

Development and Application of Biopesticides for the Management of Disease Vectors and Pests of Public Health Importance

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Abstract

The development and practical use of natural products occurred in the late 1970s when a spore-forming bacterium producing enterotoxins was discovered. This organism was designated as *Bacillus thuringiensis* ssp. *israelensis* (Bti). By 1985, large quantities of Bti formulations were employed in mosquito and black fly control programs globally. As research efforts in this area continued, a second bacterium *Bacillus sphaericus* (Bsph) was isolated and found to be highly effective against most mosquito species. This entomopathogen found its way into mosquito control around 1997. Today, both these (Bti and Bsph) microbial control agents are produced by large-scale fermentation processes in many countries. Recent advances in formulation technology of both bacteria have increased their usefulness in vector control programs. Another soil bacterium known as *Saccharopolyspora spinosa* (Actinomycetes), was discovered, producing bioactive components known as spinosyn A and spinosyn D. Large-scale fermentation technology has been developed and the product known as spinosad was produced and developed in agriculture, and recently for use in public health. However, spinosad has not been used to any great extent in public health as yet. All three microbial control agents have a good margin of safety for mammals, birds, and wildlife and are environmentally friendly. Considerable efforts were directed toward developing plant extracts for vector control, especially mosquitoes. In this context, plant-based extracts were formulated for the control of larval and adult mosquitoes. Some of the plant parts were used in manufacturing mosquito coils which number into the billions on a worldwide basis; however, only a small proportion of coils are manufactured with plant-derived products. A major area for the development of plant-based products is the use of plant essential oils which are formulated in various ways for personal protection from the attack of hematophagous insects. A number of such insect-repellent products are commercialized in many countries. The public has the perception that plant-based and other natural products are environmentally friendly and safer to

use for vector control or apply to human skin as personal protectants than synthetic chemicals. In this paper, we will dwell upon the research and development efforts leading to the development, production, and application of microbial control agents, and phytochemicals for the control of adult and preimaginal stages of disease vectors, as well as the development and use of plant essential oils for personal protection from anthropophilic insects.

Keywords: Biopesticides, phytochemicals, entomopathogens, microbial control agents, pest control

INTRODUCTION

In recent times, many terms and expressions have been coined to describe a new and improved breed of pesticides now known as “Biopesticides.” Equivalent terms describing this group of agents are biorational pesticides, biopesticides, biocides, biological pest control agents, natural products, botanicals, and naturalists (Menn 1999, Mulla 1997). The two terms that have greater use and acceptance are “biopesticides” and “natural products.” We have been promoting and holding “The International Conference on Biopesticides” for some 14 years and this conference here in Delhi, India is the 5th such one. These conferences have been quite successful and attendance and research presentations have been increasing. Proceedings of the past four conferences have been published.

Since biopesticides in the broad sense include tools and strategies for many pests and disease problems caused by pathogens and insects, the subject now covers insect pest management, weed control, semiochemicals, antagonistic bacteria and fungi and finally transgenic crop plants or mosquitoes. The core disciplines of biopesticides research have been bearing on the management of crop and public health insects, fungal and bacterial diseases of crops and other injurious agents through the use of “biopesticides” or natural products. This subject covers many other themes as you can gather from thematic subjects in this conference.

Mulla (1997) at the first International Conference on “Biopesticides” defined and characterized “biopesticides”. A couple of years later, a book edited by Hall and Menn (1999) titled “Biopesticides” presents a comprehensive treatment of this subject. Various experts in the area of “biopesticides” research and development provided chapters and critical reviews of this subject matter. This volume covers topics related to the discovery, isolation, safety, toxicology, development, registration, labeling, commercialization, and application of biopesticides in pest control programs. Although the earlier attempts were on finding and developing biopesticides for plant pest control, recent worldwide research efforts have come up with effective and viable “biopesticides” for the control of insects of public health importance.

In this paper, we will discuss the development and use of microbial control agents and their toxins and plant-based products for the management of insects of public health importance. In the past 30 years or so, great strides have been made in discovering and developing entities with a novel mode of action that provides viable strategies for solving the major resistance problems encountered in the management and control of medical insects.

Entomopathogenic Bacteria – Their Fermentation Products Employed in Vector Control

At the advent of synthetic insecticides after World War II, DDT and related compounds were largely used in vector control programs until the emergence of resistance in many target species. The organophosphorus group of insecticides provided substitutes for the organochlorine compounds. Synthetic pyrethroids and insect growth regulators were the next generations of insecticides developed for pest control. Resistance to some or all of these groups appeared soon after use in some areas of the world, but at a much slower rate than IGRs. Also, the inherent mammalian and wildlife toxicity and environmental contamination problems of synthetics caused the disuse of some of these once highly effective insecticides.

By the early 1980s, vector control programs in some areas of the world felt the impact of resistance to insecticides. The World Health Organization at the end of the 1970s launched a worldwide effort to find and develop biological control agents with special emphasis on the isolation, characterization, and development of microbial control agents for use in public health. As a result of this international effort, many entomopathogenic organisms were isolated and evaluated against disease vectors. In addition to the WHO global initiative, industrial firms in many countries launched their own search to isolate and characterize entomopathogens. At the end of the 1970s, at the peak of resistance in mosquitoes to most synthetic insecticides, the spore-forming bacterium *Bacillus thuringiensis* ssp. *israelensis* (Bti) was discovered. Rapid development, registration, and labeling of this microbial agent promoted advances in fermentation, production, and formulation technologies. Within 5 years after its discovery, its formulations were employed in large-scale mosquito and black fly control programs. The development of Bti was soon followed by the discovery of another spore-forming bacterium *Bacillus sphaericus* (Bsph), which was isolated from black flies from Africa. This bacterium showed exceptional activity against most mosquitoes. Bti was labeled for mosquito control in 1980, while Bsph was registered in 1997. These two microbial control agents and their formulations are used at present in large quantities in mosquito and other vector control programs.

Recently, another soil bacterium *Saccharopolyspora spinosa* (actinomycete) was isolated and studied for activity against insects in the agriculture and public health. This bacterium on fermentation produces two bioactive compounds known as spinosyn A and spinosyn D. This product is named spinosad, which is currently used in pest control in agriculture, but it will be launched for use in public health insect control programs in 2009/ 2010. All three bacterial products are developed for the control of larval mosquitoes and other public health insects. We will discuss the development and efficacy of various formulations of the three microbial control agents (Bti, Bsph, and spinosad) with details of the efficacy and evaluation of tailor-made formulations for use in a variety of habitats supporting immature stages of mosquitoes. It has been documented that in mosquitoes there is no cross-resistance to these biological agents and further that no resistance has emerged to Bti. In some polluted habitats, some mosquito species have developed resistance to *B. sphaericus* after six to twenty applications. A solution to this resistance problem was found quickly, by using a mixture of Bsph and Bti, the latter used in small proportions.

Bacterial Larvicides

About three decades ago, worldwide research was launched to find, isolate, and develop entomopathogenic bacteria for the control of disease vectors, especially mosquitoes. As a result

of this initiative and intensive search, several isolates and strains of two bacterial species which produced parasporal protein toxins showing high levels of activity against mosquito larvae were discovered (de Barjac 1978, 1990a, 1990b, de Barjac and Sutherland 1990, Mulla 1991, Weiser 1984, WHO 1985). Although *Bacillus thuringiensis* Berliner had been discovered as an entomopathogen several decades ago, the strains discovered up to the 1970s showed activity against herbivorous insects only. It was not until the late 1970s, that a highly mosquitocidal strain of this bacterium was found. This pathogen later described and designated as *Bacillus thuringiensis* spp *israelensis* (deBarjac 1978, 1990a) was isolated by Goldberg and Murgalit (1977). This pathogen was studied intensively and developed quickly for the control of mosquito and black fly larvae (de Barjac and Sutherland 1990, Mulla 1990).

***Bacillus Thuringiensis Israelensis* (Bti) Evaluations Against Mosquitoes**

Soon after its discovery, research on production and formulation technology was initiated by academic institutions, research institutes, and the industry. Tremendous advances were made in this regard and a variety of formulations (liquid, powder, WDG, granules, briquettes, pellets, and others) were developed for different habitats and various species. A comprehensive tome on Bti (deBarjac and Sutherland 1990) was published on the very basic and applied aspects of bacterial pathogens. There are many publications covering research on laboratory and field evaluations of Bti and its formulations against various species of mosquitoes and black flies around the world. This information is all contained in this volume of "Bacterial Control of Mosquitoes and Black Flies" (deBarjac and Sutherland 1990). Information on laboratory activity and field efficacy is contained in this volume compiled by Mulla (1990). We will decline from reviewing or analyzing this published information, except to say that significant advances were made in the application of Bti and other microbes for the control of medically important insects. It should also be mentioned that a great deal of research has been conducted on the safety and environmental aspects of Bti (deBarjac and Sutherland 1990, Mulla 1995, Su and Mulla 1999b). For details, one is referred to these publications. We will, however, present and discuss our research findings on the evaluation of Bti formulations against *Aedes aegypti* (dengue vector) larvae breeding in water-storage containers commonly used in developing countries. Most of the other testing and evaluations have been published and contained in deBarjac and Sutherland 1990).

Bti formulations were rigorously subjected to field evaluation in water-storage containers where *Aedes aegypti*, a dengue vector breeds. This entomopathogen when tested in open bodies of water and impoundments as WDG formulation, showed short residual activity (Mulla 1990). However, controlled-release formulations were developed which increased the longevity of Bti against mosquito larvae. These formulations consisted of briquettes (Fansiri et al 2006), donuts, pellets, and tablets. The tablet formulation was tested and evaluated in water-storage containers (200 L of water) against *Ae. aegypti* larvae (Figure 1). The 200L earthen jar is the most common jar used for water storage in Thailand and elsewhere. This formulation showed exceptional activity and longevity, giving prolonged control (>80% inhibition of emergence of adults) for about 112 days at the rate of 1 tablet (0.37 g 2700 ITU/mg) per 50L water (Mulla et al 2004). Under the experimental conditions in water-storage containers, various factors such as sunlight, wind, water use practices (removal and addition), and water pollution were controlled, thus obtaining longevity for 90-112 days. Under normal use conditions; however, we expect that the duration of efficacy will be shorter; most likely lasting for 2 months or so, a period which is still adequate from the standpoint of operational control programs. We can get

this inference from village trials of a temephos granular formulation applied to water-storage containers where its longevity was 2-3 months (Thavara et al 2004) as compared to the longevity of 6-8 months, using the same formulations in the current experimental set up (Mulla et al 2004).

In open bodies of water and impoundments, there are a number of other habitat factors which adversely affect the efficacy and longevity of Bti as well as other mosquito larvicides. Depth of water can reduce effectiveness against surface frequenting larvae. Vegetation and plant standing in water also reduce effectiveness and longevity. Exposure of habitat to sunlight and wind can also reduce the efficacy and longevity of larvicides. Flowing water with currents will require higher dosages as the movement of water dilutes the concentration. Mosquito breeding sources with these factors will require higher dosages of Bti and others than habitats not possessing these features. On account of these considerations applications of Bti (WDG) formulations produced mosquito larval control for only 7-12 days with .027-.053 lbs/acre of WDG in open sunlit tubs (Su and Mulla 1999a). Granular formulations similarly used yielded only short-term control.

***Bacillus Sphaericus* (Bsph) Evaluations Against Mosquitoes**

The second entomopathogenic bacterium discovered was *Bacillus sphaericus* strain 2362 from black flies in Nigeria (Weiser 1984). Other strains namely 2297 (Sri Lanka), 1593 (Indonesia), and C3-41 (China) were also isolated (deBarjac 1990b). All these strains exhibited similar levels of activity against mosquito larvae, with strain 2362 in general showing somewhat higher level of activity (deBarjac and Sutherland 1990, Mulla 1991). None of the strains showed activity against *Aedes aegypti* (breeding in artificial containers) and *Aedes albopictus* breeding in natural containerized water such as tree holes, bromeliads, leaf axles, etc. Therefore, *B. sphaericus* (Bsph) strains were not evaluated against the two container-breeding mosquitoes.

B. sphaericus formulations were extensively tested against culicine and anopheline species in impoundments and open bodies of water. This entomopathogen proved highly effective against culicine mosquitoes in polluted water (deBarjac and Sutherland 1990, Mulla et al 1988, 1997, 1999b, 2001a, 2003 Nicolas et al 1987, Skovemond and Bauduin 1997). It was evaluated against *Culex* and *Anopheles* species throughout the world. The formulations tested were corn grit granules (VectoLex CG) and water dispensable granules (VectoLex WDG) and others. In the volume of deBarjac and Sutherland (1990), there are 3 chapters dealing with the evaluation and practical application of *B. sphaericus* formulations. The chapter by L.A. Lacey (10 pages) with persistence and formulations of *B. sphaericus*, and the chapter by JM Hougard (12 pages) covers formulations and persistence of Bsph in *Culex* larval sites in tropical Africa. Another chapter by HH Yap (14 pages) compiles information on the efficacy of Bsph formulations against *Aedes*, *Anopheles*, *Culex* *Mansonia*, and *Psoropora* species. We will not evaluate and analyze this information but present some recent studies of our own on Bsph.

We evaluated Bsph formulations against the southern house mosquito *Culex quinquefasciatus* breeding in highly polluted waters in urban areas in Bangkok, Thailand. In surface drainage klongs (with slow-moving water in the absence of rain), both GR and WDG (water disposable granules) yielded 14-28 days of control of larvae at the dosages of 2 g/m² of GR and 0.1-0.25 g/m² of WDG. Heavy precipitation increased the volume of water through the klongs and washed away mosquito larvae, resulting in longer control of larvae (Mulla et al 1997). In some

stagnant polluted water habitats, Bsph provided *Culex quinquefasciatus* larval control for almost one to two months, a performance not shown by other larvicides, with successive treatments of Bsph (Mulla et al 2001a, 2003). The efficacy and longevity declined due to high levels of resistance and this high level of resistance was managed by using a mixture of Bti and Bsph against resistant larvae (Mulla et al 2003).

The efficacy and longevity of *B. sphaericus* depended on the quality of formulations, species of mosquitoes, and factors of habitats. In general, it provided persistent control of *Culex* species in highly polluted water and water not exposed to sunlight and winds. Other factors of habitat that influenced efficacy and longevity were vegetation, dilution by incoming water, and solid wastes. The two formulations of Bsph commonly used are corn grit granules and water dispersible granules (WDG). The effective rate of application is 2-5 lb/acre of the GR formulation and 0.25 – 1.0 g/m² of the WDG formulations. These rates may have to be increased in habitats with unfavorable features or where less susceptible species is prevalent.

***Saccaropolyspora Spinosa* – Fermentation Products**

Spinosad is a metabolic and fermentation product of the actinomycete bacterium *Saccharopolyspora spinosa*. This naturally occurring bacterium was isolated from the soil in 1988. It was identified as a new species (Mertz and Yao 1990, Thompson et al 1997). Actinomycete bacteria exhibit fungus-like characteristics and they are responsible for the decomposition of complex organic materials. In 1989, the most bioactive metabolites from the spinosad formulation broth were identified and designated as spinosins A & D (Sparks et al 1998). In the mixture, spinosin A and spinosin D constitute 85 and 15 % respectively (Kirst et al 1992). Both spinosins are non-volatile, slightly soluble in water, and stable under a wide range of temperatures and pH (Dow AgroSciences Tech Information).

Spinosad has a novel mode of action. It alters the formation of nicotine and GABA-gated ion channels and consistently results in neuronal excitation (Salgado 1998, Watson 2001). Spinosad with its novel mode of action is an ideal product for the management of resistance. It has shown no cross-resistance to currently used insecticides and can be rotated with all other classes of existing mosquito larvicides (Dow AgroSciences Tech Information). The mode of entry is by ingestion as well as cuticular penetration. Spinosad has been registered for use on over 250 crops. It has a good margin of safety for mammals. Short-term toxicity as determined in rats was LC₅₀ >3738 to >5000 mg/kg body weight for male and female rats respectively. Dermal and inhalation tests also showed a high level of safety. It has a good margin of safety for birds and fish and most aquatic macroinvertebrates. It does, however, impact some groups of aquatic crustaceans. Therefore, caution should be exercised in making applications in aquatic habitats where mosquitoes breed and where sensitive macroinvertebrates might prevail.

Spinosad – Evaluation and Efficacy Against Mosquito Larvae

Soon after the isolation of the actinomycete bacterium *Saccaropolyspora spinosa* (producing spinosad) from the soil, it was cultured and the products were first tested against mosquito larvae (Kirst et al 1992). It was noted that the product had considerable activity against mosquito larvae. Notwithstanding this information, spinosad was first developed for the control of crop insects. It is now registered for insect pest control of some 250 crops. Interest in testing and developing this natural product for mosquito control did not materialize until 2003/2005. We conducted preliminary studies on the activity of spinosad against mosquito

larvae in 2000 (Mulla unpublished report). During this period and thereafter numerous studies were carried out on the activity of spinosad technical and formulated materials against mosquito larvae in different regions. A flurry of studies followed evaluating various formulations of spinosad against larvae of mosquitoes: *Aedes aegypti*, *Ae. albopictus*, *Culex quinquefasciatus*, *Cx. pipiens*, *Anopheles albimanus*, *An. stephensi*, and *An. quadrimaculatus* in the laboratory and field (Bond et al 2004, Cetin et al 2005, Darriet et al 2005, Darriet and Corbel 2006, Paul et al 2006, Romi et al 2006, Thompson and Hutchins 1999). In these studies, various levels of activity were noted in the laboratory against various species. In field studies, efficacy, and longevity were dose-dependent, species variables, and habitat conditions.

Spinosad Against *Culex* Mosquito Larvae in California, USA

We initiated detailed studies on the evaluation of technical spinosad and SC 120 emulsifiable formulation against larvae of *Culex quinquefasciatus* in the laboratory in 2005 (Jiang and Mulla 2010). We determined in repeated replicated studies that spinosad showed a high level of activity against the larvae. We determined the LC₅₀ and LC₉₀ (lethal concentrations killing 50% and 90%) of the exposed larvae. We carried out tests at multiple dosages against 2nd and 4th instar larvae and the mortality was assessed at 24 and 48 hr post-exposure. Constant temperature (26-28° C) and photoperiod (14 L:10hr D) were used.

The LC₅₀ of the technical material against 2nd instar larvae for 24 hr exposure was 0.021 mg/L AI for the 4th instars it was 0.033 mg/L. The LC₅₀ of the SC120 (11.6%) was lower being 0.012 (2nd instar) and 0.014 (4th instar). The LC₉₀ for the technical material was 0.051 (2nd instar) and 0.060 (4th instar) and for the SC120 the LC₉₀ values were 0.026 (2nd instar) and 0.032 (4th instar). The data showed that mortality increased at the 48-hr exposure than the 24-hr exposure for both the 2nd and 4th instars. These tests showed that larvae of *Culex quinquefasciatus* are quite sensitive to both technical and emulsifiable formulations, the latter being more active than the former. With this data at hand, we got encouraged to take spinosad to simulated field set-ups using standard microcosms and mesocosms supporting natural populations of *Culex* mosquitoes.

Microcosms and Mesocosm Studies on Spinosad Against *Culex* Mosquito Larvae in California, USA

For the simulated field studies, we used the emulsifiable (SC 120, 11.6% AI) formulation. The studies were carried out first in fiberglass tubs (24 tubs, 1 m², water depth 30 cm containing 240 l water), followed by experiments in earthen ponds (38 non-vegetated and 64 vegetated; 27 or 37.5 m² surface, water depth 30 cm) filled with 8100 L (non-vegetated) or 11250 L (vegetated) of reservoir water. The SC120 was diluted in distilled water and aliquots were sprayed on the water's surface. The material was applied to obtain multiple dosages, 0.05, 0.1, 0.25 and 0.5 mg/L AI in microcosms and 0.025, 0.05, and 0.1 mg/L for the mesocosms (Figure 2).

Treated and untreated units (tubs) and ponds (mesocosms) replicated 4 times were sampled for larvae using a standard dipper before and at intervals after treatments. The samples were categorized into early instar larvae (1st and 2nd), late instar, (3rd and 4th), and pupae (Jiang and Mulla 2010).

In the microcosm tests (tubs), we obtained 100% initial control of the immatures of 3 species of *Culex* mosquitoes. The longevity of treatments was dose-dependent, the higher dosages providing long-lasting control for 21-35 days, depending on the dose. The results were similar in the mesocosm (ponds), where the longevity was also dose-dependent. The low dosage (0.025mg/L AI) yielded complete control for 4-7 days, while the 0.05 mg/L AI gave complete control of larvae for 7 days and pupae for 14 days. The highest dosage of 0.1 mg/L AI yielded both larval and pupal control for 14 days.

It should be pointed out that both the tubs and ponds were in open areas (Fig. 2) exposed totally to sunlight and wind. As mentioned earlier, sunlight photolysis degrades spinosad rapidly. From these studies, it can be deduced that field application rates of 0.05 to 0.10 lb/acre of active ingredients will be necessary for satisfactory control of *Culex* mosquitoes in open bodies of water. In cases where mosquitoes are breeding in polluted water or habitats with vegetation, higher rates might be necessary. It should be realized that formulation technology can improve the initial activity as well as the longevity of bioactive agents.

Spinosad Field Activity and Longevity Against *Aedes Aegypti* Larvae in Thailand

The activity of spinosad against *Ae aegypti* in the laboratory is similar to *Culex quinquefasciatus* (Bond et al 2004, Jiang and Mulla (2010). In 2005/ 2007, we evaluated 3 formulations of spinosad against *Ae aegypti* in water-storage containers. These studies were carried out under simulated field conditions.

The tests were carried out in 200 L (filled with water) earthen jars or plastic barrels (Fig 1). Spinosad formulations (SC 11.6%, Granules 0.5%, and directly applied DT tablets 7.5% AI) were applied to the containers at multiple dosages. Before the treatments, 25 *Ae aegypti* 3rd instar larvae were added to each container and 1 g of ground-up larval food was added to each and subsequently, 0.5g was added weekly. Larval survivorship was assessed using a flashlight. We also assessed the emergence of adult mosquitoes by counting the pupal skins (reflecting adult emergence). The pupal skins float on the surface and they were removed by a suction pipette and counted in a white plastic tray. Pupal skins are easier to remove and count, and their number yielded accurate data on the successful emergence of adults. Inhibition of emergence % (IE%) was calculated based on the number of larvae (25) added to each container (100 larvae in total per 4 replicates).

To determine the efficacy and longevity of each treatment, cohorts of new 25 late instar larvae were added weekly to each container until the IE% reached 80%. The water-storage containers were placed in shaded arenas on a concrete slab roofed over but open on all sides. The jars were covered either with cellocrete sheets aluminum covers or lids with a screened area in the center of the lid. They were protected from sunlight, wind, and evaporation. Usually, 3 water regimens were used: full jars all the time, ½ full jars, and full jars – ½ removal and refilled weekly. Water loss, which was minor, was replenished monthly. The results of these experiments are presented here.

Comparisons of Spinosad SC120 and GR 0.5

This experiment was started on March 2, 2005. The SC120 was used at high dosages (8.4, 3.9, 2.23, and 0.89 mg/L AI), and the GR 0.5 was used at the realistic dosages of 1, 0.5, 0.25, and 0.1 mg/L AI. The SC120 used at high rates provided almost IE 100% (control) for 104 days at all

dosages. The two lower dosages were exhausted after 125 days. The GR 0.5 used at realistic dosages gave close to 100% control for 83 days at the 3 lower dosages, with the high dosage (1.0 mg/L AI) still producing close to 100% control for 111 days (Mulla, unpublished report)

The second experiment using the SC120 alone at realistic dosages in water-storage jars was started on May 2, 2005. The spinosad doses were 0.5, 0.25, and 0.10 mg/L AI. We used two water regimens, full jars and full jars ½ removed. At these lower dosages, the longevity as expected was low; being 100% control at all 3 dosages for 39 days in both water regimens. The high dosage (0.5 mg/L) still gives an 85% reduction for 46 days in full water. Thereafter the level of control declined in all dosages (Mulla 2005, unpublished report)

Comparison of Spinosad DT Tablets and GR 0.5

The next experiment comparing the DT (directly applied tablet 7.5% AI) and GR 0.5% was started on April 18, 2007. Using the same setup and the same procedures, the dosage for each formulation was 1.0, 0.5, and 0.25 mg/L AI. As before, each treatment and control were replicated 4 times and the treated and untreated jars were challenged with 25 *Ae. aegypti* larvae (lab-reared) and added weekly until the IE% (control) dropped below 80%. Two water regimens were used, full jars and full jars-1/2 emptied and refilled weekly (Thavara et al 2009).

The DT formulations at all 3 dosages produced 100% control for 13 days in full jars, but the level of 100% control lasted longer for 20 days with the DT highest dosage (1.0 mg/L). The DT at the highest dosage (1.0 mg/L) yielded 79-100% IE for 48 days in full jars, the efficacy declining beyond this period. However, the longevity in jars with water exchange was higher, with 90-100% IE for 62 days (Thavara et al 2009). The manufacturer's recommended rate of 0.5 mg/L AI gave 79-100% IE for 27 days in the water exchange jar. It is evident that water removal and filling facilitate the release of the active ingredients from the tablet, which sinks and sets on the bottom of the jar.

All dosages of GR 0.5 produced IE 90-100% for 34 days in both full jars and full jars with water exchange. The highest dosage (1.0 mg/L) showed longer persistence yielding more than 80% IE for 62 days in both water regimens (Thavara et al 2009). The longevity at the lower dosages was shorter. It appears that the most appropriate dosages of spinosad for *Ae. aegypti* in water-storage containers will be in the range of 0.25 to 1.0 mg/L, with higher dosages providing long-lasting control.

Plant-Based Bioactive for Use as Repellents and Protectants

The use of repellents and protectants containing plant materials and synthetic chemicals is common throughout the world. The most common devices sold for personal protection are mosquito coils and preparations of plant essential oils as repellents against biting insects. It has been estimated that the number of mosquito coils sold worldwide reach 29 billion, where 95% are used in Asia (WHO 1998). Mosquito coils are used primarily by low-income communities while vaporizing mats and aerosols are used by the middle and high-income sectors. Mulla et al (2001b) noted that in mosquito-infested areas of Thailand, the residents spent about U.S. \$12.50 to \$25 per residence per annum for protection from biting mosquitoes. Similar levels of expenditures were reported for some communities in India (Snehalatha 2003). In addition to these uses, plant products are used as repellents on skin and as larvicides and adulticides, in field situations and residences.

There are numerous publications containing information on plant-based repellents and protectants and it will not be possible to list and review them here. Three publications offer an enormous amount of information on the research and development of personal protectants and repellents (Debboun et al 2007, Moore et al 2007, Sukumar et al 1991). These publications have an impressive list of published works on this subject. A considerable amount of research has been carried out on the neem tree (*Azadiracta indica*) and its various products for the control of medically important insects, including reports on the usefulness of neem products as repellents, protectants and deterrents (Mulla and Su 1999). The recent volume published on the neem tree by the Neem Foundation (Schmutterer 2002) contains valuable information on the neem tree and the unique products derived from this tree which are used in pest control and many industrial, medicinal, and agricultural enterprises. Bioactive materials from neem trees have multiple modes of action, such as repellency, deterrence, sterility, growth regulation, and toxicity (Isman 1999, Mulla and Su 1999, Schmutterer 2002).

The repellent chemicals found in different parts of plants have been identified in some plant extracts. These constituents not only act as repellents but also as feeding deterrents, toxicants, growth regulators, etc. The major groups of chemical substances, identified are categorized as alkaloids, phenols, terpenoids, and others. Different plant species and their parts yield different groups of chemicals in varying quantities. Some plants may have one or a few phytochemicals possessing repellent properties while others may have quite a few principles. The citronella group of grasses originating in India has a widespread distribution. These plants have varying amounts of repellent chemicals (mostly terpenoids), but the most abundant repellent chemicals contained are citronella, citronellol, and geraniol. The grass *Cymbopogon nardus* or citronella is the most common species used in the commercial blending of repellent products. Several other species of *Cymbopogon* are used in commercially available plant products.

Osimum species (*basilcum* in particular) contains a number of repellent compounds which also act as mosquito larvicides. Species of *Hyptis*, *Mentha*, and *Thymus* also contain insect-repellent principles and they are used in various ways by the local communities. *Tagetes* species have exhibited larvicidal and insecticidal properties, their essential oils act as repellents in some species and not others. *Artimisia* spp has both essential oils which act as repellent and other principles acting as larvicides. The neem tree *Azadiracta indica* has attracted a great deal of attention as a source of agricultural biopesticides. In India, neem tree parts have been used for thousands of years as insect repellents and protectants. In recent years, materials from this plant have been developed for the control of medical insects. The neem tree has about 35 bioactive compounds which show toxicity, causing sterility, and decreased fecundity as well as repellency and deterrence. Proper formulations can increase the toxicity and repellency of neem and other plant products. Bioactivity is related to the concentration of bioactive principles and the extent of release and absorption (Isman 1999, Mulla and Su 1999, Schmutterer 2002).

Lemon eucalyptus extracts from *Corymbia citriodora* (from China), have been the subject of many tests. The essential oil extract was noted to have repellency to mosquitoes, slightly better than most *Eucalyptus* spp. The compound p-menthane-3,8-diol (PMD) was identified as a byproduct. This material was proven to be highly repellent, equaling the repellency of deet (commercial repellent). The Chinese name of this repellent is Quwenling "effective repellent of mosquitoes."

Essential oils of plants are extracted by steam distillation. They show different levels of repellency against mosquitoes. Moore et al (2007) tabulate the protection time for essential oils in extracts of some 37 plants. The protection time increased with concentration. Most of the protection times for 50% concentration were from 0 to 80 minutes. The longest protection time was 88 minutes for *Zanthoxylum limonella* and 60 minutes for each *Apium graveolens*, *Cymbopogon nardus*, and *Pogostemon cablin*. The rest of the plant essential oils showed low levels of repellency. The same authors provide a comparison in protection time among 8 commercial plant-based products with Skin-sensation (7% deet). Six commercial products were inferior to Skin-sensation, while 2 commercial plant products (Bite Blocker and Repel) proved superior to deet. Bite Blocker contains oil from coconut, geranium, and soybean (Consep Inc, Bend Oregon) and Repel contains oil from lemon eucalyptus and PMD (Wisconsin Pharmacol Co, Jackson WI). As mentioned earlier, appropriate formulations will greatly enhance the effectiveness of plant essential oils as insect repellents. Gerberg and Novak (2007) list 50 plants and the names of repellent products derived from them. Apparently, there are other plants and products used locally that have not been commercialized.

Studies on Plant-Based Repellents and Protectants in Thailand

A systematic laboratory and field research program is underway at the National Institute of Health, Ministry of Public Health, Thailand. Experimental and commercial plant products have been evaluated against mosquitoes, black flies, land leeches, and cockroaches. A synopsis of results from these studies during the past 7 years is presented below. Landing counts of blood-sucking organisms on human volunteers were used for field assessment.

Tests Against Mosquitoes

In the first study (Tawatsin et al 2001) tested volatile oils from plants against three mosquito species determining medium protection time. Essential oils from turmeric, citronella, and hairy basil gave essentially the same protection time (6-8 hrs) as deet for *Ae. aegypti*, *An. dirus*, and *Cx. quinquefasciatus*. In another field study, the extracts of citronella oil (10-13%), eucalyptus oil (15%), and tea tree oil (5%) provided protection (93-100%) for 4 hrs from night biting mosquitoes (*Culex* and *Mansonia* spp) (Thavara et al 2002). Again, the extent of protection and duration were similar to the standard chemical repellent deet.

In a comprehensive study (Tawatsin et al 2006a) extracted and formulated essential oils from 18 plant species and compared them for protection time with deet and IR 3535 against 4 species of mosquitoes. The chemical composition of the oils for each plant species was determined. The oil yield from the 18 plants ranged from 0.04 to 0.80%. The oils were extracted from leaves, flowers, or rhizomes, depending on the plant. The plant-based products afforded low to medium protection time (0.3 to 2.8 hrs) against *Ae. aegypti* as compared to deet (7.5 hrs) and IR 3535 (6.7 hrs). It seems that *Ae. aegypti* was not sensitive to these plant extracts. On the other hand, all plant extracts with the exception of *Litsia cubeba*, *Aglaia odorata*, *Myristica fragrans*, *Psidium guajava*, *Murraya paniculata*, and *Zingiber officinale*, provided 7+ hr protection against *Ae. albopictus*, equaling that of deet or IR3535. All 18 plant extracts gave high levels of protection time (7-8 hrs) against *An. dirus*, equaling that of deet or IR3535. Only 4 plant species, yielded around 6 hrs protection time against *Cx. quinquefasciatus*, the remaining 4 species yielded a protection time of 7-8 hrs, comparable to the time afforded by the two synthetic chemicals. The plants giving 7.8 to 8 hrs protection against this mosquito were *Piper nigrum* and *Cucurma longa*. It is possible that formulation manipulation might increase the activity and duration of

plants giving less protection using unformulated material. But this would not be necessary, as the number of active plants currently known is adequate.

A field study with 4 plant-based repellents was carried out against night-biting (*Culex* and *Mansonia*) and day-biting (*Ae aegypti* and *Ae albopictus*) mosquitoes (Tawatsin 2006b). The four plant oils: fingerroot (10%), guava (10%), turmeric (10%), a commercial product Repel Care 9.5% (turmeric oil 5% and *Eucalyptus citriodora* 4.5%) were compared with deet. All repellents provided 100% protection for 9 hrs. The same products tested on human volunteers against day-biting mosquitoes gave almost 100% protection for 5 hrs and provided 76-93% protection for 8 hrs. In these tests, the efficacy of the plant extracts was equal to that of deet against both day and night-biting mosquitoes.

Field Tests Against Black Flies

The four plant-based repellents tested against mosquitoes above (Tawatsin et al, 2006b) were tested on human volunteers against black flies (*Simulium nigrogilvum* 99% of the flies collected). All four plant-based repellents gave 100% protection from the bites of black flies for nine hrs, with protection decreasing from 90 to 93% after 10 and 11 hrs of application of the repellents. The extent and duration of protection were the same for the deet and the plant products.

Field Tests Against Land Leeches

Land leeches are severe pests of man and animals in national parks, wilderness, and wetlands. They could be quite a deterrent to outdoor activity and enjoyment of wilderness and open spaces. The same 7 plant-based repellents were tested on human volunteers against land leeches. The leeches were in the genus *Haemadipsa* (*Haemadipsidae*). We noted that the leeches on contact with repellent-treated surfaces shriveled and died within minutes. All four plant-based repellents and deet provided 100% protection from leeches for 8 hrs (Tawatsin et al 2006b). Not only the products were repellent to leeches but also highly toxic to them. The leeches were highly sensitive to plant-based repellents and these can be used as sprays for clearing leech infestations in trails and wilderness areas. More studies are needed on land leech control.

Field Tests Against Cockroaches

Seven plant-based repellents were tested against the cockroaches *Periplaneta americana*, *Blattella germanica*, and *Neostylopyga rhombifolia* (Thavara et al 2007) in the laboratory. All seven products reduced cockroach populations drastically. The last species is less susceptible than the other two species. Among the seven plants Kaffir lime (*Citrus hystrix*) was the most effective giving the highest repellency (87-100%), higher than that of naphthalene used as a standard. *C. hystrix* extracts were formulated (28%) for field tests. In 3 village tests in Pitsanulok province, treatments reduced cockroaches by 50-70% in 9-12 days post-treatment. Similarly, in two areas in Bangkok, Kaffir lime formulations (20%) treatment caused a 60-80% reduction in cockroaches. This level of reduction is equivalent to that of some insecticidal treatments. More detailed studies are warranted here.

Efficacy of Mosquito Coils

Mosquito coils are used in large quantities on a worldwide basis. In most of the coils, the active ingredients consist of synthetic pyrethroids. The use of plant materials in mosquito coils is attracting greater attention. In actual practice, many people in low-income communities burn

plant parts to achieve protection from biting insects. This practice serves the same purpose as a manufactured mosquito coil sold on the market.

There have been numerous studies on the efficacy of mosquito coils based on the active ingredients consisting of synthetic pyrethroids. There are very few studies published on the efficacy of mosquito coils containing plant materials. In one study Tawatsin et al 2002, incorporated plant materials into the coil and tested them in the field against night-biting mosquitoes. The plant material constituted 25% of the coil. A blank coil was run along with the test coils. In total 9 plants were evaluated. The mosquitoes collected were species of *Culex*, *Mansonia*, *Armigeres*, and *Anopheles*. All 9 plant materials tested individually provided protection of 50-71% in 3 hrs of testing. Mosquito coils containing leaves of citronella grass showed the highest efficacy while the coils with rhizomes of turmeric were the least effective. By comparison, coils containing synthetic pyrethroids showed 84-86% repellency. It seems that further studies are needed to increase the efficacy of plant-based coils through formulation technology.

CONCLUSIONS

In conclusion, it can be said that plant phytochemicals have multiple modes of action against insects. They can be used for the control of larval and adult mosquitoes. The essential oils extracted from plants offer a rich source of products that can be used in preparations to repel insects and protect humans from biting insects. They can be employed in mosquito coils or in aerosols and lotions properly formulate for personal protection. Further research is needed to find and develop more active principles and formulations.

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Figure 1. Placement of 200 L water storage earthen jars and plastic barrels used for testing microbial pest control agents and other insecticides against larvae of the mosquito *Aedes aegypti*, in Thailand.



Figure 2. Layout of microcosms (tubs-upper) and mesocosms (lower-ponds without vegetation and ponds with vegetation) for simulated field evaluation of larvicides against mosquitoes at University of California, Riverside, Aquatic and Vector Control Research Facility.