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MINI-REVIEW

Advances in submerged liquid fermentation and formulation of entomopathogenic fungi

Gabriel Moura Mascarin[1] · Patrícia Silva Golo[2] · Cárita de Souza Ribeiro-Silva[3] · Elen Regozino Muniz[3] · Artur de Oliveira Franco[3] · Nilce Naomi Kobori[4] · Éverton Kort Kamp Fernandes[3]

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Abstract

Entomopathogenic fungi (EPF) can be defined as beneficial multifunctional eukaryotic microorganisms that display pivotal ecological services in pest management, with some species possessing the special ability to establish mutualistic relationships with plants. Mass production of these fungi is critical to support affordable widespread commercialization and worldwide field application. Among the mass production methods explored mainly by industry, submerged liquid fermentation is a robust and versatile technology that allows the formation of different types of propagules designated for various applications in pest control. Many hypocrealean EPF are easily culturable on artificial substrates by producing single-celled structures (hyphal bodies, blastospores, and submerged conidia) or multicellular structures (mycelium and microsclerotia). Less frequently, some EPF may form environmentally resistant chlamydospores, but these structures have almost always been overlooked. A continued research pipeline encompassing screening fungal strains, media optimization, and proper formulation techniques aligned with the understanding of molecular cues involved in the formation and storage stability of these propagules is imperative to unlock the full potential and to fine-tune the development of robust and effective biocontrol agents against arthropod pests and vectors of diseases. Finally, we envision a bright future for the submerged liquid fermentation technology to supplement or replace the traditional solid substrate fermentation method for the mass production of many important EPF.

Key points

- *Submerged liquid fermentation (SLF) allows precise control of nutritional and environmental factors*
- *SLF provides a scalable, robust, and cost-effective platform for mycopesticide production*
- *Enhancing formulation, shelf life, and field efficacy of submerged propagules remain crucial*
- *Understanding the molecular mechanisms behind submerged propagule formation is key to advancing SLF technology*

Keywords Biological control · Mass production · Blastospores · Microsclerotia · Submerged conidia · Bioreactor

Introduction

The direct and indirect damages to agronomically important plants caused by arthropod pests cause economic losses estimated to exceed hundreds of billions of dollars annually (Savary et al. 2019). These statistics underscore the impact of these pests on food production and the global economy, affecting not only farmers and agricultural industries but also food security and the welfare of communities worldwide. Non-agricultural arthropod pests (e.g., livestock ectoparasites) are equally significant since they can negatively impact animal production and consequently increase the costs of animal-origin products (Grisi et al. 2014). Arthropods can also impair food stocks and act as vectors of pathogens that cause diseases affecting humans and other animals (Perveen

✉ Gabriel Moura Mascarin
gabriel.mascarin@embrapa.br; gmmascar@gmail.com

✉ Éverton Kort Kamp Fernandes
evertonkort@ufg.br

[1] Laboratório de Microbiologia Ambiental, Embrapa Meio Ambiente, SP 340 Road, Km 127.5, Tanquinho Velho, Jaguariúna, SP 13918-110, Brazil

[2] Departamento de Parasitologia Animal, Instituto de Veterinária, Universidade Federal Rural Do Rio de Janeiro, Seropédica, RJ 23897-000, Brazil

[3] Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Avenida Esperança S/N, Campus Samambaia, Goiânia, GO 74605-050, Brazil

[4] Independent Researcher, Jaguariúna, SP 13912-478, Brazil

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et al. 2023). The use of entomopathogenic microorganisms as biocontrol agents of arthropods is an attractive and sustainable tool for many integrated pest management programs in forestry, agricultural, and veterinary settings. The effectiveness of these biocontrol agents is strongly affected by environmental factors, propagule type, mass production method, species and strains, formulation, and delivery strategies, as well as by the susceptibility of the arthropod host.

Brazil's biologicals market is rapidly expanding, with numerous players introducing novel mycopesticides, thereby contributing significantly to the global biopesticide industry. The country stands out as one of the most prominent and expansive markets for biologicals due to its unique diversity in climate, soil, and vegetation. This diversity fosters dynamic indigenous microbial communities with a wide range of ecological functions, from biocontrol agents to plant growth promoters and beyond. The size of the biologicals market in Brazil has doubled since the launch of the National Bioinputs Program (NBP) in 2019, according to the Ministry of Agriculture (*Ministério da Agricultura e Pecuária* (MAPA)) and laid the foundation for remarkable growth in this pest control sector. For the period 2023–2027, DunhamTrimmer estimates an 18.3% CAGR (compound annual growth rate) for biocontrols, a 12.3% CAGR for biostimulants, and a total biological market CAGR of 16.7% (DunhamTrimmer 2023). At the end of 2023, there were almost 700 registered biological products in Brazil. Microbial bioinsecticides and bionematicides account for more than half of Brazil's biocontrol market and about one-third of its biological market overall. Particularly, entomopathogenic fungi (hereafter referred to as EPF) represent a significant portion of this biopesticide market and are led by *Beauveria bassiana* and *Metarhizium anisopliae* (Fig. 1). Although liquid fermentation technology has been utilized in companies in the USA and Europe, Brazilian EPF producers still utilize mostly aerial conidia derived from the traditional solid substrate fermentation using pre-cooked and moistened cereal grains, mainly rice, for the mass production of mycopesticides used to tackle arthropod pests (Mascarin et al. 2019). In contrast, non-regulated, non-commercial on-farm producers generally utilize liquid fermentation, adapting technologies designed for bacterial production (Faria et al. 2023).

Most mycopesticides based on hypocrealean EPF (Ascomycota: Hypocreales) are comprised of airborne conidia (Faria and Wraight 2007), the infective unit of key genera, including *Metarhizium*, *Beauveria*, *Hirsutella*, *Akanthomyces* (formerly *Lecanicillium*), and *Cordyceps* (formerly *Isaria*). Typically, these infective spores are produced by simple low-tech solid-state fermentation technique, in which

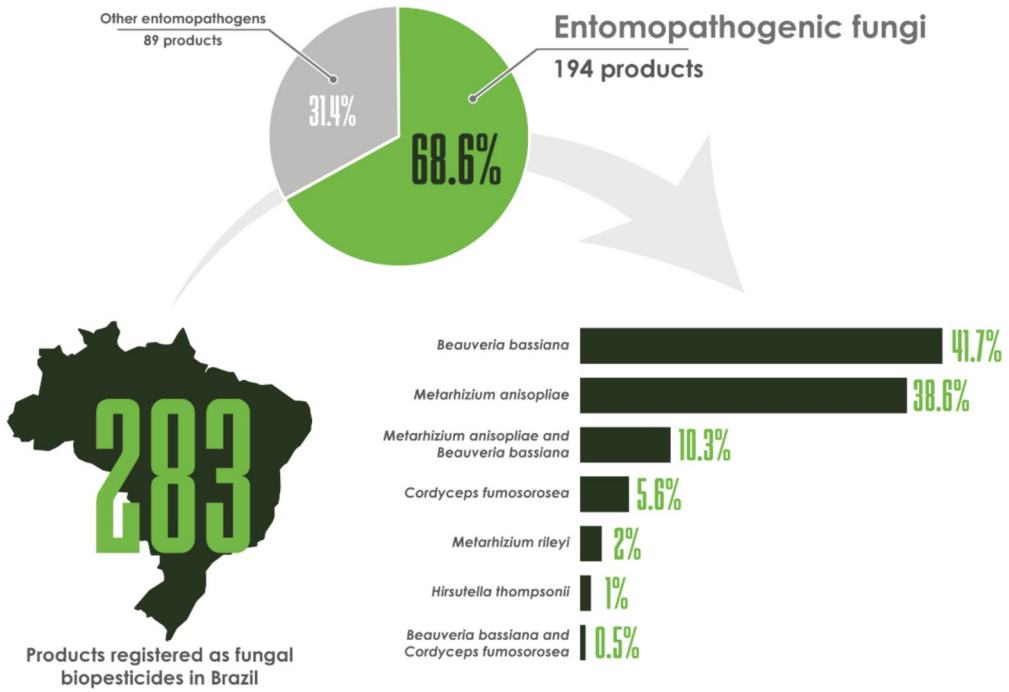

Fig. 1 A snapshot of the commercial fungal biopesticides registered in Brazil by March 2024 for controlling arthropod pests, including insects and mites. Source: AGROFIT, 2024. Even though recent evidence indicates that commercialized Brazilian fungal strains previously thought to be *Cordyceps fumosorosea* are shown to be *Cordyceps javanica* (Lopes et al. 2023b), we have chosen to refer to them as *C. fumosorosea* in this infographic. This is because *C. fumosorosea* is currently listed as the active ingredient in mycopesticides registered in Brazil, and, therefore, this is the species found in the Agrofit database

the scale-up process is performed by growing the fungus on moistened cereal grains kept in plastic bags or trays for several weeks under controlled environmental conditions. This production method is practiced in countries like Brazil, China, and the USA, where they present a myriad of programs in microbial pest control using EPF in extensive open field areas and protected environments (Arthurs and Dara 2019; Li et al. 2010). Interestingly, *B. bassiana* strain GHA and *M. brunneum* strain MetF52 are still largely produced by solid substrate fermentation in the USA, and their conidia are commercially distributed in formulated products around the globe (Jaronski 2023). In Brazil, for instance, more than 80% of the registered mycopesticides are based on two fungal strains namely IBCB-66 of *B. bassiana* and IBCB-425 of *M. anisopliae*, mainly produced by a single company that processes daily approximately 40 tons of rice to produce conidia (G. M. Mascarin, personal information). On the other hand, many cases support the advantages of submerged liquid fermentation over solid-state (or solid substrate) production (Iwanicki et al. 2021; Mascarin et al. 2015a,b, 2018, 2022). The solid-state fermentation technique has several drawbacks related to (*i*) the cost of cereal grain substrates, (*ii*) labor cost related to intensive manual handling, (*iii*) poorly regulated nutritional and environmental conditions which increase contamination by undesired microorganisms and jeopardize product quality, (*iv*) low energy efficiency due to the extended production time, often exceeding 10 days and sometimes taking over 3 weeks, coupled with the high energy and space requirements needed to autoclave large volumes of solid substrates, (*v*) large environmentally controlled rooms required to accommodate tons of fungus-colonized substrate, and (*vi*) another genuine risk related to the human involvement which is the safety issues raised by working in facilities where the production technique virtually assures airborne conidial exposures and possible allergenic reactions mainly without PPE (personal protective equipment). Conversely, the submerged liquid fermentation allows better control of the nutritional and environmental conditions that lead to a reduction of the fermentation time (30–72 h), lower labor and energy costs, and, ultimately, a higher quality EPF products.

Submerged liquid fermentation (SLF) takes place in deep-tank bioreactors that are automated, easily scalable to thousands of liters, and is less labor-intensive compared to solid substrate fermentation (SSF) methods (Fig. 2). Manipulation of the nutrient composition and physical factros (viz., carbon, nitrogen, minerals, vitamins, carbon-to-nitrogen ratio, aeration rate, temperature, rheology, osmotic pressure, and pH) makes this process easy to evaluate proper nutritional and environmental conditions for producing high amounts of active fungal propagules, including submerged conidia, blastospores, mycelium, microsclerotia, and chlamydospores. In liquid fermentation technology, a wide array of templates has been designed for the food, bioenergy, and biotechnology industries that can be adapted to the production of EPF (Fig. 3). Low-cost medium components are crucial for the economic success of the process and must be optimized to meet the quality standards of the final product produced in the downstream process. Globally, there are few mycopesticides based on liquid-grown propagules available

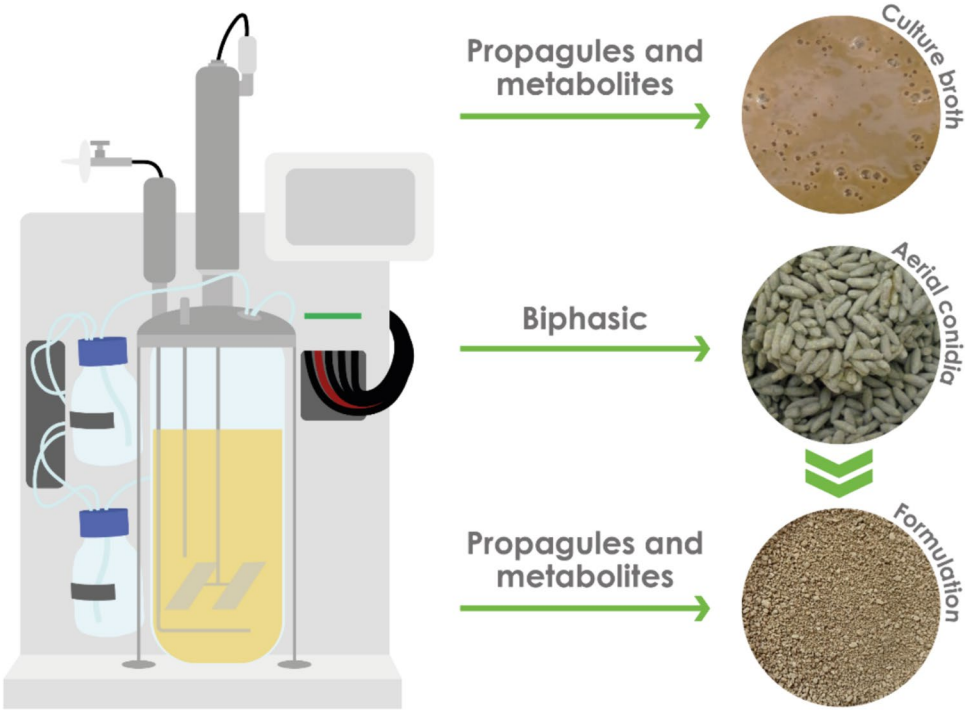

Fig. 2 Multifaceted capabilities afforded by submerged liquid fermentation technology in mass production of EPF for use as mycopesticides. After fermentation, culture broth can produce many enzymes, secondary metabolites, and active biomasses. Biphasic or two-phase fermentation can explore the culture broth as the primary inoculum for the subsequent solid-substrate fermentation process, which contributes to shortening the fermentation time while minimizing contamination risks. Biomass obtained by submerged cultivation offers different infective or resistant propagules that are amenable for formulation

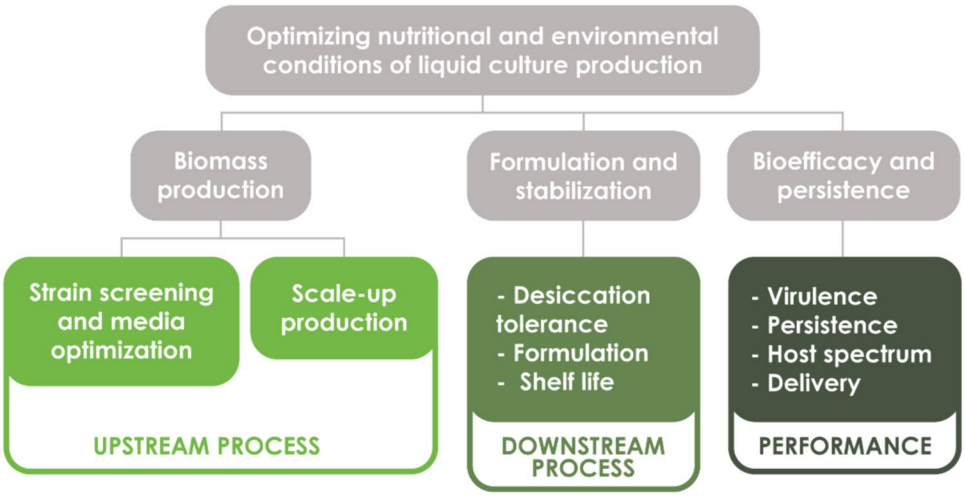

Fig. 3 Strategies employed when developing mycopesticides based on the submerged liquid fermentation process. Firstly, emphasis is placed on the optimization of the nutritional and environmental conditions during liquid culture growth tailored by the species and strain of the fungus, followed by scale-up production to validate the fermentation conditions. Secondly, formulation and stabilization are designed to focus on desiccation tolerance and extended shelf life. Thirdly, bioefficacy and persistence tests are carried out to assess product virulence, persistence, host spectrum, and delivery strategies for field application

on the market, primarily from *C. fumosorosea*, *C. javanica*, and *Akanthomyces muscarius*. However, none of these preparations is registered in Brazil. This domestic scenario in Brazil creates a unique opportunity for investments to allow submerged liquid fermentation to become Brazil's main biotechnological mass production platform for mycopesticides.

The formulation has a role in boosting the value of the active ingredient by altering the chemical and physical attributes of a fungal propagule for improved insecticidal/acaricidal activity under varied environmental conditions. The stabilization method used during formulation, i.e., the drying technology and careful selection of compatible additives to the formulation, is a critically important step when developing a shelf-stable mycopesticide. Formulated fungal propagules have advantages over unformulated products, including the following: (*i*) to enhance biological activity, (*ii*) to improve persistence in the field, (*iii*) to aid handling and application of the product, (*iv*) to provide biosafety, and (*v*) to stabilize living cells during distribution and storage (Brar et al. 2006; Burges 1998). Therefore, these beneficial characteristics ensure robustness and consistent performance to mycopesticides. Formulation costs are variable and rely on various factors, including ingredients, equipment, drying method, and active ingredient nature and volume. To reduce runoff by rain and deleterious effects caused by ultraviolet (UV) exposures, high temperatures, and low relative humidity under field conditions, fungal cells can be strategically formulated with exogenous protective agents such as oils (Alves et al. 1998; Bateman et al. 1993) and lignin (Leland and Behle 2005); the addition of nutrients or surfactants to the formulated EPF can also provide enhanced germination and speed of kill (James 2001). The toxicity of formulation components should also be carefully addressed for different fungal propagules using standard and reliable compatibility protocols usually performed by in vitro tests. Blastospores and other vegetative fungal cells are usually more sensitive than aerial conidia to some chemical adjuvants, such as long-chain alkyl-based surfactants (Jackson et al. 2009). Therefore, a thorough basic understanding of how fungal propagules interact with their host target or respond to their target environment should lay the foundation for designing proper formulations of these fungi.

Resistance to anhydrobiosis stress and storage stability of fungal propagules is also relevant when developing appropriate formulations. Some formulation components, such as exogenous nutrients, osmoprotectants, thermoprotectants, and UV blockers, can be added during growth or drying to improve desiccation tolerance, shelf life, and field persistence (Behle and Birthisel 2023). Moreover, a compatible drying method is essential for fungal viability and storage stability. Damage by dehydration can adversely affect fungal cell integrity and metabolism, thereby hindering the maintenance of viability. The type of fungal cell and its nutritional status, speed of water removal, temperature, relative humidity, and water content in the final product are among the main factors that affect cell integrity, viability, physiological vigor, and stability.

The packaging system deserves special attention after formulation and is considered a bottleneck for prolonged mycopesticides' shelf life. The poor shelf life of mycopesticides poses a barrier to their broad commercialization, especially in the tropics (Faria et al. 2022). Generally, low water

content (< 5% moisture), low temperature, and reduced oxygen levels are critical for extended storage stability of fungal propagules. Active packaging can be performed by including exogenous oxygen and moisture scavengers to prolong fungal survival during nonrefrigerated storage, which is of great interest for the widespread commercialization of mycopesticides (Faria 2011; Faria et al. 2012; Iwanicki et al. 2021; Jin et al. 1999; Mascarin and Jackson 2016; Mascarin et al. 2016). This technology has been used for many decades in food and pharmaceutical products; now, several examples of its success are available and could be adapted for storage-sensitive fungal cells. An optimal formulation still constitutes a significant bottleneck for biological control using EPF. Still, in recent years, quite a few formulated products based mainly on oil dispersion of aerial conidia have appeared in several mycopesticides traded in Brazil (Mascarin et al. 2019). Conversely, formulation of blastospores faces a challenge to its use, and most of their formulations correspond to wettable powders or wettable dispersible granules (< 5% moisture content) with improved shelf stability, although more encouraging results are achieved when they are maintained under cold storage (Iwanicki et al. 2021; Lima et al. 2024; Mascarin et al. 2015a, 2016, 2018). In downstream processing, pre-existing techniques from the food and biotechnology industry provide a resourceful template tailored to mycopesticide manufacturing that includes different drying methods, such as spray drying, fluid-bed drying, vacuum rotary drum drying, air drying, or a combination of these techniques.

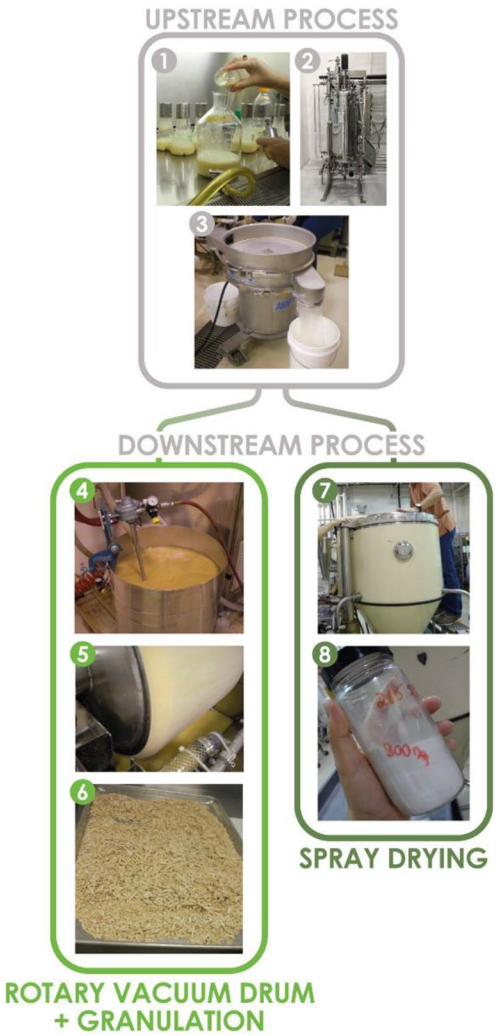

Fig. 4 Pilot-scale production platform of fungal blastospores, microsclerotia or submerged conidia followed by downstream processing via two routes: (1) liquid preculture preparation from shake flask cultures. (2) 100-L sterilizable-in-place, stainless steel, stirred-tank bioreactor commonly used for scale-up studies and to provide fungal inoculum for field tests. (3) Rotary vibrating sieve/screen for separation of fungal propagules by desired sizes, for instance mycelium is filtered out from blastospores, microsclerotia or submerged conidia. (4) Tank mix where formulation components are mixed with fungal biomass and kept under constant mechanical agitation to keep the mixture as uniform as possible. (5) Rotary vacuum drum dryer capturing fungal biomass through a layer bed based on an inert carrier (e.g., diatomaceous earth, bentonite) along with other additives of the formulation. (6) Slices of the dewatered mix comprising fungal biomass + carrier + formulation additives obtained after rotary drum filtration and ready for granulation and drying. (7) Industrial spray-dryer with rotary disc. (8) Spray-dried microencapsulated fungal propagules mixed with skim milk and other additives

Submerged liquid fermentation and formulation

EPF as biocontrol agents in large-scale field applications, especially for annual, semi-permanent, and perennial crops cultivated from thousands to millions of hectares, require sufficient biomass production in quantity and quality to meet global market demands for these products. Submerged liquid fermentation has numerous advantages over traditional solid substrate fermentation to meet this high inoculum demand of EPF for biocontrol purposes. Readers can also refer to previous review papers and book chapters addressing this topic with complementary details to this current review (Jackson 1997; Jaronski 2023).

EPF biomass produced by submerged liquid fermentation allows for easy downstream processing using different techniques or methods already employed at industrial scales in food science and pharmaceuticals. Downstream processing refers to the steps involved in the purification, separation, formulation, and drying of mycopesticides after they have been produced through fermentation or other biological processes. It is a crucial stage in producing mycopesticides, ensuring the final product meets quality standards and regulatory requirements. The downstream processing of a mycopesticide typically includes several key steps (Fig. 4):

1. Harvesting: The first step involves collecting the biopesticide-producing fungus. This is usually done once the fungus has reached its production peak during cultivation.
2. Filtration: In case the final biomass is blastospores, the desired fungal propagule may need to be separated from other unwanted biomass, such as hyphae. Thus, filtration, mainly performed by sieving, separates propagules of different sizes from the fermentation broth.
3. Concentration: After filtration, the desired fungal propagule can be concentrated using centrifugation or a rotary vacuum-filter system. This step is crucial for obtaining a high-quality and concentrated final product.
4. Formulation and drying: Once the biopesticide is purified and concentrated, it needs to be formulated into a product that can be easily applied in the field. Formulation generally involves mixing the fungal biomass with carriers, stabilizers, and other additives aiming to enhance its shelf life, stability, delivery and effectiveness. After formulating the biomass, drying reduces water content in the final product and contributes to extended shelf life.
5. Quality Control: Various quality control measures are implemented throughout the downstream processing to ensure the final mycopesticide meets the required specifications and regulatory standards. This includes testing for purity, potency, viability, vigor and other relevant parameters.

Effective downstream processing is critical in ensuring that the fungal biopesticide complies with regulatory requirements for safety and environmental impact, as well as safeguarding the final product's quality, efficacy, and commercial viability. Comparatively, when the speed of infection exerted by different submerged propagules of EPF is examined, it is reasonable to identify a higher speed for blastospores than for submerged conidia and aerial conidia generated from sporulated microsclerotia (Fig. 5). Many papers reported the remarkable high infection speed displayed by blastospores in comparison to aerial or submerged conidia (Alkhaibari et al. 2016; Mascarin et al. 2015a,b; Iwanicki et al. 2023a, b), which in some cases is very advantageous when targeting insect vectors or immature stages that may escape infections by frequent molts.

Blastospores

Once the fungus breaks through the cuticular barrier and invades the host's hemocoel, it multiplies as hyphal bodies and blastospores. Some authors confuse hyphal bodies with blastospores; however, these cell types have distinct morphological and structural characteristics, where the hyphal bodies result from the breakdown of hyphae and are crucial for the spread within host tissues, whereas the blastospores are polymorphic cells formed by budding or fission and are linked to rapid multiplication and dissemination within the host (Bitencourt et al. 2023b). Particularly, blastospores are produced under laboratory conditions using artificial culture medium rich in nutrients such as glucose, nitrogen, and dissolved oxygen. These propagules are vegetative yeast-like unicellular structures presenting a thin, single-layered cell wall forged by the pathogenic fungus to provide a rapid multiplication fashion during the colonization stage inside the host, accompanied by the production of a myriad of mycotoxins (Boomsma et al. 2014). Even in the presence of cellular and chemical defenses in arthropods (hemocytes and antifungal molecules), blastospores deceivably grow and multiply quickly, and survive the high osmotic pressure in the hemocoel (300–500 mOsmol L^{-1}) (Mascarin et al. 2015b).

The first production of blastospores using liquid culture fermentation was described by Samsinakova (1966);

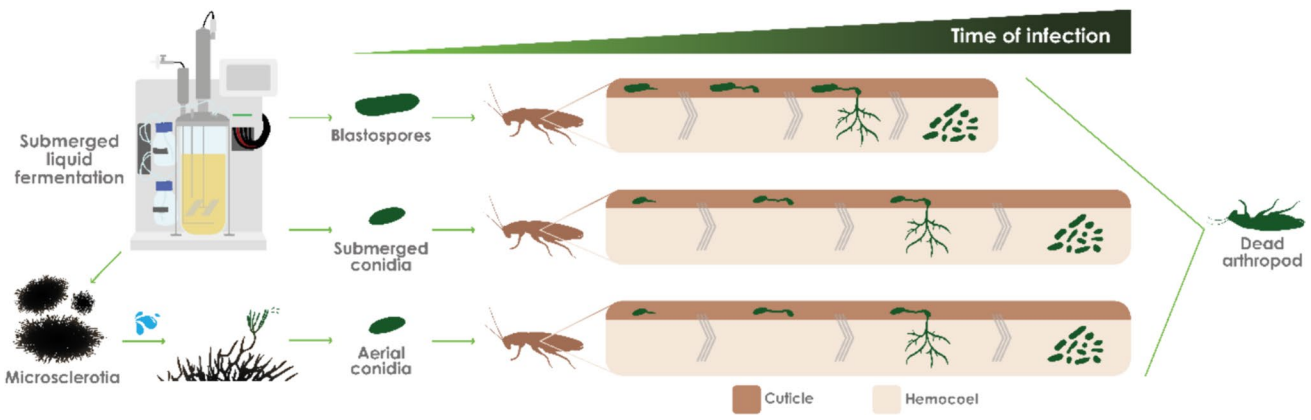

Fig. 5 Submerged fungal propagules (e.g., blastospores, submerged conidia, and microsclerotia) as potential active ingredients in mycopesticides and their mode of action via direct cuticle infection and their time of infection

in this study, the author observed an output of 6.5×10^8 blastospores mL^{-1} over 6–8 days produced in 25 g L^{-1} glucose and 25 g L^{-1} starch supplemented with 20 g L^{-1} corn steep liquor. Recently, Mascarin et al. (2023) demonstrated the effect on blastospore growth, morphology and yield when grown in hyperosmotic media generated by either high glucose concentration, polyethylene glycol (PEG), or ionic salts. These authors identified morphological changes in cell size and shape and increased proliferation of blastospores ($> 2 \times 10^9$ blastospores mL^{-1}) within 2–3 days of cultivation, accompanied by reduced hyphal proliferation in hyperosmotic liquid cultures. A negative effect on blastospore desiccation tolerance was observed for one strain of *B. bassiana* due to excess of PEG added to the medium. However, increasing the culture medium's osmolarity could enhance this propagule's productivity. This approach warrants further investigation on a strain-specific basis.

The reduction in water activity of the liquid media, along with a highly aerated environment, has been associated with increased blastospore production by various EPF. Highly aerated cultures (350 rpm with shake flask cultures and reduced liquid volumes, 25-30% v/v) of *B. bassiana* grown in the presence of high glucose concentration (140 g L^{-1}) produced considerably smaller, spherical blastospores with improved virulence (lower LC_{50} and LT_{50}) to whitefly nymphs in comparison to the larger, oblong blastospores obtained by medium with low osmotic pressure (40 g glucose L^{-1}) (Mascarin et al. 2015b). While requiring further validation, it is plausible to correlate these findings with the intracellular accumulation of polyols in blastospores. This response mirrors observations in aerial conidia grown under water stress conditions, where increased glucose concentration in the medium aims to restore osmotic balance.

In addition to the ability to be produced on a large scale in fermentation tanks with a short incubation time (~ 3 days), blastospores have been shown to have equal or greater insecticidal efficacy than aerial conidia against several arthropod species. Despite their hydrophilic characteristic, blastospores may adhere to the host's cuticle (hydrophobic) through the production of mucus or class I hydrophobin (Alkhaibari et al. 2016), while electrostatic charge also appears to play a significant role in adhesion for blastospores (Holder et al. 2007). Blastospores have multiple routes of infection (cuticle, gut, or natural openings), which may explain why these propagules kill their host more quickly when compared to conidia (Gomes et al. 2023). In the oral infection route, blastospores were able to rupture the host's intestinal cells through the midgut of *Aedes aegypti* (Diptera: Culicidae) larvae, evidenced by the presence of hyphal bodies in the hemocoel and degraded enterocytes (Bitencourt et al. 2023a). Furthermore, *B. bassiana* blastospores were more effective in evading the hemocytes of *Ae. aegypti* larvae when compared to conidia. During the first 24 h in midgut route of fungal infection, Bitencourt et al. (2023b) observed that post-ingested conidia might stimulate oenocytoids and granulocytes recruitment, secreting antimicrobial peptides (AMPs) into the gut lumen. Meanwhile, blastospores remain covered with a collagen-like protein (e.g., MCL1 in *M. anisopliae*) and lack β-glucans in the cell wall, thus acting as camouflage and facilitating evasion of the host's immune system (Wang and St. Leger 2006). Nevertheless, the specific virulence mechanisms employed by blastospores to surpass conidia in virulence remain unclear, even without excessive production of proteases Pr1 and Pr2 (Gotti et al. 2023).

A major concern for the widespread adoption of industrial production of blastospores arises from their shorter shelf life and sensitivity to industrial formulation processes, such as desiccation (Dietsch et al. 2021). Blastospores, when applied under field conditions, quickly lose their viability (Gomes et al. 2023). However, a study developed by Bernardo et al. (2020) demonstrated that the degree of susceptibility of blastospores to abiotic stresses, such as elevated temperature and UV-B radiation, varies among fungal species and strains within the genera *Metarhizium* and *Beauveria*. Nutritional factors, such as the nitrogen source, are critical for rapid and high production of blastospores with desired desiccation and thermotolerance attributes. The carbon-to-nitrogen ratio derived from carbon and nitrogen sources and concentrations plays a crucial role in optimal output and satisfactory resistance of blastospores to desiccation and heat stress (Cliquet and Jackson 2005; Li et al. 2022; Lima et al. 2024; Mascarin et al. 2018), as well as some trace metals used as micronutrients can shape some ecological adaptations of these propagules (Li et al. 2024). Desiccation tolerance of blastospores is usually improved when fungi are grown in media with high nitrogen content (> 1.5% N content) and using complex organic sources like acid-hydrolyzed casein and cottonseed flour (Mascarin et al. 2015a, 2018). Also, the high nitrogen content is pivotal in the culture medium in producing desiccation-tolerant and shelf-stable blastospores. Another study reported that *C. fumosorosea* blastospores had higher yields and better thermotolerance when grown with soy peptone, which rendered blastospores with lower intracellular trehalose and higher mannitol content (Li et al. 2022). In line with this, a recent study from our group underscored the importance of complex organic nitrogen sources in altering the carbon–nitrogen ratio of the medium and the intracellular content, which affected production yields, virulence, desiccation tolerance, shelf life, persistence, and thermotolerance of blastospores, except UV-B tolerance (Lima et al. 2024).

Formulation of blastopores is an essential approach to improve its biocontrol activity and persistence in the field. Despite the blastospores' hydrophilic characteristic, the oil-in-water emulsions containing blastospores increased

biocontrol efficacy, probably by enhancing the adhesion of these fungal cells to the arthropod cuticle (de Paula et al. 2021) or perhaps by weakening the cuticle layer (Kaiser et al. 2020). These findings indicate that oil-in-water emulsions are compatible and hold potential synergy with blastospores, although the shelf life has not been assessed for this type of formulation. Powder and granular formulations of blastospores are still challenging, and few studies have demonstrated their efficacy. For the first time, Mascarin et al. (2016) devised a spray- and air-dried formulation of blastospores packed with both oxygen and moisture scavengers that resulted in blastospores with greater than 80% viability for longer than 12 months in unrefrigerated (i.e., 28 °C) storage conditions. The authors employed skim milk and ascorbic acid for spray-dried blastospores, while only diatomaceous earth was added to the air-dried blastospores. The key finding in this study was to maintain dehydrated blastospores with low water content and low oxygen during the storage period. In addition, formulation additives like skim milk powder combined with ascorbic acid provided both physical and chemical protection to *B. bassiana* blastospores under oxidative and osmotic stresses during the harsh spray drying process. Notably, ascorbic acid serves as a potent antioxidant by scavenging reactive oxygen species (ROS) in cells under desiccation stress, thereby reducing damage to blastospores. Skim milk, which contains lactose and various proteins (such as caseins, α-lactoglobulin, β-lactoglobulin, bovine serum albumin, and lactoferrin), offers protection to microbial cellular structures and functions during dehydration. Lactose interacts with the polar head groups of phospholipids and proteins in the cell membrane, while milk proteins help reduce membrane leakage and maintain cell integrity. This combination minimizes damage and cell inactivation during spray drying (Santivarangkna et al. 2008). This work laid a groundbreaking technology that became part of a patent (Jackson and Mascarin 2016) in a way that advanced our knowledge on blastospore stabilization. This production pipeline contemplated in this patent for blastospore processing has been implemented by some companies with consistent production yields from lab to industrial scale, resulting in a satisfactory high concentration of these propagules (e.g., up to $5\text{-}6 \times 10^9$ blastospores mL^{-1} within 2-3 days) (Fig. 6).

In some cases, depending on the fungal species or even the isolate, there might be challenges in stabilizing blastospores after harvesting. For instance, to stabilize blastospores of *M. robertsii* after fermentation, these cells were mixed with carriers and additives, including fructose, skim milk, and bentonite, which afforded cell survival > 75% after spray drying. However, the half-life times of all blastospore-based formulations were shorter than 3 months, regardless of the storage temperature tested (Iwanicki et al. 2021). These formulation components have multi-purpose functions ranging from chemical and physical protection to blastospores against oxidative and osmotic stresses during the drying process, mainly due to the presence of reducing sugars and disaccharides such as fructose, maltose, sucrose, lactose, and trehalose, while clays provide an inexpensive filling and anti-caking material that facilitate further dispersion of the formulation into water in sprayer tanks. Clays, metal (iron, silicon, etc.) dioxides, talc, lignin, and charcoal can also provide physical UV radiation protection to fungal blastospores

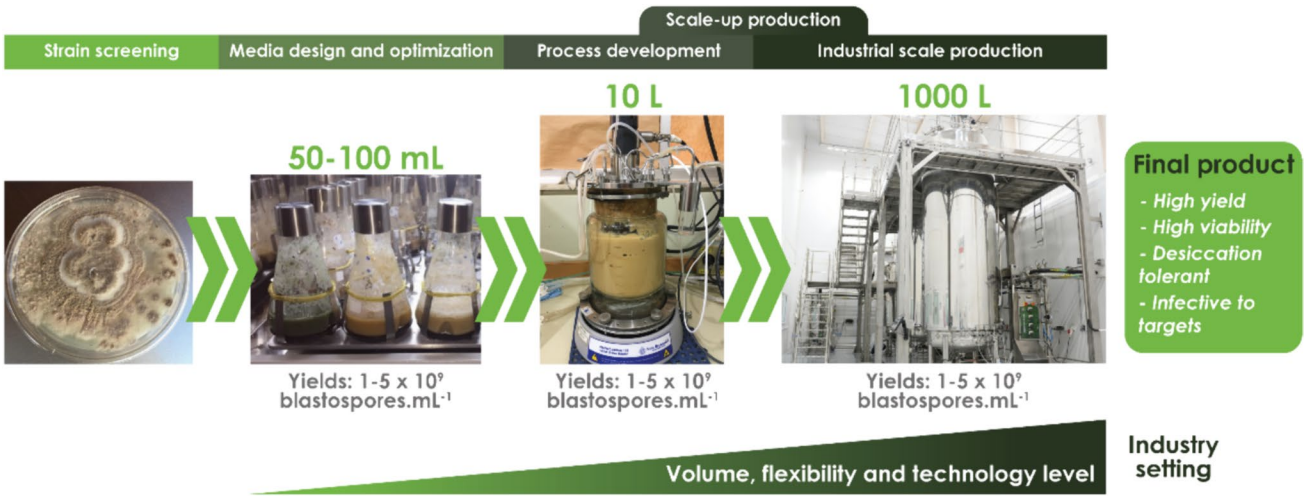

Fig. 6 Submerged liquid fermentation of filamentous entomopathogenic fungi: from small- to large-scale production. In this example, a biotechnological platform supporting cost-effective high yields of viable and desiccation-tolerant blastospores has been implemented after several years of research, including a cutting-edge patent technology (Jackson and Mascarin 2016). Industry partners can now produce blastospores of *B. bassiana*, *C. javanica*, and other related fungal species explored as mycopesticides, in 1000 to 5000-L bioreactors in only 2–3 days, maintaining high cell yields at the end of the bioprocess (GM Mascarin, personal information)

(Behle et al. 2011; Behle and Birthisel 2023). As a matter of fact, there is a scarcity of studies investigating lignin nanoparticles as a natural UV protection ingredient in formulations of fungal blastospores with the aim to maximize their post-application survivability (persistence) in the field. Despite the limited information available on blastospores that are less recalcitrant to desiccation and have poorer shelf life than other spore types, strategies that manipulate the nutritional and environmental factors during pre-harvesting liquid cultivation and post-harvesting formulation are means to improve the desiccation tolerance and shelf life of these cells.

Other less studied EPF species, such as *Fusarium* spp., *Hirsutella* spp., *Lecanicillium* spp., and *Akanthomyces* spp., are also capable of producing blastospores under submerged cultivation. Blastospores of *Hirsutella citriformis* were produced through submerged liquid fermentation with optimized conditions, yielding approximately 1.5×10^9 blastospores L^{-1}, and demonstrated insecticidal activity against the Asian citrus psyllid, *Diaphorina citri* (López et al. 2023). Entomopathogenic *Fusarium* species has been considered a potential entomopathogenic fungus that can effectively control a broad range of agricultural pests (Santos et al. 2020). According to the recent study by Zhao et al. (2023), optimized submerged culture for *Fusarium equiseti* blastospores was obtained using potato sucrose liquid medium (pH 4.5), with a primary inoculum density of 1.3×10^7 conidia mL^{-1} and a medium-to-flask ratio at 0.35 (52.5 mL in each 150 mL flask) for 6.3 days. The resultant *F. equiseti* blastospores killed almost 100% of aphids (*Myzus persicae*) by 7 days post-spraying. In another work, blastospore titters of *A. muscarius* reached from 1.72×10^9 (day 2) to 3.90×10^9 (day 5) cells mL^{-1} during submerged cultivation and displayed a similar speed of kill to aerial conidia after spraying nymphs of the whitefly *Bemisia tabaci* biotype B (Lopes et al. 2023a). Recently, Bodino et al. (2024) observed that blastospores of *Lecanicillium aphanocladii* formulated with adjuvants displayed excellent efficacy against the spittlebug *Philaenus spumarius* by killing nymphal instars and reducing the emergence rate of adults, reaching mortality levels (90%) comparable to those obtained with the commercial conidia-based mycopesticide named Naturalis® (Biogard, Grassobbio, Bergamo, Italy). In Europe, there are two commercial blastospore-based mycopesticides based on *A. muscarius* and *C. fumosorosea* traded as Mycotal® and PreFeRal® presenting label concentrations of 1×10^{10} and 2.10×10^9 CFU g^{-1}, respectively. In the USA, *C. javanica* blastospores are traded as commercial mycopesticide named PFR-97®, which contains 1×10^9 CFU g^{-1}. Field studies on the efficacy of blastospores are still limited compared to aerial conidia. However, promising results have been observed with the commercial formulation of *C. javanica* (Apopka-97 strain, PFR-97™ 20% WDG) against *D. citri* in citrus orchards, achieving up to 90% control post-application (Avery et al. 2021). A 2-year study comparing the persistence and efficacy of spray-dried blastospores of *C. javanica* (Wf GA17) with the Apopka-97 strain in cotton and vegetable crops showed that oil adjuvants enhanced whitefly control but also highlighted a significant loss of blastospores' viability within 24 h. Although blastospores and conidia of both strains demonstrated similar efficacy in reducing whiteflies, conidia consistently persisted better on plant surfaces (Wu et al. 2023). Despite the higher virulence of blastospores under controlled conditions, further research is needed to optimize their field application.

Entomopathogenic fungi can endophytically colonize plants through different inoculation routes. Blastospores applied via soil drenches appear to be more successful than applications of aerial or submerged conidia for initiating this symbiosis and conferring disease resistance to the receiving plants (Sui et al. 2022). Furthermore, the plant *Arabidopsis thaliana* treated with *B. bassiana* blastospores reduced the incidence of infections by *Botrytis cinerea*, because of the endophytic potential of this fungus to trigger host systemic defenses against a plant pathogen (Sui et al. 2022). More studies are needed to unveil the driven factors during this symbiosis process.

Despite recent efforts to unravel the molecular mechanisms underlying the formation, production, and virulence of blastospores in some EPF (Gotti et al. 2023; Iwanicki et al. 2020, 2023a, b; Mascarin et al. 2021; Zhang et al. 2019), further research is needed to identify genes suitable for manipulation to enhance production, desiccation tolerance, shelf life, and virulence. By integrating genomic, transcriptomic, metabolomic, and proteomic approaches, there is significant potential to accelerate and refine the selection of strains, culture media, and formulation compositions. This would involve identifying genes and quantitative trait loci associated with improved virulence, desiccation tolerance, reproduction, and storage survival.

Microsclerotia

In response to such adverse environmental conditions as contaminated soil or decomposing plant materials with low nutrient availability, many fungi may initiate the formation of specialized resistance structures as an adaptive response that augments their survival capacity. These conditions, often associated with microclimatic factors and the absence of hosts, may induce the production of sclerotia, an overwintering type of robust, compact, and usually melanized structure (Jackson and Jaronski 2009). Production of microsclerotia by submerged liquid fermentation was discovered for the biocontrol fungi *Colletotrichum truncatum* and *Mycoleptodicus terrestris* in the 1990s and early 2000s

(Jackson and Schisler 1994; Jackson et al. 1996; Shearer and Jackson 2006). Small sclerotia or simply microsclerotia production by some species of EPF using submerged liquid fermentation has attracted considerable attention of scientists, primarily owing to the remarkable compatibility of these structures with dry granular formulations and various natural polymers, in addition to their notorious natural resistance to adverse ecological factors alongside their capacity to produce thousands of infective conidia, ideal for developing more stable biopesticides (Jackson and Jaronski 2009; Gardescu et al. 2017; Goble et al. 2016; Marciano et al. 2021).

Microsclerotia are densely compacted hyphal aggregates typically 50–600 µm in diameter, usually dark pigmented due to melanin, with a variety of morphotypes across fungal species and strains that are induced by nutritional and environmental factors of the artificial liquid culture (Jackson and Payne 2016). This multicellular propagule is distinguished by its notable resistance to abiotic factors such as temperature variations, UV radiation and desiccation, probably related to their dark pigmentation attributed mainly to melanin, considered "the fungal armor" (Corval et al. 2021; García-Riaño et al. 2024; Paixão et al. 2021). These metabolically quiescent vegetative structures sequester significant nutritional reserves mobilized upon restoring favorable environmental conditions, notably those of moisture and temperature. The effectiveness of this production in liquid culture media, achievable within 3 to 4 days, has been meticulously documented in previous studies (Flor-Weiler et al. 2018; Jaronski 2023; Jaronski and Jackson 2008; Jackson and Jaronski 2009; Mascarin et al. 2014).

Although mycelial pellets and microsclerotia are both composed of aggregate hyphae, they differ in their attributes (Paixão et al. 2021). Microsclerotia are smaller and more compact than mycelial pellets, as the latter propagule presents a colorless medulla of thin-walled hyphae and always assumes a larger size than the former. Paixão et al. (2021) and Santos et al. (2021) pointed out the remarkable abiotic stress tolerance of microsclerotia over mycelial pellets obtained through submerged fermentation, which later was confirmed by comparing with aerial conidia of *M. robertsii* (García-Riaño et al. 2024). In this context, microsclerotia demonstrated superior resilience and conidial productivity when exposed to UV-B radiation (1283 mW m^{-2}) and heat stress (45 °C). Despite a time-dependent decrease in conidial production, microsclerotia consistently outperformed mycelial pellets, showcasing its enhanced tolerance and viability upon environmental challenges.

Liquid production methods targeting microsclerotia have been developed for various EPF, such as *Metarhizium* and *Beauveria*, as well as for non-EPF biopesticides like *Trichoderma* (Huarte-Bonnet et al. 2019; Jaronski and Jackson 2008; Jackson and Jaronski 2009; Kobori et al. 2015; Mascarin et al. 2013; Song et al. 2014). Regardless of the fungal strain or specie, microsclerotia biogenesis and production yield are notably affected by nutritional components and physical factors such as carbon sources, nitrogen sources, carbon concentration, carbon-to-nitrogen ratio, calcium and iron concentrations, aeration rate, initial inoculum density, culture age, pH, osmotic pressure, and temperature (Jackson and Jaronski 2009; Rivas-Franco et al. 2020). Glucose is the primary carbon source studied and used for growing fungal microsclerotia, while various complex nitrogen sources have been investigated, including yeast extract, glutamate, and hydrolyzed acid casein resulting in varied production yields, storage stability, and bioinsecticide activity (Behle and Jackson 2014; Jackson and Jaronski 2009; Mascarin et al. 2014). Jackson and Schisler (1994) reported that nitrogen depletion and glucose exhaustion in culture media are both critical cues for microsclerotia formation, followed by melanization.

While numerous studies aimed to optimize large-scale production, some authors note that the average output is around 10^7 microsclerotia L^{-1}. Following the evaluation of five *Metarhizium* strains, Mascarin et al. (2014) achieved yields ranging from 6.1 to 7.3×10^6 microsclerotia L^{-1} after 3 days of growth, with maximum yields of 0.7 to 1.1×10^7 microsclerotia L^{-1} after 5 days of cultivation, using a medium containing a C:N ratio of 50:1. Jaronski and Jackson (2012) achieved higher concentrations of microsclerotia by strains of *M. anisopliae* ($2.7–2.9 \times 10^8$ L^{-1}) with liquid cultures with C:N ratios of 30:1 and 50:1. Considering the production per liter, Villamizar et al. (2018) achieved an average of 7×10^6 microsclerotia L^{-1} using *Beauveria* strains in a medium with C:N ratios of 4:1 and 5:1. On the other hand, these and several other results diverge dramatically from those obtained by Yousef-Yousef et al. (2022) demanding careful attention due to the exceptionally high values obtained after 8 days ($4.6–8.2 \times 10^{11}$ microsclerotia L^{-1}). The presence of dark pigments in microsclerotium was proved by showing evidence they were made of 1,8-dihydroxynapthalene-melanin-like compounds (Espín-Sánchez et al. 2023) and is supported by the presence of a gene *hmgA* encoding the enzyme homogentisate 1,2-dioxygenase, which is essentially involved in the process of melanin synthesis in *M. brunneum* (Hu et al. 2014). Melanin-like compounds are readily identifiable in the dark-colored mature microsclerotia produced by certain fungal biocontrol agents, particularly *Metarhizium* species (Jackson et al. 2009). Typically, these microsclerotia germinate by producing hyphae (myceliogenic germination), which eventually leads to conidial production (sporogenesis). The microsclerotia sporogenesis can take more than 10 days to produce infective conidia (Marciano et al. 2021), but this event varies with fungal species, strain, formulation, incubation temperature, moisture level of the substrate, among other ecological factors (Behle and Jackson 2014; Jackson and Jaronski 2009; Mascarin et al. 2014). Given that microsclerotium proves to be

more effective for soil application as a microbial control agent (Mascarin et al. 2014), the study of handling and storage conditions before field application requires effort. As mentioned earlier, microsclerotia can be used in addition to adjuvants aiming at producing granules, pellets, and other related dry formulations (Behle and Jackson 2014; Mascarin et al. 2014). This enhances protection against abiotic factors and facilitates rehydration and, consequently, myceliogenic germination, while extending shelf life.

Behle and Jackson (2014) suggest that media with higher nitrogen concentrations produce microsclerotia with improved storage fitness. Still, the best storage stability they demonstrated at that time was around a 4-week half-life, which was considered poor for commercial products. In the same year, Mascarin et al. (2014) formulated granules of *Metarhizium* spp. with diatomaceous earth. These granules exhibited stability and excellent efficacy in generating viable conidia, even after storage at 26 °C or − 20 °C for up to 3.5 months. Although *Trichoderma* species are not generally regarded to be entomopathogenic, some species and strains can be opportunistic and facultative arthropod parasites (Poveda 2021). The stability of air-dried *Trichoderma harzianum* microsclerotia derived from different C:N ratios was found to be excellent at 4 °C and room temperature (25 °C), remaining viable for at least 12 months, as reported by Kobori et al. (2015).

Microsclerotia granules that maintain a significant shelf life under cool and unrefrigerated storage conditions, with minimal or no loss of conidial production, are the optimal objectives for applications in the field as integral components of biological pest control strategies. The composition of pellets presented by Santos et al. (2021) included vermiculite powder, diatomaceous earth, and colloidal silicon dioxide. Furthermore, its potential efficacy in controlling cattle ticks, *Rhipicephalus microplus*, was investigated. Engorged tick females were exposed to soil treated with sporulated microsclerotial pellets (0.007 g) of *M. anisopliae* s.str. As a result, these ticks exhibited a shorter oviposition time length, reduced lifespan, and lower number of hatched larvae compared to mock-untreated ticks (Santos et al. 2021). Formulations with microcrystalline cellulose (MC) granules containing microsclerotia of *M. robertsii* were also effective in reducing the number of larvae of the same tick species during the humid season (64.8% relative efficacy), following soil applications in the semi-natural pasture (Marciano et al. 2021).

The activity of microsclerotia from *Metarhizium* spp. against *Ae. aegypti* has been reported for adults under different relative humidity conditions (75% and 90%) and, when formulated with both vermiculite (VE) and MC, could cause mortality, regardless of the condition investigated, within 6 days. Even though more conidia were produced from pellets formulated with VE (Rodrigues et al. 2021), Paixão et al. (2024) also conducted a comparative analysis of microsclerotia production from two *M. robertsii* strains for the control of mosquito larvae in aquatic environments. Both strains exhibited similar biomass production and could induce mortality rates of up to 70% (Paixão et al. 2024).

In-depth fundamental research on the molecular characterization of metabolic pathways involved in the formation and development of EPF microsclerotia is ongoing but has been largely confined to a few species, such as *M. rileyi* (Song et al. 2013), *M. robertsii* (Paixão et al. 2021), and *B. bassiana* (Huarte-Bonnet et al. 2019). A comprehensive review of the biochemical and molecular mechanisms driving fungal microsclerotia biogenesis has identified key genes related to pigment biosynthesis, ion transport (primarily iron and calcium), intracellular storage, and antioxidation (Song 2018). However, further research is needed to elucidate the interplay of signaling pathways regulated by transcriptional factors in microsclerotia formation. The increasing availability of fungal genomic data, combined with other omics approaches, will be crucial for advancing our understanding and improving the development, production, and stabilization of microsclerotia in various EPF species. The findings indicate that microsclerotia preparations can deliver infective conidial inoculum directly in situ, even though they take longer to produce infectious spores (Fig. 5). This represents a significant advancement compared to other fungal propagule types, especially those produced through solid fermentation, despite research efforts devoted to enhancing effectiveness, optimizing production yields, and developing better formulations. Surprisingly, microsclerotia-based EPF products remain unregistered worldwide. Consequently, further investment and research efforts are necessary to advance our understanding and utilization of microsclerotia as a valuable active ingredient for mycopesticides.

Submerged conidia

Conidia can also be obtained by submerged cultivation, produced on typical conidiophores arising from hyphal filaments or directly from the spore through a sporulation microcycle. This microcyclic conidiation, typically induced by nutrient and/or temperature manipulation, has been developed as a model to study the biochemical events occurring during the sporulation of the conidial fungi. This potential for microcyclic sporogenesis has been of particular interest in the case of EPF since it shortens the culture time and increases spore yields.

Submerged conidia of EPF are hydrophilic propagules morphologically like aerial conidia. The latter are typically produced on the surface of the insect host or in the surrounding environment and can be dispersed by air to meet potential hosts. Conversely, submerged conidia are produced in a liquid medium like blastospores, although the latter relies

on nutrient-rich liquid cultures to form. Accordingly, submerged conidia appear an important propagule alternative for fungal production under nutrient-limiting conditions. Despite aerial and submerged conidia being morphologically similar, atomic force microscopy revealed the absence of bundles or fascicles in *B. bassiana* submerged conidia, present in the aerial conidia counterpart (Holder et al. 2007). Regarding their hydrophobicity, surface tension values and the free energies of the interaction of the cell types with surfaces indicated that the *B. bassiana* aerial conidia were hydrophobic, whereas submerged conidia were hydrophilic. A recent study compared phenotypically *B. bassiana* submerged conidia, aerial conidia, and blastospores by microscopic observation of calcofluor white-stained cells (Iwanicki et al. 2023a, b). These authors reported that aerial conidia are smaller than submerged conidia and have more deposits of chitin than the latter, followed by blastospores.

Few reports have been published on the virulence of submerged conidia of EPF towards arthropods. Some studies have shown that the virulence of submerged conidia may vary compared to other entomopathogenic fungal propagules, such as aerial conidia and blastospores (Holder et al. 2007; Iwanicki et al. 2023a, b; Basso et al. 2024). Iwanicki et al. (2023a) demonstrated the virulence of submerged conidia of the fungus *B. bassiana* against the cotton boll weevil, *Anthonomus grandis* (a destructive pest of cotton), and the fall armyworm, *Spodoptera frugiperda* (a devastating pest in maize and many other agronomic crops). These authors compared the virulence among blastospores, aerial, and submerged conidia. They observed that, in general, blastospores and submerged conidia killed these insects faster than aerial conidia for both insect species. On the other hand, according to Javar et al. (2023), aerial conidia and blastospores were slightly more virulent compared to submerged conidia when sprayed on whitefly nymphs *Trialeurodes vaporariorum.* The virulence to insects of submerged conidia of non-conventional EPF was also reported by Mascarin et al. (2022). These authors demonstrated promising results in the bioefficacy of submerged conidia of *Clonostachys rosea*, a necrotrophic mycoparasite of numerous plant pathogenic fungi, against nymphs of the whitefly *Bemisia tabaci*.

The survival of *B. bassiana* spores produced in solid and liquid media was studied by Javar et al. (2023). According to these authors, the survival of submerged conidia decreased to about 50% after 9 months at 4 °C of storage. This result implies the need for improved formulations and storage conditions to extend this propagule's shelf life. Other important attributes of fungal propagules to the market of biological products are their production yield, cost, and possible adverse effects caused by drying techniques, particularly for propagules produced by liquid fermentation. Leland et al. (2005) reported the role of the medium osmolarity in the morphology, yield, germination, virulence, and drying stability of *M. acridum* submerged conidia. Conidia from high osmolarity medium (HOM) had thin cell walls, increased production, and were more stable to drying. HOM conidia also had faster germination rates than submerged conidia, similar to blastospores, and they were more pathogenic to American grasshopper *Schistocerca americana* than submerged conidia and aerial conidia. According to the literature, the nitrogen source and the C:N ratio also play a significant role in supporting the production of submerged conidia (Mascarin et al. 2022). For *B. bassiana*, the maximum submerged conidial yield (5×10^8 mL) was obtained when glucose was the carbon source and when the glucose-to-nitrate ratio was 5:1. Regarding the pH and dissolved oxygen impact on the production of *B. bassiana* submerged conidia, Basso et al. (2023) reported that the pH fixed at 4.5 and 10% dissolved oxygen maintained in the first 24 h followed by 50% until 48 h and 30% until the end of the cultivation provided the highest concentrations of conidia within 4 days.

Despite the limited studies outlined here, further research involving diverse entomopathogenic fungal species is imperative to address knowledge gaps concerning the virulence and tolerance of submerged conidia to abiotic factors. Additionally, optimal conditions for achieving maximum yield production within a short timeframe and at minimal cost must be elucidated. Enhanced drying techniques, tailored packaging atmospheres, and formulations should be developed with a specific focus on the traits of submerged conidia to improve storage conditions, aiming for higher viability and prolonged shelf life.

Mycelium

EPF are characterized by a biphasic biological cycle: a mycelial vegetative phase and a reproductive phase yielding sexual and/or asexual spores. The vegetative phase for filamentous fungi is mainly characterized by the formation of mycelium, a mass of branching, thread-like hyphae, and it plays a crucial role in transporting nutrients and colonizing substrates. The mycelium of plant-mutualistic fungi such as *Metarhizium* and *Beauveria* is also essential for establishing colonization of the root system and trading nutrients with plants in a symbiotic interaction manner (Hu and Bidochka 2021). As a rule of thumb, all EPF are capable of forming mycelium when growing in liquid cultures, likewise when they colonize the insect body, along with the formation of yeast-like cells able to multiply by fission or budding. After host death, the mycelium also covers the mummified arthropod cadavers and later produces asexual spores.

A further disadvantage of using mycelial biomass from in vitro cultivation, or even those formed in vivo, concerns

their short ecological viability and poor resilience to adverse environmental factors, especially when compared to aerial conidia. However, formulation can significantly improve the longevity of the in vitro mycelium. In this context, dry mycelium has been extensively studied for EPF, including hypocrealean and entomophthoralean species (Jaronski 2023). The principle lies in producing the maximum of mycelium in a shorter fermentation time with the lowest media formulation cost. Different harvesting procedures and post-harvesting treatments (e.g., 10% maltose solution) were tested to obtain dry mycelium formulations that remained actively sporulating to generate inoculum source for initiation or augmentation of epizootics in target insect populations, as documented for *Zoophthora radicans* (Wraight et al. 2003; Pell et al. 1998). After that, the final objective is to promote, upon rehydration, the profuse sporulation of this biomass in a way that allows the fresh production of infective conidia with their native cell coverings in the target environment of the pest (Krueger et al. 1992). These propagules have not been subjected to any sort of degradation through production, formulation, and application technologies; they are the real deal produced naturally where you want them to be available to the hosts. Attempts to produce large quantities of mycelial biomass have been reported for various EPF such as *Hirsutella thompsonii* (McCoy et al. 1975), *C. farinosa* (Agudelo and Falcon 1983), *Purpureocillium lilacinum* (Rombach et al. 1986), *M. rileyi* (Holdom and van de Klashorst 1986), *M. anisopliae* (Krueger et al. 1992), and *Z. radicans* (McCabe and Soper 1985).

Another subject to consider when producing mycelium-based mycopesticides concerns the impact that media nutritional and environmental factors can have on the accumulation of certain endogenous (intracellular) reserves in the mycelium, such as polyols and simple sugars (e.g., trehalose), which can directly influence the mycelium's resilience to desiccation and shelf life. Interestingly, culture age can influence endogenous arabitol, erythritol, mannitol, and trehalose contents in *M. brunneum* mycelium, and elevated levels of these compounds improve drying survival and shelf life of encapsulated mycelium coupled with enhanced fungal virulence against *T. molit*or larvae (Krell et al. 2018). As a result, there has been substantial evidence about the beneficial effects of endogenous polyols in improving encapsulated mycelium's shelf life, which may prompt the development of more robust mycelium-based bioproducts.

Importantly, there is a trend in exploring mycelial preparations of EPF using a strategy entailing the encapsulated mycelium supplemented with exogenous cellulase to enhance the endophytic colonization of host plants. Krell et al. (2018) observed heightened enzymatic activity in *M. brunneum* when cellulase was co-encapsulated with mycelium. This co-encapsulation led to a notable shift from mycelial growth to spore formation, reaching a maximum count of 2.5×10^8 conidia per bead, and led to enhanced endophytic association in potato plants, exhibiting a 61.2% improvement compared to non-supplemented beads. These findings offer valuable insights into developing tailored formulations for EPF that incorporate enzymes to enhance endophytic capabilities. Such a strategy holds promise for increasing the efficacy of plant protection measures against herbivorous pests. However, a note of concern arises when plant tissues treated with cellulase may potentially facilitate the entry of opportunistic plant fungal pathogens and parasitic nematodes.

There are limited reports on the mass production of the epizootic fungus *Aschersonia aleyrodis* and several other species of its genus. Zhu et al. (2008) studied the optimal nutritional requirements to grow mycelium of *A. aleyrodis.* The maximum production of mycelial growth achieved was 20.05 g L^{-1} after 7 days of fermentation with 1.16% lactose, 0.394% tryptone, 0.4 mmol L^{-1} Fe^{2+}, and 0.00125% vitamin B1. This mycelium was further used to induce sporulation on a solid substrate, but no virulence test with any target hosts was assessed in this study. Interestingly, little research on the liquid fermentation of *Aschersonia* spp. may hinder its large-scale production and commercialization worldwide. Nonetheless, the primary biocontrol approach for the use of this fungus against target pests has been the conservative approach or inoculative and augmentative releases, where both do not require high amounts of fungal inoculum to be produced for application in the agroecosystems.

Unlike the prominent members of ascomycetous EPF, highly important species with a more fastidious diet and, in most cases, host specialists belong to Entomophthoromycotina. As they are considered obligatory pathogens of arthropods, there are obstacles regarding their mass production using both solid-state or submerged fermentation. Nevertheless, mycelial production has also been contemplated, especially in the case of Oomycota and Entomophthoromycotina, as it is simply a step to directly or indirectly reach an infective state that is useful for the intended purpose. Thus, the successful use of a mycelial formulation in biological control hinges on the ability of the mycelium to sporulate under natural conditions. Entomophthoromycotina represents several species within the genera *Batkoa*, *Furia*, *Erynia*, *Pandora*, *Massospora*, *Entomophthora*, *Entomophaga*, *Zoophthora*, and *Neozygites*, all pathogenic to insects or mites and often causing outstanding epizootics in host populations. Still, some have been difficult (or, in a few cases, impossible) to culture in vitro and, therefore, to be developed as effectively applicable mycopesticides.

The mycelial production by entomophthoralean fungi has been an exciting and feasible alternative when using submerged liquid fermentation with nutrient-rich media. For instance, a liquid culture medium for the common aphid entomophthoralean pathogen, *Pandora* (formerly *Erynia*)

neoaphidis, was determined by Gray and Markham (1997) and consisted of glucose, yeast extract, mycological peptone, KH_2PO_4, Na_2HPO_4, and 0.01% oleic acid carried out in 1.5-L fermentation volumes. They obtained considerable mycelial biomass in batches but not continuous fermentations. Although the mycelial phase can be produced in vitro, it cannot be used directly in biocontrol formulation as this type of propagule is not infective, but is a vital step towards the production of primary conidia. Therefore, alginate provides an alternative formulation method through the entrapment of mycelia in the alginate beads to further sustain the conidia production of this fungus for the biocontrol of aphids (Shah et al. 1998). In a recent work by Muskat et al. (2022), they found that skim milk supplemented with yeast extract and low-cost protein hydrolysate from animal by products were the best combination to maximize the mycelial biomass yield in liquid shaking culture of the apple psyllid pathogen, *Pandora cacopsyllae*. Further, this fungus produced finely dispersed mycelium by increasing osmotic pressure in the liquid culture media by adding sodium chloride. When this fungus was grown into a stirred tank bioreactor with a working volume of 8 L, maximum mycelial biomass reached a dry weight of 21 g L^{-1} in 48 h. Although this strategy appears exciting and economically viable, applying dry encapsulated mycelium under field conditions brings climatic obstacles, such as the high enough humidity required for the dry mycelium to produce infective spores.

Complex sources of nitrogen can significantly affect the production of mycelium or hyphal bodies in liquid media of all three species of entomophthoralean fungi, *Batkoa* sp., *Furia* sp., and *Neozygites floridana*, known for their epizootic potential against insect pests. According to Leite et al. (2005), yeast extract allowed the highest production of *Batkoa* sp., with a concentration of 0.5% being the most suitable for vegetative (mycelial) growth. The combination of 0.33% each of yeast extract + beef extract + skim milk allowed the highest production of *Furia* sp. mycelium. The combination of yeast extract + skim milk (0.5% of each) allowed the second highest production of *Furia* sp. and was the most suitable for mass production due to the lower cost. The combination of 1% each of yeast extract + peptone + skim milk was the most appropriate for producing *N. floridana* hyphal bodies. Leite et al. (2003) also noted that nitrogen sources remarkably influenced the growth pattern of these three fungal species compared to different carbon sources. Still, glucose seemed to be the preferred carbon source, increasing biomass production for these three fungal species.

The advancement of mycelial formulations for commercial use requires further investigation to develop storage techniques at room temperature without compromising the quality of mycelium-based products. The key challenge lies in resolving issues related to preserving dry-mycelium formulations. Successfully addressing these challenges could unlock significant potential for pest control. However, considering the heightened resilience to adverse abiotic factors and prolonged shelf life of microsclerotium compared to mycelium, the former should be prioritized as the preferred propagule in mycopesticides intended to target soil-dwelling arthropod pests. Mycelium production by entomophthoralean species seems to remain the prime strategy for their mass production. Nevertheless, the requirement of high environmental moisture for their sporulation to generate infective conidia, combined with their poor shelf life, makes this group of EPF a challenge for commercial exploitation.

Zygospores and oospores

A different type of mass-producible spore is the resting spore of the flagellate water molds from Oomycota and entomophthoralean fungi, encompassing two groups of arthropod pathogenic fungi with a role in disease carryover. These spores offer the advantage of being highly resistant and can survive for several months both in vitro and in nature. They can also be produced in liquid culture and on solid media. However, these spores, like the mycelium, are not directly infectious; their pathogenicity depends on their potential to produce infective spores by germination. These lower fungi sexually produce resistant oospores or zygospores commonly used in biocontrol formulations.

The in vitro cultivation of entomophthoralean fungi is quite complicated as it exhibits significant variability in difficulty, a factor influenced by the specific species and even the strain being dealt with. Generally, *Neozygites* species pose more critical challenges in cultivation, while *Conidiobolus* is considered the more easily cultivable species. The initial breakthroughs in in vitro production of Entomophthorales involved utilizing Grace's insect cell culture medium amended with fetal bovine serum (5% v/v), mimicking the insect hemolymph (Dunphy et al. 1978). This method, such as the one employed by Kogan and Hajek (2000), who produced azygospores of *Entomophaga maimaiga*, continues to be used. As an attempt to lower the production costs of these fastidious entomophthoralean fungi, other research groups devised media formulations based on organic carbon and nitrogen compounds. In this way, Latgé et al. (1977) outlined optimal media for zygospores of the aphid pathogen *Entomophthora virulenta* comprising dextrose and corn syrup as carbon sources, and yeast extract, soybean flour, or cottonseed flour as the most effective nitrogen sources, resulting in a high number of zygospores obtained in 9 days having 70% germination rate. Therefore, these zygospores can be used in mycopesticide formulations.

In their quest for a mass production medium for *Z. radicans* azygospores, Senthilkumar et al. (2011) conducted experiments testing various concentrations and ratios of

sunflower oil or dextrose as a carbon source and yeast extract or peptone as a nitrogen source in submerged cultivation. They identified the optimal ratio as 4:8 yeast extract to sunflower oil medium. *Leptolegnia chapmani*, *Coelomomyces* spp., and *Lagenidium giganteum* are obligate arthropod pathogenic species causing diseases in larval mosquitoes. Due to their intricate life cycles and high host specificity, these fungal species pose daunting hurdles for mass production through liquid or solid fermentation processes, which have consequently limited their widespread commercialization as mycopesticides.

Chlamydospores

Chlamydospores are resistant fungal propagules formed by asexual reproduction that differentiate directly from a hyphal compartment (Watkinson et al. 2015; Deshpande 1999). Although these studies suggest chlamydospores are infective propagules that can cause disease in arthropods, infection may be limited via the gut by the reactivation of chlamydospores after ingestion by the arthropod host. The route of infection through cuticle or natural opening penetrations might not be feasible because chlamydospores are metabolically quiescent propagules invested in a thickened three-layer cell wall (Ment et al. 2010). A potential impact of *M. anisopliae* chlamydospores for controlling arthropod pests may lie in the ability of this fungus to naturally produce chlamydospores in infected targets (e.g., tick eggs in the soil) under stressful environmental conditions and thereby overcome challenging periods to produce infective conidia under optimum conditions in the field (Ment et al. 2010).

Research on the production of chlamydospores from EPF is scarce, with a noticeable absence of studies focusing on the fermentation of chlamydospores of *Beauveria* spp., *Metarhizium* spp., or *Cordyceps* spp. Consequently, there is a shortage of bioproducts utilizing chlamydospores from these fungi. Deep studies are necessary to clarify the efficacy of chlamydospores of EPF in controlling target arthropod pests and their massive production for industrial purposes.

Obstacles and perspectives

Building fundamental knowledge and effectively targeting technical gaps around the optimization of submerged liquid fermentation processes aligned with proper formulation strategies for EPF are paramount for their successful, widespread commercial development as biocontrol agents of arthropod pests and as plant growth promoters supported by their ability to act as endophytes in some cases, notably for *Metarhizium* and *Beauveria*. This paper currently addresses a variety of propagules produced by submerged liquid fermentation, including information about their morphological characteristics, biocontrol efficacy, growth requirements, scale-up challenges, formulation, and shelf life. Compelling differences in biomass production among EPF can be observed at different hierarchical groups and taxonomical levels, which means the natural diversity among species and even strains can play an important role in outcomes from this mycopesticide development pipeline. Biopesticide companies will need to invest in optimizing medium components and growth parameters as well as formulation technologies. Although still overlooked, the formulation of secondary metabolites and cuticle-degrading enzymes that are also produced during liquid fermentation, in combination with a variety of fungal propagules, can be better explored to ensure that the final product renders synergistic outcomes for pest control.

Mass production is required to deliver a large amount of viable and active fungal biomass for use in the inundative biological control strategy, which is one of the advantages of using cost-effective submerged liquid fermentation technology. However, efficient mass production of EPF relies on a deep understanding of their morphogenesis, nutritional-environmental requirements, and high-quality operational standards to ensure batch-to-batch uniformity and purity. These represent significant challenges for the subject, particularly concerning the on-farm production practiced by farmers where contamination is recurrent and can consequently jeopardize the success of the biocontrol strategy (Faria et al. 2023). It is also true that the final product depends on proper formulation and packaging, which can render another bottleneck, especially when dealing with liquid formulations that still pose a cumbersome challenge for stabilization and long-term shelf life of submerged fungal propagules, especially submerged conidia and blastospores. For instance, instead of developing an emulsifiable oil formulation of blastospores with poor shelf life, it could be more convenient to adopt the delivery approach by mixing blastospores with an emulsifiable oil in the tank mix before application (Kaiser et al. 2020). Additionally, appropriate formulations must also enhance the protection of these fungal propagules against UV radiation, elevated temperature, desiccation, and other adverse environmental factors to increase the persistence of vigorous EPF in the field and, therefore, improve their efficacy against target arthropods. Further research is essential to optimize field applications and develop delivery strategies better suited for mycopesticides based on submerged propagules. This would enhance their attractiveness and competitiveness compared to aerial conidia in terms of persistence and control efficacy. Urgent investigation is needed to address this gap, as many companies remain skeptical about the successful performance of liquid-grown fungal propagules in real-world conditions.

Equally significant is the practical and technological advancement that submerged liquid fermentation brings to Brazil's biopesticide industry, particularly in the mass production of EPF. Increasingly, companies are adopting this technology through a biphasic or two-stage fermentation process at an industrial scale. This approach not only shortens incubation time but also enhances conidia production on cereal grains via stationary solid substrate fermentation. Therefore, the insights and information presented in this paper may prove valuable for companies utilizing biphasic fermentation in their mycopesticide production.

Biotechnological tools offer vast potential to enhance the virulence, tolerance to adverse environmental conditions, and application flexibility of EPF for pest control. In addition to manipulating nutritional and environmental conditions during fungal growth, we see significant promise in the use of precise genetic engineering techniques, like CRISPR-Cas technologies. These advancements could be key to unraveling the complexities of EPF genomes, providing deeper insights into the molecular mechanisms driving primary growth. Such knowledge could significantly improve the production of specific propagules through submerged cultivation, offering valuable solutions to overcome challenges posed by recalcitrant fungal strains that struggle with mass production, desiccation tolerance, and shelf life, thereby meeting the urgent needs of the industry.

Research into novel formulation techniques, such as encapsulation, granulation, Pickering emulsion, and coacervation, combined with innovative packaging systems, aims to improve the stability, dispersal, efficacy, and shelf life of EPF products. When paired with advances in fermentation technology—encompassing bioreactor design, process optimization, automation, and targeted genetic strain improvement—these innovations have the potential to revolutionize the scalability, efficiency, and cost-effectiveness of submerged liquid fermentation. This could economically surpass the solid substrate fermentation products currently on the market, reinforcing the paradigm shift towards submerged fermentation as the preferred method for the mass production of EPF worldwide.

Finally, there is still progress to be made in advancing submerged liquid fermentation technology for EPF, particularly in understanding the physiological, biochemical, and molecular mechanisms across different species and strains. By adopting an integrated, multidisciplinary scientific approach, we can address the practical challenges faced by the industry. This effort should be guided by fundamental knowledge arising from close collaboration between academia and the private sector.

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Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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