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Limiting factors of mycopesticide development

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ABSTRACT

Phytosanitary crop protection products have shown their impact on the environment. They may not be very selective and their excessive use in agriculture causes pollution of soil and groundwater, destruction of many beneficial insects and the emergence of resistant pests. Hence there is strong public and political pressure driven by consumers to implement phytosanitary alternatives that are less aggressive to the environment, such of biopesticides. Biopesticides and, in particular, mycopesticides are frequently used as an environmentally friendly tools to reduce plant diseases by inhibiting the growth of pathogens and inducing resistance in plants.

In this review, we will first analyze the current evolution of the global market of biopesticides. Boosted by different political initiatives all over the world, this market has increased 6-fold between 2005 and 2016. In 2016, it represented 6% of the global pesticide market with a compound annual growth rate (CGAR) of 14.1%. The mycopesticides accounted for only 10% of the global biopesticide market of 2016.

We then focused on two main factors which should contribute to the future development of mycopesticides: the large panel of their modes of action and the physiological state of the active product. Indeed, several modes of action could, in some instances, increase the efficacy of a biopesticide and postpone the emergence of resistance mechanism. The broad set of mechanisms use by *Trichoderma harzianum* and *T. atroviride* to reduce plant diseases: nutrient competition, direct antagonism, mycoparasitism and induction of resistance mechanism in plants were developed as an example. Stability of the active substance is another essential factor for creating competitive mycopesticides. For this, the choice of propagule types could be a major factor in enhancing their stability. This choice was discussed highlighting the advantages of conidia or sporidia from solid-state fermentation.

To end with, the registration process in European Union was described revealing another limiting factor that delays the development of mycopesticides in this zone.

1. Introduction

Microbial pathogens affecting plant health and causing plant disease are a major and chronic threat to food production and ecosystem stability throughout the world. Crop protection chemicals, also known as synthetic chemical pesticides, help farmers to control or kill insects, weeds, microbial pathogens and other potentially harmful pests. Although their effectiveness is clear, their negative impact on the environment is becoming ever more widely discussed.

Indeed, they may not be very selective and their excessive use in agriculture is the cause of soil and groundwater pollution, development of weed-, insect- or pathogen-resistances, as well as the destruction of many useful organisms including insects (such as pollinators) (Sarwar, 2015). There is also a potential deleterious effect on the farmer's health (García-García et al., 2016; Shammi et al., 2018).

With intensification of agricultural production in recent decades, producers have become increasingly reliant on these products as a fairly reliable method of protecting crops, helping to ensure the economic stability of their operations (de Weger et al., 1995; Gerhardson, 2002; Postma et al., 2003). In parallel, due to the deleterious effects of these products, different policies of pesticide reduction have been implemented in several places in the world.

All of this has contributed to increased interest in the implementation of phytosanitary alternatives that are less aggressive to the environment, such as biopesticides also referred to as biocontrol or bioprotection agents. The latter have several definitions. For example,

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according to the Environmental Protection Agency (EPA) in the USA, "Biopesticides include naturally occurring substances that control pests (biochemical pesticides), microorganisms that control pests (microbial pesticides), and pesticidal substances produced by plants containing added genetic material (plant-incorporated protectants) or PIPs" (US EPA, OCSPP, 2016), while in EU biopesticides are a form of pesticide based on micro-organisms or natural products.

There are several advantages to the use of biopesticides. Biopesticides are often less toxic than chemical products and decompose quickly. This can avoid pollution problems and residue concerns. Targeting specific pests, biopesticides generally affect only the target pest and closely related organisms; this protects other organisms living in the same environment (Leahy et al., 2014; Villaverde et al., 2014). With their complex mode of action, biopesticides can also delay the development of resistance.

There are various types of biocontrol agents based on viruses or bacteria: *Cypovirus,* also known as cytoplasmic polyhedrosis virus, a pathogen against predator insects *Helicoverpa armigera* (Marzban et al., 2013); *Baculoviridae* infecting larvae or adult insects of arthropods as *Cydia pomonella granulovirus* (CpGV) and used as biological control agent of *Cydia pomonella* in apple orchards (Stará and Kocourek, 2018); *Spodoptera exigua multiple nucleopolyhedrovirus* that infect *Spodoptera exigua* larvae (Lasa et al., 2007); *Bacillus thuringiensis*, which is the best known among bacterial bioinsecticides (Beas-Catena et al., 2014; Melo et al., 2016); *Bacillus* sp. *(including B. subtilis, B. licheniformis, B. amyloliquefaciens, B. velezensis* and recently *Bacillus nakamurai)* which is one of the main biofungicides and *B. firmus* which is authorized in soil treatment against nematodes (Jacques et al., 2014; Jiang et al., 2018; Johnsson et al., 1998, Leathers et al., 2020, Ongena and Jacques, 2008; Wilson et al., 2013).

In addition to viruses and bacteria, several strains of filamentous fungi are used for the biological protection of plants. Among these strains, the genus *Trichoderma* is widely used as a mycofungicide. It comprises a group of imperfect saprophytic filamentous fungi which have antifungal activities against several rhizosphere and phyllosphere pathogens.

Trichoderma has a beneficial effect on growth of several plant species (Cavalcante et al., 2008; De los Santos-Villalobos et al., 2013; Dodd et al., 2013). In this genus, the species *T. harzianum* is considered to be the most effective biological control agent (Dodd et al., 2013; Naher et al., 2014). For example, *T. harzianum* T35 controls *Fusarium oxysporum* by competing for both rhizosphere colonization and nutrients (Rajesh et al., 2016), *T. harzianum* T22 induces systemic resistance against *Fusarium verticillioides* in maize (Ferrigo et al., 2014). *Trichoderma atroviride* (strains SC1 and USPP-T1) have shown good efficacy for the protection of pruning wounds in the grapevine (Mondello et al., 2018). *Pseudozyma flocculosa* is also one of the few fungal products that has antagonistic effects against powdery mildew (Laur et al., 2018; Siddiqui, 2006). This disease attacks many predominant plant species among which the three important greenhouse crops: tomato by *Oidium neolycopersici*, cucumber by *Sphaerotheca fulginea* and roses by *Sphaerotheca pannosa* (Hajlaoui et al., 1992; Jarvis et al., 1989; Jones et al., 2001). There are other fungi that have biofungicidal activity such *Coniothyrium minitans*. This fungus is able to parasitise *Sclerotinia sclerotiorum* that is a destructive pathogen of a broad range of plant hosts (Bennett et al., 2003; de Vrije et al., 2001).

The fungus *Paecilomyces lilacinus* is one of the most studied nematicides in biological control. *P. lilacinus* penetrates nematode eggs by secreting lytic enzymes such as chitinases and proteases (Abbas et al., 2016; Dong et al., 2007; Lamovšek et al., 2013). *Beauveria bassiana* and *Metarhizium anisopliae*, two species considered as alternatives to insecticides, attack *Tetranychus cinnabarinus*, cockroaches, termites and mosquitos as *Anopheles arabiensis* and *Culex quinquefasciatus* (El Abas El Agali et al., 2017) and many other agricultural pest insects.

There are other fungi that have effects in fumigation such as *Muscodor albus*. This fungus has the ability to produce a mixture of volatile compounds (alcohols, esters, ketones, acids and lipids) which can kill pathogens like molds and bacteria (Mitchell et al., 2010).

The benefits of biopesticides for the environment are progressively increasing their market share as a substitute for chemical pesticides. After analyzing the evolution of these markets and the political initiatives taken in different countries to promote biopesticide development, this review focuses on a single type of biopesticide that is a mycopesticide. The goal is to analyse the main factors that could help in speeding up their commercial development. We first highlighted their numerous modes of action. We then discussed the physiological state of the fungus cells used as active products and its influence on the product stability Finally, we described the complexity of the registration process in the EU for biopesticides.

2. Politics to develop biopesticide market

Over the past few years, attention has been directed towards the introduction of biopesticides as an alternative solution to chemical pesticides to reduce their presence in the environment and their predominance on the international market. Several measures have been taken in different countries to accelerate the achievement of this objective. For example, in order to reduce the use of conventional chemical pesticides, the Environmental Protection Agency (EPA) of the United States established the Biopesticides and Pollution Prevention Division (BPPD) in 1994. The goal of BPPD is to facilitate the registration of biopesticides in order to encourage the development and use of low risk biological pesticides as alternatives to the chemical pesticides. Since biopesticides tend to be considered as a low risk product, EPA requires less data to register a biopesticide than to register a conventional pesticide, and EPA's review times are shorter for biopesticides. The process for biopesticide starts with a first level of toxicity and ecotoxicity evaluation. If there are no direct toxic effect then no further tests are required. But if there are direct toxic effects the review process is equally rigorous as compared to the review of conventional pesticides.

In 2004, the United States Congress passed the Pesticide Registration Improvement Act (PRIA) and established a registration system with specific fees and decision times by type of action. PRIA has been renewed and extended three times and the law establishing the current fees and decision times is the Pesticide Registration Improvement Extension Act of 2018 (PRIA 4) which will expire in 2023. Timeframes to register biopesticides products vary dependent on the PRIA code assigned to the submission. For example, according to the PRIA 4 decision review timelines, a new registration of new biopesticide active ingredient for non-food use (PRIA Code B600) is 13 months and addition to the standard 21 days for initial technical screening of the application. Also, a new active ingredient for food use and the establishment of a tolerance (PRIA code 580) the EPA review time is 20 months and 21 days for technical screening. The EPA fees to register a new biopesticide can be as high as $53,606 USD which is low as compared to a conventional chemical whose PRIA fee may go as high as $790,737 USD (according to TSG consulting, www.tsgconsulting.com). In addition, the EPA awards grants each year for registration-related R&D for biopesticides through an interagency agreement with the USDA's Interregional Research Project 4 (IR-4) (Leahy et al., 2014; Seiber et al., 2014).

In 2009, the entry in force of the (EC) 1107/2009 regulation, superseding the Council Directive 91/414/EEC, has led to the withdrawal of more than 200 substances from the European market during the last decade. This new regulation gave biopesticide manufacturers an opportunity to fill the void left by synthetic pesticides (Chen, 2017). In addition, in order to facilitate the registration of the new alternatives, the Regulation CE 1107/2009 defined a low risk class of products in which the mycopesticides could enter. However, the low risk status is only granted after a full evaluation procedure as it is done for all other substances. IBMA (International Biocontrol Manufacturer Association) made some propositions on the actual way to facilitate the registration of active substances and plant protection products under the low risk definition. The full process from active substance dossier submission to final registration of product could be reduced from a typical duration of 4.5–5 years to 3–3.5 years. But actually, in practice the timelines are longer and can be 5–10 years.

In France, the “EcoPhyto Plan” was launched in 2008 by the French government, aiming to reduce the use of plant protection products by 50% within ten years, if possible. Several years later, as this objective was far to be reached, the French government launched the project EcoPhyto Plan II which supports the objectives of EcoPhyto Plan I. The target of a 50% reduction in the use of plant protection products in France within ten years has been renewed, under a two-phase timeframe. The first phase aims for a 25% reduction by 2020 through mainstreaming and optimizing currently available techniques. The second phase aims for a 50% reduction by 2025. It will focus on major changes to production systems and sectors, supported by medium- and long-term policy determinants and by scientific and technical advances (Guichard et al., 2017).

The Chinese government actively encourages the development and use of alternative measures, including biopesticides. In 2005, China developed regulations for the implementation of organic products certification (Chen, 2017).

In addition, to encourage the production of biopesticides a number of changes in national and international regulations of the chemical pesticides have been realized. The objective of these changes is the reduction of the adverse effects of indiscriminate use of chemical pesticides on the environment and human health. For this reason, regulation is becoming more and more demanding in terms of toxicological tests and monitoring of possible adverse effects on human health over time (Ongena and Jacques, 2008). These changes have complicated the registration procedures for new chemicals, but did they strengthen the biopesticide market? And despite all the benefits of biopesticides, have they succeeded in replacing chemical pesticides?

3. The global market for biopesticides compared to chemical pesticides and agricultural biotechnology products

The evolution of the global market for pesticides shows that the demand for chemical pesticides during the period 2012–2014 was increasing. According to reports published by BCC Research in 2012–2014, global demand for chemical pesticides reached 26 billion dollars in 2006, with an estimate that it would decrease to 24 billion dollars in 2013. But the results have been disappointing, the global turnover made with chemical pesticides doubled in 2013 to $52 billion and reached $58 billion in 2014. Then this demand was slightly decreased during the period 2014–2016 to reach 56 billion (Fig. 1) (Chen, 2017; Lehr, 2015, 2012; Tharkore, 2006).

The demand for biopesticides increased 6-fold during the last decade from $ 0.68 billion in 2005, it reached $4 billion in 2016, and the compound annual growth rate (CAGR) was increasing from 12 to 14.1%. While promising, this value should increase to speed up the expected strong reduction of chemical pesticides. Indeed in 2016 the biopesticides only represent 6% of the market of chemical pesticides (Chen, 2017; Lehr, 2015; Tharkore, 2006). The development of Integrated Pest Management (IPM) program could help in this development (Leahy et al., 2014).

According to EPA “the IPM is an effective and environmentally sensitive approach to pest management that relies on a combination of common-sense practices. IPM programs use current, comprehensive information on the life cycles of pests and their interaction with the environment. This information, in combination with available pest control methods, is used to manage pest damage by the most economical means and with the least possible hazard to people, property, and the environment”.

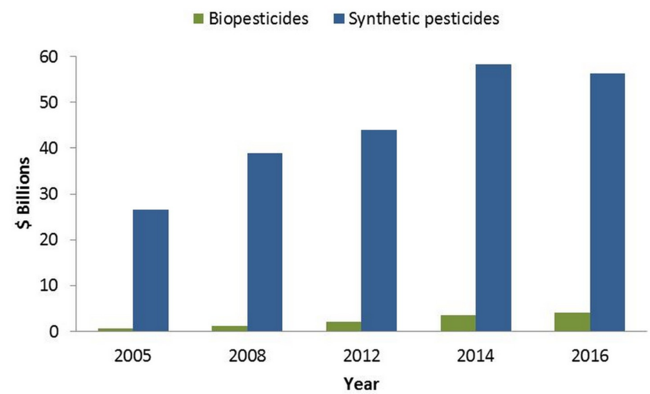


Fig. 1. The evolution of the global market for chemical pesticides compared to biopesticides during the last decade (2004–2016). Data adapted from last reports of BCC Research (2006, 2012, 2015 and 2017) (Chen, 2017; Lehr, 2015, 2012; Tharkore, 2006).

4. Global market evolution of mycopesticides

Mycopesticides are pesticides whose main active ingredient is composed of a fungus. They are divided into several types. On the one hand, mycoinsecticides such as *Beauveria bassiana* represent approximately 60% of the total market for mycopesticides, while *Verticillium lecanii* and *Metarhizium sp* account for 16% of this market. On the other hand, mycofungicides such as *Trichoderma sp*, *Ampelomyces quisqualis, Coniothyrium minitans, Gliocladium* spp. *and nematicides such as Purpureocillium lilacinum* make up the rest of the mycopesticide market (Chen, 2017; Lehr, 2015; Singh et al., 2013; Tharkore, 2006). In 2016, the mycopesticides accounted for 10% of the global biopesticide market compared to 75% for bacterial biopesticides (Fig. 2) (Chen, 2017). This lower market share has to be correlated to the characteristics of a successful commercial mycopesticide.

5. Characteristics of a successful commercial mycopesticide

The characteristics of a competitive mycopesticide are: efficacy comparable to its chemical counterpart or ability to be used as a component of an IPM program, stability of the product (long shelf life during transport and storage) but biodegradable, lower toxicity and ecotoxicity than chemical pesticides, simplicity of production on an industrial scale, simplicity in application, compatibility with agronomic use and equipment, cost/benefit price and the ability to be registered (Kaewchai et al., 2009; Spadaro and Gullino, 2005). When one of these characteristics is not met, the biopesticide directly loses its commercial potential (Fig. 3). The efficacy and the stability of mycopesticides are two essential factors to warranty their effectiveness.

6. Mycopesticides: mode of action and its relation to biopesticide efficacy

The mechanisms of action of biological control agents with pathogens are numerous and complex. They are influenced by several soil ecosystem factors, namely pH, moisture, temperature and the presence of some other microorganisms which have effects against the mycopesticide (Nicot, 2011). A good knowledge of the modes of action of fungi with a biocontrol activity is of utmost importance to determine their modes of application as mycopesticides. In addition, products that have a single action site, like chemical pesticides, are characterized by a higher risk of resistance than those with multiple action sites. Most microbial pesticides and precisely the mycopesticides offer multiple modes of action and are generally considered to have low risk for developing resistance (Hubbard et al., 2014).

The biocontrol activity of fungus against pathogens can be due to

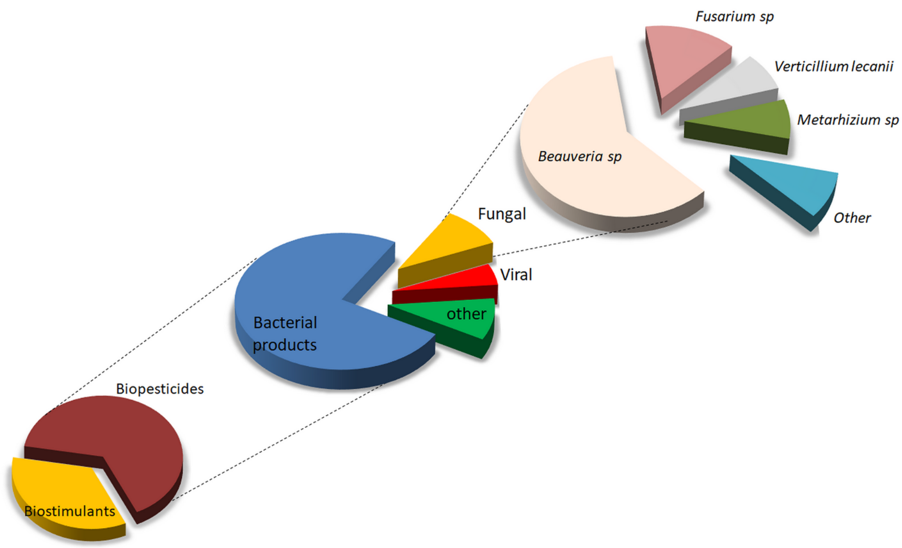


Fig. 2. Market share for biological control agents. Mycopesticides accounts for 10% of the biopesticides that exist in the global market (Bergin, 2014; Chen, 2017).

(1) nutrient and space competition (2) direct interaction by the production of an antimicrobial substance and mycoparasitism and (3) induction of systemic plant resistance (Viterbo et al., 2007).

6.1. Competition

Nutrients and space are two major limiting factors for microbial growth. Therefore, competition strategies are based on them to prevent other microbe's growth. Competition is divided in two types: (1) nutrient competition in the soil where biological control agents compete with other pathogenic fungi for food elements (carbohydrates, nitrogen, oxygen, and micronutrients such as iron) (Chet et al., 1990; Fravel, 2005; Irtwange, 2006); (2) space competition where fungi defend their space against other microorganisms by, for example acidifying the rhizosphere, to prevent growth of other microorganisms (Benítez et al., 2004).

6.2. Production of an antimicrobial substance and mycoparasitism

Direct antagonism is defined as being the inhibition or destruction of organisms by the production of metabolites synthesized by the fungal biocontrol agents. These metabolites encompass volatile or non-volatile toxic molecules such as antimicrobial substances or lytic enzymes. Production of these metabolites by fungus is sometimes triggered by its substrate depletion. This prevents other microorganisms from competing for scarce resources and colonizing its niche (Irtwange, 2006; Viterbo et al., 2007).

Several substances produced by fungi act as antimicrobial compounds. These substances inhibit the growth of the pathogen. For example, glyovirin synthesized by *Gliocladium virens* inhibits the growth of *Pythium ultimum* by causing the coagulation of its protoplasm (Esser, 2002).

Parasitism and, more precisely mycoparasitism in fungi, is a mode

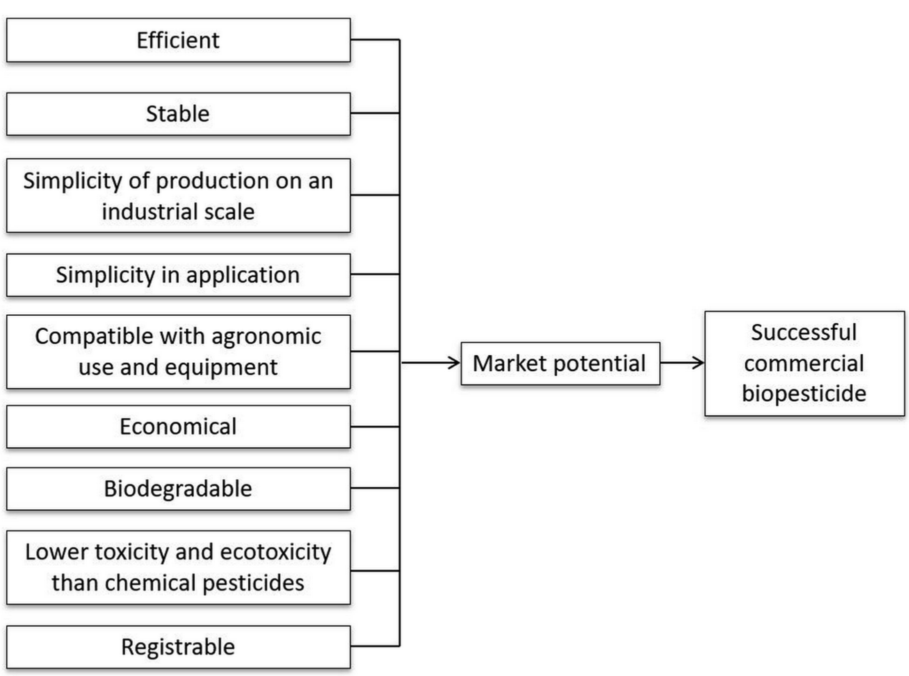


Fig. 3. The most important characteristics of successful commercial mycopesticides.

of action which plays a role in the reduction of plant diseases where the target species serves as a nutrient source for the biocontrol fungi. This mode of action is called Mycoparasitism. This is a very complex process that involves sequential events. The first step includes the recognition of the host. The second step is direct contact with the host, followed by the secretion of extracellular lytic enzymes to destroy the cell wall (Irtwange, 2006; Viterbo et al., 2007). For example, *Chaetomium sp* is a fungal species that produces β-1,3 glucanase. This enzyme has the ability to degrade the cell walls of plant pathogens including *Rhizoctonia solani* or *Gibberella zeae* (Sun et al., 2006). This characteristic makes it a potential mycopesticide.

Mycoparasitism is divided in two types, differentiated according to parasite aggressiveness towards its host. Necrotrophic, where parasites have a destructive mode of action, leads to the death of the host organisms. In the biotrophic mycoparasitism, the development of the parasite is favored by the survival of the hosts rather than their death (Benítez et al., 2004; Kaewchai et al., 2009).

6.3. Induced systemic resistance

To fight a pathogen, plants can activate a wide range of biochemical and molecular defenses: this system is called systemic resistance. With this system, in the presence of deleterious microorganisms, plants implement preventive mechanisms by producing secondary metabolites that have antimicrobial activities such as phytoalexin or by cell wall thickening with lignin deposition (Harman et al., 2004; Monfil and Casas-Flores, 2014). The interaction of plants with non-pathogenic microorganisms can also induce systemic resistance. One can take advantage of using these microorganisms in biological control (van Loon, 2007).

6.4. Trichoderma sp., a mycopesticide with multiple modes of action

The biological control mechanism in *Trichoderma* is a combination of the different modes of action (Fig. 4). *Trichoderma* is considered as an aggressive competitor. In order to avoid starvation, *Trichoderma* species grow very fast to colonize substrates and to exclude pathogens such as *Fusarium* (Kumar and Gupta, 2016). Under iron-limiting conditions, *Trichoderma* excretes low molecular weight ferric iron specific chelators termed as siderophores, which aid in sequestering and transport of iron. This competition decreases the germination rate of *Fusarium* spores. In addition, the colonization of the roots and precisely their extremity by *Trichoderma* reduces the infection by pathogens such as *Fusarium* and *Pythium* (Felix et al., 2014; Mohiddin et al., 2011).

Trichoderma is well known for its production of a series of molecules that have inhibitory or destructive effects against competitive microorganisms. They include pyrones such as 6-pentyl-alpha-pyrone (6-PP), polyketides (PKs), mycotoxins such as L and M koninginins produced by *T. koningii*, antimicrobial substances and lytic enzymes (Lang et al., 2015; Monfil and Casas-Flores, 2014).

Gliotoxin, harzianic acid, viridin and viridiol are examples of antimicrobial substances produced by *Trichoderma sp* (Kaewchai et al., 2009; Viterbo et al., 2007).

The mechanism of mycoparasitism of *Trichoderma sp*. relies on the production of enzymes that have lytic effects such as cellulases, glucanases, proteases and chitinases (De los Santos-Villalobos et al., 2013). The proteases produced by *T. harzianum* T-39 are involved in the degradation of membranes of the hyphae and cell walls of *Botrytis cinerea* (Elad and Kapat, 1999).

More recent studies have shown that application of *Trichoderma* on cotton roots induces local, but not systemic, resistance by increased accumulation of terpenoids and induction of peroxidase activity in treated plants (Harman et al., 2004; Lamovšek et al., 2013). Secondary metabolites produced by *T. harzianum* and *T. atroviride* induce a systematic defense in tomato plants against *Botrytis cinerea* and *Leptosphaeria maculans* (Vinale et al., 2008).

Peptaibol antimicrobial agents produced by *T. pseudokoningii* SMF2 and inducing a systematic defense and resistance reaction against the tobacco mosaic virus is another mechanism featured by *Trichoderma* (Luo et al., 2010).

6.5. The increase in the complexity of the mycopesticide mode of action, the example of Pseudozyma flocculosa

There are fungi, such as *Pseudozyma flocculosa*, that have biocontrol effects but their mode of action remains complex to decrypt. Antibiosis was initially suggested as its mode of action (Hajlaoui et al., 1992). This rapid action on mildew is achieved by the production of glycolipid molecules named flocculosin. This latter was purified from a solid culture medium of *P. flocculosa* and was shown to possess an important antifungal activity *in vitro* (Cheng et al., 2003). The sequencing of a group of complex genes that regulates the synthesis of flocculosin showed that *P. flocculosa* synthesized flocculosin in response to the variable availability of substrates on the leaf surface (Hammami et al., 2011).

Later, Hammami et al. (2011) showed that the specificity of the biocontrol activity of *P. flocculosa* appears to be more complex than simple antibiosis toward *Erysiphales* (Hammami et al., 2011).

Recently a transcriptomic analysis has highlighted a complex phenomenon in *P. flocculosa* called hyperbiotrophy. This phenomenon is the result of a tritrophic interaction between the biocontrol agent, the plant and the pathogen. The infection by the pathogen is an essential factor in stimulating the *P. flocculosa* action. After infection, *P. flocculosa* indirectly parasitizes the plant (barley), albeit transiently, by diverting nutrients extracted by the pathogen (*Blumeria graminis)* from barley leaves through a process involving unique effectors. The interaction culminates with a decline of the pathogen, thereby stopping *P. flocculosa* growth and enabling higher metabolic activity of the plant (Laur et al., 2018).

7. The impact of propagule types on the stability and the effectiveness of mycopesticides

Despite their versatile and complex modes of action, the efficacy of mycopesticide is still in most cases under the expected results. Some studies have shown that the stability of mycopesticides could play an important role in their effectiveness (Muñoz et al., 1995; Zaki et al., 2018). They indicate that an unstable product means a low efficiency product.

After an *in vitro* study, Hibar et *al.* showed in 2007 that there is no difference in efficiency between the *B. subtilis* bacterial biopesticides and a *T. harzianum* mycopesticide. But an *in vivo* study demonstrated that the mycopesticides have lost their efficacy (Hibar and Daami-Remadi, 2007). This loss of efficacy could have been due to the stability and susceptibility of the propagules of *T. harzianum* in environmental conditions. The choice of resistant propagules is thus essential to create a stable and effective mycopesticide.

There are three types of propagules that are used as an active substance in mycopesticides: small fragments of mycelium called hyphae, blastospore (also known submerged conidia) and aerial conidia (from the *Deuteromycota*) or aerial sporidia (from the *Basidiomycota*) (Laur et al., 2018; Zaki et al., 2018). The type of propagules differs according to the mode of production used (submerged fermentation or solid-state fermentation).

7.1. Hyphae and blastospores

Asexual conidia or spores are the primary means of protection and preservation of fungal genomes due to their resistance to environmental conditions such as extreme temperatures, UV light and desiccation (Morozova et al., 2001; Thomas et al., 1987). The preferred method for industrial scale production of conidia or spores is submerged

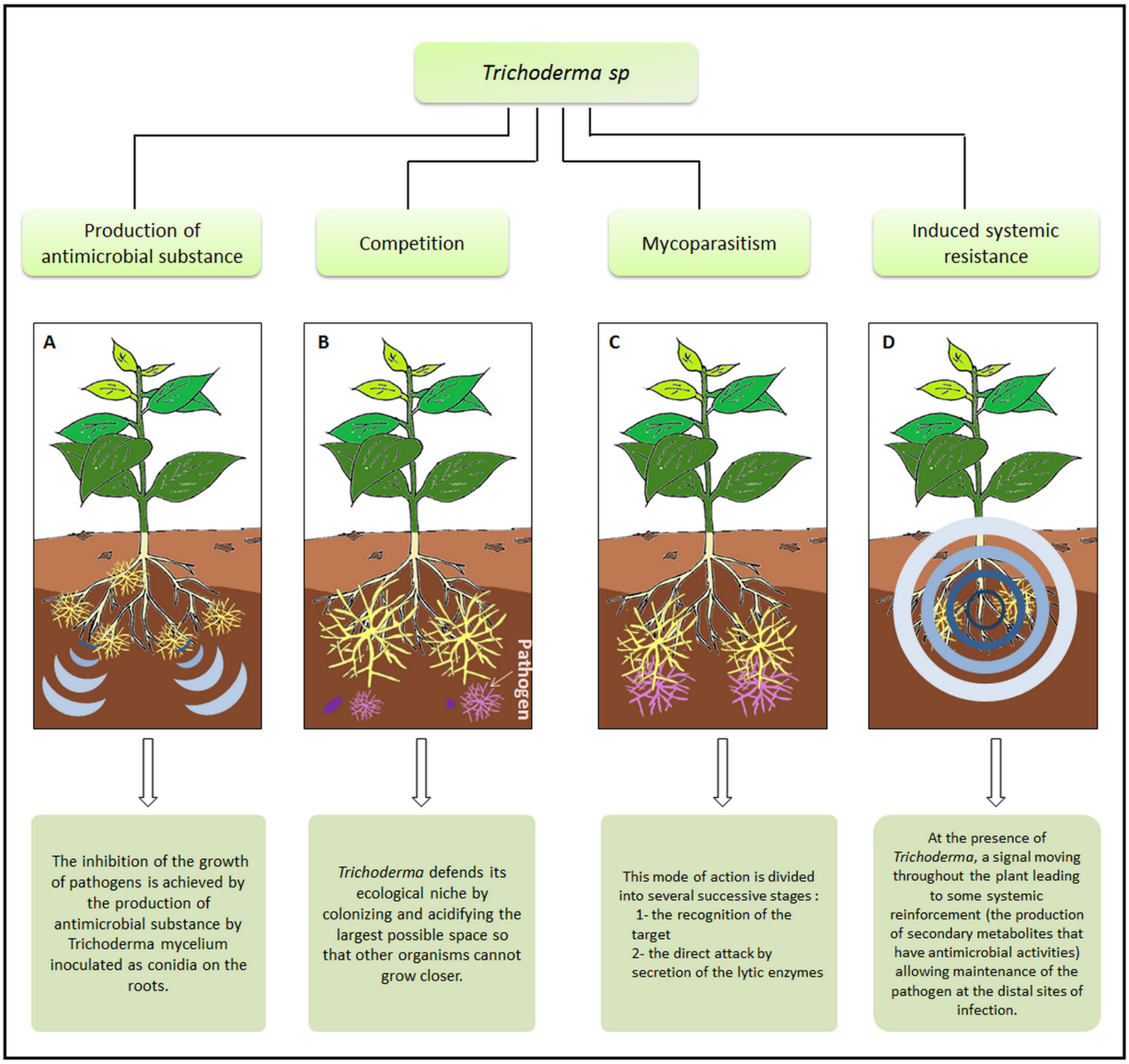


Fig. 4. View of the interactions of *Trichoderma sp*. in the context of biological control of plant diseases. The four illustrations describe the modes of action of this filamentous fungus. A: the inhibition of the growth of pathogens is achieved by the production of an antimicrobial substance by *Trichoderma* mycelium inoculated as conidia on the roots. B: competition where *Trichoderma* defends its ecological niche by colonizing and acidifying the largest possible space so that other organisms cannot grow closer. C: mycoparasitism. This mode of action is divided into several successive stages, 1- the recognition of the target, 2- the direct attack by secretion of the lytic enzymes. D: induced systemic resistance. This mode of action relies on a signal moving throughout the plant leading to some systemic reinforcement (the production of secondary metabolites that have antimicrobial activities) allowing maintenance of the pathogen at the distal sites of infection.

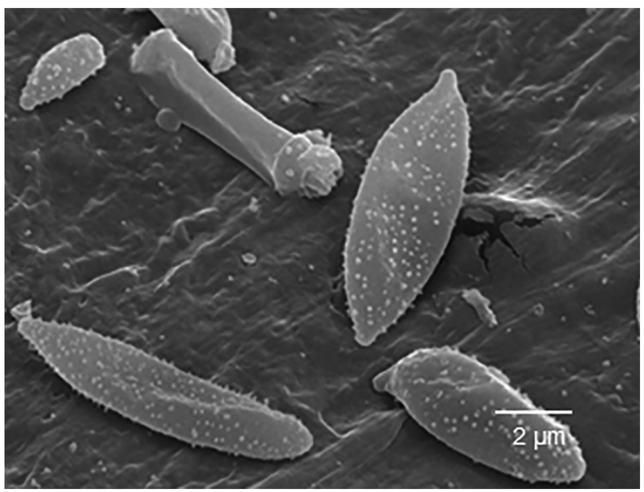
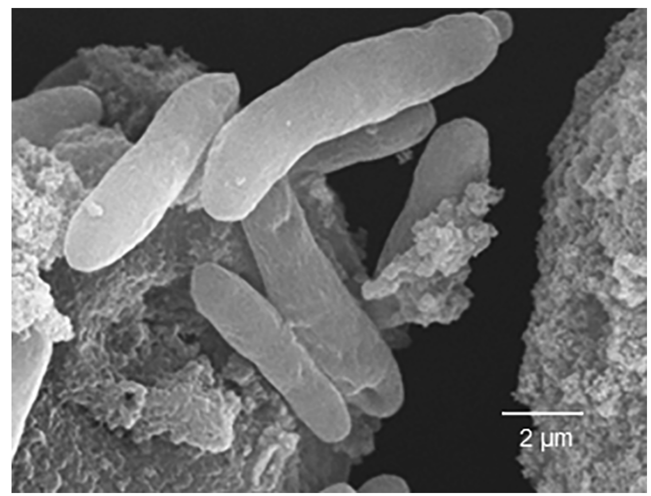
fermentation due to its high profitability, short production time and the availability of all materials and equipment required for liquid-state fermentation (LSF). In addition, process parameters such as pH, aeration and temperature can readily be controlled at the desired levels.

With LSF and according to species, the Hyphomycetes may be brought to develop in various forms, including, unicellular hyphae which often form cylindrical blastospores. The latter are obtained directly from other blastospores, or from conidiogenous cells which form on submerged hyphae (De La Cruz Quiroz et al., 2015; Zaki et al., 2018). They are called submerged conidia in *Deuteromycota* and submerged sporidia in *Basidiomycota*.

Despite all the advantages mentioned above, the propagules produced by this technique have a very short life in adverse environmental conditions. Therefore, most biocontrol agents based on blastospores require immediate use on plants. Muñoz et al. (1995) has shown that the viability of *T. Hazianum* blastospores decreased drastically to 15% after 45 days of storage at room temperature (Muñoz et al., 1995). In addition, they are very sensitive to UV. A recent study showed that the submerged sporidia that come from liquid fermentation are devoid of an outer melanin layer (Zaki et al., 2018). This pigment plays a very important role in the protection of fungal cells against unfavorable environmental factors such as UV rays, lytic enzymes and toxic metals (Nosanchuk et al., 2015). Probably, the absence of melanin may be responsible for their low stability and their sensitivity to UV.

Several formulations have been initiated since 1970 that can be used to maintain the stability of mycopesticides. For example, *Beauveria brongniartii* blastospores mixed with sugar and stored at 23 °C under vacuum maintain viability for 8 months (Butt et al., 2001). In 2006, Jakson showed that the addition of whole milk to the formulation, which is composed of a starch-oil mixture (Fantesk ™), significantly improved the stability of lyophilized *Paecilomyces fumosoroseus* blastospores stored at 20 °C up to 3 months and up to 12 months if stored at 4 °C (Jackson et al., 2006). Mascarin *et al* 2015 showed that storage stability of blastospore varied with nitrogen source and fungal strain. Air-dried blastospores of *B. bassiana* strains showed half-lives > 14 months at 4 °C in contrast to 9.2–13.1 months for *Isaria fumosorosea*. But the storage of biopesticides in refrigerators is not convenient on the farm. In addition, the stability needs to reach 18 months to consider it as a successful commercial mycopesticide (Aak et al., 2018). Several studies have shown that the encapsulation of blastopores with

Table 1
Comparison between aerial conidia and submerged conidia. SSF: solid-state fermentation, LSF: liquid-state fermentation.

Property	Aerial conidia by SSF	Submerged conidia by LSF	References
Viability at 20 °C	Stable	Reduced	(Muñoz et al., 1995; Watanabe et al., 2006)
UV resistance	Resistant	Sensible	(Muñoz et al., 1995)
Drying resistance	Resistant	Sensible	
Wall	Thick	Thin	(Muñoz et al., 1995; Watanabe et al., 2006)
Germination	Slow	Fast	(Feng et al., 2002)
Hydrophobicity	Marked	Average	(Pascual et al., 2000)
Ornamentation	Characteristics: warty, thorny, rough wall (depends on the species)	Absent	(Watanabe et al., 2006; Zaki et al., 2018)
Mitochondria	Rare	Present	
Vacuole	Rare	Present	(Watanabe et al., 2006)
Exp: *Pseudozyma flocculosa*			

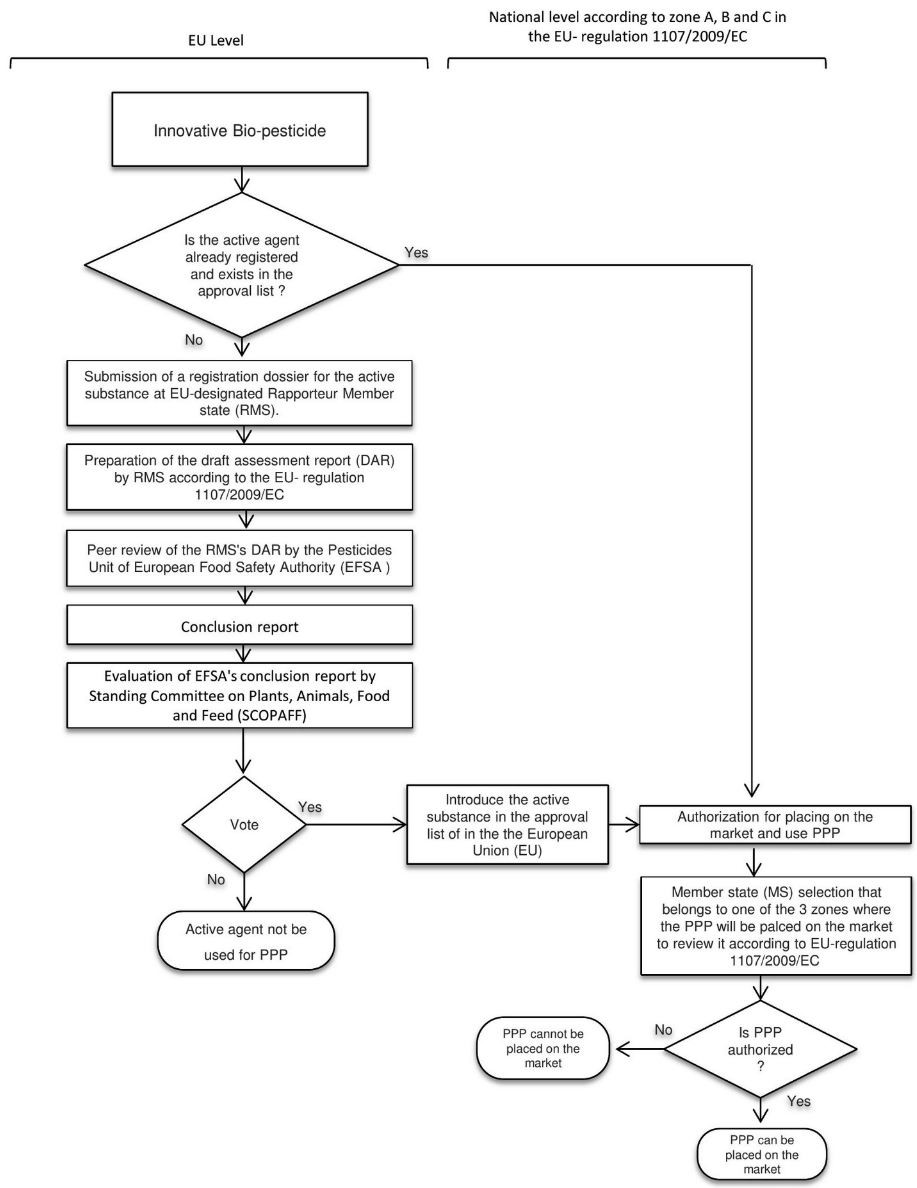


Fig. 5. The evaluation sequence followed for the registration of a new biopesticide product in the European Union according to the (EC) N° 1107/2009. The registration takes place in two stages. EU level: to introduce the active substance in the EU approved list and national level: for the registration of Plant Protection Products (PPPs) in one of 3 zones according to EU regulation 1107/2009.

polysaccharides by the spray drying technique followed by storage under vacuum condition maintains their viability for one year at 20 °C (Goettel et al., 1990; Wandersch et al., 1990). However, none of these techniques has been successfully applied on an industrial scale. Low stability, cold preservation and absence of melanin indicate that blastospores are not suitable forms for biopesticide application.

7.2. Aerial spore or conidia

Aerial conidia (AC) or aerial sporidia (AS) are spores produced in solid-state fermentation (SSF). This system of fermentation resembles the natural way of microbial life, especially that of fungi. In this process the substrate has the ability to support microbial growth and metabolism production (De La Cruz Quiroz et al., 2015; Thomas et al., 2013). This induces the differences in the morphological, functional and biochemical properties of the spores produced by SSF and LSF (see Table 1). In addition, AC and AS from SSF survive longer under natural and drastic environmental conditions. Watanabe et al (2006) has shown that AC of *T. asperellum* SKT-1 maintained high viability under conditions in which blastospores produced by LSF lost their viability (Watanabe et al., 2006).

Several studies have shown that there is a difference in surface topology and internal organization between aerial conidia and blastospores. The cell walls of the aerial conidia of *T. asperellum*, *T. harzianum* and *V. lecaniis* are thicker and more verrucous than those of blastospores which have a smooth wall. In addition, *T. harzianum* aerial conidia are more resistant to UVc radiation (Feng et al., 2002; Muñoz et al., 1995; Watanabe et al., 2006). There is a large morphological difference between the aerial sporidia *Pseudozyma flocculosa* from LSF and the blastospores from SSF. The cell wall of submerged sporidia was thinner than that of aerial sporidia. The thickness of the aerial sporidia wall is due to the presence of a melanin- rich outer layer (Zaki et al., 2018). The presence of melanin in aerial spores and its absence in blastospores could explain the respectively high and low stability in both types of propagules.

The advantages of SSF are responsible for the commercial success of some mycopesticides, but it presents difficulties in controlling the

Table 2
Mycopesticides products approved in Europe (EU). Formulation; SC: suspension concentrate, WP: wettable powder, GR: Granular, EC: emulsifiable concentrate, OD: Oil Dispersion and WG: Water dispersible granule. Propagules; C: Conidia, B: Blastospores and H: Hypha. EU: European Union and US: United States.

Product	Strain	Target	Formulation	Propagules	Producer	Approval in EU or US	References
Mycoinsecticides							
Naturalis L	*Beauveria bassiana strain ATCC 74040*	Japanese beetles, chinch bugs, mole crickets, and sod webworms	OD	C	Troy Biosciences Inc., USA	EU and US	(Fleury et al., 1984; Lehr, 2015)
BotaniGard 22 WP	*B. bassiana GHA strain*	Whiteflies, thrips, aphids, psyllids, mealybugs, scarab beetles, plant bugs and weevils.	WP	–	lam International Corporation, USA	EU and US	(Ehlers, 2011; Fleury et al., 1984; Lehr, 2015; Parker et al., 2015; Shipp et al., 2012)
Mycotal	*Verticillium lecanii Ve6*	Whiteflies (*Bemisia tabaci*, *Trialeurodes vaporariorum*) and thrips (*Frankliniella occidentalis*).	WP/SC	C/B	Koppert Biological Systems, The Netherlands	EU	(Alavo, 2015)
BIO 1020	*Metarhizium anisopliae*						
ATCC 90448	*Otiorhyncus sultacus, Exomala orientalis, Fungus gnats, Sciaridae*	GR	H	Bayer AG, Germany	EU	(Ehlers, 2011; Fleury et al., 1984; Poidatz et al., 2018)	
GranMet WP	*M. anisopliae BIPESCO 5/F52*	*Otiorhynchus sulcatus, Strophosoma melanogram, Phyllopertha horticola, Amphimallon solstitialis, Diabrotica virgifera*	WP	–	Agrifutur s.r.L, Italy		
	EU	(Benheim et al., 2012)					
Met52EC	*M. anisopliae BIPESCO 5/F52*	*Tetranychus urticae, Bemisia argentifolii, Trialeurodes vaporariorum, Franklinieall occidentalis*	EC	–	Novozymes Biologicals, Inc., Denmark	EU	(Fischhoff et al., 2017)
Met 52 granular	*M. anisopliae BIPESCO 5/F52*	*Otiorhyncus sultacus, Exomala orientalis, Fungus gnats, Sciaridae*	GR	–	Novozymes Biologicals, Inc. Denmark	EU and US	(Fischhoff et al., 2017; Lehr, 2015; Mauchline and Stannard, 2013)
Tick-Ex G	*M. anisopliae BIPESCO 5/F52*	*Otiorhyncus sultacus, Exomala orientalis, Fungus gnats, Sciaridae*	GR		Novozymes Biologicals, Inc., Denmark	EU and US	(Balusu and Fadamiro, 2012; Ehlers, 2011; Irtwange, 2006; Saxena, 2015)
Tick-Ex EC	*M. anisopliae BIPESCO 5/F52*	*Otiorhyncus sultacus, Exomala orientalis, Fungus gnats, Sciaridae*	EC		Novozymes Biologicals, Inc., Denmark	EU and US	(Saxena, 2015)
PreFeRal	*Isaria fumosorosea* (ATCC 20874 or Apopka 97 strain)	Greenhouse whitefly (*Trialeurodes vaporariorum*)	WG	B	Biobest n.v., Belgium (under license from Certis, Inc., USA)	EU	(Aak et al., 2018; Ehlers, 2011)
Mycofungicide							
TRIANU M-P	*T. harzianum T-22 (ATCC 20847)*	*Pythium* spp. *Rhizoctonia* spp. *Fusarium spp*	WP	–	Koppert BiologicalSystems, The Netherlands	EU	(Cruz et al., 2018; Lehr, 2015)
VIRISAN	*T. asperellum TV1*	Preventive action against soil pathogens such as *Pythium* spp., *Rhizoctonia* spp., *Phytophthora* spp., *Phoma* spp., *Verticillium* spp., and *Fusarium* spp.	WP	–	Sagro, USA	EU	(Woo et al., 2014)
REMEDIER	*T. gamsii ICC080*	Preventive action against soil pathogens such as *Pythium* spp., *Rhizoctonia* spp., *Phytophthora* spp., *Phoma* spp., *Verticillium* spp., and *Fusarium* spp.	WP	–	Sagro, USA	EU.	(Asad et al., 2014; Woo et al., 2014)
TUSAL	*T. asperellum strain T25*	*Phythophthora sp, Fusarium* sp., *Rhizoctonia solani, Pythium* sp., *Sclerotinia sclerotiorum*	WG	–	Sagro, USA	EU	(Woo et al., 2014)
T34 Biocontrol	*T. asperellum strain T34*	*Fusarium oxysporum f.sp. dianthi*	WP	–	Biocontrol Technologies S.L., Fargro Ltd Spain	EU and US	(Segarra et al., 2013)
ESQUIVE	*T. atroviride* strain I-1237	Wood decay diseases	WP	–	Agrauxine, France	EU	(Woo et al., 2014)
BINAB TF WP	*T. atroviride* ATCC 20476	*Botrytis cinerea, Chondrostereum purpureum*	WP	–	BINAB Bio-Innovation AB, Sweden	EU	(Woo et al., 2014)
BINAB T Vector	*T. atroviride* ATCC 20476	*Botrytis cinerea, Chondrostereum purpureum*	P	–	BINAB Bio-Innovation AB, Sweden	EU	
AQ-10	*Ampelomyces quisqualis* strain AQ10	Powdery mildew	WG	B/H	Intrachem France	EU and US	(Fondevilla and Rubiales, 2012; Siozios et al., 2015)
AQ-10 WG	*A. quisqualis* strain AQ10	Powdery mildew	WG	B/H	Intrachem France	EU	

physicochemical parameters (De La Cruz Quiroz et al., 2015). For example, *Paecilomyces fumosoroseus* needs light for optimal production of aerial conidia (Wraight et al., 2001). The production of conidia by solid fermentation requires a large surface area, periodic agitation and particulate solid substrates. Moreover, the increase in temperature and the pH variation during the vegetative growth phase is a critical problem in the solid fermentation domain (Harman et al., 2004; Sun et al., 2006).

8. Regulation of mycopesticides in the European Union

The last obstacle to develop mycopesticides in the European Union is the registration dossier. Mycopesticide products may also have non-beneficial effects. For this reason, the commercialization and use of mycopesticides in the European Union requires authorization or approval in order to avoid hazards to humans, animals and the environment. However, the regulations do not differ between biopesticides and pesticides. Even the low risk substances need to go through the complete evaluation procedure.

To be registered, mycopesticides must comply with three regulations in the European Union. The first is Regulation N° (EC) 1107/2009 dictated by the European Commission and regarding the placing of plant protection products on the market. This Regulation replaces the previous framework Directive 91/414/EEC. The second is the Regulation (EC) N° 1185/2009 on pesticide statistics. The third is the Directive (EC) N° 2009/128 on the sustainable development and use of pesticides (Deravel and François Krier, 2014; Villaverde et al., 2014). The aim of these regulations is to ensure a high level of protection for human and animal health and for the environment while at the same time preserving the competitiveness of European Community agriculture. It is applied to all phytosanitary products. The precautionary principle is applied and these regulations ensure that industry demonstrates that substances or products manufactured or placed on the market have no adverse effect.

Regulation (EC) N°1107/2009 explains the procedures and requirements concerning the placement of plant protection products (PPPs) on the market. In this regulation, the microorganisms are subject to the same approval procedure as the active substances of chemical products. A dossier must be submitted to a Member State in the European Union (EU) designated as the Rapporteur Member State (RMS). After completeness check and evaluation, an initial draft assessment report (DAR) will be produced and sent to the European Food Safety Authority (EFSA).

The DAR must contain all the information required by article 3 on the identity of the active substance, on its biological efficacy, its toxicity on the environment, against animals and the risk to consumers. After peer review of the RMS's DAR by the Pesticides Unit of EFSA, it produces a public conclusion report. The Risk managers of the Standing Committee on Plants, Animals, Food and Feed (SCOPAFF) will analyze the EFSA's conclusion report and they will vote to decide whether or not to register the compound on the EU's list of approved active substances (European Council, 2009; Storck et al., 2017). The biopesticide registration chain is described in Fig. 5.

The last step is the registration of the finished product PPP in different member states in the EU. In order to avoid registering the PPP in each country according to Directive 91/414 / EEC directives, the (EC) n° 1107/2009 divides Europe into three geographical zones according to pedoclimatic criteria (Zone A, B and C) in order to speed up the regulatory process in different Member States. The review procedure of PPPs is similar to that of active substances.

The biological origin of the substances and products proposed for approval does not guarantee their approval. Despite the low risk status in the Regulation CE 1107/2009, this status is only granted after a full evaluation procedure, because to register the active ingredient of biopesticide, CE 1107/2009 requires the same data as the registration of a conventional pesticide. The long duration and the high cost of registration lead SME (small and medium-sized enterprises) to look for their own markets outside the EU. Table 2 shows some mycopesticides registered in the European Union.

9. Conclusions and prospects

As shown in this review, the commercial evolution of biopesticide market is promising with a recent compound annual growth rate (CGAR) of 14.1%. In addition, different countries all over the world have taken different political initiatives to speed-up their development. Among the broad set of biocontrol agents, the mycopesticides are interesting products because they use several modes of actions to reduce plant diseases related to phytopathogenic fungi: nutrient competition, direct antagonism, mycoparasitism and induction of resistance mechanism in plants. Indeed, this diversity of mode of actions could reduce or delay the emergence of resistance mechanism. In 2016, the mycopesticides accounted for only 10% of the global biopesticide market.

The stability of the active substance is also an essential factor for creating good successful mycopesticides. For this to increase the adoption of mycopesticides, the chosen propagule types and their mode of production are major factors to be considered in enhancing the stability of a mycopesticide. The orientation towards the use of the solid-state fermentation technique could be a good choice to guarantee the stability of the conidia or sporidia of fungi. For this, the development of this technology has become necessary to increase the volume of production and profitability. For example, the development of different new supports or substrates is necessary to make fermentations more accessible and more flexible for users worldwide.

In European Union, the complexity of registration of biopesticide products is another limiting factor that delays the development of mycopesticides. Facilitating the attribution of the low risk substance status in the EU for biopesticides and mycopesticides with fast track registration procedure would be helpful to promote their development and success.

CRediT authorship contribution statement

Omran Zaki: Conceptualization, Methodology, Writing - original draft. **Frederic Weekers:** Supervision. **Philippe Thonart:** Validation, Writing - review & editing. **Erin Tesch:** Validation. **Philippe Kuenemann:** Validation. **Philippe Jacques:** Supervision, Validation, Writing - review & editing.

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