table V) despite being so late in the season and the water activity of the pistachios likely decreasing to lower levels (*17*). The high s.e.m. of the limonene in the control was indicative of the overall decrease of the monoterpene content for this late season sampling. Also, 6 of the 10 compounds in the *ex situ* control performed two months earlier (Table IV) are still detected from the *in situ* control nut (limonene, terpinolene, bornyl acetate, α-thujene, Δ^3-carene, α-pinene). The levels of monoterpenes may have an important role in the activity of fungi since many monoterpenes have been reported to possess antifungal activity (*27*). The presence of the fatty acid breakdown products decanal and nonanal may be of interest. As noted earlier, nonanal has been shown to have navel orangeworm semiochemical activity. Finally, if navel orangeworm female moths are searching for suitable hosts for overwintering, the volatiles from the naturally damaged pistachios may represent a good starting point for evaluating the emitted volatiles.

Semiochemicals Specific to Pistachios?

The noted host range of the polyphagous navel orangeworm is diverse and spans across five different families of agricultural commodities. Each of these crops likely has a distinctive odor associated with them. As discussed previously, this was particularly true for the two major California crops, almond and pistachio. Work in our laboratories has demonstrated an apparent relationship among the navel orangeworm, its almond host, and a semiochemical produced by fungal spores when on the almond host. Our recent work has taken this apparent relationship and extended its possibility to pistachios as the host plant, and whether a new volatile or set of volatiles will act as an orchard-specific semiochemical of the navel orangeworm.

Several experiments recently performed in our laboratories have been discussed: emissions from pistachio and almond mummies and the variability of their emission from season to season as well as their ability to influence female navel orangeworm to oviposit on traps baited with pistachio mummies; the volatile emissions of split pistachios; and, the volatile emission of naturally damaged pistachios. Each of these experiments has resulted in a consistent set of monoterpenes that appear to represent the basic background volatile profile of pistachios. Additionally, each experiment has highlighted either substantial increases in volatile amounts or volatiles that are unique to the varying conditions of the pistachio tissues. Results from these experiments have provided numerous candidate volatiles that will be considered through a series of electrophysiological and behavioral assays. Ultimately, the goal of these projects is to develop a synthetic blend of host plant volatiles that is based on pistachio tissues and their resident fungal spores. Moreover, it is hoped that any resultant blend will be effective in pistachio orchards just as the almond-based synthetic blend is effective in almond orchards.

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Chapter 15

Development of Specialized Pheromone and Lure Application Technologies (SPLAT®) for Management of Coleopteran Pests in Agricultural and Forest Systems

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Since ISCA Technologies' market introduction of Specialized Pheromone and Lure Application Technology (SPLAT®) in 2004, it has been implemented for effective pest control through a variety of techniques, including mating disruption, mass trapping, attract and kill, and repellency. The majority of SPLAT®-based pest control products have targeted lepidopteran (e.g., SPLAT® OFM, for oriental fruit moth, *Grapholita molesta*; SPLAT® GM for gypsy moth, *Lymantria dispar*; SPLAT® EC, for carob moth, *Ectomyelois ceratoniae*) and dipteran pests (e.g., SPLAT® MAT Spinosad ME for

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tephritid fruit flies, Tephritidae). This chapter will describe a relatively new family of SPLAT® formulations designed to attract or repel coleopteran pests. Following a brief description of the characteristics of SPLAT® and its advantages over other forms of semiochemical-based pest control, each section will begin with an introduction to each product's method of action, specifically mating disruption, attract and kill, and repellency, and will then summarize current research on the application of each technique to combat a specific coleopteran pest: oriental beetle (OrB), *Anomala orientalis* (SPLAT® OrB & SPLAT® OrB A&K), red palm weevil (RPW), *Rhynchophorus ferrugineus* (Hook™ RPW), and mountain pine beetle (MPB), *Dendroctonus ponderosae* (SPLAT® Verb).

Specialized Pheromone and Lure Application Technology (SPLAT®)

Semiochemical methods of pest control have numerous advantages over the use of conventional insecticides, both in terms of efficacy and environmental sustainability. Semiochemicals, such as sex pheromones used by insects to locate a mate, and host plant kairomones, which serve as important cues to help insects navigate their environment, are naturally occurring, often species-specific substances. Artificial introduction of such substances into a field environment often allows the grower to elicit a specific response from a specific insect pest, leaving desirable nontarget organisms, like natural enemies and pollinators, often unaffected. Behavioral manipulation by means of semiochemical applications has been shown to involve less risk of the target species developing resistance to the active ingredient (AI), a common shortcoming among conventional insecticides (*1*). Furthermore, unlike conventional insecticides, which are often applied as cover sprays over the entire crop, semiochemicals tend to be deployed in discrete point sources. This reduces the likelihood of nontarget organisms coming into contact with the compound, and enables growers to avoid contamination of food crops.

Despite these advantages, it has proven difficult to develop and commercialize effective, economically-viable semiochemical-based pest control technologies. The primary obstacle to wide-spread adoption of these technologies is the high cost of semiochemicals, which are generally more expensive than conventional pesticides (*2–4*). In many cases, however, it is not the intrinsic cost of the semiochemical itself that makes the end product economically unfeasible, but rather the method by which it must be deployed in the field. Past and current semiochemical formulations have typically been developed as devices, which by their very nature require manual application. This can lead to prohibitively high application costs, especially in large and/or heavily infested fields, requiring high application densities. These costs can be further compounded by the physical limitations of the active semiochemical (i.e., chemical instability/volatility, sensitivity to light or heat), as well as by the mechanism of behavior manipulation

in which it is employed. Mating disruption formulations, particularly those that work by camouflage (defined later in this chapter), generally require large quantities of pheromone to be deployed in order to be effective. In addition, many semiochemical products have demonstrated inconsistent capability to maintain the release rate and period necessary to achieve effective pest control. For example, an early bubblecap formulation developed for management of MPB infestations failed to release its AI, verbenone – the same AI contained in ISCA's MPB repellent product, SPLAT® Verb, to be discussed later in this chapter – at levels sufficient to compete with pheromones naturally emitted during an infestation, and therefore failed to adequately repel the pest (*5*).

These general shortcomings in semiochemical formulations make them prone to failure under high pest pressure. In these situations, successful application of these products often requires treatments of conventional insecticides prior to deployment, in order to knock down the pest population in the field to sufficiently low numbers for the semiochemical AI to exert the desired influence over the pest's behavior. This is a particularly severe hindrance to the acceptance of semiochemical technology as a viable method of pest control, since the health and environmental hazards associated with conventional insecticides represent, from the viewpoint of stakeholders, researchers, and the general public, some of the most persuasive justifications for developing such technologies in the first place.

Mindful of these prior deficiencies, ISCA Technologies has developed an innovative and versatile alternative: Specialized Pheromone & Lure Application Technology (SPLAT®). Rather than a single device, SPLAT® is an amorphous, flowable controlled-release emulsion, with chemical and physical properties that may be adjusted by small changes in composition, in processing, or in application method to suit a wide variety of pest management techniques, while minimizing application costs. It is a shear-thinning, thixotropic, non-Newtonian fluid that enters a liquid state when agitated, such as by stirring, but quickly solidifies when the source of agitation is removed. This property allows for easy manual or mechanical application in the field, enabling the user to customize point source size and distribution to the requirements of each field, while still ensuring that the product will persist in the field for a sufficient period to achieve effective pest control. SPLAT® formulations typically become rainfast within 3 hours following application, and can provide continuous controlled release of semiochemical AIs for intervals ranging from 2 weeks to 6 months. The rate of release of the incorporated AIs into the field is determined by the AI molecules' rate of diffusion through the SPLAT® matrix, which occurs in accordance with Fick's First Law of Diffusion (*6*), stating that molecules move from regions of high concentration to regions of low concentration at a rate that is directly proportional to their concentration gradient. This rate may be precisely controlled or adjusted by subtle changes in the formulation's characteristics and application method. Because of its controlled-release capacity, SPLAT® formulations typically use smaller amounts of AIs than other semiochemical products, reducing application costs and environmental impact.

This product's unique adaptability makes SPLAT® formulations amenable to a virtually unlimited range of application methods. In previous applications, SPLAT® products have been applied by hand, using such simple tools as a

spatula or knife to protect relatively small numbers of high-value plants; delivered through metered syringes and caulking guns; sprayed mechanically via modified applicator vehicles, including tractors, all-terrain vehicles, and motorcycles; loaded into tanks of airplanes and sprayed over hundreds of thousands of hectares of forests; and even fired in pellets from paintball guns. This technology boasts additional advantages in terms of reduced environmental impact. SPLAT® is biodegradable, and all of its inert ingredients have been certified as food safe by the US Environmental Protection Agency (US EPA). A number of commercial SPLAT® formulations have even been granted organic status, including SPLAT® GM Organic for mating disruption of the gypsy moth and SPLAT® EC Organic for the carob moth, *Ectomyelois ceratoniae*.

While SPLAT® formulations have been developed for control of dozens of insect pests, including multiple representatives of the orders Lepidoptera, Diptera, and Hemiptera, this chapter focuses solely on those designed to target coleopteran pests, specifically: SPLAT® OrB for mating disruption and attract and kill of OrB in blueberries, *Vaccinium* spp.; Hook™ RPW for attract and kill of the RPW in date palms, *Phoenix dactylifera*; and SPLAT® Verb, a repellent for MPB in lodgepole pine, *Pinus contorta*.

SPLAT® Technologies for Control of the Oriental Beetle, *Anomala orientalis*

Mating Disruption

Mating disruption, currently the most commonly employed method of semiochemical-based pest control, is a technique of insect behavior manipulation that seeks to reduce the reproductive success of the target pest, thereby diminishing its population and impact on crops over time. In order to deter individuals from locating a mate, this technique requires synthetic sex pheromones, or chemically and biologically similar analogs, to be dispensed into the environment at levels that disrupt males' abilities to detect and respond to the natural pheromone plume emitted by a calling female.

In addition to reducing pest populations by interfering with mate finding, and consequently reducing the number of successful mating events, the artificial introduction of sex pheromones required to achieve mating disruption may also result in loss of fitness among females of the target species, as a consequence of delayed mating. Females have only a limited amount of time in which to reproduce and to select an appropriate site for oviposition. Recent studies have shown that a delay in mating, such as this would be caused by a decrease in males' abilities to locate females due to the presence of synthetic pheromone, can negatively impact females' levels of reproductive fitness by decreasing fecundity (number of eggs carried by the female), fertility (capability of eggs to produce viable offspring), and pre-oviposition period (*7*).

A number of mechanisms by which mating disruption can occur have been proposed and investigated. These may operate separately or in combination, depending on the target pest and the pheromone used to control it. The most widely accepted of these mechanisms are described below.

1. **Competitive attraction**, also referred to as "false trail following" occurs when males orient and respond to synthetic pheromone plumes produced by semiochemical dispensers, rather than the natural plume emitted by a calling female. This mechanism of mating disruption is population density dependent, and decreases in efficacy as the population of the pest increases.
2. **Camouflage**, unlike competitive attraction, is density independent, and requires that the environment be saturated with synthetic pheromone to the extent that mate-seeking males are incapable of homing in on females.
3. **Desensitization** takes place when males' responses to the female sex pheromone are significantly diminished due to over-exposure, and may occur either through adaptation of the males' olfactory receptor neurons, or by habituation of the insects' central nervous system (*7*, *8*).
4. **Sensory imbalance** achieves mating disruption by impeding the male's ability to recognize the female sex pheromone within the treated environment, thereby preventing him from responding to it.

Understanding which of these mechanisms operates predominately in a given scenario may help to determine how to deploy a pheromone formulation in such a way as to achieve the highest level of mating disruption, and consequently, the largest reduction of pest population and damage to host plants (*9*). Unfortunately, the majority of past research has been primarily aimed at evaluating the quality and reliability of control achieved by mating disruption techniques for various insect species, rather than identifying the precise mechanism by which they function.

In the development and testing of SPLAT® OrB, a mating disruption formulation for the oriental beetle, ISCA Technologies and its collaborator, Dr. Cesar Rodriguez-Saona, Assistant Professor of Entomology at Rutgers University, endeavored not only to verify the product's efficacy against the target pest, but also to determine how its application disrupted the insect's reproduction.

SPLAT® OrB for Mating Disruption

Native to Asia, OrB was introduced to the United States from Japan in the early 20th century. It was first detected in Connecticut in 1920, following a shipment of infested nursery stock (*10*, *11*), and has since expanded its range through the northeastern United States, from Maine west to Ohio, and south to South Carolina. It is a major pest of several plant varieties throughout this region, including soft fruits such as blueberries, raspberries, **Rubus** spp., and cranberries, **Vaccinium** spp., as well as ornamental nursery stock and turfgrass. Of particular concern is the threat posed by OrB to the blueberry industry of New Jersey, a crop recently valued at approximately $82 million (*12*, *13*). As with other susceptible crops, the damage inflicted by OrB results exclusively from the feeding of the larvae on the roots of the plant (adults reportedly do not feed). Infested blueberry bushes produce fewer fruits, have smaller leaves and generally reduced vigor, and tend to have shorter life spans than uninfested bushes (*14*). In severe infestations, OrB grubs can virtually destroy the entire root system of the plant, leading to its death (*14*).

SPLAT® OrB was developed in order to provide blueberry growers with an alternative OrB control method to standard treatments, which currently consist of soil applications of the neonicotinoid insecticide, imidacloprid. Growers have expressed a number of concerns regarding the use of this insect neurotoxin, including its high cost of application; the possibility of OrB developing resistance to its mode of action; and the harmful effects it may exert on beneficial nontarget organisms, for example, the OrB natural enemy, *Tiphia vernalis* (*15*), and the honeybee, *Apis mellifera,* a pollinator necessary to ensure proper fruit set in blueberry crops (*16*). These concerns have generated increasing interest in the investigation of mating disruption as a tool to manage OrB, an opportunity made possible in the early 1990s by the identification and successful synthesis of the sex pheromone for this species, a 9:1 blend of (*Z*)-7-tetradecen-2-one and (*E*)-7-tetradecen-2-one (*17–19*). SPLAT® OrB relies solely on the major component, (*Z*)-7-tetradecen-2-one, for its behavior-modifying capabilities, in light of the findings of Facundo et al. (*20*), which demonstrated that this compound alone was equally attractive to OrB males as the 9:1 blend composing the natural pheromone.

SPLAT® OrB was tested in 2008-2009 in three commercial blueberry farms, located in Atlantic Co., New Jersey, compared against a plastic "bubble" dispenser formulation using the same AI [(*Z*)-7-tetradecen-2-one], for efficacy of mating disruption in OrB. The two treatments were applied at two corresponding rates in the field: 2.5 g AI/ha and 5 g AI/ha. Plastic pheromone dispensers were hung directly from blueberry plants, at a height of approximately 20 cm above the ground. SPLAT® OrB was applied in 0.5-g dollops (ca. 3.5 cm long, 1.5 cm wide, 2 mm thick), each containing 5 mg AI, deposited on wooden stakes (15 x 1.5 cm plant labels; Gemplers, Bellville WI) using a plastic spatula. The stakes were inserted into the soil, each close to the base of a blueberry bush, so that the SPLAT® OrB dollop was positioned 5-10 cm above the ground. This placement was intended to enable maximum impact on OrB mating, which has been shown to occur at the surface of the soil (*21*). Both pheromone treatments were applied throughout a 1-ha plot, along an evenly-spaced grid between blueberry bushes. Each treatment was replicated three times, with treatments within each blueberry farm assigned to plots in a randomized complete block design. Plots were separated from one another by a minimum distance of 50 m. Both pheromone-treated and untreated (control) plots (equivalent in size) were treated with fungicides and insecticides according to the growers' standard program. None, however, were treated with imidacloprid.

Treatment efficacy for both SPLAT® OrB and plastic pheromone dispensers were evaluated based on the following factors:

1. Trap shutdown was measured by the number of OrB caught in pheromone-baited traps. Three traps were placed in each plot, and monitored on a weekly basis for the duration of the study.
2. Female mating status was evaluated by placing 5 screened cages in each plot, each containing a virgin female OrB. These cages were designed to permit entry by OrB males, allowing them access to the caged female, but preventing escape from the cage by either sex. Cages remained in the

field for 3 nights, and were then retrieved to evaluate OrB mating success, via egg laying by caged females. Cages were deployed 3 times over an interval of 2 weeks, on June 20 and 24, 2009, and on July 1, 2009.

3. Grub density (number of OrB larvae per blueberry bush) was evaluated using 5 potted 2- to 3-year-old bushes, placed in each plot. One virgin female was tethered in each pot (n = 75 virgin females), replaced every week for a period of 4 weeks. Larval density for each potted blueberry plant was ascertained through destructive sampling of root systems.

A disruption index (DI) was calculated for this experiment according to the following equation: (average OrB capture/trap in control plots) - (average OrB capture/trap in treatment plots) / (average OrB capture/trap in control plots) x 100. Data on OrB trap captures, female mating status (percentage of mated OrB females retrieved from cages), and grub density in potted blueberry bushes, as well as calculated DI values were subjected to analysis of variance (ANOVA) (Minitab 13.32; Minitab Inc., State College, PA). Trap capture and grub density data were log-transformed for normality when required. Female mating status data and DI values were arcsine square root-transformed. Means for all data were separated using Tukey tests ($\alpha = 0.5$).

Field observations were conducted in one of the blueberry farms to ascertain whether male OrB approached pheromone point sources in the field (Figure 1), a pattern which would be useful in determining the mechanism by which SPLAT® OrB disrupted mating in OrB. Observations were made for a 30-minute period in each treatment plot every 4 h, starting at 8:00 and ending at 20:00 (all observations were carried out on July 3, 2009). Ten SPLAT® OrB-treated stakes, chosen at random, were observed per plot for 3-5 minutes. Similar observations were carried out in random locations in control plots as well. The number of OrB detected within 1 m of each observation point was recorded. Data for OrB attraction to pheromone point sources were log-transformed ($x + 0.1$), analyzed using ANOVA, and then subjected to Tukey tests.

In June-July 2008, a mark-release-recapture study was conducted in a blueberry field at Rutgers Blueberry/Cranberry Center, to ascertain the distance at which OrB males respond to both artificial and natural pheromone sources. Four sources were evaluated: virgin OrB females, SPLAT® OrB (0.5-g dollop containing 5 mg AI), plastic dispensers (containing 0.1-g AI), and rubber septa (300 µg AI). SPLAT® OrB dollops, plastic dispensers, and rubber septa were placed inside individual Japanese beetle traps, while virgin females were placed in wire cages attached to the trap. Male OrB to be used for this study were trapped out of the field and taken to the laboratory to be marked according to their intended release site. Markings were made using enamel paints (The Testor Corp., Rockford IL) on the pronotum and/or left or right elytra. Laboratory observations indicated no effects of the paints on beetle behavior. Marked OrB were released at the following distances from each treatment: 3.75, 7.5, 15, 30, and 60 m, on 3 different days in 3 consecutive weeks (240 OrB released in total). Resulting data were arcsine transformed and analyzed via ANOVA. Means were separated via Tukey test.

Figure 1. Male OrB approaching a dollop of SPLAT®OrB in a blueberry field. The beetle walking towards, and then visiting, a dollop of SPLAT®OrB (inset, arrow points to the beetle).

In order to determine the effect of SPLAT® OrB application density on beetle attraction to pheromone sources, an additional study was conducted at the Atlantic Co., NJ blueberry farms evaluating OrB trap captures in plots treated with 4 different densities of SPLAT® OrB dollops: 0 dollops (0 g AI)/ha, 25 dollops (0.125 g AI)/ha, 250 dollops (1.25 g AI)/ha, and 500 dollops (2.5 g AI)/ha. All SPLAT® OrB dollops were applied to wooden stakes and deployed in the field as previously detailed. Data for the 3 pheromone-baited traps placed in each plot (20 m between traps) was summed and log-transformed. This was followed by ANOVA analysis and Tukey test ($\alpha = 0.05$). DI values were arcsine square root-transformed prior to ANOVA analysis and Tukey test. The data from this study were also plotted in order to test the predictions put forward by Miller et al. (*8*) for the competitive attraction mechanism of mating disruption.

Trap captures in pheromone-treated plots (both hand-applied plastic dispensers and SPLAT® OrB dollops) were found to be significantly lower compared to those in untreated plots ($F = 31.8$; df = 4,8; $P < 0.001$). The higher treatment rate of 5 g AI/ha produced disruption index (DI) values 6-9% higher than the lower treatment rate of 2.5 g AI/ha. Fewer caged females were found to have mated ($F = 14.9$; df = 4,8; $P = 0.001$), and a smaller number of males were found in cages that were placed in pheromone treatment plots ($F = 22.5$; df = 4,8; $P < 0.001$). Grub density was also reduced in SPLAT® OrB-treated plots at both application densities, as well as in plots treated with the higher density of plastic dispensers ($P \leq 0.05$). Field observations conducted in SPLAT® OrB and plastic dispenser plots revealed that beetles did approach pheromone sources in the field,

and did so at all hours of the day at which observations were made – a finding of particular significance, given the fact that these insects normally exhibit cryptic behaviors. In contrast, observers stationed at random locations within control plots reported no OrB sightings for the duration of the study. The results of the mark-release-recapture study carried out at the Rutgers Blueberry/Cranberry Center further confirmed OrB attraction to point sources. Both SPLAT® OrB and plastic pheromone dispensers were found to exert a stronger level of attraction in OrB than rubber septa and virgin females, and were capable of drawing the beetles to the traps from distances of more than 60 m. Fewer marked OrB were recovered as the distance between the release site and the pheromone source increased.

The SPLAT® OrB density study showed that the higher application rates for this formulation (250 dollops/ha and 500 dollops/ha) resulted in larger reductions in OrB trap captures, and DI values approximately 10% higher than were seen in the lower density plots (25 dollops/ha), suggesting that the higher application rate is required to achieve the most effective mating disruption against this pest. In addition, the data produced by the density study were found to conform to Miller et al.'s (*8*) predictions for competitive attraction (Figure 2). OrB trap capture data (un-transformed), when plotted against SPLAT® OrB dollop density, yielded a graph that descends concavely as density increases, asymptotically approaching zero. This suggests that the ability of males to successfully detect and locate a pheromone source decreases as the density of the pheromone sources increases – a strong indication that competitive attraction is taking place, especially taking into account the field observations conducted in the pheromone-treated plots, which visibly confirmed OrB attraction to point sources. Furthermore, when the data was plotted as a Miller-Gut plot [1/OrB male trap capture (y-axis) vs. point source density (x-axis) and as a Miller-de Lame plot [OrB male trap capture (y-axis) vs. (point source density x OrB male trap capture) (x-axis)], it produced straight lines, the former with a positive, and the later with a negative slope, also in accordance with predictions for the competitive attraction mechanism for mating disruption.

Conclusions

SPLAT® OrB applied at low densities in the field was equally as effective at disrupting mating in the OrB as plastic pheromone dispensers, but proved more effective at reducing grub density in potted blueberry plants than plastic dispensers at the lower application rate. SPLAT® OrB is amenable – indeed, is designed for – mechanized application, and would therefore represent a significant advantage over hand-applied dispensers in terms of labor costs, since the amount of pheromone applied (and its associated cost) is the same for both formulations. However, due to restrictions on the experimental use of the OrB sex pheromone [a ketone, not exempt from the tolerance requirements established by the US EPA for use on fruit crops (*12*, *22–24*)], current formulations must be retrievable from the field, effectively ruling out mechanical application for the present. Should the US EPA approve registration of SPLAT® OrB, thereby releasing it from the limitations of an experimental use product, this formulation may prove to be a valuable OrB management tool for growers of blueberries and other susceptible crops.

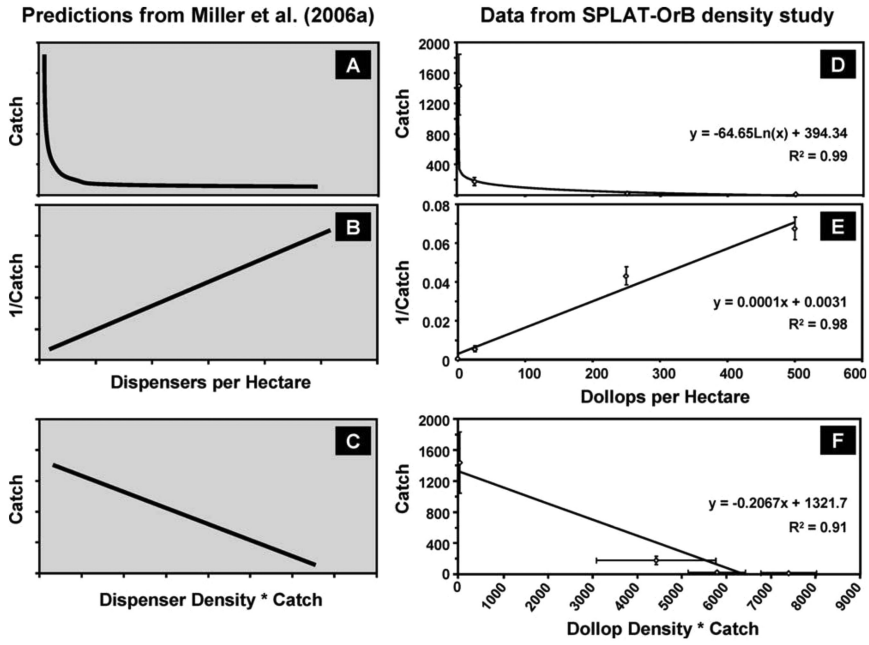

Figure 2. SPLAT® OrB promotes mating disruption through the mechanism of competitive attraction. Predictions for competitive attraction as described in Miller et al. (8): untransformed number of trap captures against dispenser density (A); Miller-Gut plot (B); Miller-de Lame plot (C). These plots are compared with data from our SPLAT® OrB density study: plot of untransformed oriental beetle male captures per trap versus SPLAT® OrB dollop density (D), Miller-Gut plot for oriental beetle data (E), and Miller-de Lame plot for oriental beetle data (F). Data are means ± SE, and equations for best-fit curve and lines are presented in graphs DEF. (Reproduced with permission from reference (12). Copyright 2010 The Entomological Society of America.)

A mechanical applicator for SPLAT® OrB in blueberries has been developed and tested (Figure 3). Precise applications of dollops to the soil at the base of blueberry plants can be adjusted by changing the speed at which the user drives the vehicle through the field.

Field observations in pheromone-treated plots, as well as the results of the mark-release-recapture and SPLAT® OrB density studies all point to competitive attraction as a key mechanism in OrB mating disruption. Beetles were found to perceive and approach pheromone sources, and to be preferentially attracted to SPLAT® OrB over virgin females. These findings not only indicate SPLAT® OrB's potential as an effective mating disruption product, but also suggest an alternative means of control for this pest: the incorporation of an insecticide into SPLAT® OrB, to create a novel attract-and-kill formulation.

Figure 3. The mechanical applicator mounted on a 'gator' in a blueberry field. The applicator has two adjustable arms that protrude from the back of the gator towards the ground that contain valves that open and close periodically, at pre-set intervals and pre-set opening times, allowing the SPLAT® OrB under pressure in the pipes, to exit and fall onto the ground. By setting the SPLAT®OrB pressure, the time interval between openings, and the time that the valve is opened, the user is able to precisely deposit dollops at regular intervals simply by adjusting the speed of the vehicle. Inset shows a gray dollop of SPLAT® OrB near a US $0.10 coin, for scale.

Attract and Kill

While attract and kill (A&K) is at its heart a simpler, more straightforward technique of insect pest management than mating disruption, which, as previously illustrated, can occur by a variety of different mechanisms, it can be deceptively difficult to implement effectively in a field environment. At its most basic level, this technique can be defined as follows: an attractant, which may be a crude bait or semiochemical, or even a visual or auditory cue, lures the target insect (males, females, or both) to a point source either containing or directly adjacent to a toxicant. This control agent is generally an insecticide, but sterilants (as in lure and sterilize) and insect pathogens (as in lure and infect) have also been used. Upon contact or ingestion of the toxicant at or within the point source, depending on its mechanism of action, the target insect either dies or suffers detrimental effects that, over time, result in reduced pest populations. In some

cases, the toxicant does not necessarily have to kill the insect in order to effect population suppression, as long as its sublethal effects sufficiently impair the insect's reproductive capacity (*25*). For example, one study found that sublethal exposure to an A&K formulation for codling moth, *Cydia pomonella*, caused males of the species to undergo autotomy (self-amputation) of the thoracic limbs, significantly impeding their physical ability to mate and reducing their general fitness (*26*, *27*).

In order to successfully manage a pest population, an effective A&K system calls for a highly-optimized product, in terms of the attractant and the insect control agent to be used. The lure must be a strong enough attractant to draw the target insect directly to the point source and induce contact, or in the case of some stomach-poison insecticides, to induce feeding. The insecticide, too, must be highly potent, and as fast acting as possible, ensuring that all or nearly all of the insects that are attracted to the point source are either killed or incapacitated shortly after contact/ingestion, before they have the opportunity to mate. In a review on the potential of A&K strategies for insect pest management, El-Sayed et al. (*27*) put forth 3 crucial events that must take place in order for any A&K formulation to achieve effective control. First, the insect must successfully locate and contact the point source, which should ideally be more attractive to the target insect than any natural attractant source present in the field. Second, the attracted insect must then ingest an adequate quantity, or spend a sufficient period of time in contact with the insecticide, or other toxicant, to achieve the desired detrimental effect. Third, the resulting level of mortality/debilitation must be such that it has a significant negative effect on the pest population in treated areas (*27*).

In addition to the high standards of efficacy demanded for successful application, there are a number of other drawbacks to the implementation of A&K methods of pest control. The addition of an insecticide into a semiochemical formulation, such as SPLAT® OrB, creates complications regarding product efficacy, as well as public opinion and acceptance of the product. Interactions between the toxicant and the attractant within the formulation may negatively impact is effectiveness, most obviously if the former component is an insecticide that is repellent to the target species (*27*). The general public may be more reticent to accept A&K control techniques than those that function by mating disruption, out of concern that the toxicants they employ may harm the environment or nontarget species (*28*).

However, A&K formulations differ from traditional applications of chemical insecticides on several critical points. Developers of A&K products almost invariably select low-risk insecticides, those that have demonstrated minimal hazards to mammals and other non-target species, as well as to the environment, and deploy them in miniscule amounts compared to conventional cover sprays (*25*, *29*, *30*). Also, the application of A&K products in lieu of conventional insecticides eliminates the danger of spray drift (*25*). In many cases, as with the use of sex pheromones as lures, the attractant is species-specific, further reducing the risk of exposure to non-targets. While the efficacy of A&K declines as pest population density increases, as does mating disruption (*29*, *31–34*), the former technique has been shown to be less subject to the limitations of environmental factors, such as the topography and size of the treatment plot (*27*).

The results of the Rodriguez-Saona et al. (*12*) trial of SPLAT® OrB for mating disruption, described above, identified this product as having a high potential for use as an A&K formulation for OrB. During these studies, the beetles were observed to approach SPLAT® OrB dollops in the field at all times of the day, and to preferentially pursue odor plumes produced by this treatment over those produced by virgin female OrB. As a result, ISCA scientists designed SPLAT® OrB A&K, a SPLAT® formulation combining the same AI used in the mating disruption product (the major component of the OrB sex pheromone) with the reduced-risk contact insecticide, cypermethrin. This product was tested by the same research team as the 2010 mating disruption study, to determine 1) whether SPLAT® OrB A&K was capable of delivering the same efficacy and reliability of pest control as its mating disruption counterpart, 2) whether efficacy of either formulation increased with higher point source density, and 3) the field longevity of each formulation. Their work is described in the following section.

SPLAT® OrB for Attract and Kill

A field study evaluating the efficacy of SPLAT® OrB and SPLAT® OrB A&K for control of the OrB was conducted by Dr. Rodriguez-Saona and his research team in 2011, in two commercial blueberry farms in Atlantic Co., New Jersey (Atlantic Blueberry Co., 7201 Weymouth Road #A, Hammonton; and Bellbay Farms, 131 Main Road, Hammonton). Four pheromone treatments were applied over 1-ha experimental plots, separated from one another by a minimum distance of 90 m.

The following treatments were assigned to plots within blocks according to a randomized complete block design:

1. SPLAT® OrB [1% (*Z*)-7-tetradecen-2-one] applied as 250 1-g dollops
2. SPLAT® OrB [1% (*Z*)-7-tetradecen-2-one] applied as 500 1-g dollops
3. SPLAT® OrB A&K [1% (*Z*)-7-tetradecen-2-one + 2% cypermethrin] applied as 250 1-g dollops
4. SPLAT® OrB A&K [1% (*Z*)-7-tetradecen-2-one + 2% cypermethrin] applied as 500 1-g dollops
5. Untreated control

In accordance with the EPA's restrictions regarding the experimental use of unregistered pest control products, all SPLAT® OrB formulations were applied to wooden stakes (the same as were used in the 2010 mating disruption study), using a caulking gun (Newborn X-lite, Newborn Brothers Co., Inc., Jessup MD) calibrated to dispense a 1-g dollop of material. SPLAT® OrB-treated stakes were inserted into the ground along an evenly spaced grid between blueberry plants. This method of application ensured that the treatments could be easily removed at the conclusion of the study (per EPA requirements). All treatments were replicated

3 times (1 replicate/block). The application of pheromone treatments commenced on June 3, 2011, with SPLAT® OrB for mating disruption, and ended 3 days later on June 6, 2011, with SPLAT® OrB A&K. No applications of imidacloprid were made to any of the treatment or control plots, though all were treated with other insecticides and fungicides according to the growers' standard program.

The efficacy of the 4 SPLAT® OrB treatments was evaluated based on the following criteria:

1. Trap shutdown was measured by the number of OrB males captured in three Japanese beetle traps (Trécé, Salinas, CA) baited with 300 µg (*Z*)-7-tetradecen-2-one (ISCA Technologies, Riverside, CA) placed in each plot. Traps were deployed on June 10, 2011 and were monitored weekly. Pheromone lures were replaced at 3 week intervals. Trap capture data was log transformed ($\log_{10}$ + 0.01) and subjected to ANOVA (GLM Univariate; IBM SPSS v19, Statistics Base) and Tukey test. A disruption index (DI) was also calculated, using the formula, [(Average OrB captured/trap in untreated plots + 0.01) – (Average OrB captured/trap in SPLAT®OrB-treated plots + 0.01) / (Average OrB captured/trap in untreated plots + 0.01) x 100]. The resulting DI values were arcsine square root-transformed before undergoing ANOVA and Tukey test.
2. Grub density was measured by tethering virgin OrB females to potted 2- to 3-year-old blueberry bushes, as described in the 2010 mating disruption study. Four of these potted bushes were placed near the center of each plot at the end of June 2011, and were removed in mid-July 2011. After their removal from the field, the plants were stored in a greenhouse until the following November, allowing any OrB larvae present within the plots to attain a sufficient size as to be easily detectable before being counted.
3. Behavioral observations were conducted to verify that OrB approached the pheromone point sources in the field. Observations were conducted in the treatment plots on July 19, 2011 at three times of the day, 800, 1200, and 2000. Ten SPLAT® OrB point sources, selected at random, were visually monitored in each plot for a period of 3-5 minutes. Similar observations were also carried out at randomly chosen locations within the untreated plots. As in the 2010 mating disruption study, observers stationed in both the treatment and control plots recorded the number of OrB detected within 1 m of the observation point.

A laboratory study was also performed on the residual activity of SPLAT®, evaluating the following treatments: 1) SPLAT® OrB [1% (*Z*)-7-tetradecen-2-one], 2) SPLAT® OrB A&K [1% (*Z*)-7-tetradecen-2-one + 2% cypermethrin], and 3) SPLAT® Blank (no AI) as a control. One fresh, unaged 1-g dollop of SPLAT® was applied at the center of a plastic petri dish (95 mm in diameter, 15 mm in height; Fisher Scientific, Pittsburgh, PA). A total of 20 dishes were prepared for each treatment, with each dish representing one replicate. Beginning

at Day 0, one OrB male was placed in each treatment dish with the SPLAT® dollop, and the lid of the petri dish was placed on loosely to allow for adequate airflow. Mortality among OrB due to contact with the formulation was recorded at 2, 12, 24 and 48 h of exposure. The procedure was repeated with all 3 SPLAT® formulations, aged in a 25°C incubator for 15, 21, or 30 d. For the 30-d aged dollops, 16 rather than 20 replicates were made. Cotton balls soaked with water were included in the treatment dishes along with the SPLAT® dollops for the 21- and 30-d aged samples, following abnormally high mortality for the 15-d assay, presumably due to lack of moisture. The significance of the relationship between SPLAT® formulation and OrB mortality was compared for each sample-age category, via a Chi-square goodness of fit test with a Bonferroni correction for multiple pairwise comparisons. OrB trap captures were significantly lower in SPLAT® OrB treatment plots than in control plots ($F = 178.5$, 4 df, $P < 0.0001$) in all cases, irrespective of formulation or point source density. There was no significant difference in trap captures of OrB between plots treated with SPLAT® OrB and those treated with SPLAT® OrB A&K. Beetle captures in all treatment plots remained at or close to zero, with consistently high DI values (87.8–89.5%) throughout the entire study. No significant differences were observed in DI values between SPLAT® treatments. The equivalently high levels of efficacy achieved by the low (250 dollops per ha) and high application density (500 dollops per ha) in both formulations (Figure 4) suggest that OrB could potentially be managed below threshold levels in blueberry fields using the more cost-effective lower application rate.

As also occurred in the previous mating disruption study, OrB were observed near point sources during all observation periods in treatment plots, whereas none were seen in the control plots for the length of the study. Both living and dead beetles were seen, and most were observed close to point sources near plot borders as opposed to the interior of the treated field. These findings provide strong evidence that SPLAT® OrB A&K maintains the same level of attraction as the mating disruption formulation, in spite of the addition of cypermethrin, a compound which, according to the results of the residual activity study, exerts a fairly reliable degree of mortality in the target insect. A period of 48 h of exposure to SPLAT® OrB A&K resulted in 100% mortality of OrB, regardless of the age of the material. In contrast, the final mortality rates for beetles exposed to SPLAT® OrB (without insecticide) were not significantly different from those of OrB exposed to a blank sample of SPLAT®. Exposure to SPLAT® OrB A&K resulted in significantly higher levels of final mortality than SPLAT® OrB or SPLAT® (blank), in all cases except those assayed on Day 15, when, as previously stated, OrB exposed to the control treatment suffered an abnormally high level of mortality.

While sample age did not appear to affect OrB mortality rate at 48 h of exposure to SPLAT® OrB A&K, it does appear to have impacted the amount of time required for mortality to reach 100%. Plots of OrB mortality over time suggest that as the age of the material increases, so too does the length of time required to achieve mortality in exposed beetles.

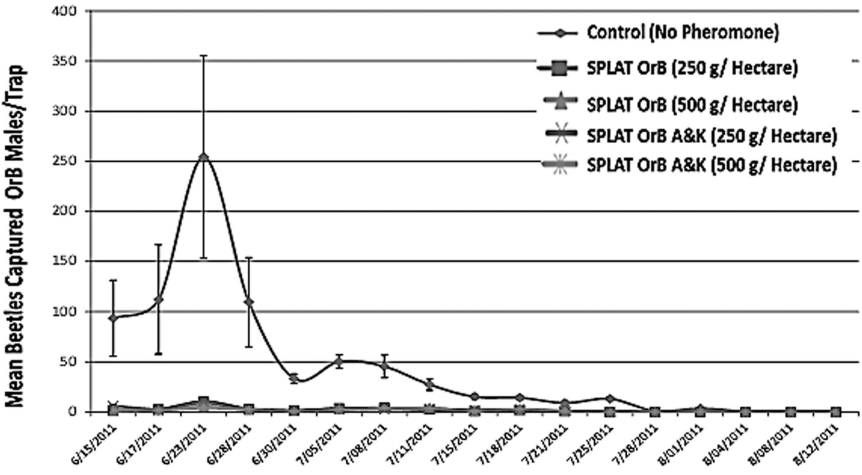

Figure 4. Trap catches of male beetles in pheromone traps in OrB pheromone-treated and control fields. Mean (± SE) number of male oriental beetles captured in pheromone-baited traps on each observation date. Three traps were located in each plot; data represent the average from nine traps per treatment. SPLAT® OrB (1% (Z)-7-tetradecen-2-one) applications were made on June 3 and SPLAT® OrB A&K (1% (Z)-7-tetradecen-2-one + 2% Cypermethrin) applications were made on June 6, 2011. Traps were installed on June 10 and lures were replaced every three weeks.

Conclusions

Treatments of SPLAT® OrB and SPLAT® OrB A&K resulted in equivalent reductions in OrB trap capture in treated blueberry fields. While it remains unclear from these results whether control by SPLAT® OrB A&K occurs through attract and kill or by mating disruption, the data generated by this study are encouraging. SPLAT® OrB A&K retained the necessary degree of attraction to lure OrB towards the point source in the field, and the results of the residual activity study strongly indicate that this formulation's insecticidal component, cypermethrin, functions as an adequate killing agent against this pest species.

ISCA Technologies and its collaborators are continuing research on the optimization of the SPLAT® OrB attract and kill formulation. Dr. Rodriguez-Saona and his research team are currently engaged in a study to evaluate the frequency that OrB approach, locate and contact SPLAT® dollops in the field. Experiments are also being conducted on the use of alternative varieties of insecticide in SPLAT® OrB, to improve the formulation's field efficacy and longevity.

Hook™ RPW: Attract and Kill for the Red Palm Weevil, *Rhynchophorus ferrugineus*

The RPW is an internal tissue borer known to prey on 40 palm species around the world (*35*, *36*). Originally reported as a pest of coconut palm in India in the early twentieth century (*37*), RPW has spread rapidly across the globe since the 1980s, presumably through transport of infested plant material, and is now present on every continent except Antarctica. It is considered a major pest of date palm throughout the Middle East and Mediterranean regions, and has been reported in half the date-producing countries of the world (*38*).

The weevil's activity as an internal tissue feeder makes its presence in a field notoriously difficult to detect. The most obvious symptoms of infestation, such as the presence of chewed plant material (frass), tunnels bored through the tree trunk or frond petiole base, and the fermented odor and gnawing sounds produced by feeding larvae (*39*, *40*), only become evident after the weevils infesting the tree have already completed at least one life cycle. Because infested trees can only be saved if treated early, through the application of stem-injected insecticides, a number of early detection systems have been developed since RPW attained global pest status, including endoscope technology (*41*); auditory equipment to detect and amplify the sound of RPW grubs feeding on internal plant tissues (*42*, *43*); and the use of sniffer dogs trained to identify and respond to the characteristic fermented odor of an infestation (*44*). Late-stage infested trees may contain multiple generations of the pest, resulting in such extensive damage to the plant that it is not uncommon for the trunk of the tree to break off at the point of the infestation, or even topple over. Palms that have reached this severe level of infestation must be removed from the field and destroyed.

Ferrugineol (4-methyl-5-nonanol), an aggregation pheromone produced by male RPW, was first identified and synthesized in the early 1990s (*45*), and has since proven a valuable tool for the management of this destructive insect. Though the pheromone is attractive to both sexes, traps baited with ferrugineol tend to capture significantly more females than males, with the average reported ratio of captured RPW being 2:1, female to male (*46–51*). Furthermore, RPW females captured in these traps tend to be young, gravid, and/or fertile individuals, indicating excellent potential for RPW population suppression through application of this semiochemical (*52*, *53*).

Ferrugineol has traditionally been deployed among susceptible host plants in food-baited pheromone traps (FBPTs), either for monitoring purposes or mass trapping in order to reduce populations. FBPTs typically take the form of bucket traps, partially buried in the ground among palm trees, to facilitate entry by the weevils and to prevent their escape. Traps are commonly baited with sugar-rich foods (dates are commonly used) and a sufficient quantity of water to keep the bait material from drying out, in addition to a ferrugineol lure. The presence of the food bait is thought to synergize the attractive effect of the pheromone. Insecticide solutions are usually included as well, in order to ensure that captured weevils do not return to the field. One study conducted in Costa Rica found that 90% of test weevils (in this case, the South American palm weevil, *Rhynchophorus palmarum*, a close relative of RPW) placed in similar FBPTs escaped in 24 h or less when no

toxicant was included (*54*). While these FBPTs have been employed successfully to manage RPW in several countries (*38*), they require frequent servicing (i.e., regular replacement of food baits and water) in order to maintain efficacy. Faleiro (*40*) reported that the efficiency of FBPTs diminished appreciably if food baits were not replaced with fresh material every 1 to 2 weeks. This requirement makes FBPTs a costly and labor-intensive method of RPW management, especially in cases of severe infestations, which demand that traps be placed in the field at higher densities (*55*).

Hook™ RPW, an attract-and-kill formulation that combines the aggregation pheromone ferrugineol with the insecticide cypermethrin, was designed as a lower maintenance, more cost-effective strategy to control RPW populations. With this product, ISCA Technologies sought to develop a more attractive, longer-lasting lure than any formulation currently available – one that would function in the absence of food baits, rendering the maintenance associated with FBPTs unnecessary, and when coupled with a potent, fast-acting insecticide, might enable users to do away with the trap altogether, instead applying the product directly to palm trees.

Dr. H.A.F. El-Shafie and Dr. J.R. Faleiro, of the Date Palm Research Centre of Excellence, King Faisal University, Saudi Arabia, et al. (*55*) carried out field trials in an RPW-infested date plantation, located in Al-Guaibah, Al-Hassa, comparing the efficacy of Hook™ RPW to that of the standard mass-trapping program currently implemented to manage the pest in that region. The date plantation selected for this study was planted with date palms of the "Khalas" cultivar; palms comprising the study sites were approximately 10 years in age, 3 m in height, and stood at a density of 150 trees per ha. The test plantation was being treated in accordance with a standard mass trapping program, at a density of one FBPT per ha, at the time the study was conducted.

Hook™ RPW was applied in 100 3-g dollops over a 0.4-ha treatment plot, containing roughly 60 date palms (250 dollops per ha). Although this formulation was intended for direct application to the surface of palm trees, for this study each Hook™ RPW dollop was deposited at the bottom of its own individual bucket trap, to allow accurate measurement of the number of RPW successfully attracted to and killed by the formulation. Each Hook™ RPW trap consisted of a 5-L bucket with 8 3-cm openings immediately below the rim, evenly spaced around the circumference of the bucket, to allow RPW to enter the trap. Traps were evenly spaced along 4 100-m rows, each with 25 traps positioned 4 m apart. Hook™ RPW-treated rows were separated by a distance of 10 m (equivalent to 5 rows of planted date palm trees). The number of RPW caught and killed in Hook™ RPW-treated plots was compared to RPW captures resulting from a standard FBPT program, represented in this study by 2 FBPTs positioned 60 m from either edge of the Hook™ RPW-treated plot. Each FBPT contained 200 g of dates in 1 L of water spiked with 0.01% Carbofuran 10G, along with a ferrugineol lure (Ferrolure + 700 mg), in a 5-L bucket trap with 4 openings; the food bait was renewed on a weekly basis. RPW trap catches for both the Hook™ RPW- and FBPT-treated plots were recorded every week for 13 weeks. This period of time was intended to correspond with the period of peak weevil activity at this location, as identified by El-Garhy (*56*), from March 29 to June 28, 2011.

To compensate for the discrepancy in trap placement density between treatments (Hook™ RPW vs. FBPTs), data on the total number of RPW caught per week in treatment plots was converted to number of RPW caught per ha per week. The resulting data were subjected to a non-parametric Mann-Whitney test to ascertain the impact of treatment on rate of weevil capture. All statistical tests were performed at $\alpha = 0.05$.

In order to evaluate any potential bait-lure synergy attending the application of Hook™ RPW, an additional field study was conducted using the following treatments:

1. Hook™ RPW, applied as a 3-g dollop in a bucket trap without food bait, in the same manner as in the Hook™ RPW/FBPT comparison study.
2. Traditional FBPT, containing the same components as those deployed in the comparison study.
3. Hook™ RPW with food bait, consisting of a traditional FBPT (same as treatment 2), with the addition of a 3-g dollop of Hook™ RPW hung from the lid of the bucket trap in a plastic container.

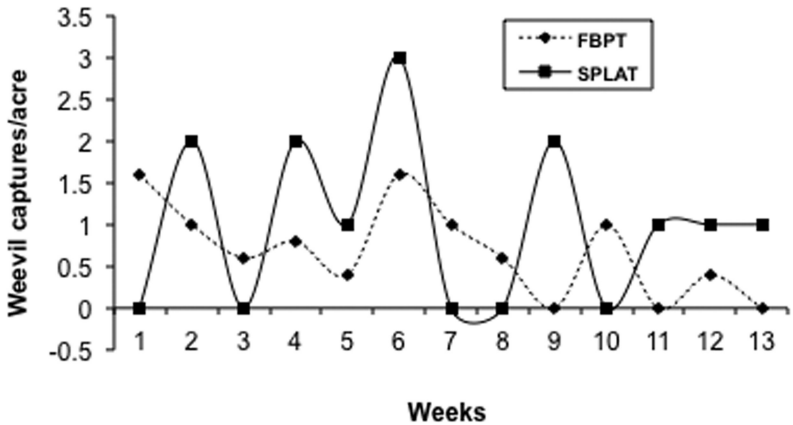

Figure 5. Weekly capture of red palm weevil per 0.4 ha (acre) over 13 weeks of the period of peak weevil activity Al-Guaibah, Al-Hassa, Saudi Arabia in 5-L bucket trap with 4 openings baited with Hook™ RPW, applied as 3-g dollops once at day zero, compared to number of RPW caught and killed in standard FBPT traps with a ferrugineol lure (Ferrolure + 700 mg) and a standard food lure. The food lure of the FBPT traps contained 200 g of dates in 1 L of water spiked with 0.01% Carbofuran 10G, renewed every week throughout the 13 weeks of the experiment.

The experimental site for the bait-lure synergy study was divided into 7 blocks, and a segment of each block assigned to each of the 3 treatments, resulting in 7 replicates per treatment. Treatments within the same block were separated by a distance of 15 m, and blocks were spaced 25 m from one another. Treatments were rotated to different positions within their block each week, to correct for

positional bias, and RPW captures were recorded at the same interval for a period of 3 weeks. Data on mean number of RPW captured per trap per week were subjected to a non-parametric Kruskal-Wallis test.

Analysis of trap capture data (Figure 5) revealed no significant difference in trapping efficacy between traps baited with Hook™ RPW (mean = 2.5 RPW per ha per week) and traditional FBPTs (mean = 3.5 RPW per ha per week), suggesting that the Hook™ formulation is capable of delivering the same quality of RPW control as FBPTs, without the need for the companion inputs of food baits, water, or additional insecticide required with the use of currently-available ferrugineol lures. These findings are further supported by the results of the bait-lure synergy study, which indicated that Hook™ RPW sustained the same level of attraction to RPW regardless of the presence or absence of food baits. Data on mean ranks of RPW caught per week per trap, produced by the Kruskal-Wallis test, revealed no significant difference in trapping efficacy among traps baited with Hook™ RPW alone and FBPTs with and without Hook™ RPW. This SPLAT®-based formulation also demonstrated excellent field longevity, maintaining efficacy for the full 3-month test period despite the hot, dry conditions of Saudi Arabia.

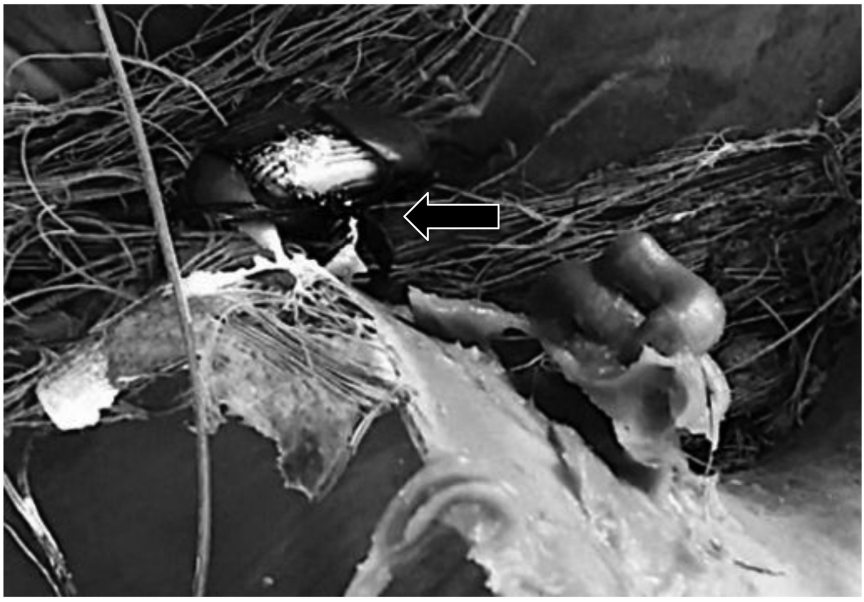

Figure 6. Red palm weevil (RPW) visiting a Hook™ RPW dollop (dollop bottom right). While contacting the Hook™ RPW dollop, beetles receive a transfer of material (arrow points to a white blob attached to the elytra and leg). Field and laboratory observations indicate that the weevils receive lethal doses of insecticide when contacting Hook™ RPW point sources. They are found dead or dying near the dollop, usually on the ground. Direct observations indicate that after a few minutes of contact with Hook™ RPW, the beetles become sedentary, dying within a few hours.

Conclusions

The results of this study verified that Hook™ RPW exerts an equivalent level of RPW attraction to that achieved by currently employed food-baited pheromone traps. Ongoing and future research on this formulation will focus on its application directly in the field, without the use of a trap. In order to make such an application feasible without posing any danger of inadvertently triggering infestations in treated palms, the killing efficacy of the insecticidal component of Hook™ RPW must be confirmed. To this end, ISCA Technologies has engaged the cooperation of a number of research partners for additional field testing and bioassays in Europe, South East Asia and the Middle East. Preliminary testing has produced persuasive evidence of Hook™ RPW's attract-and-kill capacity (Figure 6).

SPLAT® Verb: A Repellent for Mountain Pine Beetle, *Dendroctonus ponderosae*

Repellents

For purposes of this chapter, we define an insect repellent as any compound that manipulates an insect's behavior in such a way as to prevent its locating, feeding, or ovipositing on an otherwise appropriate substrate. Such compounds may exert repellent effects through a variety of mechanisms, and include antifeedants, oviposition deterrents, and other inhibitors. Repellents may be used alone, to repel insects from a host or environment the user desires to protect, or in tandem with an attractant component deployed as part of a "push-pull" strategy intended to intensify the effect of the repellent (*57*). By means of such a coordinated system, an insect may be diverted away from its preferred host or habitat to a location where its presence can do less harm; where it can be trapped and subsequently removed from the field; or where it can be eliminated through the application of insecticides, with less risk of contamination to the protected crop or harmful effects to other organisms.

While a number of repellents have been developed for control of hematophagous insects (*58*), chiefly for pests of medical or veterinary importance (*57*, *59*), few have been developed for agricultural or forest pests despite a growing body of research demonstrating their potential use in these sectors. Repellent formulations using semiochemicals face the same obstacles to commercialization as other semiochemical pest management techniques. They must compete with relatively effective, comparatively inexpensive insecticides that are usually available to manage the target pest (*58*), and unlike such insecticides, they work effectively only against a limited number of insects for a few select host plants. Semiochemical repellents also require the user to have a thorough understanding of the target pest's behavior and its place within its ecosystem compared to conventional pesticide use, or even other forms of semiochemical pest control, like mating disruption or attract and kill (*4*, *57*). The niche market that repellent

products occupy, coupled with the large investment of time and resources required to secure product registration, often make the research and development of these products a costly venture, especially for small businesses.

However, repellents can be of great value in the management of forest pests, where the massive, largely inaccessible areas that characterize the susceptible "crop" make applications of conventional insecticides impractical or socially unacceptable at any appreciable scale. ISCA Technologies is currently expanding its involvement in the forestry sector. This commenced with the launch of SPLAT® GM for gypsy moth mating disruption – since adopted by the USDA Forest Service's Slow the Spread Program – and with the introduction of SPLAT® Verb. This product is designed to protect susceptible pines from attack by MPB, an aggressive species of bark beetle responsible for devastating losses in forests throughout much of western North America. SPLAT® Verb has shown a consistently high degree of efficacy during all field tests in areas with moderate to heavy MPB population pressure. This research, conducted in collaboration with teams from the USDA Forest Service led by Dr. Christopher J. Fettig of the Pacific Southwest Research Station and Mr. A. Steven Munson of Forest Health Protection, is summarized below.

SPLAT® Verb for Mountain Pine Beetle

Unlike most bark beetles, MPB is a primary tree killer of several tree species, including lodgepole pine, ponderosa pine, *P. ponderosa*, whitebark pine, *P. albicaulis*, and sugar pine, *P. lambertiana*. MPB utilize the phloem tissues of their host trees as a food source, a place of shelter, and a nursery in which to establish and rear their brood. In order to gain access to these tissues, the attacking beetles must first overcome the tree's formidable physical and chemical defenses. Pines and other conifers have evolved to respond to an attack by boring insects by flushing large amounts of oleoresin, in some cases containing insecticidal and antimicrobial components, to the site of the attack to enmire or drown the attacking insects, as well as to seal any wounds caused by their activity (*60*, *61*).

The MPB's method of overcoming this defense mechanism is through mass attack behavior. Like other aggressive (host-killing) bark beetles, this species has evolved a highly sophisticated and complex system of chemical communication that synchronizes mass attack on a susceptible host tree, consisting of a number of aggregation pheromones (*62*, *63*), such as *cis*- and *trans*-verbenol, and *exo*-brevicomin (*64–66*), and host-produced kairomones, such as α-pinene and myrcene (*67*). This system enables recruitment of an overwhelming number of beetles (hundreds to thousands, depending on the species, size, and vigor of the host) to attack the tree within a limited time frame (*61*, *68–73*). If MPB recruitment reaches this threshold, the tree's defensive responses are effectively disabled. Beetle-killed trees are easily identifiable within 1 year of a successful mass attack by the appearance of fading crowns.

Outbreaks of MPB have now impacted >8.9 millon ha in the western United States, appearing to have peaked in 2009 (*74*). These mortality events are part of the ecology of western forests and influence many processes, but the economic

and social implications are significant. An outbreak in British Columbia, Canada has affected >18 million ha, resulting in the most extensive MPB outbreak in recorded history (*75*). Since it began in 2000, an estimated 723 million m^3 of timber, corresponding to roughly half this region's marketable pine, has been killed (*75*). This drastic increase in MPB's impact on western forests has largely been attributed to recent warming trends (*76*) and an abundance of susceptible hosts (*77*). Warmer temperatures favor MPB outbreaks by facilitating the survival of overwintering larvae and by increasing the frequency and severity of drought stress in host trees, limiting their production of resin and increasing their susceptibility to attack (*60*, *78–81*). Forest management practices have also been suggested as contributing factors, as years of fire suppression efforts and reductions in levels of harvesting have resulted in overly-dense forests of mature trees – optimal conditions for an MPB outbreak (*82–84*).

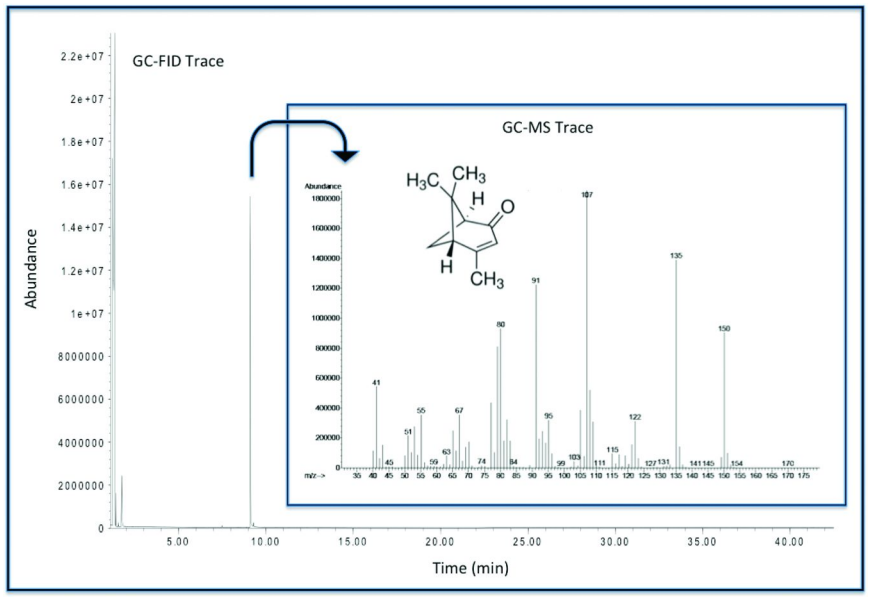

Figure 7. A representative analysis using gas chromatograph (GC) and the inset showing the mass-spectrometrometer (GC-MS) run of the solvent extraction of a single SPLAT® Verb dollop, aged for a month under direct sun exposure in Riverside, California. The trace shows a single large peak, characterized as verbenone (inset).

Previous methods of control of this widespread pest, such as forest thinning to reduce stand density, sanitation treatments to remove infested hosts (*82*, *84–87*), and preventative insecticide treatments – the carbamate carbaryl is among the most commonly used (*88*) – are often limited to small spatial scales. Insecticide

treatments in particular are costly and labor-intensive to implement. Repellent applications of the principal anti-aggregation pheromone of MPB, verbenone (4,5,5-trimethylbicyclo [3.1.1] hept-3-en-2-one), have long been viewed as a promising alternative to protect individual trees, stands or small landscapes of susceptible hosts. Produced during the latter stages of mass attack, this pheromone indicates to approaching MPB that the tree currently undergoing colonization is already overcrowded, and is therefore an undesirable host. The anti-aggregative behavior induced by verbenone is thought to contribute to survival and health of MPB brood by preventing overcrowding within the host tree (*89*). Verbenone may also be an indicator of host tissue quality and its quantity a function of microbial degradation. Verbenone is a natural, safe compound, approved by the US EPA as a management tool for MPB (and other bark beetles species), and by the US Food and Drug Administration (FDA) as a food additive (*90*).

Though verbenone has shown potential as an MPB repellent in several studies (*91–96*), it has yet to be widely adopted by stakeholders in the forestry sector, largely due to the inconsistent efficacy of available formulations, particularly if deployed in areas under heavy MPB population pressure. Most have taken the form of devices, which as previously discussed, entails certain limitations of range and flexibility of application, and many have demonstrated inconsistent capacity to sustain adequate release at rates necessary to impart desired behaviors. The performance of these repellents in the field may have been further compromised by the propensity of verbenone to photoisomerize to chrysanthenone, a chemical that exerts no effect on MPB behavior (*97*). SPLAT® Verb was designed to correct for these deficiencies (Figure 7), to release biologically-active quantities of verbenone long enough to cover the entire MPB flight period in most locations (8-12 weeks), through its UV-protected, rainfast flowable matrix.

Field studies of SPLAT® impregnated with verbenone began in the summer of 2011 and are ongoing. A pilot study to assess the capacity of a prototype of SPLAT® Verb to protect individual lodgepole pines from MPB attack commenced in mid-July 2011 on the Grey's River Ranger District, Bridger-Teton National Forest, Wyoming. Twenty-one lodgepole pines, selected at random, were treated with SPLAT® Verb applied in 4 large dollops spaced evenly around the circumference of the tree trunk at a height of ca. 4 m. The SPLAT® material was deposited onto the bark through the spray nozzle of a Graco 15:1 automotive-style grease pump, fitted with a 5-m hose. Fifteen of the SPLAT® Verb-treated trees received approximately 533 g of the formulation (ca. 32 g verbenone), while the remaining 6 trees received approximately 650 g of SPLAT® (ca. 39 g verbenone). Thirty additional lodgepole pines were also randomly selected to serve as untreated baited controls. One MPB tree bait (Contech Inc., Delta, BC, Canada) was attached to each treated and untreated tree, at a height of ca. 2.4 m from the ground on the north side of the tree, to ensure adequate MPB pressure. Tree baits were attached immediately following SPLAT® application, and were removed ca. 1 month later. Treatment efficacy was assessed by recording tree mortality rates due to MPB attack between treated and untreated trees, as evidenced by crown fade, 1 year after treatment. About 93% of untreated baited controls (28 out of 30) died from MPB attack, while no mortality was observed in SPLAT® Verb-treated trees.

Figure 8. Manual application of SPLAT® Verb for individual tree protection using a caulking gun. Arrow points to a SPLAT® Verb dollop containing ca. 1.75 g of verbenone; an individual tree receives four dollops.

Encouraged by these results, a second field study was conducted in the same area the following year, with an enhanced prototype of SPLAT® Verb and an application procedure adjusted to correct for deficiencies identified during the 2011 study. The optimized SPLAT® Verb formulation (10% AI) was applied to 30 randomly-selected lodgepole pines at a reduced application rate of 7 g of verbenone per tree (i.e., a load comparable with commercial pouch formulations) (Figure 8). The material was deposited in 4 evenly-spaced, evenly-sized dollops per tree using a caulking gun (Newborn XLite) at a height of ca. 2.5 m. As in the 2011 study, all treated trees were baited with an MPB tree bait (Contech Inc.), which was left in place for the first 113 d of the study. Thirty lodgepole pines were also randomly selected and baited to serve as untreated controls. All experiment trees were inspected for signs of MPB infestation before treatment; any trees displaying such signs were excluded from the study. Treatments and tree baits were applied prior to the beginning of MPB flight in the area. No SPLAT® Verb-treated trees showed signs of MPB attack, while 28 of 30 untreated baited controls (93.3%) died. An interesting pattern was observed concerning the frequency of MPB attacks in the vicinity of treated and control trees. Two trees growing within an 11-m radius of SPLAT® Verb-treated trees experienced MPB mass attack, compared to an additional 61 mass-attacked trees within the same distance of untreated trees. These data indicate that not only does SPLAT® Verb

effectively prevent mass attack on the tree to which it is applied, it also casts a protective "halo effect" of repellency over trees growing within at least 11 m of treated trees.

The efficacy of the enhanced prototype of SPLAT® Verb has also been examined on 0.4-ha plots in the Montpelier Ranger District, Caribou-Targhee National Forest, Idaho. SPLAT® Verb, applied at a cumulative rate of 875 g verbenone per ha at a height of ca. 2 m on tree boles in appropriate dollop sizes to attain adequate coverage over the experimental plot, was examined against untreated control plots and plots treated with a verbenone pouch formulation (Contech Inc., 7 g AI). Verbenone pouches were stapled to the tree bole at the same height as SPLAT® Verb dollops along a 9.1 by 9.1-m grid (125 U per ha). A single MPB tree bait (Contech Inc.) was affixed to 1 tree in the center of each plot. Each treatment was replicated 5 times. All treatments were applied before peak MPB flight. Tree mortality, based on crown fade, was determined in July 2013. Analyses are ongoing.

Conclusions

SPLAT® Verb has demonstrated excellent performance as a repellent for MPB in all field studies. As a result, ISCA Technologies began commercialization of SPLAT® Verb in 2012, and has since obtained unconditional registration with the US EPA (Reg. No. 80286-20), as well as state registrations in Colorado, Idaho, Montana, Nevada, Oregon, Washington and Wyoming. Registration is pending in California.

ISCA Technologies and its collaborators are continuing research and development of SPLAT® Verb. Dr. Fettig and his research team are currently evaluating SPLAT® Verb as an MPB repellent for protection of individual ponderosa pines, a host species in which verbenone has generally been regarded as ineffective (*5*, *96*, *98*). Preliminary results indicate similar levels of efficacy can be expected in ponderosa pine as have been observed for lodgepole pine. For example, in one study 89.7% of trees (26 out of 29) were mass attacked by MPB in the untreated baited control compared to 14.3% (4 out of 28 trees) in the SPLAT® Verb-treated plots. In 2014, experiments are planned to determine optimal (minimum) release rates and distribution patterns for applying SPLAT® Verb to individual trees and small-scale stands. In addition, based on research by Fettig et al. (*99–101*), ISCA Technologies is developing an alternative verbenone formulation, SPLAT® Verb Plus, which combines a 3-component blend of non-host angiosperm volatiles (NAVs) with verbenone to increase its repellent effect. Our primary goal for SPLAT® Verb Plus is to create an effective deterrent to mass attack by a species of bark beetle closely related to MPB, the western pine beetle, *Dendroctonus brevicomis*, which has been identified as a primary tree killer of ponderosa pine and has proven difficult to manage with verbenone alone (*102–106*). However, the addition of NAVs to SPLAT® Verb may also improve its potency against MPB (*100*), in addition to other bark beetle species that respond to verbenone.

Conclusions and Future Directions

Effective pest management often requires a multifaceted, integrated approach that (a) combines management strategies, such as monitoring and trapping programs, mating disruption and other behavioral-manipulation tactics, as well as the conscientious and selective use of insecticides; (b) considers the behavior and ecology of the target pest, nontarget organisms and the host(s); and (c) addresses the welfare of workers, user groups and consumers, as well as the natural environment. The versatility and adaptability of SPLAT® technology can complement such a strategy, in terms of "filling in the gaps" of existing pest management programs. To that end, SPLAT® formulations have been successfully applied using a wide range of tactics, including sex pheromones (e.g., SPLAT® OrB), reduced-risk insecticides (e.g., SPLAT® OrB A&K, Hook™ RPW), and repellents (e.g., SPLAT® Verb), against an equally varied array of insect species, including members of the orders Lepidoptera, Diptera, Hemiptera, and as outlined in this chapter, Coleoptera. Development of new SPLAT® formulations for agricultural, forest and medical/veterinary pests is one of ISCA's most active areas of research. Our organization will continue to engage the cooperation of expert researchers and stakeholders, both within the United States and abroad, to explore and evaluate new opportunities for the implementation of SPLAT® technology in semiochemical-based pest control applications.

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Chapter 16

The Market and Potential for Biopesticides

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Biopesticides represent approximately $2-3 billion of the $56 billion pesticide market. Growth of biopesticides is projected to outpace that of chemical pesticides, with compounded annual growth rates of more than 15%. With global population expected to increase to 9 million by 2050, there is an increasing need to produce more food more sustainably. When incorporated into crop production and pest management programs, biopesticides offer the potential for higher crop yields and quality than chemical-only programs. Added benefits include chemical pesticide residue and resistance management, shorter field re-entry, biodegradability, and low risk to beneficials, including honeybees. For these reasons, large agrichemical companies have become involved in biopesticides largely through acquisitions and licensing deals. Recently, several companies have started microbial biopesticide discovery programs. Challenges to the adoption of biopesticides include inappropriate testing regimes without considering biopesticides' unique modes of action and lingering perceptions of cost and efficacy.

The Agrichemical Market

Crop Protection

Conventional Production. Growers are constantly challenged to supply the escalating global demand for food, while reducing the negative impact of crop protection practices on consumers, farm workers and the environment. The dominant technologies for crop protection are conventional chemical pesticides

In Biopesticides: State of the Art and Future Opportunities; Coats, et al.;

and genetically modified crops. Major agrichemical companies have invested billions of dollars to develop genetically modified crops that resist pests or are tolerant to non-selective herbicide sprays. The market for genetically modified crops was estimated at $15 billion in 2012 and is predicted to grow 5% annually through 2015, according to Nature (*1*). In addition, according to the International Service for the Acquisition of Agri-biotech Applications, a third-party not-for-profit organization, in 2013, 175.2 million hectares (445 million acres) were planted with genetically modified crops (*2*). Soybean, cotton, and maize plantings have made the greatest inroads, accounting for 79%, 70% and 32%, respectively, of genetically modified seeds planted globally.

Conventional chemical pesticides and genetically modified crops have historically been effective in controlling pests. However, there are increasing challenges facing the use of conventional chemical pesticides such as pest resistance and environmental, consumer and worker safety concerns. Governmental agencies are further pressuring growers by restricting or banning certain forms of conventional chemical pesticide usage (*3–9*).

At the same time, a number of supermarket chains and food processors, key purchasers of specialty fruits, nuts and vegetables, are imposing synthetic chemical residue restrictions, limiting options available to growers close to harvest. Consumers, scientists and environmental groups have also voiced concerns about the unintended effects of genetically modified crops, including pest resistance and contamination of non-genetically modified crops. In response to consumer and environmental group concerns and restrictions by importing countries, several large-scale food purchasers have demanded that their contracted growers supply them only non-genetically modified crops (*10–16*).

These factors are significant market drivers for conventional producers, and their impact is continuing to grow. An increasing number of growers are implementing integrated pest management (IPM) programs that, among other things, combine bio-based pest management products and crop cultivating practices and techniques such as crop rotation, with conventional chemical pesticides and genetically modified crops. Bio-based pest management products are becoming a larger component of IPM programs due in part to the challenges associated with conventional chemical pesticides and genetically modified crops (*17*, *18*).

Phillips McDougall as reported by Agrow (*19*) estimates the 2013 agrichemical market was $59.2 billion at the distributor level (including non-crop pesticides), up from 2012 by 10%.

Non-crop pesticide sales rose by 1.9% to $6.5 billion, while the crop protection market grew by 11.2% to $52.7 million. In real terms, taking currency effects and inflation into account, crop protection sales were up by 9%, which was similar to the growth seen in 2012.

All regions showed increased crop protection sales last year (Table 1), but growth was led by Latin America with a 26.9% (18.9% in real terms) rise to $14.5 billion. Sales in Europe grew by 9.5% (6.2%) to $13.6 billion.

The NAFTA region recorded 7.8% (6%) growth to $9.95 billion. Most Asian markets were stronger than in 2012, with sales up by 1.3% (14.6%). Growth was again driven by China, Indonesia and India.

Table 1. Crop Protection Sales by Region ($ Billion)

Region	*2012*	*% change*	*2013*
Latin America	11.467	+26.9	14.547
Europe	12.426	+9.5	13.608
Asia	12.478	+1.3	12.634
NAFTA	9.238	+7.8	9.955
Rest of the world	1.751	+9.7	1.921
Total	**47.360**	**+11.2**	**52.665**

Source: Adapted from Agrow and Phillips McDougall (19, 20).

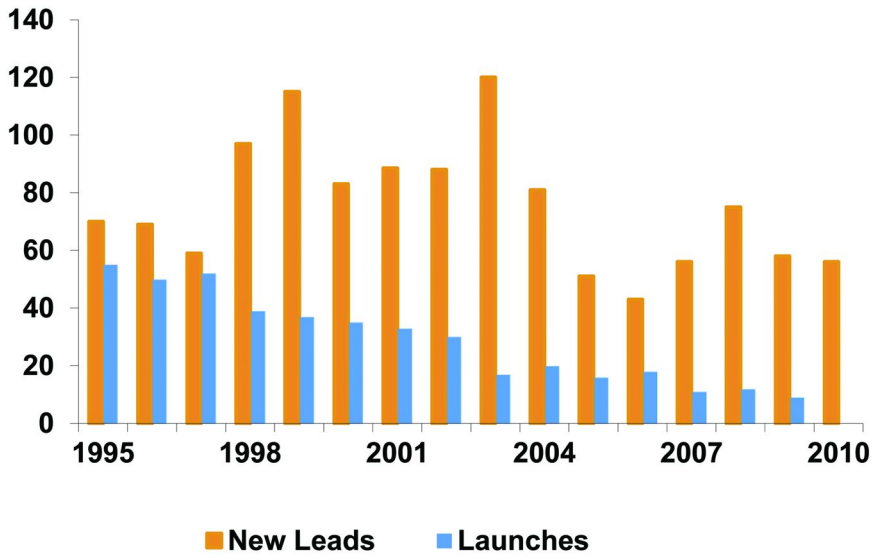

Figure 1. Number of new chemical leads versus number of synthetic pesticide product launches from 1995-2010 (21). (Reproduced with permission from ref. (22). Copyright 2012 Elsevier.) (see color insert)

The discovery of new synthetic pesticides has become increasingly difficult and costly. It is estimated that companies must screen at least 140,000 chemicals to find one new, commercially acceptable, synthetic pesticide (*20*). The discovery of new chemical leads has decreased since 2005 and it is increasingly more difficult to convert a new lead into a new product launch, as indicated by the trending decline in new product launches from 2002 to 2010 (Figure 1) (*21*, *22*). Since it now requires more than $250 million to develop one new synthetic pesticide (Figure 2) and takes ten years, fewer and fewer new chemical active ingredients will be launched over the next 10-20 years (*4*, *22*). In contrast, the cost to develop a biopesticide is in the order of $3-7 million and takes approximately three years

to get to market in the U.S. (*23*). The key reason for this is that a commercial biopesticide can be discovered by screening many fewer microorganisms than the number of compounds necessary to find a commercial synthetic chemical pesticide. For example, *Bacillus subtilis* strain 713 (Serenade®) and *Bacillus pumilus* strain 2808 (Sonata®) used for the control of fungal disease of plants, were discovered after screening 713 and 2,808 microbial strains, respectively (P. Marrone, pers. commun.). Venerate™ bioinsecticide was discovered after screening about 400 microorganisms (*24*).

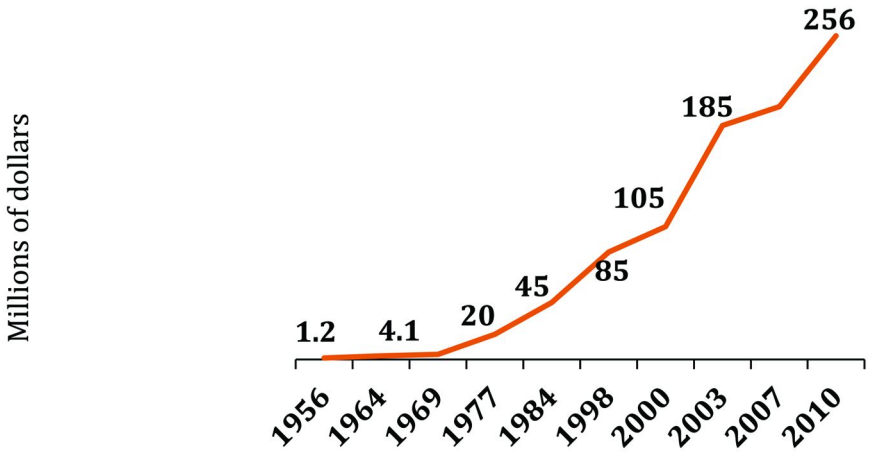

Figure 2. Increasing cost to develop one new chemical pesticide (4). (Reproduced with permission from ref. (22). Copyright 2012 Elsevier.)

Biological Pesticide Market

Demand for effective and environmentally responsible bio-based products for crop protection continues to increase. Biopesticides include microorganisms that are pathogens of insects, nematodes or other pests and microorganisms that produce metabolites that kill pests, as well as low-risk biochemicals, such as pheromones, fatty acids and some plant extracts The global market for biopesticides ,was valued at $1.6 billion for 2009 and is expected to reach $3.3 billion by 2014, with a 15.6% compound annual growth projected during that period, according to BCC Research, an independent market research firm (*25*).

Another market research firm, Lux Research, estimates that the nascent biopesticide market will more than double to $4.5 billion in 2023, or about 7% of the total pesticide market. "Amid concerns over climate change and environmental impacts of farming, "greener" agricultural technologies are drawing the attention of regulators, producers, and consumers."

"Outside pressures will provide new growth opportunities in agriculture. In the case of biopesticides, the EU's ban on neonicotinoid pesticides and health crises like recent accidental poisonings in India will push adoption of these safer

products in the future," said Sara Olson, Lux Research Analyst and the lead author of the report titled, "Green Dreams or Growth Opportunities: Assessing the Market Potential for 'Greener' Agricultural Technologies (*26*)."

MarketsandMarkets (*27*) estimates the global market for biopesticide was valued at $1.3 billion in 2011 and is expected to reach $3.2 billion by 2017, growing at a compound annual growth rate of 15.8% from 2012 to 2017. North America dominated the global biopesticide market, accounting for around 40% of the global biopesticide demand in 2011. Europe is expected to be the fastest growing market in the near future because of the stringent regulation for pesticides and increasing demand for organic products.

The global market is dominated by bacterial-based products (Table 2). Around 50% of biopesticides are used in horticultural trees and crops, 30% on grazing and dry land, with the remaining 12% in field crops (*25*).

Table 2. Estimated Total Global Market for All Types of Biopesticides (Including Biochemicals), through 2014 ($US Millions) (*25*). (Reproduced with permission from ref. (*22*). Copyright 2012 Elsevier.)

Product	2008	2009	2014	CAGR [b] % 2009–2014
Bacteria	893.6	1,194.3	2,516.5	16.1
Fungi	113.3	150.6	288.9	13.9
Predators	98.4	128.0	247.2	14.1
Virus	60.1	80.0	155.5	14.2
Others [a]	34.6	47.1	91.9	14.3
Total	1,200.0	1,600.0	3,300.0	15.6

[a] Include protozoa, nematodes

[b] Compound Annual Growth Rate

Large Companies Move into Biologicals

Large agrichemical companies have become involved in biopesticides through in-licensing of technology and products, joint ventures and acquisitions. Table 3lists the acquisitions of companies with biologicals focus since 2009. These large companies have paid significant dollars to acquire companies with no or modest revenues (e.g., Pasteuria, Devgen, Divergence, AgraQuest). In addition, several companies have started microbial biopesticide discovery programs such as Monsanto and Novozymes (*28*) and FMC and Chr. Hansen (*29*).

Table 3. Acquisitions and Joint Ventures of Biological Companies by Larger Companies and Agrichemical Companies since 2009

Company	Year acquired	Price (mil USD)	Acquirer or Partner	What they do
Novozymes	2014	$300 into Novozymes	Monsanto	Biologicals joint venture
Chr. Hansen	2013	Not disclosed	FMC	Microbial screening joint venture
Novozymes	2013	Not disclosed	TJ Technologies	*Bacillus*-based plant health products
Center for Agricultural and Environmental Biosolutions	2013	Not disclosed	FMC	Microbial endophyte discovery
Prophyta	2013	$35	Bayer	Fungi-based biopesticides
Devgen	2012	$523	Syngenta	RNAi, rice germplasm
AgraQuest	2012	$425 + $75 earnout	Bayer	Biofungicides, Bioinsecticide
Pasteuria	2012	$123	Syngenta	Bionematicide
Becker Underwood	2012	$1000	BASF	Seed treatments, biopesticides
Divergence	2011	Not disclosed	Monsanto	RNAi, chemical nematicide
EMD	2011	$275	Novozymes	Microbial inoculants
AgroGreen	2009	Not disclosed	Bayer	*Bacillus firmus* bionematicide

In addition, large companies have signed deals with smaller ones to gain access to technologies to distribute. Table 4 below shows some of the distribution deals in the last few years (compiled from company press releases).

Why have these larger companies become interested in biologicals? Biopesticides are increasingly required in pest management programs to meet customer needs.

For example, produce exporters and importers monitor the levels of chemical pesticide residues as their customers such as supermarket retailers demand no or few residues. These supermarkets have put restrictions on the number and types of chemical residues allowed on vegetables produced under cover in southern Spain. In the United States, SYSCO, Wal-Mart and other food companies are developing sustainable farming requirements of their grower-suppliers. In 2010, Wal-Mart announced its global sustainable agriculture goals to require sustainable best practices throughout its global food supply chain (*30*). These sustainable

programs incorporate requirements for natural resource and energy stewardship, reductions and restrictions of chemical pesticides and fair treatment of farm workers.

Table 4. Distribution Deals of Biopesticide Companies with Agrichemical Companies

Company with Technology	Partner	Technology	Distribution Region
Marrone Bio Innovations	Syngenta	Regalia® Biofungicide	Europe, Africa, Middle east
Marrone Bio Innovations	FMC	Regalia® Biofungicide	Latin America
Biopreparáty	Gowan	Microbial fungicide	North America
Ecoflora	Gowan	Pesticidal Plant Extracts	North America
Tyratech	AmVac	Insecticidal Essential Oils	North America
Novozymes	Syngenta	Taegro® Biofungicide	Global
CEV	FMC	Problad® Biofungicide	North America

Biopesticides provide added benefits or fit where few chemicals exist due to government restrictions or phase-outs. For example, many chemicals have been eliminated or restricted for plant parasitic nematode control and biologicals are becoming a leading technology. A biopesticide company in Germany (now acquired by Bayer), Prophyta, developed a strain of *Paecilomyces lilacinas* fungus for nematode control in high value fruits and vegetable crops. Bayer purchased Agrogreen's bacterial product based on *Bacillus firmus*, which they developed into a seed treatment for plant stand establishment and yield increase. This Poncho®/ Votivo® seed treatment is a combination of a chemical insecticide (Poncho) and the *Bacillus* (Votivo). Bayer has stated that their expectation for this corn and soybean seed treatment is in the hundreds of millions of dollars in annual sales.

Syngenta acquired the biopesticide company Pasteuria Biosciences thus gaining access to various species of the bacterium *Pasteuria* that infect and kill nematodes. Through a multi-year collaboration the preceded the acquisition, the two companies developed and field-tested a seed treatment of *Pasteuria* for controlling soybean cyst nematodes. Syngenta has publicly stated that they expect this seed treatment to generate $200 million in annual revenues.

Today, agrichemical companies are faced with the development of resistance to important chemical pesticides such as the strobilurin fungicides and neonicitinoid insecticides and several herbicides, including glyphosate (*31–33*).

Most of these modern chemical pesticides are single site mode of action and hence pests develop resistance to these chemical classes relatively quickly. Given the large investment to develop a chemical pesticide, companies develop resistance management strategies to delay resistance. This includes rotation or tank mixtures with other chemicals, but also with biopesticides. Most biopesticides have complex modes of action – living microorganisms that infect and kill a pest, or microorganisms and plant extracts that contain mixtures of multiple classes of chemical compounds, providing longer durability. This complex mode of action can also delay the development of resistance to chemical pesticides (*22*, *34*).

Biopesticides meet the growing consumer demand for health and wellness, e.g., low or no residues and to expand the garden pesticides segment where consumers increasingly ask for natural products (*35*) Several Canadian provinces have banned "cosmetic" uses of pesticides, which are applications to gardens, rights of way, schools and golf courses. (*36*)

Companies can develop biological solutions that fill unmet market needs and gaps in their crop protection portfolios caused by restrictions on chemical pesticides. For example, the European Union passed legislation 91/4/14 (*37*) that restricted and removed hundreds of pesticide active ingredients. This was followed by passage of the Sustainable Use Directive, (*38*) which legislated reductions in chemical pesticides and increasing use of integrated pest management and alternatives such as biopesticides. Several countries passed programs to accelerate biologicals, for example, in Brazil (*39*) and China (*40*), allowing biopesticides to get to the market more quickly and with less capital than chemicals.

A final reason why large companies have shown interest in getting into biologicals is that biologicals are one of the fastest growing input segments, e.g., 10-16% CAGR, as referenced above.

Benefits of Bio-Based Pest Management

Bio-based pest management products are gaining popularity and represent a strong growth sector within the market for pest management technologies. Today's biopesticides are more effective, easier to use, more cost effective, and have better shelf life than biopesticides of decades ago. Growers are increasingly incorporating bio-based pest management products into IPM programs, and bio-based pest management products help create the type of sustainable agriculture programs that growers and food companies increasingly emphasize, driven by consumer demands for low or no-residue produce, fair labor treatment and stewardship of natural resources.

Many bio-based pest management products perform as well as or better than conventional chemical pesticides (*17*, *18*, *23*, *26*, *28*). When used in alternation or in spray tank mixtures with conventional chemical pesticides, bio-based pest management products can increase crop yields and quality over chemical-only programs (*41*). Bio-based pest management products can affect plant physiology and morphology in ways that may improve crop yield and can increase the

efficacy through synergistic action with conventional chemical pesticides (*41*, *42*). In addition, only in a small number of cases have pests developed resistance to bio-based pest management products (*43*). When used together, bio-based pest management products have been shown to extend the product life of conventional chemical pesticides by providing an additional mode of action, which delays the development of pest resistance to both control tactics.

Most bio-based pest management products are listed for use in organic farming, providing those growers with compelling pest control options to protect yields and quality. Given their generally lower non-target organism toxicity compared with many conventional chemical pesticides, bio-based pest management products can add flexibility to harvest timing and worker re-entry times and can improve worker safety. Most bio-based pest management products are also exempt from conventional chemical residue tolerances, which are permissible levels of chemical residue at time of harvest set by governmental agencies. These exemptions mean that many bio-based pest management products are not subject to restrictions by food retailers and governmental agencies limiting chemical residues on produce, which enables growers to export to wider markets.

In addition to performance attributes, bio-based pest management products registered with the EPA as biopesticides can offer other advantages over conventional chemical pesticides. From an environmental perspective, biopesticides have low non-target toxicity, posing low risk to most non-target organisms, including humans, other mammals, birds, fish and beneficial insects such as honeybees. Biopesticides are biodegradable, resulting in less risk to surface water and groundwater and generally have low air-polluting volatile organic compounds content. Because biopesticides tend to pose fewer risks than conventional pesticides, the EPA offers a more streamlined registration process for these products, which generally requires significantly less toxicological and environmental data and a lower registration fee. As a result, both the time and money required to bring a new product to market are reduced.

What Makes a Successful Biopesticide?

1) Good level of consistency – even if lower efficacy than the chemical, customers will use it if they know what it does each and every time
2) Easy to use with existing practices – is compatible with existing spray equipment and spray timing
3) Cost competitive
4) Differentiated with additional value-add (controls pests not controlled by chemicals, unique mode of action, fast field re-entry, zero pre-harvest interval)
5) Compatible with chemical pesticides in spray tank mixtures
6) Synergistic with chemical pesticides such that the combination of chemical and biological can be calculated to be more than additive and is significantly better than either alone (*41*, *42*).
7) Significant efficacy data package is generated before product launch
8) Customer education on mode of action, specific timing, integration etc.

The United States leads the way with the most streamlined biopesticide regulations. Harmonization with Canada has had limited success. Submission of a product concurrently to both the EPA and the PMRA (Pest Management Regulatory Authority) of Canada for joint review has not reduced the time for approval, and often increased the time (Personal experience and Personal Communication with Bill Stoneman, Executive Director of the Biopesticide Industry Alliance). Although Europe would like to accelerate more biological tools to the market due to the restriction and elimination of so many chemical active ingredients, Complex European case by case rules, cost several millions of dollars more than a U.S. registration and may take several years for approval.

Challenges of Bio-Based Pest Management

- Biopesticides are a small part of the crop protection market
- Perceptions persist about efficacy & cost
- Biopesticides are not tested properly based on their modes of action
- Regulations are increasing in complexity and cost in some regions
- There is no global regulatory harmonization.

The single biggest barrier to adoption and growth of biopesticides is the fact that they are often not tested based on their modes of action and are usually tested stand alone rather than in rotations and tank mixes as is customary farmer practice with pesticides. For example, insecticide testing schemes in the greenhouse and field are often designed to test contact insecticides that kill in 48 hours (Marrone, personal experience). There are many instances where biological insecticides are sprayed and rated like chemicals despite the fact that the most successful biopesticide ever develop is based on *Bacillus thuringiensis* (B.t.), which slowly kills small but not large caterpillar larvae after several days through ingestion but not by contact (*44*). One wonders if B.t. would ever make it to market today based on the narrow testing regimes that biopesticides are subject to. Test protocols including observations of plant damage, yield and quality should be incorporated into testing regimens. For example, Marrone Bio Innovations launched Grandevo® bioinsecticide, which is based on a new species of bacterium, *Chromobacterium substugae*. This product has no contact activity on insect pests and must be ingested. After ingestion, the insects become agitated, are repelled and stop feeding and reproduction. Death of the insect pests may take as long as 7 days. Well-designed and carefully implemented test protocols can maximize the efficacy of a product with a unique mode of action like this. (*45*)

Biologicals are frequently tested stand alone, compared to the best chemical program of chemical rotations and tank mixes. More appropriate is to test each chemical and biological stand alone and then incorporate the biological into the program with the chemicals to show the benefit of the biological in the program. Oftentimes, you will see better efficacy and quality of the crop with the biological in the program. Even when the control is equal to the chemical program (no improvement in efficacy), the added benefits of resistance and residue management, shorter worker re-entry and zero day pre-harvest intervals can make a compelling value proposition to growers (*17*, *18*, *22*, *41*, *42*).

The following summarizes the activities that can drive further adoption of biologicals.

- Formulation innovation – new inerts; formulations to extend field residual life & improve consistency
- Premixes of chemicals + biopesticides (e.g., Poncho/Votivo) for better efficacy and functionality
- Premixes of multiple biopesticide Active Ingredients (e.g. Becker Underwood's Biostacked® seed treatments of *Rhizobium* + *Bacillus*.)
- More education & training about how the products work and specifically how to integrate them into IPM programs; understand their unique modes of action
- Support from University Extension – field trials include BOTH: a) stand alone compared to b) integrated into tank-mix and alternation programs
- More on-farm demonstrations – show a block of the biopesticide in the program compared to block of grower's chemical-only program

Resources and Trade Associations

The Biopesticide Industry Alliance (BPIA), http://www.biopesticideindustryalliance.org created in 2000, is dedicated to fostering adoption of biopesticide technology through increased awareness about their effectiveness and full range of benefits to a progressive pest management program. The BPIA members typically meet twice per year, rotating locations in Washington, DC, Sacramento, CA and Ottawa Canada. Committees. The BPIA Regulatory and Government Affairs Committees are active in insuring that regulations remain transparent and meet statutory timelines for approvals.

The International Biocontrol Manufacturers' Association (IBMA) http://www.ibma-global.org is the worldwide association of biocontrol industries producing microorganisms, macroorganisms, semiochemicals and natural pesticides for plant protection and public health. IBMA was created in 1995 to represent the views of these biological control producers, which are mainly small companies with limited resources: Manufacturers, research organisations, extension services, consultants, and distributors, all contribute to the development of biocontrol and participate in IBMA activities. IBMA actively seeks to form a global federation of likeminded regional associations and has already formed a working link with BPIA in North America. IBMA holds an annual member meeting in October in Basel, Switzerland.

IR-4 (USDA program housed at Rutgers University) http://ir4.rutgers.edu/biopesticides.html The primary objective of the IR-4 Biopesticide and Organic Support Program is to further the development and registration of biopesticides for use in pest management systems for specialty crops or for minor uses on major crops. IR-4 has an efficacy grant program that researchers can apply to get funds to do early field trials with biopesticides and also to demonstrate their performance in IPM programs. IR-4 has a searchable biopesticide label database. Through its many years of registering biopesticides and supporting biopesticides through its

efficacy and other educational initiatives, IR-4 has been instrumental in helping educate users and researchers about the best use of biopesticides and their benefits in IPM programs. IR-4 has a close collaboration with BPIA.

Conclusions and Prospects

Biopesticides continue to grow at a pace that exceeds chemical pesticides and when incorporated into pest management programs can provide benefits that customers are increasingly recognizing, such as residue and resistance management, shorter worker re-entry, and low risk to beneficial organisms, including honeybees. Most important, however, is that biopesticides can make conventional programs better, increasing yield and quality compared to chemical-only programs. Biopesticides meet consumer demands for health and wellness. GM crops and chemical pesticides currently dominate pest management programs and are largely seen as essential requirements to feed the world. Restrictions on chemical pesticides are expected to continue and resistance has become a factor in the deployment and sustainability of GM crops. As such, biopesticides can be the third leg of technology inputs, and over time can increase the output, durability and sustainability of IPM programs.

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Chapter 17

Biopesticide Registration Successes of the IR-4 Project and Changes in Regulatory Requirements

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Since 1982, Interregional Research Project Number 4 (IR-4) has assisted in the registration of biopesticides with the United States Environmental Protection Agency (EPA). An explanation is provided of some of the registrations recently obtained by IR-4 and how the regulations in EPA have changed. Additionlly, an update is included how new toxicology tests are being evaluated which are more animal friendly. Regulations regarding RNAi are still under development. Finally, how the fee for service system of EPA under the Pesticide Registration Improvement Act operates is described.

Introduction

IR-4 Overview

The IR-4 Project is funded by the United States Department of Agriculture (USDA) agencies National Institute of Food and Agriculture (NIFA) and the Agricultural Research Service (ARS) provides financial and human resources as well, in addition to financial support from the directors of state agricultural experiment stations. IR-4 is an applied research program whose mission is to assist specialty crop producers by giving them access to safe and effective pest control products. The program was initiated in 1963 and historically has focused on registration and reregistration of pesticides for use on specialty crops or for minor uses on major crops.

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IR-4 broadened its scope in 1982 to include research leading to registration of a wide range of biopesticides including microbials, nonviable microbials, biochemicals, genetically-altered microbials, and transgenic plants. The program is committed to promoting development of alternative pest control products for use on specialty food crops and ornamentals by working cooperatively with public and private sector individuals and organizations. IR-4 interacts with the USDA, EPA, and product registrants to determine the requirements for registration of proposed uses. The program has the resources to develop research protocols, assist with Experimental Use Permits, coordinate and fund field and greenhouse research, assist in the development of Tier I toxicology and non-target organism waivers, and prepare data packages for submission to the EPA.

The EPA under FIFRA regulates all materials that claim to have pesticidal properties. In the biopesticide area, these include microbials such as fungi, bacteria, and viruses, low toxicity biochemicals, pheromones, minerals, plant growth regulators, genetically modified plants and microbials, and pesticidal plant extracts. In general, the number and type of studies required to register biopesticide products are different from the studies required to register conventional products.

Biologicals such as arthropod (insect) parasites and predators or predacious nematodes are not regulated under FIFRA and do not fall under EPA regulation.

The primary objective of the IR-4 Biopesticide Research Program is to further the development and registration of biopesticides for use in pest management systems for specialty crops or for minor uses on major crops. IR-4 assistance has included developing research protocols to meet EPA data requirements as well. The program has also funded small and large scale field efficacy trials and residue trials, if needed. Depending on the stage of development, IR-4 has assisted in obtaining Experimental Use Permits from the EPA. IR-4 regularly prepares and submits petitions and registration documents to the EPA to support registrations and label expansions.

Registration Activities

The registration activities of IR-4 were reviewed in 2006 (*1*). Among IR-4's recent successes in registration include natural products, microbials and most recently, transgenic products. Within the last 10 years IR-4 has registered 15 new active ingredients. When combined with label expansions facilitated through 391 efficacy projects funded by IR-4, over 6,000 new pest-crop combinations have been labelled. A few of those acheivements are decribed in the following summary.

New Active Ingredient Registrations

Aflatoxin is a potent liver carcinogen produced by the fungus *Aspergillus flavus*. The product AF36 contains a specific strain of *Aspergillus flavus* designated as AF36 which had previously been registered on cotton to prevent aflatoxin contamination of cottonseed meal. The AF36 strain has subsequently

been expanded to prevent aflatoxin in pistachio and corn. Weed control is one of the greatest challenges in organic crop production. A form of concentrated vinegar containing the active ingredient acetic acid causes weed cell membranes to collapse, affording contact weed control. The fungus *Trichoderma hamatum* strain 382 was registered primarily for the management of soil diseases in ornamental crops and vegetable seedlings. Dates are a specialty crop in the desert Southwest which are attacked by the larvae of the carob moth. This has traditionally been controlled with malathion dust utilizing plumes of the powder to fill paper wrapped date clusters. The larvae develop from eggs laid by the female moths. A new active ingredient, (Z,E) -7,9-11- dodecatrienyl formate, is the pheromone of the carob moth and the presence of its scent in the air makes it difficult to locate receptive females by the male carob moth. This disruption in mating results in a reduction in mated females, thereby leaving infertile eggs that do not produce larvae to attack the date crop. Utilizing the same approach, IR-4 helped register an oriental beetle pheromone in blueberry and ornamental crops. Bacterial diseases are difficult to control even with conventional pesticides. A new form of biopesticide utilizes a bacteriophage which is a virus that infects a bacteria. Utililizing this technology, IR-4 helped to register bacteriophage of *Clavibacter michiganensis* subsp. *michiganensis* in tomato. Biotechnology -based approaches are becoming more common in row crops but still lagging in fruit crops. IR-4 assisted USDA with registering C5 HoneySweet Plum. The C5 plum is resistant to plum pox virus because it produces the viral coat protein thereby preventing the virus from replicating.

Label Expansions

The IR-4 efficacy program funds research ranging from early stage products that are not yet registered through products that are already registered. Utilization of biopesticides by growers is facilitated by on-farm demonstrations. Within this program a majority of the emphasis is geared primarily toward adding new uses (new crops or pests) for products that are already registered.

Some label expansions supported by the IR-4 efficacy projects for insect management include verebone attractant in pine forests, and the plant extract *Chenopodium ambrosoides* in impatiens and petunia. Label expansions for disease management include *Reynoutria sachanilensis* on blueberry, cucumber, apple, peach and tomato. The fungicide *Trichoderma asperellum on* strawberry, summer squash and pepper, *Trichoderma virens* G41 on Pointsettia and

Polyoxin-D Zinc salt on ginseng and pepper, and *Bacillus subtilus* GB03 in Peanut and Pepper. *Bacillus mycoides* isolate Bac J was registered in pecans, sugar beet, and tomato. Primarily for root and systemic fungal rots, potassium phosphite had the crops greenhouse tomato, pepper and cucumber added to the label. Biopesticides for nematode management have included *Paeciliomyces lilacinus* strain 251 on pineapple and tomato. The bacteria *Bacillus firmus* had the crops apple, carrot, celery, cherry, lettuce, soybean, sugarbeet, and ornamentals added to the label. For managing weeds in turf, efficacy data was supported for the organism *Phoma macrostoma* and the biochemical ammonium nonanoate in

pepper. In addition to the fully registered uses, a specific type of registration for emergency use, known as a Section 18, was supported for 9,10- anthraquinone for managing birds in corn, rice and sunflower.

Regulatory Changes

Biopesticide Registration Data Guidelines

The biopesticide registration data requirements were initially developed in 1984 and were updated in 2007 (*2*). Pesticides often are manufactured in different stages. For example a microorganism may be fermented or a plant extracted. These active ingredients may later be mixed with surfactants, diluents, colorants, stabilizing agents, etc., to improve biological activity, distribution on the plant following application, or another property of the final product. The 2007 guidelines provided clearer guidance on what type of tests needed to be performed on the active ingredient versus the final formulated product. The newer guidelines also gave updated definitions for a biochemical and microbial biopesticides. In many instances data requirements are often fulfilled by citing information in the public literature sure as natural ocurrence, history of exposure to man and the environment, persistence, etc. While this is still true, the updated regulations codified this by explaining the process for discussing the applicability of public literature in pre-registration meetings. In practice, pre-registraton meetings have become more formalized in that a specific form proposing how data requirements will be fulfilled is submitted before a pre-registration meeting, which has helped to ensure that all the applicable guidelines have been discussed and has facilitated a more rapid turn around time for meeting minutes. It also provided for coordination with USDA-APHIS regarding registration of products requiring permits for interstate transport. The updated guidelines also provided additional information under which data for use patterns (food crops, forestry, indoor, etc) was required, conditionally required or not required. It also clarified that even when data was required, the data requirements could still be fulfilled through waivers. The terms waivers and waived are not synonymous under the regulations since waived means that data is not required while a waiver of data is a scientific justification on why information in the public literature, the application technique or usually a combination of supporting pieces of information satisfy the data requirement. Therefore a waiver is an alternate form of fulfilling a data requirement in lieu of conducting direct toxicology studies. This risk assessment includes both hazard and exposure. For microbials, there has been added emphasis on the deposition of samples into a nationally recognized collection. In addition the updated guidelines separated the data requirements for biopesticides pursuing an Experimental Use Permit (EUP) versus a full registration. The trend has been for the EUP data requirements to be fairly similar to a full registration with its purpose being more strictly intended for product performance data collection. Within microbial products, there has been greater emphasis on the deposition of strains into a nationally-recognized culture collection.

Regulatory Guidelines under Development

In addition to the current guidelines, there are discussions about expanding the guidelines to encompass the newer RNAi- based biopesticides. The most common form of biotechnology is the incorporation of genetic material from *Bacillus thuringiensis* (Bt) to code for the production of CRY proteins in plants that control certain lepidopteran insect larvae. Because of the history of biotechnology coming through the microorganism Bt, these products are considered to be biopesticides. These are commonly referred to as Plant Incorporated Protectants (PIPs) and are regulated in EPA's Biopesticide and Pollution Prevention Division (BPPD).

IR-4 first met with EPA about RNAi technology in 2008 for the dsRNA of Israeli acute paralysis virus of honeybees. The term RNAi stands for RNA interference because it interfers or silences processes such as protein synthesis. Without certain proteins, pests may not develop or certain processes cannot continue and so the process fails to function or the organisms fail to reproduce. There are many potential applications to this technology such as controlling pathogens and insects, including those that transmit pathogens. Just like one's genetic makeup, this technology is highly specific. One of the newer approaches to RNAi -based technology has been delivery of dsRNA through feeding insects.

RNAi is the name of the process, but it is double stranded RNA that does the work. RNA is normally single stranded, not double stranded. One of the main functions of RNA is in protein synthesis which involves several steps . A single strand of messenger RNA (mRNA) is made off of the template provided by DNA. The mRNA then causes amino acids to form chains in the exact order to produce a certain protein. RNAi interferes between these two processes by interfering with or cutting up the target mRNA (*3*). The result is that the proteins are not formed and unmodified genes are only interfered with or silenced. While most current biotechnological traits involve incorporating specific genetic material into the plants genome, some RNAi - based technology can be incorporated into plants or sprayed onto plants and it does not modify the plant genome itself. RNAi is also referred to as double stranded RNA (dsRNA) technology since the interference is actually initiated due to the presence of dsRNA and it is the dsRNA that is applied or functions.

EPA does not have an existing set of regulations appropriate for RNAi technology, but regulations are being formed (*4*). EPA recently held a Science Advisory Panel meeting to discuss the formation of regulations for RNAi technology. The regulations are likely to be similar to those currently used for Plant Incorporated Protectants (Genetically Modified Crops). There may be greater emphasis on environmental fate since some are sprayed onto plants or may be mobile within insects or other organisms although data indicates that RNA is rapidly degraded. RNAi technology regulation may be based on the way in which they are delivered and their intended activity and specificity.

Changes in Toxicity Testing

The overall trend in toxicology testing has been to reduce the potential for animal stress and reduce unecessary testing (*5*). EPA has always had a policy of considering the need to test if there is adequate data in the public literature or if the nature of the product (such as a strong acid) already indicates it is known to be caustic to skin or eyes. Due to the types of active ingredients registered as biopesticides they tend to be less toxic, however the requirement that needs to be met is that there be adequate information for EPA to make a risk assessment.

The alternatives to traditional animal studies can be categorized into three main areas including refinement, reduction and replacement. The refinement alternative involves new or modified test methods that refine procedures to lessen or eliminate pain or distress in animals or enhances animal well-being. The reduction alternative involves new or modified test methods that reduces the number of animals required for a test method, while remaining consistent with sound scientific practices necessary to obtain valid results. Finally, the replacement alternative involves new or modified test methods that replace animals with non-animal systems or replaces an animal species with a phylogenetically lower species.

Two specific examples of data guidelines that EPA is trying to consider alternatives include ocular and dermal sensitization. The traditional ocular toxicity data is commonly developed in the Draize rabbit test. For ocular tests there is a voluntary pilot program involving three alternative approaches. The Bovine Corneal Opacity and Permeability Assay (BCOP) - is an assay that uses bovine (cow) eyes which are received shortly after the slaughter of the animal so the cells are still viable. The corneas are excised and treated with a chemical to determine its potential to damage the eye. The BCOP model is a model with endpoints similar to many human corneal responses. The EpiOcular™ (EO) Model is an *in vitro* model of the human corneal epithelium composed of normal human derived epidermal keratinocytes and is used to evaluate the eye irritation potential of chemicals, particularly surfactants. This test is probably applicable to many biopesticides since membrane or cuticle permeability is a common mode of action. The third method is the Cytosensor Microphysiometer (CM) Assay. This assay evaluates the potential eye toxicity of a chemical by measuring the dose required to reduce the metabolic rate in treated cells in vitro. A microphysiometer is used to electronically measure the metabolic rate of cell populations through small changes in acidic metabolites in the medium. The rate is constant in an undamaged cell population and if the cells are injured, an altered metabolic rate is found.

For dermal sensitization, the most common study is the Guinea-Pig Maximization Test or Buehler test. An alternative known as the Local Lymph Node Assay is based on cellular proliferation in mouse ear cells. The reduced LLNA test method can be used in situations where dose-response information is not required. For example, it can be used for a single level limit dose test.

Pesticide Registration Improvement Act

Often referred to by the acronym PRIA, this act was initiated in 2007 and set registration review timelines and included a fee structure that EPA charges to review a data package and tolerance petition. The result has been greater costs of registration, but with a more predictable period of time that EPA has to render a decision. The amount of fees and length of review depend on the type of product and the complexity of the registration. PRIA is currently under its third version, which was updated in June 2012 (*6*). For some of the more common registrations such as a brand new active microbial or biochemical active ingredient, the fees are about $30,000 with a 17 month review time, while for plant incorporated protectants it is more commonly in the $300,000 price range with a 21 month timeline. Comparing this to conventional chemical pesticides, which have about a $600,000 fee with a 24 month timeline, therefore it still holds true that the fees and timelines are reduced as an incentive to register biopesticides. Over the course of the three revisions to this program, the number of types of actions requiring fees has increased. Initially the act was focused on active ingredient registration applications but has expanded to registration of inert ingredients. Other actions now include fees for external review, review of protocols and data protection. The act provides an online decision tree to help applicants understand which category is applicable to their action. Furthermore, there are provisions for reducing the fees for small companies and more specifically, IR-4 is exempt from fees for registrations associated with a new tolerance or tolerance exemption petition.

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Chapter 18

Regulation of Biopesticides by the Environmental Protection Agency

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The Biopesticides and Pollution Prevention Division of the Office of Pesticide Programs in the Office of Chemical Safety and Pollution Prevention of the Environmental Protection Agency registers and regulates reduced risk, safer, biologically based pesticides, known as biopesticides. This paper describes EPA BPPD's current regulatory processes for biopesticides, and possible future regulatory challenges.

General Overview of Biopesticides Regulation

The United States Environmental Protection Agency (EPA) regulates the sale and distribution of pesticides in the United States pursuant to authority granted under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). (*1*) "Biopesticides" are a specific subgroup of pesticides. At EPA, the term "Biopesticides" encompasses three different types of pesticide products: biochemical pesticides, microbial pesticides, and plant-incorporated protectants, or "PIPs." (*2*) A "biochemical" pesticide is defined as a pesticide that: (1) is a naturally occurring substance or is structurally similar and functionally identical to a naturally occurring substance; (2) has a history of exposure to humans and the environment demonstrating minimal toxicity; and (3) has a non-toxic mode of action to the target pest(s). (*3*) Examples of biochemical pesticides include pheromones, plant regulators, attractants, anti-feedants, dessicants, and other biologically based chemicals that have a non-toxic mode of action (*4*, *5*).

A "microbial" pesticide is defined as a microbial agent that is intended to prevent, destroy, repel, or mitigate any pest, or that is intended for use as a plant regulator, defoliant, or desiccant that is (1) a eukaryotic microorganism, including, but not limited to, protozoa, algae, and fungi; (2) is a prokaryotic

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microorganism, including, but not limited to, Eubacteria and Archaebacteria; or (3) is a parasitically replicating microscopic element, including, but not limited to, viruses." (*6*) Microbial pesticides can be either naturally occurring or genetically engineered. Modes of action of microbial pesticides include ecological competition, growth inhibition, direct toxicity, and use of the pest as a growth substrate.

A "plant-incorporated-protectant" is defined as "a pesticidal substance that is intended to be produced and used in a living plant, or in the produce thereof, and the genetic material necessary for the production of such substance. It also includes any inert ingredient contained in the plant, or produce thereof (*7*)."

In addition to FIFRA, pesticides in general, and biopesticides in particular, are also subject to regulation under the Federal Food, Drug, and Cosmetic Act (FFDCA) (*8*) - which requires, *inter alia*, that EPA ensure that any residues of pesticides occurring on edible food occur at a concentration that is no greater than that for which the Agency has determined that there is a reasonable certainty of no harm resulting from dietary exposure to such residues; the Endangered Species Act (ESA) (*9*) – which, in pertinent part, prohibits Federal agencies from authorizing, funding, or effectuating actions that may jeopardize a listed species or modify the critical habitat of a listed species; the Migratory Bird Treaty Act (MBTA) (*10*) – which affords migratory birds broad protections from human interference, except when authorized by a Federal permit; and the Clean Water Act (*11*) – which authorizes EPA to regulate the discharge of pollutants emanating from "point sources" into "waters of the United States."

Basic Steps in the Registration Process

At EPA, regulatory actions regarding the sale, distribution, and use of chemicals are the responsibility of the Office of Chemical Safety and Pollution Prevention (OCSPP). Within OCSPP, the Office of Pesticide Programs (OPP) implements FIFRA authority to regulate pesticides. The Biopesticides and Pollution Prevention Division (BPPD) of OPP focusses on regulating reduced risk, safer biopesticides.

The biopesticides registration process at BPPD ideally begins with a "pre-submission" meeting. This is a meeting where the potential biopesticides registration applicant meets with BPPD staff prior to submitting a pesticide registration application, to apprise BPPD of the forthcoming registration application and to discuss issues that may be relevant to the application and to BPPD's review of the application (*12*). BPPD staff invest significant time in preparing for pre-submission meetings in the hope that the product specific guidance provided to the potential applicant will ultimately result in a stronger and more substantively complete registration application. This, in turn, hopefully will decrease the number of applications that have significant deficiencies that delay the application decison (*13*). To get the most out of the pre-submission meeting, it is useful for a potential applicant to provide a draft label and confidential statement of formula (CSF) at the pre-submission meeting.

The formal registration process begins with submission of a pesticide registration application (*14*). The first step in the review process is the 21-day initial content screen, wherein OPP staff have up to 21 days to complete an initial screen of the contents of the application. The purpose of the initial content screen is to determine if the application package appears to contain all of the required forms, relevant data, and draft labeling, and if all submitted materials are properly formatted (*15*). If the application passes the 21-day initial content screen, it is forwarded to the registering division and the regulatory time period mandated by the Pesticide Registration Improvement Act (PRIA) begins.

Upon receiving an application that has passed the 21-day initial content screen, BPPD staff conduct a "preliminary technical screen (*16*)." The preliminary technical screen determines if the application, data, and information submitted as part of the application are "accurate and complete" and are consistent with the proposed label and any necessary FFDCA action (*17*). The purpose of the preliminary technical screen is to assess whether the contents of the application are sufficient to support a full regulatory review (*18*).

If a biopesticide registration application fails either the 21-day initial content screen or the preliminary technical screen, the applicant will have 10 business days to correct the deficiency. FIFRA mandates that failure to correct the deficiency within 10 business days of notification of such failure is to result in rejection of the application (*19*).

Upon passing the 21-day initial content screen and the preliminary technical screen, the application is placed into formal science review. During the formal science review process, BPPD assesses data and information addressing specific data requirements pertaining to the chemistry and composition of the product, the potential for human health effects, and the potential for environmental effects based on the product's proposed use patterns (*20*).

The Biochemical Pesticides data requirements are set forth at 40 C.F.R. 158.2000, Subpart U. These data requirements include: verification of product identity, composition, and physicochemical characteristics; human health toxicity; nontarget organism toxicity; and environmental fate.

The data requirements are tiered, i.e., Tier 1 baseline data are required and, depending upon results of the Tier 1 studies, additional data may or may not be required. The Tier 1 data requirements for human toxicity encompass acute and subchronic testing. The Tier II and Tier III data requirements may be triggered by adverse results in Tier I testing. The Tier 1 environmental effects data requirements encompass avian, aquatic, nontarget plant, and insect testing. Again, Tier II and Tier III data requirements may be triggered by adverse results in Tier I testing. In addition to the human toxicity and environmental effects data, submission of efficacy data is required if the product label makes claims as to public health pests (e.g., ticks, fleas, mosquitoes, etc.). And, to meet the food safety requirements of the FFDCA, for food uses, either residue data are required or BPPD must determine that the active ingredient qualifies for an exemption from the requirement of a tolerance.

The Microbial Pesticides data requirements are set forth at 40 C.F.R. 158.2100, Subpart V. Microbial pesticides are more varied and may present greater potential concerns than biochemical pesticides. Thus, the microbial

data requirements are more complex than are the data requirements for biochemicals. In addition to the basic data required generally, e.g., product identity, physicochemical characteristics, human health toxicity, and non-target effects, the microbial pesticide data requirements include deposition of a sample into a nationally recognized culture collection; testing to determine pathogenicity; and cell culture study for viruses. Typically, subchronic data are not required in Tier I for microbial pesticides. Similarly, as microbial pesticides that meet the FIFRA registration standard almost invariably qualify for an exemption from the requirement of a tolerance, residue data are generally not required.

As with biochemical pesticides, Tier II and Tier III data requirements may be triggered by adverse results in Tier I testing. In addition to the human toxicity and environmental effects data, submission of efficacy data is required if the product label makes claims as to public health pests (e.g., ticks, fleas, mosquitoes, etc.).

If BPPD reviewers determine that the data and information submitted are inadequate to address the applicable data requirements, a "75-day deficiency letter" may be sent to the applicant. The 75-day letter identifies the unsatisfied data requirement, specifies the deficiency, and informs the applicant of what is needed to cure the deficiency. The applicant is given 75 days to cure the deficiency, or to request extension of the PRIA regulatory timeframe. Failure to do so may result in EPA denying the application for pesticide registration. In lieu of a formal denial of the application, BPPD may determine that the data and information submitted in support of an application is insufficient to grant a pesticide registration, and it may inform the applicant that it "cannot grant" the registration on the basis of the data and information on file. If BPPD issues a "cannot grant" determination, that application is taken out of the PRIA queue and enters a regulatory netherworld where it is neither denied, nor has any reasonable likelihood of being granted. The applicant may, however, commit to submitting the necessary data and negotiate a new date for EPA to make a registration decision.

It should be noted that the data requirements for biopesticides are significantly truncated from those applicable to conventional chemical pesticides. The biopesticide data requirements were developed with the understanding that these are reduced risk, safer pesticide products that, typically, do not present risks to humans or non-target organisms that are of equivalent magnitude as the risks presented by conventional chemicals. This is also reflected in the significantly shorter decision timeframes established under PRIA for biopesticides.

Plant Incorporated Protectants

A "plant incorporated protectant" is defined by EPA as a "pesticidal substance that is intended to be produced and used in a living plant, or in the produce thereof, and the genetic material necessary for production of such a pesticidal substance. It also includes any inert ingredient contained in the plant, or produce thereof (*21*)." It is important to note that EPA does not regulate the genetically modified plant. The plant is not a pesticide. The pesticide is the material, produced in the plant, that

has the actual pesticidal effect, and, in addition, the genetic material, introduced into the plant, that is necessary for production of the pesticidal substance. EPA has regulations specifically addressing certain aspects of PIP regulation at 40 C.F.R. Part 174.

There are not PIP-specific data requirements. The data requirements applicable to PIPs are derived from the microbial pesticides data requirements, although, in practice, the actual requirements applicable to a particular PIP product are developed on a case-by-case basis. In 1999, EPA's FIFRA Science Advisory Panel (SAP) opined on the data requirements that EPA should apply to PIPs (*22*).

The data requirements imposed on PIP registration applications are comprehensive. These requirements will include:

- Information on the genetic engineering techniques used to transform the plant;
- The identity of the inserted or deleted gene segment (base sequence data or enzyme restriction map of the gene);
- Information on the control region of the gene in question;
- Description of the new traits or characteristic that are intended to be expressed;
- Tests to evaluate genetic stability and exchange;
- Characterisation data on the expressed protein;
- Mammalian toxicity data;
- An assessment of potential allergenicity;
- Non-target organism toxicity data;
- Information relevant to an assessment of the possibility of gene flow;
- Environmental fate characteristics;
- Other information BPPD that deems relevant.

The voluminous data and information demonstrating (1) that PIP constructs approved by EPA for sale and distribution do not present risks either to human health or the environment, and, (2) that there is a reasonable certainty of no harm if either the PIP or food containing a PIP are ingested, are publicly available from EPA's Office of Pesticide Programs.

In addition, for *B.t.* PIPs, EPA conducts a comprehensive analysis of the likelihood of resistance developing to the Cry protein expressed by the PIP. EPA considers the potential development of insect resistance to a Cry protein to constitute an unreasonable adverse effect under FIFRA (*23*). EPA's analysis considers the likelihood of resistance developing under the expected conditions of use, given the specific physiologic profile of the particular PIP, i.e., how high a dose is produced by the plant, where is the protein expressed in the plant, how great is the selection pressure that can be reasonably expected of the target pest, is there a possibility of cross-resistance between this Cry protein and one or more other Cry proteins, and other relevant factors.

In the context of the *B.t.* PIPs, EPA has implemented a "structured refuge" strategy to address the likelihood of resistance developing to expressed Cry proteins. In the most favorable scenario, the structured refuge strategy combines (1) PIP plants that express a high dose of the lethal Cry protein with (2) a structured refuge where transformed plants are not not grown. EPA refers to this as the High Dose Refuge Strategy (HDRS). The HDRS is based on the theory that the non-*B.t.* fields (i.e., refuges) will not provide selection pressure for resistance alleles effective against the relevant *B.t.* Cry toxin. If that is correct, the overwhelming majority of the target insects emerging from the refuge field will be homozygous recessive for resistance alleles. Thus, if there are homozygous dominant resistant insects emerging from the *B.t.* field, they will most likely encounter and mate with homozygous recessive insects emerging from either the *B.t.* field or the non-*B.t.* refuge. This mating will give rise to susceptible heterozygotes whose progeny should not survive exposure to the *B.t.* Cry toxin.

As noted, the ideal structured refuge scenario is a high-dose PIP, combined with a structured refuge that is placed in close proximity to the *B.t.* crop field. In the event that the PIP is not high dose, the approach requires a larger refuge. Over the years, EPA has approved different refuge configurations. There have been single-trait corn products with 20% refuge; single-trait corn products with 50% refuge when grown in cotton growing areas where the target pest is polyphagous; single-trait cotton products with 5% non-pesticide treated refuge; single-trait cotton products with 20% pesticide treated refuge; pyramided corn products with a 5% refuge (*24*); pyramided cotton products using natural refuges; and, more recently, products that included non-*B.t.* seeds in the same bag with the *B.t.* seed, so that the refuge is necessarily planted simultaneously with the *B.t.* crop.

As a legal matter, there is a strong argument that EPA does not have authority under FIFRA to mandate the utilisation of any refuge requirement as a mandatory requirement of registration of these pesticide products. It is not at all clear that loss of efficacy of a particular pesticidal active ingredient is the type of effect that Congress contemplated as an "adverse effect" under FIFRA – even if that active ingredient is a safe, extremely low risk biopesticide active ingredient. Therefore, given that the regulated industry has willingly cooperated with EPA in requiring growers to plant refuges, and, subsequently, in developing products that incorporate simultaneously planted refuges, it is fair to describe the structured refuge strategy and its manifestly successful implementation (*25*), as a notable example of government-industry cooperation in the service of the common good. The regulated community, and, in particular, the Agricultural Biotechnology Stewardship Technical Committee (ABSTC), which was formed by the PIP producing registrants to promote the development and implementation of effective insect resistance management strategies, are to be commended, along with EPA, for their commitment to preservation of the efficacy of this vital technology.

Additional information on BPPD regulation of PIPs is available at EPA's Introduction to Biotechnology Regulation for Pesticides website at: http://www.epa.gov/pesticides/biopesticides/regtools/biotech-reg-prod.htm (accessed April 30, 2014).

Biopesticides Regulatory Challenges

Novel Genetic Technologies

A significant issue facing BPPD is developing an appropriate approach to regulating novel genetic technologies. The current regulatory scheme for regulating biotech pesticides was devised to regulate plants genetically engineered to produce exogenous proteins. New genetic technologies do not necessarily require the transformation of plants with exogenous transgenic DNA to confer desirable traits. Examples of novel genetic techniques that can be utilised for pest or weed control functions include zinc-finger nuclease technology, oligonucleotide directed mutagenesis, and RNA interference methodologies (*26*). BPPD's current regulatory approaches may not be appropriate for genetic methodologies that, rather than incorporating novel DNA sequences or producing new proteins, alter phenotypes by regulating gene expression to modulate protein expression or other metabolic functions. As noted above, EPA regulates the products of genetic technologies – not the plant, and not the techniques. Given the character of these new technologies, the relevant question is 'how should the products of these techniques be regulated in the context of the existing statutory authorities?' Examining potential products that utilise RNA interference technology, oligonucleotide directed mutagenesis, and zinc finger nuclease mutagenesis will serve as examples of the regulatory challenges that BPPD faces.

RNA interference (RNAi) technology currently being developed for pesticidal applications functions by suppression of gene expression by double-stranded RNA molecules. RNAi techniques are being developed that utilise either genomic constructs expressing gene silencing sequences or exogenously applied RNAi that works after transport into the cell nucleus. While RNAi constructs do not result in the production of new lethal active ingredients, they do alter the physiological function of plants and when directed against arthropod pests, destroy, repel, or mitigate them. Thus, they fall under the FIFRA definition of "pesticide." 7 U.S.C. 136a(u). As pesticides, these constructs should either be regulated under FIFRA, or exempted from FIFRA regulation (*27*). It is not clear, however, that these constructs should be categorically exempted from FIFRA regulation. To more fully examine the issues surrounding appropriate regulation of RNAi technologies, e.g., mammalian toxicity, environmental fate, hazards to non-target organisms, in January, 2014, EPA convened a meeting of the SAP to address issues related to problem formulation for risk assessment and regulation of RNAi constructs. http://www.epa.gov/scipoly/sap/meetings/2014/012814meeting.html (it should be noted that the RNAi SAP meeting was one of the most heavily attended SAP meetings ever, and that a number of very thoughtful and informative comments were submitted to the public docket for this meeting) (*28*).

The Meeting Minutes of the FIFRA Science Advisory Panel's January 28, 2014, meeting on "RNAi Technology as a Pesticide: Problem Formulation for Human Health and Ecological Risk Assessment" (*29*) were transmitted to the Office of Pesticide Programs on May 1, 2014. In sum, the Panel opined that, while additional study must be done, pest control products that are developed that utilize RNA interference (RNAi) technology are not likely to result in adverse effects to humans through ingestion exposures. The Panel recommended further

study of the possibility of inhalation and dermal exposures, and of possible effects to individuals with certain medical conditions. Regarding possible ecological effects, the Panel noted significant uncertainties based on the currently available data and information. In essence, the Panel recommended that EPA develop a new ecological risk assessment paradigm for RNAi products.

At the January 28 SAP meeting, the Panel was asked 7 broad questions related specifically to problem formulation for RNAi risk assessment. Three of these broad questions related to human health considerations, and four addressed ecological risks. Regarding human health considerations, the Panel opined that current knowledge of the human transcriptome is sufficient that bioinformatic analysis can identify nucleotide sequences with shared identity with specific siRNA sequences (*30*); the Panel also concluded that there is no convincing evidence in the literature supporting a concern that ingested RNAi sequences could survive mammalian digestive processes in a form that could produce biological effects (*31*); and that the RNases and acids found in the human digestive system will likely ensure that all forms of RNAs, no matter their intrinsic structure, will be degraded (*32*). The Panel recommended additional study to evaluate RNAi constructs for potential for exposure through inhalation and dermal routes, and that the possibility of adverse effects in individuals with certain health conditions be examined (*33*).

Regarding potential ecological impacts of RNAi constructs, the Panel raised significant concerns regarding whether EPA's current ecological testing framework is adequate to address the unique risks that may be posed by RNAi products. The Panel concluded that there are insufficient data and information concerning the degradation, persistence, and bioavailabililty of RNAi constructs in the environment (*34*). The Panel specifically noted that EPA's current non-target testing paradigm for PIPs addresses expression of novel proteins and that this is a mode of action that is distinct from RNAi. Thus, the Panel recommended that EPA's RNAi ecological risk assessment framework specifically address the "unique environmental fate and exposure scenarios posed by dsRNA PIPs (*35*)." Moreover, the Panel concluded that the potential scope of use of RNAi formulations in agricultural systems "warrants exploration of the potential for unintended ecological effects (*36*)." The Panel also discussed the significant deficiencies of the Agency's current ecological risk assessment framework as it relates to assessment of RNAi products. The Panel made numerous recommendations to the Agency to assist it in developing a scientifically valid ecological risk assessment scheme for these products (*37*).

RNAi technology provides significant challenges to BPPD in the context of ecological risk assessment. While EPA's regulatory processes for the current set of genetically engineered plant-incorporated-protectants (PIPs) is well established and the risk assessment process for these products is now routine, it is clear that, at least with respect to ecological risk assessment, the SAP is recommending that EPA almost start from scratch to develop a new RNAi risk assessment paradigm, based on RNAi-specific data and information. If adopted, these recommendations will have significant impact in terms of the time it takes to obtain a registration, cost of additional data development, and could impact product development decisions by technology developers.

Another genetic technology that may present novel regulatory questions is oligonucleotide directed mutagenesis. Oligonucleotide directed mutagenesis uses short sequence-specific nucleotide chains (20 to 100 nucleotides) to target specific genes and induce point mutations. The point mutations are generated by the organism's endogenous nucleic acid repair apparatus, and are heritable. Using this technology, genome alterations can include nucleotide substitutions (thus altering codons), insertions (thus adding codons), and deletions (thus deleting codons). Oligonucleotide directed mutagenesis has been demonstrated successfully in a host of plants, including canola, corn, tobacco, rice, and wheat. Plants genetically altered in this manner may be considered to meet the FIFRA definition of a pesticide, i.e., if they have been altered to destroy, mitigate, or repel a pest. This, of course, would be particularly true if the producer intends to sell or distribute the transformed plant with accompanying pesticidal claims. But, that said, what would be the best way to assess whether such product could have an unreasonable adverse effect on the environment? Could EPA determine that it is appropriate to issue a categorical exemption for such products that, for example, incorporate codon deletions in a protein that is vital for plant function, but not for nutritional purposes, and the absence of which does not convey any deleterious human or environmental effect? Or, given the uncertainty of the developmental capacity for such products, would it be better for EPA to regulate such products on a case-by-case basis, such that constructs that can be clearly demonstrated not to have adverse effects on human health or the environment could be regulated in such a manner that constitutes a de facto exemption (as EPA has done with certain virus resistant fruits). These issues will almost certainly require close examination by EPA, and EPA may decide to convene an SAP meeting to fully vet the relevant issues.

Similar to oligonucleotide directed mutagenesis, zinc finger nuclease mutagenesis mediates highly specific genetic alterations. These nucleases can be employed in different ways to cause nucleotide deletions, specific nucleotide sequence alterations (e.g., specific codon changes can be effected), or insertion of an entire gene in a precise chromosomal location. The alterations effected by zinc finger nuclease mutagenesis also are heritable. Thus, similar to oligonucleotide directed mutagenesis, plants genetically altered through the technique of zinc finger mutagenesis may be considered to meet the FIFRA definition of a pesticide, i.e., if they have been altered to destroy, mitigate, or repel a pest. Again, this would be true if the producer intends to sell or distribute the transformed plant with accompanying pesticidal claims. Could EPA also determine that it is appropriate to issue a categorical exemption for products produced using zinc finger nuclease mutagenesis that incorporate codon deletions in a protein that is vital for plant function, but not for nutritional purposes, and the absence of which does not convey any deleterious human or environmental effect? Or, could EPA determine that certain specific or categorical protein insertions should be exempt from FIFRA regulation. As with oligonucleotide directed mutagenesis, EPA may need to convene an SAP to fully vet the risk assessment issues relevant to zinc finger nucleases.

Conclusion

The Biopesticides and Pollution Prevention Division in EPA's Office of Pesticide Programs was established in 1994 to to provide category-specific regulatory focus on reduced risk, safer, biologically based pesticides. During my tenure as Director of BPPD, I encouraged and exhorted BPPD's staff and management to strive to be the number one regulatory body in the world with that particular and exclusive focus, and to meet that goal without compromising adherence to the statutory and regulatory mandates applicable to pesticide regulation in the United States. I feel confident in the conclusion that BPPD did so, and I hope that it will continue to do so. With respect to PIPs and genetic biochemicals, BPPD will be challenged, however, by the continued development of ever more technologically sophisticated pest control technologies. In regulating the first generation of genetic pest control technologies, BPPD has performed admirably in adapting to that purpose a hoary statute intended to address mid- to late-20th Century chemical products. Whether BPPD can build on and extend this regulatory legerdemain to the next generation of genetics-based pest control products remains to be seen.

References

1. 7 U.S. Code (U.S.C.) §§ 136–136w.
2. U.S. EPA. *Pesticides: Regulating Biopesticides*; http://www.epa.gov/oppbppd1/biopesticides (accessed April 30, 2014).
3. 40 Code of Federal Regulations (C.F.R.) § 158.2000(a)(1).
4. Note that a "non-toxic" mode of action does not mean a "non-lethal" mode of action. An efficacious desiccant will result in the death of the target pest; it does so, however, by means of a physical effect on the pest's physiologic characteristics.
5. A subset of biochemical pesticides that has raised interesting issues in the context of biopesticides registrations are plant extracts. Applications for the registration of plant extracts often raise complex issues because the composition of the extracts may be complex. As discussed later in the paper, registration requires identification of the specific active ingredient conferring the pesticidal effect, and also product characterisation data identifying the composition of the formulation. There have been instances where registration has been delayed because of inadequate compositional characterisation of complex plant extracts.
6. 40 C.F.R. § 158.2100(b).
7. 40 C.F.R. 174.3.
8. 21 U.S.C. §§ 301−399(d).
9. 16 U.S.C. §§ 1531−1544.
10. 16 U.S.C. §§ 703−712.
11. 33 U.S.C. §§ 1251−1387.
12. See, U.S. EPA *Implementing the Pesticide Registration Improvement Act – Fiscal Year 2012 Ninth Annual* Report (March 1, 2013), http://www.epa.gov/pesticides/fees/2012annual_report/improve-registration-fy2012.pdf at 4

(accessed April 30, 2014). The pre-submission meeting is not mandatory. An applicant can submit a pesticide registration without engaging in a pre-submission meeting. BPPD strongly recommends, however, that applicants schedule a pre-submission meeting, as it is an excellent opportunity for applicants to receive valuable input from BPPD staff concerning the application.

13. The Pesticide Registration Improvement Act, the third iteration of which was enacted in 2012, Pub. L. No. 112-177 (PRIA III), established a fee-for-service system for pesticide registrations. Thus, pesticide registration applicants pay an application fee (see 7 U.S.C § 136w-8) that varies depending upon the type of application submitted to EPA. In addition, PRIA established set time periods for EPA to act on these applications. If EPA determines that an application is deficient and does not support an approval decision, EPA and the applicant may negotiate an extension of the decision time period. These extensions generally encompass sufficient time for the applicant to supplement the deficient application and for EPA to complete its assessment of the supplemented application package. The various applications fees are substantially reduced for biopesticides applications, as are the review times.
14. Useful information on preparing a biopesticide registration application may be found at http://www.epa.gov/oppbppd1/biopesticides, *supra* note 2.
15. 7 U.S.C. § 33(f)(4)(B)(iii).
16. 7 U.S.C. § 33(f)(4)B)(i).
17. 7 U.S.C. § 33(f)(4)(B)(iv).
18. 7 U.S.C. § 33(f)(4)(B)(iv).
19. 7 U.S.C. § 33(f)(4)(B)(ii).
20. The environmental effects evaluated include environmental fate characteristics (e.g., persistence, potential to bioaccumulate) and the potential to cause harmful effects on nontarget organisms, including endangered species.
21. 40 C.F.R. § 174.3.
22. *December 8−9, 1999: Characterization and Non-Target Organism Requirement for Protein Plant Pesticide and Cumulative Risk Assessment Methodology Issues of Pesticide Substances that have a Common Mechanism of Toxicity*; (http://www.epa.gov/scipoly/sap/meetings/1999/120899_mtg.htm) (accessed April 30, 2014).
23. Note that EPA's regulation of PIPs is unique in this respect. EPA typically does not consider likelihood of pest resistance developing to a pesticide in its regulatory assessment.
24. A "pyramid" refers to a PIP that incorporates two or more active ingredients efficacious against the same pest (preferably with different modes of action).
25. By describing the HDRS as "manifestly successful," it is not meant to discount the reports of apparently resistant western corn rootworm (WCRW) populations in certain areas of the "Red Zone." In fact, the appearance of these isolated populations of resistant WCRW proves the point. Investigations into the emergence of these populations indicate that the following factors have proven significant: a relatively low-dose PIP

variety, growers that have not fully implemented the required refuges, and high selection pressure resulting from multi-year corn-on-corn plantings.

26. See Podevin, N.; Davies, H.; Hartung, F.; Nogue, F.; Casacuberta, J. Site-directed nucleases: a paradigm shift in predictable, knowledge-based plant breeding. *Trends Biotechnol* **2013**, *31*, 375–383. These techniques have been demonstrated to effectively transform crop plants, e.g., meganuclease mutagenesis in maize; TALENs have been used to transform tobacco and rice; and zinc-finger nucleases have been used to transform tobacco and soybean. *Id*.
27. FIFRA Section 25(b)(2) provides EPA with authority to exempt a pesticide from FIFRA regulation, if such pesticide is of a character that is unnecessary to be subject to requirements of FIFRA.
28. Note that the European Food Safety Agency (EFSA) also is taking proactive steps to address how best to consider the novel risk assessment issues raised by this new technology. The Agency has scheduled a meeting of leading scientists and risk assessment experts to discuss (1) RNAi mechanisms in plants, mammals, and invertebrates; (2) current and anticipated RNAi applications in genetically engineered plants; and (3) appropriate risk assessment approaches for such plants.
29. SAP Minutes No. 2014-02, *RNAi Technology as a Pesticide: Problem Formulation for Human Health and Ecological Risk Assessment* (4/28/14) (http://www.epa.gov/scipoly/sap/meetings/2014/january/012814minutes.pdf) (accessed May 8, 2014).
30. SAP Minutes No. 2014-02, *RNAi Technology as a Pesticide: Problem Formulation for Human Health and Ecological Risk Assessment* (4/28/14) (http://www.epa.gov/scipoly/sap/meetings/2014/january/012814minutes.pdf) (accessed May 8, 2014), p 27.
31. SAP Minutes No. 2014-02, *RNAi Technology as a Pesticide: Problem Formulation for Human Health and Ecological Risk Assessment* (4/28/14) (http://www.epa.gov/scipoly/sap/meetings/2014/january/012814minutes.pdf) (accessed May 8, 2014), p 30.
32. SAP Minutes No. 2014-02, *RNAi Technology as a Pesticide: Problem Formulation for Human Health and Ecological Risk Assessment* (4/28/14) (http://www.epa.gov/scipoly/sap/meetings/2014/january/012814minutes.pdf) (accessed May 8, 2014), p 38.
33. SAP Minutes No. 2014-02, *RNAi Technology as a Pesticide: Problem Formulation for Human Health and Ecological Risk Assessment* (4/28/14) (http://www.epa.gov/scipoly/sap/meetings/2014/january/012814minutes.pdf) (accessed May 8, 2014), p 41.
34. SAP Minutes No. 2014-02, *RNAi Technology as a Pesticide: Problem Formulation for Human Health and Ecological Risk Assessment* (4/28/14) (http://www.epa.gov/scipoly/sap/meetings/2014/january/012814minutes.pdf) (accessed May 8, 2014), p 42.
35. SAP Minutes No. 2014-02, *RNAi Technology as a Pesticide: Problem Formulation for Human Health and Ecological Risk Assessment* (4/28/14) (http://www.epa.gov/scipoly/sap/meetings/2014/january/012814minutes.pdf) (accessed May 8, 2014), p 45.

36. SAP Minutes No. 2014-02, *RNAi Technology as a Pesticide: Problem Formulation for Human Health and Ecological Risk Assessment* (4/28/14) (http://www.epa.gov/scipoly/sap/meetings/2014/january/012814minutes.pdf) (accessed May 8, 2014), p 53.
37. SAP Minutes No. 2014-02, *RNAi Technology as a Pesticide: Problem Formulation for Human Health and Ecological Risk Assessment* (4/28/14) (http://www.epa.gov/scipoly/sap/meetings/2014/january/012814minutes.pdf) (accessed May 8, 2014), pp 57−64.

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