

# BIOINSECTICIDES AS FUTURE MAINSTREAM PEST CONTROL AGENTS: OPPORTUNITIES AND CHALLENGES

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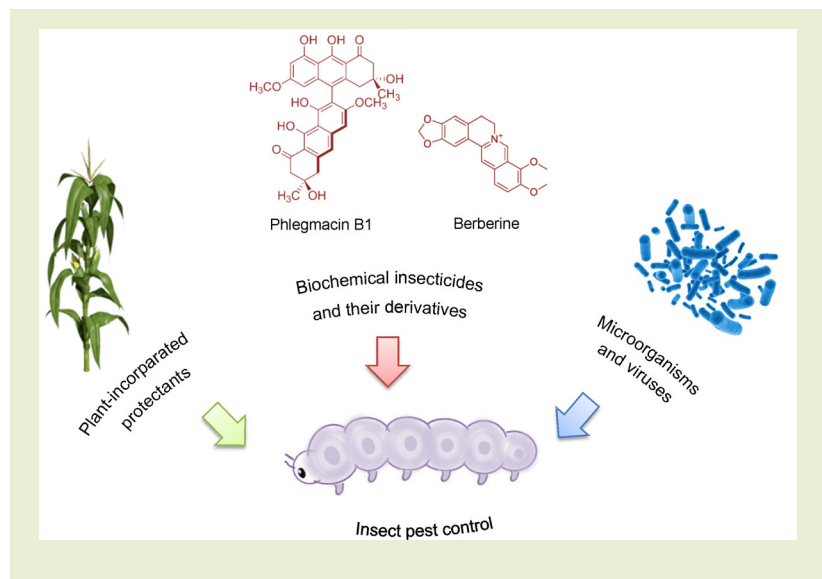
## KEYWORDS

biochemical insecticide, bioinsecticide, microbial bioinsecticides, plant-incorporated protectant, RNA insecticide

## HIGHLIGHTS

- Wide use of botanical insecticides is limited by the availability of certain plants.
- Studies are needed to improve RNAi efficiency and to assess their safety risk.
- Microbial insecticides are promising, but they only control a narrow range of pests.
- Multitarget approach should be a promising strategy in future pest control.
- Nanoformulation could enhance stability and control the release of bioinsecticides.

## GRAPHICAL ABSTRACT



## ABSTRACT

Bioinsecticides are naturally-occurring substances from different sources that control insect pests. Ideal bioinsecticides should have low toxicity to non-target organisms. They should also be easily degraded in sewage treatment works and natural environments, highly effective in small quantities and affect target pests only. Public concerns about possible side-effects of synthetic pesticides have accelerated bioinsecticide research and development. However, to develop bioinsecticides into mainstream products, their high production costs, short shelf-life and often uncertain modes of action need to be considered. This review summarizes current progress on bioinsecticides which are categorized as

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biochemical insecticides and their derivatives, plant-incorporated protectants, and microbial bioinsecticides. The current constraints that prevent bioinsecticides from being widely used are discussed and future research directions are proposed.

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## 1 INTRODUCTION

Insecticides are agents used to control insects by killing them or by otherwise preventing their undesirable or destructive behavior. Wider use of insecticides guarantees an adequate food supply for humans especially in times of increasing soil losses due to drought and salinization. However, many synthetic insecticides have turned out to be toxic or carcinogenic contaminants in the food chain and the environment. There is increasing public pressure for safer insecticides. Bioinsecticides are a promising choice because they are believed to have a low environmental risk, negligible mammalian toxicity, high species-selectivity (i.e., safety to non-target organisms such as honeybees), low risk of development of resistance and a low groundwater vulnerability<sup>[1]</sup>. The size of the global bioinsecticide market is projected to grow from an estimated value of 2.2 billion USD in 2020 to reach 4.6 billion USD by 2025<sup>[2]</sup>, making the development and production of bioinsecticides a very rewarding field.

The definition of bioinsecticide is inconsistent from country to country because of variation in conclusions on the concept of safety<sup>[3]</sup>. Terms frequently used as synonyms of the term bioinsecticide include biological insecticide, biorational insecticide, biopesticide and biocontrol agent. Following the definition of biopesticide by the United States Environmental Protection Agency (US-EPA), bioinsecticides can be defined as naturally-occurring substances from microbes, plants or animals that control pests (biochemical insecticides and derivatives), insecticidal substances produced by plants containing added genetic material (plant-incorporated protectants, PIPs), and microorganisms and viruses that control pests (microbial bioinsecticides). The inconsistent definition of bioinsecticide may cause problems and delays throughout the authorization process and drastically impede the rapid implementation of bioinsecticides. The term bioinsecticide here is used to encompass biological insecticide, biorational insecticide, biopesticide and biocontrol agents. In this review the advantages and disadvantages of applying bioinsecticides are summarized and discussed.

## 2 BIOCHEMICAL INSECTICIDES AND THEIR DERIVATIVES

The US-EPA defines biochemical pesticides as naturally-occurring substances that control pests and include pheromones, attractants and repellents but exclude many botanical insecticides that act by toxic mechanisms. Here we do not exclude these agents as they are accepted as biochemical insecticides in some other countries.

### 2.1 Biochemical insecticides produced by animals

Animal-produced bioinsecticides usually refer to hormones, semiochemicals and animal toxins, most of them are chemically synthesized in biomimetic approaches (Table 1).

Insect hormones control a wide range of physiological processes and orchestrate the development of insects. The treatment of insects with ecdysteroids or juvenile hormones (JH) that control molting and metamorphosis is known to disrupt development and finally kill insects. These insect hormones hence possess insecticidal activity and therefore are potential bioinsecticides but they are chemically unstable. Some stable analogs binding to the corresponding hormone receptors have been chemically synthesized for use as insecticides. These include 20-hydroxyecdysone (20E) agonists (e.g., tebufenozide, methoxyfenozide and halofenozide) and JH analogs (e.g., fenoxycarb, pyriproxifen, hydroprene and methoprene)<sup>[4]</sup>. Together with chitin synthesis inhibitors they are also known as insect growth regulators<sup>[5]</sup>. These compounds have been intensively used over recent decades and insect pests have developed resistance in many cases<sup>[4]</sup>. Advances in structural biology have led to detailed insights into the interactions between hormones and their receptors (in particular 20E and JH) and novel chemicals can be designed to mimic hormones through novel modes of action to overcome resistance.

Semiochemicals include sex pheromones, aggregation

Table 1 Type, target and application of different bioinsecticides

Bioinsecticide	Type	Target	Application	References
<b>Biochemical pesticides</b>				
Animal	Animal hormones	Juvenile hormone receptor	These hormones are chemically unstable, thus their agonists and antagonist are widely used as insecticides	[4,5]
	Semiochemicals	Ecdysone receptor	They are applied to suppress insect pests using strategies such as attract-and-kill, mass trapping, mating disruption, monitoring and push-pull	[6–10]
Microbial	Animal toxins	Postsynaptic receptors	They are mostly used as PIPs generating transgenic plants or for viral vector construction	[11]
	Chemicals	Insect voltage-gated sodium channels	They are widely applied as insecticides. The high resistance by the pests has been developed, and toxicity to humans and animals has been reported	[12]
Botanical insecticides	Chemicals	Glutamate-gated Cl <sup>-</sup> channels	They are widely applied as insecticides. The high resistance by the pests has been developed, and toxicity to humans and animals has been reported	[13,14]
	Microbial toxins	Nicotinic acetylcholine receptor	It could be developed as new insecticide	[15]
Botanical insecticides	Microbial toxins	L-glutamate-gated chloride channels	They have been sprayed to control mosquito larvae in breeding areas. More frequently, they have been introduced into transgenic crops or sold with live spores to control insect pests	[16–18]
	Essential oils	Receptor in the insect midgut cells	They have been widely used as bioinsecticides. However, many essential oils are restricted to local, indigenous use because of their lack of widespread cultivation	[19–22]
Botanical insecticides	Pyrethrins	Multiple targets such as P450 cytochromes, octopamine receptors	It has been widely used in agricultural pest control, structural pest control and for public health	[23]
	Azadirachtin	Voltage-gated sodium channels	It has been widely used for decades against insect pest	[24–26]
Botanical insecticides	Rotenone	Insect growth regulator	It has been used as an insecticide for more than 150 years, but fallen out of favor in most industrialized countries because of mammalian toxicity	[14]
	Plant alkaloids	Site I respiration in mitochondria	It has been used for many years as a fumigant for the control of many insects. But it is very toxic to humans by inhalation or skin contact	[27]
Botanical insecticides	Plant alkaloids	Cholinergic acetylcholine nicotinic receptor	It has limited use as insecticides because they are moderately toxic to mammals, but very toxic to fish	[28]
	Plant alkaloids	Calcium channels in the sarcoplasmic reticulum		

(Continued)

Bioinsecticide	Type	Sabadilla	Target	Application	References
Plant-Incorporated Protectants					
Insecticidal proteins-based PIPs	Bt toxins	–	Voltage-sensitive sodium channels	It is used for the control of thrips on citrus, avocados, and mangos	[28]
RNAi-based PIPs	–	–	Receptor in the insect midgut cells	Bt genes have been approved for commercialized cultivation in most of the major grain and economic crops. Other insecticidal proteins have also been evaluated as potential PIPs	[29–31]
Microbial bioinsecticides			Multiple targets	RNAi-based PIPs are highly specific and provide an environmentally friendly method to control insect pests. Public resentment toward genetically modified plants generally limits their acceptance	[32,33]
Bacterial bioinsecticides	–	<i>Bacillus thuringiensis</i> <i>Paenibacillus popilliae</i> <i>Paenibacillus lentimorbus</i> <i>Pseudomonas aeruginosa</i> <i>Pseudomonas taiwanensis</i>	Release toxins targeting receptor in the insect midgut cells	<i>B. thuringiensis</i> is the most widely used species to control a variety of insect pests in agriculture, forestry and public health. To date, over one hundred <i>B. thuringiensis</i> -based bioinsecticides have been developed	[34,35]
Fungal bioinsecticides	–	<i>Beauveria bassiana</i> , <i>Metarhizium anisopliae</i> , <i>Metarhizium rileyi</i> , <i>Paecilomyces farinosus</i> , <i>Verticillium lecanii</i>	Attack integument or gut epithelium, utilizing nutrients in the hemocoel, some release toxins	They have been widely evaluated as control agents for a diverse variety of noxious arthropods of agricultural importance	[35]
Viral bioinsecticides	–	<i>Nucleopolyhedroviruses</i> , <i>granuloviruses</i>	Cell lysis	The use of entomopathogenic viruses in global crop protection has grown in the last decade, mainly to control lepidopteran pests	[36]

pheromones, alarm pheromones and other types of compounds that are used as attractants and repellents for both monitoring and controlling insect pest populations. Chemically, the semiochemicals can be alcohols, aldehydes, alkanes, amino acids, esters, heterocyclic aromatic compounds, proteins, salts, sulfur-containing compounds, terpenes or triglycerides<sup>[37]</sup>. Semiochemicals are valuable and efficient compounds for integrated pest management including attract-and-kill, mass trapping, mating disruption, monitoring and push-pull<sup>[6]</sup>. The market for semiochemicals in insect control is advancing rapidly, especially in integrated pest management. For instance, the sex pheromone from the pyralid rice pest *Cnaphalocrocis medinalis* (Lepidoptera: Pyralidae), consists of four components, (Z)-11-octadecenal (Z11-18:Ald), (Z)-13-octadecenal (Z13-18:Ald), (Z)-11-octadecen-1-ol (Z11-18:OH) and (Z)-13-octadecen-1-ol (Z13-18:OH) in a ratio of 11:100:24:36. It was used for pheromone-baited trap in rice fields to monitor *C. medinalis*<sup>[7]</sup>. Also, sex pheromones from 19 species of gall midges (Diptera: Cecidomyiidae) have been identified and reported. Sex pheromone-based monitoring systems have been developed and commercialized for at least ten gall midge species<sup>[8]</sup>. A push-pull system that deploys an alarm pheromone (1,4-benzoquinone, 2-methyl-1,4-benzoquinone and 2-ethyl-1,4-benzoquinone) and an aggregation pheromone [(R)-limonene, 2-nonanone, (E)-ocimene, (S)-linalool, (R)-daucene and (E,E)- $\alpha$ -farnesene] has been shown to simultaneously capture a higher number of the lesser mealworm *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) than a pull system that only contains aggregation pheromone<sup>[9]</sup>. Finally, the bed bug *Cimex lectularius* (Hemiptera: Cimicidae) aggregation pheromone comprises a unique blend of five volatile components [dimethyl disulfide, dimethyl trisulfide, (E)-2-hexenal and (E)-2-octenal, 2-hexanone] and one less-volatile component (histamine). These components synergistically mediate both the attraction and arrest of bed bugs. They are highly effective at luring bed bugs into traps<sup>[10]</sup>.

Animal toxins are polypeptides or proteins usually produced by predatory arthropods such as bees, scorpions, spiders and wasps. The major components of most spider venoms are small disulfide-rich peptides that kill insects by targeting presynaptic ion channels or postsynaptic receptors<sup>[11]</sup>. The  $\beta$ -toxins from scorpions bind to voltage-gated sodium channels of insects with high affinity<sup>[12]</sup>. Given the properties of arthropod toxins, they may be more useful as PIPs generating transgenic plants or for viral vector construction.

## 2.2 Biochemical insecticides produced by microorganisms

Microbially-produced bioinsecticides have been very success-

fully developed and widely used in the past. They include avermectins, *Bacillus thuringiensis* (Bt) endotoxins and spinosyns (Table 1). Chemical modification of some of these microbial products has also been successfully employed to generate new insecticides including emamectin from avermectins and spinetoram from spinosyns. Avermectins and their derivatives are macrocyclic lactones that target glutamate-gated Cl<sup>-</sup> channels in the peripheral nervous system<sup>[13]</sup>. Spinosyns and their derivatives are also macrocyclic lactones. They target the nicotinic acetylcholine receptor at a different site from nicotine or imidacloprid as well as gamma-aminobutyric acid (GABA) receptors<sup>[13]</sup>. To date, more than 20 spinosyns and more than 800 spinosoids (semisynthetic analogs) have been isolated or synthesized<sup>[13,14]</sup>. The widespread application of these chemicals consequently leads to the high resistance by the pests and toxicity to humans and animals has been frequently reported.

Bt endotoxins, known as Cry and Cyt toxins, are pore-forming protein toxins. They bind to specific receptors in insect midgut cells and facilitate the formation of a pre-pore oligomer structure followed by membrane insertion. This membrane insertion causes the formation of large cation-selective pores that increase the water permeability of the cell membrane of the midgut cells<sup>[16,17]</sup>. The affected pests stop feeding and starve to death. Bt toxins have been sprayed in large amounts to control mosquito larvae in breeding areas. However, they have only limited use in agriculture as sprayable insecticides because of their sensitivity to solar radiation and limited activity against borer insect pests. Rather, Bt toxins have been introduced into transgenic crops or sold with live spores to control insect pests<sup>[18]</sup>. A number of Bt toxins with diverse insect selectivity have been identified that have increased insecticidal activities against important insect pests<sup>[16]</sup>. Bt strains that are used as bacterial bioinsecticides are discussed below as microbial bioinsecticides.

Some other microbes produce secondary metabolites that have insecticidal activity. As microbes are species-rich in nature, exploiting microbial secondary metabolites will be a promising direction to expand the spectrum of bioinsecticides and perhaps develop novel insecticides with new modes of action. Okaramine, for example, is an indole alkaloid discovered in fermentation preparations of *Penicillium simplicissimum* strain AK-40 which grows in soybean pulp (*okara*). Okaramine exhibits broad-spectrum insecticidal activity. Most okaramines activate L-glutamate-gated chloride channels found only in invertebrate nervous systems and muscle cells<sup>[15]</sup>.

## 2.3 Biochemical insecticides produced by plants

Essential oils are obtained from plant materials through

hydrodistillation, steam distillation, dry distillation or mechanical cold pressing. They contain mainly two classes of phytochemicals: terpenoids (monoterpenes and sesquiterpenes with low molecular masses) and phenylpropanoids, but to a lesser extent<sup>[19]</sup>. Essential oils have been intensively studied for their pesticidal activities and are described as sustainable and effective alternatives to synthetic insecticides. The diverse constituents of essential oils exert multiple effects in insect pests such as repellent, growth-reducing and insecticidal effects. For example, eugenol and cinnamaldehyde have ovicidal, larvicidal and adulticidal toxicities<sup>[20]</sup>. Dillapiole, piperamides and furanocoumarins inhibit P450 cytochromes and this makes them good synergists with other insecticides<sup>[21]</sup>. Thymol and monoterpenes are neurotoxic to insects because they interact with GABA and octopamine receptors and/or inhibit acetylcholinesterase<sup>[21]</sup>. Some constituents such as clerodane diterpenes, neem triterpenoids and sesquiterpene lactones have deterrent and fumigant activities and can also repel insects<sup>[22]</sup>. More detailed information about essential oils can be found in the excellent review by Regnault-Roger et al.<sup>[21]</sup>. The chemically redundant and diverse characteristics of essential oils may reduce the risk of the development of tolerance in insects compared to a single compound insecticide. So far, neem oil, orange oil, peppermint oil, rosemary oil and tea tree oil and have been widely used as bioinsecticides. However, many more essential oils have restricted use, i.e., they are used only by indigenous communities because of the lack of widespread cultivation.

Pyrethrins, obtained from the dried flowers of Dalmatian pyrethrum (*Tanacetum cinerariifolium*), constitute a small class of specialized metabolites with insecticidal activities. Six naturally-occurring pyrethrins were initially discovered and then synthetic pyrethroids (pyrethrin derivatives) resembling their natural counterparts were developed with increased environmental stability and toxicity toward insects<sup>[14]</sup>. Pyrethrins and their derivatives have been shown to bind to the voltage-gated sodium channels in the insect nervous system to block sodium transport, enhance channel inactivation and prolong channel opening<sup>[13]</sup>. Nearly 100 years have passed since the initial identification of pyrethrins. Pyrethrins inspired the development of photostable pyrethroids that have been highly successful and represent one prominent example of synthetic pesticide chemistry based on a natural product model<sup>[38]</sup>. However, pyrethroids have little chemical similarity to the natural products and many have different modes of action. At present, most chemical companies have discontinued pyrethroid research due to the appearance of pyrethroid-resistant insect pests.

Azadirachtin is a tetranortriterpenoid belonging to the limo-

noids. It is the main bioactive compound abundantly present in mature seeds of *Azadirachta indica*<sup>[24]</sup>. Azadirachtin has a set of properties that potentiates its application as an excellent bioinsecticide. It can be rapidly degraded by light radiation and by microbes in soil, water and on plants, and it has a negligible toxicity to mammals and the environment<sup>[25]</sup>. Azadirachtin has a broad spectrum of activities. It acts as an insect growth regulator that modifies the behavior and growth of insects<sup>[26]</sup>. It can also act as an oviposition deterrent to female insects leading to decreasing egg-size and thus impairs insect propagation<sup>[26]</sup>. Its bioactivity, environmental safety and public acceptability make azadirachtin an excellent bioinsecticide but its use is lagging far behind likely due to the high costs of production associated with growing trees<sup>[24]</sup>. Efforts to produce this very complex molecule via de novo synthesis have been suspended. The main challenge appears to be the stability of the natural product in the environment.

Rotenone has been used as an insecticide for more than 150 years. It is one of several isoflavonoids produced in the roots or rhizomes of the tropical legumes *Derris*, *Lonchocarpus* and *Tephrosia*. Rotenone is an inhibitor of site I of the respiratory chain in mitochondria preventing energy production<sup>[14]</sup>. It is used as a selective, non-systemic insecticide and can be applied as a stomach poison or by topical contact. It has been used to control a wide range of insect pests including aphids, beetles, fire ants, mosquitoes, moths and thrips<sup>[22]</sup>. However, it also exhibits toxicity in mammals and therefore has largely fallen out of favor in most industrialized countries (EU countries and the USA), at least in crop protection.

The plant alkaloids are another family of botanical chemicals that have been widely applied as insecticides. The initially-used plant alkaloids like nicotine and Rytania alkaloid have gradually faded out of usage because of their toxicity, while newly-discovered alkaloids are promising in insect pest control. Nicotine is a major representative of this family within the genus *Nicotiana*<sup>[14]</sup>. It binds to the cholinergic nicotinic acetylcholine receptor in nerve cells of insects, leading to a continuous firing of the neuronal receptor and causing depolarization of nerve cells and resulting in a neurotoxic effect<sup>[27]</sup>. Nicotine functions predominantly through the vapor phase but also with slight contact and stomach action. Nicotine has been used for many years as a fumigant for the control of many insects. However, it is very toxic to humans by inhalation or skin contact.

Rytania alkaloid extracts isolated from the stems of *Rytania* spp., particularly *Rytania speciosa*, have insecticidal properties. Ryanodine and related alkaloids affect muscles by binding to

the calcium channels in the sarcoplasmic reticulum. This causes calcium ion to flow into the cytoplasm and death follows very rapidly<sup>[28]</sup>. *Ryania* extracts have had limited use as insecticides because they are moderately toxic to mammals and highly toxic to fish. *Sabadilla* is an insecticidal preparation from the crushed seeds of the liliaceous plant *Schoenocaulon officinale*, containing a mixture of the alkaloids, cevadine and veratridine. Cevadine, veratridine and related ceveratrum alkaloids activate the voltage-sensitive sodium channels of nerve, heart and skeletal muscle cell membranes. Their binding site appears to be different from that of the pyrethroids. They are insecticides with contact action and initial effects include paralysis, with death occurring later<sup>[28]</sup>. Both veratridine and cevadine are degraded in air and sunlight and this makes them environmentally friendly. In addition to these well-known alkaloids, newer alkaloids with insecticidal activities have been identified including matrine and related quinolizidine, alkaloids extracted from *Sophora flavescens*, berberine extracted from *Phellodendron amurense*<sup>[39]</sup> and huperzine extracted from the New Zealand club moss *Phlegmariurus varius*<sup>[40]</sup>. Some of these alkaloids have been commercialized as insecticides in some countries with a considerable history of research on botanical insecticides including China and Korea.

Wide use of most botanical insecticides is limited by the available biomass of certain plants, especially of those that produce essential oils. However, they are in local use in greenhouse and indoor cultivation of food and medicinal crops, management of domestic and urban pests, and control of ectoparasites on foods and companion animals.

### 3 PLANT-INCORPORATED PROTECTANTS

PIPs are pesticidal substances produced by transgenic plants containing genetic material necessary for the plant to produce the substance (US-EPA). Both insecticidal proteins and dsRNAs based on RNAi technology can be expressed in transgenic plants as PIPs to achieve insect pest control (Table 1).

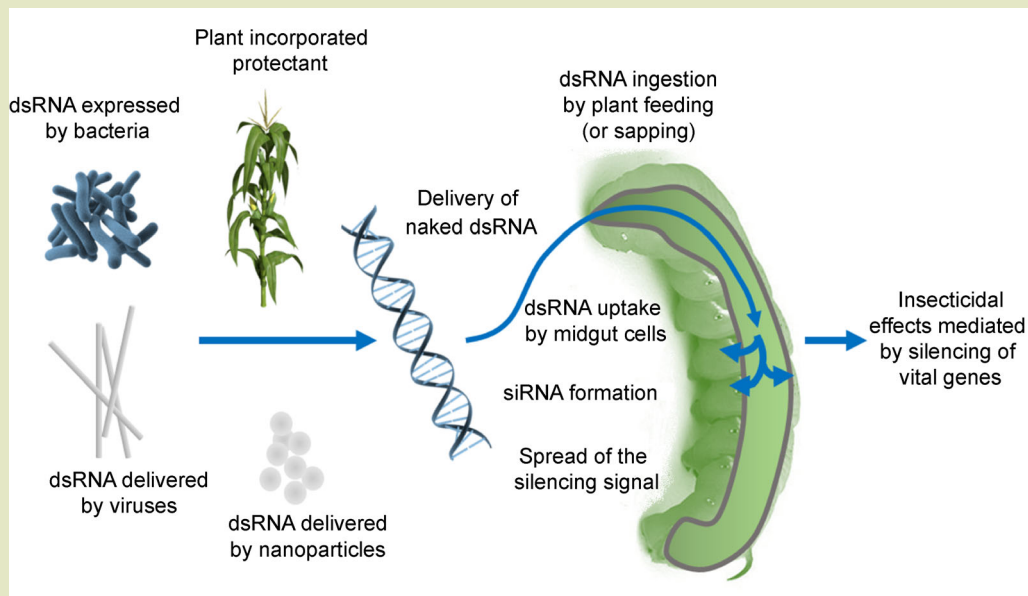
#### 3.1 Insecticidal protein-based PIPs

Bt toxins are the most common insecticidal proteins to be expressed in transgenic crops and these have been grown commercially since 1996<sup>[29]</sup>. They are expressed by the genetic insertion of *cry* or *cyt* genes from various Bt subspecies and strains that encode structurally diverse Bt toxins that differ in their activities against coleopteran, hymenopteran, dipteran and lepidopteran pests<sup>[16]</sup>. Some are active against non-insect pests

including plant pathogenic nematodes, mites and protozoans<sup>[30]</sup>. In addition, genes encoding vegetative insecticidal proteins (Vips) from Bt have been used to produce transgenic plants and they are considered to be the next generation of insecticidal proteins used in plant protection<sup>[31]</sup>. Bt genes have been approved for commercial use in most major economic crops including cotton, eggplant, maize, potato, rice, soybean, sugarcane and tomato to protect against about 30 major coleopteran and lepidopteran pests. Bt crops have been grown on over 40 Mha for more than 20 years with substantial environmental and economic benefits. However, because of the large-scale application of Bt transgenic crops, resistance or tolerance of different insect pests has been reported to various Bt toxins under either field or laboratory selection<sup>[17]</sup>. This means that resistance-management strategies must be employed to overcome this problem. Different insecticidal proteins from bacteria other than Bt have been evaluated as potential PIPs. These include proteins derived from *Photorhabdus temperata* (make caterpillars floppy, Mcf and membrane attack complex/perforin, Mpf), *Bacillus cereus* (Vip2 active component, Vpa), *Lysinibacillus sphaericus* (mosquitocidal toxin 1, Mtx1; sphaericolysin-related pesticidal proteins, Spp; and toxin\_10 pesticidal proteins, Tpp) and *Vibrio parahaemolyticus* (Photorhabdus insect-related A/B toxins, PirA/B)<sup>[41]</sup>. Furthermore, genes encoding insecticidal proteins from non-bacterial sources have been examined for their use as PIPs. These include plant proteins that affect insect digestive systems (e.g.,  $\alpha$ -amylase inhibitors<sup>[42]</sup>, some lectins<sup>[43]</sup> and various trypsin inhibitors<sup>[44]</sup>), chitinases from different sources<sup>[45]</sup>, and proteins that interfere with the uptake of essential nutrients (e.g., avidin, which sequesters dietary biotin<sup>[46]</sup>). However, the identification of novel insecticidal proteins with sufficient activity against key target pests is still highly sought but yet extremely challenging.

#### 3.2 RNAi-based PIPs

RNA interference is a powerful technology that allows silencing of specific target genes. One possibility to trigger RNAi effects in plants is to express inverted repeat transgenes. This results in the formation of dsRNA which is processed by the Dicer enzyme into short siRNA duplexes being incorporated into the RNA-induced silencing complex. One strand of the siRNA functions to guide this complex to the complementary target mRNA which is subsequently degraded. Transgenic plants expressing dsRNA to silence vital genes of plant-feeding insects have been rapidly developed for pest management (Fig. 1). The major advantage of RNAi-based PIPs over current pest management strategies is intrinsically high species-selectivity sparing non-target species. The screening of potential candidates for effective RNAi-mediated pest control has revealed various suitable target



**Fig. 1** Delivery of dsRNA to insects by different approaches. Double-stranded RNA can be delivered directly, or by nanoparticles viruses, bacteria and transgenic plants. Following ingestion dsRNA is absorbed by midgut cells as shown here for the tobacco hornworm. The absorption of dsRNA may be mediated by endocytosis or may involve specific channels. In many insects the RNAi effect is systemic, requiring the spread of the silencing signal.

genes and a wide range of transgenic crops expressing dsRNAs to silence these genes enhance resistance to the specific pests. Next to nuclear transformation, transplastomic transformation (i.e., insertion of the transgene into the chloroplast genome) has also been successful (summarized by Adeyinka et al.<sup>[32]</sup>). First proof of concept for this approach was provided by Baum et al.<sup>[47]</sup> who showed that ingestion of dsRNA to knockdown V-ATPase subunit A gene expression triggers insecticidal responses in different coleopteran species including the western corn rootworm, *Diabrotica virgifera virgifera*. Subsequently, studies by the University of Nebraska and Dow AgroSciences generated a transgenic maize line expressing dsRNA specificity for this and another V-ATPase gene and reported reduced root damage by western corn rootworm<sup>[48]</sup>. Similarly, expression of dsRNA for V-ATPase subunit A in transgenic tobacco enhanced resistance to whitefly, *Bemisia tabaci*<sup>[49]</sup>. Another obvious target gene for RNAi-based pest control encodes the ecdysone receptor, assuming that RNAi-mediated silencing of this gene will lead to abortive molting as shown in several studies analyzing different pests<sup>[50]</sup>. Accordingly, a transgenic tobacco line expressing dsRNA to silence the ecdysone receptor gene in the cotton bollworm, *Helicoverpa armigera*, enhanced its resistance toward cotton bollworm and the beet armyworm, *Spodoptera exigua*<sup>[51]</sup>. Similarly, transgenic potato lines expressing dsRNA specificity for the same gene had resistance against the Colorado potato beetle, *Leptinotarsa decemlineata*<sup>[52]</sup>. A target gene that

turned out to be highly effective when dsRNA was fed to the western corn rootworm was *DvSnf7* which encodes a vacuolar sorting protein necessary for autophagy and membrane stability in beetle midgut cells<sup>[53]</sup>. Recently, a transgenic maize line was commercialized by Monsanto which expresses a fragment of the *DvSnf7* gene in addition to three *Cry* genes aiming to reduce the risk of Bt resistance. This line was approved by the Canadian Food Inspection Agency in 2016 and the US-EPA in 2017. Notably, *DvSnf7* dsRNA has no adverse effects on larval or adult honeybees even at high concentrations<sup>[54]</sup>. Further targets used to create pest-resistant transgenic plants by dsRNA expression include genes encoding chitinase<sup>[55]</sup>, nuclear hormone receptor 3, and the P450 monooxygenase CYP6AE14<sup>[56]</sup>, and different aphid proteins<sup>[57]</sup>.

RNAi-based PIPs are highly specific and provide an environmentally friendly method of controlling insect pests. Apart from the advantages of PIPs summarized above, some disadvantages need to be discussed here. Certainly, public resentment toward genetically modified plants generally limits the acceptance of PIPs in many countries. The major concerns relate to the risk of outcrossing events spreading genetically modified genes, decreasing plant diversity and biodiversity, development of resistance in weeds and pests, damage to beneficial animals, farmer dependence on seed monopolies, and potential allergic reactions in sensitized humans<sup>[33]</sup>. As far as it relates to RNAi-



based PIPs, additional restrictions result from the facts that RNAi efficiency varies largely between different insect taxa, dsRNA is highly labile in the environment, and no computational models are available that predict the efficiency of RNAi-based approaches.

Further empirical studies are therefore needed to increase the efficiency of RNAi and to assess their associated safety risks, particularly the remaining potential for side-effects in non-target organisms.

Next to RNAi-based PIPs another RNAi approach involves the administration of topically applied dsRNAs which may be an environmentally safe alternative to some synthetic chemical insecticides because of rapid degradation in the environment (Fig. 1)<sup>[58]</sup>. Spraying actin-dsRNAs to the surfaces of potato plant leaves has been successfully used to control the Colorado potato beetle<sup>[59]</sup>. A recent study analyzing 14 beetle populations collected across various European regions also demonstrated that there is limited variation in RNAi effects after foliar application of actin-dsRNA<sup>[60]</sup>. Treatment of leaf discs with dsRNA silencing the *lesswright* gene, which is necessary for embryonic development and hemocyte production, was recently shown to be effective against the coleopteran pest *Henosepilachna vigintioctopunctata*, which feeds mainly on solanaceous plants<sup>[61]</sup>. Using a dsRNA delivery approach via leaf discs to kill the two-spotted spider mite, *Tetranychus urticae*, additional target genes have been effective including genes that encode the  $\beta$  subunit of coatomer protein complex (showing highest mortality), the M1 metalloprotease and the ribosomal protein S4, next to the already established V-ATPase subunit A<sup>[62]</sup>. Other target genes for the control of spider mites may include the *Spook* gene which encodes a P450 enzyme<sup>[63]</sup> and the chitin synthase 1 gene (*Chs1*)<sup>[64]</sup>. In most other studies, dsRNAs were used as topical applications to target genes which have been mentioned already in the context of RNAi-based PIPs<sup>[65]</sup>. Topical dsRNA administration may be effective only in feeding pests that ingest the dsRNA and may not directly be applicable to sap-feeding insects. Andrade and Hunter have developed an RNAi feeding bioassay to control the Asian citrus psyllid and other hemipterans to overcome this limitation<sup>[66]</sup>. They placed citrus shoots into a solution containing dsRNA targeting the arginine kinase gene and showed that dsRNA was transported via the xylem to the leaves where it was lethal to hemipteran species feeding on the shoots.

So far the focus has been mainly on topical administration of dsRNA solutions without considering novel delivery techniques that help to increase RNAi efficiency by protecting naked dsRNA from degrading factors present in the environment (Fig. 1).

Plasmid-transformed bacteria that express dsRNA specific for insect target genes provide an easy and affordable technique for the topical delivery of dsRNA. This approach was established for instance in the lepidopteran pest *S. exigua* for silencing the gene encoding *Chs1*<sup>[67]</sup>. Feeding these bacteria to the larvae resulted in a dose-dependent systemic RNAi effect with a significant decline in *Chs1* mRNA levels and a decrease in survival rates. This delivery system has been successfully used against various insect pests<sup>[68,69]</sup> and was optimized by sonication<sup>[70]</sup>. A novel pET28-BL21(DE3) RNase III-system was recently developed to express *vestigial*-dsRNA for the control of the Asian ladybeetle, *Harmonia axyridis*. This system was more efficient in dsRNA production than the widely used L4440-HT115(DE3) system, which was used also in the above example of *S. exigua* to silence *Chs1*<sup>[71]</sup>. Nanoparticle-mediated dsRNA delivery is another advance that improves RNAi effects by both protecting the dsRNA and possibly increasing dsRNA adsorption rates. Nanoparticle carriers that have been tested for dsRNA delivery to pest insects are chitosan and its derivatives<sup>[72,73]</sup>, branched amphiphilic peptide capsules<sup>[74]</sup>, guanylated polymers<sup>[75]</sup> and liposomes<sup>[76]</sup>. Finally, also recombinant plant viruses, e.g., tobacco mosaic and tobacco rattle viruses, have been successfully tested as natural carriers for dsRNA delivery by transforming plant tissues transiently<sup>[77]</sup>.

In summary, administration of sprayable dsRNA might become a powerful and cost-effective technology in pest control providing high species-selectivity and low environmental side-effects. Topical delivery of dsRNA-nanoparticle complexes or bacteria that express dsRNA may circumvent problems of dsRNA instability and low absorption rates as observed for naked dsRNA.

## 4 MICROBIAL BIOINSECTICIDES

Infectious agents constitute the largest group of insect pest biocontrol agents with bacteria, fungi and baculoviruses the most promising (Table 1). There are at least 1500 naturally-occurring insect-specific microorganisms, 100 of which are known to have insecticidal activities. Over 200 microbial pesticides are highly effective, species-specific and ecologically-friendly biopesticides that have been commercialized in 30 countries, contributing 90% to the whole biopesticide market<sup>[34]</sup>.

### 4.1 Bacterial bioinsecticides

Bacterial bioinsecticides are probably the most widely used biological agents for pest control. Most of these bacteria carry toxins with high specificity toward certain insect pests. Various

*B. thuringiensis* strains that contain various Bt toxins are the most widely used bacteria to control a range of insect pests in agriculture, forestry and public health. To date, over 100 *B. thuringiensis*-based bioinsecticides have been developed for coleopteran, dipteran and lepidopteran pests<sup>[16]</sup>. Also, *Paenibacillus* spp., including *Paenibacillus popilliae* and *Paenibacillus lentimorbus*, and *Pseudomonas* spp., including *Pseudomonas aeruginosa* and *Pseudomonas taiwanensis*, are also important for controlling insect pests<sup>[34,35]</sup>. Insecticidal *Chromobacterium subtsugae* and *Burkholderia rinojensis* are reported to function as stomach poisons toward different insect orders<sup>[78]</sup>.

## 4.2 Fungal bioinsecticides

Entomopathogenic fungi are also promising as they have evolved multiple pathogenicity mechanisms. The most widely used and commercialized species are *Beauveria bassiana*, *Metarhizium anisopliae*, *Metarhizium rileyi*, *Paecilomyces farinosus*, and *Verticillium lecanii*<sup>[34,35,79]</sup>. These fungi attack the host integument or gut epithelium to grow using nutrients present in the hemocoel while avoiding insect immune responses. Some species such as *B. bassiana* and *M. anisopliae* cause insect diseases such as muscardine. Entomopathogenic fungi may be applied in the form of conidia or mycelia which sporulate after application.

## 4.3 Viral bioinsecticides

The deployment of entomopathogenic viruses in global crop protection has increased over the last decade. About a dozen of these viruses have been commercialized. The lepidopteran-specific nucleopolyhedroviruses and granuloviruses are the most successful<sup>[35,36]</sup>. Their insecticidal activity is through cell lysis after virus replication in the nuclei or cytoplasm of host cells<sup>[36]</sup>. The replication of viruses within the host may create enzootics and ultimately decrease insect pest populations.

Although microbial insecticides are promising, they might only control a narrow range of pests due to host-specificity. They are also very sensitive to heat, UV light and desiccation. These limitations need to be resolved so that the widespread application of these biocontrol agents will be realized.

## 4.4 Tritrophic plant–microbe–insect interactions

Focusing on plant–insect interactions may be a simplification of the actual problem in the field. In general, the plant rhizosphere is populated by beneficial microorganisms including mycorrhizal fungi that control plant fitness. In increasing numbers of cases a recurrent scenario emerges in which the plant–fungus

interaction affects the success of pest insects in colonizing a plant<sup>[80]</sup>. For instance, chrysanthemum growing in soil containing arbuscular mycorrhizae is less susceptible to the thrips *Franklinella occidentalis* in comparison to plants grown on sterile soil<sup>[81]</sup>. Another study demonstrates that colonization of the barrel medic *Medicago truncatula* by the arbuscular mycorrhiza *Rhizophagus irregularis* attenuated growth of the aphid *Acyrtosiphon pisum*<sup>[82]</sup>. Enhanced plant defense has been postulated as a major mechanism in protection against insect pests. The genetic, molecular and cellular pathways await identification. Thus, modulating or improving plant–mycorrhiza interactions may be an elegant and harmless way of insect pest management.

## 5 FUTURE TRENDS

The increasing demand for safer food supply and the growing global concerns about pesticidal toxicity have become strong driving forces for the growth of the bioinsecticides market. Increasing numbers of countries, particularly economically-developed countries, have been highly supportive of the adoption of bioinsecticides through imposing laws and policies, but the regulatory demands have actually increased in many jurisdictions, impeding the widespread adoption of bioinsecticides<sup>[83]</sup>. Insecticide resistance and short residual action also restrict existing bioinsecticides for wider application. New strategies should be pursued in future research.

### 5.1 New modes of action

Like synthetic chemical insecticides, some bioinsecticides target proteins of the insect nervous system, in particular acetylcholinesterases, nicotinic acetylcholine receptors,  $\gamma$ -aminobutyric acid receptor/chloride channels and voltage-gated sodium channels<sup>[13]</sup>. The lower diversity of targets the more resistance by pests is observed, and neurotoxicity to human and non-target animals is unavoidable. The identification of non-neurotoxic modes of action is therefore much needed.

Some bioproducts have shown insecticidal activity by arresting pest molting. Phlegmacin B1, for example, is a microbial secondary metabolite derived from the strain *Talaromyces* sp. Phlegmacin B1 inhibits three chitinolytic enzymes: GH18 chitinases *OfChi-h*, *OfChtI* and GH20  $\beta$ -N-acetyl-D-hexosaminidase *OfHex1*, the enzymes involving in cuticle chitin hydrolysis during insect molting<sup>[84]</sup>. Another example is berberine, a phytochemical with multiple medicinal applications. Berberine exhibits useful insecticidal activity by inhibiting the growth and development of the pest *Ostrinia furnacalis*. In vitro studies show that it inhibits the activity of *OfChtI* and

*OfHex1*<sup>[85]</sup>.

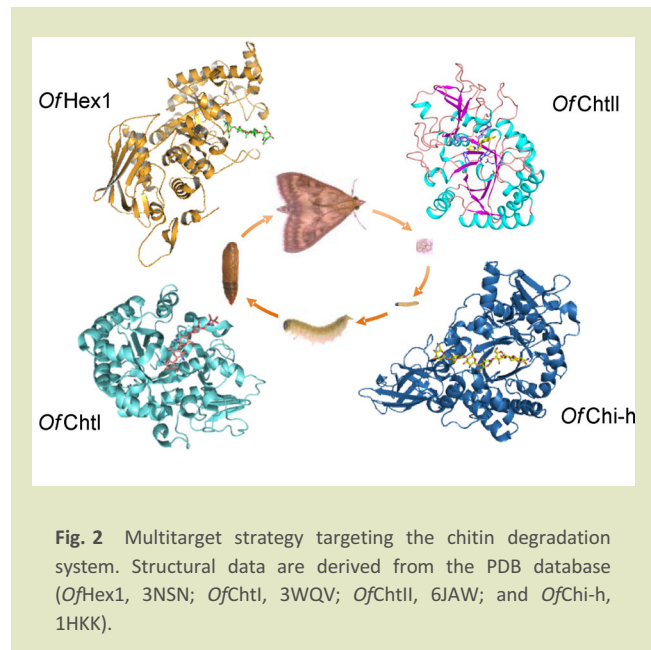
Terpenes are natural products that have been widely used for controlling insects. Recent studies show that some terpenes, e.g., thymol and carvacrol, target tyramine receptors and this is an underutilized but promising target for agrochemical discovery<sup>[86]</sup>.

## 5.2 Multitarget strategy

In clinical practice the inefficacy of certain treatments based on one molecular target has encouraged the adoption of a multitarget strategy which has been highly efficient against complex diseases including diabetes, HIV, malaria, neurodegenerative diseases and tuberculosis<sup>[87]</sup>. This multitarget approach should be considered as a promising strategy in future pest control since single-target pesticides have a high risk of leading to the development of resistance. The costs and risks in developing multitarget pesticides are in principle no different from that of any other single-target pesticide. There are also lower risks of pesticide interactions and toxicity compared to multicomponent pesticides<sup>[87]</sup>.

Functional proteins or regulatory RNAs that affect the insect cuticle by interfering with chitin biogenesis or degradation may also be good candidates for multitarget strategies. In general, these physiological processes require a number of vital enzymes and regulatory proteins. For example, cuticle chitin hydrolysis requires at least three enzymes that fall into two glycoside hydrolase (GH) families, GH18 and GH20<sup>[88,89]</sup>. The puzzle of cuticle chitin hydrolysis has recently been solved by structural biology analysis (Fig. 2) and high-speed atomic force microscopy<sup>[90,91]</sup>. Enzymes belonging to GH18 and GH20 families follow a similar substrate-assisted catalytic mechanism and it appears plausible to obtain one molecule that targets several enzymes belonging to GH18 and GH20 families. Phlegmacin B1 displays inhibitory activity against three chitinolytic enzymes comprising two GH18 chitinases and one GH20  $\beta$ -N-acetyl-D-hexosaminidase<sup>[84]</sup>. Although other compounds may have higher enzymatic inhibitory activities to a single enzyme, e.g., lower IC50 or Ki values, phlegmacin B1 with relative higher IC50 or Ki values has much higher in vivo activity<sup>[84]</sup>, also suggesting that molecules with multiple targets will be more efficient.

The essential oils extracted from some plants may also be considered as multitarget drugs. They contain hundreds of related terpenes that are both phytochemically diverse and overlapping in function<sup>[3]</sup>. They have diverse physiological targets within the insects and may delay the development of insect resistance.



## 5.3 Nanoformulations

The rapid biodegradability of bioinsecticides has advantages and disadvantages. Bioinsecticides may rapidly degrade in the environment but will have shorter half-lives, reducing their bioavailability. Recent studies indicate that bioinsecticides encapsulated in nanoparticles (NPs) have high stability against degradation and can be released to the environment in a controlled manner<sup>[92,93]</sup>.

As proposed by Jampilek, current NP platforms can be classified into three major categories, inorganic-based NPs, organic-based NPs and hybrid NPs combined of inorganic and organic components<sup>[94]</sup>. Inorganic-based NPs, such as nanocrystals, shells, quantum dots based on gold, silver, copper, iron, various semiconductors, ceramic (silica-based NPs), and carbon nanotubes or fullerenes, are non-biodegradable. Organic-based NPs such as liposomes, solid lipid NPs, polymeric NPs, micelles and capsules are frequently biodegradable. Most organic-based NPs consist of water-soluble biodegradable and biocompatible polymers such as polylactide homopolymers and polyacrylamide, or natural compounds such as chitosan, lignin, starch, cellulose and their derivatives.

Multiple bioinsecticide compounds have been encapsulated using NPs, for example botanical insecticides including essential oils, azadirachtin, rotenone, carvacrol, thymol, eugenol and curcumin<sup>[95]</sup>. An example is neem extracts encapsulated in NPs and formulated as a colloidal suspension giving 100% larval mortality in *Plutella xylostella*. The nanoparticle increases the stability of neem extracts against ultraviolet radiation<sup>[63]</sup>. The

control efficacy of polyethylene glycol NPs loaded with garlic essential oil against adult *Triblium castaneum* remained at over 80% after 5 months, while the control efficacy of free garlic essential oil at a similar concentration was only 11%<sup>[96]</sup>. Silver nanoformulations have included extracts from numerous terrestrial plants such as neem and *Annona*<sup>[23]</sup>. They have been developed into mosquito larvicides for the control of vector-borne diseases<sup>[97]</sup>. More examples are reviewed by Pradhan and Mailapalli who have summarized the main bioactive compounds and carrier systems employing nanotechnology<sup>[98]</sup>.

Nanoparticles have also been used in RNAi-based pest control as previously discussed. They can protect dsRNA/siRNA molecules from enzymatic degradation and promote their translocation across cell membranes. For example, chitosan/dsRNA nanopar-

ticle-mediated oral feeding increased RNAi efficiency to silence chitin synthase genes significantly in *Anopheles gambiae*<sup>[72]</sup>. A branched amphiphilic peptide nanomaterial facilitates cellular uptake of dsRNA by insects through feeding. This increased the mortality to 75% while dsRNA alone resulted in a mortality of about 30%<sup>[74]</sup>. Liposomes were used to deliver dsRNA of the  $\alpha$ -tubulin gene through oral feeding. Liposomes delayed the degradation of dsRNA in the midgut and increased the mortality of German cockroach by significantly inhibiting  $\alpha$ -tubulin expression in the midgut<sup>[76]</sup>.

Generally, nanoformulations of bioinsecticides may increase the stability (delay degradation), increase the efficacy (higher surface area), increase the activity (smaller particle size), decrease non-target toxicity (elimination of organic solvents) and also enable controlled release of active ingredients<sup>[93,95]</sup>.

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### Compliance with ethics guidelines

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