

# Translating Fungal Broth Metabolite Bioactivity into Market-Ready Mycoherbicide

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**Abstract--** The growing reliance on synthetic herbicides has led to escalating concerns over herbicide resistance, environmental contamination, and regulatory restrictions, underscoring the urgent need for sustainable weed management alternatives. Fungal broth-derived metabolites have emerged as promising bioactive agents for mycoherbicide development due to their diverse modes of action, target specificity, and environmental compatibility. However, translating laboratory-scale bioactivity into commercially viable products remains a major bottleneck, primarily due to challenges associated with metabolite stability, formulation compatibility, shelf life, and field efficacy.

This review critically examines recent advances in the development of metabolite-driven mycoherbicides derived from fungal fermentation broths, with a particular focus on formulation strategies that enable market readiness. Key aspects discussed include the identification and optimization of phytotoxic fungal metabolites, the advantages of using cell-free fungal broths over living propagules, and the role of formulation technologies such as emulsifiable concentrates, micro- and nano-encapsulation, adjuvant integration, and controlled-release systems. The review further explores strategies for enhancing product stability, scalability, and consistency under diverse agro-climatic conditions, while addressing regulatory and commercialization considerations.

By integrating biological efficacy with formulation science and industrial feasibility, this article highlights practical pathways for converting fungal metabolite bioactivity into field-ready mycoherbicide products. The insights presented aim to support the development of next-generation, eco-friendly weed control solutions that align with the principles of sustainable and precision agriculture.

**Keywords--** Fungal metabolites; Mycoherbicides; Sustainable weed management; Cell-free fungal extracts; Formulation technology; Microencapsulation; Eco-friendly herbicides; Precision agriculture

## I. INTRODUCTION

Weeds remain among the most persistent and economically damaging biotic constraints to global agricultural production, causing significant yield losses, increased production costs, and reduced crop quality across diverse agroecosystems (Oerke, 2006; Pimentel, 2005; Chauhan et al., 2017; Duke et al., 2019).

Yield losses due to weed interference frequently exceed 30–40% in major crops and may reach up to 70% under severe infestations (Buhler et al., 1997; Oerke, 2006; Norris et al., 2013; Werth, 2014). In addition to direct competition for resources, weeds disrupt crop phenology and serve as reservoirs for pests and pathogens, further destabilizing production systems (Holm et al., 1997; Zimdahl, 2018; Chauhan, 2020).

Synthetic herbicides have long dominated weed management due to their rapid action, broad-spectrum control, and ease of application (Duke & Powles, 2008; Powles & Yu, 2010; Beckie & Harker, 2017). While these chemicals have contributed substantially to agricultural productivity (Oerke, 2006; Duke et al., 2019), their intensive and often indiscriminate use has led to environmental contamination, adverse effects on non-target organisms, ecosystem disruption, and potential human health risks (Duke et al., 2002; Solomon & Thompson, 2003; Dayan et al., 2009; Aktar et al., 2009; Berrada et al., 2011; Goulson, 2013).

A major consequence of continued herbicide dependence is the widespread evolution of herbicide-resistant weed populations. Resistance has now been reported in over 260 weed species globally, spanning multiple herbicide modes of action, including ALS inhibitors, EPSPS inhibitors, and PPO inhibitors (Powles & Preston, 2006; Beckie, 2011; Norsworthy et al., 2012; Powles & Yu, 2010; Heap, 2024). This escalating resistance crisis threatens the long-term sustainability of chemical weed control and underscores the urgent need for alternative strategies with novel and multi-target mechanisms (Beckie & Harker, 2017; Duke et al., 2019).

Consequently, sustainable weed management approaches have gained increasing attention, particularly those integrating biological tools within integrated weed management (IWM) frameworks (Liebman & Gallandt, 1997; Mortensen et al., 2012; Cordeau et al., 2016). IWM emphasizes the coordinated use of cultural, mechanical, biological, and chemical tactics to achieve durable weed suppression while minimizing environmental impacts and resistance development (Froud-Williams et al., 2012; Shaner, 2014).

Among biological alternatives, bioherbicides derived from living organisms or their natural metabolites represent promising eco-compatible weed control tools (Charudattan, 2001; Hallett, 2005; Bailey et al., 2010; TeBeest et al., 2020). These include phytopathogenic fungi and bacteria, allelopathic plant extracts, and microbial secondary metabolites that interfere with weed growth through diverse biochemical and physiological mechanisms (Putnam et al., 1985; Duke & Dayan, 2015). Compared with synthetic herbicides, bioherbicides often exhibit improved biodegradability, reduced non-target toxicity, and novel or multiple modes of action, thereby lowering environmental persistence and resistance risks (Dayan & Duke, 2010; Duke et al., 2019).

Fungal-based bioherbicides, or mycoherbicides, have attracted particular attention due to the remarkable diversity of phytotoxic secondary metabolites produced by fungi (Abbas et al., 1995; Vurro et al., 2009; Evidente et al., 2013). Fungal genera such as *Fusarium*, *Alternaria*, *Colletotrichum*, *Phoma*, *Drechslera*, and *Trichoderma* synthesize metabolites across diverse chemical classes, including polyketides, terpenoids, alkaloids, phenolics, and cyclic peptides (Strobel, 2006; Andolfi et al., 2014; Cimmino et al., 2015). These compounds disrupt key plant processes such as photosynthesis, respiration, membrane integrity, oxidative balance, and hormone signaling (Inderjit & Duke, 2003; Dayan & Duke, 2014).

Early mycoherbicide research focused primarily on live fungal propagules. However, the field performance of living agents has often been inconsistent due to their sensitivity to environmental variability, limited shelf life, formulation constraints, and biosafety concerns (Charudattan, 2001; Hallett, 2005; Cordeau et al., 2016; TeBeest et al., 2020). Exposure to ultraviolet radiation, desiccation, temperature extremes, and microbial antagonism frequently limits their reliability under field conditions (Blakeman et al., 1998; Cook et al., 1999).

In recent years, research has increasingly shifted toward fungal cell-free culture filtrates (CFCFs), obtained by removing fungal biomass while retaining extracellular phytotoxic metabolites responsible for herbicidal activity (Glenn et al., 1996; Chee et al., 2000; Abbas et al., 2007; Cimmino et al., 2015). Unlike conventional mycoherbicides, CFCF-based systems decouple herbicidal efficacy from fungal viability and infection processes. This metabolite-driven approach offers important advantages, including enhanced formulation flexibility, improved storage stability, standardization of active constituents, reduced environmental sensitivity, and simplified biosafety and regulatory considerations (Bailey et al., 2010; Duke et al., 2019).

Despite these advantages, the practical application of fungal CFCF bioherbicides remains constrained by formulation-related challenges, including poor solubility, chemical instability, rapid environmental degradation, and inefficient delivery to target weeds (Dayan et al., 2009; Vurro et al., 2009; Duke & Dayan, 2015). Many fungal metabolites are susceptible to photodegradation, temperature fluctuations, and wash-off by rainfall, resulting in variable field efficacy (Andolfi et al., 2014).

Formulation science is therefore central to translating fungal metabolite bioactivity into market-ready mycoherbicide products. Conventional and advanced delivery systems—such as emulsifiable concentrates, adjuvant-assisted formulations, micro- and nano-encapsulation, and controlled-release matrices—have been explored to enhance metabolite stability, bioavailability, and weed control efficacy (Vurro et al., 2009; Kah et al., 2013; Duke et al., 2019; Yu et al., 2018). Nanotechnology-based approaches offer particular promise by improving plant uptake, protecting active compounds from environmental degradation, and minimizing off-target effects (DeRosa et al., 2010; Zhao et al., 2017; Singh et al., 2019a).

Mechanistically, fungal metabolites in CFCFs exhibit diverse and often multisite modes of action, including membrane disruption, inhibition of photosynthesis and respiration, induction of oxidative stress, and interference with hormone signaling pathways (Abbas et al., 1995; Dayan & Duke, 2014; Duke & Dayan, 2015). Such multisite activity enhances efficacy and reduces the likelihood of resistance development relative to single-target synthetic herbicides (Powles & Preston, 2006; Beckie & Harker, 2017). Several metabolites also demonstrate species-specific selectivity, enabling their integration into crop-specific weed management programs (Rice, 1984; Inderjit & Duke, 2003; Schmidt et al., 2007). In addition, allelopathic interactions mediated by fungal metabolites may contribute to soil seedbank suppression, providing longer-term weed control benefits (Putnam et al., 1985; Weston, 1996; Macías et al., 2007).

Nevertheless, large-scale commercialization of fungal CFCF bioherbicides remains limited by challenges such as batch-to-batch variability, fermentation scale-up constraints, regulatory complexity, and limited farmer adoption (Bailey et al., 2010; Cordeau et al., 2016; TeBeest et al., 2020). Regulatory frameworks for microbial metabolites vary widely across regions, complicating product registration and market access (Steinmetz et al., 2008; Garthwaite et al., 2012).

Adoption by farmers is further constrained by perceptions of inconsistent performance, higher costs relative to synthetic herbicides, and limited availability of formulations tailored to dominant weed problems (Mortensen et al., 2012; Norsworthy et al., 2012).

Addressing these limitations requires coordinated advances in fermentation optimization, formulation engineering, regulatory harmonization, and precision application technologies. Emerging tools in metabolomics, systems biology, and synthetic biology offer new opportunities to discover novel phytotoxic metabolites and optimize production pathways for enhanced yield, stability, and selectivity (Gross & Loper, 2009; Hagiwara et al., 2016; Palmer & Dahm, 2020).

This review synthesizes recent advances in fungal CFCF-based mycoherbicides, emphasizing metabolite diversity, formulation strategies, modes of action, safety considerations, and commercialization pathways. The extensive contributions of Singh and Pandey further underscore the promise of fungal metabolites as natural herbicides and highlight the critical role of formulation and delivery technologies in translating fungal bioactivity into effective, sustainable, and market-ready weed management solutions (Pandey et al., 2007; Singh & Pandey, 2019a, 2019b; Singh & Pandey, 2022).

## II. WEED MANAGEMENT CHALLENGES AND THE NEED FOR BIOHERBICIDES

Sustainable weed management is essential for maintaining global agricultural productivity; however, contemporary weed control systems face escalating ecological, regulatory, and socio-economic challenges. Decades of heavy reliance on synthetic herbicides have delivered diminishing returns, driving renewed interest in biologically based weed management strategies that align with sustainability and resistance management goals.

### 2.1 Limitations of Synthetic Herbicides

The most critical limitation of synthetic herbicides is the rapid and widespread evolution of herbicide-resistant weed populations. Repeated use of herbicides with identical modes of action imposes strong selection pressure, leading to the emergence of resistant biotypes capable of surviving previously effective doses (Duke et al., 2019; Heap, 2024). Resistance has now been documented across nearly all major herbicide classes, including acetolactate synthase (ALS) inhibitors, acetyl-CoA carboxylase (ACCCase) inhibitors, and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitors, severely compromising weed control efficacy and increasing production costs (Chauhan et al., 2017; Duke & Dayan, 2015).

Beyond resistance, environmental contamination associated with synthetic herbicides poses a major concern. Many herbicides persist in soil and aquatic environments and are prone to off-target movement via leaching, runoff, spray drift, and volatilization (Duke et al., 2002; Dayan et al., 2009). Such contamination negatively affects non-target plants, beneficial soil microorganisms, aquatic organisms, and pollinators, disrupting ecosystem services essential for sustainable agriculture (Cordeau et al., 2016). Residual herbicide activity can also impair subsequent crops, particularly in diversified crop rotations, thereby limiting agronomic flexibility.

Human health concerns further complicate continued dependence on chemical herbicides. Chronic exposure to certain active ingredients has been associated with toxicological risks, including endocrine disruption, carcinogenicity, and neurological effects, although risks vary among compounds and exposure scenarios (Duke et al., 2002; Dayan et al., 2009). These concerns have intensified public scrutiny and driven demand for safer weed management alternatives.

As a result, regulatory pressure on synthetic herbicides is increasing globally. Several active ingredients have been restricted or withdrawn due to environmental and health concerns, with glyphosate representing one of the most debated examples (Cordeau et al., 2016; Duke et al., 2019). Regulatory uncertainty, combined with rising development costs and prolonged approval timelines, has discouraged investment in new chemical herbicide discovery. Collectively, resistance evolution, environmental persistence, health risks, and regulatory constraints underscore the declining sustainability of chemical-only weed management approaches.

### 2.2 Advantages of Bioherbicides

In contrast, bioherbicides derived from microorganisms or their natural metabolites offer several ecological and agronomic advantages consistent with sustainable agriculture principles. A key benefit is their favorable environmental profile. Biological agents and microbial metabolites generally exhibit lower toxicity to non-target organisms and reduced environmental persistence, minimizing unintended ecological impacts (Charudattan, 2005; Hallett, 2005). Their inherent specificity often enables selective weed suppression while preserving beneficial soil and plant-associated microbiota.

Bioherbicides are also typically biodegradable, as most microbial metabolites are readily degraded by sunlight, microbial activity, and enzymatic processes, resulting in minimal residue accumulation in soil, water, or harvested produce (Bailey et al., 2010; Duke et al., 2019).

This characteristic facilitates compliance with stringent residue regulations and supports safer food production systems.

Importantly, bioherbicides frequently possess novel or multiple modes of action, distinguishing them from many synthetic herbicides that target single biochemical pathways. Fungal and bacterial metabolites can disrupt weed growth through diverse mechanisms, including membrane destabilization, inhibition of photosynthesis or respiration, induction of oxidative stress, and interference with plant hormone signaling (Abbas et al., 1995; Evidente et al., 2013; Duke & Dayan, 2015). Such multisite activity reduces the likelihood of resistance development and provides valuable tools for managing herbicide-resistant weeds.

From an agronomic perspective, bioherbicides are well suited for integration into integrated weed management (IWM) systems, where they complement rather than replace cultural, mechanical, and chemical control practices (Cordeau et al., 2016). Their compatibility with organic farming, conservation agriculture, and precision application technologies further enhances their relevance in modern cropping systems.

Despite these advantages, the widespread adoption of bioherbicides depends on overcoming challenges related to consistency, formulation stability, and field performance. Advances in microbial biotechnology, fermentation optimization, and formulation science—particularly for metabolite-based products such as fungal cell-free culture filtrates—are steadily improving product reliability and competitiveness. As regulatory and societal pressures on synthetic herbicides intensify, bioherbicides are increasingly recognized as indispensable components of sustainable and resilient weed management strategies.

### III. FUNGAL CELL-FREE BROTH: PRODUCTION, COMPOSITION, AND BIOACTIVE METABOLITES

Fungal cell-free culture filtrate (CFCF) is a metabolite-rich liquid fraction obtained from fungal cultures after removal of mycelial biomass and spores. Enriched with extracellular secondary metabolites responsible for phytotoxic activity, CFCF has emerged as a promising alternative to live fungal inoculants for mycoherbicide development. The efficacy of CFCF-based bioherbicides depends on optimized production processes, metabolite composition, and the biological activity of the compounds present.

#### 3.1 Production of Fungal Cell-Free Broth

Fungal CFCF is typically produced via submerged fermentation, in which selected fungal strains are cultivated in liquid media under controlled conditions. Key parameters—including carbon and nitrogen sources, pH, temperature, aeration, agitation, and incubation duration—strongly influence fungal growth and secondary metabolite synthesis (Abbas et al., 1995; Kim et al., 2011). Complex media containing carbohydrates (e.g., glucose, sucrose, starch) and organic nitrogen sources (e.g., yeast extract, peptone) are commonly used to enhance metabolite yield, whereas defined media can improve reproducibility and standardization (Vurro et al., 2009).

Following fermentation, fungal biomass is removed by filtration or centrifugation to obtain the cell-free broth. Membrane filtration is often employed to eliminate viable propagules, improving biosafety and regulatory acceptability (Bailey et al., 2010). Depending on formulation objectives, the broth may be used directly or subjected to downstream processing such as concentration, solvent extraction, or partial purification (Cimmino et al., 2015). Optimization of fermentation conditions is critical, as variations in nutrient availability or incubation time can substantially alter metabolite profiles and phytotoxic potency (Abbas et al., 2007; Andolfi et al., 2014). Recent advances in metabolomics and systems biology have facilitated targeted optimization of biosynthetic pathways governing secondary metabolite production.

#### 3.2 Composition of Fungal Cell-Free Broth

The chemical composition of fungal CFCF is highly complex and strain-dependent, reflecting the diversity of fungal secondary metabolism. CFCFs typically contain mixtures of low-molecular-weight secondary metabolites, organic acids, enzymes, and signaling molecules, many of which contribute to phytotoxic activity (Evidente et al., 2013; Duke & Dayan, 2015). Unlike single-compound synthetic herbicides, this multi-component nature often results in additive or synergistic bioactivity.

Secondary metabolites in CFCFs belong to diverse chemical classes, including polyketides, terpenoids, alkaloids, phenolics, lactones, and cyclic peptides (Cimmino et al., 2015; Evidente et al., 2013). Their relative abundance varies with fungal species, culture conditions, and growth phase. For example, *Phoma* and *Alternaria* species produce polyketide-derived phytotoxins such as phomalactone and tenuazonic acid, whereas *Fusarium* species synthesize trichothecenes, fumonisins, and related bioactive metabolites (Abbas et al., 1995; Andolfi et al., 2014).

In addition to small-molecule toxins, some CFCFs contain extracellular enzymes (e.g., cellulases, pectinases, proteases) that may indirectly enhance phytotoxicity by degrading plant cell walls or facilitating metabolite penetration (Vurro et al., 2009). Organic acids and reactive oxygen species generated during fermentation may further contribute to herbicidal effects.

### 3.3 Bioactive Metabolites with Herbicidal Potential

Numerous fungal metabolites isolated from CFCFs exhibit strong herbicidal activity, causing growth inhibition, chlorosis, necrosis, or plant death depending on concentration, exposure duration, and target species (Abbas et al., 2007; Evidente et al., 2013). Importantly, many metabolites demonstrate selective toxicity toward specific weed species, offering potential crop safety advantages.

Polyketide-derived compounds are among the most extensively studied fungal bioherbicides. Phomalactone from *Phoma* spp. inhibits seed germination and seedling growth in several weed species, while tenuazonic acid from *Alternaria* spp. disrupts protein synthesis and photosynthesis, resulting in rapid growth suppression (Andolfi et al., 2014; Evidente et al., 2013). Terpenoids and alkaloids contribute additional herbicidal activity by disrupting membrane integrity, mitochondrial function, and hormonal balance (Duke & Dayan, 2015). Cyclic peptides and lactones, although less common, are of particular interest due to their high potency and unique modes of action.

A defining feature of CFCF-based bioherbicides is the simultaneous presence of multiple bioactive compounds. This multi-site activity can enhance efficacy through synergistic interactions and reduce the risk of resistance development in weed populations (Duke et al., 2019). However, it also necessitates rigorous metabolite characterization to ensure standardization and regulatory compliance.

### 3.4 Advantages of Fungal Cell-Free Culture Filtrates over Live Mycoherbicides

Traditional mycoherbicides based on live fungal propagules depend on spore germination, host recognition, and successful colonization, processes that are highly sensitive to environmental conditions such as humidity, temperature, ultraviolet radiation, and microbial competition (Charudattan, 2001; Hallett, 2005; Cordeau et al., 2016). These dependencies often result in inconsistent field performance. In contrast, CFCFs bypass the requirement for fungal viability, as herbicidal activity is mediated directly by extracellular metabolites.

This decoupling of efficacy from microbial survival confers greater reliability across diverse agro-climatic conditions and reduces vulnerability to environmental stressors such as desiccation and UV exposure (Blakeman et al., 1998; Cook et al., 1999). CFCFs also offer enhanced formulation flexibility, as they are compatible with surfactants, stabilizers, encapsulation matrices, and adjuvants that are often unsuitable for live fungal agents (Bailey et al., 2010; Vurro et al., 2009). This compatibility enables the development of formulations with improved shelf life, controlled release, and optimized delivery to target weed tissues.

From a biosafety and regulatory perspective, the absence of viable propagules reduces concerns related to unintended environmental persistence, non-target colonization, and host-range expansion (Glenn et al., 1996; Steinmetz et al., 2008). Collectively, these attributes position fungal CFCFs as a more predictable, controllable, and application-ready platform for mycoherbicide development, providing a strong foundation for the formulation and delivery strategies discussed in subsequent sections.

## IV. SOURCES OF FUNGAL BIOHERBICIDAL METABOLITES

Fungi constitute a prolific source of phytotoxic secondary metabolites due to their ecological diversity and metabolic versatility. Many fungal species secrete extracellular compounds during submerged fermentation that inhibit weed seed germination, suppress seedling growth, or induce necrosis. These extracellular metabolites, accumulated in culture filtrates, form the bioactive basis of cell-free culture filtrate (CFCF)-based mycoherbicides. Identification of effective fungal taxa and metabolite classes is therefore central to rational bioherbicide development.

### 4.1 Fungal Genera Producing Bioherbicidal Metabolites

Several fungal genera consistently exhibit strong phytotoxic activity via their culture filtrates and have emerged as leading candidates for metabolite-driven bioherbicides.

*Colletotrichum* spp. are among the most extensively investigated mycoherbicidal fungi, producing polyketide and phenolic metabolites that cause chlorosis, necrosis, and growth inhibition in weeds such as *Aeschynomene virginica* and *Parthenium hysterophorus* (Charudattan, 2005; Vurro et al., 2009). Their metabolites primarily disrupt chloroplast function and induce oxidative stress, and their extracellular secretion favors CFCF-based formulations.

*Fusarium* spp. produces diverse phytotoxins, including trichothecenes, fumonisins, and fusaric acid, which inhibit weed germination and seedling development by affecting cell division, membrane integrity, and hormonal balance (Abbas et al., 1995; Duke & Dayan, 2015). Although some compounds raise biosafety concerns, strain-level selection and metabolite profiling enable targeted and safer bioherbicidal applications.

*Phoma* spp. generate potent polyketide and lactone metabolites, such as phomalactone, which accumulate in culture filtrates and inhibit grass weeds by disrupting photosynthesis and cellular metabolism (Evidente et al., 2013; Andolfi et al., 2014). Their strong extracellular activity makes them particularly suitable for CFCF-based products.

*Alternaria* spp. produce metabolites including tenuazonic acid and alternariol, which impair protein synthesis, induce oxidative stress, and inhibit photosynthesis (Abbas et al., 1995; Cimmino et al., 2015). These compounds are readily secreted into the growth medium and exhibit broad phytotoxic activity.

*Trichoderma* spp., widely known for biocontrol and plant growth-promoting traits, also produce phytotoxic metabolites such as peptaibols and terpenoids that disrupt membrane integrity and induce oxidative stress (Kim et al., 2011; Duke et al., 2019). Although generally less aggressive, their favorable biosafety profile supports use in integrated weed management systems.

Collectively, these genera highlight the diversity and functional potential of fungal metabolites for developing selective and environmentally compatible bioherbicides (Table 1).

#### 4.2 Major Classes of Bioherbicidal Metabolites

The herbicidal activity of fungal CFCFs results from complex mixtures of secondary metabolites spanning several chemical classes, often exhibiting additive or synergistic effects.

Alkaloids interfere with enzyme activity, nucleic acid synthesis, and hormonal signaling, leading to growth inhibition and chlorosis, though their potency necessitates careful selectivity assessment (Duke & Dayan, 2015).

Terpenoids disrupt membrane integrity, mitochondrial respiration, and photosynthetic electron transport, frequently causing rapid tissue necrosis (Evidente et al., 2013).

Polyketides—such as phomalactone, tenuazonic acid, and alternariol—are among the most widely reported bioherbicidal compounds, inhibiting protein synthesis, respiration, and photosynthesis while inducing oxidative stress (Andolfi et al., 2014; Cimmino et al., 2015). Peptides and lactones, including peptaibols from *Trichoderma*, form membrane pores that cause ion leakage and cell death, whereas lactones selectively inhibit early weed developmental stages (Kim et al., 2011).

While the coexistence of multiple metabolite classes enhances efficacy and reduces resistance risk, it also complicates standardization, formulation stability, and regulatory approval. These challenges underscore the need for advanced analytical tools and formulation strategies to translate fungal CFCFs into reliable, market-ready bioherbicides. Representative fungal genera, their key extracellular metabolites, target weeds, and dominant modes of action are summarized in **Table 1**.

**Table 1.**  
**Representative fungal sources of bioherbicidal metabolites in cell-free culture filtrates (CFCFs)**

<b>Fungal genus</b>	<b>Major extracellular metabolites</b>	<b>Representative target weeds</b>	<b>Dominant mode(s) of action</b>	<b>Key references</b>
<i>Phoma</i> spp.	Phomalactone, polyketides	<i>Echinochloa crus-galli</i> , <i>Amaranthus</i> spp.	Inhibition of germination, photosynthesis and protein synthesis	Evidente et al., 2013; Andolfi et al., 2014
<i>Alternaria</i> spp.	Tenuazonic acid, alternariol	<i>Chenopodium album</i> , <i>Setaria</i> spp.	Protein synthesis inhibition, oxidative stress	Abbas et al., 1995; Cimmino et al., 2015
<i>Fusarium</i> spp.	Trichothecenes, fumonisins, fusaric acid	<i>Cyperus rotundus</i> , <i>Digitaria</i> spp.	Membrane damage, inhibition of cell division	Abbas et al., 2007; Duke & Dayan, 2015
<i>Colletotrichum</i> spp.	Polyketides, phenolic toxins	<i>Aeschynomene virginica</i> , <i>Parthenium hysterophorus</i>	Chloroplast disruption, necrosis	Charudattan, 2005; Vurro et al., 2009
<i>Drechslera</i> spp.	Helminthosporol, related toxins	<i>Avena fatua</i> , <i>Lolium</i> spp.	Respiratory inhibition, growth regulation	Evidente et al., 2013
<i>Trichoderma</i> spp.	Peptaibols, terpenoids	Early-stage broadleaf weeds	Membrane permeabilization, oxidative stress	Kim et al., 2011; Duke et al., 2019
<i>Ascochyta</i> spp.	Ascochitine, phenolic metabolites	<i>Galium aparine</i> , <i>Cirsium</i> spp.	Growth inhibition, chlorosis	Hallett, 2005; Bailey et al., 2010

**Note:** *Herbicidal efficacy and selectivity depend on metabolite concentration, formulation, application strategy, and environmental conditions.*

## V. FORMULATION STRATEGIES FOR FUNGAL CELL-FREE BROTH BIOHERBICIDES

The performance of fungal cell-free broth (CFCF) bioherbicides is largely dictated by formulation, which determines metabolite stability, delivery efficiency, and consistency under field conditions. Unlike synthetic herbicides, fungal metabolites are often chemically labile, poorly soluble, and sensitive to ultraviolet radiation, temperature fluctuations, and rainfall. Consequently, formulation design is critical for translating metabolite-rich CFCFs into reliable, market-ready weed control products (Vurro et al., 2009; Duke & Dayan, 2015).

The primary objectives of CFCF formulation are to protect bioactive metabolites from degradation, enhance uptake and persistence on target weeds, and ensure compatibility with existing agricultural practices. Both conventional pesticide formulations and advanced delivery technologies have been explored to achieve these goals.

### 5.1 Conventional Formulation Approaches

Conventional formulations remain important for early development due to their simplicity, scalability, and grower familiarity. **Emulsifiable concentrates (ECs)** improve solubility and foliar availability of lipophilic metabolites but may raise environmental and regulatory concerns due to solvent use (Bailey et al., 2010). **Wettable powders (WPs)** offer improved storage stability and reduced microbial contamination but may suffer from dustiness, poor leaf adhesion, and uneven dispersion (Hallett, 2005). **Suspension concentrates (SCs)** provide a solvent-reduced alternative, offering improved handling safety and uniform application when metabolites can be concentrated or partially purified (Vurro et al., 2009).

While these approaches are useful for greenhouse and pilot-scale studies, their limited robustness under variable field conditions has driven interest in advanced formulation strategies (Table 2).

**Table 2.**  
**Formulants used in fungal cell-free broth (CFCF) bioherbicides**

<b>Formulant Category</b>	<b>Examples</b>	<b>Functional Role</b>	<b>Notes</b>
Carriers	Talc, kaolin, bentonite, diatomaceous earth, starch; water, mineral oil, vegetable oil	Provide bulk, improve handling, aid delivery and dispersion	Choice depends on liquid vs. solid formulation, target weed, and application method
Surfactants / Wetting Agents	Tween 20, Tween 80, Triton X-100, sodium lauryl sulfate	Reduce surface tension, enhance foliar spreading, improve metabolite uptake	Nonionic surfactants preferred to minimize phytotoxicity
Stickers / Adherents	Gum arabic, xanthan gum, alginate, polyvinyl alcohol, mineral oil	Enhance adhesion to leaves, improve rainfastness	Important for foliar-applied CFCFs
Humectants / Moisture Retainers	Glycerol, sorbitol, polyethylene glycol (PEG), sucrose, glucose	Maintain moisture on leaf surfaces to prolong metabolite absorption	Useful under dry or arid conditions
Stabilizers / Protectants	Ascorbic acid, tocopherols, BHT, titanium dioxide, zinc oxide, phosphate/citrate buffers	Protect metabolites from oxidative, thermal, and UV degradation; maintain pH stability	Critical for maintaining bioactivity during storage and field application
Encapsulation Materials	Alginate, chitosan, starch, cellulose derivatives, lecithin, PLGA	Controlled release, protection against environmental stress, enhanced stability	Can modulate release kinetics and reduce non-target effects
Drying Aids	Sucrose, trehalose, maltodextrin, sorbitol, mannitol, talc, kaolin	Facilitate conversion to powders, improve shelf life, prevent metabolite degradation	Important for spray-dried or freeze-dried formulations
Nanocarrier Systems	Polymeric nanoparticles (PLGA, chitosan), nanoemulsions, silica nanoparticles, metal oxides	Enhance solubility, stability, targeted delivery, and foliar or root uptake	Experimental stage; potential for controlled release

*Key considerations for formulant selection:*

- *Fungal metabolite type:* Lipophilic vs. hydrophilic metabolites require different carriers or surfactants.
- *Target weed species:* Leaf morphology, waxiness, and cuticle thickness influence formulation choice.
- *Application method:* Foliar sprays, soil incorporation, or seed treatments determine adhesiveness, solubility, and release kinetics.
- *Environmental conditions:* Temperature, UV exposure, rainfall, and humidity dictate the need for stabilizers, UV protectants, and encapsulation.

*5.2 Advanced Formulation Technologies*

Recent advances in formulation science have enabled improved protection, delivery, and persistence of fungal metabolites.

*Microencapsulation* entrains metabolites within biodegradable matrices such as alginate, chitosan, starch, or synthetic polymers, enhancing stability against UV radiation, temperature stress, and leaching while enabling controlled release (Vurro et al., 2009; Duke & Dayan, 2015). Encapsulation can prolong herbicidal activity, reduce application rates, and mitigate non-target effects.

*Nanoparticle-based delivery systems*, including polymeric nanoparticles and nanoemulsions, further enhance solubility, foliar penetration, and uptake efficiency (Dayan et al., 2009). These systems offer potential for targeted or stimuli-responsive release and improved consistency, although most applications remain at an experimental stage.



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*Adjuvants and surfactants* play a central role in improving foliar performance by enhancing wetting, spreading, adhesion, and rainfastness. Humectants prolong metabolite absorption, while UV protectants and antioxidants reduce photodegradation (Hallett, 2005; Dayan et al., 2009). Careful selection is essential to avoid antagonistic interactions or unintended phytotoxicity.

### 5.3 Integration with Application Methods

Formulation strategies must align with application mode and target weed biology. Foliar applications require rapid uptake and rainfastness, whereas soil-applied products benefit from controlled release and enhanced environmental stability. Compatibility with spray equipment, tank mixes, and precision application technologies is essential for grower adoption (Bailey et al., 2010; Duke et al., 2019).

Overall, advances in formulation science are enabling fungal CFCF bioherbicides to transition from experimental systems to practical components of integrated weed management. Continued optimization focused on stability, cost reduction, and scalability will be critical for successful commercialization and widespread adoption.

## VI. OPTIMIZATION OF BIOACTIVITY AND FIELD PERFORMANCE OF CFCF-BASED BIOHERBICIDES

The metabolite-driven nature of fungal cell-free culture filtrate (CFCF) bioherbicides enables optimization strategies fundamentally different from those used for live microbial products. Because efficacy is independent of fungal viability, CFCF bioherbicides can be standardized using metabolite concentration, enzymatic activity, or bioassay-based dose–response metrics rather than colony-forming units. This CFU-independent framework improves production consistency, formulation control, and quality assurance.

Optimization begins at the fermentation stage, where nutrient composition, carbon and nitrogen sources, pH, aeration, temperature, and incubation time strongly influence metabolite yield and composition.

Advances in fermentation engineering, coupled with metabolomic and bioassay-guided profiling, enable selective enhancement of phytotoxic metabolites while minimizing inactive or undesirable by-products (Gross & Loper, 2009; Hagiwara et al., 2016).

Downstream processing and formulation are equally critical for maintaining metabolite stability and bioavailability. Many fungal metabolites are chemically labile, poorly soluble, and sensitive to UV radiation, temperature, and moisture. Conventional and advanced formulations—including emulsifiable concentrates, wettable powders, microencapsulation, controlled-release matrices, and nanocarrier-based systems—have therefore been developed to protect metabolites, prolong field persistence, and enhance plant uptake (Vurro et al., 2009; Kah et al., 2013; Yu et al., 2018).

Nanotechnology-based delivery systems are particularly promising, as nanocarriers can shield metabolites from environmental degradation, enhance cuticular penetration, and enable controlled or targeted release. These attributes improve efficacy at lower application rates and reduce off-target effects, aligning well with precision and site-specific weed management strategies (DeRosa et al., 2010; Zhao et al., 2017; Singh et al., 2019a).

Field performance is further influenced by application parameters such as spray volume, droplet size, adjuvant selection, and timing relative to weed growth stage. Because metabolite uptake is often contact-dependent, optimizing leaf wetting, retention, and penetration is essential for consistent weed suppression. Integration with precision application technologies can further enhance efficacy while reducing non-target exposure.

Collectively, optimization across fermentation, formulation, delivery, and application capitalizes on the inherent advantages of CFCF-based bioherbicides, enabling reproducible efficacy, extended shelf life, and scalable production. These advances are central to overcoming commercialization barriers and integrating fungal CFCF bioherbicides into modern integrated weed management systems (Table 3).

**Table 3.**  
**Comparative features of conventional mycoherbicides and fungal cell-free culture filtrate (CFCF)-based bioherbicides**

<b>Attribute</b>	<b>Conventional Mycoherbicides</b>	<b>CFCF-Based Bioherbicides</b>
Biological nature	Living fungal propagules (spores, mycelia, sclerotia)	Cell-free preparations containing extracellular fungal metabolites
Requirement for fungal viability	Essential for efficacy	Not required
Standardization parameter	Colony-forming units (CFUs), spore count, or inoculum density	Metabolite concentration, enzyme activity units, or bioassay-based dose–response
Mode of action	Infection-dependent: germination, host penetration, colonization, and growth	Metabolite-mediated: direct disruption of physiological and biochemical processes
Speed of symptom expression	Generally slow (days to weeks)	Rapid (hours to days), depending on metabolite composition
Sensitivity to environmental factors	Highly sensitive to temperature, humidity, UV radiation, and microbial competition	Relatively tolerant to environmental variability
Field performance consistency	Often variable and unpredictable	More consistent and reproducible
Formulation flexibility	Limited due to viability constraints	High; compatible with surfactants, stabilizers, encapsulation, and drying
Shelf life	Short to moderate; viability declines during storage	Extended; dependent on metabolite protection and formulation
Storage requirements	Often requires cold or controlled conditions	Can be stored as liquid or dry formulations under wider conditions
Ease of handling and application	Requires careful handling to maintain viability	Easier handling; no viability maintenance required
Regulatory considerations	Release of live microorganisms; higher biosafety scrutiny	No release of viable organisms; generally reduced regulatory complexity
Biosafety and ecological risk	Potential for non-target colonization or persistence	Lower ecological risk; reduced concerns regarding environmental establishment
Production scalability	Limited by propagule quality and consistency	Highly scalable via industrial fermentation and downstream processing
Batch-to-batch variability	Often high due to biological variability	Lower with metabolite-based standardization
Compatibility with precision agriculture	Limited	High; suitable for targeted and controlled applications
Commercialization potential	Historically limited market success	Strong potential as next-generation bioherbicides
Representative examples	<i>Colletotrichum</i> , <i>Alternaria</i> , <i>Fusarium</i> -based products	Phytotoxin- and enzyme-rich fungal culture filtrates

### 6.1 Strategies for Enhancing Bioherbicidal Potency

*Optimization of culture media* is central to maximizing the phytotoxic potential of fungal cell-free culture filtrates (CFCFs). Carbon and nitrogen source type and concentration strongly influence both metabolite yield and composition. While readily assimilable sugars promote rapid biomass accumulation, nutrient limitation or complex carbon sources often favor secondary metabolite biosynthesis. Similarly, nitrogen form and carbon-to-nitrogen ratios regulate metabolic fluxes and phytotoxic profiles.

Targeted adjustment of these parameters, together with micronutrient supplementation, can substantially enhance bioherbicidal activity against specific weeds (Abbas et al., 1995; Andolfi et al., 2014).

The **use of elicitors and controlled stress** represents a scalable, non-genetic approach to further enhance metabolite production. Chemical elicitors, metal ions, plant-derived extracts, and signaling molecules can activate otherwise silent biosynthetic pathways, while controlled abiotic stresses (e.g., osmotic, oxidative, or pH stress) stimulate secondary metabolism (Cimmino et al., 2015). These strategies are cost-effective, industrially feasible, and compatible with regulatory requirements.

### 6.2 Stability of Bioactive Metabolites

Maintaining metabolite stability is essential for consistent field performance. **pH stabilization** through buffering systems prevents chemical degradation or precipitation, ensuring uniform dispersion and sustained bioactivity during storage and application (Hallett, 2005).

Protection against **oxidative and photodegradation** is equally critical, as UV radiation and reactive oxygen species rapidly inactivate many fungal metabolites. Antioxidants, UV protectants, and encapsulation technologies effectively reduce degradation and extend functional persistence following application (Dayan et al., 2009; Vurro et al., 2009).

### 6.3 Storage Stability and Shelf-Life Enhancement

Commercial viability requires extended shelf life under practical storage conditions. Although liquid formulations may require refrigeration, conversion of CFCFs into dry forms via spray-drying or freeze-drying significantly improves stability by reducing moisture content. The inclusion of protective polymers or sugars further preserves metabolite integrity during processing and storage (Bailey et al., 2010). Appropriate packaging that limits exposure to light, oxygen, and humidity is also essential for maintaining product quality.

### 6.4 Integrated Optimization for Field Consistency

Reliable field performance of CFCF-based bioherbicides depends on an integrated optimization framework encompassing fermentation control, elicitor use, metabolite stabilization, and formulation design. The CFU-independent nature of CFCFs enables flexible formulation formats and application methods without constraints imposed by microbial viability. This holistic approach minimizes batch-to-batch variability, improves performance across diverse agro-climatic conditions, and enhances translation from laboratory efficacy to field reliability (Duke et al., 2019).

Overall, metabolite-centric optimization and stabilization strategies position fungal CFCF-based bioherbicides as reliable, environmentally safe, and economically viable components of modern integrated weed management systems.

## VII. DELIVERY AND APPLICATION TECHNOLOGIES FOR FUNGAL CELL-FREE BROTH BIOHERBICIDES

Delivery and application strategies critically determine the field efficacy, selectivity, and economic viability of fungal cell-free broth (CFCF) bioherbicides.

Unlike synthetic herbicides, fungal metabolites are often environmentally sensitive and lack systemic mobility, necessitating carefully optimized application methods tailored to metabolite properties, formulation type, target weed biology, and cropping systems (Hallett, 2005; Duke & Dayan, 2015).

### 7.1 Foliar Spray Applications

Foliar application is the most common delivery method for post-emergence use of CFCF bioherbicides. Applied metabolites must overcome physical barriers such as the cuticle and epicuticular waxes to exert phytotoxic effects. Efficacy is strongly influenced by spray coverage, droplet retention, and absorption efficiency.

Adjuvants—including surfactants, oils, and humectants—enhance wetting, spreading, and penetration, particularly on waxy or pubescent weed surfaces (Hallett, 2005). Environmental conditions further modulate performance: high humidity favors absorption, whereas intense sunlight accelerates photodegradation. Accordingly, precise timing of foliar applications is essential to maximize efficacy and minimize metabolite loss.

### 7.2 Soil Application for Pre-Emergence Weed Control

Soil application offers a viable option for pre-emergence weed management by targeting germinating seeds and young seedlings. Bioactive metabolites interfere with germination, root development, and early metabolic processes.

Successful soil delivery requires formulations capable of maintaining stability under variable pH, moisture, and microbial activity. Controlled-release and encapsulated formulations can regulate metabolite availability and reduce leaching or rapid degradation (Vurro et al., 2009). Soil texture, organic matter, and microbial composition strongly influence metabolite persistence and bioavailability, highlighting the need for site-specific optimization of application rates and timing.

### 7.3 Integration with Precision Application Technologies

Integration with precision agriculture technologies offers major advantages for CFCF bioherbicide deployment. Sensor-guided sprayers, GPS-based variable-rate systems, and machine vision platforms enable site-specific application, reducing off-target exposure and total input requirements (Duke et al., 2019).

Real-time weed detection and targeted spraying are particularly beneficial for bioherbicides, which often require precise placement and timing.

Precision systems also support optimization of droplet size, spray pressure, and nozzle selection, improving coverage uniformity and consistency. As these technologies become more accessible, their integration is expected to enhance field reliability and adoption of CFCF bioherbicides within integrated weed management systems.

#### *7.4 Challenges and Future Directions*

Despite significant potential, application of CFCF bioherbicides remains constrained by environmental sensitivity, variable field performance, and logistical challenges. Future research should focus on formulation–application co-design, development of precision-compatible products, and decision-support tools tailored to diverse cropping systems and climates. Advances in sensor technology, formulation science, and digital agriculture will be pivotal in unlocking the full potential of fungal CFCF bioherbicides as sustainable alternatives to synthetic herbicides.

### VIII. MECHANISMS OF PHYTOTOXICITY OF FUNGAL CELL-FREE BROTH BIOHERBICIDES

Fungal cell-free broth (CFCF) bioherbicides suppress weeds through diverse and often multi-site phytotoxic mechanisms that reflect the chemical complexity of fungal secondary metabolites. Unlike many synthetic herbicides with single biochemical targets, fungal metabolites simultaneously affect multiple physiological and cellular processes, enhancing efficacy and reducing the risk of resistance development (Duke & Dayan, 2015; Vurro et al., 2009).

#### *8.1 Disruption of Cell Membrane Integrity*

Membrane disruption is a common mechanism of fungal metabolite phytotoxicity. Amphiphilic peptides, terpenoids, and related compounds interact with lipid bilayers, increasing membrane permeability and causing ion and metabolite leakage (Kim et al., 2011). Peptaibols from *Trichoderma* spp. form transmembrane pores, collapsing membrane potential and rapidly inducing plasmolysis, electrolyte leakage, and cell death. This mechanism typically produces fast-acting necrotic symptoms following foliar application.

#### *8.2 Inhibition of Photosynthesis*

Many fungal polyketides and phenolic metabolites inhibit photosynthesis by disrupting photosystem II or electron transport within chloroplasts, leading to reduced energy production and reactive oxygen species (ROS) accumulation (Dayan et al., 2009).

Damage to thylakoid membranes and inhibition of chlorophyll biosynthesis result in chlorosis, bleaching, and eventual necrosis, particularly in fast-growing annual weeds with high photosynthetic demand.

#### *8.3 Induction of Oxidative Stress*

Oxidative stress is a central component of fungal metabolite phytotoxicity. By disrupting electron transport or inhibiting antioxidant defenses, fungal metabolites elevate ROS levels beyond cellular detoxification capacity (Cimmino et al., 2015). Excess ROS cause lipid peroxidation, protein oxidation, and DNA damage, leading to growth inhibition and programmed cell death. Oxidative stress frequently acts synergistically with membrane disruption and photosynthetic inhibition.

#### *8.4 Interference with Plant Hormone Signaling*

Certain fungal metabolites disrupt plant hormone biosynthesis or signaling by mimicking or antagonizing endogenous regulators such as auxins, gibberellins, and abscisic acid (Duke & Dayan, 2015). Hormonal imbalance results in abnormal growth, epinasty, root malformation, and delayed germination, making this mechanism particularly relevant for pre-emergence weed control and species-selective activity.

#### *8.5 Multi-Target and Synergistic Effects*

A defining characteristic of CFCF bioherbicides is the simultaneous presence of multiple bioactive metabolites targeting different cellular processes. This multi-target action enhances herbicidal robustness and lowers the probability of resistance evolution via single-gene mutations (Vurro et al., 2009). Synergistic interactions among metabolites can further increase efficacy at reduced application rates, though chemical complexity complicates mechanistic elucidation and standardization.

#### *8.6 Implications for Resistance Management and Sustainability*

The diverse, multi-site mechanisms of fungal CFCF bioherbicides offer strong advantages for resistance management and long-term sustainability. By targeting fundamental physiological processes through multiple pathways, these bioherbicides can complement synthetic herbicides and help mitigate the spread of resistant weed populations (Duke et al., 2019). Continued integration of metabolomics, plant physiology, and molecular biology will be essential for optimizing efficacy and supporting the broader adoption of CFCF bioherbicides in integrated weed management systems.

**IX. EFFICACY OF FUNGAL CELL-FREE BROTH  
BIOHERBICIDES UNDER GREENHOUSE AND FIELD  
CONDITIONS**

Evaluation of fungal cell-free broth (CFCF) bioherbicides typically begins under controlled greenhouse conditions, where environmental variability is minimized and intrinsic bioactivity can be accurately assessed. Numerous studies report strong phytotoxic effects of fungal CFCFs against diverse weed species in greenhouse trials; however, translating this efficacy to field conditions remains challenging due to environmental complexity and biological variability (Hallett, 2005; Vurro et al., 2009).

*9.1 Greenhouse Evaluation and Proof-of-Concept*

Greenhouse studies serve as essential platforms for screening fungal strains, optimizing fermentation and formulation parameters, and establishing dose–response relationships. Under controlled temperature, humidity, and light conditions, CFCF bioherbicides frequently induce rapid inhibition of seed germination, suppression of seedling growth, and foliar necrosis in target weeds (Andolfi et al., 2014; Cimmino et al., 2015). Reduced ultraviolet exposure and high humidity favor metabolite stability and uptake, often resulting in weed control comparable to synthetic herbicides and supporting progression to field testing.

*9.2 Limitations under Field Conditions*

Field performance of CFCF bioherbicides is often reduced by environmental and biological constraints. Ultraviolet radiation, high temperatures, and rainfall can rapidly degrade or remove bioactive metabolites, particularly in unformulated products (Dayan et al., 2009). Limited rainfastness following foliar application and interspecific variability in leaf morphology, cuticle composition, and metabolic detoxification further contribute to inconsistent weed control across field environments.

*9.3 Strategies to Enhance Field Efficacy*

Bridging the greenhouse–field efficacy gap requires targeted formulation and application strategies. Incorporation of UV protectants, antioxidants, adjuvants, and encapsulation technologies can improve metabolite stability, rainfastness, and persistence (Vurro et al., 2009). Optimizing application timing under high humidity and low irradiance, combined with precision delivery systems, enhances absorption, selectivity, and overall field performance while reducing application rates and non-target effects (Duke et al., 2019).

*9.4 Practical Implications*

Although fungal CFCF bioherbicides may exhibit greater performance variability than synthetic herbicides, formulation refinement and strategic application can substantially improve reliability. Their environmental safety, novel modes of action, and compatibility with integrated weed management make them particularly valuable in organic systems, high-value crops, and resistance-prone agroecosystems. Continued field-scale validation and formulation innovation remain critical for their successful adoption in sustainable agriculture.

**X. SAFETY AND REGULATORY CONSIDERATIONS FOR  
FUNGAL CELL-FREE BROTH BIOHERBICIDES**

Successful commercialization of fungal cell-free broth (CFCF) bioherbicides depends on thorough evaluation of safety, environmental behavior, and regulatory compliance. Compared with synthetic herbicides, CFCF bioherbicides generally present lower ecological risk due to biodegradability and target specificity; however, rigorous risk assessment remains essential to ensure protection of non-target organisms, ecosystems, and human health (Vurro et al., 2009; Duke and Dayan, 2015).

*10.1 Non-Target Organism Safety*

Regulatory approval requires assessment of potential toxicity to non-target organisms, including beneficial insects, soil microbiota, aquatic species, birds, and mammals. Although CFCFs lack viable fungal propagules, some secondary metabolites may exhibit cytotoxic or antimicrobial activity (Andolfi et al., 2014). Standardized acute and chronic bioassays are therefore used to establish no-observed-effect concentrations and define safe application rates. Overall, most CFCF bioherbicides demonstrate favorable non-target toxicity profiles relative to chemical herbicides, supporting their compatibility with integrated weed management systems.

*10.2 Environmental Fate and Persistence*

Understanding the environmental fate of fungal metabolites is critical for both safety and efficacy. Key parameters include degradation in soil and water, adsorption to soil particles, leaching potential, and susceptibility to photodegradation and microbial metabolism (Dayan et al., 2009; Cimmino et al., 2015). Fungal metabolites typically exhibit short environmental half-lives and low bioaccumulation risk. However, advanced formulations such as encapsulated or controlled-release systems may alter persistence and require targeted environmental risk evaluation.

### *10.3 Regulatory Frameworks across Major Regions*

Regulatory pathways for CFCF bioherbicides vary globally. In the United States, fungal metabolite-based products are regulated by the EPA under the Biopesticides and Pollution Prevention Division, often benefiting from streamlined registration compared to conventional herbicides. The absence of live microorganisms reduces concerns related to environmental persistence and non-target colonization.

In the European Union, regulation is more complex, with CFCF products evaluated under either the Plant Protection Products Regulation (EC No. 1107/2009) or the Biocidal Products Regulation. Regulatory ambiguity surrounding complex metabolite mixtures and multi-component actives often results in higher data requirements and longer approval timelines.

In India, bioherbicides are regulated by the Central Insecticides Board and Registration Committee under the Insecticides Act, 1968. While policy support for biopesticides is increasing, specific guidelines for cell-free, metabolite-based products are still evolving. Clear alignment with emerging biopesticide frameworks could significantly accelerate registration of CFCF bioherbicides with demonstrated safety and sustainability benefits.

### *10.4 Risk Mitigation and Best Practices*

Safe deployment of CFCF bioherbicides relies on best management practices, including optimized application timing, targeted delivery systems, avoidance of conditions favoring runoff or leaching, and clear labeling for handling and disposal. These measures, combined with inherently low toxicity and biodegradability, reinforce the environmental advantages of CFCF bioherbicides.

Overall, fungal CFCF bioherbicides exhibit strong safety profiles and favorable regulatory potential. Early integration of non-target toxicity testing, environmental fate studies, and regulatory strategy into product development will be essential for achieving both commercial success and ecological sustainability.

## **XI. COMMERCIALIZATION STATUS OF FUNGAL CELL-FREE BROTH BIOHERBICIDES**

Fungal cell-free broth (CFCF) bioherbicides offer eco-friendly weed control, yet their commercialization remains limited. A few products derived from *Colletotrichum* or *Phoma* metabolites have reached pre-commercial or pilot-scale deployment, demonstrating efficacy against annual grasses and broadleaf weeds (Charudattan, 2005; Andolfi et al., 2014).

However, CFCF bioherbicides remain less prevalent than live microbial or chemical herbicides due to technical, economic, and social constraints.

### *11.1 Scale-Up and Production Challenges*

Large-scale metabolite production is sensitive to fermentation conditions—nutrients, pH, temperature, and aeration all affect yield and bioactivity (Bailey et al., 2010; Cimmino et al., 2015). Batch-to-batch variability in metabolite profiles can compromise efficacy, while production costs remain higher than for conventional herbicides. Advances in metabolic engineering, elicitor use, and process automation are expected to improve yields and economic feasibility.

### *11.2 Quality Control*

Consistency in metabolite composition is essential for regulatory compliance and reliable field performance. CFCFs contain complex metabolite mixtures whose concentrations vary with strain, growth stage, and culture conditions. Analytical methods such as HPLC, mass spectrometry, and metabolomics, combined with bioassay-guided potency testing, are critical for batch standardization and quality assurance.

### *11.3 Market Awareness and Farmer Adoption*

Farmer adoption depends on awareness, confidence in efficacy, ease of application, and cost competitiveness (Hallett, 2005). Education, extension programs, and demonstration of environmental benefits and crop safety are key to increasing acceptance. Integration within existing weed management systems enhances practical relevance.

### *11.4 Future Prospects*

CFCF bioherbicides hold strong potential amid rising herbicide resistance, regulatory restrictions, and demand for sustainable agriculture. Opportunities include development of stable formulations, integration with precision application technologies, exploitation of novel fungal metabolites, and expansion into organic or low-input cropping systems. Collaborative efforts across research, industry, and regulatory agencies are essential to overcome technical and market barriers, enabling wider adoption of these next-generation bioherbicides.

In summary, commercialization is currently constrained by production scale, metabolite consistency, and market awareness. Advances in fermentation, formulation, and farmer education will be pivotal in translating CFCF bioherbicides into practical tools for integrated weed management.

**XII. CHALLENGES AND RESEARCH GAPS IN FUNGAL CELL-FREE BROTH BIOHERBICIDE DEVELOPMENT**

Fungal CFCF bioherbicides show promise as sustainable alternatives to synthetic herbicides, but adoption is limited by technical, biological, and operational constraints. Addressing these gaps is critical for consistent efficacy, regulatory compliance, and integration into modern weed management.

*12.1 Standardization of Metabolite Profiles*

Batch-to-batch variability in fungal metabolite composition can lead to inconsistent bioactivity (Andolfi et al., 2014; Cimmino et al., 2015). Research priorities include:

- High-throughput metabolite profiling (HPLC, LC-MS, NMR).
- Selection or engineering of stable, high-yield fungal strains.
- Optimization of fermentation parameters for reproducible metabolite production.

Robust quality control and standardization are essential for regulatory approval and commercial reliability.

*12.2 Formulation Stability under Field Conditions*

Fungal metabolites are sensitive to UV, temperature, and microbial degradation (Dayan et al., 2009; Vurro et al., 2009). Key strategies include:

- Advanced formulations: microencapsulation, nanocarriers, controlled-release matrices.
- Adjuvants and surfactants for improved leaf adhesion, rainfastness, and metabolite uptake.
- Shelf-life extension via drying methods, stabilizers, and optimized packaging.

Enhanced stability ensures predictable field performance and farmer confidence.

*12.3 Expanding Target Weed Spectrum*

Most CFCF bioherbicides are highly effective against specific weeds, particularly fast-growing annuals, but less so against perennials or resistant species (Duke and Dayan, 2015). Research directions include:

- Discovery of novel fungal strains and metabolites with broader activity.
- Understanding weed susceptibility at physiological and molecular levels.
- Synergistic metabolite blends or combination formulations to extend spectrum without reducing selectivity.

*12.4 Integration into Integrated Weed Management (IWM)*

Effective use requires incorporation into IWM systems alongside chemical, mechanical, and cultural controls (Hallett, 2005; Bailey et al., 2010). Research needs include:

- Optimized timing and sequencing protocols.
- Compatibility studies with other herbicides and control methods.
- Assessment of long-term impacts on weed populations, resistance management, and soil health.

*12.5 Additional Research Priorities*

- Mechanistic studies: Molecular targets and signaling pathways for selective bioherbicide design.
- Regulatory science: Harmonization of metabolite-based product guidelines and registration pathways.
- Economic feasibility: Cost-benefit analyses and scalable industrial production.
- Farmer adoption: Socio-economic studies to promote awareness and uptake.

Addressing these challenges through formulation innovation, field validation, and education is critical to realize the full potential of CFCF bioherbicides as effective, eco-friendly, and commercially viable weed management tools.

**XIII. FUTURE PROSPECTS OF FUNGAL CELL-FREE BROTH BIOHERBICIDES**

Fungal CFCF bioherbicides are poised for growth due to rising demand for sustainable weed control, regulatory restrictions on synthetic herbicides, and advances in microbial biotechnology. While challenges in metabolite standardization, formulation stability, and field efficacy persist, emerging technologies offer promising solutions.

*13.1 Genomic and Metabolomic Discovery*

Genomics, transcriptomics, and metabolomics enable targeted identification of biosynthetic gene clusters and novel bioactive metabolites (Evidente et al., 2013; Andolfi et al., 2014). High-resolution mass spectrometry and NMR profiling facilitate comprehensive metabolite characterization, optimized fermentation, and standardized, high-efficacy formulations, reducing trial-and-error development.

### 13.2 Precision Formulation

Advanced delivery strategies—microencapsulation, nanocarriers, and controlled-release matrices—protect metabolites from degradation, enhance uptake, and extend field persistence (Dayan et al., 2009; Vurro et al., 2009). Tailoring formulations to weed ecology and environmental conditions improves selectivity, efficacy, and adoption, particularly when combined with smart spraying and variable-rate application technologies.

### 13.3 Synergistic Integration with Beneficial Microbes

CFCF bioherbicides can be combined with plant growth-promoting rhizobacteria, mycorrhizal fungi, or microbial consortia to enhance weed suppression and support crop health (Kim et al., 2011; Duke et al., 2019). Such synergistic approaches improve metabolite persistence, root absorption, and overall soil ecosystem services.

### 13.4 Integration with IWM and Digital Agriculture

Integration into digital agriculture platforms allows real-time weed detection and targeted application, optimizing bioherbicide delivery and minimizing environmental losses. Combined with cultural and mechanical controls in integrated weed management, CFCF bioherbicides contribute to holistic, sustainable weed suppression strategies.

### 13.5 Market Expansion and Regulatory Facilitation

Favorable regulatory trends, combined with standardization, field validation, and farmer education, can accelerate adoption in organic, low-input, and specialty cropping systems. Collaboration among researchers, industry, and policymakers can streamline registration and commercialization, enhancing market confidence in these eco-friendly alternatives.

Fungal CFCF bioherbicides, leveraging metabolite discovery, precision formulation, synergistic biocontrol, and digital agriculture, offer effective, environmentally safe, and commercially viable solutions for sustainable weed management.

## XIV. CONCLUSION AND FUTURE OUTLOOK

Fungal cell-free culture filtrate (CFCF) bioherbicides represent a next-generation approach to sustainable weed management, relying on extracellular metabolites rather than living fungi. This metabolite-driven strategy enables precise standardization, formulation flexibility, and compatibility with diverse delivery systems, overcoming limitations of conventional mycoherbicides such as short shelf life, environmental sensitivity, and inconsistent field performance.

Key advantages include multi-target phytotoxicity, reduced resistance risk, environmental safety, and seamless integration into integrated weed management (IWM). The absence of living organisms minimizes biosafety concerns and simplifies regulatory approval, while chemical diversity allows selective and synergistic weed control, including herbicide-resistant populations.

Challenges remain in batch-to-batch metabolite variability, environmental degradation, large-scale production, and regulatory ambiguity for complex metabolite mixtures. Addressing these requires advances in fermentation optimization, metabolomic profiling, formulation engineering, and harmonized regulatory frameworks.

Looking forward, CFCF bioherbicides are well suited for precision and climate-smart agriculture. Integration with sensor-guided sprayers, variable-rate dosing, and digital decision-support systems can enhance targeting, reduce application rates, and limit non-target exposure. Their compatibility with organic and reduced-input systems further strengthens their role in sustainable and resilient cropping systems.

Overall, fungal CFCF bioherbicides offer a scalable, eco-friendly, and scientifically robust platform for modern weed control, with continued interdisciplinary research essential for translating their potential into commercially viable solutions.

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