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CRISPR-BASED MOLECULAR DETECTION OF FUNGAL PATHOGENS AFFECTING COMMERCIAL MUSHROOM CULTIVATION

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Abstract

Commercial mushroom cultivation faces persistent threats from fungal pathogens including Trichoderma species (green mold), Lecanicillium fungicola (dry bubble), Mycogone perniciosa (wet bubble), and Cladobotryum species (cobweb disease), causing yield losses ranging from 20% to complete crop destruction. Current disease management strategies rely heavily on chemical fungicides and visual symptom recognition, yet both approaches are increasingly inadequate due to fungicide resistance development, regulatory restrictions, and the delayed manifestation of disease symptoms that appear only after irreversible yield loss and pathogen dissemination have occurred. This review critically examines the emerging application of CRISPR-Cas-based molecular diagnostics for rapid, on-site detection of fungal pathogens in commercial mushroom cultivation systems. The fundamental mechanisms of CRISPR-Cas diagnostic platforms, particularly Cas12a and Cas13a effectors that exhibit target-activated collateral cleavage activity, are explored alongside their integration with isothermal amplification technologies including Recombinase Polymerase Amplification (RPA) and Loop-Mediated Isothermal Amplification (LAMP). Key considerations for diagnostic assay development are addressed, including target genomic locus selection (ITS, TEF1-α, RPB2, β-tubulin), crRNA engineering parameters, off-target mitigation strategies, and the challenges posed by substrate-derived inhibitors such as humic acids and polysaccharides present in mushroom compost and casing materials. Comparative analysis reveals that CRISPR-Cas coupled isothermal biosensors achieve superior analytical specificity through dual-recognition architecture (isothermal primers plus crRNA verification), ultra-high sensitivity (1 fg/μL to 100 aM), and rapid turnaround times (25–50 minutes) while demonstrating tolerance to crude sample lysates and field-deployable point-of-care compatibility. This integrated molecular detection approach offers a transformative solution for early pathogen surveillance, facilitating timely biosecurity interventions and reducing economic losses in global mushroom production systems.

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1. Introduction

Commercial mushroom cultivation represents an intensive, highly controlled agricultural sector producing high-value edible and medicinal basidiomycetes, including *Agaricus bisporus* (button mushroom), *Pleurotus ostreatus* (oyster mushroom), *Lentinula edodes* (shiitake), and *Ganoderma* species (Grimm & Wösten, 2018). The artificial microenvironments required for optimal mushroom growth characterized by high relative humidity, elevated temperature, and nutrient-dense organic substrates simultaneously create ideal conditions for the proliferation of specialized fungal mycoparasites and aggressive substrate competitors. Fungal diseases such as green mold, dry bubble, wet bubble, and cobweb disease pose persistent threats to global mushroom production, causing yield losses ranging from minor economic reductions to total crop destruction (Grogan, 2008).

Historically, disease management in mushroom farms has relied on strict hygiene, environmental control, and the application of chemical fungicides. However, the management of fungal pathogens in mushroom crops presents a unique biological challenge: because both the host crop and the pathogen belong to the Kingdom Fungi, developing selective chemical agents that suppress the pathogen without exhibiting host phytotoxicity is intrinsically difficult (Moore et al., 2011). Furthermore, the continuous use of limited fungicide classes has driven the emergence of resistant pathogen strains, while regulatory frameworks, such as those in the European Union, have progressively restricted or withdrawn approval for key chemical active ingredients like prochloraz (McKay et al., 1999).

A central vulnerability in commercial mushroom disease management is the delayed manifestation of visual symptoms. Symptoms such as stipe deformation, cap spotting, or undifferentiated tissue masses typically appear late in the infection cycle often during primordial formation or post-casing by which time microscopic conidia have already proliferated and dispersed throughout the cultivation facility via air currents, water splash, personnel, and insect vectors. Consequently, visual diagnosis occurs after irreversible yield loss has occurred and secondary spread is established (Pardo-Giménez et al., 2022).

2. Major Fungal Pathogens and Economic Impacts in Mushroom Cultivation

Commercial mushroom farms face major threats from four principal fungal pathogen groups: Trichoderma species (green mold), Lecanicillium fungicola (dry bubble), Mycogone perniciosa (wet bubble), and Cladobotryum species (cobweb disease). Each pathogen exhibits distinct ecological adaptations, pathogenic mechanisms, and symptom profiles (Romaine et al., 2005).

2.1 Green Mold Disease (Trichoderma species)

Green mold is widely regarded as the most destructive fungal disease affecting commercial mushroom production worldwide. Epidemics caused by aggressive strains of Trichoderma severely impacted the mushroom industries of Europe and North America in the 1980s and 1990s and continue to cause substantial yield reductions globally. The primary causative agents include Trichoderma aggressivum (specifically T. aggressivum f. aggressivum and T. aggressivum f. europaeum) in A. bisporus cultivation, as well as T. pleurotum and T. pleuroticola in Pleurotus ostreatus production, and T. harzianum, T. viride, and T. virens across various cultivated edible fungi (Wasser, 2014).

Trichoderma species are fast-growing saprophytes and aggressive mycoparasites. Upon colonizing Phase II or Phase III compost or casing soil, Trichoderma mycelium competes aggressively with host mushroom hyphae for space and nutrients, secreting cell-wall-degrading enzymes such as chitinases and glucanases alongside antifungal secondary metabolites that inhibit host growth and induce severe oxidative stress (Royse et al., 2017). Symptoms manifest initially as dense, white, uncolonized patches of mycelium within the substrate, rapidly followed by massive green sporulation. In severe outbreaks, yield loss reaches 60% to 100%, rendering substrate bags or beds entirely unproductive. A diagnostic complexity arises from the dual ecological role of Trichoderma: while certain species act as devastating agricultural pests in mushroom

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houses, other non-pathogenic Trichoderma strains are widely applied in arable crop agriculture as biological control agents and biostimulants. Molecular detection systems must therefore achieve strain- or species-level resolution to differentiate aggressive mushroom pathogens from beneficial background isolates (Chang & Miles, 2004).

2.2 Dry Bubble Disease (Lecanicillium fungicola)

Dry bubble disease, caused by the ascomycete Lecanicillium fungicola (formerly Verticillium fungicola), represents one of the most widespread fungal diseases in white button mushroom (Agaricus bisporus) production. Two principal varieties are recognized: L. fungicola var. fungicola and L. fungicola var. aleophilum. The pathogen accounts for estimated global crop losses of 2% to 20%, translating to hundreds of millions of euros in annual damages within the European sector alone (Noble et al., 2009).

Symptom development in host mushrooms is strictly dependent on the developmental stage at which infection occurs. Primary infection occurring at or prior to the initiation of mushroom pinheads prevents normal tissue differentiation, resulting in the formation of amorphous, deformed masses of tissue termed "dry bubbles". Infection occurring at later developmental stages leads to stipe blowout, characterized by stipe splitting and curling, or localized cinnamon-brown necrotic lesions known as cap spotting (Fletcher & Gaze, 2008). Lecanicillium fungicola produces abundant conidia held in sticky mucilaginous droplets, facilitating rapid mechanical dispersal across growing rooms via watering operations, dust, farm workers, and dipteran vectors such as phorid and sciarid flies. Peat moss used in casing material serves as a primary environmental reservoir for L. fungicola inoculum (Carrasco & Preston, 2020).

2.3 Wet Bubble Disease (Mycogone perniciosa)

Wet bubble disease is caused by the mycoparasite Mycogone perniciosa (teleomorph Hypomyces perniciosus). The disease affects A. bisporus production globally, causing severe crop failure during primordial formation and early harvesting flushes. Infection transforms developing primordia into large, misshapen, sclerodermoid tissue masses. As the disease progresses, these undifferentiated tissue masses exude amber-colored droplets of liquid containing extracellular enzymes and fungal spores, ultimately undergoing soft, foul-smelling wet rot due to secondary bacterial colonization (Savoie, 2016).

Mycogone perniciosa produces two distinct spore types: short-lived, thin-walled bicellular conidia responsible for rapid secondary spread during crop watering, and thick-walled, highly resilient chlamydospores capable of persisting in soil, casing debris, and farm structures for extended periods. Contaminated casing soil, untreated water supplies, and airborne dust are primary infection vectors. Because A. bisporus commercial strains lack resistance to M. perniciosa, unmitigated outbreaks often lead to complete yield loss in affected growing rooms (Shamtsyan et al., 2010).

2.4 Cobweb Disease (Cladobotryum species)

Cobweb disease, caused by Cladobotryum species (syn. Dactylium spp., teleomorph Hypomyces spp.), including C. mycophilum, C. dendroides, and C. protrusum, is characterized by rapid hyphal extension over casing beds. The pathogen forms a course, white, cottony mycelial mat that envelops developing mushroom pins and mature fruiting bodies. Infected mushroom tissues turn light brown, decay, and become completely covered by fungal growth, which subsequently turns pink or red as conidia mature (Romaine & Schlagnhaufer, 1995).

Conidia of Cladobotryum are dry and easily aerosolized, allowing rapid airborne transmission throughout cultivation rooms and between climate zones within a facility. Delayed containment of localized cobweb patches leads to widespread farm contamination. Furthermore, emerging strains of Cladobotryum have developed resistance to key commercial fungicides, including metrafenone, underscoring the need for early non-chemical detection and biosecurity protocols (Eastwood et al., 2011).

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Table 1. Primary Fungal Pathogens of Commercial Mushrooms, Associated Diseases, Pathogenesis Targets, and Economic Losses.

Pathogen Species	Disease Name	Host Species	Key Symptoms	Primary Inoculum Vector	Typical Economic Loss
Trichoderma aggressivum, T. pleuroticola, T. harzianum	Green Mold Disease	A. bisporus, P. ostreatus, G. sichuanense	Substrate colonization, white mycelial patches turning green, host growth inhibition, toxic metabolite production	Substrate, casing, airborne dust, phorid/sciarid flies, equipment	20% to 100% loss
Lecanicillium fungicola (vars. fungicola, aleophilum)	Dry Bubble Disease	A. bisporus, P. ostreatus, A. bitorquis	Undifferentiated tissue masses (dry bubbles), stipe blowout, cinnamon-brown cap spots	Peat casing material, water splash, farm workers, fly vectors	2% to 20% globally (~€300M in EU)
Mycogone perniciosa (Hypomyces perniciosus)	Wet Bubble Disease	A. bisporus	Sclerodermoid lumps, amber liquid exudation, soft wet decay of fruiting bodies	Soil, casing, dust, persistent chlamydospores, watering	Up to 100% in unmitigated rooms
Cladobotryum mycophilum, C. dendroides, C. protrusum	Cobweb Disease	A. bisporus, L. edodes, P. ostreatus	White cottony mycelium covering beds and pins, rotting host tissue, pink/red conidial mats	Dry airborne conidia, casing, contaminated equipment	Localized to total flush destruction

3. Principles and Mechanisms of CRISPR-Cas Diagnostic Systems

CRISPR-Cas systems, originally identified as adaptive immune mechanisms in bacteria and archaea, rely on RNA-guided endonuclease complexes to recognize and cleave foreign nucleic acids in a sequence-specific manner. In molecular diagnostics, these systems have been repurposed to act as biosensors, where specific nucleic acid target recognition triggers an enzymatic signal readout (Nussenzweig & Marraffini, 2020). The fundamental workflow proceeds through target DNA or RNA recognition by a crRNA-Cas complex, specific genomic site binding, target-activated non-specific trans-cleavage of labeled reporter oligonucleotides, and subsequent signal transduction readable via fluorescence or colorimetric lateral flow formats (Sorek et al., 2013).

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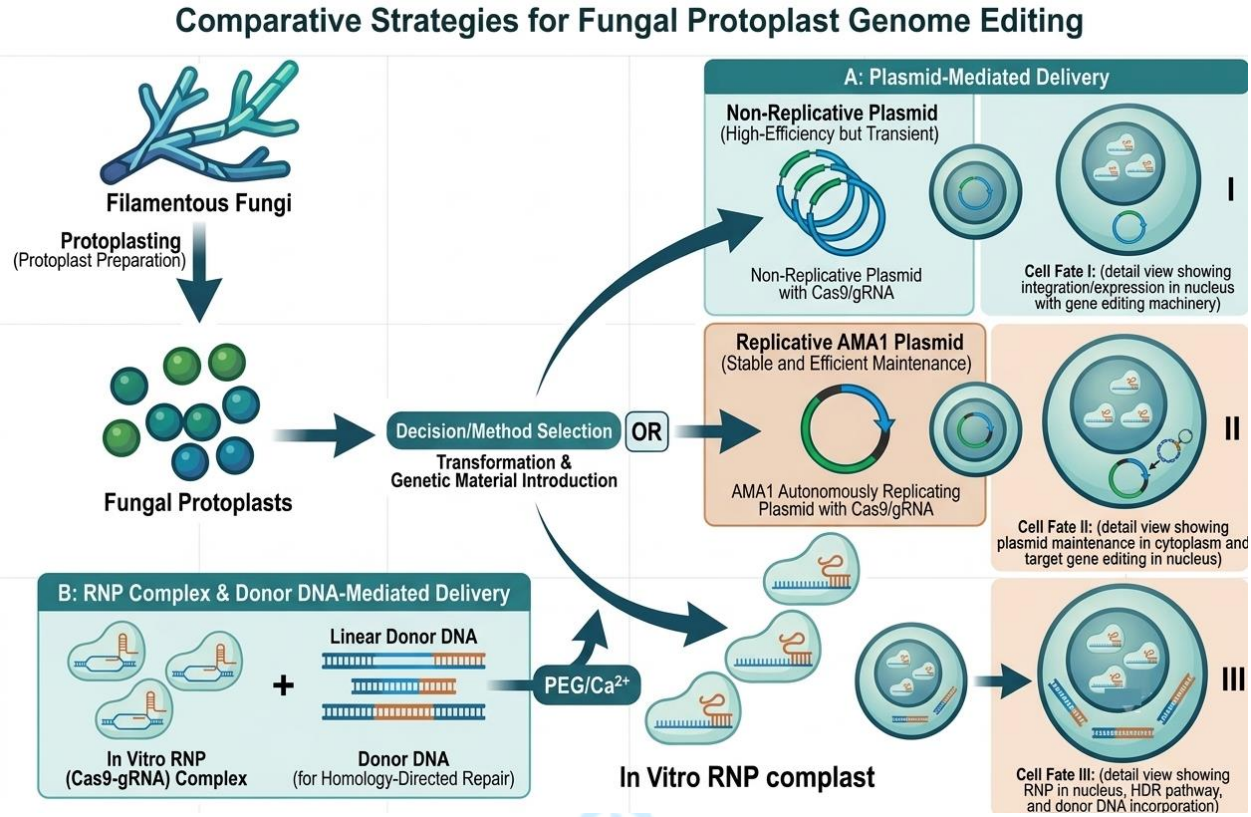
Figure 1. Comparative delivery strategies and intracellular cell fates of plasmid- versus RNP-mediated CRISPR-Cas genome editing in fungal protoplasts.



3.1 Effector Cas Nucleases in Nucleic Acid Sensing

Diagnostic CRISPR platforms utilize specific Class 2 Cas effectors, categorized primarily into Type II (Cas9), Type V (Cas12), and Type VI (Cas13) systems. Type II Cas9 nucleases are guided by a chimeric single-guide RNA (sgRNA) or a crRNA:tracrRNA duplex to recognize double-stranded DNA (dsDNA) adjacent to a 3' Protospacer Adjacent Motif (PAM), typically 5'-NGG-3' (Puig-Serra et al., 2022). Target binding induces site-specific double-strand breaks (DSBs) via its RuvC and HNH nuclease domains. While Cas9 is predominantly used for target gene editing, catalytically inactive Cas9 (dCas9) or engineered Cas9 complexes are utilized in diagnostic platforms through targeted sequence binding to facilitate isothermal amplification or target capture (Li et al., 2019).

Type V Cas12a (Cpf1) nucleases are single-RNA-guided endonucleases that target dsDNA or single-stranded DNA (ssDNA) sequences containing a 5' T-rich PAM motif, typically 5'-TTTN-3'. Upon binding to its complementary target sequence, Cas12a forms a ternary R-loop complex that activates its RuvC catalytic domain (Jolany Vangah et al., 2020). Catalytic activation induces targeted cis-cleavage of the target DNA strand followed by rapid, non-specific, promiscuous trans-cleavage (collateral cleavage) of nearby non-target ssDNA reporter molecules. This indiscriminate ssDNase activity forms the core operational mechanism of Cas12a-driven diagnostic platforms such as DETECTR (DNA Endonuclease-Targeted CRISPR Trans Reporter) and HOLMES (Hourglass On-Demand Lethal Microbe Detection System) (Yuan et al., 2022).

Type VI Cas13a (C2c2) nucleases are RNA-guided endonucleases that target single-stranded RNA (ssRNA) sequences containing a Protospacer Flanking Sequence (PFS). Hybridization of the crRNA guide to the target RNA sequence induces

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a conformational shift that assembles two Higher Eukaryotes and Prokaryotes Nucleotide-binding (HEPN) domains into a single active catalytic site. Activated Cas13a displays robust collateral trans-cleavage of surrounding non-target ssRNA molecules, a property harnessed in platforms such as SHERLOCK (Specific High-Sensitivity Enzymatic Reporter UnLOCKing) (Verma et al., 2022).

3.2 Biophysical Mechanism of Collateral Signal Amplification

The signal amplification cascade of Cas12a and Cas13a diagnostics relies entirely on target-activated collateral cleavage. Synthetic reporter oligonucleotides are introduced into the reaction mix alongside the Cas protein, crRNA, and sample nucleic acid. These reporters consist of short ssDNA (for Cas12a) or ssRNA (for Cas13a) probes coupled to detection moieties (Li et al., 2018).

In fluorophore-quencher (FQ) reporting systems, a fluorescent dye (such as FAM, HEX, or ROX) is conjugated to one terminus of the short reporter oligonucleotide, while an appropriate dark quencher (such as Iowa Black or BHQ-1) is attached to the opposing terminus. In the intact, uncleaved state, proximity between the molecules causes Fluorescence Resonance Energy Transfer (FRET), quenching the fluorescent signal (Broughton et al., 2020). When a target pathogen sequence triggers Cas trans-cleavage, the single-stranded oligonucleotide linker is degraded, physically liberating the fluorophore from the quencher and generating a high-intensity fluorescent signal detectable via hand-held UV/LED devices or plate readers (Ding et al., 2020).

In chromogenic or lateral flow dipstick (LFD) reporting systems, reporter oligonucleotides are dual-labeled with distinct affinity tags, such as 5'-fluorescein (FAM) and 3'-biotin. In the absence of target pathogen nucleic acids, intact reporters bind anti-FAM antibody-conjugated gold nanoparticles and are fully sequestered at a primary streptavidin control line. Conversely, target-activated Cas collateral cleavage degrades the reporter sequence, releasing free FAM-nanoparticle complexes that migrate further up the nitrocellulose strip to be captured by species-specific secondary antibodies at the test line, producing a visible band readable by the naked eye (Patchsung et al., 2020). Because a single target-activated Cas protein can cleave thousands of reporter probes per minute, collateral activity acts as a catalytic signal amplifier, enabling high analytical sensitivity (Gootenberg et al., 2018).

4. Diagnostic Loci Selection and crRNA Engineering

The selection of target genomic regions and the structural design of CRISPR RNAs (crRNAs) dictate the ultimate specificity and sensitivity of CRISPR diagnostics in mushroom cultivation. Diagnostic assays must reliably distinguish target mycoparasite genomes from background DNA originating from the host mushroom (A. bisporus, P. ostreatus), non-pathogenic substrate microflora, and related non-pathogenic fungal species within the same genus (Lucia et al., 2020).

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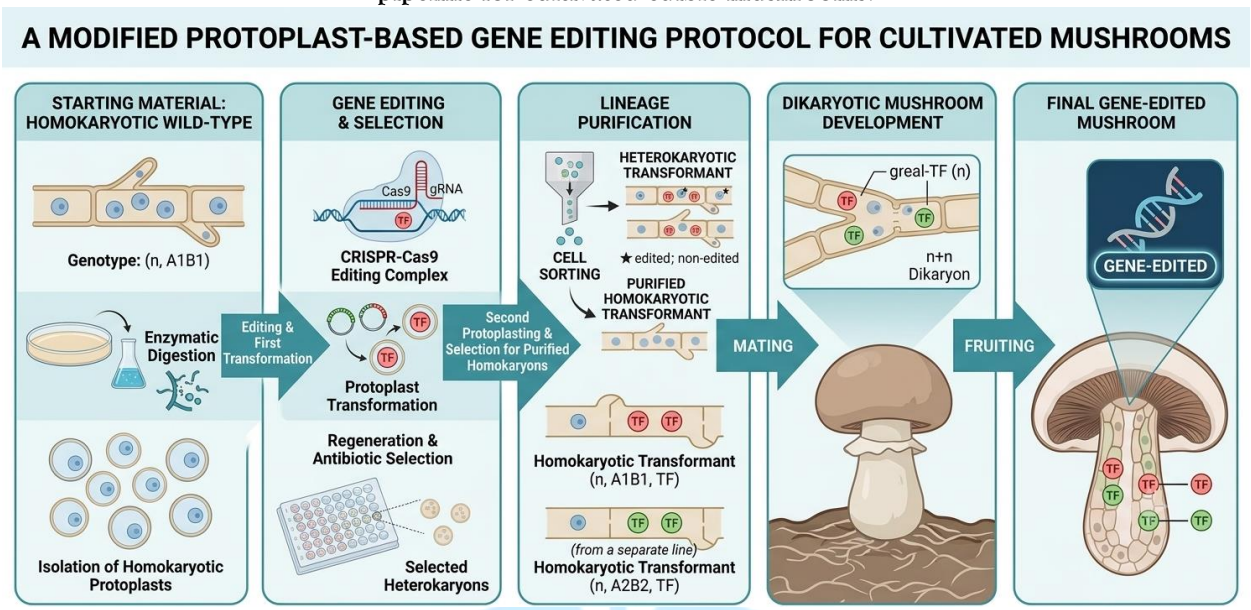
Figure 2. A modified protoplast-based CRISPR-Cas gene editing and homokaryotic lineage purification pipeline for cultivated edible mushrooms.



4.1 Target Genomic Loci Evaluation

Selecting the appropriate target gene locus involves balancing genomic copy number with sequence divergence across closely related species. The Internal Transcribed Spacer (ITS) region of the nuclear ribosomal RNA gene locus (encompassing ITS1, 5.8S, and ITS2) is the standard fungal barcode. Because hundreds of tandem ribosomal repeats exist within each fungal genome, targeting the ITS region provides a high template copy number, significantly boosting raw analytical sensitivity (Kwon & Shin, 2021). However, closely related hypocrealean fungi such as distinguishing Trichoderma aggressivum from Trichoderma harzianum, or differentiating varieties within Lecanicillium fungicola frequently exhibit high ITS sequence identity. Consequently, ITS-targeted crRNAs require thorough in silico screening and empirical validation against non-target database sequences to prevent false-positive cross-reactivity (Chen et al., 2018).

The Translation Elongation Factor 1-alpha (TEF1-α) gene offers higher phylogenetic resolution than the ITS region due to variable intron sequences. TEF1-α is widely recognized as the gold-standard diagnostic marker for resolving Trichoderma species, allowing crRNAs to easily distinguish aggressive green mold agents (T. aggressivum, T. pleuroticola, T. pleurotum) from non-pathogenic environmental or commercial biocontrol isolates (Broughton et al., 2020).

Protein-coding loci such as the RNA Polymerase II Subunit B2 (RPB2) and β-tubulin (TUB) genes provide exceptional species-level discrimination within complex mycoparasitic genera including Mycogone and Cladobotryum. These single-copy genes possess variable exon-intron boundaries that facilitate the design of highly species-specific amplification primers and crRNAs, ensuring absolute target discrimination even in complex environmental matrices (Kellner et al., 2019).

4.2 crRNA Design Parameters and Off-Target Mitigation

Designing crRNAs for diagnostic Cas12a or Cas13a platforms requires strict adherence to structural and biophysical parameters. For canonical LbCas12a or AsCas12a nucleases, candidate protospacer target sequences must be located immediately 3' to a 5'-TTTN-3' PAM motif. The discovery and engineering of Cas12a variants with relaxed PAM requirements, such as LbCas12a-Ultra (which can recognize 5'-TTN-3' or non-canonical PAM sites), significantly expands the selectable targeting space across AT-rich or

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GC-rich genomic regions of fungal targets (Tang et al., 2021).

The spacer sequence of the crRNA is typically engineered to a length of 20 to 24 nucleotides. Within this spacer, the "seed region" encompassing nucleotides 1 to 10 directly adjacent to the PAM is exceptionally sensitive to sequence mismatching. A single nucleotide mismatch within the seed region significantly impairs Cas complex target binding and suppresses collateral activation, providing single-nucleotide polymorphism (SNP) discrimination capability. This structural intolerance allows researchers to design crRNAs that deliberately target species-specific SNPs within conserved fungal genes (Molina Vargas et al., 2024).

In silico secondary structure folding evaluations (such as RNAfold analysis) are necessary to prevent the formation of stable internal hairpins or self-dimerization within the crRNA spacer, ensuring that the guide RNA remains fully accessible for target hybridization. Furthermore, emerging cascading diagnostic strategies deploy multi-crRNA pools wherein three or four distinct crRNAs targeting different regions of the same gene locus are combined in a single reaction to dramatically accelerate Cas activation rates and lower the overall limit of detection (LOD) without introducing non-specific background noise (Effah et al., 2026).

5. Integration with Isothermal Amplification and Point-of-Care Readouts

While highly optimized CRISPR systems can achieve target detection in high-copy contexts, reliable sensing of trace fungal pathogen loads in commercial mushroom substrates requires pre-amplification. Integrating CRISPR detection with isothermal nucleic acid amplification strategies bypasses the need for laboratory thermal cyclers, allowing rapid, sub-femtomolar detection at constant ambient temperatures (Taemaitree, 2018).

5.1 Isothermal Amplification Technologies

Recombinase Polymerase Amplification (RPA) is the most widely integrated isothermal method for field-deployable CRISPR biosensors. Operating at mild temperatures between 37°C and 42°C, RPA achieves exponential nucleic acid amplification within 15 to 30 minutes. The reaction mechanism uses three primary enzymes: a recombinase (such as T4 UvsX) that pairs oligonucleotide primers with homologous double-stranded DNA, a single-stranded DNA-binding protein (SSB, such as T4 UvsY) that stabilizes the displaced DNA strand, and a strand-displacing DNA polymerase (such as Sau or Bsu polymerase). RPA exhibits high tolerance to common organic inhibitors, making it an ideal pre-amplification engine for crude mushroom substrate extracts (Singh et al., 2022).

Loop-Mediated Isothermal Amplification (LAMP) serves as another powerful isothermal driver, operating at elevated temperatures between 60°C and 65°C. LAMP utilizes a strand-displacing DNA polymerase (such as Bst polymerase) alongside four to six specialized primers designed to recognize six to eight distinct regions of the target gene, producing stem-loop concatemers within 30 to 50 minutes. While LAMP primer design is structurally complex, the multi-primer requirement delivers exceptional analytical specificity prior to Cas cleavage (Feng et al., 2022).

Table 2. Representative Isothermal CRISPR-Cas Diagnostic Platforms for Fungal Pathogen Detection.

Pathogen Target	Cas Effector Variant	Pre-Amplification Method	Reaction Temp. & Time	Limit of Detection (LOD)	Readout Modality
Cladobotryum mycophilum	Cas12a	RPA (Double-layer Hydrogel)	37°C, <40 min	1 fg/µL	Visual hydrogel fluorescence
Fusarium graminearum	LbCas12a	PCR / RPA	37°C, ~58 min	1 fg/µL	Microplate reader / Fluorescence

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Alternaria triticina	Cas12a	RPA	35–37°C, 25–30 min	10 pg/µL	Fluorescence / Lateral Flow Strip
Verticillium dahliae	Cas13a	RPA	37°C, 50–60 min	100 aM	Fluorescence / LFD
Magnaporthe oryzae Triticum	Cas12a / dCas9	RPA	37–39°C, 22–30 min	1 pg/µL (10 pg/µL)	Lateral Flow Strip (PCRD)
Candidatus Phytoplasma	LbCas12a-Ultra	Pre-amp Free	37°C, 30 min	Sub-picomolar (10-fold > LbCas12a)	7nt Stem-loop Fluorescence / LFA

6. Matrix Interferences and Nucleic Acid Extraction from Substrates

Executing molecular diagnostics directly within commercial mushroom facilities requires overcoming significant biochemical challenges posed by cultivation substrates. Diagnostic samples evaluated for fungal pathogen contamination include Phase I–III composted straw and poultry manure, casing soil composed of decomposed peat moss and chalk, spent mushroom substrate, and environmental air dust (Grimm & Wösten, 2018).

6.1 Inhibitors Present in Mushroom Substrates

Mushroom growth substrates contain complex chemical compounds that act as potent enzymatic inhibitors. Humic and fulvic acids, generated during the microbial decomposition of straw, peat, and manure, represent the primary inhibitors co-extracted with fungal genomic DNA. Due to their structural similarities, size, and charge density, humic substances co-purify alongside nucleic acids (Sorek et al., 2013). Once in solution, humic acids directly bind and denature DNA polymerases, recombinases, and Cas proteins, or chelate necessary divalent metal cofactors (Mg^{2+}), leading to partial or complete inhibition of amplification and CRISPR cleavage. Additionally, high background concentrations of fungal cell-wall polysaccharides (such as β-glucans and chitin) and polyphenols from organic casing materials cause steric hindrance and prevent efficient crRNA-target hybridization (Chang & Miles, 2004).

6.2 Field-Compatible Extraction and Inhibitor Removal Strategies

To transition CRISPR diagnostics from specialized laboratories to farm settings, traditional multi-step spin-column or phenol-chloroform nucleic acid purifications must be replaced with rapid, field-compatible extraction protocols (Jolany Vangah et al., 2020).

Simplified crude extraction buffers based on alkaline polyethylene glycol and sodium hydroxide (PEG-NaOH) enable rapid nucleic acid release. Brief physical homogenization (1 to 2 minutes) of compost or casing samples in PEG-NaOH ruptures fungal cell walls and liberates genomic DNA while simultaneously precipitating coarse humic acid fractions. Subsequent dilution of the crude lysate lowers residual inhibitor concentrations below inhibitory thresholds, allowing direct input into RPA-CRISPR reactions (Verma et al., 2022).

Inclusion of chemical adsorbents and chelating agents within lysis buffers further mitigates matrix interference. Polyvinylpolypyrrolidone (PVPP) and cetyltrimethylammonium bromide (CTAB) effectively bind polyphenols and humic contaminants. Pre-treatment with divalent salt additives, such as calcium chloride ($CaCl_2$) or calcium carbonate ($CaCO_3$), promotes the selective precipitation of humic complexes from acidic peat casing extracts prior to amplification (Grogan, 2008). Furthermore, formulating isothermal master mixes with inhibitor-tolerant polymerases and protective protein additives (such as bovine serum albumin or skim milk powder) sequesters trace inhibitors, preserving Cas cleavage efficiency (Noble et al., 2009).

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7. Comparative Analysis of Detection Technologies

Evaluating CRISPR-based molecular detection alongside established diagnostic techniques highlights its relative performance and operational capabilities in commercial mushroom pathology (Pardo-Giménez et al., 2022).

7.1 Morphological and Culture-Based Diagnostics

Traditional identification relies on isolating fungal pathogens on selective agar media, followed by incubation and microscopic examination of conidiophore architecture and spore morphology. While visually direct, culture-based methods require 5 to 14 days for colony development and demand specialized mycological training. Morphological discrimination between closely related species (such as distinguishing pathogenic T. aggressivum from benign T. harzianum) is notoriously difficult, and non-culturable or dormant resting structures (such as soil-bound chlamydospores) are frequently missed (Fletcher & Gaze, 2008).

7.2 Polymerase Chain Reaction (PCR) and Quantitative PCR (qPCR)

Conventional PCR and quantitative real-time PCR (qPCR) targeting ITS, TEF1-α, or RPB2 loci serve as benchmark molecular standards. These methods deliver exceptional sensitivity (LOD ranging from 0.01 to 1 pg/µL) and enable quantitative tracking of pathogen loads. However, qPCR relies heavily on pristine, highly purified DNA samples to prevent humic acid inhibition, requires costly, delicate thermal cyclers, and takes several hours to complete, rendering on-site testing impractical (Royse et al., 2017).

7.3 Standalone Isothermal Amplification (LAMP / RPA)

Standalone isothermal platforms (such as LAMP or RPA coupled to turbidimetric or visual dye readouts) eliminate the need for thermal cyclers and reduce assay turnaround times to 20–40 minutes. While highly field-adaptable, standalone isothermal methods are susceptible to false-positive results caused by non-specific primer binding or primer-dimer amplification, particularly when evaluating complex, unpurified organic substrate lysates (Moore et al., 2011).

7.4 CRISPR-Cas Coupled Isothermal Biosensors

Coupling RNA-guided CRISPR-Cas cleavage with isothermal pre-amplification solves the specificity limitations of standalone isothermal assays. In this dual-recognition architecture, even if isothermal primers generate off-target amplicons, the Cas effector will not undergo collateral activation unless the crRNA spacer sequence specifically hybridizes with the internal target locus (Sorek et al., 2013). This layer of sequence verification delivers exceptional analytical specificity, sub-femtomolar sensitivity, and strong tolerance to substrate matrix inhibitors, providing visual results within 25 to 50 minutes under ambient farm conditions (Li et al., 2019).

Table 3. Comparative Evaluation of Diagnostic Methodologies in Mushroom Pathology.

Diagnostic Parameter	Cultivation & Microscopy	Quantitative PCR (qPCR)	Standalone Isothermal (LAMP / RPA)	CRISPR-Cas Coupled Biosensors
Turnaround Time	5 to 14 days	2 to 4 hours	20 to 45 minutes	25 to 50 minutes
Equipment Requirement	Incubators, light microscopes	Quantitative thermal cycler	Simple heat block / water bath	Heat block or ambient tube format
On-Site Adaptability	Poor (requires lab)	Poor (requires lab setup)	Moderate to High	High (field-deployable POCT)
Sensitivity (LOD)	Low (requires visible growth)	High (0.01–1 pg/µL)	High (1–10 pg/µL)	Ultra-high (1 fg/µL – 100 aM)

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Specificity Level	Low to Moderate	High	Moderate (susceptible to false positives)	Superior (dual primer + crRNA verification)
Inhibitor Susceptibility	High (suppresses culture)	High (requires purified DNA)	Moderate	Low to Moderate (tolerant to crude lysate)
Skill Level Required	High (mycological training)	High (molecular biology lab)	Moderate	Low (farm staff with test strips)

8. Conclusions

CRISPR-Cas-based molecular diagnostics represent a paradigm shift in the detection and management of fungal pathogens affecting commercial mushroom cultivation. The integration of RNA-guided Cas effectors with isothermal amplification technologies addresses the critical limitations of conventional diagnostic approaches, including prolonged turnaround times, laboratory dependency, and susceptibility to substrate-derived inhibitors. The dual-recognition architecture of CRISPR-Cas coupled biosensors provides exceptional analytical specificity that effectively discriminates between closely related pathogenic and non-pathogenic fungal species, a capability particularly essential for *Trichoderma* pathogen identification given its dual ecological role as both agricultural pest and biological control agent. The successful deployment of these diagnostic systems in mushroom production facilities will require continued optimization of field-compatible nucleic acid extraction protocols, development of lyophilized reagent formulations for ambient-temperature stability, and establishment of standardized sampling protocols across different cultivation phases (compost, casing, and post-harvest environments). Economic feasibility assessments and cost-effectiveness analyses compared to traditional culture-based and qPCR methods remain important areas for future investigation. Additionally, the integration of CRISPR diagnostics with digital data platforms and smartphone-based readout systems could enable real-time disease surveillance networks across mushroom production regions, facilitating early outbreak warnings and coordinated biosecurity responses. Collectively, CRISPR-Cas molecular diagnostics offer a transformative tool for precision disease management in mushroom cultivation, shifting the paradigm from reactive visual inspection to proactive, molecular-based surveillance that can substantially mitigate crop losses, reduce fungicide reliance, and enhance the sustainability and resilience of commercial mushroom production systems worldwide.

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