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Rayhane Hamrouni, Flor Regus, Anne Marie Farnet da Silva, Thierry Orsière, Jean-Luc Boudenne, et al.. Current status and future trends of microbial and nematode-based biopesticides for biocontrol of crop pathogens. *Critical Reviews in Biotechnology*, In press, 10.1080/07388551.2024.2370370 . hal-04690560

HAL Id: hal-04690560







<https://hal.science/hal-04690560v1>

Submitted on 1 Oct 2024

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Current status and future trends of microbial and nematode-based biopesticides for biocontrol of crop pathogens

Rayhane Hamrouni^{a,b} , Flor Regus^{a,c}, Anne-Marie Farnet Da Silva^a , Thierry Orsiere^a , Jean-Luc Boudenne^b , Isabelle Laffont-Schwob^c , Pierre Christen^a, and Nathalie Dupuy^a 

^aAix Marseille Univ, Avignon Univ, CNRS, IRD, IMBE, Marseille, France; ^bAix Marseille Univ, CNRS, LCE UMR 7376, 13331, Marseille, France; ^cAix Marseille Univ, IRD, LPED, Marseille, France

ABSTRACT

The increasing public demand to avoid the use of synthetic pesticides and fertilizers in agricultural production systems, causing serious environmental damages, has challenged industry to develop new and effective solutions to manage and control phytopathogens. Biopesticides, particularly microbial-based biopesticides, are a promising new alternative with high biodegradability, specificity, suitability for incorporation into integrated pest management practices, low likelihood of resistance development, and practically no known human health risks. However: expensive production methods, narrow action spectra, susceptibility to environmental conditions, short shelf life, poor storage stability, legislation registry constraints, and general lack of knowledge are slowing down their adoption. In addition to regulatory framework revisions and improved training initiatives, improved preservation methods, thoughtfully designed formulations, and field test validations are needed to offer new microbial- and nematode-based biopesticides with improved efficacy and increased shelf-life. During the last several years, substantial advancements in biopesticide production have been developed. The novelty part of this review written in 2023 is to summarize (i) mechanisms of action of beneficial microorganisms used to increase crop performance and (ii) successful formulation including commercial products for the biological control of phytopathogens based on microorganisms, nematode and/or metabolites.

Introduction

Modern agricultural practices cause many environmental issues and one of their toughest challenges is to become more sustainable. No standardization in agriculture practices can be observed all over the world, but the intensive production methods and the use of indiscriminate agrochemical pesticides and fertilizers are more or less common [1,2]. The amounts of pesticides used in agriculture are growing sharply, especially in the developing world [3]. Around the globe, approximately 890 synthetic chemicals are approved as pesticides, whereas marketed products are estimated to be approximately 20 700 [4]. The use of pesticides is not evenly distributed among various crops, and data indicates that 93% of all row crops, such as corn, cotton, and soybeans, are treated with some type of pesticide, whereas the percentage of forage crops is less than 10. The reasons for this include the increased demand for food products [5] due to the increasing global population [6]. Synthetic pesticides provide

significant benefits to human populations and enjoy a great reputation for enhancing crop productivity and controlling several plant diseases. Nevertheless, notable environmental as well as health risks are linked to their use. In recent years, their role in damaging agroecosystems has been well documented and the occurrence of major pest resistance development, such as the Fall armyworm in 2016 in West Africa [3], has increased. Meanwhile, the scenario in “developing countries” is worrying: recent studies show that although these regions use only 20% of the world’s agrochemicals, they suffer 99% of deaths induced by pesticide poisoning [7]. According to the World Health Organization [8], the victims are overwhelmingly farmers. Occupational exposure to very high levels of pesticides is of concern to farmers in agricultural countries (Spain, China) mainly due to a lack of technical education. The annual deaths due to pesticide exposure are approximately 20 000 [7]. Recent studies and reports showed epidemiological and toxicological evidence for

the various health effects associated with pesticides [2,7]. Evidence of the short-term negative effects of pesticides on human health includes rashes, headaches, nausea and vomiting, disorientation, shock, respiratory failure, coma, and the most severe cases leading to death [9,10]. Toxicological studies conducted in animals as well as some epidemiological studies showed that several pesticides to which workers and the general population may be chronically exposed are potentially: carcinogens, neurotoxins, reproductive toxins, and immunotoxins. Many scientists also report the impacts of pesticides on biochemical parameters, particularly on protein metabolism, and endocrine and reproductive systems [11]. In addition to human health concerns, aquatic organisms are directly exposed to pesticides resulting from surface run-off [12].

Another major dependency of agriculture is on synthetic fertilizers, which are widely used to enhance soil productivity. However, the application of synthetic fertilizers is currently increasing with the increase of sustainable crop production and the population's demand for a better nutritional quality of food [13]. However, the persistent use of synthetic fertilizers affects long-term soil fertility. Over the last few years, a great number of experiments have been initiated to examine the effects of synthetic fertilizers on soil quality and soil fertility [14,15]. These studies concluded that in one way, the use of synthetic fertilizers was necessary, and continuous application increased concentrations of soil organic matter, and total nitrogen. On the other hand, the use of those fertilizers also resulted in the decline of soil fertility and productivity, causing air, soil, and water pollution in the long run. The work of Zenget al. [13]

showed that excessive use of nitrogen and phosphorus fertilizers induced various negative effects in the environment, such as nitrate accumulation in plant products and soil acidification during vegetable production.

Additionally, Meena et al. [16] reported that microbial diversity and activity are also affected by the application of fertilizers. These findings and reports of the adverse effects of conjugated use of synthetic pesticides and fertilizers on natural resources, animal and human health, are driving projects for greener and safer alternatives compatible with both crop productivity and control of diseases at a global scale. Concomitantly, there is increasing public pressure to replace, if possible, synthetic fertilizers and pesticides by sustainable alternatives. For example, the French government (Ecophyto II+plan) has encouraged the use of biopesticides in order to reach a 50% reduction of synthetic pesticide use by 2025 [17].

Among the biological control strategies under development, the most important ones are biopesticide and biofertilizer formulations using biological control agents (BCA), such as bacteria, fungi, viruses, and their combinations (Figure 1) [18]. Particularly, the frequent use of fungi in bioformulations reflects their role in nature as balancers of the populations of ticks, aphids, and other insects, and many other pests affecting crops. Worldwide, the biofungicide market is growing annually at a rate of 44% in North America, 20% in Europe and Oceania, 10% in Latin and South American countries, and 6% in Asia [19]. Meanwhile, various researchers all over the world are focused and continuously engaged in developing new formulation products which could: (i) be easy to use, (ii) show enhanced

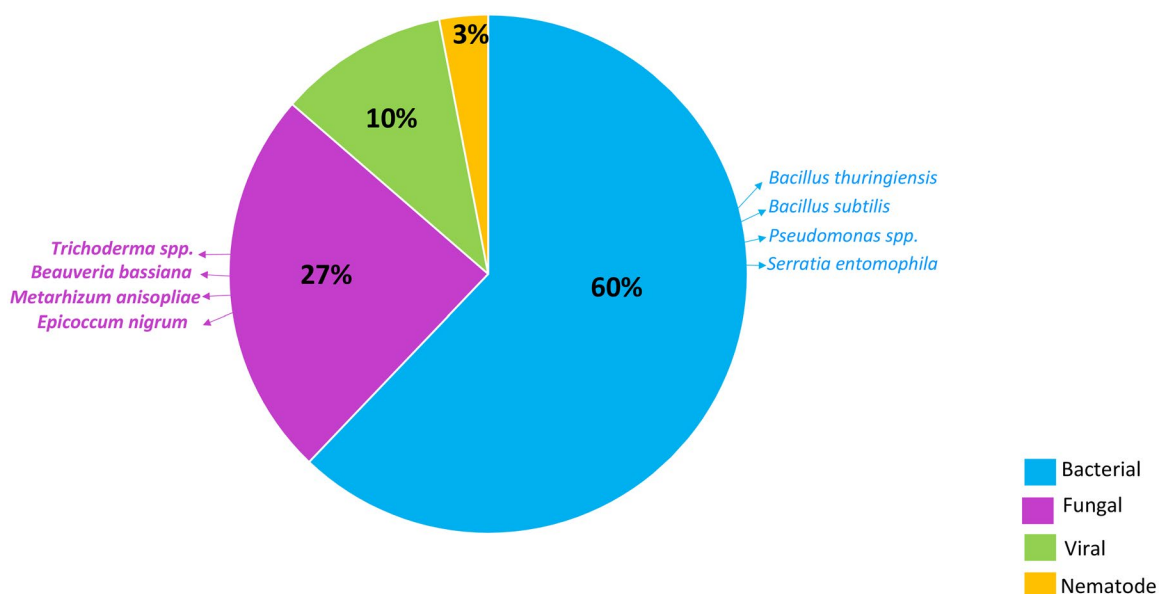


Figure 1. Types of biopesticides used worldwide.

activity toward phytopathogens, (iii) cover more target crops, and (iv) be safer by design for the ecosystems including humans. In 2016, a comprehensive overview of microbial formulations, including those containing fungi, was presented by Kumar et al. [20]. However, the formulation methodologies used to preserve functionality during storage and distribution have not been fully reviewed.

The aim of this review is to summarize the progress to date in the use of biocontrol agents for agricultural application and to describe the most important metabolites produced by these biocontrol agents while also emphasizing their biological mechanism as biological control.

A brief history

The strategy of disease management and reduction of phytopathogen pressure using biocontrol agents represent a part of historical events that took place in the field of soil microbiology. Describing all of these events is a heavy task and is beyond the scope of this review. However, some of the discoveries which strengthen the field of biological control and of biofertilizers are discussed here. The major events in biopesticide history are summarized in Figure 2.

In 1838, the French chemist Boussingault first recognized that leguminous plants could use nitrogen (N₂) from the air [21] and this information was confirmed by Hellriegel and Wilfarth in Germany, who demonstrated that pea plants in association with bacteria in their root nodules are capable of using gaseous nitrogen [22].

Historical trends that have opened opportunities in developing biopesticides first began in Italy when Bassi discovered that a fungus, *Beauveria bassiana*, caused an infectious disease in silkworms [23]. The bacterium *Bacillus thuringiensis* was the initial microorganism-based biopesticide used and has had the most widespread use to date. In 1901, *B. thuringiensis* was isolated from

a diseased silkworm by Japanese biologist Ishiwata [24]. In 1920, *B. thuringiensis* was first used in France as a biological insecticide. In early 1938, the initial commercially available *B. thuringiensis* product, sporeine, appeared in France [25]. Commercial success stories from the 1980s and 1990s include products containing *Agrobacterium radiobacter* to prevent crown gall on woody crops, as well as *Pseudomonas fluorescens* used for the prevention of firelight in orchards where the streptomycin had been over-used and resistant pathogen populations were abundant. Since 1995, over 100 active ingredients have been registered by the United States Environmental Protection Agency (USEPA). Many of the active ingredients currently approved for use in the US can be found in publicly available databases. Nowadays, approximately 1400 biopesticide products are being sold and the number of registered products is increasing day by day.

Biological control agents: an improved alternative

Bacteria

Bacillus thuringiensis is a well-known biocontrol agent (BCA) against several insects in agriculture, such as *Plutella xylostera*. This bacterium's main characteristic is the synthesis of crystalline inclusions containing proteins called "crystalline proteins" (δ endotoxins), or Cry proteins, during sporulation. These proteins have insecticidal properties [26]. The mode of action is the following (Figure 3): after ingestion, the crystals are solubilized by the alkaline conditions in the insect midgut and are subsequently proteolytically converted into a toxic core fragment [27].

There are over 40 *B. thuringiensis* products (e.g., LARVECT 50, Mosquito Dunks, Monterey B.t.) available in the market for controlling: caterpillars, beetles and blood-feeding flies, such as mosquitoes. Altogether, this accounts for 1% of the total insecticide market [28].

Bacillus subtilis is a lipopeptide producer that has antibiotic properties against several phytopathogenic

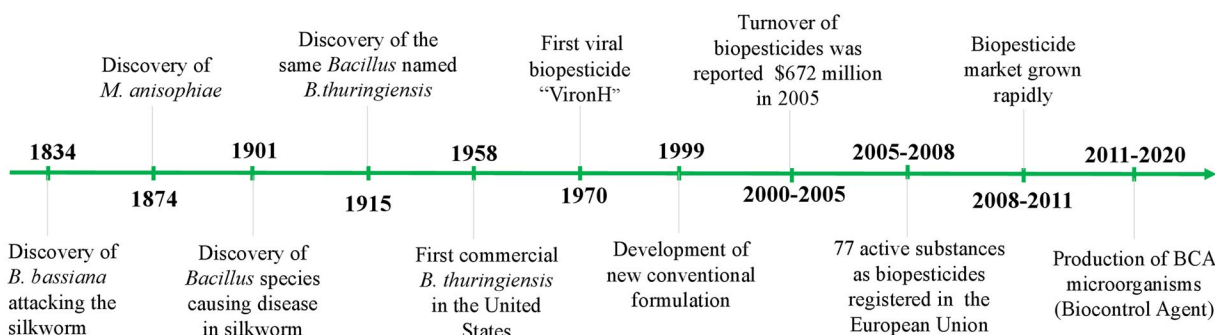


Figure 2. Timeline of key events in biopesticide development.

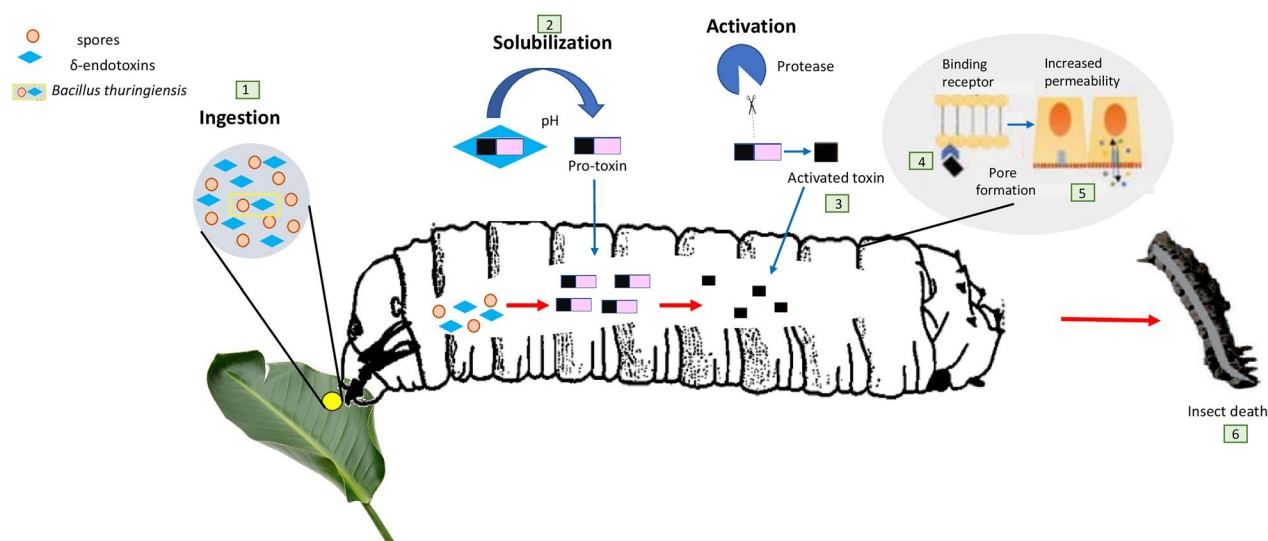


Figure 3. Mechanism of action of Cry proteins with insecticide action produced by *Bacillus thuringiensis*.

fungi [29] and has been used in biopesticide formulation. *B. subtilis* has been reported as an endophyte and has the ability to inhibit soil phytopathogens, such as *Xanthomonas campestris*, *Ralstonia solanacearum*, *Fusarium oxysporum* and *Sclerotium rolfsii* [30]. Shih et al. [29] showed that a *B. subtilis* strain achieved a production yield of 11.435 mg of Iturin A, a lipopeptide antibiotic, per gram of fermented material when using solid-state fermentation (SSF) including gluten-containing flour and rice bran as substrates. They reported that this production was 10-fold over submerged fermentation, showing that SSF is an efficient technology.

Another BCA is *Serratia entomophila*, a naturally controlling agent for the grass grub *Costelytra zealandica*, and against Lepidopteran pests [31]. Grimont et al. [32] have first reported *S. entomophila* as a natural biocontrol agent against *C. zealandica*. Later, a Mexican strain (*S. entomophila* Mor4.1) was also found to be active against another white grub, *Phyllophaga blanchardi* [33]. *S. entomophila* AB2 was also reported to be a biocontrol agent against *Lepidopteran* pests. Recent studies have shown no significant effect of this bacterium and its toxins on the vertebrates. However, a few toxic effects on some non-target arthropods and crustaceans have been recorded.

Actinobacteria represent another class including species with a significant potential of biocontrol agents. An insecticidal *Streptomyces* species exhibited the production of a variety of insecticidal toxins, such as macrocyclic lactone derivatives, acting on the insect's peripheral nervous system [34]. Similarly, *Burkholderia rinojensis*, a beta proteobacterium, has been recently discovered and developed into a product acting against diverse chewing and sucking insects and mites following ingestion

and/or contact. Their insecticidal action relies on different metabolites and the commercial product is based on heat-killed cells and spent fermentation media [35].

Fungi

Filamentous fungi have generated a special interest because they have a higher spectrum of disease control and high biomass yield [36]. They are phytopathogen's natural enemies.

To be considered a good BCA candidate, a fungus must have more than one mechanism of action against phytopathogens. There are three main mechanisms of control: (i) hyperparasitism: when entomopathogenic fungi come in contact and adhere to the insect cuticle, their spores germinate, then, assisted by the secretion of lytic enzymes, the germ tube penetrates the cuticle (24–48 h). Fungi grow inside the insect, finally fully taking over its host's body (5–7 days). Once infected, the insect body supports the development of conidia which eventually spreads new spores reaching new hosts [37,38], (ii) antibiosis: the production and release of several compounds that delay or inhibit the microorganism's growth, (iii) competition for ecological niches, or the induction of local and systemic defence responses (Figure 4).

Trichoderma spp. are ubiquitous soil fungi, which can play an important role as BCA. They are very popular microorganisms in agriculture mainly due to their capabilities to act as highly efficient biofertilizer and biopesticide. Thus, the *Trichoderma* species could be a good model for biocontrol application.

After the publication of *Trichoderma viride* acting as a parasite on other fungi in 1932 [39], knowledge

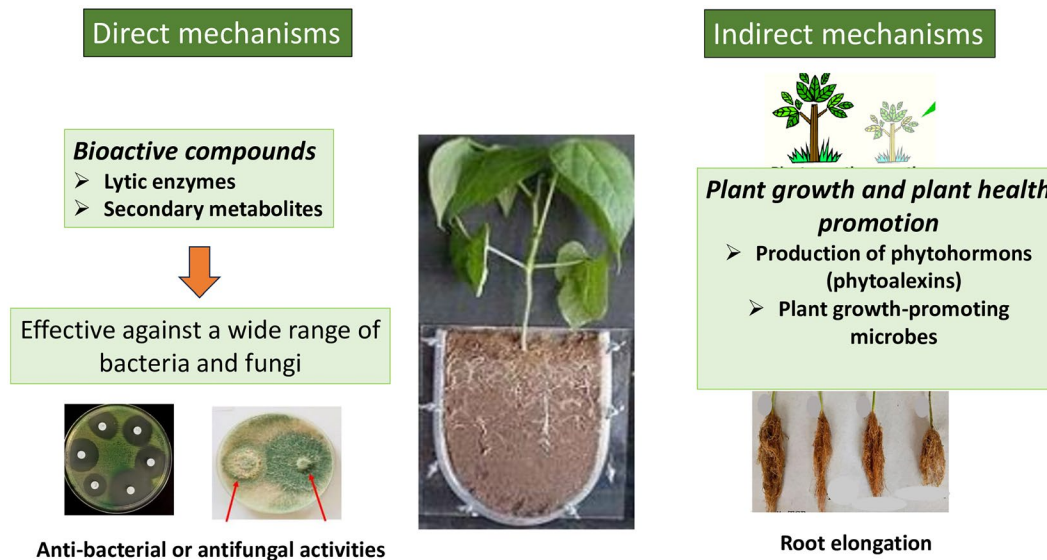


Figure 4. Biopesticides: application and possible mechanism of action.

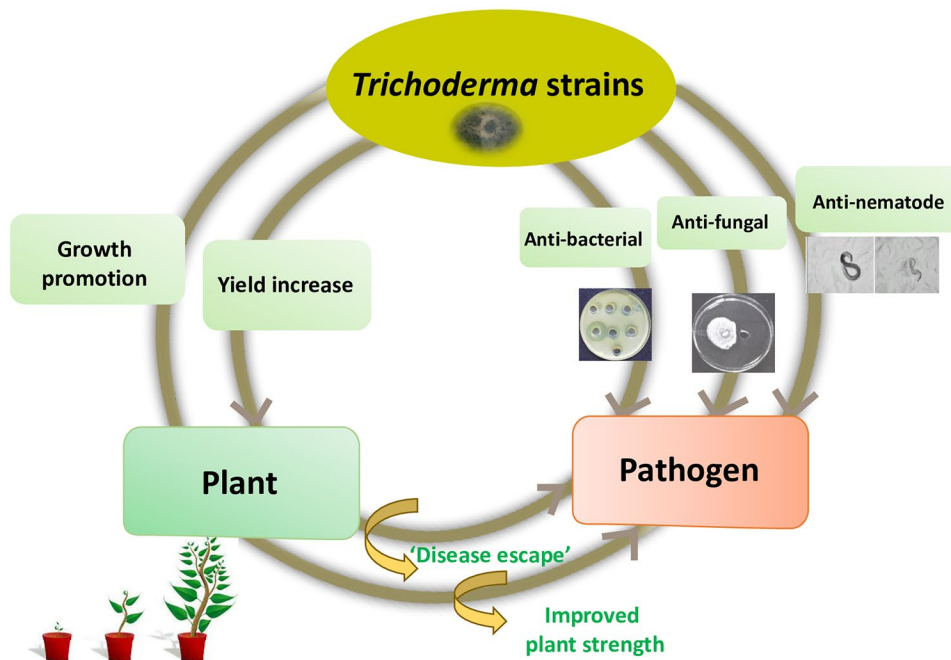


Figure 5. Potentially useful biocontrol activities of *Trichoderma* spp. Strains.

relative to the antagonistic properties of *Trichoderma* strains has progressed rapidly. To date, the most important species in this field are *T. harzianum* (in earlier reports sometimes misidentified as *T. atroviride*), *T. virens*, *T. viride* and *T. asperellum*. In their defensive actions, *Trichoderma* strains apply lytic enzymes [40], proteolytic enzymes [41], ABC (ATP-binding cassette) transporter membrane pumps [42], and secondary metabolites (mycotoxins, fragrance, etc.) [43].

The *Trichoderma* genus is considered as one of the most popular BCA against several plant pests due to

the diversity of its mechanisms of action (Figure 5). The competition for nutrients and space, the production of antibiotics, such as volatile metabolites and other secondary metabolites, are active tools against their hosts and mycoparasitism mediated by the production of lytic enzymes. The *Trichoderma* genus can inhibit phytopathogenic fungi, such as *Fusarium oxysporum*, *Botrytis cinerea*, *Crinipellis perniciosus*, and *Rhizoctonia solani*, etc. [44,45].

Metarhizium is a genus of entomopathogenic fungi distributed globally from the Arctic to the tropics. It

has been widely studied because of its: narrow host range, safety, environmental friendliness, and ease of mass production [46]. An example is BIO 1020, a mycoinsecticide developed by Bayer (Germany), which contains *Metarhizium anisopliae* as an active ingredient to target pests from the *Coleoptera* species. Another example is Green Guard[®]; a mycoacaricide developed by Becker Underwood Inc. (Ames, IA, United States) that contains *Metarhizium acridum* as an active ingredient to target pests from the Orthoptera family [47].

Beauveria bassiana is one of the most used entomopathogenic fungi. Many laboratory assays showed its great effectiveness against various crop pests and it was the first example of microbial control on insects by the end of the nineteenth century. ***B. bassiana*** products represent 33.9% of all produced biopesticides aiming at herbivore insect treatments, e.g., *Lygus lineolaris*, *Cosmopolites sordidus*, *Xylosandrus germanus*, and *Panonychus citri*, that cause economic losses to farmers. Recent studies have highlighted the potential of some *B. bassiana* and *B. brongniartii* strains as endophytes in biological control applications [48].

Epicoccum nigrum is a natural enemy of several phytopathogen fungi, such as *Leucostoma cincta*, *Sclerotinia sclerotium*, *Colletotrichum gloeosporioides*, *Botrytis cinerea* and *Monilinia laxa* [49].

Coniothyrium minitans is commonly used for fungal disease control in beans, lettuce, potatoes, oil seeds, caused by *S. sclerotium* [50].

Clonostachys rosea represents another well exploited fungal species. It is a non-pathogen and saprophytic fungus, but it is a mycoparasitic microorganism considered to be a highly effective antagonist against *B. cinerea* [51].

Nematodes

Entomopathogenic nematode (EPN) species from genera *Heterorhabditis* and *Steinernema* have been effectively used as biological insecticides in pest management programs because they are considered nontoxic to humans, relatively specific to their target pests, and can be applied with standard pesticide equipment [52]. Early-stage juveniles penetrate the host insect *via* spiracles, mouth, anus, or, in some species, through intersegmental membranes of the cuticle, and release their symbiotic bacteria in the hemocoel. The life cycle of nematode–bacteria association is composed of two stages: (i) a free stage in the soil, where the infective juveniles carry bacteria in their guts and search for new insect hosts, and (ii) a parasitic stage, where the infective juveniles infect the insect, release

their bacterial symbionts and reproduce in order to produce new infective juveniles (IJs) [53].

Nematodes can also directly control various orchard insects. The larvae of the plum curculio, *Conotrachelus nenuphar*, is an important pest of pome fruits in North America. A single *Steinernema riobrave* ($0.4\text{--}4.0 \times 10^6/\text{m}^2$) application to the soil reduced fruit infestation by up to 80% [54].

The larvae of the false codling moth, *Thaumatotibia leucotreta*, an important pest of citrus in South Africa, cause infested fruits to fall to the ground. The mature larvae leave the fruit and burrow in the ground (1–8 cm depth) to pupate. The application of *Heterorhabditis bacteriophora* (10–20 infective juveniles (IJs)/cm²) to the soil reduced fruit infestation by up to 81%, but monthly applications at 5–10 IJs/cm² provided greater consistent control [55].

Research on the use of EPN against insect pests of corn, vegetable, and tuber crops, has been extensively summarized in Gassman and Clifton [56], Georgis et al. [57], Cabanillas et al. [58] and Bélair et al. [59]. The larvae of corn rootworms (CRW), *Diabrotica* spp., are among the most destructive pests of corn. In field scale experiments, effective control with EPNs ($2.8\text{--}3.5 \times 10^9$ IJ/ha) has also been observed. (75%–72%) with *H. bacteriophora*, (69%–67%) with *H. megidis*, (32%–91%) with *S. feltiae* [60].

The introduction of commercial nematode formulations has accelerated in recent years, but new formulation technology that produces stable, effective, and consistent products would expand markets even further [61,62]. Spray application using a nematode suspension in water is straight forward, inexpensive, and often effective. However, capsules, bait pellets, and granular formulations that protect and/or release nematodes in the soil are also desirable for insect control [62]. The quality of the final formulation plays a major role in the efficacy of nematode-based biological control applications against pests. This formulation must ensure the viability of the microorganisms, preserving their germinating power; and it must help the microorganisms keep their virulence against the pest involved. It is also essential to protect the BCA from the ultraviolet (UV) rays of the sun and to stabilize the product for both storage and utilization conditions.

Secretion of lytic enzymes from microorganisms

Diverse microorganisms secrete and excrete antifungal compounds that can interfere with pathogen growth and/or activities.

Biopesticides can have direct actions on the phytopathogen or, indirect effects by promoting plant health and thus its resistance to the pathogenic agent. The production of antibiotics, lytic enzymes, and secretion of substances can reinforce the host immune system or promote growth (Figure 4). Thus, many fungi produce lytic enzymes that hydrolyze a wide variety of polymeric compounds, including cellulose, hemicellulose, chitin, proteins, and DNA.

Ultrastructural and histochemical studies demonstrate that the expression and secretion of these enzymes by different microorganisms cause localized lysis of cell walls of phytopathogenic microorganisms at points of contact between the antagonist and the host, directly resulting in the suppression of plant pathogen activities [63].

Chitinases, glucanases, proteases, amylases, lipases, cellulases, and other hydrolytic enzymes are the most common cocktails of lytic enzymes capable of degrading cell walls. These enzymes are usually extracellular, of low molecular weight, and highly stable. They have been reported mainly in isolates of mycoparasitic fungi, such as *Trichoderma harzianum*, *Trichoderma viride*, *Trichoderma reesei* and *Trichoderma asperellum* [19,64].

Chitinases

The antagonistic activity of the chitinases of mycoparasitic fungi might be involved in the biocontrol of phytopathogens. They are considered as an effective tool for complete degradation of the cuticle of pathogen insects. Chitinolytic enzymes are classified into three types: (i) endochitinases, that cleave internal bonds within chitin, releasing chitotetraose, chitotriose, and chitobiose, (ii) exochitinases, that catalyze the release of chitobiose without the formation of monosaccharides and (iii) N-acetylhexosaminidases that cleave chitobiose, chitotriose, and chitotetraose into N-acetylglucosamine monomers similarly to exochitinases [65].

In 1992, De La Cruz et al. [66] were the first to isolate, purify, and characterize chitinases of the mycoparasitic fungus *T. harzianum* used as a mean of biocontrol. The strain was grown in minimal liquid medium supplemented with chitin as the sole source of carbon, and three proteins exhibiting chitinase activity were isolated from the culture supernatant. Following protein purification using ammonium sulfate precipitation and adsorption to colloidal chitin, two endochitinases were characterized with molecular weights of 42 and 37 kDa, respectively.

Although the purification and molecular properties of chitinase enzymes have been proven in multiple reports, data on the efficiency of chitinase producers in

exerting biocontrol are limited to only few publications. So far, several chitinases, including endo, exochitinases and Nacetylglucosaminidases, have been tested on suppression of phytopathogenic fungi. It has been reported that three endochitinases (33, 37, and 42 kDa) of *T. harzianum* CECT 2413 are able to lyse the cell walls of *Botrytis cinerea* and inhibit spore germination and germ-tube elongation in several fungi [67].

Glucanases

β -1,3-Glucanases are widespread in fungi, bacteria, and higher plants. The fungal cell wall is generally composed of 90% polysaccharides (glucan and chitin), which are the main polymers of carbohydrates, as well as other polymers, such as α -1,3-glucan, α -1,4-glucan, β -1,3-glucan, β -1,4-glucan, β -1,6-glucan, chitosan, mannan, and galactomannan [68]. Glucans are also present in the cuticle of nematode eggs, such as in the phytoparasitic nematodes from the *Meloidogyne* genus, whose viability decreases severely when treated with the *Trichoderma* species [69]. β -glucanases play crucial roles in the morphogenetic-morphological processes during the development and differentiation stages of fungal life. Several types of these enzymes exist, classified according to the type of β -glucosidic linkage that they cleave and the mechanism of substrate attack: (i) exo- β -1,3-glucanases, hydrolyze the substrate by sequentially cleaving glucose residues from the non-reducing and (ii) endo- β -1,3-glucanases cleave β -linkages at random sites along the polysaccharide chain, releasing smaller oligosaccharides [70]. These enzymes also play a key role in the mycoparasitic activity of filamentous fungi against several plant pathogenic fungi, allowing their use as biocontrol agents [71]. There are several studies that report the production of glucanases enzymes under SSF. De la Cruz Quiroz et al. [72] reported an improvement in endoglucanase activity applying an aeration rate of 2 ml min⁻¹ g⁻¹ using an SSF with five *Trichoderma* strains (*T. longibranchiatum*, *T. harzianum*, *T. yunnanense*, *T. asperellum* T2-10 and *T. asperellum* T2-31). These indicated that forced aeration during the fermentation process increased the endoglucanase activity, and the best value was observed with *T. yunnanense* (38.32 U/g).

Cellulases

Cellulases are among the most important enzymes used in modern biotechnology, especially in the process of plant biomass hydrolysis that supports numerous biotechnological applications. These enzymes are extensively produced by the genus *Trichoderma* [19].

Certain biopesticides as previously stated, play a real in promoting plant health, resulting in a better response to pathogenic agents: The known function of cellulases is to degrade cellulose-rich plant cell walls. Enzyme cellulases play essential roles in the specificity, recognition, and adhesion of certain symbiotic fungi that produce lytic enzymes and antibiotics [73–75]. These enzymes can cause defense reactions in tobacco leaves, such as cytoplasmic contraction and nuclear accumulation. Major impediments to commercial exploitation of cellulases are currently: yield, stability and cost of production [76]. In the past few decades focus has been made on liquid fermentation and very little attention has been given to SSF [19,72].

Proteases

Proteases (peptidases or proteolytic enzymes) constitute a large group of enzymes that catalyze the hydrolysis of peptide bonds which can be specific, leading, for instance, to selective protein cleavage for post-translational modification and processing [77].

Microbial proteases have attracted a great deal of attention in the last decade due to their biotechnological potential in various industrial processes, such as in detergent, textile, leather, dairy and pharmaceutical preparations. In the past decade, this group represented approximately 60% of total enzyme sales in the world [78]. In biocontrol, proteases produced by *Trichoderma spp.* may be involved in the inactivation of extracellular enzymes of phytopathogenic fungi. In this context, it was demonstrated that certain hydrolytic enzymes produced by *Botrytis cinerea*, namely endo- and exo-polygalacturonases, were partially deactivated by proteases from *T. harzianum* isolates [79].

Fungal proteases have attracted the attention of environmental biotechnologists because fungi can grow on low-cost substrates and secrete large numbers of enzymes into the culture medium which could ease downstream processing [80]. In recent years, there have been attempts to produce different types of proteases through liquid fermentation or SSF. Each technique has particular advantages that the others are unable to match [81]. A great number of fungal strains have been used to produce proteases, such as *Aspergillus*, *Penicillium*, *Rhizopus*, *Thermoascus*, *Trichoderma*, among others.

Secondary metabolites that are toxic to other fungi

Biocontrol microorganisms have a key role to play as alternative solutions in agriculture due to their ability

to produce a wide range of antifungal compounds and for their ability to parasitize other (micro)organisms including fungi, bacteria, or insects. These bioactive molecules could be used world-wide for the protection of various crops and/or bio-fertilization (Figure 4) [82].

Microorganisms, particularly fungi, produce various SMs, showing several biological activities possibly related to biotic and abiotic factors, such as competition against other micro- and macro-organisms, symbiosis, and metal transport [83]. In addition, SMs are biosynthesized from primary metabolites in specialized pathways (polyketide or mevalonate pathways derived from Acetyl Coenzyme A, or amino acids) and some genes are clustered together. Some examples of SMs are **mycotoxins** (produced by fungi that colonize crops capable of causing disease and death to humans and other animals), **phytotoxins** (produced by fungal pathogens that attack plants), **pigments** with antioxidant activity and **antibiotics** (capable of inhibiting or killing microbial competitors) [19]. Based on their structure, known metabolites with antibiotic activity can be classified into two main types: (i) low-molecular-weight and volatile metabolites and (ii) high-molecular-weight and polar metabolites. Low molecular weight and volatile metabolites include simple aromatic compounds, volatile terpenes, and isocyanides, and relatively non-polar substances with significant vapor pressure [84].

It must be stressed that the following paragraphs and Table 1 report the most important SMs isolated from microorganisms that have shown a significant antifungal activity.

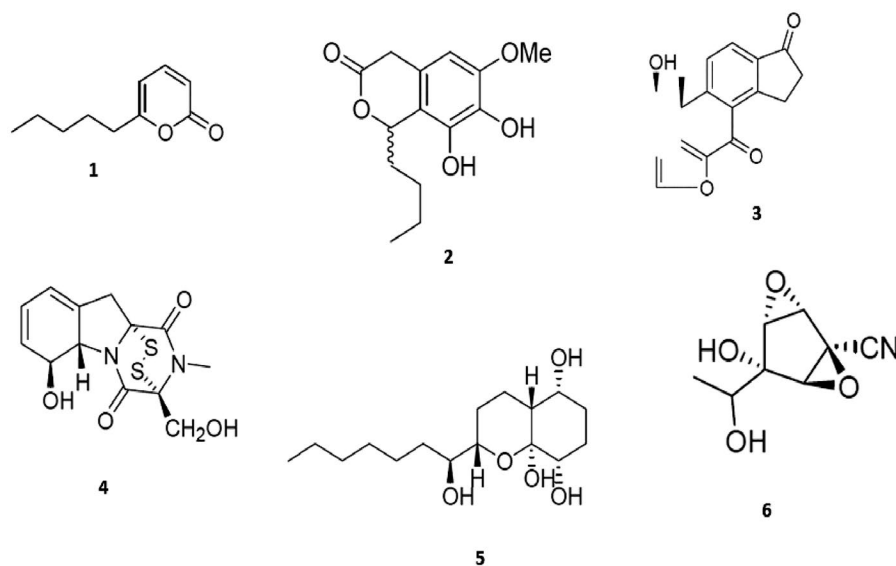
Pyrones

6-Pentyl- α -pyrone (6-PP) (Figure 6) is one of the major pyrones produced by *Trichoderma* strains. It was first isolated from *T. viride* and was then identified in the culture medium of different *Trichoderma* species (*T. asperellum*, *T. viride*, *T. atroviride*, *T. harzianum*, *T. koningii*). 6-PP is a volatile compound, responsible for the coconut aroma released by axenically developed colonies [85,86]. It is derived from the oxidation of the fatty acid linoleic acid. Although the main steps of 6-PP synthesis in *T. harzianum* have been elucidated, the enzymes involved in its biosynthesis still need to be identified [97].

6-PP is an example of biopesticides with direct effect on phytopathogens: the antifungal activities of this volatile compound have been proved both *in vivo* and *in vitro* toward several plant pathogenic fungi, such as *Rhizoctonia solani*, *Botrytis cinerea*, and *Fusarium spp.*, and a strong relationship has been found

Table 1. Examples of molecules with biopesticides activities for the biological control of phytopathogen.

Biopesticide sources	Microorganism species	Molecules with biopesticide activities	Phytopathogen	Target crop of the pathogen	Reference
Fungi	<i>Trichoderma harzianum</i>	Pyrones: 6-pentyl- α -pyrones, Cytosporone S Peptaibols: Trichorzianines A, B Trichokindins I–VII Harzianins HC Koninginins: Koninginins A, B, D, E and G Azaphilones: Harziphilone, fleepphilone:	<i>Rhizoctonia solani</i> <i>Acremonium cucurbitacearum</i> <i>Fusarium oxysporum</i>	Cocoa plant Bean plant Wheat Apple Maize	[85–87]
	<i>Trichoderma viride</i>	Peptaibols : Suzukacillin A Trichotoxin A40 Viridins: viridiol	<i>Fusarium caeruleum</i>	Spruce Pine Cowpea	[19,88]
	<i>Trichoderma virens</i>	Viridins: viridiol	<i>Rhizopus oryzae</i>	Cotton seeds	[89]
	<i>Trichoderma asperellum</i>	Pyrazines: Tetramethylpyrazine	<i>Fusarium oxysporum</i>	Tomato	[90]
	<i>Trichoderma aureoviride</i> <i>Trichoderma koningii</i> <i>Trichoderma longibrachiatum</i> <i>Trichoderma gamsii</i>	Peptaibols: Trikoningin KA, KB Atroviridins A–C Viridins: viridiol Koninginins: Koninginins A, B, D, E and G Secondary metabolites: β -exotoxin δ -endotoxins Lytic enzymes: Chitinases	<i>Sclerotinia sclerotiorum</i>	Sunflower	[91–93]
Bacteria	<i>Bacillus thuringiensis</i>	Plant hormone: Indole-3-acetic acid Secondary metabolites: Pantocin A, dapdiamide E Nonribosomal peptides: Surfactins, fengycins, iturins	<i>Spodoptera frugiperda</i> <i>Helicoverpa zea</i>	Maize Corn Sugarcane	[94]
	<i>Pantoea agglomerans</i>	Plant hormone: Indole-3-acetic acid Secondary metabolites: Pantocin A, dapdiamide E	<i>Erwinia amylovora</i>	Apples Pears	[95]
	<i>Bacillus subtilis</i>	Nonribosomal peptides: Surfactins, fengycins, iturins	<i>Listeria spp.</i> <i>Legionella spp.</i>	Bulb Rot	[96]

**Figure 6.** Chemical structures of (1) 6-pentyl- α -pyrone, (2) cytosporone S, (3) chaetoviridin, (4) Gliotoxins, (5) Koninginins, (6) Trichorzianin TA.

between the biosynthesis of this metabolite and the biocontrol ability of the producing fungi.

Rosales et al. [88] reported auxin-like activity of 6-PP on pea, tomato, and canola, with a plant-growth promoting effect at low concentrations and a plant-growth inhibiting effect at higher concentrations. Bello et al. [98] observed that 6-PP significantly affects: germination, germ tube growth, colony formation, and colony size of phytopathogens. The major limitation to its field use is related to the cost of its synthesis. Hamrouni et al. [40] reported a comparison of 6-PP production under SSF and liquid fermentation. The authors obtained a significant difference in 6-PP biosynthesis with a higher 6-PP production using SSF.

Cytosporone S (Figure 6) is another pyrone, recently isolated from a *Trichoderma* strain. It has been reported to have *in vitro* antibiotic activity against several bacteria and fungi [84].

Other pyrones with antifungal activity, named solanopyrones C, N, and O, were identified from the endophytic fungi *Nigrospora* sp.YB-141 and *Botryosphaeria dothidea* KJ-1. The *in vitro* antifungal activities of these SMs were demonstrated against *B. cinerea* and *Penicillium islandicum*.

Peptaibols

The name “peptaibol” is formed from the names of the components: peptide, aib (α -aminoisobutyric acid) and amino alcohol. Peptaibols are short linear amphipathic polypeptide chains containing a high proportion of non-proteinogenic amino acids, such as alpha-aminoisobutyrate and isovaline. They are classified into three groups depending on their chain lengths [99]: (i) short peptaibol sequences with 11–16 amino acid residues (e.g., harzianins), (ii) lipopeptaibols with 6 or 10 residues (e.g., trichogins), (iii) long peptaibol sequences with 18–20 residues (e.g., trichorzianins).

Peptaibols are solely described in filamentous fungi exhibiting a mycoparasitic lifestyle, with a high abundance of over 80% of all known substances being derived from *T. harzianum* [100]. Other mycoparasites, such as *Tolypocladium ophioglossoides*, *T. viride*, *Escovopsis weberi*, *Gliocladium*, *Acremonium*, *Emericellopsis*, and, *Paecilomyces* genera also comprise peptaibiotic-associated gene clusters, which are absent in plants and other entomopathogenic species of the fungal order of the Hypocreales, suggesting the restriction of these genes to mycoparasitic species.

Peptaibols are thought to act on membranes of the target fungus to inhibit membrane-associated enzymes involved in cell wall synthesis [101]. Previous

reports described the conformational properties and biological activities of the peptaibols of filamentous fungi. It has been demonstrated that peptaibols inhibit β -glucan synthase activity in the host fungus, preventing the synthesis of its cell walls, and it can act synergistically with *T. harzianum* β -glucanases [87]. Tamandegani et al. [102] demonstrated that the peptaibol « Trichorzianin TA » (Figure 5) produced by *T. harzianum* inhibited *in vitro* *B. cinerea* β -1,3-glucan synthase activity. Two antifungal peptaibols (particularly against *Stromatinia cepivora* and *B. cinerea*) named Trichorzins HA, MA and Trichorzianin TA were isolated from two isolates of the mycoparasite *T. harzianum*. The most widely known peptaibol is the *T. viride* alamethicin [88]. Similar evidence suggests the antifungal activity of the peptaibol Koninginins (Figure 6) from *Trichoderma pseudokongii* causes extensive apoptosis by loss of the mitochondrial transmembrane, potentially resulting in cell death of *F. oxysporum*. The terms peptaibiome and peptaibiomics (peptide antibiotics or peptaibiotics) have been suggested to describe the analysis of peptaibols expressed in microorganisms using spectrometric methods, such as LC/ESI-MS or intact-cell MALDI-TOF [103]. Moreover, these authors produced peptaibols by SSF, and they identified the peptaibols composition of *Trichoderma koningiopsis* and *T. gamsii* (syn. *T. viride*) using spectrometric methods, such as HPLC-ESI-MS measurements, which revealed a total of 30 peptaibol sequences.

Gliotoxins

Gliotoxins (Figure 6) are sulfur-containing mycotoxins belonging to the class of diketopiperazines. They are produced mainly by the *Trichoderma* species and several other fungi, such as *Gliocladium fimbriatum*, *Aspergillus fumigatus*, *Penicillium obscuro*, and *Acremonium* strains [104]. Gliotoxins have antimicrobial, antiviral, antifungal, and immunomodulating properties [105]. Particularly, most of the studies indicated their potential role of antifungal activity in the biocontrol mechanism of *T. virens* and *Acremonium spp.* It is synergistically enhanced by the cell-wall-degrading enzymes of the biocontrol agents *T. harzianum* and *T. virens*. Little is known regarding the modes of action of gliotoxins in fungal cells during antagonistic interactions.

The fungal mycoparasite agent *Trichoderma virens* consists of P (gliovirin producing) group and Q (gliotoxin producing) group strains, based on their antibiotic profiles. « Q group » strains of *T. virens* produce the antifungal gliovirin which is active against *R. solani*,

but less effective against *Phytophthora ultimum*. Strains of the « P group » produce gliotoxin, which is very active against *P. ultimum* but not so active against *R. solani* [106]. Despite this, it has been demonstrated that strains of the “P group” are able to effectively control *Phytophthora* spp. damping off on cotton, while “Q group” strains give better results toward the same disease caused by *R. solani* [106].

Polyketides

Polyketides represent a highly diverse group of secondary metabolites that have carbon skeletons, including polyphenols, macrolides, polyenes, enediynes, and polyethers. They are the main class of secondary metabolites derived from fungi. The spectrum of substances ranges from spore, pigments, and antibiotics to toxins [107]. Polyketides play important roles in the life cycles of microorganisms that produce them, but little is known about their role in antagonism. Among polyketides, chaetoviridin (Figure 6) and chaetoglobosin from *Chaetomium globosum* are particularly well documented [108]. Zhang et al. [109] demonstrated that chaetoviridin A could significantly inhibit the spore germination of *Verticillium dahliae*. Transcriptome analysis showed that the differentially expressed genes gradually increased from 33 to 47 after spores of *Verticillium dahliae* treated with chaetoviridin A 1 and 3 h, and the inhibitory effect gradually increased.

Terpenoids

Terpenoids are volatile to nonvolatile substances constituting the highest abundant natural products on earth. Terpenes are classified into seven groups depending on their number of carbon atoms: hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), sesterterpenes (C25), triterpenes (C30), and tetraterpenes (C40). Each class includes linear and cyclic molecules; cyclization is generated by terpene cyclases [110]. Terpenoids of fungal origin comprise molecules phytohormone-like, mycotoxins as well as antibiotics and antitumor substances [111]. Trichotecin from *Trichothecium roseum* and *Stachybotrys elegans*, harzianum A from *Trichoderma arundinaceum*, as well as trichoviridin from *T. viride* and trichothecenes from *Stachybotrys cylindrospora* exhibit *in vitro* antifungal properties [112]. However, the isolation and separation of these compounds are very difficult due to their instability. The production of several terpenoids has been proven for *Trichoderma* species [113], but their biosynthetic pathways mostly remain to be determined.

Nonribosomal peptides (NRPs)

Bacillus subtilis and other *Bacillus* strains produce various SMs. The most prominent and bioactive SMs are nonribosomal peptides (NRPs), of which isoforms belong to the families of surfactins, fengycins, or iturins. The surfactin bioactivity consists of a surfactant activity triggering cell lysis due to the penetration of the lipid bilayer membranes and the formation of ion-conducting channels. It is presumed that the antifungal plipastatin (a surfactine), expressed from the *ppsA-E* gene cluster, acts as an inhibitor of phospholipase A2, forming pores in the fungal membrane and causing morphological changes in the fungal membrane and cell wall. This antifungal potential was demonstrated primarily against various filamentous fungi.

The quality and the quantity of SMs synthesized depend on (i) the compound considered; (ii) the species and the strain; (iii) the occurrence of other microorganisms; (iv) the equilibrium among elicited biosynthesis and biotransformation rate and (v) growth conditions, such as temperature, pH, substrate, moisture, phytopathogen biomass and incubation time. Technology is an opportunity for molecules with biopesticide activities production, using several kinds of substrate. The following paragraphs report recent data about the state of the art regarding SSF technology.

Challenges of biopesticide production using solid-state fermentation technology

The market for biopesticides is growing quickly in comparison with conventional synthetic pesticides. Biopesticides offer a more ecological way for the management of pests, but they also face several challenges, such as many barriers for the use of biological products, a lack of knowledge of agricultural biological products, and, most importantly, a lack of global availability [114]. To enhance the use of biopesticides, it is necessary to optimize the antibiotic, antifungal metabolites, and spore production of species of interest.

Among the available microbial culture methods, SSF is a method that is thousands of years old, which was first used by mankind for food production. This technology is receiving renewed attention because of the growing need for new metabolites in various industries, such as agriculture, human health, food, and cosmetics [103,115]. Today, the most important applications of SSF are biomass production (mycelium, spores, inoculum, and biopesticides), secondary metabolites production (aromatic compounds, mycotoxins, and volatile organic compounds, etc.), enzyme production

(pectinases, chitinases, lipases, cellulases, etc.), and primary metabolites production (citric acid, lactic acid) [19,114]. SSF is an alternative that offers the production of high quantities of antifungal compounds, lytic enzymes, and spores, from different species of filamentous fungi with a minimal cost of separation. Species that have been cultivated using SSF include *T. harzianum*, *B. bassiana*, and *M. anisopliae* [19,72]. This technological approach allows the use of different agro-industrial residues, such as plant bagasse, rice bran, rice husk, corncobs, tea waste, maize and wheat bran, and fruit seeds [116]. They are mainly constituted by cellulose, which is naturally rich in carbohydrates, and by other nutrients (nitrogen sources, mineral salts, etc.) as a source for microorganism development and for the induced production of value-added products [117].

Despite this, the byproduct volumes generated by the agro-industry are massive and, if left to decay, can constitute environmental pollution. In recent years, numerous studies have been published supporting the use of SSF in valorization of agricultural byproducts for microbial growth [118,119]. De la Cruz-Quiroz et al. [72] reported the production of lytic enzymes as pectinases, chitinases, cellulases, lipases and virulent *Trichoderma* spores' production by SSF using a mixture of sugarcane bagasse, wheat bran, chitin, and potato flour, as a substrate for six *Trichoderma* strains. In France, the intensive production of wine generates tons of vine shoots every year and some of these byproducts enter in a primary valorization with polyphenol extraction. This high value-added molecule recovery does not reduce the volume of the vine shoots but generates materials exhausted in chemically active compounds. Recently, Maiga et al. [76] evaluated the use of exhausted vine shoots as support for fungal growth for conidia and secondary metabolites productions using SSF. The carbon/nitrogen ratio of the obtained fermented material has also been evaluated to propose an ultimate way of valorization through composting. Likewise, Hamrouni et al. [119] highlighted the possibility to produce spores, lytic enzymes and antifungal metabolites using SSF technology and *T. asperellum* TF1 cultivated on a mixture of vine shoots, *Jatropha* cake, olive pomace and olive oil as substrates.

In comparison with its liquid equivalent, SSF has several advantages:

1. The culture media is easier to prepare (solid substrates added with water and some salts), with unnecessary agitation. In addition, a non-sterilized substrate can be used.

2. SSF allows the production of high value-added molecules from unused resources, thus decreasing the pollution volume and its toxicity (e.g., *Jatropha curcas* byproducts).
3. Molecules produced by SSF have different and more interesting properties than those obtained with liquid fermentation, such as the more thermostable characteristics or higher pH stability.
4. Lack of free water reduces liquid volumes in fermentation reactors, downstream-effluent treatment costs and prevents bacterial contamination [19].

Table 2 shows the most studies on solid and liquid fermentation use to produce biopesticides employing microorganisms.

Biopesticide formulations and application methods

The United States Environmental Protection Agency (EPA) defines biopesticides as "certain types of pesticides derived from such natural materials as animals, plants, bacteria, and certain minerals". The most commonly used biopesticides are living organisms, which are pathogenic for the pest of interest. These include biofungicides (*Trichoderma* spp.), bioherbicides (*Phytophthora* spp.), and bioinsecticides (*B. thuringiensis*) [50]. Biopesticides fall into three major categories: (i) Biochemical pesticides (substances such as plant extracts, fatty acids, or pheromones), (ii) Plant-Incorporated-Protectants (substances produced by plants and the genetic material necessary for the plant to produce the substance), and (iii) Microbial pesticides (naturally occurring microorganisms including bacteria, fungus, virus, protozoan or alga).

Product formulation is a critical area for commercial purposes. Typically, formulation refers to the preparation of a product including active substances and inert ingredients. However, regarding bioformulation, no uniform definition is available and authors define it individually. Ojuederie et al. [134] define this term as the formulation of BCA that can be used to (i) stabilize the selected organisms during production, distribution, and storage, (ii) protect the agent from harmful environmental factors, and (iii) enhance the activity of these organisms. Samadaet al. [64] state that bioformulation is the preparation of microorganism(s) that may be a partial or complete substitute for chemical fertilization/pesticides. De la Cruz Quiroz et al. [75] explain that the bioformulation process leads to a final product by mixing the microbial component with different carriers and

Table 2. Literature on solid and liquid fermentation use to produce biopesticides employing microorganisms.

Species	Growth substrates	Maximum biomass yield	Disease phytopathogen	References
Solid-state fermentation				
<i>Bacillus amyloliquefaciens</i>	Feed-grade, soybean, corn flour, wheat bran	7.24×10^{10} CFU/g DM	<i>Erysiphe cichoracearum</i>	[120]
<i>Bacillus sphaericus</i>	Wheat bran, rice hull, wheat straw, corn stover, corn cobs, cotton stakes, olive mill by products, date stone, pea peels, potato peels	116×10^9 CFU/g DM	<i>Culex pipiens</i>	[121]
<i>Bacillus thuringiensis</i>	bulking agent was mixed with digestate and biowaste	4×10^8 spores/g DM	<i>Coleopteran pests</i>	[122]
<i>Pseudomonas aeruginosa</i>	Tung tree		<i>Colletotrichum lini</i> , <i>Rhizoctonia solani</i> <i>Fusarium oxysporum</i>	[123]
<i>Streptomyces Similanensis</i>	Rice bran, cassava chips, and coconut husks	151×10^7 CFU/g DM	Phytophthora palmivora	[124]
<i>Streptomyces griseorubens</i>	Peat soil with 2% w/w rice husk	1.69×10^9 CFU/g DM	<i>Neoscytalidium dimidiatum</i>	[125]
<i>Trichoderma atroviride</i>	Agricultural digestate	689.8 mg of mycelium/g DM	Cress	[126]
<i>Trichoderma asperellum</i>				
<i>Trichoderma harzianum</i>				
<i>Trichoderma reesei</i>	Apple, banana, and grape fruits wastes	689.8 mg of mycelium/g DM	Cress and tomato	[127]
<i>Trichoderma atroviride</i>				
<i>Aspergillus flavipes</i>	Soybean		<i>Eucalyptus clone</i>	[128]
<i>Trichoderma guizhouense</i>	Rice straw + amino acids	4.62×10^{10} CFU/g DM	Pepper	[129]
<i>Fusarium redolens</i>	Wheat bran	38×10^{12} CFU/g DM	<i>Coleus forskohlii</i>	[130]
<i>Phialemoniopsis cornearis</i>	Wheat bran	4×10^{11} CFU/g DM	<i>Coleus forskohlii</i>	[130]
<i>Macrophomina pseudophaseolina</i>	Wheat bran	21×10^{12} CFU/g DM	<i>Coleus forskohlii</i>	[130]
<i>Kosakonia pseudosacchari</i>	Vermiculite, exhausted yeasts and vinasse	6.9×10^7 CFU/g DM	Corn	[131]
Liquid fermentation				
<i>Clonostachys rosea</i>	peptone, sucrose, dextrose, K_2HPO_4 , KCl, $MgSO_4$, $FeSO_4$	1.78×10^7 CFU/ml	<i>Botrytis cinerea</i>	[132]
<i>Bacillus thuringiensis kurstaki</i>	Glucose, soybean peptone, yeast extract, mineral salts: $MgSO_4 \cdot 7H_2O$, $MnSO_4$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, KCl, $FeSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 6H_2O$	2.31×10^{12} CFU/ml	<i>Anagasta kuehniella</i>	[133]

additives for better preservation from environmental conditions. However, any operative definition must include an active ingredient, a carrier material, and an additive. The active ingredient is mostly a viable organism; it may be living microorganisms, spores, or cells. The viability of these organisms will have to be maintained at acceptable levels during the formulation process and storage. According to Kumar et al. [20], bioformulations are biologically active products containing one or more beneficial microbial strains, commonly used to increase plant growth, and soil fertility and to reduce phytopathogens. A suitable carrier material: is inert, supports active ingredients, and assures that a microorganism is easily established in or around the plant, thus providing better plant growth [135]. In addition, the criteria for selection of biopesticides are mainly: high pathogen specificity, infrequent pest resistance development, suitability for use in integrated pest management schemes, rapid biodegradation of resi-

dues, and minor or no health risk to workers (Figure 7). Different factors have to be taken into account considering the application of the biopesticides: early plant exposure to such biopesticides enhances its response against pathogens, while long-term applications can even act as a plant growth stimulant, enhancing root growth and development, conferring tolerance to abiotic stresses, and improving uptake and use efficiency of nutrients, and thus increasing crop productivity [136].

Formulation designs and application methodologies directly affect the: efficacy, consistency, shelf life, and virulence of commercial biopesticides. For instance, formulations applied as sprays are generally expected to exert an immediate effect on plant pests and infections [137]. Moreover, many different resources are available and the quantities applied as recommended by the supplier, are very variable according to the biopesticides: BASF (TrichoPlus™, Green Muscle™, and

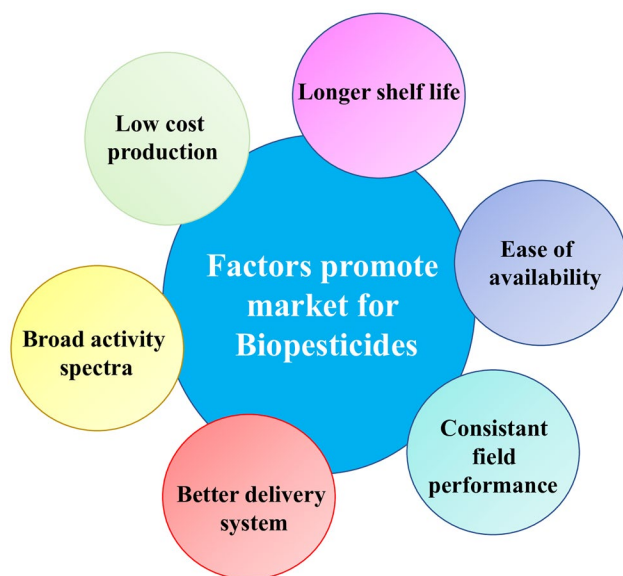


Figure 7. Diagrammatic representation of factors affecting market of biopesticides.

Green Guard™), AgriLife (Biokuprum™, Downycare™, Powderycare™, and Racer™), Dudutech (Trichotech™, Beauvitech™, Lecatech™, and Mytech™), LAM International (Mycostar™), and Verdera (Rotstop™) do not disclose key information on the strains and the quantity used in their products (Table 3).

The most important characteristics of the developed formulations are (i) stabilization of microbial agent during distribution and storage, (ii) easy handling and application of the product, (iii) protection of the bio-agent from adverse environmental conditions, and (iv) enhancement of the bioagent's activity by increasing contact and interaction with the target pest. Broadly, biopesticide formulations are classified into dry and liquid formulations [139]. Solid formulations for direct application include granules (G), microgranules (MG), water dispersible granules (WDG), powders for seed dressing (DS), and wettable powders (WP). Liquid formulations include emulsifiable concentrates (EC), suspension concentrates (SC), oil dispersions (OD), and capsule suspensions (CS). Commercial success of these formulations is based on the capability of a microorganism to survive and proliferate in the: field condition, shelf life, efficiency to control pests and diseases, market price, ease of handling, and application [140]. Granule (G) particles have been designed to make wettable powders friendly for both users and environment, the free-flowing granules being quickly dissolving in water [141]. Granular formulations are usually safe, having no risk of inhalation and being mostly used in soil treatment. They contain wetting agents and the dispersing agent is usually at a high

Table 3. Commercial products for the biological control of phytopathogens [75, 138].

Microorganisms producing biopesticides	Phytopathogen	Target crop	Product name and source
<i>Bacillus subtilis</i>	<i>Pythium ultimum</i>	Damping-off and root	Kodiak (A-I 3), Kodiak HB, Kodiak AT and Epic (MBI600) (Gustafson, Inc., TX, USA)
	<i>Rhizoctonia solani</i>	rots of cotton and	
	<i>Fusarium spp.</i>	legumes	
<i>Pseudomonas Fluorescens</i>	<i>Erwinia amylovora</i>	Fruit trees, potato, strawberry, tomato	BlightBan A506 (Plant Health Technologies, ID, USA).
<i>Pseudomonas chlororaphis</i>	Seed borne and soilborne pathogens	Cereals	Cedomon (BioAgri. Stockholm, Sweden)
<i>Pseudomonas syringae</i>	<i>Botrytis cinerea</i>	Pome fruit and/or	Bio-save \00 (ESC-\0), Bio-save \000 (ESC-IO), Biosave 110 (ESC-II) (Ecogen, Inc.PA, USA or Israel). BioBlast (Eco Science Corp, NJ, USA)
	<i>Geotrichum candidum</i>	citrus	
	<i>Mucor pyroformis</i>		
<i>Trichoderma harzianum</i> + <i>Trichoderma polysporum</i>	Soilborne pathogens	Protected crops	BINAB-T WP (Bio-InnovationAB, Bredholmen, Sweden)
<i>Trichoderma harzianum</i> + <i>Trichoderma viride</i>	Various fungi (root protectants and soil conditioners)	Various hosts	Trichopel, Trichoflow - Turf and Trichoboost (Agrimm Technologies Ltd, New Zealand)
<i>Trichoderma viride</i>	<i>Phytophthora spp.</i>	Ornamentals	Bip T (Poland)
<i>Trichoderma harzianum</i> + <i>Trichoderma polysporum</i>	<i>Botrytis cinerea</i>	Strawberry	BINAB-T WP (Bio-Innovation AB, Bredholmen, Sweden)
<i>Coniothyrium minitans</i>	<i>Sclerotia sclerotinia</i>	Vegetables Sunflower	Contans (Prophyta, Germany, Russian Government)

concentration. The concentration of microorganisms in granules ranges from 5 to 20%. Microgranules (100–600µm) and coarse particles size (100–1000µm) are made from mineral materials, such as silica, kaolin, polymers, ground plant, and dry fertilizers [142]. Granule particles have a long shelf life [143].

Three types of granule formulations are currently available: (i) the microorganism is fixed to the surface of a granular carrier with a sticker, (ii) the microorganisms are attached to a carrier paste or powder as a matrix, (iii) the microorganisms are sprayed onto a rotating granular carrier without a sticker.

Lignite silage was used to produce granules containing fungi e.g., *Gliocladium roseum* and *T. harzianum* to control *Rhizoctonia solani* in soil causing damping-off of peanut [144]. Lignite granules (425–2000µm diameter) were amended with the product of sorghum fermentation. Then, isolates of *T. harzianum* and *Gliocladium roseum* were allowed growing on these

granules for 7 days. These granules were allowed to air-dry followed by incubation before application in *Rhizoctonia solani* infested soil [145].

Soesanto [146] studied shelf life, antagonism and the effect of granular application of *Pseudomonas fluorescens* to control *Ralstonia solanacearum* infested tomato. They mentioned that all the granular suppressed the disease indicated by the lengthening incubation period of 22.77–26.25%, reducing the disease incidence to 60–85%.

Although granular formulations are very effective, their application is also limited due to the inactivation of active ingredients following exposure to UV light [147,148].

Conclusion and future steps

Recent multi-billion acquisitions and ongoing collaborations between agrobiotechnology companies and research centers suggest that biopesticides and biofertilizers may represent an agronomic sector with strong growth potential.

From the investigations described above, most of the microorganisms (fungi, bacteria, actinomycetes, etc.) clearly have great potential for the future, due to their ability to produce a wide range of SMs with antifungal activities and to parasitize other microorganisms.

Optimization of bioformulation is certainly dependent on the detailed knowledge of microbial as well as plant physiology, but several technological challenges such as the fermentation process, and formulation type would enhance the performance of the final product.

The SSF may be favored to synthetic media or liquid culture for bioformulation production of BCA's as biopesticides. Indeed, SSF facilitates the development of biopesticide formulations used in field crops and may decrease process costs due to (i) possible use of natural substrates (mainly by-products), (ii) low aeration rate, and (iii) easier preparation of culture media.

Finally, there is today ever-increasing information about microbial-based biopesticides used in biological control. However, more investigations are needed to enhance our knowledge on antibiotic and antifungal compounds as well as on process of bioformulation at large scales. Last but not least, efforts should be performed in several directions: (i) isolation and selection of efficient antagonist strains, (ii) correlation between bioformulations and their effectiveness as biological control agents, (iii) establishment of biological indicators to control biopesticides virulence and, (iv) establishment of tools for traceability for monitoring biopesticides in soil. To allow this intention to succeed fully, the natural substrates should be derived from

local secondary products, and therefore valued in a short circuit logic, and the safety concerns of the finished products should be evaluated during the development of the formulations both for humans and at the level of ecosystems.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

Funding for this study was received from Excellence Initiative of Aix Marseille University, A*MIDEX, a French "Investissement d'Avenir" Program, under the project ALCOVE from ITEM (AMX-19-IET-012).

ORCID

Isabelle Laffont-Schwob  <http://orcid.org/0000-0003-3630-6450>

Rayhane Hamrouni  <http://orcid.org/0000-0002-7000-4352>
Anne-Marie Farnet Da Silva  <http://orcid.org/0000-0001-6603-1174>

Thierry Orsiere  <http://orcid.org/0000-0002-6106-7757>

Jean-Luc Boudenne  <http://orcid.org/0000-0001-6150-4171>

Nathalie Dupuy  <http://orcid.org/0000-0002-8739-2689>

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