Chapter

Secondary Metabolites of Entomopathogens as Biotechnological Tools for the Biological Control of Agricultural Insect Pests

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Abstract

In recent years, the irrational application of chemical insecticides has caused the appearance of pest insect populations that are resistant to the active principles of commercial insecticides. In addition, these chemical compounds cause significant damage to the environment and to the people who apply them. The use of secondary metabolites produced by entomopathogenic microorganisms is a viable alternative that could mitigate the damage caused by chemical insecticides. Actually, the secondary metabolites of entomopathogens microorganisms have been studied; however, there are few reports on their massive production and their direct application as biological control agents. The aim of this book chapter is to describe, in a very general way, some of the secondary metabolites produced by entomopathogenic microorganisms, their potential application as bioinsecticides as well as their mass production.

Keywords: secondary metabolites, beauvericin, cry proteins, destruxins, biological control

1. Introduction

The presence of pest insect populations resistant to some chemical insecticides, caused by indiscriminate and irrational use, has produced enormous agricultural losses throughout the world. For example, a solution to combat the increase of pest insect populations has been to increase the recommended doses and the application times of the chemical insecticide, with consequent damage to the environment [1].

On the other hand, the impact on the exports of agricultural products is due to the restrictions given in the European Union and the United States regarding residual chemicals in vegetables and fruits [2].

Actually, an alternative to the use of chemical insecticides, is the application of entomopathogenic fungi and bacteria. These microorganisms have been used as biological control agents for pest insects since the beginning of the last century. For example, in Mexico, the use of Entomopathogenic Microorganisms (EM) has not become widespread, although the application of these microorganisms as part of plant health began more than 50 years ago; however, a significant increase in the use and commercialization of biological products have been observed since 1990 in all the world [3].

In fact, the reason for the increase in the use and applications of EM throughout the world has been because of its efficiency in killing insect pests, remaining long in the field after application, in addition to its specific interaction with the insect pest and be relatively safe in terms of the environment. On the other hand, the mechanisms of pathogenicity of EM have also been extensively studied and some of the compounds, called secondary metabolites, that participate in infectious processes have been described. This knowledge has allowed establishment of strategies to improve the production of secondary metabolites and their application as biological control agents [4].

The secondary metabolites are a group of compounds that have a vital role in infective and control processes. These compounds synthesized by EM do not play a direct role in growth or reproduction but rather have an adaptation function to the environment which surrounds them. Furthermore, they have their origin as derivatives of various intermediate compounds in primary metabolism. EM secrete a wide range of secondary metabolites that can be used in biological control [5].

For example, the secondary metabolites synthesized by Entomopathogenic Fungi (EF), such as oxalic acid, beauvericins, and dextrixins, which are toxic against insects. These compounds are produced when the fungus has penetrated the exoskeleton and has reached the hemocele, that is when it is considered that have insecticidal properties [6, 7]. Equally, some bacteria can produce proteins with insecticidal capacity, as is the case with the bacterium *Bacillus thuringiensis*. These proteins called Cry proteins and are capable of controlling insects, such as Lepidoptera, Coleoptera, and Diptera, among others. Certain virulence factors, such as VIP proteins, S-layer proteins, and some enzymes with insecticidal capacity, have also been described [8].

The aim of this book chapter is to describe the potential of the main secondary metabolites produced by entomopathogenic microorganisms, as biological control agents, as well as analyze the main routes and bioprocesses of biotechnological production of these compounds.

2. Secondary metabolites of entomopathogenic fungi

Currently, many secondary metabolites generated by different entomopathogenic fungi have been reported. Some secondary metabolites may be of simple organic structure, but regularly they are compounds of a slightly more complex structure. Furthermore, many secondary metabolites are cyclic and linear peptide toxins, which are derived from primary metabolites, and in some cases with unusual structures and occasionally accompanied by processes of specific biosynthesis [9, 10].

2.1 Low molecular weight metabolites of entomopathogenic fungi

In recent research, a considerable number of low molecular weight secondary metabolites have been reported, these compounds have been isolated from insect pathogens. **Figure 1** shows some secondary metabolites with insecticidal activity produced by entomopathogenic fungi. These metabolites have simple structures, such as oxalic acid, 2,6-pyridinedicarboxylic acid (dipicolinic acid), 4-hydroxymethylazoxybenzene-4-carboxylic acid. Some reports describe that the secondary metabolites of entomopathogenic fungi can alter the permeability of insect cell membranes, inducing the loss of fluids in the cells, they also modify the molting and metamorphosis process, change in fertility, and interferes with interactions. Ligand-receptor occur in the plasmatic membrane, deformations in the wings, and finally, cause the death of the insect [11].

2.1.1 Oxalic acid

The production of this secondary metabolite with insecticidal activity has been reported in *Beauveria* sp., *Lecanicillium (Verticillium) lecanii*, *Paecilomyces fumosoroseus* and *Metarhizium anisopliae*. This substance has been described as a virulence factor in phytopathogenic fungi, in addition to being studied as an element that participates in the solubilization of cuticular protein [12]. There are some reports where oxalic acid has been used to control *Varroa destructor*; this compound has been applied to the colony using the spray technique (obsolete method due to its complicated handling), by dripping and sublimation. All techniques have been highly effective (90–95% or more) in broodless colonies and therefore meet the requirements for a winter treatment [13]. According to Ref. [14], a solution of oxalic acid and ethanol-water in a ratio of 0.05, 0.1, 0.2, and 0.4 w/v; can be administered in amounts of 200 μL per treatment; in a single application and therefore meets the needs of the large-scale beekeeping industry.

2.1.2 2,6-pyridinedicarboxylic acid

This important compound (dipicolinic acid) has been produced by some entomopathogenic fungi, among which *Isaria* sp., *M. anisopliae, B. bassiana*, and *L (V) lecanii* [15, 16]. Its sodium salt has insecticidal properties against *Spodoptera frugiperda* larvae [16].

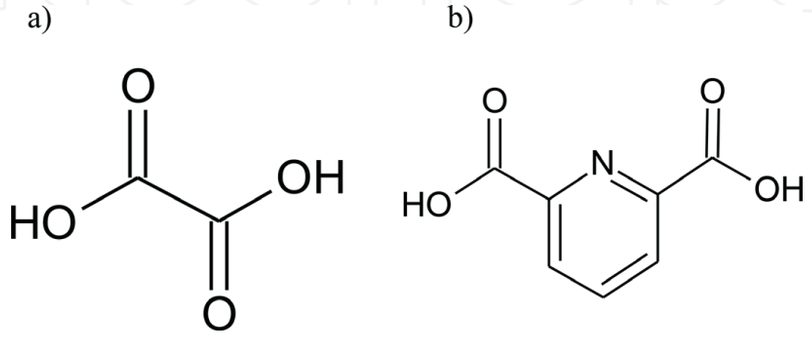

Figure 1.
Structures of low molecular weight secondary metabolites. (a) Oxalic acid produced by Beauveria spp., Lecanicillium (Verticillium) lecanii, Paecilomyces fumosoroseus, and Metarhizium anisopliae. (b) Dipicolinic acid produced by Isaria spp., M. anisopliae, B. bassiana, and L (V). Lecanii.

2.1.3 4-hydroxymethylazoxybenzene-4-carboxylic acid

It has been isolated together with its oxidation product (azoxybenzene-4,4-dicarboxylic acid) from the filtrate of the culture broth of *Entomophthora virulenta*, proving to be responsible for the insecticidal activity exhibited by this fungus against *Calliphora erythrocephala* [17].

2.2 Toxins with a peptide nature

Currently, cyclic and linear peptide toxins have been reported (**Figure 2**). The insecticidal action of these compounds has been described as very specific for certain groups of insects and their toxicity is due to the synergistic action of a complex group of compounds [18].

2.2.1 Beauvericin

Beauvericin has been the first molecule to be characterized due to its natural insecticidal properties, this compound was isolated for the first time from the mycelium of *Beauveria bassiana*, and in subsequent works, it has been extracted from different species of *Fusarium* and *Paecilomyces* species [18, 19]. Furthermore, beauvericin has been described as a cyclopeptide and its biosynthesis involves a multifunctional enzyme known as eniatin synthetase, whose expression is constitutive [18, 19]. In addition, it is known that this compound is an ionophore agent capable of forming Ca^{+} and K^{+} ion complexes that increase the natural and artificial permeability of membranes, inducing dehydration of tissues due to fluid loss from cells. Some reports indicate that this would be the main cause of the death of the insect, besides causing changes in the nucleus of the cells. This molecule has also been described and forms alterations in the molting and metamorphosis processes, as well as in fertility [19]. Some studies have reported that it has insecticidal properties against *Aedes aegypti* and *Hypothenemus hampei* mosquito larvae, producing paralysis after 6 hours of treatment and 73% mortality at 72 hours [20].

2.2.2 Efrapeptins

These molecules constitute a highly varied mixture of antibiotic peptides generated by some fungi, such as *Tolypocladium niveun*, *Beauveria nivea*, and *Tolypocladium*

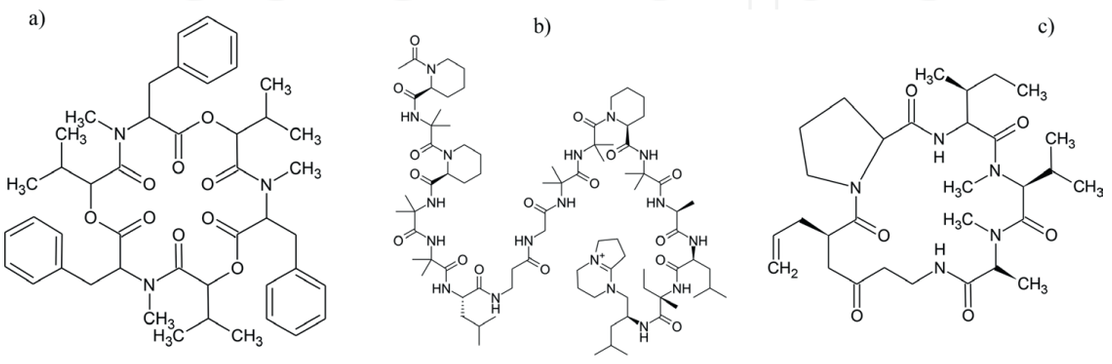

Figure 2.
Chemical structures of some toxins of a peptide nature. (a) Beauvericin, produced by Beauveria bassiana and Paecilomyces sp. (b) Efrapeptin F generated by Tolipocladium niveun, Beauveria nivea, and Tolipocladium cylindrosporum. (c) Destruxin a produced by Metarrizhium anisopliae.

cylindrosporum [21]. A nonpolar extract from a culture broth of *Beauveria bassiana* showed toxic activity against the red potato beetle. In fact, this extract, after appropriate fractionation, resulted in a peptide fraction identified as a mixture of efrapeptins, which was isolated by HPLC in five different peaks. These peaks were purified and characterized chromatographically, turning out to be rich in alpha-aminoisobutyric amino acid, and contained at least 15 other amino acid residues, in addition to having the acetylated N-terminus [22]. Efrapeptins produced by T. *cylindrosporum* have been shown to interfere with agglutinin in *Galleria mellonella* larvae, suggesting that these metabolites may interfere with ligand-receptor interactions that occur at the plasma membrane, allowing us to infer that most interactions between cells and humoral components of the insect immune system are receptor-mediated [23].

2.2.3 Destruxins

They are the best-characterized compounds since their mode of action also inhibits DNA, RNA, and protein synthesis in insect cells. One of the first studies aimed at the detection of fungal toxic substances has been on *Metarhizium anisopliae* strains, leading to the isolation of two cyclic depsipeptides that were called destruxins A and B. Today, 14 species of destruxins with variable insecticidal activities are known. This type of toxin has been analyzed and is capable of inhibiting the secretion of fluids through the Malpighi tube in *Schistocerca gregaria* [24]. The destruxins A, B, and E that have been produced by *M. anisopliae*, exhibited insecticidal properties when tested in *Plutella xylostella* larvae, with a high level of mortality in insect populations. They also caused deformations in the elytra and forewing of the insect. The influence of destruxin E on calcium flux and protein phosphorylation has also been observed *in vitro* cultures of Lepidoptera [25].

On the one hand, bassiacridin is a toxic protein, it has been purified from a strain of Beauveria bassiana by chromatographic methods. In a recent study, bassiacridin showed no affinity for anion exchangers and was characterized as a 60 KD monomer and an isoelectric point of 9.5. Furthermore, this molecule was shown to have β-glucosidase, N-galactosidase, and N-acetylglucosaminidase activity, as well as a proven insecticidal action [26].

3. *B. thuringiensis* secondary metabolites

δ-endotoxins produced particularly by *B. thuringiensis* (Bt) are parasporal protein bodies made up of polypeptide units of different molecular weights, from 27 to 140 kDa. Currently, 300 holotypes of *B. thuringiensis* toxins have been reported, classifying them into 73 Cry and 3 Cyt families. Bt toxins are produced during the sporulation phase, the Cry (crystal) protein is known for its specific toxic effects on a target organism (most belong to the order of insects), likewise the Cyt (cytolytic) proteins have been related with toxic effects on a wide variety of insects, mainly Diptera; however, its cytotoxicity against mammalian cells has also been proven [27].

Furthermore, during the vegetative cycle, *B. thuringiensis* produces soluble virulence factors of various types; some researchers have proposed that they be considered additional virulence factors to δ-endotoxins, which would explain the high pathogenicity of this bacterium toward its target organisms. In addition, some of these alternative virulence factors include -β-Exotoxins (Thuringiensins), nucleotide analogs of ATP and UTP that are synthesized by enzymes whose genes are found on

conjugative plasmids, compete for RNA polymerases and thus block rRNA synthesis in mammals; by inhibiting transcription, they exert mutagenic activity. They are used for house fly control in Africa. VIP proteins (Vegetative Insecticidal Proteins), these proteins have crystallized and contain a domain similar to the active site of proteins with ADP-ribosylation activity SIP proteins [28].

3.1 Cry proteins

Cry toxins were the first described proteins in *B. thuringiensis*, being the most diverse group, with 78 different protein families and a total of 817 described proteins. Cry proteins are active against a wide range of insect species (Lepidoptera, Coleoptera, Aphids, Nematodes, etc.). The continuous and growing discovery of a wide variety of Cry toxins then demanded the creation of a nomenclature system that would allow them to be classified in an orderly manner. The classification system for these toxins was designed and proposed by the *B. thuringiensis* Toxin Nomenclature Committee, which was based on the identity percentage of the amino acid sequence of each protein. In this way, when a new *B. thuringiensis* toxin is discovered, it is assigned a name by comparing it with its closest counterpart [29].

Additionally, the mechanism of action of Cry proteins was mainly described in Lepidoptera as a multistep process. *B. thuringiensis* crystals are ingested and then solubilized in the insect's midgut, after which the crystal proteins are released as protoxins. These will not cause damage *per se* but must be processed by intestinal proteases to generate active toxins that will lead to the death of the larva. In their monomeric form, the toxins cross the peritrophic membrane and bind univalent to cadherin, with great affinity for the apical face of the epithelial membrane. Then, according to studies carried out in insect cell cultures, a magnesium ion-dependent signaling cascade is initiated that would be responsible for cell death [30].

Furthermore, the initiation of this signaling cascade stimulates the exocytosis of cadherin from intracellular vesicles to the apical membrane of the cell and increases the number of receptors; therefore, it recruits a greater number of free toxins that would amplify the initial signal. On the other hand, based on *in vitro* experiments and bioassays, it is proposed that the binding of monomers to cadherin facilitates proteolytic cleavage on the N-terminus of the toxin. This last cleavage induces the assembly of the monomers and a pre-pore oligomeric form is established. This structure increases the affinity for a secondary receptor, such as aminopeptidase N or alkaline phosphatase. Finally, binding to this second receptor facilitates the formation of a pore in the epithelium of the midgut, which causes an osmotic imbalance and the consequent cell lysis. The intestinal tissue is seriously damaged, which prevents the assimilation and retention of vital compounds for the larva and leads to the death of the insect. Death can be accelerated by germinating spores and proliferating vegetative cells in the insect's hemocoel [31].

Due to the success in biological control, some brands have developed commercial products based on *B. thuringiensis*. For example, The Abbott Company has, among others, products based on the kurstaki serovars like Dipel (cry1Aa, cry1Ab1, cry1Ac1, cry2Aa1, cry2Ab1 genes) and Xentari (*B. thuringiensis* aizawai with cry1Aa1, cry1Ab1, cry1Ba1, cry1Ca1, cry1Da1 genes) that they are lethal against lepidopteran insects. Ecogen Inc. has promoted the biopesticides Lepinox (based on *B. thuringiensis* kurstaki) and Crymax (*B. thuringiensis* strain ED7826 with cry1Ac, cry2A, and cry1C genes) for lepidopteran lethality. Biochem has developed a formula based on *B. thuringiensis* israelensis for the control of Diptera (Cry4Aa1, Cry4Ba1,

Cry10Aa1, Cry11Aa1 toxins). Mycogen developed M-trak, a product for which it was sued by the company Novo Nordisk for misuse of the strain [32].

3.2 Cyt proteins

Cyt proteins have been found in the parasporal crystal produced by *B. thuringiensis* and they are active against Diptera. These proteins present cytolytic activity *in vitro* assays so that Cyt proteins break intestine cells due to their cytolytic activity and not by forming pores like Cry proteins. So far, known members of the Cyt proteins include the Cyt1, Cyt2, and Cyt3 protein families. Cyt proteins have a molecular weight of 28 kDa, which is a cytolysin of nonspecific action, produced mainly by the israelensis variety, accumulating in the crystal, together with the endotoxins typical of this variety. Because they have no homology to the other Cry proteins, the Cyt toxins are not classified as endotoxins. The general structure of Cyt proteins has an α/β-like domain with a β-sheet in the center surrounded by two α-helicals. The central β-sheet consists of six antiparallel β-strands, flanked by a layer of α-helices [33].

According to the studies of Ref. [34], the amplification of fragments of the expected size was obtained by PCR using pairs of oligonucleotide primers that detect the genes cry4Aa, cry4Ba, cry11Aa, cry11Ba, cyt1Aa, cyt1Ab, and cyt2Aa. The analyzes confirmed that, of the 1073 isolates subjected to PCR, only 45 (4.2% of the total) presented amplification for a single gene or the combination of them among some isolates. Of the 45 isolates (specific to Diptera) of *B. thuringiensis* subjected to selective bioassays, 13 (28.9%) presented 100% mortality against *A. aegypti* larvae, which were subjected to quantitative bioassays for the calculation of LC_{50} and LC_{90}.

The relationship between toxicity and gene content of the *B. thuringiensis* isolates studied suggests a joint action of the toxins in the control of *A. aegypti*. Isolates carrying three or more Diptera-specific genes (cry and cyt) were effective in selective bioassay tests, causing 100% mortality, which is equivalent to treatment with the standard strain *B. thuringiensis* var. israelensis. Some isolates showed three combinations of genes and showed no control efficacy against insect larvae. In these combinations, the cry4Aa and cry4Ba genes were not present and, in isolates 574 and 582, the cry11Aa gene was not present either [33, 34].

In studies carried out by [35], the strains LBT-63 and LBT-87 have shown amorphous crystals very similar to those found in *B. thuringiensis* subs. Israelensis; however, strains LBT-62 and LBT-99 showed very small inclusions attached to the spores. The evaluation of the biological activity of strains LBT −62 and LBT 83 and LBT-99 against *D. melanogaster* have shown how the LBT-87 strain has a 70% mortality in the larvae. Of the larvae that managed to survive and form pupae, they never reached the adult stage. The remaining strains only slightly delayed pupal formation, but adult emergence eventually occurred. Although the LBT-87 strain showed an effect against *D. melanogaster*, the virulence was lower than that obtained with the standard strain *B. thuringiensis* subs. Israelensis, specific for Diptera.

3.3 VIP proteins

VIP proteins (Vegetative Insecticidal Proteins) constitute another family of insecticidal proteins produced by some strains of *B. thuringiensis*. Such proteins do not resemble Cry proteins and are produced during the vegetative phase of the bacterium, in addition to being soluble, do not form crystals and have the same magnitude of toxicity as Cry proteins. In addition, the insecticidal spectrum of VIP proteins includes

Coleoptera (VIP1 and VIP2) and Lepidoptera (Vip3), including some species of insect pests that are insensitive to the action of Cry proteins. The *Vip2A(a) and Vip1A(a)* genes of some *B. thuringiensis* strains are known to be located in plasmids. Also, the proteins encoded by these genes contain a signal peptide typical of secretory proteins in the N-terminal region. In addition, it is known that both Cry and VIP proteins, toxic to beetle larvae, have similar sequences and probably a similar mode of action, since both are based on binding to specific membrane receptors of midgut epithelial cells of the target insects and in the consequent formation of pores [36].

Through histopathological observations, it was possible to verify that the epithelial cells of the midgut of susceptible insects are the main target of the insecticidal protein VIP3A, which causes intestinal paralysis, complete cell lysis, and the consequent death of the larvae. Thus, disruption of intestinal cells appears to be the main mechanism for the lethality of VIP proteins. In addition, the production of the VIP3A protein by vegetative cells after spore germination is an important factor in combined spore toxicity in insect species in which the Cry proteins are relatively inactive [37].

3.4 S-layer proteins

The surface layers (S-layers) proteins are known as monomolecular crystalline arrays of proteinaceous subunits, these compounds are over the surfaces of many bacteria and archaea [38]. Besides, S-layer proteins frequently have demonstrated a big capacity for self-assembly during their formative stage. Several signal peptides and three S-layer homology (SLH) domains, which are anchored to the cell surface are found at the N-terminal part of S-layer proteins and various S-layer genes from *B. thuringiensis* have been cloned and sequenced [39]. Among these *B. thuringiensis* strains, only one S-layer of *B. thuringiensis* subsp. finitimus strain CTC has been thoroughly studied for its biochemical characterization [40].

According to Ref. [41], four *B. thuringiensis* strains whose parasporal inclusions contained the S-layer protein (SLP) and cloned two slp genes from each strain. So, phylogenetic analysis indicated these SLPs could be divided into two groups, SLP1s and SLP2s. To confirm whether SLPs were present in the S-layer or as a parasporal inclusion, strains CTC and BMB1152 were chosen for further study. Western blots with isolated S-layer proteins from strains CTC and BMB1152 in the vegetative phase showed that SLP1s and SLP2s were constituents of the S-layer.

On the other hand, Ref. [42] reported the identification of an S-layer protein by the screening of *B. thuringiensis* strains for activity against various insect pests as the coleopteran *Epilachna varivestis* (Mexican bean beetle; Coleoptera: Coccinellidae). Some of the *B. thuringiensis* strains assayed against *E. varivestis* showed moderate toxicity. However, a *B. thuringiensis* strain (GP1) that was isolated from a dead insect showed remarkably high insecticidal activity. The parasporal crystal produced by the GP1 strain was purified and shown to have insecticidal activity against *E. varivestis* but not against the lepidopteran *Manduca sexta* or *Spodoptera frugiperda* or the dipteran *A. aegypti*.

4. Development and escalation in the production of secondary metabolites

In general lines, the development of biopesticides has a close relationship with the study and exploitation of modern biotechnology, therefore, so that the entities related to such initiatives (industry, universities, and research centers, among others) can

generate innovation and technological development requires a highly qualified human resource of a multidisciplinary nature [43]. In this context, technological development can be understood as the use of existing scientific knowledge for the production of new materials, devices, products, procedures, systems, or services, as well as for their substantial improvement, which includes prototyping and pilot installations [44]. However, the technological development of a microbial biopesticide is a complex task that involves not only technical-scientific stages but also stages associated with the analysis of economic viability and market potential, as well as compliance with national regulations and regulations for bioproducts [45].

Other important aspects in the development of a biopesticide are the costs of implementation and execution, the relative complexity, and the duration of each stage. Samada et al. [46] have described as technological development progresses, possible microorganisms or candidate isolates are screened, but, in turn, the costs, complexity, and time required increase due to multiple tests, bioassays, analysis, and studies that must be carried out to ensure the efficiency and reproducibility of the bioproduct under controlled, semi-controlled, and field conditions. Once all the stages of the development of a biopesticide have been executed (which can take between 4 and 5 years), it can be ensured that the technological development is efficient, effective, and stable, which allows projecting the commercialization and distribution of the biopesticide in the emerging market of bioproducts worldwide.

The need for sustainable development of agriculture has fostered many initiatives that have stimulated the development of alternative methods that reduce the use of chemical pesticides in pest control. Microbial biopesticides, therefore, represent one of the most promising alternatives and, although their commercialization remains marginal, the demand is constantly increasing in all parts of the world. This significant increase coincides with the growth of biological pest control in high-value crops, such as greenhouse-produced vegetables, fruit crops, vineyards, and forestry, among others. On the other hand, despite the fact that biological control has shown an important development in organic agriculture, the most promising future of biopesticides is found in integrated pest management (IPM) programs [47].

Even though there are significant advances in scientific and technological knowledge on the development of biopesticides, there are few cases in which formulations with a high and consistent biocontrol activity have been produced, which also have a wide spectrum of use and respond to the challenges cheaply. Although many bioproducts have been developed, several of them have been withdrawn from the market before achieving commercial success. In recent years, there have been important advances in the development and industrial production of biopesticides, but they still occupy a small percentage of the products used for crop protection. In many cases, research centers or companies develop excellent biopesticides but fail to position them before producers. For this reason, a product is only effective if it generates impact in field conditions and if it is supported by a solid market strategy that guarantees reliability and profitability [48].

5. Conclusions and perspectives

The production of a biopesticide not only requires detailed knowledge of the microbiology and physiology of the biocontrol microorganism but also knowledge of the biology of the pest, the epidemiological aspects that define its harmful effect on the crop, as well as the physiology of the pest. Plant. In addition to this, several

technological challenges related to the fermentation process, the type of formulation, the pest population versus the biocontrol microorganism population, and the application systems used must be addressed. Therefore, before the development and application of a formulation, it is necessary to understand the ecology of the interaction between the biocontrol microorganism, the host plant, and the pest. Likewise, in the development of biopesticides, each step must be considered: the selection of the microorganism, the production method, the delivery system, the application technology, the factors that affect its development and persistence in the environment and, ultimately, instance, the availability of the product in the market and the various positioning actions of this to achieve recognition and acceptance by farmers.

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Conflict of interest

The authors declare no conflict of interest.

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