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Production and Stabilization of Mycoherbicides

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Abstract

Despite the urgent need for alternatives to chemicals in plant protection, biological herbicides are not widely used as biofungicides and bioinsecticides. The review is devoted to connections between fungal biology, biochemistry, their ability to survive in extreme environment and development of effective mycoherbicides. Advanced studies on the production and stabilization of mycofungicides and mycoinsecticides were analyzed too in order to obtain ideas for the improvement of efficacy and technology of mycoherbicides in the future. The analysis of research data published within last 20 years showed following trends. First, more attention is paid for production both effective and stress tolerant propagules especially based on the submerged fungal mycelium and its modifications (blastospores, chlamydospores and microsclerotia). Second, the construction of bioreactors, in particular, for solid-state fermentation is continuously being improved that allows producing highly stress tolerant fungal aerial conidia. Third, based on studies of biochemical mechanisms of viability of fungi in extreme environment, the approaches of stabilization and storage of fungal propagules were developed. However, the positive reply to the question, if biopesticides including mycoherbicides, will become a serious alternative to agrochemicals, will be possible when they demonstrate stable efficacy in the field conditions and safety for both environment and end users.

Keywords: biopesticides, fungi, biology, biochemistry, ecology, stress tolerance, mycoherbicides, mycoinsecticides, mycofungicides, production, stabilization, formulation

1. Introduction

With gradual increase of restrictions for use of chemical pesticides, the role of natural regulators of pest organisms including weeds and invasive plants will grow up. The development of weed biocontrol is stimulated due to their increasing resistance to chemical herbicides and

slow down development of novel herbicidal active components with new mode of action [1]. There are a few mycoherbicides among biopesticides registered in the last years [2].

Despite biocontrol efficacy is generally lower than application of pesticides, biologicals have some advantages over chemicals: (1) biopesticides can be used for resistance management, especially since they may have multiple modes of action, which would reduce the chance of resistance occurring in a particular crop pest; (2) many biopesticides have no or low restricted entry intervals, meaning that post-application, restricted entry into the field is very low and there are often no limitations prior to harvest and (3) there are generally exemptions of biopesticides from maximum residue limits because they are considered acceptable and relatively safe [3].

More than half a century had passed since the first mycoherbicide was registered. Dispute raged, and still rages today, about whether “Have bioherbicides come of age?”, “What is their really contribution to crop protection?” or “Athletes foot or Achilles heel?” [4–9]. This is partly because the biological herbicides as distinct from chemical preparations are not “stand alone” products. There are significant differences in their origins (biological vs. chemical), modes of action (multiple vs. singular), manufacturing methods (fermentation vs. synthesis), requirements to storing and application conditions, etc. [10]. Efficacy of mycoherbicide strategy depends on thorough understanding of host-pathogen-environment interactions. The biological herbicides are more effective when they are incorporated into integrated weed management programs [11]. For example, it was demonstrated that the bioherbicide *Myrothecium verrucaria* (7.5×10^{12} spores/ha) used along with mowing allows to quickly eradicate kudzu (*Pueraria montana* var. *lobata*) [12]. Bioherbicidal efficacy also can be improved using bio-based formulation [13].

Currently, it highlighted 18 of the most serious weeds in agriculture and 50 troublesome ones in cultivated crops, pastures and waterways [11]. Mycoherbicides are mainly used to prevent and control the spread of such worst parasitic weeds as *Orobancha*, *Phelipanche*, *Striga* and *Cuscuta* [3, 14]. Most of them are invasive species. Invasive plants do not only displace the indigenous species, but also change soil biota over considerable territories. Therefore, the presence of particular arbuscular mycorrhizal fungi may determine the success of their invasion [15–19]. Herbicide contamination also can cause deleterious effects on soil biota. Therefore, it is supposed that mycoherbicides used along with other biological and mechanical methods of plant protection might make a more positive impact on restoring native plants population than chemicals. For example, it was shown the promotion effects of *Fusarium oxysporum* f. sp. *strigae*, a soil-borne biocontrol agent against *Striga hermonthica*, on total fungal and arbuscular mycorrhizal fungal taxa in rhizospheres native plants *Gigaspora margarita* [20, 21]. Application of mycoinsecticide *Metarhizium anisopliae*, for leafroller (*Cnaphalocrocis medinalis*) control increased the relative distribution of bacterial species (*Methylobacterium*, *Sphingobium* and *Deinococcus*) implicated in organic pollutant degradation and plant growth promotion [22].

Key features of mycoherbicides are host specificity, crop tolerance, efficacy, environmental fate, temperature and moisture spectrum, mode of action and toxicology [23]. It is important to realize that not only the choice of the strain, but also types of propagules (conidia, mycelium, sclerotia, etc.), production and application method is influenced by mycoherbicide features. Fungal propagules are influenced by a number of environmental factors (temperature, humidity etc.) that affect their biocontrol efficacy. It was demonstrated that the propagules' choice,

formulation and application strategy potentially reduce the dew period requirement [24, 25]. Another possible approach would be a manipulation with fermentation conditions up to product infection materials with set-up parameters [7, 26]. Similarly, during fungal growth physical, chemical and nutritional conditions can be altered to manipulate endogenous reserves for production of propagules with improved stress tolerance to abiotic factors and virulence to host [7, 27–29]. Depending on production method conidia significantly differ by the content of compatible solutes and resistance to environmental influences. The maximum difference is observed when comparing conidia obtained on artificial nutrient media and in nature [28, 30].

Despite of considerable progress in technologies of production and application of mycoherbicides, biopesticides for control of phytophagous insects and plant pathogens have showed much higher commercial success. In some cases, the useful experience for development commercially viable mycoinsecticides and mycofungicides can be tested for the improvement of potential mycoherbicides. For this reason, in this review we analyzed the approaches for producing both mycoherbicides and other types of biopesticides based on fungi.

2. Production

2.1. Choice of propagule types

Various kinds of fungal propagules often fulfill different purposes. In nature, the typical infectious propagules of the pathogenic Ascomycetes are the aerial conidia that facilitate distribution and spreading of these fungi. Generally, aerial conidia can be cost-effectively produced under laboratory conditions [31]. Blastospores, submerged (microcycle) conidia, sporogenically competent mycelia and microsclerotia may be used as the infectious agents as well. They often have a higher survival capability as well as the increased genetic diversity, which probably enhances survival in unstable environments [32, 33]. The morphological and physiological features of submerged conidia can significantly differ from properties of aerial conidia produced by a solid-state culture. For example, submerged conidia and blastospores of *Metarhizium anisopliae* var. *acridum* is characterized by lower surface-hydrophobicity and faster germination as compared to air conidia [34, 35]. Choice of the appropriate propagule is defined by the quality specifications (life-time requirements, desiccation, thermal and UV tolerance, speed of germination and infection, environmental stability and reproduction and the inherent ability) [26, 27, 36–38]. If the conidia production is technologically quite complex or expensive (e.g. due to UV requirements, low viability of propagules during storage and drying, expensive substrates, low-yield spore production, etc.), the mycelium is used as infection material. Application of vegetative mycelium was more effective than conidia in several “fungus-weed” pathosystems *Alternaria cassiae* Jurair & Khan/*Cassia obtusifolia* L. [39], *Chondrostereum purpureum* (Pers. ex Fr.) Pouzar/*Prunus serotina* Erhr. [40], *Phoma herbarum* Westend/*Taraxacum officinale* G.H. Weber ex Wiggers [41], *Sphaceloma poinsettiae* Jenkins & Ruehle/*Euphobia heterophylla* L. [42], *Stagonospora cirsii*/*Cirsium arvense* [43] and *Alternaria alternata* (Fr.) Keissler/*Eupatorium adenophorum* Spreng. [44]. Possibly, in some cases the fungal mycelium is able to complete the infection process faster than conidia [44, 45]. At the same time, the mycelium is generally less tolerant to the abiotic

stress. Nevertheless, the mycelium modifications like chlamydospores, microsclerotia and sclerotia can keep vitality of the fungus for a longer time and can infect the host under suitable weather conditions [46]. Fungal chlamydospores and microsclerotia are evaluated as infection materials for mycoherbicides as well as for other mycopesticides. In nature, chlamydospores formed by *Fusarium oxysporum* play a significant role in long-term survival of the fungus due to their resistance to temperature extremes and desiccation [47, 48]. Chlamydospores of *F. oxysporum* are more thermotolerant than microconidia, it makes them suitable for dry mycoherbicidal formulation. A liquid culture medium was developed for their production [47–49]. A formulation based on dried chlamydospores *F. oxysporum* f sp. *strigae* was developed to control *Striga hermonthica* and *S. asiatica*. It was registered in 2008 in Africa [5, 20]. Another mycoherbicide, DeVine is a liquid formulation of *Phytophthora palmivora* (*P. citrophthora*) chlamydospores for control of milkweed vine (*Morrenia odorata*) in Florida citrus groves. One of the possible weaknesses of such propagules is the uneven germination. Arabic gum in a liquid formulation of chlamydospores of *F. oxysporum* stimulated germ tube elongation and the production of secondary chlamydospores [52]. Nevertheless, the germination rate of conidia of *Rhynchosporium alimatis* was two times lower than the germination of chlamydospores [53]. In the practice, conidia of *Mycoleptodiscus terrestris* cannot be produced using submerged fermentation. At the same time, microsclerotia of this fungus are capable to remain stable in dry conditions and to germinate both hyphally and sporogenically upon rehydration that enhances the potential of this fungus for its use as biological control agent for hydrilla [54]. The mycoherbicide Sarritor was developed on the base of microsclerotia of *Sclerotinia minor*. It demonstrated its high efficacy against dandelion (*Taraxacum officinale*) (78 and 97% by pre- or post-emergence application correspondingly) [55]. The microsclerotia of *Colletotrichum truncatum* can be produced by both submerged and solid-state fermentation and to be effectively used for *Sesbania exaltata* control [56–58]. The development of “multi-propagule” formulations of mycoherbicides is possible as well [58].

However, a few of successful field experiments with microsclerotia-based mycoinsecticide were described. The field efficacy of solid and liquid formulations of microsclerotia *Metarhizium brunneum* F52 was lower or comparable with its conidial preparations. However, microsclerotia of the fungus can be applied with a harsh hydro-mulch technique [59].

2.2. Mass production of mycoherbicide propagules

High spore density (about 10^{12} – 10^{14} CFU per ha) is required for use of mycoherbicides in the field. Therefore, one of the main technological goals is to obtain cost-effective, viable and aggressive infectious material [3, 26]. The secondary use of substrates is a solution of their decontamination and utilization. For example, multi-step waste wood bio-recycling includes the cultivation of *Lentinula edodes* and *Pleurotus ostreatus* followed by *Trichoderma*, *Beauveria* and *Brachygladium* biocontrol strains [60–62].

The loss of viability of the infectious material is usually observed during its drying and storage. Moreover, in nature, the combination of temperature and humidity optimal for rapid germination of fungal spores is relatively rare. Germination of spores can be also suppressed by the action of solar irradiation. Thus, the techniques and conditions for cultivation of bio-control fungi and the selection of the nutrient media composition should be directed both to reach high biomass yields and to improve their activity in the field [63, 64].

There are several approaches to improve fitness of biocontrol fungi: strain selection, optimization of media composition, addition protectors (compatible solutes such as trehalose, sucrose, glycine-betaine, etc.) and treatment of growing cultures with sub-lethal doses of stress factors (e.g. oxidative stress and temperature) [26, 65, 66]. However, on the practice sub-optimal water activity of the substrates are widely used and helpful [67, 68].

Propagules can be produced by solid-state and liquid fermentation or two-phase system.

2.2.1. Liquid submerged fermentation (LSF)

LSF is the most commonly used technology for microbial inoculum production. Collego and DeVine, the first commercially produced bioherbicides, had been manufactured this way. The ability to fully control the cultivation process and its relatively short duration (several days) is an undoubted advantage of LSF over solid-state fermentation. The composition of a culture medium is an important parameter in the biotechnological process because it is 30–40% of the production costs. A commercial LSF medium for *C. truncatum* conidia production includes glucose (20 g/l), yeast extract (2.5 g/l), cottonseed flour (7.5 g/l) and various salts. After 72 h cultivation, more than 6×10^7 conidia/ml is produced [69]. To obtain a high titer of *Paecilomyces fumosoroseus* blastospores resistant to lyophilization, a nutrient medium was optimized, allowing to receive $1\text{--}2 \times 10^9$ spores/ml after a 48-h fermentation. The key factors were high inoculum concentration, amino acid-rich nitrogen source and trace elements [70]. The nutrient medium composition and fermentation parameters (2% of inoculum, duration 120–160 h) for production of mature chlamydospores (more than 1×10^8 CFU/ml) of *Gliocladium virens* GL-21 (SoilGard™ biofungicide) were selected [71].

To obtain a high yield of viable and stress tolerant infectious material, the composition of the liquid nutrient medium requires optimization. Its algorithm can include three main steps: (1) selection of the basal medium with a set of vitamins and trace elements, on which the fungus grows and/or sporulate well; (2) selection of carbon and nitrogen sources and their optimal concentration and ratio determination and (3) replacement of artificial carbon and nitrogen sources by cheap natural ones [72]. Application of factorial design and response surface methods were successfully used to optimize the growth parameters required for large scale conidia production of potential mycoherbicides based on *Gloeocercospora sorghi* and *Septoria polygonorum* [73, 74].

To obtain high titers of *Colletotrichum coccodes* conidia, the optimal carbon concentration in the medium was 20 g/l and C/N ratio of 10:1 [75]. In the case of *C. truncatum*, microcyclic sporulation was induced at carbon concentration in the medium up to 4–16 g/l and C/N ratio in the range 10:1–80:1. At carbon concentration of more than 25 g/l in the submerged culture of this fungus, microsclerotia were formed. The maximum yield of *C. truncatum* conidia was obtained at carbon concentration up to 4–8 g/l and C/N ratio of 30:1, but conidia from media with C/N ratio of 10:1 were more pathogenic and resistant to drying. The conidia obtained in the latter medium contained more proteins, trehalose and polyols, but less glucose and lipids than from C/N ratios in the range 30–80:1 [64, 76, 77]. The influence of carbon concentration and C/N ratio on fungal growth and sporulation is not only species, but also strain dependent [78].

The liquid nutrient medium tonicity has a significant effect on the yield and quality of propagules. Sporulation of *Ulocladium atrum* in a liquid medium was obtained with a reduced water

potential ($\Psi = -2.1$ MPa) by adding glycerol (7.3% w/v) and calcium chloride (20 mM) to the medium. Biomass from liquid cultures responded to water-stress by accumulating increased concentrations of polyols (glycerol) and trehalose [79]. Increased liquid nutrient medium tonicity (osmolality 804–1454 mOsm) of the made by 50–150 g/l of PEG 200 polyethylene glycol increased the yield of submerged *Metarhizium anisopliae* var. *acridum* conidia up to 25%. Spores from high osmolality medium had increased pathogenicity and tolerance to drying. Interestingly, relative drying stability did not appear to be the result of differences in polyol or trehalose concentrations [35].

Non-optimal carbon sources also stimulated *M. anisopliae* formatting resistant to long-range ultraviolet (<290 nm) and accumulating about two times more mannitol and trehalose conidia [80]. The effect of alkane-growth induction of the entomopathogenic fungus *Beauveria bassiana* on the virulence was demonstrated. That alkane supplementation of culture media does not affect the fatty acid composition but change the unsaturated/saturated ratio. However, the unsaturated/saturated ratio diminished markedly from 4.32 to 2.47 [81].

At the same time, liquid substrates are uncommon one for fungal growth.

2.2.2. Solid-state fermentation (SSF)

Solid-state fermentation is the most suitable for cultivation of fungi because their habitats are chiefly solid substrates. In fact, SSF imitates the yields aerial conidia as the final product of conidiation processes. For example, 98% of marine fungi were isolated from submerged solid substrates [82]. In the most cases, spore yields and viability are higher than they are produced by SSF [83]. Hydrophobic air conidia are best suitable for oil formulations, since prolong the conidial viability and decreases UV radiation sensitivity [84–86]. Indeed, numerous studies have shown that conidia produced in an SSF culture are tolerant toward environmental factors (dehydration, drop of temperature and solar irradiation) than spores obtained by SmF [87]. Conidia and blastospores are the main infective units used in biological control with entomopathogenic fungi. There is no absolute advantage between both infective units. However, most formulations of mycoinsecticides are based on aerial conidia obtained in solid-state culture, since these propagules are more resistant to abiotic factors found in open fields [88].

A polysaccharide matrix often surrounds the spores produced by SSF and protects them during desiccation opposite the spores produced by LSF [89]. The choice of substrate, its humidity and growing time also affect the quality of propagules [90]. For example, dried conidia of *Colletotrichum truncatum* produced on vermiculite tended to retain efficacy during storage better than spores recovered from perlite culture [91]. Sometimes the fermentation can be terminated after the fungus has penetrated the nutritive substrate but before conidiation has begun [92, 93]. Dried grain kernels colonized by *Beauveria* or *Metarhizium* remain competent for regrowth and sporulation upon rehydration. The colonized grains are also viable for lengthy periods in the soil, germinating when suitable conditions arise. For example, after such granules are applied into soil or mixed into horticultural soil the conidia were produced within the habitat of the target insect [92, 93]. At the same time, SSF is not widely used earlier in bioherbicide production due to higher costs, more chances of contamination and the complexity of spores' recovery from the substrate [94].

In the case of small manufacturers, the propagules traditionally produced in the plastic bottle or perforated polypropylene carrier bags [95, 96]. This process was the first designed to meet the biological requirements of genus *Metarhizium* fungi. Technology allowed obtaining the conidial yield 1.5×10^9 conidia/g rice and substrate handling capacity was 82 kg rice/production cycle [97]. Later it has been used to produce conidia of the other entomopathogenic fungi like *Beauveria bassiana*, *Lecanicillium lecanii* and *Penicillium frequentans*, and phytopathogenic fungus *Lasioidiplodia pseudotheobromae* [98–101]. However, this process presents difficulties in terms of monitoring and various process parameters control, which directly affect the production yields and quality. These problems are already apparent at small scale in the laboratory and are exacerbated with increase in scale. For example, in the most of SSF bioreactors constructions it is extremely difficult to eliminate the temperature gradient and the oxygen concentration in the substrate [83, 95]. Elevating of CO₂ levels in substrate can suppress conidiation of *Alternaria cassiae* and *A. crassa* [102]. These problems can be solved by selecting water-retaining additives to the substrate (e.g. cannabis trusses), appropriate stirring and aeration of the substrate [96]. Use of tray bioreactors results in similar or higher production and productivity of conidia than those obtained with the traditional. That is now possible because of advances in the construction of SSF bioreactors [87, 104]. For example, a stirred bioreactor with aeration supply has been designed for *Paecilomyces lilacinus* conidia production [103]. Laverlam International Corporation developed *B. bassiana* in SSF column bioreactors. As well as traditional approach shows the process consists of biphasic system. Producing by submerge fermentation inoculum is used for SSF [97]. The method was developed to produce conidia *Coniothyrium minitans* in internally agitated bioreactor on the oats, as substrate, providing the volumetric conidia yield more than 5×10^{14} conidia/m³. Significant yield increase probably is provided for the internal agitation caused mechanical damage to mycelium, which directly affected conidia production [105].

It is well known the positive effect of near ultraviolet radiation on sporulation of certain phytopathogenic fungi from genera *Ascochyta* and *Alternaria*. In the application of UV during fermentation and the employment of microbial mixed cultures, SSF can offer this option that cannot be achieved by SmF. However, a direct comparison between the SSF and SmF cultivation modes of fungi is difficult to make because the two processes differ [82].

Naturally occurring substances can be applied for bioherbicide production [106, 107]. SSF allows to obtain bioherbicides utilizing the agroindustry waste such as bagasse, soybean bran and corn steep liquor [108].

3. Stabilization of fungal propagules

Biological material produced by fermentation and separation from a substrate as a rule cannot be stored for a long time. Even at a low temperature of the storage fungal spores, the mycelium can germinate slowly under appropriate wetness that is unpromising without a plant substrate. Many locally produced biopesticides should be used within several weeks after fermentation was finished as DeVine™, a mycoherbicide based on spores of *Phytophthora palmivora* [64, 109].

At the high-productivity biotech companies, the microorganisms should be stabilized to prevent germination of propagules for a long time (months, years). This can be achieved basically

by concentration, drying or encapsulation of biomaterial on polymer layer and storage under appropriate conditions. In the ideal situation, the modern biopesticides can be stored not less than 2 years at the temperature 4°C, 3 months at 30°C and several days at 40–50°C [64].

There are quite simple and cheap techniques of stabilization and storage of some microorganisms. For instance, infection material of *Fusarium oxysporum* antagonistic strains is produced, dried and stored in the peat. The fungal spores did not lose viability for several years [110]. There are no universal recipes. An optimal stabilization technique should be developed for any fungal biocontrol agent.

It is well known that fungal growth and development are depend on temperature, free water availability, pH and oxygen concentration. For stabilization of the fungal propagules, these factors are manipulated by lowering pH, water activity, temperature and oxygen concentration [67, 68].

In many fungi, spores or spore matrix contains the inhibitors that prevent their germination in fruiting bodies, conidiomata, pustules even at the favorable wetness and temperature. These compounds isolated from some rust and anthracnose fungi were demonstrated to be fungistatic [111–115]. Probably, they can be used as natural preservatives and for stabilization of spores of biocontrol fungi.

Spores of many different fungi aggregated in conidiomata can survive over a season and longer under stress and varied environmental conditions including drying, UV-irradiation and low winter temperature. As a rule, such spores are pigmented or/and surrounded by thin shell (as teliospores of rust and smut fungi) or incorporated into spore matrix (as in coelomycetous fungi). Chemical analysis of the matrix in *Ascochyta* and *Phoma* spp. showed that it consists of pigments, glucose, polysaccharides, tyrosine and proteins [116, 117].

Protective compounds, such as pigments and compatible solutes, in fungal cells as well thickness of cell wall and plasma membrane lipid composition play important role in their resistance to artificial drying. Pigments, especially phenolic ones, utilize reactive oxygen species (ROS) which production is induced in drying process [28, 46]. Taking in account this consideration protective compounds are added to the biomaterial (at the concentration about 5–20%) before drying to prevent deleterious effects of ROS and to regulate osmotic pressure. Dried biomass should be stored at the darkness and lower oxygen concentrations. The rehydration is the important step too. It should be gradual and be made in wet atmosphere, warm water (30–37°C) in order to prevent the injury of fungal plasma membranes [46, 118].

3.1. Biomass concentration and preservation

The preparation of the concentrated suspensions or emulsions, pastes with addition of preservatives (germination inhibitors, antibiotics, etc.) is the simple techniques of stabilization and storage of fungal propagules, especially, if the it sensitive to drying.

3.1.1. Concentrates

A liquid formulation of the biofungicide was developed on the base of the yeast *Rhodotorula minuta* for biocontrol of mango anthracnose. The addition of glycerol (20%) and xanthan

(5%) to the concentrated spore suspension (10^9 CFU/mL) prevented a preparation contamination and cell sedimentation. At the temperature 4°C , CFU number was decreased 100 times after 6 months of the storage. For stabilization of a bioinsecticide based on the mycelium of *Lagenidium giganteum* a concentrated emulsion was developed containing 40% of refined corn oil and 0.5% AEROSIL (Fumed Silica, R974). The latter prevented mycelium sedimentation and aggregation. This formulation can be stored under room conditions for 12 weeks without loss of efficacy against mosquitos [119].

Some components of emulsion concentrates (for instance, plant or paraffinic oils) affect efficacy of biopesticides including mycoherbicides. They prevent fast water evaporation from spray droplets and improve thermotolerance of fungal cells as it was shown for *Metarhizium anisopliae* s.l. (IP 46) and *Metarhizium robertsii* (ARSEF2575) [120]. Application of *Microsphaeropsis amaranthi* against the weed, *Microsphaeropsis amaranthi* in Sunspray 6E oil (10% v/v) resulted in improved disease impact under low moisture conditions [121].

3.1.2. Pastes

The mycelium of *Trichoderma asperellum* GSS 03-35 produced by submerged liquid fermentation was stabilized by concentration to 6–10% of dry matter into paste containing corn starch (5%) as stiffener. The paste had pH 3 and contained copper sulfate (20 mg/L). During the course of storage, the fungus formed chlamydospores and conidia. After 6 months of storage at the temperature 20°C the fungus remained effective against head blight of wheat [122].

3.2. Drying

The drying is the most popular technique of inoculum stabilization. Besides simple drying by warm heat on trays (convection drying), spray drying, fluid bed drying and lyophilisation (freeze-drying) are used. The selection of the drying technique depends on availability, costs and sensitivity of the biomaterial.

3.2.1. Convection drying

The biomaterial mixed with preservatives and fillers is dried on trays in thin layer. This technique is used for production of the biofungicide *Trichodermin* (Biotechmash, the Ukraine) as a wettable powder. This simple technique can be applied for drying of low-scale amounts of the biomass and for preliminary experiments. Corn starch, rice flour, talc, diatomaceous earth and kaolin were evaluated as preservatives and fillers during drying of blastospores of *Beauveria bassiana*. Kaolin (at the concentration of 5% w/v of spore concentrate) allowed to maintain satisfactory viability of spores ($\geq 50\%$) for 7 weeks storage at 4°C [123]. Conidia of *Stagonospora convolvuli* LA39 produced on V8 agar and dried with kaolin as a filler (1 g per 10^9 conidia) by air flow kept high viability ($>70\%$) and pathogenicity about 5 months under the temperature 3°C . After 17 months of the storage just 5% of total conidia were viable, when the conidia were stored under the temperature 20°C conidia their viability decrease to 20% for a week [124].

In some inoculum stabilization protocols, convection drying was proposed for formulation of conidia and microsclerotia of *Beauveria*, *Metarhizium*, *Colletotrichum*, *Mycropletodiscus* and *Trichoderma*. Some useful additives can be used to improve of viability of the infection units:

skimmed milk or/and glycerol (ca. 1–2%, nutrient sources, humectants), clay (ca. 5%, kaolin or peat to protect conidia against UV) and plant oil (4–10%, adhesive) [126, 127].

The drying technique “Stareze” is based on the addition of a membrane stabilizer (sucrose) during the fermentation. High concentration of sucrose (400 g/L) was added to 96-h submerged culture of *Metarhizium anisopliae*. The fermentation was stopped after 168 h and a filler (silica, HiSil™-233, 35 g/L) was added. The filtered product was dried on the trays at ambient temperature. The blastospores of *M. anisopliae* stayed alive for 6 months at 2–4°C [128].

3.2.2. Spray drying

The spore suspension with some adjuvants and additives is sprayed in heated air followed by fast drying (5–30 s). In the case of fluid bed drying, the suspension follows to the bed from dried material babbling by air that forms pseudo-boiling layer. Particles of the drying material stick to gradually form granules (www.niroinc.com).

Submerged conidia of *M. anisopliae* mixed with defatted milk (20%) and sucrose (2.5%) survived better spray drying than freeze-drying process. However, inlet and outlet temperature caused significant effect on their viability [129]. Granules of the commercial biofungicide Contans® are produced by drying conidia of *Coniothyrium minitans* in glucose solution in a spray drier; the product contains about 95% of glucose and 5% of conidia (ca. 1×10^{13} conidia/kg) and remains effective for 2 years when stored at 4°C [96]. In some cases, this technique of drying is not appropriate. Conidia of the epiphytic fungus, *Epicoccum nigrum*, produced by solid-state fermentation lost viability after spray drying at inlet temperature 150°C. However, fluid bed drying was favorable: dried at 30–40°C conidia remained viable even without any preservative and can be stored for 90 days and more [130].

A method was developed for microencapsulation of *Trichoderma* conidia with sugar through spray drying. Microencapsulation with sugars, such as sucrose, molasses or glycerol, significantly ($P < 0.05$) increased the survival percentages of conidia after drying. Microencapsulation of conidia with 2% sucrose solution resulted in the highest survival percentage when compared with other sucrose concentrations and had about 7.5×10^{10} CFU in each gram of dried conidia, and 3.4 mg of sucrose added to each gram of dried conidia. The optimal inlet/outlet temperature setting was 60/31°C for spray drying and microencapsulation. The particle size of microencapsulated conidia balls ranged from 10 to 25 µm. The spray dried biomass of *T. harzianum* was a flow-able powder with over 99% conidia, which could be used in a variety of formulation developments from seed coatings to sprayable formulations [131].

3.2.3. Freeze-drying

Under liophylisation, water vapors from ice under low pressure bypass the liquid state. Conidia of *Septoria passiflorae* survived well after freeze-drying when 10% of skimmed milk was added to the conidial suspension. The fungus stayed viable for >1 year when stored in a vacuum package at 1°C [132]. Blastospores of *Paecilomyces fumosoroseus* together with protectors (10% lactose + 1% bovine albumin, or composition of starch, vegetable oil, sucrose and milk) remain viable after freeze-drying at the level 75% for 50 weeks at –20°C, while at 4°C their viability decreased to the level of 10% [133].

3.3. Encapsulation

Concentrated biomaterial can be incorporated into different polymer matrices that protect fungal cells from effects of some factors such as UV-irradiation and microbial contamination. Products that are resulted from encapsulation process include gel, granules, capsules and microcapsules. There are various industrial equipment for their production [134].

3.3.1. Alginate granules

The process is based on the polymerization of sodium alginate in the solution calcium chloride. For instance, the suspension of the biomaterial (1 part) is mixed with sodium alginate (1.3% solution, 4 parts) and kaolin (5% of total weight); the mixture is dropped into 0.25 M solution of calcium chloride; the resulted granules are filtered and dried. The technique was used for the first time to formulate conidia of *Alternaria cassiae* [135]. Intensity of the fungal sporulation on the granules depended on inoculum production method, fillers and adjuvants; kaolin can be effectively replaced by corn flour [136].

Various compositions of alginate granules were evaluated for many potential and commercial biopesticides. Chitin (2% of granules weight) together with wheat bran (2%) significantly increased spore production of *Beauveria bassiana* on the granules [137]. Starch addition accelerated the rupture of granules and colonization of the peat substrate by *Trichoderma* sp. [138]. For field experiments of biofungicide based on the non-toxicogenic *Aspergillus flavus* different adjuvants (1% of granules weight) and fungicides (0.5–1.25 mg per 50 g of the mixture of sodium alginate and 2.5 g of corn flour) were evaluated. Triptone and peptone addition significantly stimulated spore production of the fungus on the granules. The fungicides did not inhibited the antagonist development and preserve the granules against contamination [136].

Composition of alginate formulation of *Trichoderma* sp. conidia optimized by factorial design experiments included glycerol (2% w/v), sodium polyphosphate 2% (w/v) and citrus pectin that allows to maintain the satisfactory titer of conidia for 14 weeks at 28°C. Formulation quality was monitored by Fourier-transform infrared spectroscopy and some chemical interactions between polymers were found [138]. For production of complex mycoinsecticides (“attract and kill”) based on *Saccharomyces cerevisiae* (used as an attractant for wireworms) and *Metarhizium brunneum* (as an insect killer), the technical scale technology was developed that included jet cutting of droplets and bed drying of granules at 40–50°C till aw 0.1–0.2 [139].

3.3.2. Microencapsulation

Fungal biomaterial (e.g. conidia and mycelia) suspended in sodium alginate solution or in the mixture of agar-agar (1%) and gelatin (1:1, v/v) is emulsified in corn oil with n-hexadecan (6:4) and lecithin as emulsifier. Gelatin-agar globules were gelated in the emulsion while alginate microcapsules were polymerized when dropped into calcium chloride solution. The size of microcapsules varied from 10 to 400 µm depending on ratio of the mentioned components. The microcapsules were separated from the liquids by vacuum filtration and used by spraying. The microencapsulation technique was successfully used in model experiments for development of artificial conidia based on conidia of *Fusarium avenaceum* and mycelium of *Bipolaris sorokiniana* [10–141].

3.3.3. "Pesta" granules

The production of Pesta granules is based on the technology of pasta production. Inoculum suspension (52 mL), wheat semolina flour (80 g) and kaolin (20 g) are mixed to produce dough. The dough is passed through a pasta maker after that it is dried, crashed and sieved. The technique was tried for encapsulation of conidia of potential mycoherbicides (*Alternaria cassiae*, *A. crassa*, *Colletotrichum truncatum* and *Fusarium lateritium*) as well for stabilization of entomopathogenic nematodes [142, 143]. Melanized fungal structures as pigmented conidia, chlamydospores, microsclerotia and sclerotia granules are generally compatible to Pesta process while non-pigmented conidia of *F. oxysporum*, *C. truncatum*, *Trematophoma lignicola* were not viable in the final product [49, 57, 143–145].

Microsclerotia of *C. truncatum* survived in Pesta granules and remained to produce virulent conidia (for biocontrol of weed *Sesbania exaltata*) for 52 weeks at 25°C low water activity (aw 0.18–0.29), and for 10 years at 4°C [57, 146] while the fungal conidia can be stored no more than 32 weeks [147]. Interestingly, that during the process of encapsulation of *Alternaria alternata* conidia with Pesta process, the number of colony forming units increased due to destroying their aggregations. The virulence of the fungus was stable at a low relative air humidity (12%) for more than 2 years [145].

The composition of Pesta granules can be easily modified. Shabana et al. [148] evaluated various compositions for *Fusarium oxysporum* f. sp. *orthoceras* using 3% (w/w) sucrose, corn flour, glycerol, starch WaterLock B209, cellulose and yeast extract. The last component improved viability of chlamydospores as well as of microconidia in the granules. However, the prepared samples showed appropriate viability (60–80% for 12 months) under 25°C and relative humidity 11–12%; under higher temperature (25°C) and humidity (51–53%) viability of the fungus dramatically decreased by the 4–8th month of storage [148]. The biocontrol efficacy of *Aspergillus alliaceus* against parasitic weeds (*Orobancha* spp.) incorporated in Pesta granules was improved by addition of potato broth or sorghum meal [149].

For encapsulation of conidia of potential mycopesticides (*C. truncatum*, *Alternaria* sp., *Paecilomyces fumoroseus*, *Aspergillus flavus*, *A. parasiticus*) produced by solid and liquid state fermentation the twin-screw extrusion was successfully tested. Ingredients were mixed in the mixer of an extruder and resulted Pesta granules were dried by fluid bed drying at 50°C. The inoculum produced by solid-state fermentation was shown to be less sensitive to whole the stabilization process than the biomaterial from the liquid culture [150].

3.3.4. Stabilize granules

The main components of these granules are a membrane stabilizer (for instance, sucrose at the concentration 10–65% from granules weight), a water absorbance agent (starch), a filler (diatomaceous earth, silica Hi-Sil® at the concentration 5–20%). Additionally, the granules can include vegetable oil (ca. 20%), UV-protectant, preservatives and other inert fillers [151]. For example, sucrose (4 parts), starch (1 part), unrefined vegetable oil (1 part), silica gel (1.5 parts) and biological suspension (4 parts) are mixed and extruded; the resulted pasta is conventionally dried and

crashed or milled. This technique was successfully used for potential bioherbicides based on *Fusarium oxysporum* (microconidia and mycelium) and *Pseudomonas* spp. that remain viable for a long time [152–154]. However, submerged conidia of *Metarhizium anisopliae* (the producer of the bioinsecticide Green Muscle™) survived better when the above-mentioned process Satreze was used [128].

The safety and evaluation of postponed risks of mycopesticides are still under question. An agroecosystem is inundated by a fungus at very high concentrations and there is a risk of the crop injury. Some plant pathogens can survive in the soil or plant debris. They are able of producing biologically active compounds (mycotoxins, antibiotics, phytotoxins, etc.). The number of safety research on the safety of mycoherbicides is limited to *Sclerotinia sclerotiorum*, *S. minor*, *Colletotrichum coccodes*, *Fusarium oxysporum* f. sp. *strigae*, *Phoma macrostoma* and *Stagonospora convolvuli* [7, 23, 55, 155–159]. The experience of field observations is limited to several years.

Molecular marking of biocontrol strains is an approach for their post-application tracking and quantification. For instance, the strain *Fusarium oxysporum* f. sp. *strigae* F2, which is potential mycoherbicide against *Striga* spp., was compared with several strains *F. oxysporum* using fluorescent AFLP. Based on this comparison a specific PCR primer was developed for making F2 only in the soil [20, 21, 160].

In conclusion, the approaches for stabilization and storage of biopesticides based on fungal propagules were discussed in this review. In order to produce both virulent and stress tolerant propagules for mycoherbicides based on the submerged fungal mycelium as well as on conidia, chlamydospores and microsclerotia a liquid medium should be optimized. The construction of bioreactors, in particular, for solid-state fermentation is continuously being improved that allows of producing highly stress tolerant fungal aerial conidia. Various recipes for liquid (e.g. suspension and emulsion concentrates) and solid (like alginate and stabilize granules) formulation of mycoherbicides were developed to be stored for a long time and effectively used. However, the efficacy of mycoherbicides is still unstable and their safety is not proved clearly to be widely commercialized.

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