Chapter 5
Phytochemicals for Pest Management: Current Advances and Future Opportunities

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Abstract As with pharmaceuticals, a significant proportion of commercial pesticides are natural molecules or are derived from natural compounds. This review describes some of the past commercial successes of phytochemicals as pesticides by pesticide class as well as current work and future prospects for development of pesticides from plant-derived natural compounds. For example, two compounds isolated by assay-guided fractionation of the essential oil of American beautyberry (*Callicarpa americana* L.) (Verbenaceae), callicarpenal and intermediol, were found to have very potent insect repellent properties. An analysis of the number of new phytochemicals being discovered yearly and the relatively few bioassays for potential pesticidal activity that most of the known phytochemicals have been subjected to, indicates that this area still has a bright future. Furthermore, chemical modification of these compounds and their use to discover new modes of action greatly expand the scope for future work. In addition, the use of transgene technology holds great promise, not only to protect crops from pests, by imparting production or manipulation of production of pest management phytochemicals, but also for crop/weed allelopathy, as success in this effort would greatly decrease the most used form of synthetic pesticides, herbicides.

5.1 Introduction

A large fraction of phytochemical secondary compounds owe their existence to the coevolution of the producing plant with its biotic threats, such as herbivorous arthropods and mollusks, plant pathogens, and competing plant species. At least one of their functions in nature is to repel, inhibit, kill, or otherwise avoid damage from

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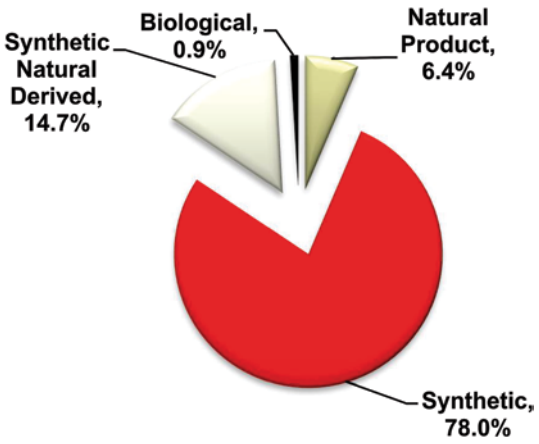

Fig. 5.1 Proportions of pesticides approved by the United States Environmental Protection Agency (USEPA) for use in the USA that are synthetic, natural compounds, derived from natural compounds, and biological agents [1]

these biotic hazards. Thus, these compounds are much more likely to have utility as a pesticide or as a molecular scaffold for pesticide design than compounds in synthetic libraries that have not been designed around compounds with known biological activity. Indeed, as with pharmaceuticals, a significant proportion of commercial pesticides are natural molecules or are derived from natural compounds [1]. From 1997–2010, about 20 % of the new pesticide active ingredients approved for use in the USA were natural products or natural product derivatives (Fig. 5.1). Some of the purely synthetic pesticides were discovered after the discovery of the molecular target site of natural inhibitors. These are not counted in the proportions in Fig. 5.1.

Historically, most of the natural products that have been useful as pesticides or as leads in pesticide discovery have come from plants. Yet, the pesticide industry seems to have focused recently on microbes as sources of leads. Our group spends most of its efforts on discovering potential pesticides from plants. We are also interested in the genetics and synthesis of these compounds as transgene technology allows us to impart production of these natural pesticides into crops.

Several aspects of natural products have reduced interest in them for pesticide discovery. The structural complexity of many natural products is too great for economically feasible production on a commercial scale. Much effort can be wasted in rediscovering known compounds [e.g., 2]. Obtaining enough of some phytochemicals for adequate evaluation can be time consuming and expensive. Sustainable harvest of botanical sources for a compound is often problematic. Natural does not equal nontoxic. We do not cover the mammalian toxicity of the compounds discussed in this short review. Except for materials used in traditional Chinese medicine (TCM), there is very little of this type of information available for the compounds that we mention. The half-lives of many natural compounds are often very short in the environment. This is an environmental advantage, but pesticides must persist sufficiently long to have their desired effects. Patenting can be more complex with natural products for several reasons. Legal complexities with countries or even populations of origin have grown, especially with plant species. This is one

reason that even the interest of pharmaceutical companies in phytochemical sources has waned [3]. Lastly, the physicochemical properties of natural compounds are often unsuitable for agricultural use.

Still, there are numerous advantages to phytochemicals in pesticide discovery. They are generally more environmentally benign. They are often sources of new molecular target sites, an aspect that is increasingly important as evolution of pesticide resistance to current modes of action increases [4]. They are often a source of novel chemical structures that differ from those more likely to be devised by traditional pesticide chemists. In some cases, pesticidal phytochemicals have evolved useful selectivity. In addition, modern technology has made discovery of these compounds and their biological activity simpler, faster, and less expensive than a few years ago. Finally, production of these compounds can be transferred from one plant species to another via transgene technology. Crops have sometimes been bred for phytochemical-based pest resistance in the past, but this process has been limited by the phytochemical makeup of related species with which the crop can interbreed.

In this chapter, we briefly describe some of the past commercial successes of phytochemicals as pesticides by pesticide class. Then, we discuss some of the promising work from our group within these pesticide classes.

5.2 Insecticides and Arthropod Repellents

Of all commercial pesticide classes, insecticides have the highest fraction of natural product or natural product-derived products [1]. Slightly more than 30 % of the conventional arthropod pesticides and repellent new active ingredients registered and approved for use by the United States Environmental Protection Agency (USEPA) from 1997 to 2010 were natural products or natural product-derived products [1]. The biggest classes of these compounds, the pyrethroids and the neonicotinoids, originated from phytochemicals. There are also quinoline- and pyrrole-derived commercial insecticides. A very important new class of insecticides are those that target the ryanodine target site in insects. Ryanodine is a compound from the plant *Ryania speciosa* that binds Ca channels of insect muscles [5, 6]. This phytochemical provided the clue for a much-needed new insecticide target site with which to fight evolution of insecticide resistance. Veratridine sulfate from the sabadilla lilly (*Schoenocaulon officinale*) is sold as an insecticide [7]. The scientific literature is full of reports of insecticidal phytochemicals that have not been widely or successfully commercialized.

Several formulations of plant extracts such as neem (*Azadirachta indica*) containing the insect-active compound azadirachtin are sold. There are numerous reviews of neem and azadirachtin as an insecticide [e.g., 8]. Many plant essential oils are available as bioinsecticides, a category of pesticides that does not require the stringent toxicological and environmental testing required of conventional pesticides [9].

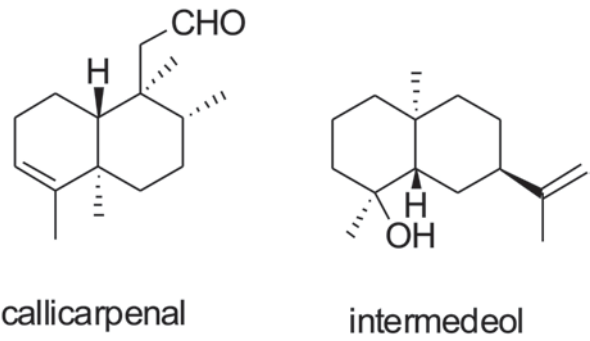

Fig. 5.2 Structures of two insect repellent compounds from *Callicarpa americana*

Our laboratory has focused on insect repellents. One of the more potent repellents is a constituent of the essential oil obtained from American beautyberry (*Callicarpa americana* L.) (Verbenaceae), a common shrub in the US southeast. In Mississippi, crushed leaves of *C. americana* were placed under the harnesses of draft animals as a traditional means to protect the animals from hematophagous insects [10, 11]. Specific identification of the compounds responsible for the mosquito (*Aedes aegypti*) biting deterrence in the leaves of this folk remedy was recently completed using a bioassay-directed fractionation approach. Ultimately, the study identified the compounds callicarpenal and intermediol as those responsible for the biting deterrence from the leaves and hence the folk remedy (Fig. 5.2). Both compounds were evaluated in laboratory bioassays for repellent activity against host-seeking nymphs of the blacklegged tick, *Ixodes scapularis*. Callicarpenal and intermediol, at 155 nmol/cm^2 of cloth, repelled 98 and 96% of *I. scapularis* nymphs, respectively. Dose–response tests with *I. scapularis* nymphs showed no difference in repellence among callicarpenal, intermediol, and *N, N*-diethyl-*m*-toluamide (DEET) [12]. Callicarpenal, at 155 nmol/cm^2 of cloth, repelled 100 and 53.3% of *I. scapularis* nymphs at 3 and 4 h, respectively. Both compounds also repel imported fire ants (*Solenopsis* spp.) [13].

More recently, two additional arthropod repellent folk remedies, breadfruit (*Artocarpus altilis*) and *Jatropha* sp., were investigated by Cantrell and colleagues [14, 15]. These two folk remedies are administered traditionally as spatial arthropod repellents by both burning seed-pressed oil in the case of *Jatropha* sp. and burning the dried male inflorescence of breadfruit.

A systematic bioassay-directed study of *Jatropha* sp. oil using adult *Aedes aegypti* females indicated that oleic, palmitic, linoleic, and stearic acids were all active at 25 nmol/cm^2 above a solvent control and were partially responsible for the activity of the oil itself. Evaluation of the triglycerides containing each of these fatty acids revealed that tripalmitin, tristearin, trilinolein, and triolein all demonstrated significant activity above a solvent control at 10 μg/cm^2, with tripalmitin the most active. This study was the first report on the insect repellent activity of triglycerides.

A similar approach to that used for *Jatropha* sp. identified capric, undecanoic, and lauric acids as primary deterrent constituents from the male inflorescence of

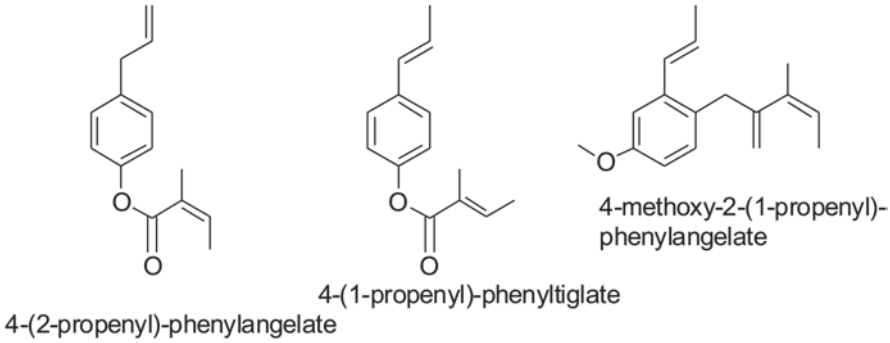

Fig. 5.3 Structures of three major phenylpropanoid constituents of *P. isaurica* oil that were bioassayed against *Liphaphis pseudobrassicae*

breadfruit. A synthetic mixture of fatty acids present in the most active fraction and individual fatty acids was significantly more active than DEET.

Essential oils and plant extracts from plants used in traditional medicines worldwide still continue to provide us with new and unique biological activity. Our group evaluated essential oils from 23 plant species comprising 14 genera and 4 plant families obtained from 26 locations in Turkey [16]. Essential oils obtained by Clevenger distillation were mixed with dimethyl sulfoxide and evaluated for insecticidal activity against adult turnip aphids (*Lipaphis pseudobrassicae* Davis). Aphids were quickly incapacitated by aliphatic aldehydes, phenols, and monocyclic terpenes contained in *Biflora* and *Satureja* species at concentrations as low as 0.3–1.0 mg/ml. *Pimpinella isaurica* essential oil and its three pure phenylpropanoids were tested at a single concentration of 10 mg/ml. Individually, the three major phenylpropanoids—4-(2-propenyl)-phenylangelate, 4-(1-propenyl)-phenyltiglate, and 4-methoxy-2-(1-propenyl)-phenylangelate (Fig. 5.3)—were not toxic to turnip aphids; however, when they were combined they killed aphids. The intact *P. isaurica* oil killed aphids faster than a mixture of the three phenylpropanoids.

We are studying TCM plants to find new agrochemicals with exceptionally low mammalian and environmental toxicity. *Angelica sinensis* (Apiaceae) is one such plant. Dong quai is the Chinese name for the roots of *A. sinensis*, which is a TCM treatment for gynecological disorders. Bioassay-guided fractionation of *A. sinensis* root extract led to the isolation of (*Z*)-ligustilide as an effective insect repellent [17]. This compound had previously been found as an insecticidal constituent of the essential oil of *Ligusticum mutellina* [18]. A mosquito biting deterrence assay showed that (*Z*)-ligustilide (Fig. 5.4) was more potent than the commercial standard DEET to *Ae. Aegypti* and *Anopheles stephensi.*

Essential oils of *Cupressus funebris*, *Juniperus communis*, and *J. chinensis* were evaluated for repellence against adult yellow fever mosquitoes, *Ae. Aegypti*; host-seeking lone star tick nymphs, *Amblyomma amerincanum*; the blacklegged tick, *I. scapularis*, and for toxicity against *Ae. aegypti* larvae and adults [19]. All oils were repellent to both species of ticks. The EC_{95} values of *C. funebris*, *J. communis,*

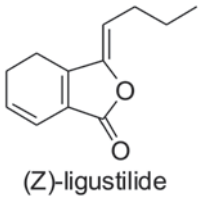

Fig. 5.4 Structure of a mosquito repellent compound from *Angelica sinensis*

and *J. chinensis* oils against *A. americanum* were 0.43, 0.51, and 0.92 mg oil/cm^2 filter paper, respectively, compared to 0.68 mg DEET/cm^2 filter paper. All *I. scapularis* nymphs were repelled by 0.10 mg oil/cm^2 filter paper of *C. funebris* oil. At 4 h after application, 0.83 mg oil/cm^2 filter paper, *C. funebris* and *J. chinensis* oils repelled ≥ 80 % of *A. americanum* nymphs. The oils of *C. funebris* and *J. chinensis* did not prevent female *Ae. aegypti* from biting at the highest dosage tested (1.50 mg/cm^2). However, the oil of *J. communis* had a minimum effective dosage (estimate of ED_{99}) for repellence of 0.029 ± 0.018 mg/cm^2; this oil was nearly as potent as DEET. The oil of *J. chinensis* showed a slight ability to kill *Ae. aegypti* larvae, at 80 and 100 % at 125 and 250 ppm, respectively.

5.3 Fungicides

Almost 30 % of the new fungicide active ingredient registrations in the USA from 1997 to 2010 were either natural products (11.4 %) or natural product-derived synthetic compounds (17.1 %) [1]. Several of the latter are derived from phytochemicals, such as benzothiazdiazole, acibenzolar-S-methyl, and the alkaloid sanguinarine (Fig. 5.5).

During the past 15 years, we have learned that biological activity of plant extracts against filamentous plant pathogenic fungi does not parallel activity against human pathogenic fungi. We have found that fungicidal chemistry from plants is more common in plants obtained from tropical, moist environments. Medicinal and aromatic plants used in traditional medicine often provide rich sources of novel activity against fungi. A discovery strategy based on this information has led to patenting of sampangine and novel cyclopentenedione compounds for the control of agriculturally important fungal plant pathogens [20, 21].

As part of our ongoing studies on the essential oils, we evaluated *Pimpinella* essential oils that are characterized by high concentrations of pseudoisoeugeneol-type phenylpropanoids. Trinorsesquiterpenes (geijerenes and azulenes) were also found to be characteristic constituents of *Pimpinella* oils [22]. Of the 22 isolated compounds during this investigation, two phenylpropanoids, 4-(3-methyloxiranyl) phenyl 2-methylbutyrate and epoxypseudoisoeugenyl 2-methylbutyrate, showed better antifungal activity than the trinorsesquiterpenes, 4-(6-methylbicyclo[4.1.0]

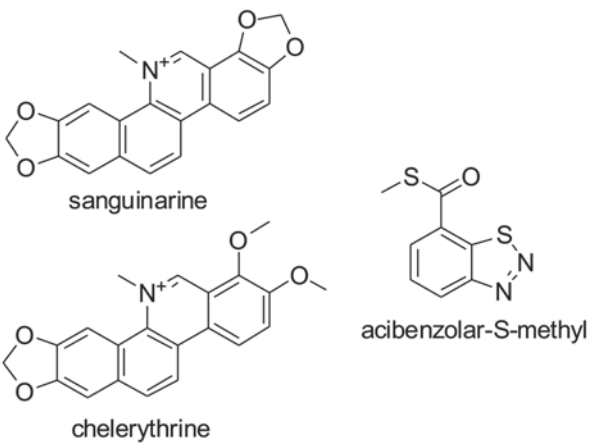

Fig. 5.5 Phytochemical-derived commercial fungicides

hept-2-en-7yl)butan-2-one (tragione) and dictamnol (Fig. 5.6), using direct bioautography against *Collectotrichum acutatum*, *C. fragariae*, and *C. gloesporioides*. The compounds were subsequently evaluated in a 96-well microtiter assay that showed that 4-(3-methyloxiranyl)phenyl 2-methylbutyrate and epoxypseudoisoeugenyl 2-methylbutyrate (Fig. 5.6) produced the most significant growth inhibition in *Phomopsis* spp., *Colletotrichum* spp., and *Botrytis cinerea* [23].

The peanut plant (*Arachis hypogaea* L.), when infected by a microbial pathogen, is capable of producing stilbene-derived compounds that are considered antifungal phytoalexins. In addition, health benefits of some stilbenes from peanuts, including resveratrol and pterostilbene, have been and are being established. Since peanut stilbenoids appear to play roles in plant defense mechanisms, they were evaluated for their effects on economically important plant pathogenic fungi of the genera *Colletotrichum*, *Botrytis*, *Fusarium*, and *Phomopsis*. The results of these studies reveal that peanut stilbenoids, as well as related natural and synthetic stilbene derivatives, display a diverse range of biological activities against fungal plant pathogens [24].

A preparative overpressure layer chromatography (OPLC) method was used for the separation of two new natural compounds, 4-hydroxy-5,6-dimethoxynaphthalene-2-carbaldehyde and 12,13-didehydro-20,29-dihydrobetulin, together with nine known compounds from the acetone extract of the roots of *Diospyros virginiana*. All isolated compounds were evaluated for their antifungal activities against *Colletotrichum fragariae*, *C. gloeosporioides*, *C. acutatum*, *Botrytis cinerea*, *Fusarium oxysporum*, *Phomopsis obscurans*, and *P. viticola* using an *in vitro* micro-dilution broth assay. The results indicated that the compounds methyl-juglone and isodiospyrin (Fig. 5.7) were highly active against *P. obscurans* at 30 μM with 97.0 and 81.4% growth inhibition, respectively, and moderate activity against *P. viticola* (54.3 and 36.6%, respectively). OPLC is a rapid and efficient method of exploiting bioactive natural products [25].

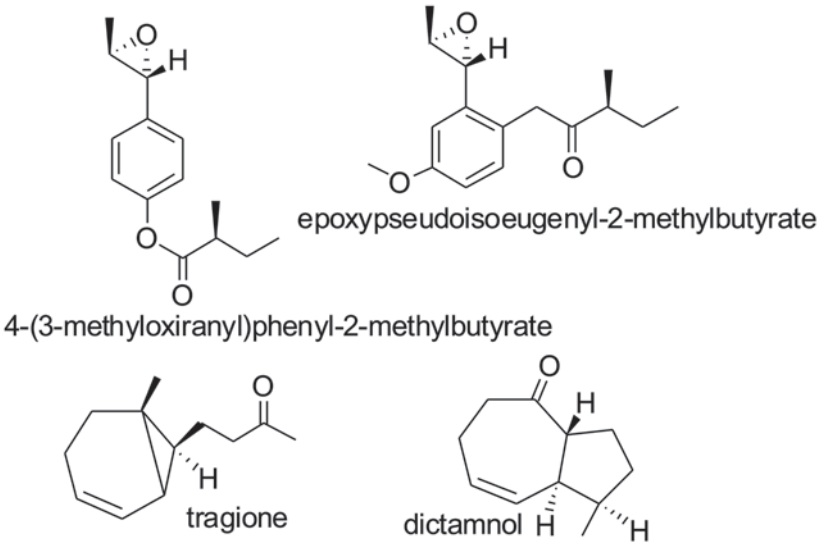

Fig. 5.6 Active antifungal compounds from the essential oil of *Pimpinella* species

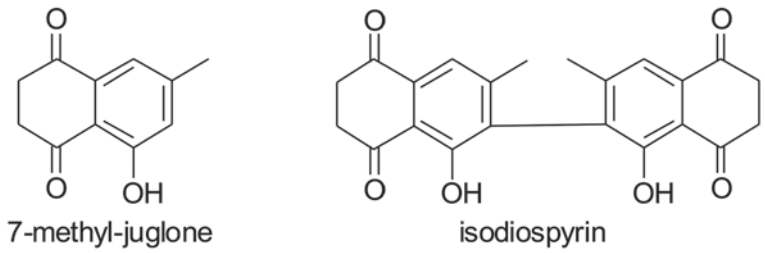

Fig. 5.7 Active antifungal compounds from the roots of *D. virginiana*

5.4 Molluscicides

There are relatively few effective, commercial molluscicides available. However, there are many reports of molluscicidal effects of crude extracts of plants and phytochemicals. For example, a crude butanol extract of *Phytolacca dodecandra* (endod) is effective against *Biomphalaria* snails [26]. Many of these are reviewed by Marston and Hostettmann [27].

Development of the berries of endod as a molluscicide to control schistosomiasis has been very successful in Ethiopia [28]. The active constituents have been isolated and identified as saponins [27]. *Lonicera nigra, Hedera helix, Cornus florida,* and *Asparagus curillus* are among some other plants that have been investigated

for molluscicidal saponins [27]. *H. helix* contains a hederagenine glycoside with an LC_{100} of 3 ppm against *B. glabrata* snails.

Our efforts are geared toward development of natural product-based molluscicides to control snails that are harmful for agricultural commodities such as channel catfish (*Ictalurus punctatus*), rice, taro, and orchids. Catfish is one of the main farm-raised fish in the USA. The ram's horn snail (*Planobdella trivolvis*) is an intermediate host for the trematode *Bolbophorus confusus* that was discovered to be a significant problem in 1999 in commercial channel catfish production ponds in the Mississippi Delta region [29, 30], and it has been reported from other states (Arkansas, Louisiana, Alabama, and California). These trematodes have a digenetic life cycle that involves two intermediate hosts, the snail and the catfish, and the American white pelican (*Pelecanus erythrorhynchos*). Catfish infested by the parasitic metacercariae develop cysts, have impaired growth, and are prone to other diseases that can weaken and kill the catfish. The annual economic loss to the catfish industry in the USA due to the trematode problem is estimated to be in millions of dollars. At present, there is no cure or treatment for infected fish. One practical approach to eradicate or control this problem is to interrupt the life cycle of the parasite by eliminating the snails, which are essential to the life cycle.

We have shown that vulgarone B (Fig. 5.8), isolated from the steam distillate of the aerial parts of the plant *Artemisia douglasiana* (Asteraceae), is active toward the snails with an LC_{50} of *ca* 24 μM [31]. The snails showed severe hemolysis associated with lethality when treated with vulgarone B. Channel catfish toxicity studies indicate an LC_{50} of *ca* 207 μM. Thus, vulgarone B may be an environmentally acceptable alternative for snail control in aquaculture when applied within the margin of safety [31]. 2*Z*,8*Z*-matricaria methyl ester (Fig. 5.8) isolated from *Erigeron speciosus* (Asteraceae) has also shown molluscicidal activity against *P. trivolvis* with a LC_{50} of 50 μM associated with marked hemolysis of the snail [32]. In laboratory experiments, Yucca extract at 10 ppm caused 100 % mortality of *P. trivolvis*, but ethanol extracts of *Phytolacca americana* (American poke weed) berries and *Lonicera nigra* (black-berried honeysuckle) were inactive (unpublished data). On the other hand, hederagenin 3-O-β-D-glucopyranoside (Fig. 5.8), isolated from English ivy (*H. helix*, an invasive plant in the Northwestern states, showed activity against *P. trivolvis* with an LC_{50} of 30 μM in laboratory studies (unpublished data). This compound has also shown activity against *B. glabrata* snails [27].

The golden apple snail (GAS), *Pomacea canaliculata* (Lamarck), is a major pest of rice in all rice-growing countries outside the USA, where it was either intentionally or accidentally introduced [33]. In the Philippines, the government promoted GAS production for human consumption [34, 35]. However, the demand dropped because GAS was found to transfer the rat lungworm parasite (*Angiostrongylus cantonensis*) to humans if undercooked GAS was consumed. Thus, snail farmers growing GAS abandoned their cultures, and the snails were disposed of without precautions. GAS soon invaded the rice fields, where it found an ideal habitat and abundant food supply. The economic losses were estimated to be up to $ 1.2 billion by 2003 [36]. GAS is also a problem in taro plantations in Hawaii, where $CuSO_4$ is currently used to control the snail population [37].

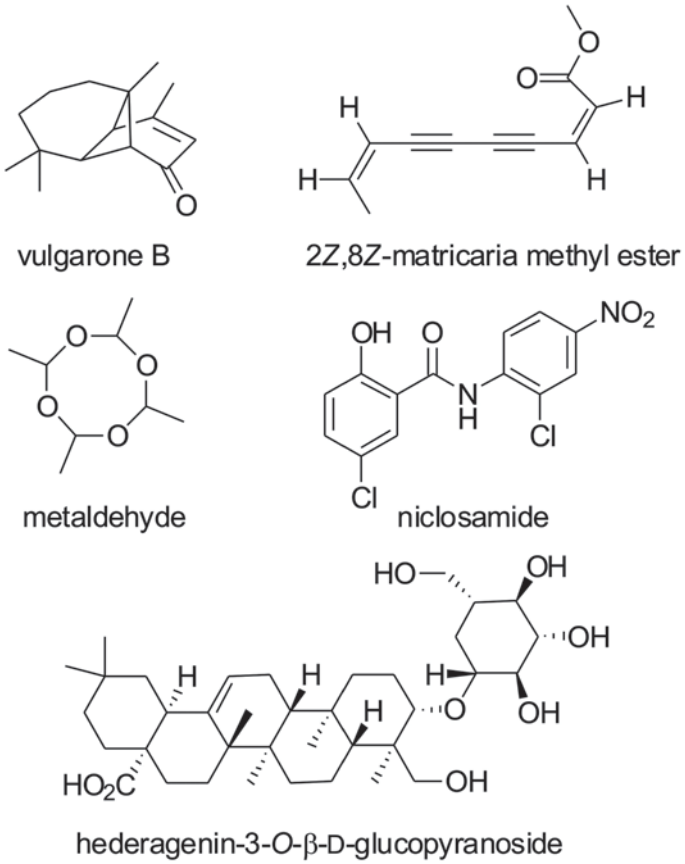

Fig. 5.8 Molluscicidal compounds mentioned in the text

Integrated management methods are recommended for GAS, but many farmers depend on commercially available synthetic molluscicides (niclosamide and metaldehyde—Fig. 5.8). There are numerous cases of poisoning caused by metaldehyde [38, 39]. Therefore, cost-effective, target-specific, and environmentally friendly molluscicides are needed, due to the economic burden and undesirable effects of currently available commercial molluscicides. Vulgarone B is a potential molluscicide with an LC_{50} value of about 30 μM at 24 h for GAS [40]. In the same bioassay, the standard commercial molluscicide, metaldehyde, also had an LC_{50} of about 30 μM. This corresponds to about 6.5 and 4.4 mg/L of the vulgarone B and metaldehyde, respectively. The concentrations needed for 100% mortality at 24 h were about 75 and 200 μM, respectively, for vulgarone B and metaldehyde. In practical terms, a rice farmer who uses about 250 liters of water for spraying one hectare will require 4.8 g of pure vulgarone B for GAS control [40]. Vulgarone B did not cause further mortality at 48–96 h after treatment, unlike the observed increased mortality

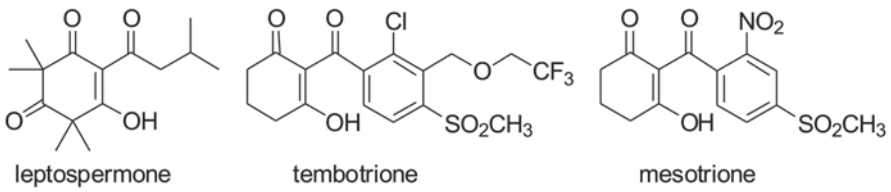

Fig. 5.9 Structures of the natural triketone leptospermone and two synthetic analog that are sold as commercial herbicides

with time with metaldehyde. This indicates that the vulgarone B is fast acting, unlike metaldehyde.

There was no phytotoxicity 10 days after treatment to 14-day-old rice plants at concentrations of vulgarone B that cause complete or nearly complete mortality of GAS. However, when incorporated into agar growth medium, pronounced chlorosis occurred after 14 days of growth. Therefore, vulgarone B should be used after the germination of rice seeds. Field and laboratory experiments have also shown the potential of vulgarone B as a molluscicide in taro paddies in Hawaii (unpublished data).

5.5 Herbicides and Algicides

5.5.1 Herbicides

Only about 8 % of the new active ingredient registrations for conventional herbicides from 1997 to 2010 in the USA have been synthetic, natural product-derived compounds [1]. These have all been triketones that were partially inspired by the plant allelochemical leptospermone (Fig. 5.9) [41]. The discovery and development of these herbicides resulted from a convergence between astute chemical ecology observations made by Reed Gray of the Western Research Center in California (Stauffer Chemical at the time) and independent chemical synthesis efforts in the same laboratory. In 1977, Gray observed that the bottlebrush plant (*Callistemon citrinus*) appeared to repress the growth of plants in its surroundings. Crude extracts from this plant caused the bleaching of grass weeds. He identified the active component as leptospermone, a natural triketone structure with no known biological activity, although it had been reported in a number of Australasian shrubs several years earlier [42]. Leptospermone was moderately active in greenhouse tests, controlling mostly small-seeded grass weeds. This natural product and a small number of synthetic structural analogs were patented as herbicides in 1980 [43]. A few years later, a separate group at the Western Research Center was generating analogs of the cyclohexanedione herbicide sethoxydim, an inhibitor or acetyl-coenzyme-A carboxylase. Some of the second-generation herbicidal derivatives with a dimedone backbone caused bleaching symptoms similar to that from leptospermone. Combination of the syncarpic

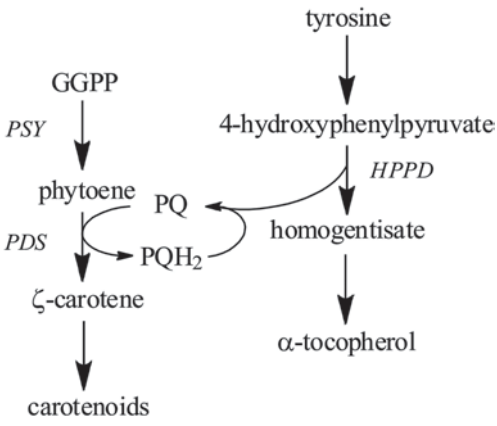

Fig. 5.10 Role of HPPD in carotenoid synthesis. *PSY* phytoene synthase, *PDS* phytoene desaturase *HPPD p*-hydroxyphenylpyruvate dioxygenase, *PQ* plastoquinone

acid of leptospermone to this chemistry ultimately served as the basis for the development of the triketone synthetic herbicides (Fig. 5.9) [44].

β-Triketones (e.g., leptospermone, flavesone, agglomerone, tasmanone, papuanone, and grandiflorone) are common in many Australasian woody plant genera (e.g., *Leptospermum*, *Eucalyptus*, *Callistemon*, *Xanthostemon*, *Backhousia*, *Calytrix*, *Baeckea*, *Melaceuca*, and *Corymbia*) [42, 45, 46]. On average, steam-distilled manuka oil accounts for 0.3 % of the dry weight of *L. scoparium* [47]. However, the amount of β-triketone present in these oils varies widely across New Zealand. Some chemotypes contain as little as 0.1 % triketone while others can accumulate up to 33 % [47]. More than 200 individual manuka plants from 87 sites throughout New Zealand were analyzed and the triketone-rich chemotypes were almost exclusively limited to the East Cape region [47]. Cluster analysis of the composition of these samples identified 11 geographical chemotypes distinguished by different levels of monoterpenes and sesquiterpenes, methyl cinnamate, and triketones. The reason for this chemotaxonomic geographical distribution is not well understood.

Little is known about the chemotypes outside of New Zealand, though clearly the *Callistemon* samples studied by Gray in 1977 contained sufficient amounts of leptospermone for isolation and purification. Interestingly, this adds to the serendipity of the discovery of its herbicidal activity since the analysis of a number of *Callistemon* species either did not report the presence of detectable amounts of triketones [42] or only trace amounts [48]. The primary constituent of the essential oil of this genus is the monoterpene 1,8-cineole [49].

Synthetic β-triketone herbicides (e.g., sulcotrione and mesotrione) cause bleaching of newly emerging tissues [50]. This symptom was traditionally associated with inhibitors of phytoene desaturase, but triketone herbicides do not inhibit this enzyme. It was later found that these herbicides inhibit *p*-hydroxyphenylpyruvate dioxygenase (HPPD), a key enzyme involved in the biosynthesis of prenyl quinones and tocopherols [50]. Plastoquinone (a prenylquinone) is an essential cofactor for phytoene desaturase [51] (Fig. 5.10). In the absence of plastoquinone, phytoene desaturase activity is reduced which results in the bleaching of young foliage and accumulation

of phytoene customarily associated with phytoene desaturase inhibitors [52]. Chlorophyll levels are also affected because the photosynthetic apparatus is no longer protected from reactive oxygen species generated under high light intensity.

Gray observed that leptospermone caused bleaching of plant tissues [43]. Work with the bioactive components of manuka oil demonstrated that some natural β-triketones also inhibit plant HPPD [53]. Most of the activity of manuka oil was attributed to leptospermone because it was the most abundant triketone in the examined oil. However, grandiflorone, a minor constituent that has a more lipophilic side chain, was a much more potent inhibitor of HPPD. Conversely, the short methyl side chain of flavesone nullified the activity of this triketone. The important role of the lipophilicity of the side chain was confirmed by a structure–activity study using a series of natural and synthetic leptospermone analogs [54].

Greenhouse experiments using agricultural soils showed that manuka oil was active both when applied to the foliage and to the soil surface. While most essential oils have little to no soil activity, preemergence application of manuka oil controlled the growth of large crabgrass at a rate of 3 L/ha. The soil activity of manuka oil is due in part to the relatively slow dissipation of leptospermone, which remained active in soil for at least 2 weeks [55].

Triketones and other phytotoxic natural products are often produced and stored in specialized structures, which may serve in part as a mechanism to prevent autotoxic effects [56, 57]. In the leaves of members of the Myrtaceae family, which encompasses most of the known herbicidal triketone-producing species, specialized secretory glands consisting of roughly spherical secretion-filled spaces lined with specialized glandular cells are found (Fig. 5.11). In the genus *Leptospermum*, the gland is typically covered by two to four cells, which have thin, straight walls and are generally of the same approximate size. These cells are encircled by 5–14 unspecialized epidermal cells in a spiral orientation. Although there has been a debate on the method of glandular cavity production, evidence suggests that in the Myrtaceae this formation occurs schizogenously. Schizogenous formation proceeds by the division of single cells within the epidermis or mesophyll layer with the oil cavity forming as an intracellular space [58]. The schizogenous cavity is lined with a single layer of four to six epithelial cells that are thought to be responsible for the production of the volatile oils stored within the cavity [59, 60]. In *Melaleuca* species, the cells lining the immature gland cavity were shown to be metabolically active by osmophilic staining, supporting their role in oil synthesis. In this species, the mature glands contain highly vacuolated epithelial cells lining the gland cavity that are unlikely to lead to continued oil synthesis and accumulation [60]. It has also been demonstrated that essential oil has the potential for release through the modified epidermal cells covering the gland, although the physiological and ecological aspects of this phenomena remain to be investigated.

The *in vitro* chemical synthesis of leptospermone and many other triketones has been well studied [61], but much work remains to unravel the *in vivo* biosynthesis of these compounds. Although an *in planta* biosynthetic route has yet to be established, a hypothetical pathway can be proposed based on the structure of the final compounds (Fig. 5.11). In a series of conversions analogous to the well-examined

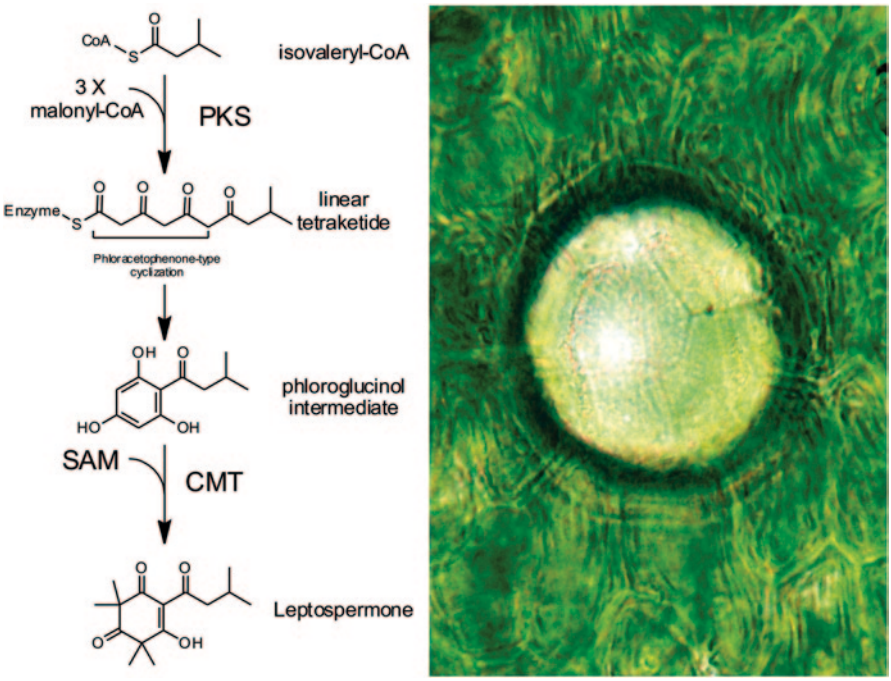

Fig. 5.11 Micrograph of a representative *Leptospermum scoparium* (manuka) schizogenous gland, and a proposed biosynthetic pathway to leptospermone

chalcone synthase enzyme [62], a type III polyketide synthase (PKS) sequentially condenses three malonyl CoA molecules into a polyketide chain extending from an isovaleryl CoA starter molecule. The enzyme subsequently cyclizes the linear tetraketide intermediate via a Claisen-type condensation to generate a phloroglucinol intermediate. A PKS enzyme, valeropenone synthase (VPS), with this activity has been purified to homogeneity and biochemically characterized from *Humulus lupulus* L. (hops) cone glandular hairs [63]. VPS is thought to be involved in the production of the beer flavoring iso-acids of hops which have been shown to contain a β,β-triketone moiety [64]. Subsequently, a gene for this enzyme has been identified and characterized [65]. Efforts are currently underway to isolate and characterize enzymes homologous to VPS from *Leptospermum scoparium* as an initial effort to characterize the leptospermone biosynthetic pathway.

After the production of the phloroglucinol intermediate, the compound would be proposed to undergo spontaneous keto–enol tautomerization, and subsequently to undergo methylation by an as-of-yet unidentified C-methyltransferase (CMT). Early work with methionine-methyl-C^{14} labeled adult *Dryopteris marginalis* ferns demonstrated that the C- and O-methyl substituents of isolated phloroglucinols were derived from methionine [66]. If these findings are consistent with leptospermone, the biosynthetic methyltransferases are likely to be similar to S-adenosylmethionine using CMTs identified in other species.

Table 5.1 Bioassay evaluation results of 9,10-anthraquinone and the analog anthraquinone-59 against the cyanobacterium *Planktothrix perornata* and the green alga *Selenastrum capricornutum*.

Compound	LOEC[a]	LCIC[b]	IC50[c]
P. perornata			
9,10-Anthraquinone	100	100	nd
Anthraquinone-59	10	100	6.3
S. capricornutum			
9,10-Anthraquinone	>100,000	>100,000	nd
Anthraquinone-59	10,000	100,000	5,623

nd not determined
[a] Lowest-observed-effect concentration (nM)
[b] Lowest-complete-inhibition concentration (nM)
[c] 50% inhibition concentration (nM)

5.5.2 Algicides

Some blue-green algae (cyanobacteria) synthesize secondary compounds that can impart unsavory flavors to pond-cultured fish. Currently, aquaculturists are using copper sulfate and chelates, as well as diuron, a synthetic herbicide, to kill cyanobacteria. However, these products generally kill all algae, including beneficial eukaryotic species that are better oxygenators of the water and are not associated with off-flavor compound production. Our laboratory has had a research program to discover natural product-based compounds that are selective for killing cyanobacteria. Among thousands of plant crude extracts and pure compounds tested in the laboratory, 9,10-anthraquinone was one of the most promising compounds [67], with about a thousand times greater activity against a noxious cyanobacterium than on a green alga (Table 5.1). However, the physicochemical properties of this compound were not suitable for use in aquaculture ponds, so the molecule was modified to impart water solubility, while retaining its biological activity [68]. The best modification was the analog anthraquinone-59, which has been patented to control cyanobacteria (Fig. 5.12) [69].

5.6 Transgenic Approaches to Phytotochemical-Based Pest Resistance

All phytochemicals are produced by enzymes encoded by plant genes. With transgenic approaches, we can impart production of new pest management compounds into crops or manipulate the production of such compounds that already exist in crops. Our laboratory has been interested in using these methods to alter the production of sorgoleone in *Sorghum* spp. and other crop species such as rice.

Sorgoleone, a major component of the hydrophobic root exudate of sorghum [*Sorghum bicolor* (L.) Moench], represents one of the most extensively studied

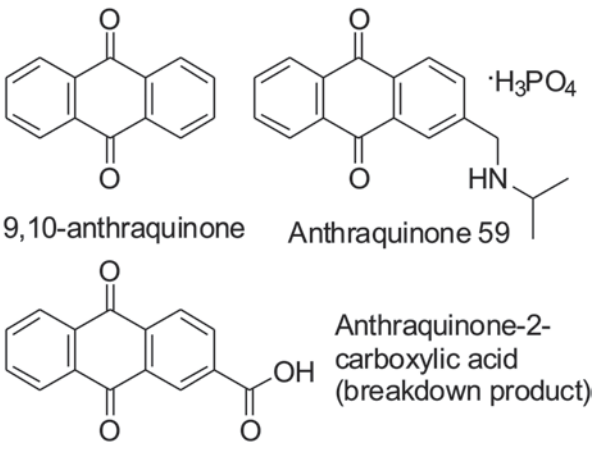

Fig. 5.12 Structures of 9,10-anthraquinone, patented water-soluble analog anthraquinone-59, and the major breakdown product of anthraquinone-59

allelochemicals. In contrast to phenolic lipid-type compounds found in other plants, sorgoleone is an uncommon lipid benzoquinone possessing significant herbicidal activity and is produced exclusively by *Sorghum* spp. [70]. Sorgoleone suppresses the growth of a large number of plant species and appears to be most active on small-seeded plants [71–76]. Additionally, sorgoleone has a relatively long half-life in soil and has been reported to inhibit multiple cellular targets, including plastoquinone/photosystem II, *p*-hydroxyphenylpyruvate dioxygenase, and mitochondrial respiration [77–83]. Thus, evolution of resistance to sorgoleone would presumably be less likely in comparison with a phytotoxin possessing a less-complex mode of action. For the above-mentioned reasons, significant interest has been generated in the potential development of sorgoleone as a natural product-based herbicide.

The sorgoleone biosynthetic pathway appears to exclusively or predominantly reside in root hair cells, with the end product sorgoleone comprising a major portion of the hydrophobic exudate material released into the rhizosphere [80, 84–87]. The biosynthetic pathway of sorgoleone has been previously investigated, and these studies have shown that the aromatic moiety within sorgoleone's structure is synthesized via iterative condensation reactions catalyzed by alkylresorcinol synthase (ARS) enzymes utilizing a C16:3 fatty acyl-CoA precursor [88, 89]. The resulting 5-pentadecatrienyl resorcinol intermediate (produced by ARS) is next methylated by a root hair-specific *O*-methyltransferase [90] and subsequently dihydroxylated by a P450 monooxygenase to yield sorgoleone (Fig. 5.13).

A strategy for the cloning and functional characterization of genes and enzymes involved in sorgoleone biosynthesis has been pursued, involving the screening of candidate gene sequences derived from a root hair-specific *S. bicolor* expressed sequence tag (EST) database [90]. Using this approach, two root hair-specific fatty acid destaurase enzymes were identified, designated SbDES2 and SbDES3, which are likely responsible for the generation of the C16:3 fatty acyl-CoA precursor by consecutive desaturation reactions, starting with palmitoleoyl-CoA (Fig. 5.13) [86]. Heterologous co-expression of *SbDES2* and *SbDES3* in yeast cells resulted in the

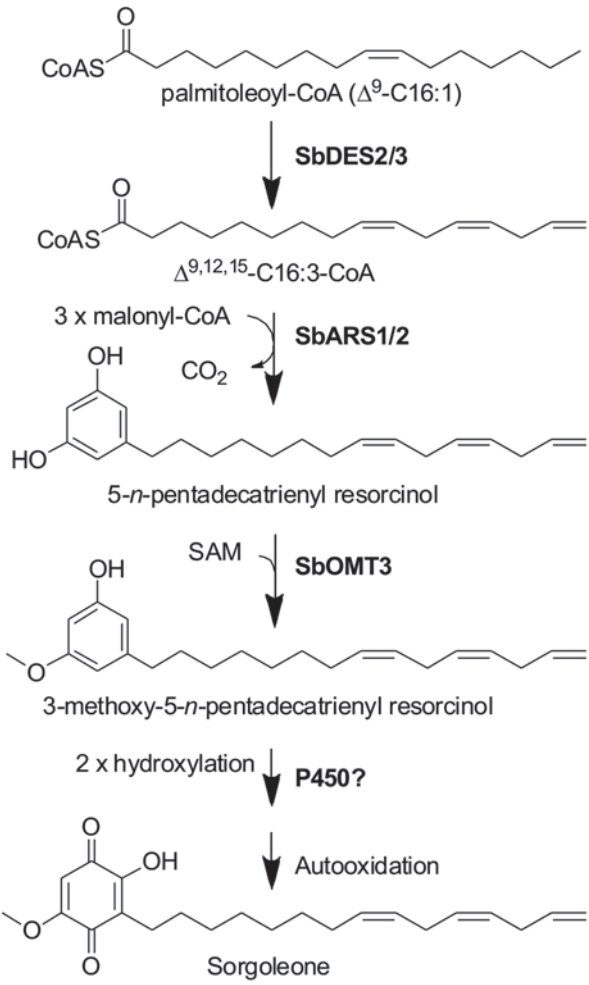

Fig. 5.13 Sorgoleone biosynthetic pathway

production of hexadecatrienoic acid (16:3$\Delta^{9,12,15}$; Fig. 5.13). Co-expression of the two enzymes was required, given that SbDES2 was found to convert endogenous palmitoleic acid (16:1Δ^{9}) to hexadecadienoic acid (16:2$\Delta^{9,12}$), thus providing a substrate that recombinant SbDES3 was capable of converting into hexadecatrienoic acid in vivo [86].

Two root hair-specific ARSs (designated SbARS1 and SbARS2), representing the first such enzymes described from higher plants, have also been characterized and linked to the biosynthesis of sorgoleone [87]. The recombinant SbARS1 and SbARS2 enzymes have both been shown to accept a variety of fatty acyl-CoA starter units using *in vitro* enzymatic assays, including the physiological substrate hexadecatrienyl-CoA (C16:3$\Delta^{9,12,15}$-CoA; Fig. 5.13). The 5-pentadecatrienyl resorcinol intermediate produced by SbARS1 and SbARS2 *in planta* is likely next methylated

by a root hair-specific *O*-methyltransferase designated SbOMT3 [90]. Recombinant SbOMT3 was found by *in vitro* enzymatic assays to exhibit a marked preference for alkylresorcinolic substrates among a panel of phenolic compounds tested [90]. As mentioned, the final steps in the sorgoleone biosynthetic pathway involve the dihydroxylation of the 3-methoxy-5-*n*-pentadecatrienyl resorcinol intermediate (Fig. 5.13), and work on several candidate root hair-specific P450 monooxygenase sequences identified within the root hair ESTs is ongoing (Z. Pan, unpublished).

Initial efforts to alter sorgoleone levels in transgenic *S. bicolor* events have been successfully performed with RNA interference (RNAi), using SbARS1/2 3′ coding and contiguous untranslated region (UTR) sequences incorporated within hairpin RNA (hpRNA)-forming binary transformation vectors [87]. For these experiments, segregating T_1 populations representing multiple independent transgenics were first analyzed by quantitative real time polymerase chain reaction (qRT-PCR) for the presence of the transgene-derived hpRNA and individual seedlings were scored as hpRNA "+" or "–". Pooled samples from hpRNA "+" or "–" individuals were analyzed by gas chromatography–mass spectrometry (GC-MS), and sorgoleone levels were found to be reduced to unquantifiable levels in the hpRNA-expressing transformants (Fig. 5.14; see also [87]). The work performed to date on sorgoleone biosynthesis, involving heterologous expression experiments and RNAi in transgenic *S. bicolor*, has provided compelling evidence that SbDES2, SbDES3, SbOMT3, SbARS1, and SbARS2 participate in sorgoleone biosynthesis *in vivo*. These sequences should provide a powerful new toolbox for the manipulation of sorgoleone biosynthesis in *S. bicolor* and the potential transfer of this trait to other crop species.

5.7 Summary

We have provided examples of natural product-based pesticides that are now commercially successful, as well as a few examples of the many natural compounds that we have studied which are active against pests. Our group's research is but a small sampling of the extensive, international effort to discover natural product-based pest management products. Some have argued that we have reached diminishing returns with this approach, but an analysis of the number of new phytochemicals being discovered yearly and the relatively few bioassays for potential pesticidal activity that most of the known phytochemicals have been subjected to, indicates that this is not the case. Furthermore, chemical modification of these compounds and their use to discover new modes of action greatly expands the scope for future work.

It is even clearer that we have only scratched the surface of the possibilities of using transgene technology to protect crops from pests by imparting production or manipulation of production of pest management phytochemicals. We are especially interested in using this method for crop/weed allelopathy [91, 92], as success in this effort would greatly decrease the most used form of synthetic pesticides, herbicides. Furthermore, these efforts provide much-needed experimental verification of the plant/plant allelopathy role of putative allelochemicals. For example, strong support

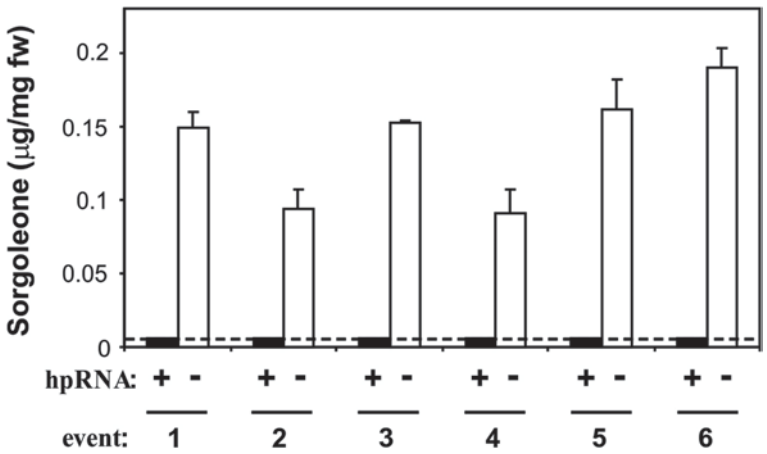

Fig. 5.14 Sorgoleone levels in different *S. bicolor* RNAi transgenic events. Sorgoleone levels were determined by GC-MS analysis of root exudates prepared from pooled hpRNA "+" and hpRNAi "–" seedlings. Data are expressed as mean ± SD

for the allelopathic role of momilactones in rice was recently generated by using gene knockouts to reduce expression of two genes encoding enzymes in the momilactone pathway [93]. These early efforts are promising and should stimulate further research along these lines. Lastly, there can be unexpected spin-off from such research. For example, members of our group have used an *O*-methyltransferase gene of the sorgoleone pathway [90] with a peanut stilbene synthase gene to impart the production of pterostilbene in plants [94]. Pterostilbene is a phytochemical fungicide [e.g., 95], so this technology could be used to improve fungal pathogen resistance in crops. Additional potential benefits of such a transgenic crop are the health-promoting properties of pterostilbene [e.g., 96–98]. Such creative use of the genetics of phytochemical production bodes well for the future of meshing phytochemistry with transgene technology.

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