Extracting Protoplasts from Filamentous Fungi Using Extralyse, An Enzyme Used in the Wine Industry

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The ability to extract protoplasts has contributed significantly to the study of fungi and plants. Protoplasts have historically been used to determine chromosome number via pulsed-field electrophoresis and for the functional characterization of genes via protoplast transformation. More recently, protoplasts have been used to extract the high-molecular-weight DNA required for long-read sequencing projects. The availability of efficient protoplast extraction protocols is thus integral to the study and experimental manipulation of model and non-model fungi. One major hurdle to the development of such protocols has been the discontinuation of enzymes and enzyme cocktails used to digest the fungal cell wall. Here, we provide five protoplast extraction protocols for use in various filamentous ascomycete species spanning the genera *Ceratocystis*, *Fusarium*, *Metarhizium*, *Ophiostoma*, and *Sclerotinia*. These protocols all use an inexpensive, readily available enzyme cocktail called Extralyse, a commercially available product commonly used in the wine making industry. Using this enzyme cocktail overcomes reliance on the laboratory-grade enzymes that have frequently been discontinued and are often cost prohibitive at the concentrations required. The protocols described here will allow further research, including genome editing, to be conducted in these fungal genera. Importantly, these protocols also provide a starting point for the development of protoplast extraction techniques in other filamentous fungi. This resource can therefore be used to expand the molecular toolkits available for fungi beyond the species described here, including those with relevance in both medical and biotechnological industries.

Basic Protocol 1: Protoplast extractions from *Ceratocystis eucalypticola* and *Ceratocystis fimbriata*
Basic Protocol 2: Protoplast extractions from *Fusarium circinatum*
Basic Protocol 3: Protoplast extractions from *Metarhizium acridum*, *Metarhizium brunneum*, and *Metarhizium guizhouense*
Basic Protocol 4: Protoplast extractions from *Ophiostoma novo-ulmi*
Basic Protocol 5: Protoplast extractions from *Sclerotinia sclerotiorum*

Keywords: filamentous fungi • genome editing • long-read sequencing • protoplasts

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INTRODUCTION

The fungal cell wall is a complex, dynamic extracellular matrix that shapes the cell and provides rigidity and structure (Gow et al., 2017). This cellular barrier, comprising various polymers, including chitin as well as 1,3-β- and 1,6-β-glucans, protects the cell from osmotically unstable environments, prevents the uptake of toxic substances, and defends against mycoviruses and other pathogens (Peberdy & Ferenczy, 1985). Additionally, the cell wall is metabolically active and is embedded with many proteins that facilitate interactions between the intracellular space and the extracellular environment and play important roles in cell wall reorganization and cell growth (de Groot et al., 2005; Peberdy & Ferenczy, 1985).

The cell wall poses several challenges to the study of fungal biology (Lichius et al., 2020). Most notably, it prevents the uptake of transforming DNA as well as the extraction of large, intact DNA fragments and chromosomes, thus hindering the use of molecular transformation techniques and genomic studies in many fungi. Over the past six decades, various protocols have been developed for the removal of the cell wall (Bachmann & Bonner, 1959; Daly et al., 2017; Eddy & Williamson, 1957; Hamlyn et al., 1981). This is typically achieved by the enzymatic digestion of the fungal cell wall using enzymes like chitinases and β-glucanases, usually in complex enzyme mixtures (Rodriguez-Iglesias & Schmoll, 2015). These enzymes break down the primary components of the cell wall, releasing a membrane-bound cell, called a protoplast, that is more amenable to manipulation (Peberdy & Ferenczy, 1985).

Protoplasts have been used for numerous applications in fungal biology research. Protoplast-mediated transformation is one of the most common forms of transformation used for genome editing in filamentous fungi (Rodriguez-Iglesias & Schmoll, 2015). This is primarily because no highly specialized equipment or materials are needed as they are for other techniques, such as electroporation (Lichius et al., 2020). Additionally, protoplast-mediated transformation does not rely on the construction of complex binary vectors, such as are required for *Agrobacterium*-mediated transformation (Lichius et al., 2020). Thus, the transformation of protoplasts is frequently used for gene knockout and/or gene knock-in experiments that aim to characterize gene function (Phasha et al., 2021a; Wilson et al., 2020; Yamato et al., 2019). The transformation of protoplasts can also be used for reporter analysis experiments, in which novel promoter regions can be identified and tissue- or time-specific expression determined using a reporter gene such as green fluorescent protein or luciferase (Sugano et al., 2017; Na et al., 2024; Fuji & Takaya, 2008).

Protoplasts have also been used for the extraction of high-molecular-weight (HMW) DNA, including long DNA fragments and whole chromosomes. Because the fragile protoplasts can be lysed gently, the DNA present within these cells can be released without harsh mechanical and chemical disruption (Griffiths et al., 2006; Wötemeyer & Wöstemeyer, 1998). Protoplasts have thus historically been used to determine chromosome number and karyotypes in fungi. This is achieved using clamped homogeneous

electric field gel electrophoresis, in which whole chromosomes are separated on large agarose gels (Debets et al., 1990; Hamer et al., 1989; Shi & Miao, 2005). Protoplasts have also been used to extract DNA for use in high-throughput long-range PCR, particularly for the screening of positive transformants (van Zeijl et al., 1998). More recently, protoplasts have been used to extract DNA for long-read sequencing (Li & Wang, 2021), an approach that is likely to be increasingly used as third-generation sequencing and other next-generation sequencing (NGS) technologies improve and become more cost effective.

Protoplast extraction protocols have been used in the genetic modification of a wide variety of model and non-model fungi. This includes human pathogens (Garre et al., 2015; Zhao et al., 2019), plant pathogens (Antonio et al., 2010; Phasha et al., 2021a, 2021b), saprobes (Wilson et al., 2020), mycorrhizal fungi (Wang & Liu, 2013), edible mushrooms (Chang et al., 1985), and mushrooms used in traditional medicinal practices (Wang et al., 2020). The widespread utility of protoplasts has, however, been threatened by the ever-decreasing availability of the enzymes used to extract these cells. Commercially available enzyme mixes such as Protoplast F, Glucanex, Lysing Enzymes from *Trichoderma harzianum*, Novozym 234, and others have been used to develop protoplast extraction protocols from a variety of fungi (Daly et al., 2017; Lakshmi & Chandra, 1993; Varavallo et al., 2004; Wilson & Wingfield, 2020). However, over time, these cocktails have been discontinued and are no longer available for purchase (Table 1). This requires the re-optimization of these otherwise established extraction protocols using alternative enzymes. Because of the discontinuation of enzyme cocktails in particular, researchers have resorted to purchasing individual, highly purified enzymes to produce their own digestion mixtures, making protoplast extraction a very costly endeavor.

It has recently been shown that enzymes used in the production of wine are suitable substitutes for the laboratory-grade enzymes previously used for protoplast extraction (Roux & Chooi, 2022; Syme et al., 2018). One such enzyme mix, Extralyse, is produced by Laffort (Bordeaux, France) and contains enzymes with pectinase, β-glucanase, and cinnamoyl esterase activity. In addition to being widely available, this enzyme mix is also a cheaper alternative and can be purchased in large quantities. The following sections describe optimized Extralyse-based protoplast extraction protocols for five different fungal genera (Table 2). Each protocol details the suitable starting material from each species, the digestion parameters, and protoplast collection techniques. Some of the protocols also include pre-treatment and post-digestion steps that increase the quality and quantity of the extracted protoplasts. Although the fungi for which we provide these protocols have relevance in forestry and agriculture, we emphasize that the methodology can readily be adapted and applied to other filamentous fungi. As such, we also provide detailed descriptions of how the individual steps of these protocols can be optimized for other fungal species (Fig. 1).

Table 1 Examples of Cell-Wall-Degrading Enzymes That Are No Longer Commercially Available

Enzyme	Description	Link
Protoplast F	A mixture of cell-wall-degrading enzymes, including exo-1,3-β-glucanase and endo-1,3-β-glucanase	*https://www.megazyme.com/protoplast-f*
Lysing enzymes from *Trichoderma harzianum*/Glucanex	A mixture of enzymes with β-1,3-glucanase activity	*https://www.sigmaaldrich.com/DK/en/product/sigma/l1412*
Novozym 234	A mixture of β-glucanase, cellulase, protease, chitinase, and α(1,3)-glucanase	*https://pubmed.ncbi.nlm.nih.gov/19041907/*

Table 2 Species Included in This Study

Class	Order	Family	Genus and species	Relevance
Sordariomycetes	*Microascales*	*Ceratocystidaceae*	*Ceratocystis eucalypticola*	Pathogen of *Eucalyptus* species; causal agent of Ceratocystis Wilt
			Ceratocystis fimbriata	Pathogen of sweet potato
Sordariomycetes	*Hypocreales*	*Nectriaceae*	*Fusarium circinatum*	Pathogen of *Pinus* species; causal agent of Pitch Canker Disease
Sordariomycetes	*Hypocreales*	*Clavicipitaceae*	*Metarhizium acridum, Metarhizium brunneum, Metarhizium guizhouense*	Pathogens of various agriculturally important insect pests, such as locusts and caterpillars
Sordariomycetes	*Ophiostomatales*	*Ophiostomataceae*	*Ophiostoma novo-ulmi*	Pathogen on *Ulmus* species; causal agent of Dutch elm disease
Leotiomycetes	*Helotiales*	*Sclerotiniaceae*	*Sclerotinia sclerotiorum*	Pathogen on a wide variety of agricultural crops, including canola, soybean, and sunflowers

Common Considerations

The five basic protocols below are separated into species-specific sections because they differ in several important ways. However, there are some important factors that are common to all five protocols and are detailed here.

Protoplasts are fragile

During all protoplast extraction protocols, it is important to ensure that the protoplasts are handled gently. Use only 1-ml pipet tips when resuspending or transferring protoplast suspensions to ensure that the protoplasts are not exposed to excessive mechanical disruption during pipetting. When resuspending the protoplasts, pipet slowly and gently to avoid damage to the protoplasts. Avoid jostling tubes holding the protoplasts.

Monitoring the digestion

It is recommended that the digestion be monitored so that an optimal number of protoplasts can be extracted. Depending on the duration of the enzymatic digestion, the progress can be monitored in 1- to 2-hr increments. To do this, remove up to 10 µl of the digestion solution and visualize using standard light microscopy methods. If only a small amount of material is visible, remove up to 1 ml of the enzyme digestion and centrifuge as described in the protocol. Discard the supernatant, resuspend in at least 10 µl of buffer, and visualize using standard light microscopy methods. The digestion is complete when the majority of visible cells are protoplasts. It is important not to filter the digestion solutions through Miracloth at this step as that will remove any remaining mycelium and make it difficult to determine the ratio of protoplasts to mycelium. In turn, this makes it difficult to assess how far the digestion has progressed. Filtration through Miracloth should be done only once the digestion is complete, and the protoplasts need to be separated from the mycelium for use in downstream applications.

Protoplast counting

One of the final steps in each of the protocols is to count the number of protoplasts produced. This can be done using a hemocytometer and standard counting methods. Many tutorials and methodologies have been written on this, as exemplified by Absher (1973).

Once the protoplast concentration is known, the suspension can either be concentrated through centrifugation and resuspension in a smaller volume or diluted using the relevant buffer.

Aseptic techniques and sterile equipment

All the equipment and materials used in the below protocols should be sterile, having been autoclaved or otherwise sterilized ahead of time. This includes, for example, Erlenmeyer flasks, scalpel blades, and Miracloth, as well as buffers and media. Aseptic technique should also be used throughout, ensuring that no external material contaminates the protoplasting solutions.

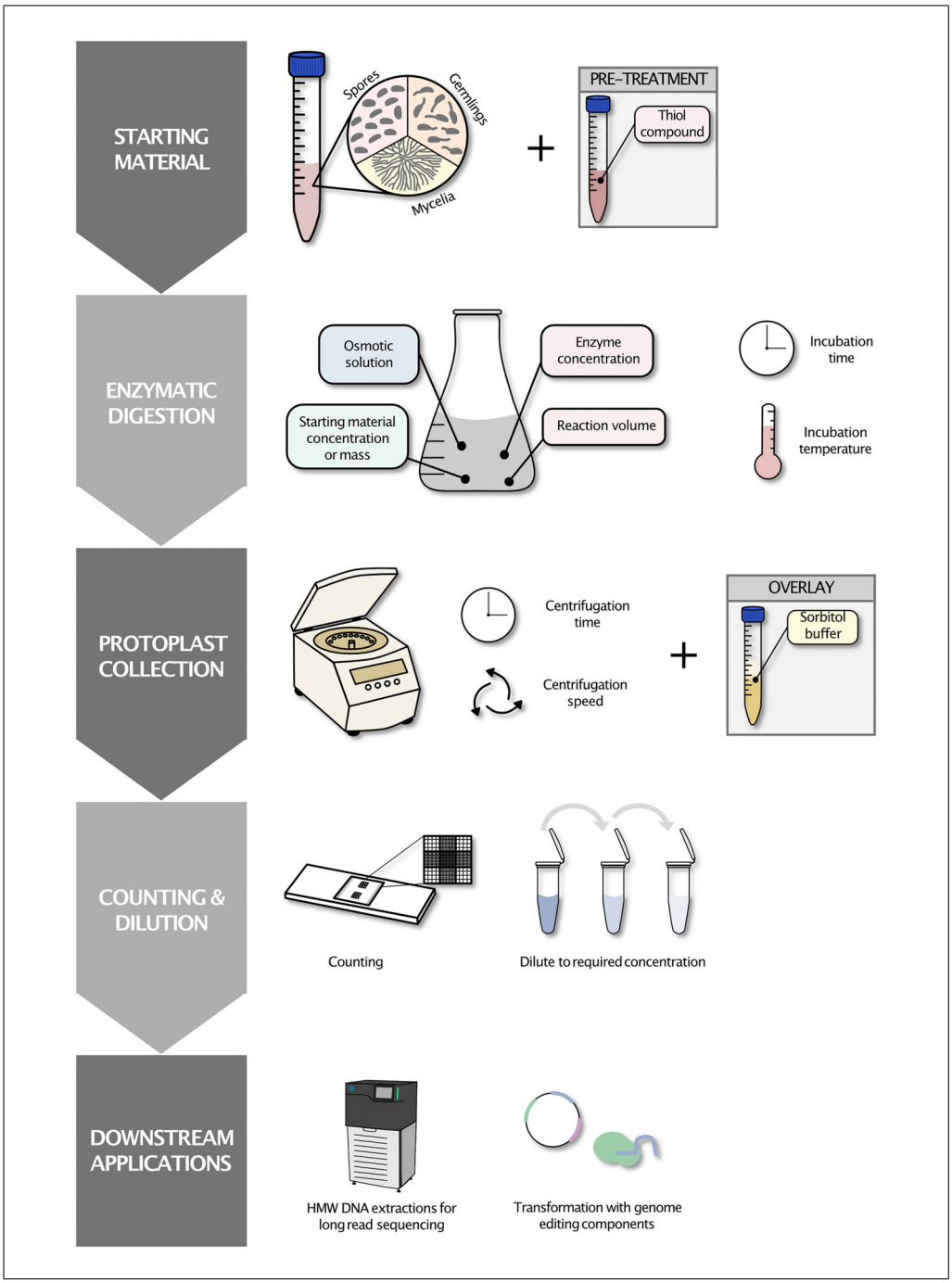

Figure 1 Flow chart representing the protoplast extraction protocol. Factors that may require species-specific optimization are illustrated at each step, and optional steps that may improve protoplast yield and quality are included in light grey boxes.

BASIC PROTOCOL 1

PROTOPLAST EXTRACTIONS FROM *CERATOCYSTIS EUCALYPTICOLA* AND *CERATOCYSTIS FIMBRIATA*

Ceratocystis is a genus of filamentous, plant pathogenic fungi in the phylum *Ascomycota*. *Ceratocystis* species are extensively researched pathogens of woody plants and are known to cause many wilt diseases (Bailey & Meinhardt, 2016). In particular, *Ceratocystis* species have devastating effects on agricultural and forestry industries, making the understanding of these fungal species important for disease prevention and control. *Ceratocystis fimbriata* and *Ceratocystis eucalypticola* are among the most studied species of *Ceratocystis*. *C. fimbriata* infects sweet potato (*Ipomoea batatas*), one of the most widely cultivated tuber crops in the world (Paul et al., 2018). In contrast, *C. eucalypticola* was isolated from *Eucalyptus grandis* × *urophylla* hybrids in South Africa (van Wyk et al., 2012), where it causes vascular wilt, as well as discoloration in the cambium and sapwood on the root and stem tissue (Roux et al., 2020). This discoloration results in a significant decrease in the quality and economic value of the wood. The emergence of *C. eucalypticola* in the past decade and the continued destruction caused by *C. fimbriata* have made the study of these phytopathogenic fungi vitally important to the forestry and agricultural sectors in numerous countries worldwide. It is therefore necessary to have robust techniques for the study of these fungi. Here, we present a protocol for the efficient extraction of protoplasts from these two species using Extralyse.

Materials

Malt extract agar (MEA; see recipe)
Cultures of *Ceratocystis eucalypticola* (isolate CMW 51360) and *Ceratocystis fimbriata* (isolate CMW 14799), both maintained in the culture collection (CMW) of the Forestry & Agricultural Biotechnology Institute (FABI) at the University of Pretoria
Potato dextrose broth (PDB; see recipe)
1.2 M magnesium sulfate ($MgSO_4$; see recipe)
Enzyme solution 1 (see recipe)
Ice
Protoplast overlay solution (POS; see recipe)
Separation buffer B (SBB; see recipe)
Sorbitol/Tris·Cl/$CaCl_2$ (STC) buffer A (see recipe)
Dimethyl sulfoxide (DMSO; see recipe)
Liquid nitrogen

Laminar flow hood
60-mm petri plates
100-µl to 1-ml pipetes and appropriate pipet tips
Plate spreader
Miracloth (Merck, cat. no. 475855): place 1 layer of Miracloth in a funnel, cover with foil, and autoclave before use
250-ml Erlenmeyer flask
Shaking incubator
50-µm-pore-size nylon mesh (Merck, cat. no. NY4100010): place one layer of mesh in a funnel, cover with foil, and autoclave before use
50- and 15-ml Falcon tubes
Light microscope
Microscope slides and cover slips
Centrifuge
Hemocytometer
2-ml cryotubes, precooled in liquid nitrogen

1. Inoculate each of 5-10 MEA plates (prepared in 60-mm petri plates) with a block of actively growing culture of *C. fimbriata* or *C. eucalypticola*. The block should be approximately 5 mm × 5 mm in size and can be excised using a sterile scalpel blade. Incubate the plates at 25°C for 5-7 days.
2. Wash the 5- to 7-day old sporulating culture with 5 ml PDB and loosen the conidia by gently scraping the mycelium with a plate spreader. Collect the PDB/conidium solution and filter it through a layer of Miracloth into a 250-ml Erlenmeyer flask.
3. Wash the Miracloth with additional PDB, producing a liquid culture at a final volume of 50 ml.
4. Incubate the liquid culture at 25°C overnight (~12-16 hr) in a shaking incubator at 120 rpm, to produce mature mycelium (Fig. 2A).
5. Filter the resultant liquid culture through nylon mesh and wash the mycelium with 20 ml of 1.2 M $MgSO_4$.
6. Transfer the mycelial clump to a sterile 50-ml Falcon tube and resuspend it in 16 ml enzyme solution 1. Incubate at room temperature (between 22°C and 25°C) with shaking at 25 rpm for 2.5-3.5 hr.
7. Monitor the progress of the digestion under a light microscope at 30-min intervals for 2 hr and then at 15-min intervals, until most of the mycelium is digested and abundant protoplasts are observed (see Common Considerations in article introduction).
8. Aliquot 4 ml of the protoplast suspension into individual 15-ml Falcon tubes (4 tubes in total) and place the tubes on ice.

   These protoplasts are fragile as they lack a cell wall. Ensure gentle pipetting and do not jostle the Falcon tubes.

9. Gently overlay the protoplast suspension with 6 ml POS, taking care not to disturb the interphase.

   This step begins the density gradient centrifugation collection step, which enables the separation of the protoplasts from other cell types and cellular debris.

10. Centrifuge the solution for 15 min at 1500 × *g*, 4°C.

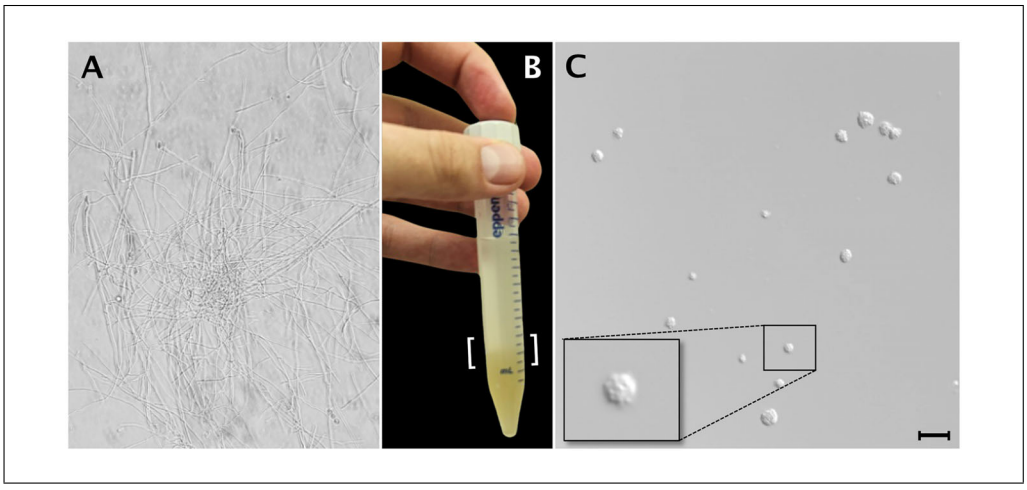

Figure 2 Protoplasting of *Ceratocystis* species. (**A**) Mature mycelia grown from *C. fimbriata* (CMW 14799) conidia, to be used as the starting material for the digestion. (**B**) Sorbitol overlay of the protoplasts, showing distinct layers. The region wherein the protoplasts are expected to settle (between 3 and 4 ml) is indicated by the white bracket. (**C**) Extracted protoplasts after ~3 hr of digestion and isolation. Scale bar, 10 µm. Inset shows a zoomed-in protoplast (C).

11. Transfer ~1 ml of the cloudy interface layer from each tube into a single new 50-ml Falcon tube (resulting in a total of 4 ml) and add 4 ml SBB (Fig. 2B).

    The cloudy interface layer may be difficult to visualize. The protoplasts are typically found in the layer between the 3- and 4-ml measurement lines in a 15-ml Falcon tube.

12. Centrifuge the samples for 10 min at 1000 × *g*, 4°C, and discard the supernatant.
13. Wash the pellet with 20 ml SBB and centrifuge again.
14. Wash the pelleted protoplasts with 5 ml STC buffer A and centrifuge 10 min at 1000 × *g*, 4°C. Perform this step twice.
15. Gently resuspend the remaining protoplasts in 200 µl STC buffer A (Fig. 2C).
16. Count the protoplasts using a hemocytometer (see Common Considerations in article introduction). Dilute to the required concentration using STC buffer A.
17. If the protoplasts are to be used for future experiments, add DMSO to a final concentration of 7% (v/v), aliquot into cryotubes precooled in liquid nitrogen, snap-freeze in liquid nitrogen, and store the protoplast solution at –80°C for up to 6 months. If not stored, use directly.
18. If stored, thaw the protoplasts on ice, pellet by centrifugation for 10 min at 1000 × *g*, 4°C, and wash once with STC buffer A prior to use for downstream applications.

BASIC PROTOCOL 2

PROTOPLAST EXTRACTIONS FROM *FUSARIUM CIRCINATUM*

Fusarium circinatum (Nirenberg & O'Donnell, 1998) is the causal agent of pine pitch canker and one of the most destructive pathogens of *Pinus* species globally (Wingfield et al., 2008). The fungus is thought to have originated in Mexico and the Caribbean, from which it has spread to many countries, posing a significant threat in natural forests and plantation forestry (Drenkhan et al., 2020). *F. circinatum* can infect all life stages of the plant. In mature pine trees, infection results in dieback and the formation of resinous cankers, leading to the common name "pitch canker disease." Infection in nursery seedlings often results in a high mortality rate due to wilting, damping-off, and root and collar rot (Wingfield et al., 2008). As a result, disease epidemics have negative economic impacts due to yield losses and reductions in timber quality (Mitchell et al., 2011; Wingfield et al., 2008). Although much is known about this pathogen, there is currently a gap in understanding its virulence mechanisms, with only three pathogenicity-associated genes having been functionally characterized to date (Phasha et al., 2021a, 2021b; van Dijk et al., 2025). Here, we present a protocol for the extraction of protoplasts using Extralyse, which can be used in downstream applications like genome editing, in turn enabling deeper insights into this important plant pathogen.

Materials

Potato dextrose agar, half strength ($\frac{1}{2}$PDA; see recipe)
Cultures of *Fusarium circinatum* (isolate CMW 350), maintained in the culture collection of the Forestry & Agricultural Biotechnology Institute (FABI) at the University of Pretoria
Potato dextrose broth, quarter strength ($\frac{1}{4}$PDB; see recipe)
Distilled water, autoclaved
Dithiothreitol-ethylenediamine tetraacetic acid (DTT-EDTA; see recipe)
Enzyme solution 2 (see recipe)
1.2 M potassium chloride (KCl; see recipe)
Sorbitol/Tris·Cl/$CaCl_2$ (STC) buffer B (see recipe)
Dimethyl sulfoxide (DMSO; see recipe)

Laminar flow hood
60-mm petri plates
Scalpel and blade
250-ml Erlenmeyer flask
Shaking incubator
100-µl to 1-ml pipets and appropriate pipet tips
50-ml Falcon tubes
Centrifuge
1.5-ml Eppendorf tubes
Microscope slides and cover slips
Light microscope
Hemocytometer
2-ml cryotubes

1. Inoculate the center of 60-mm petri plates containing $\frac{1}{2}$PDA with a mycelial block and incubate for 7 days at 25°C.
2. With a scalpel, excise a pea-sized amount of mycelium from the leading edge of growth on an active culture and use this to inoculate 100 ml PDB in a 250-ml Erlenmeyer flask.
3. Incubate the culture with shaking at 150 rpm for 36 hr at 25°C (Fig. 3A).
4. Transfer the resultant culture into 50-ml Falcon tubes and centrifuge for 15 min at $>3200 \times g$, 4°C.
5. Discard the supernatant, resuspend the pellet with 10 ml distilled water, and centrifuge as in step 4.
6. Discard the supernatant, resuspend the pellet in 1 ml distilled water, transfer to a 1.5-ml Eppendorf tube, and centrifuge as in step 4.
7. Discard the supernatant, resuspend the mycelia in 1 ml of DTT-EDTA solution, and incubate 1 hr at room temperature.

This step is a form of pre-treatment that is essential for formation of protoplasts (see Commentary for further details).

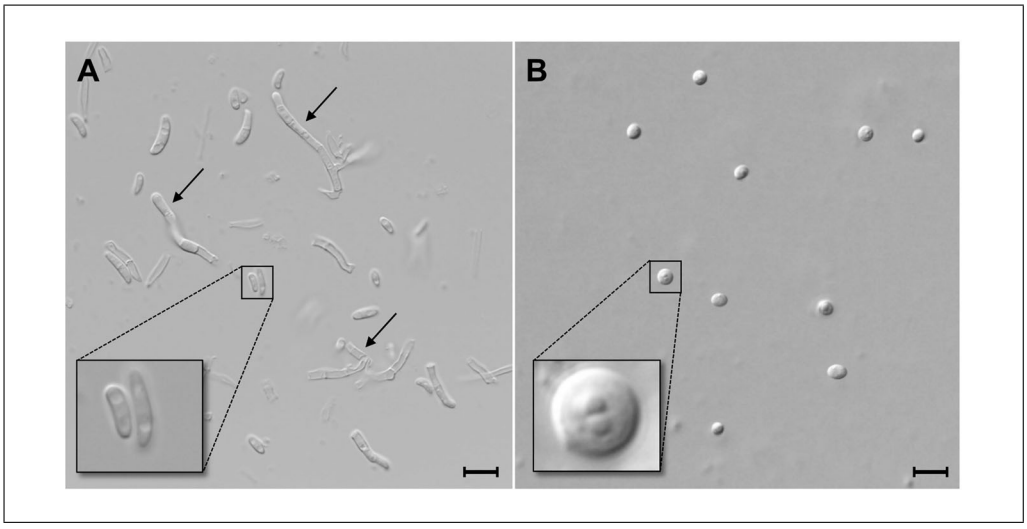

Figure 3 Protoplasting of *Fusarium circinatum*. (**A**) A combination of conidia and mycelia from *F. circinatum* (CMW 350) after a 36-hr incubation in potato dextrose broth. This is the material to be used as the starting material for the digestion. (**B**) Extracted protoplasts after digestion for ~24 hr. Scale bars, 10 µm. Insets shows two zoomed-in conidia (A) and a zoomed-in protoplast (B), and arrows point at mycelium (A).

8. Centrifuge the solution for 15 min at >3200 × *g*, 4°C, and discard the supernatant.
9. Resuspend the mycelium (Fig. 3A) in 1 ml of enzyme solution 2 and incubate for 24 hr at 25°C.
10. Check the progress of the digestion at 20, 22, and 24 hr (see Common Considerations in article introduction).
11. Pellet the protoplasts by centrifugation for 10 min at 1000 × *g*, 4°C, and discard the supernatant.

    These protoplasts are fragile as they lack a cell wall. Ensure gentle pipetting and do not jostle the Falcon tube.

12. Gently resuspend the protoplasts in 1 ml of 1.2 M KCl and centrifuge 10 min at 1000 × *g*, 4°C. Discard the supernatant.
13. Gently resuspend the protoplasts in 1 ml of 1.2 M KCl (Fig. 3B).
14. Count the protoplasts using a hemocytometer (see Common Considerations in article introduction). Dilute to the required concentration using STC buffer B.
15. If the protoplasts are to be used for future applications, add DMSO to a final concentration of 7% (v/v) and store at –20°C in suitable cryotubes for up to 1 year. If not stored, use directly.
16. If stored, thaw the protoplasts on ice prior to use for downstream applications.

BASIC PROTOCOL 3

PROTOPLAST EXTRACTIONS FROM *METARHIZIUM ACRIDUM, METARHIZIUM BRUNNEUM*, AND *METARHIZIUM GUIZHOUENSE*

The genus *Metarhizium* accommodates a wide variety of fungal species which act as both insect pathogens and plant symbionts (St. Leger, 2024). These species can contribute to plant health, particularly in agricultural settings, by suppressing insect pests like locusts and other arthropods (Kabaluk & Ericsson, 2007; Wang & Feng, 2014) while promoting plant growth by facilitating nutrient transfer to the plant (Behie et al., 2012). Several species in the genus *Metarhizium* are widely used as biocontrol agents against insect pests (Brunner-Mendoza et al., 2019; Wang & Feng, 2014) and are being investigated as plant-growth-promoting agents as well. The ability to functionally characterize genes putatively associated with complex biological traits such as pathogenicity and virulence (Sevim et al., 2012; Tong et al., 2020), host range (Zhang et al., 2019), and root-colonizing capacity (Fang & St. Leger, 2010; Hu & Bidochka, 2021) is thus essential for a more in-depth understanding of these species and how they interact with their plant and insect hosts. We present here an efficient protoplast extraction protocol using Extralyse for these species.

Materials

Cultures of *Metarhizium acridum* (isolate ARSEF 3391) and *Metarhizium guizhouense* (isolate ARSEF 4153, both maintained in the culture collection (ARSEF) of the U.S. Department of Agriculture's Agricultural Research Service
Cultures of *Metarhizium brunneum* (isolate KVL 12-30) maintained at the culture collection (KVL) of the Section for Organismal Biology at the University of Copenhagen)
Sabouraud dextrose agar with yeast, quarter strength ($\frac{1}{4}$SDAY; see recipe)
0.5% Triton-X (see recipe)
0.05% Triton-X (see recipe)
Sabouraud dextrose yeast broth (SDY; see recipe)
1.2 M potassium chloride (KCl; see recipe)
Enzyme solution 3 (see recipe)
Sorbitol/Tris·Cl/$CaCl_2$ (STC) buffer C (see recipe)

Laminar flow hood
90-mm petri plates
Plate spreader
100-µl to 1-ml pipets and appropriate pipet tips
50-ml Falcon tubes
Centrifuge
250-ml Erlenmeyer flasks
Shaking incubator
50-ml Erlenmeyer flask, sterile
Microscope slides and cover slips
Light microscope
Miracloth (Merck, cat. no. 475855), one layer: place one layer of Miracloth in a funnel, cover with foil, and autoclave before use
Miracloth (Merck, cat. no. 475855), two layers: place two layers of Miracloth in a funnel, cover with foil, and autoclave before use
15-ml Falcon tube
Hemocytometer
2-ml cryotubes

1. Harvest conidia (Fig. 4A) from 10- to 14-day old cultures grown on $\frac{1}{4}$SDAY medium. Wash the plate with 10 ml of 0.5% (v/v) Triton-X, scrape gently with a plate spreader, and transfer the conidial solution into a 50-ml Falcon tube.
2. Centrifuge for 5 min at 5000 × *g*, room temperature, and wash with 10 ml of 0.05% (v/v) Triton-X. Repeat this step twice, resuspending the final conidial mass in up to 10 ml of 0.05% Triton-X.
3. Inoculate 100 ml SDY medium in a 250-ml Erlenmeyer flask with $\sim 10^5$-10^6 conidia.
4. Incubate the liquid culture at room temperature with shaking at 180 rpm for up to 4 days. This produces a mix of conidial germlings and well-established mycelial strands (Fig. 4B).

   Different isolates of the three Metarhizium *species may have different optimal growth times. Ideally, a mixture of conidial germlings and more established mycelia should be present within the liquid culture before the digestion is performed (see Fig. 4B). This requires monitoring every 24 hr.*

5. Collect the mycelia by filtration through one layer of Miracloth. Wash the mycelial mat with up to 5 ml of 1.2 M KCl, and transfer the mat from the Miracloth into a 50-ml Falcon tube.

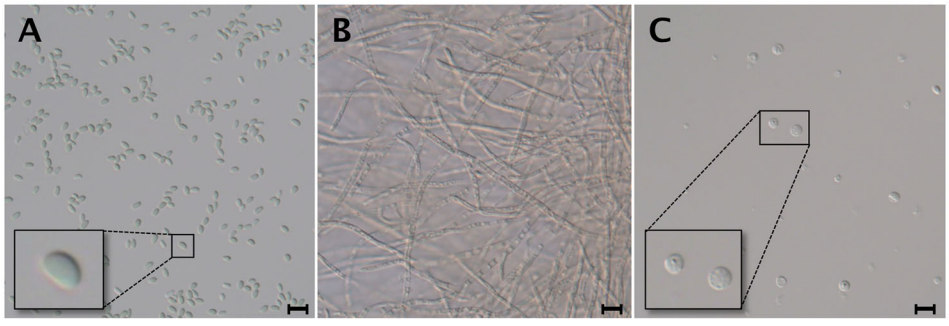

Figure 4 Protoplasting of *Metarhizium* species. (**A**) Conidia from *Metarhizium acridum* (ARSEF 3391). (**B**) Mature mycelium from *Metarhizium brunneum* (KVL 12-30) to be used as the starting material for the digestion. (**C**) Extracted protoplasts from *Metarhizium acridum* (ARSEF 3391) after ~1 hr; some cellular debris is also visible. Scale bars, 10 µm. Insets show a zoomed-in conidium (A) and two zoomed-in protoplasts (C).

6. Resuspend the collected mycelia in up to 10 ml of 1.2 M KCl.

   Depending on the yield of germlings and mycelia, up to 10 ml of KCl may be necessary to resuspend the collected mycelia. Use enough buffer to produce a suspension that is easy to pipet using a standard 1-ml pipet tip.

7. Add 9 ml of enzyme solution 3 to a 50-ml Erlenmeyer flask and add 1 ml of the collected mycelia.
8. Incubate the enzyme mixture at room temperature with shaking at 120 rpm for 1-2 hr.
9. Check the progress of the digestion at 1 and 2 hr (see Common Considerations in article introduction).
10. Filter the enzyme mixture through two layers of Miracloth into a 15-ml Falcon tube. Centrifuge for 10 min at 3000 × *g*, 4°C, to collect the protoplasts (Fig. 4C).
11. Gently resuspend the protoplasts in 2 ml STC buffer C.
12. Count the protoplasts using a hemocytometer (see Common Considerations in article introduction). Dilute to the required concentration in STC buffer C.
13. If the protoplasts are to be used for future applications, store at –80°C in suitable cryotubes for up to 6 months. If not stored, use immediately.
14. If stored, thaw the protoplasts on ice before using them for downstream applications.

BASIC PROTOCOL 4

PROTOPLAST EXTRACTIONS FROM *OPHIOSTOMA NOVO-ULMI*

Various species within the *Ophiostomatales* are recognized for their pathogenic effects on humans, plants, and animals, with some being capable of forming symbiotic associations with insects (de Beer et al., 2022). One prominent tree pathogen belonging to the genus *Ophiostoma*, *Ophiostoma novo-ulmi*, is responsible for Dutch elm disease (DED). The disease occurs due to an interaction between *O. novo-ulmi* and bark beetles, which bore galleries in trees to lay eggs, spreading fungal spores. These spores germinate within the galleries, forming dense mycelial masses that serve as food for beetle larvae. As the beetles move through the galleries, spores adhere to their exoskeletons, facilitating transmission to other trees as mature beetles emerge and infest new hosts (Comeau et al., 2015). Over the past five decades, DED has wreaked havoc on European and North American elm forests on a pandemic scale, while viable treatment options have remained elusive (Martín et al., 2023). To effectively manage and potentially eradicate DED, obtaining an in-depth understanding of the fungal pathogen is imperative. This can potentially be accomplished through functional characterization studies, including gene-editing techniques using protoplast-mediated transformation. As most protocols for extracting protoplasts from *O. novo-ulmi* date back to the 1990s (Richards, 1994; Royer et al., 1991; Temple et al., 1997), we present an updated and optimized protocol utilizing Extralyse for protoplast extraction from *O. novo-ulmi*.

Materials

Cultures of *Ophiostoma novo-ulmi* (isolate CMW 10573), maintained at the culture collection (CMW) of the Forestry & Agricultural Biotechnology Institute (FABI) at the University of *Pretoria*
Malt extract agar supplemented with cycloheximide (MEA+C; see recipe)
Standard medium (see recipe)
Distilled water, autoclaved
Dithiothreitol-ethylenediamine tetraacetic acid (DTT-EDTA; see recipe)

Enzyme solution 4 (see recipe)
MCE solution (see recipe)

Ice
Laminar flow hood
60-mm petri plates
Scalpel and scalpel blade
250-ml Erlenmeyer flasks
Shaking incubator
Whatman no. 1 filter paper (Cytiva, cat. no. 3001-917)
50-ml Falcon tubes
Centrifuge
100-µl to 1-ml pipets and appropriate pipet tips
Microscope slides and cover slips
Light microscope
Hemocytometer

1. Scrape a small section of mycelium from a fully grown plate culture of *O. novo-ulmi* and place it on the surface of a fresh MEA+C plate. Incubate for 7 days at 25°C.
2. Excise an agar block from the leading edge of an actively growing culture using a scalpel and use it to inoculate 200 ml standard medium in a 250-ml Erlenmeyer flask.
3. Incubate the culture with shaking at 150 rpm for 7 days at 25°C.
4. Filter the resultant culture into a new 250-ml Erlenmeyer flask through Whatman no. 1 filter paper to remove the mycelia and harvest the conidia (Fig. 5A). Divide the spore suspension across four 50-ml Falcon tubes.
5. Centrifuge the filtrate for 10 min at 1000 × *g*, 4°C.
6. Discard the supernatant and resuspend the pellet in 5 ml distilled water.
7. Centrifuge the sample for 10 min at 1000 × *g*, 4°C. Discard the supernatant. Repeat this step three times.

Repeating this step ensures the pellet is free from any remaining medium.

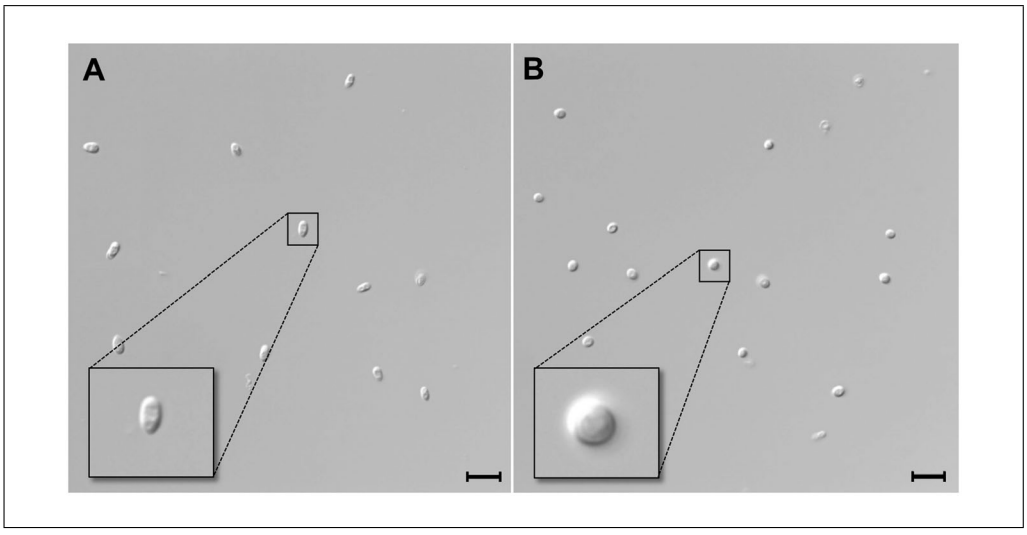

Figure 5 Protoplasting of *Ophiostoma novo-ulmi*. (**A**) Conidia from *O. novo-ulmi* (CMW 10573) to be used as the starting material for the digestion. (**B**) Protoplasts extracted after ~3 hr of digestion. Scale bars, 10 µm. Insets show a zoomed-in conidium (A) and a zoomed-in protoplast (B).

8. Resuspend the cleaned spores in 1 ml DTT-EDTA and incubate at room temperature for 1 hr.

   This step is essential for formation of protoplasts (see Commentary for further details).

9. Collect the treated spores by centrifugation for 15 min at 1000 × *g*, 4°C.
10. Resuspend the spores in 1 ml enzyme solution 4 and incubate for 3 hr at 28°C with shaking at 120 rpm.
11. Check the progress of the digestion at 2 and 3 hr (see Common Considerations in article introduction).
12. Pellet the protoplasts by centrifugation for 10 min at 1000 × *g*, 4°C, and discard the supernatant.
13. Gently resuspend the protoplasts in 1 ml MCE solution and centrifuge as in step 12. Perform this wash step twice to ensure the pellet is free from any remaining enzyme solution.

    These protoplasts are fragile as they lack a cell wall. Ensure gentle pipetting and do not jostle the Falcon tube.

14. Resuspend the protoplasts in 1 ml MCE solution (Fig. 5B).
15. Count the protoplasts using a hemocytometer (see Common Considerations in article introduction). Dilute to the required concentration in MCE solution.
16. Once diluted, transfer the tubes onto ice and use the protoplasts immediately; do not store.

    Storage is not recommended as this will interfere with the quality of the protoplasts.

BASIC PROTOCOL 5

PROTOPLAST EXTRACTIONS FROM *SCLEROTINIA SCLEROTIORUM*

The necrotrophic fungus *Sclerotinia sclerotiorum* is an agriculturally and economically important plant pathogenic fungus affecting many food crops globally (Bolton et al., 2006). It is notable for having a host range exceeding 400 plant species, with disease reports spanning many countries from every continent (Bolton et al., 2006; Purdy, 1979). Currently, there is no single treatment plan for the complete control of *S. sclerotiorum*, with most success involving an integrated management strategy that relies primarily on fungicides (O'Sullivan et al., 2021). Achieving control with fungicides is challenging due to disease variability across planting seasons, weather conditions affecting effectiveness, and the emergence of resistant genotypes (Moellers et al., 2017). A better understanding of the biology of *S. sclerotiorum* may in future lead to more effective disease management strategies (Lan et al., 2023). Target genes that can be useful in managing disease or mitigating the effect of infection can be identified through functional gene studies that rely on efficient transformation protocols (Fan et al., 2017; Lan et al., 2023; Liang et al., 2018; Xiao et al., 2014). Here we report a method for generating *S. sclerotiorum* protoplasts using Extralyse, which can be used for transformation and other downstream applications.

Materials

Cultures of *Sclerotinia sclerotiorum* (isolate CMW 60861), maintained in the culture collection (CMW) of the Forestry & Agricultural Biotechnology Institute (FABI) at the University of Pretoria
Potato dextrose agar, half strength ($\frac{1}{2}$PDA; see recipe)
Potato dextrose broth, full strength (PDB; see recipe)
1.2 M potassium chloride (KCl; see recipe)
Enzyme solution 5 (see recipe)
Sucrose/Tris·Cl/$CaCl_2$ (STC) buffer D (see recipe)

Ice
Laminar flow hood
90-mm petri plates
Cellophane, cut into 80-mm-diameter discs (Bio-Rad, cat. no. 1650922)
Incubator
250-ml Erlenmeyer flasks
Shaking incubator
Cheesecloth: place one layer of cheesecloth in a funnel, cover with foil, and autoclave before use
Lab spatula
Scale
100-µl to 1-ml pipets and appropriate pipet tips
Microscope slides and cover slips
Light microscope
Miracloth (Merck, cat. no. 475855): place two layers Miracloth in a funnel, cover with foil, and autoclave before use
50-ml Falcon tubes
Centrifuge
Hemocytometer

1. Place a cellophane disc on a 90-mm petri plate with $\frac{1}{2}$PDA medium and inoculate with a mycelial block. Incubate the cultures at 25°C for ∼3 days. Monitor growth, as the mycelium should be harvested before pigmentation occurs (Fig. 6A).

   Multiple plates may be needed to obtain the 5 g of mycelia required for later steps, especially if growth is variable. In addition, pigmentation may interfere with the enzyme digestion process of the mycelial cell wall. Therefore, it is best to harvest before pigmentation occurs.

2. Transfer all unpigmented mycelia from the cellophane to a 250-ml Erlenmeyer flask containing 100 ml PDB and incubate overnight at 25°C with shaking at 120 rpm.

3. Filter resultant culture through cheesecloth and wash the mycelial clump with 5 ml of 1.2 M KCl. Remove excess liquid by gently pressing on the mycelium with a lab spatula.

4. Add 5 g (wet weight) of the mycelium to 50 ml enzyme solution 5.

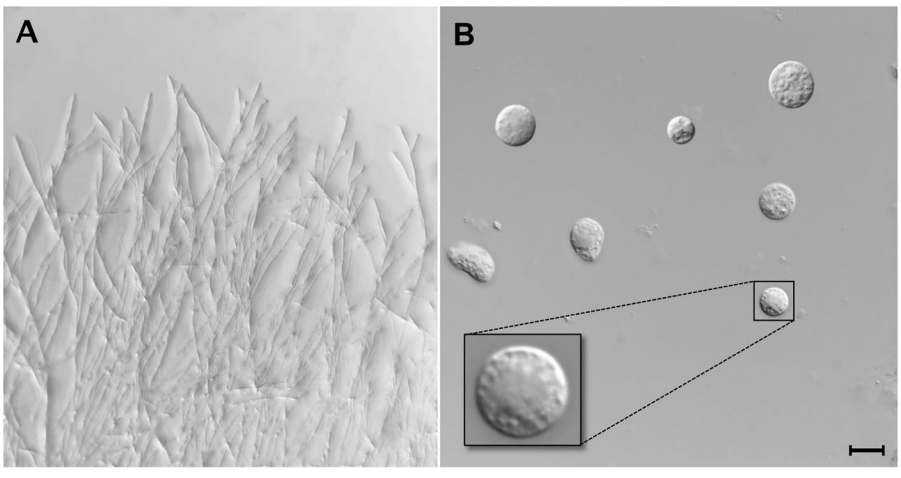

Figure 6 Protoplasting of *Sclerotinia sclerotiorum*. (**A**) Unpigmented mycelia from *S. sclerotiorum* (CMW 60861) to be used as digestion starting material. (**B**) Extracted protoplasts after 3 hr of digestion. Scale bar, 10 µm. Inset shows a zoomed-in protoplast (B).

5. Incubate at 25°C for ∼2 hr with shaking at 90 rpm.
6. Check the progress of the digestion at 1 and 2 hr (see Common Considerations in article introduction).
7. Filter the resulting solution through Miracloth into a 50-ml Falcon tube to remove mycelial debris.
8. Collect protoplasts by centrifugation for 15 min at 2500 × *g*, 4°C. Discard supernatant. From this point on, keep the protoplasts on ice.
9. Gently resuspend the protoplast pellet in 1 ml STC buffer D (Fig. 6B). Centrifuge as in step 8.
10. Remove the supernatant, resuspend in 1 ml STC buffer D, and centrifuge as in step 8.
11. Remove the supernatant and resuspend in 1 ml STC buffer D.
12. Count the protoplasts using a hemocytometer (see Common Considerations in article introduction). Dilute to required concentration using STC buffer D.
13. Use immediately for downstream applications; do not store the protoplasts.

Storage is not recommended as this will interfere with the quality of the protoplasts.

REAGENTS AND SOLUTIONS

Dimethyl sulfoxide (DMSO)

140 µl dimethyl sulfoxide (14% [v/v] final; New England Biolabs, cat. no. 12611S)
Up to 1 ml distilled water
Store up to 2 years at –20°C

Dithiothreitol-ethylenediamine tetraacetic acid (DTT-EDTA)

0.77 g dithiothreitol (50 mM final; Sigma Aldrich, cat. no. 3860-OP)
0.73 g ethylenediamine tetraacetic acid, pH 8 (25 mM final; Sigma Aldrich, cat. no. EDS)
Up to 100 ml distilled water
Filter sterilize using a 0.22-µm-pore-size filter
Store up to 1 year at room temperature

Enzyme solution 1

Dissolve 750 mg Extralyse in up to 15 ml solution A (see recipe; 0.05 g/ml final; Laffort)
Filter sterilize using a 0.22-µm-pore-size filter
Make fresh for each use

Enzyme solution 2

Combine 500 mg Extralyse with 1 ml 1.2 M KCl (0.5 g/ml final; Laffort)
Vortex until dissolved
Filter sterilize using a 0.22-µm-pore-size filter
Make fresh for each use

Enzyme solution 3

Dissolve 500 mg Extralyse in 9 ml 1.2 M KCl (0.5 g/ml final once 1 ml of fungal material is added; Laffort)
Filter sterilize using a 0.22-µm-pore-size filter
Make fresh for each use

Enzyme solution 4

Dissolve 500 mg Extralyse in 1 ml MCE solution (0.5 g/ml final; Laffort)
Filter sterilize using a 0.22-µm-pore-size filter
Make fresh for each use

Enzyme solution 5

Dissolve 5 g Extralyse in 50 ml 1.2 M KCl (0.1 g/ml final; Laffort)
Filter sterilize using a 0.22-µm-pore-size filter
Make fresh for each use

Magnesium sulfate ($MgSO_4$), 1.2 M

144.44 g $MgSO_4$ (1.2 M final; Sigma Aldrich, cat. no. M7506)
Up to 1 L distilled water
Autoclave before use
Store up to 1 year at room temperature

Malt extract agar (MEA)

20 g malt extract (2% [w/v] final; Neogen Culture Media, cat. no. NCM0093)
20 g agar (2% [w/v] final; Neogen Culture Media, cat. no. NCM0238)
Up to 1 L distilled water
Autoclave before use
Store up to 3 months at room temperature

Malt extract agar supplemented with cycloheximide (MEA+C)

20 g malt extract (2% [w/v] final; Neogen® Culture Media, cat. no. NCM0093)
20 g agar (2% [w/v] final; Neogen® Culture Media, cat. no. NCM0238)
Up to 1 L distilled water
Autoclave before use

To supplement with cycloheximide, prepare a 10 mg/ml solution of cycloheximide in water (Sigma Aldrich, cat. no. 239763-M) and filter sterilize using a 0.22-µm-pore size filter. When the media has cooled enough to pour, add 1 ml cycloheximide (0.01 mg/ml final; Sigma Aldrich, cat. no. 239763-M) and pour into 60 mm Petri plates. Store up to 3 months at 4°C.

MCE solution

54.65 g mannitol (0.6 M final; Sigma Aldrich, cat. no. M4125)
12.90 g sodium citrate (0.1 M final; Sigma Aldrich, cat. no. 25114)
1.46 g EDTA (0.01 M final; Sigma Aldrich, cat. no. EDS)
Up to 500 ml distilled water
Adjust pH to 5.8
Autoclave before use
Store up to 1 year at room temperature

Potassium chloride (KCl), 1.2 M

89.46 g KCl (1.2 M final; Sigma Aldrich, cat. no. 529552)
Up to 1 L distilled water
Autoclave before use
Store up to 1 year at room temperature

Potato dextrose agar, half strength ($\frac{1}{2}$PDA)

12 g Potato Dextrose Broth (1.2% [w/v], final; BD Difco, cat. no. 254920)
15 g agar (1.5% w/v, final; Sigma Aldrich, cat. no. 05040)

Autoclave before use and pour into 60-mm Petri plates
Store up to 6 months at 4°C

Potato dextrose broth, full strength (PDB)

25 g Potato Dextrose Broth (2.5% [w/v] final; BD Difco, cat. no. 254920)
Up to 1 L water
Autoclave before use
Store up to 3 months at 4°C

Potato dextrose broth, quarter strength ($\frac{1}{4}$PDB)

6 g Potato Dextrose Broth (0.6% [w/v]; BD Difco, cat. no. 254920)
Up to 1 L distilled water
Autoclave before use
Store up to 3 months at 4°C

Protoplast overlay solution (POS)

Dissolve 54.65 g sorbitol (1.2 M intermediate; Sigma Aldrich, cat. no. 1617000) in up to 250 ml distilled water. Dissolve 0.79 g Tris·Cl, pH 7.5 (20 mM intermediate; Sigma Aldrich, cat. no. 648313) in up to 250 ml distilled water. Autoclave sorbitol and Tris·Cl separately.

Combine 250 ml sorbitol and 250 ml Tris·Cl to reach final concentrations of 0.6 M and 10 mM, respectively. Make fresh and keep at 4°C until use.

Sabouraud dextrose agar with yeast, quarter strength ($\frac{1}{4}$SDAY)

10 g dextrose (1% [w/v] final; Sigma Aldrich, cat. no. 1181302)
2.5 g peptone (0.25% [w/v] final; Sigma Aldrich, cat. no. 90765)
2.5 g yeast extract (0.2% [w/v] final; Sigma Aldrich, cat. no. 07533)
20 g agar (2% [w/v] final; Sigma Aldrich, cat. no. 05040)
Up to 1 L distilled water
Autoclave before use and pour into 90-mm Petri plates
Store up to 3 months at room temperature

Sabouraud dextrose yeast broth (SDY)

40 g dextrose (4% [w/v] final; Sigma Aldrich, cat. no. 1181302)
10 g peptone (1% [w/v] final; Sigma Aldrich, cat. no. 90765)
10 g yeast extract (1% [w/v] final; Sigma Aldrich, cat. no. 07533)
Up to 1 L distilled water
Autoclave before use
Store up to 3 months at 4°C

Separation buffer B (SBB)

Dissolve 91.10 g sorbitol (2 M intermediate; Sigma Aldrich, cat. no. 1617000) in up to 250 ml distilled water. Dissolve 0.79 g Tris·Cl, pH 7.5 (20 mM intermediate; Sigma Aldrich, cat. no. 648313), in up to 250 ml distilled water. Autoclave sorbitol and Tris·Cl separately.

Combine 250 ml sorbitol and 250 ml Tris·Cl to reach final concentrations of 1 M and 10 mM, respectively. Make fresh and keep at 4°C until use.

Solution A

144.44 g magnesium sulfate (1.2 M final; Sigma Aldrich, cat. no. M7506)
Potassium phosphate buffer, pH 5.8 (10 mM final)
1.28 g potassium phosphate monobasic (Merck, cat. no. 529568)

101.73 mg potassium phosphate dibasic (Merck, cat. no. 60353)
Up to 1 L distilled water
Store up to a year at room temperature

Standard medium

10 g sucrose (1% [w/v]; Sigma Aldrich, cat. no. S0389)
20 g L-asparagine (2% [w/v]; Sigma Aldrich, cat. no. 1.01566)
2 g yeast extract (0.2% [w/v]; Sigma Aldrich, cat. no. HG000BX6)
2 g monopotassium phosphate (KH_2PO_4; 0.2% [w/v]; Sigma Aldrich, cat. no. P5655)
0.1 g magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$; 0.01% [w/v]; Sigma Aldrich, cat. no. SAAR4124000)
0.44 mg zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$; 0.000044% [w/v]; Sigma Aldrich, cat. no. 31655)
0.48 mg iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$; 0.000048% [w/v]; Sigma Aldrich, cat. no. 1.03943)
0.36 mg manganese(II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$; 0.000036% [w/v]; Sigma Aldrich, cat. no. 203734)
Up to 1 L distilled water
Autoclave before use
Store up to 3 months at 4°C

STC buffer A

109.30 g M sorbitol (1.2 M final; Sigma Aldrich, cat. no. 1617000)
0.79 g Tris·Cl, pH 7.5 (10 mM final; Sigma Aldrich, cat. no. 648313)
0.55 g $CaCl_2$ (10 mM final; Sigma Aldrich, cat. no. C5670)
Up to 500 ml distilled water
Autoclave before use
Make fresh and keep at 4°C

STC buffer B

109.30 g sorbitol (1.2 M final; Sigma Aldrich, cat. no. 1617000)
0.79 g Tris·Cl, pH 7.5 (10 mM final; Sigma Aldrich, cat. no. 648313)
2.77 g $CaCl_2$ (50 mM final; Sigma Aldrich, cat. no. C5670)
Up to 500 ml distilled water
Autoclave before use
Store up to 1 year at room temperature

STC buffer C

54.65 g sorbitol (0.6 M final; Sigma Aldrich, cat. no. 1617000)
0.79 g Tris·Cl, pH 7.5 (10 mM final; Sigma Aldrich, cat. no. 648313)
0.55 g $CaCl_2$ (10 mM final; Sigma Aldrich, cat. no. C5670)
Up to 500 ml distilled water
Autoclave before use
Store up to 1 year at room temperature

STC buffer D

100 g sucrose (0.6 M final; Merck, cat. no. S0389)
3.94 g Tris·Cl, pH 7.5 (50 mM final; Roche, cat. no. 10708976001)
2.77 g $CaCl_2$ (50 mM final; Merck, cat. no. 1.02378.0500)
Up to 500 ml distilled water
Autoclave before use
Store up to 1 year at room temperature

Triton-X, 0.05%

0.5 ml Triton X-100 (0.05% [v/v]; Merck Millipore, 108603)
Up to 1 L distilled water
Store up to 1 year at 4°C

Triton-X, 0.5%

5 ml Triton X-100 (0.5% [v/v]; Merck Millipore, 108603)
Up to 1 L distilled water
Store up to 1 year at 4°C

COMMENTARY

The protocols presented here detail the extraction of protoplasts from diverse fungal species with relevance in both agricultural and forestry contexts (Brasier, 1991; Drenkhan et al., 2020; Kabbage et al., 2015; Nasution et al., 2019; St. Leger, 2024). Given the positive and negative impacts of these fungi, it is essential to develop a deeper understanding of their biology as well as their interactions with plants and insects within these ecosystems. As such, the ability to extract high-quality protoplasts for downstream applications, such as genome editing and long-read sequencing, is a pivotal step towards filling such knowledge gaps.

The protocols detailed above use a commercially available enzyme mixture produced by Laffort (France), a company that provides various biotechnological solutions for the wine industry. This enzyme mixture, Extralyse, contributes to the wine-making process by accelerating yeast autolysis, likely via enzymatic action on the yeast cell walls. For this reason, Extralyse is suitable for the extraction of protoplasts in other fungi as well. Extralyse has previously been used for protoplast extraction in *Aspergillus nidulans* (Roux & Chooi, 2022) and *Pyrenophora teres* (Syme et al., 2018). A similar wine industry enzyme cocktail called VinoTaste, produced by Novonesis (previously Novozymes), has also been used for protoplast extraction in various filamentous fungi, including *Botrytis cinerea* (Coca-Ruiz et al., 2024) and *Aspergillus fumigatus* (Zhao et al., 2019). The wide use of these enzymes and their cost-effective availability provides an opportunity to establish efficient protoplast extraction protocols for use in a broad range of fungi, expanding the molecular toolkits available to model and non-model fungi alike.

Protoplasts are an important tool for the study of fungal biology, having long been used for karyotyping and genetic transformation in fungi ranging from notorious pathogens to highly sought after truffles. Recent advances in long-read sequencing and genome editing technologies have reinvigorated the use of fungal protoplasts, as they represent a suitable starting material for the extraction of HMW DNA and for genetic transformation. This study provides an important resource for the study of fungi from five different genera and will thereby contribute directly to the further investigation of species with relevance in the agricultural and forestry sectors. The protocols presented here will not only facilitate the extraction of protoplasts for use in numerous downstream applications but also will serve as a foundation for the development and optimization of similar protocols in other fungal species.

Critical Parameters

As a consequence of the taxonomic distribution of the species included here (Table 2), the protocols presented in this study can be used as a key starting point for the establishment, development, and optimization of protoplast extraction protocols for many other filamentous ascomycetes. There are several individual steps in the extraction protocols presented here that may require species-specific optimization. These are described in detail below.

Starting material

The starting material—which encompasses both the cell type and the age of the culture—used for the enzyme digestion can have a significant impact on the quality and yield of the protoplasts (Table 3). There are three common cell types used for protoplast extraction: spores (Cheng & Bélanger, 2000; Moore & Peberdy, 1976); germlings, i.e., germinated conidia (Amalamol et al., 2022; Wilson & Wingfield, 2020); and mycelia (Phasha et al., 2021a, 2021b; Varavallo et al., 2004). Each of these cell/tissue types have different cell wall constituents, which also vary from species to species, and will respond differently to the

Table 3 Starting Material Commonly Used for Protoplast Extraction

Species	Starting material	Age	Selected references
Huntiella omanensis	Germlings	12 hr	Wilson et al. (2020)
Fusarium circinatum	Germlings	10 hr	Phasha et al. (2021a, 2021b)
Gibberella fujikuroi	Mycelia	46 hr	Brückner et al. (1990)
Colletotrichum falcatum	Germlings	24 hr	Amalamol et al. (2022)
Penicillium brevicompactum	Mycelia	24 hr	Varavallo et al. (2004)
Coccidioides immitis	Germlings	12 hr	Reichard et al. (2000)
Dichomitus squalens	Mycelia	16-20 hr	Daly et al. (2017)
Coprinus cinereus	Oidia	N/A	Binninger et al. (1987)
Rhizoctonia solani	Mycelia	63.5 hr	Robinson & Deacon (2001)
Agaricus bisporus	Mushroom gills	N/A	Chen & Hampp (1993)

specific enzymatic capacity of the chosen enzyme solution.

When mycelium is used as the starting material, the age of the culture will also significantly impact the efficiency of the digestion (Table 3). Protoplast yield is typically highest when actively growing mycelium that has not reached stationary phase is used (Farina et al., 2004; Peberdy et al., 1976; Robinson & Deacon, 2001; Wubie et al., 2014). This may be due to the immature nature of the cell wall in younger, actively growing mycelial strands and the deposit of metabolites like melanin in the cell walls of older mycelium (Peberdy et al., 1976). Because fungi grow at different rates and thus reach the stationary phase of growth at different times, the precise age of mycelium suitable for protoplast isolation differs from species to species. For efficient protoplast extraction, young mycelium is needed for some species (e.g., *F. circinatum* and *Coccidioides immitis*), whereas others, such as *Rhizoctonia solani*, require older mycelium (Table 3).

Enzyme concentration and enzyme solution volume

The digestion of the fungal cell wall takes place within a liquid enzyme solution, providing at least two variables for optimization: the enzyme's concentration and the solution's volume. In the protocols presented above, the concentration of Extralyse ranged from 50 to 500 mg/ml and the volume of the digestion ranged from 1 to 50 ml. Notably, the optimal enzyme concentration and solution volume may also be affected by both the type and density of the starting material (Brückner et al., 1990; Farina et al., 2004). For example, a larger digestion volume may be necessary when using mature mycelia as opposed to spores as a starting material. Similarly, a lower concentration of enzyme may be sufficient for digestion of immature mycelium, whereas a higher concentration may be necessary for the digestion of hardy spores. The starting material, enzyme concentration, and digestion volume may thus need to be co-optimized.

Digestion incubation time

The length of time required to release an optimal number of protoplasts will depend on the species and the starting material, and may also depend on the osmotic solution used (see below). Incubation times that are too short will decrease the protoplast yield by preventing the efficient digestion of the cell wall, whereas extended digestion times will decrease the yield due to lysis of the protoplasts formed early in the digestion (Chen & Hampp, 1993). The shortest incubation time proposed in the protocols detailed above was 1 hr (*Metarhizium* spp.) and the longest was 24 hr (*F. circinatum*), suggesting a wide range of potentially suitable incubation times and emphasizing the need for species-specific optimization.

Osmotic solution

The osmotic solution in which the protoplasts are extracted maintains a suitable environment for the fragile cells by reducing the osmotic pressure and stabilizing the cellular membrane. Given that different species have different cellular constituents, the optimal environment, and thus the osmotic solution, will differ from species to species (Peberdy et al., 1976; Peberdy & Ferenczy, 1985).

In general, osmotic stabilizers are comprised of sugars, sugar alcohols, and/or inorganic salts (Peberdy & Ferenczy, 1985). Certain solutes, such as sorbitol and potassium

Table 4 Osmotic Solutions Commonly Used for Protoplast Extraction in Fungi

Species	Primary solute	Concentration	Selected references
Huntiella omanensis	Sorbitol	1 M	Wilson et al. (2020)
Fusarium circinatum	KCl	1.2 M	Phasha et al. (2021a, 2021b)
Colletotrichum falcatum	$MgSO_4$	1.2 M	Amalamol et al. (2022)
Aspergillus nidulans	$MgSO_4$	1.2 M	Koukaki et al. (2003)
Penicillium brevicompactum	NaCl	0.8 M	Varavallo et al. (2004)
Coccidioides immitis	$MgSO_4$	1.2 M	Reichard et al. (2000)
Saccharomyces cerevisiae	Sorbitol	1 M	Ezeronye & Okerentugba (2001)
	Mannitol	0.8 M	Ezeronye & Okerentugba (2001)
Candida albicans	KCl	0.6 M	Marriott (1975)
Kluyveromyces marxianus	Sorbitol	1.5 M	Lyu et al. (2021)
Dichomitus squalens	KCl	0.7 M	Daly et al. (2017)
	$MgSO_4$	0.6 M	
Coprinus cinereus	Mannitol	0.5 M	Binninger et al. (1987)
Agaricus bisporus	KCl	0.35 M	Chen & Hampp (1993)

chloride, are used for a wide variety of species and are thus a suitable starting point for optimization (Table 4; Daly et al., 2017; Dhar & Kaur, 2009; Sharma et al., 2021; Wilson & Wingfield, 2020). In general, inorganic salts are used for filamentous fungi, whereas sugar or sugar alcohol solutions are used for yeasts (Peberdy & Ferenczy, 1985). In three of the five species included here, potassium chloride was used as the osmotic stabilizer.

The concentration of the solute is also essential for creating an osmotically stable environment for the protoplasts (Peberdy et al., 1976). Concentrations of ~0.8 M are typically used, although they can ranges from as low as 0.35 M to as high as 1.2 M and will depend on the species as well as the solute (Table 4).

Additional steps

In addition to the common steps detailed above, some protoplast extraction protocols include pre-treatment or post-digestion steps to improve the purity and/or yield of the extracted protoplasts. Pre-treatment of the fungal material with thiol compounds such as dithiothreitol (DTT) or β-mercaptoethanol before cell wall digestion is a necessary step for species such as *Acremonium chrysogenum* (Hamlyn et al., 1981), *Beauveria bassiana* (Pfeifer & Khachatourians, 1987), and *Saccharomyces cerevisiae* (Ezeronye & Okerentugba, 2001). These compounds are thought to disrupt disulfide linkages within cell wall proteins, thereby making the cell wall more susceptible to enzymatic digestion (Anderson & Millbank, 1966; Peterson et al., 1976). Interestingly, pre-treatment with thiol compounds has no effect on the protoplast yield in some species, such as *Aspergillus nidulans* or *Podospora anserina* (Ferrer et al., 1985; Hamlyn et al., 1981).

A density gradient centrifugation step can be added as a post-digestion step to increase the purity of the extracted protoplasts (Wang & Liu, 2013). Separating the protoplasts from the mycelium, conidia and other cellular debris once the enzymatic digestion is complete can be difficult, and this additional material may negatively impact the downstream applications for which the protoplasts were isolated. The inclusion of a density gradient centrifugation step, as described in the *Ceratocystis* spp. protocol above, allows the separation of cell types with different densities, thus purifying the protoplasts from the denser cellular debris (Ridenour et al., 2012). This step is crucial in protocols for extracting protoplasts in species like *Botrytis cinerea* (Cai & Jin, 2021), *Fusarium verticillioides* (Ridenour et al., 2012), and *Oidiodendron maius* (Bardi et al., 1999) and may thus be necessary for other species where additional cellular material has contaminated the protoplast suspension.

Troubleshooting

Table 5 lists problems that may arise with these procedures along with their possible causes and solutions.

Table 5 Troubleshooting Guide for Protoplast Extractions in Filamentous Fungi

Problem	Possible cause	Solution
Conidia are not being digested	The cell wall is too tough for the enzyme	Incubate the conidia in liquid medium to obtain fresh germlings, which may be more susceptible to digestion
Mature mycelia are not being digested	The cell wall is too tough for the enzyme	Decrease the strength of the liquid medium in which the mycelium is grown to produce weaker mycelial cell walls
Incomplete digestion of the fungal cell wall	Enzyme concentration may be too low	Increase the enzyme concentration
	The concentration of starting material may be too high, exhausting the enzyme available	Decrease the concentration of starting material added
	The reaction volume is too large, preventing proper interaction of the enzyme with the conidia or mycelia	Decrease the reaction volume
Conidia/mycelia have disappeared and no protoplasts are present when enzymatic digestion progress is checked	Over-digestion of the starting material	Decrease the digestion time
	The concentration of the enzyme is too high	Decrease the enzyme concentration
Protoplasts don't form a pellet during centrifugation for collection	Protoplasts are less dense than conidia and mycelia and don't pellet as easily	Decrease the centrifugation temperature to 4°C
Protoplasts are seen in enzymatic digestion but disappear after collection	Improper osmotic solution leading to bursting of the protoplasts	Increase or decrease osmotic concentration; change the solute
	The protoplasts were resuspended too roughly, resulting in degradation	Cut the tip off the pipet tip and resuspend gently
Too much background mycelia after collection	The mycelia were not removed sufficiently during digestion, or the digestion time is too short	Increase the number of layers of Miracloth during filtration; extend the digestion time; add a sorbitol overlay centrifugation step

Time Considerations

Basic Protocol 1: Protoplast extractions from Ceratocystis eucalypticola and Ceratocystis fimbriata

The initial step in this protocol is to produce sporulating cultures from which conidia can be harvested. This step requires only 30 min of hands-on time followed by a 5- to 7-day incubation period. The following step entails harvesting mycelia and inoculating PDA broth for germination, and requires ∼30 min of hands-on time and an overnight incubation period. The process of extracting protoplast takes ∼6–8 hr. Thus, this entire protocol could take up to 8 days to complete.

Basic Protocol 2: Protoplast extractions from Fusarium circinatum

The initial step in this protocol is to produce the culture from which a mycelial block is harvested. This step takes up to 7 days, and only requires ∼1 hr of hands-on time to prepare. Preparation and inoculation of the broth with the mycelial block requires ∼1 hr, after which the culture is incubated for a further 36 hr. This is followed by another 3 hr of hands-on time, during which the mycelium is harvested and transferred into the enzyme solution. An additional incubation step at this stage takes 24 hr, after which the protoplasts are harvested, taking ∼2 hr. In total, the protocol should take a maximum of 11 days, but this predominantly comprises incubation steps, which can be minimized if the researcher has access to actively growing cultures.

Basic Protocol 3: Protoplast extractions from Metarhizium acridum, Metarhizium brunneum, and Metarhizium guizhouense

The initial step in this protocol is to produce sporulating cultures from which the conidia can be harvested. This step takes between 10 and 14 days, but requires only ∼1 hr of

hands-on time to prepare the relevant cultures. The following step includes harvesting and counting the conidia, which should take 2-4 hr. This is followed by a second growth step of up to 4 days and only requires ∼30 min of hands-on time to prepare the liquid cultures. The final steps relate to the actual extraction protocol and should take a maximum of 6-8 hr. Thus, in total, this protocol could take up to 19 days but predominantly comprises incubation steps, the first of which can be minimized if the researcher already has actively growing and sporulating cultures.

Basic Protocol 4: Protoplast extractions from Ophiostoma novo-ulmi

The first step in this protocol involves producing the *O. novo-ulmi* culture from which mycelia is harvested. This step includes <1 hr of hands-on time, but 7-10 days of incubation. The following step involves transferring mycelium to liquid medium, which includes less than 1 hour of hands-on time and a further 7 days of incubation. This is followed by a filtration step, which can take up to 6 hr or be left overnight but only includes ∼30 mins of hands-on work. The remainder of the protocol relates to the protoplast extraction steps and can take 6-9 hr due to various incubation steps. Thus, in total, this protocol will take up to 19 days, most of which consists of various incubation steps that can be minimized if the researcher has access to actively growing cultures.

Basic Protocol 5: Protoplast extractions from Sclerotinia sclerotiorum

The first step in this protocol involves growing fungal mycelia on PDA overlaid with cellophane. Preparing these cultures requires ∼1 hr of hands-on time followed by 3-4 days of incubation. Harvesting of the mycelium and transferring it to PDB requires 1 hr of hands-on time, which is followed by a 15-hr incubation. The final steps relate to the extraction of the protoplasts and collectively take between 4 and 5 hr of hands-on time. Thus, overall, this protocol can be completed in a week, depending on the growth of the initial culture on PDA.

Understanding Results

The primary aim of the protocols presented above is to extract high quality, pure protoplasts. This is achieved through the enzymatic digestion of conidial or mycelial cell walls with a commonly available enzyme cocktail used in the wine industry. The anticipated result of these protocols is thus a solution harboring a high yield of protoplasts that can be used in downstream applications. This yield differs significantly from extraction to extraction, depending on the concentration of the starting material, which is difficult to measure and standardize, particularly when mycelium or a spore/mycelium mixture is used. However, a successful result for these protocols constitutes a pure protoplast solution with limited cellular debris, as is illustrated in Figures 2 to 6.

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Conflict of Interest

The authors confirm that no conflicts of interest exist with regard to the publication of this research. None of the authors are in any way professionally or personally associated with Laffort, the producer of the enzyme used for these protocols.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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