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# Apple Bitter Rot: Biology, Ecology, Omics, Virulence Factors, and Management of Causal *Colletotrichum* Species

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## ABSTRACT

Apple bitter rot is caused by various *Colletotrichum* spp. that threaten apple production globally resulting in millions of dollars in damage annually. The fungus causes a decline in fruit quality and yield, eventually rotting the fruit and rendering it inedible. The pathogen is difficult to keep out of orchards because of its broad host range and transmissibility by rain splash and insects. Once the disease manifests, pathogen identification is difficult due to evolving taxonomy and similar morphology between species. Current management strategies are threatened by an increase in fungicide resistance and regulations on many multisite fungicides, leading to a pressing need for new management options for control. This review aims to summarise the most current knowledge regarding the biology, virulence factors, ecology, omics and emerging management strategies for *Colletotrichum* species that cause apple bitter rot.

**Taxonomy:** *Colletotrichum* species—Domain Eukaryota, Kingdom Fungi, Phylum Ascomycota, Class Sordariomycetes, Order Glomerellales, Family Glomerellaceae, Genus *Colletotrichum*.

**Biology:** Hemibiotrophic pathogen with a wide host range that establishes a biotrophic interaction where it penetrates host plants using appressoria followed by a switch to necrotrophy causing rot symptoms.

**Toxins:** Cercosporin, colletotrichins, colletotric acid, ferricrocin.

**Host Range:** The host range varies by species but largely occurs on dicotyledonous plants and is less prevalent on monocots as well as gymnosperms, ferns, mosses and animals (e.g., insects).

**Disease Symptoms:** Symptoms often manifest as flat to sunken necrotic areas on fruit. Lesions on leaves and fruit can have concentric rings with abundant pathogen sporulation.

**Disease Control:** *Colletotrichum* spp. are primarily managed by single-site quinone outside inhibitor (QoI), methyl benzimidazole carbamate (MBC), demethylation inhibitor (DMI) fungicides, and multisite dithiocarbamate and phthalimide fungicides. Susceptibility may vary with species, strain specificity, or geographic region. Other management options include clean stock production, cultural practices, resistance breeding, and biological control through the introduction of protective or competing microorganisms.

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## 1 | Introduction

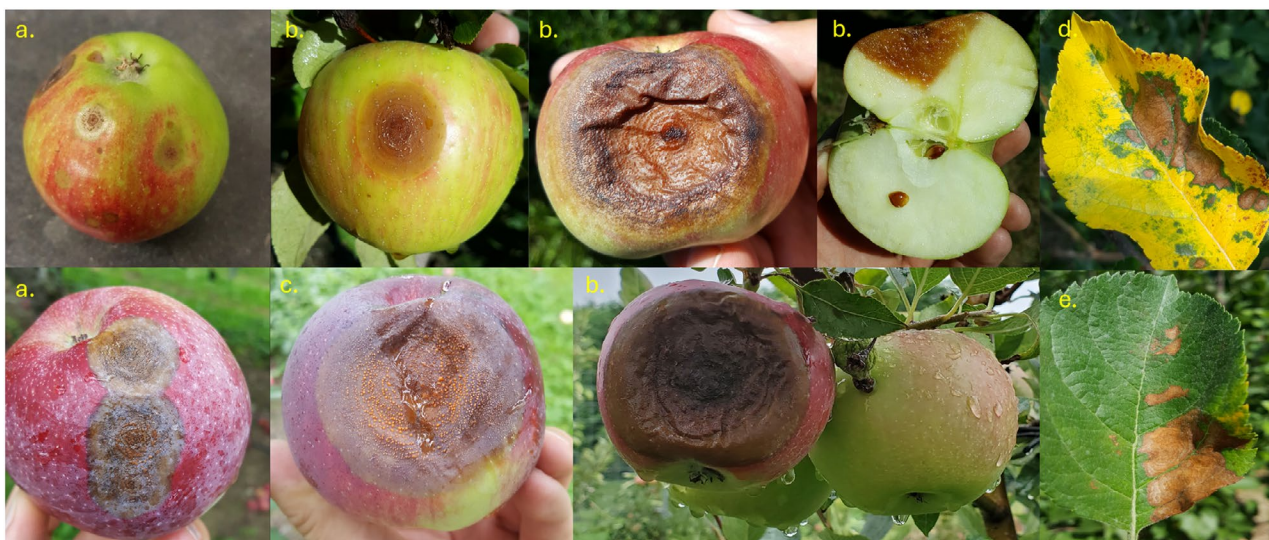
Since the 2022/23 season, apple production in the United States is expected to increase by 1.5% to 4.4 million tonnes in 2023/24 and has an estimated value of \$3.1 billion in 2022 (US Apple 2023; USDA 2023). Bitter rot is caused by several species in the *Colletotrichum* genus and can have significant impacts on the production of apples. The disease can cause significant losses in apple production, ranging from 14% to 100% losses in some organic and conventional orchards (Aćimović 2018; Iungerman 2013). *Colletotrichum* spp. are on the rise across the United States, especially in the Mid-Atlantic region where an estimated \$500 million worth of apples are produced. The spread of *Colletotrichum* spp. is troubling considering recent fungicide resistance studies, which exhibit  $EC_{50}$  values of over 1000 ppm for *C. fioriniae*, *C. chrysophilum*, *C. noveboracense* and *C. siamense* in Group 7 of the Fungicide Resistance Action Committee (FRAC) fungicides (Martin et al. 2022). The FRAC 11 group fungicides that have long been used to control apple bitter rot are also becoming less effective, with some strains of *C. siamense* requiring  $EC_{50}$  values exceeding 100 ppm to suppress growth (Chechi et al. 2019). Alternative management strategies for bitter rot in apples are critically needed as the Environmental Protection Agency (EPA) and United States Department of Agriculture (USDA) are re-evaluating registrations of multisite fungicides effective against apple bitter rot including captan, ziram, ferbam and thiram and implementing new pesticide toxicity evaluation for mammals (EPA 2024).

Investigations of effectors of *Colletotrichum* spp. are growing in prevalence (Groß et al. 2024; Lu et al. 2022; Yuan et al. 2024) and may lead to the development of targeted treatments aimed at disrupting the action of necessary effectors and thus preventing infection. Studies into the classification and detection of *Colletotrichum* spp. are alleviating taxonomic discrepancies and providing avenues for faster, more sensitive detection of these pathogens. Combined with the discovery of fundamental effectors, biological controls and the advent of biorational materials, the avenues for managing bitter rot are promising. However, more work is needed

for research breakthroughs to yield effective solutions in the field and postharvest conditions. We summarise the most up-to-date knowledge regarding the biology, geographic distribution, genomics, effector profiles and management strategies for apple bitter rot causal pathogens. We focus on providing readers with specific details about relevant studies and pooling the findings from several studies to draw more generalised conclusions.

## 2 | Bitter Rot Biology and the Postharvest Lifestyle

Symptoms of bitter rot are typically characterised by circular lesions ranging from 0.5 to 6 cm. Lesions are flat to sunken, light brown to brown and progress to the core of the fruit in a V-shaped pattern. Orange to brown fruiting structures can be visualised as concentric rings developing during high relative humidity and are typically produced from small black acervuli on the surface of the fruit (Figure 1) (Alaniz et al. 2012). On leaves, the disease is called Glomerella leaf spot (GLS) and displays different symptoms to those on fruit. GLS appears as small purple spots on the surface of the leaf. As symptoms progress, concentric rings in angular brown lesions become visible on the leaf's surface and chlorotic halos develop around them (Villani 2018). Importantly, however, *Colletotrichum* infections on leaves and fruit are unlikely to overlap (i.e., infection of leaves may not serve as an inoculum source for fruit) because of differences present in the transcriptome during the development of these two diseases (Andrello et al. 2024; Gan et al. 2013; Rockenbach et al. 2016). *Colletotrichum* reproduction is mainly asexual and typically relies on an endophytic lifestyle until the switch triggering the necrotrophic phase occurs. The disease is spread primarily by conidia transported by rain splash and could move into orchards from nearby forests that serve as a reservoir for some species. Some insects, such as honeybees, can spread spores of *Colletotrichum* through faecal matter. Spores transmitted in this way can survive in the digestive tract of the bees for at least 24 h and have a viability of around 1%. Though this seems small, the transmission



**FIGURE 1** | *Colletotrichum* infections on fruit and leaves of *Malus domestica*. (a) Empire apples displaying symptoms of bitter rot. (b) Honeycrisp apples displaying symptoms of bitter rot. (c) McIntosh apples displaying symptoms of bitter rot. (d) Jonagold apple leaf displaying symptoms of Glomerella leaf spot (GLS). (e) Fuji apple leaf displaying symptoms of GLS.

probability exponentially increases towards 1.0 if over 10 workers consume at least 60,000 spores which are viable in sucrose solutions (50% sucrose) up to 3 days (Parish et al. 2019). In rare instances, when the sexual stage of *Colletotrichum* is present during infection in apples, airborne ascospores could also serve as a vehicle for fungal spread (Martin and Peter 2021). The formation of ascospores is only likely during highly favourable environmental conditions and budding ascospores have only recently been observed in vivo on a closely related but nonpathogenic to apple species *C. lindemuthianum* (Martins et al. 2023).

Many species of *Colletotrichum* follow a general hemibiotrophic lifestyle. This involves an initial biotrophic development where the fungus infects the host plant through the development of appressoria that penetrate the host's cells. The pathogen then spreads by growing hyphae designed to destroy and feed on host tissues (McDowell 2013). During early biotrophy (22 hours post-infection [hpi]), effectors and secondary metabolic pathways are at their highest expression, with effectors continuing later into the biotrophic phase (40 hpi). Necrotrophy is indicated by the downregulation of secondary metabolism and effector genes while membrane transporters and carbohydrate-active enzymes are upregulated (O'Connell et al. 2012). The exact molecular mechanisms that induce the transition from late biotrophy to necrotrophy are still unknown and only a few proteins have been shown to be critical for the transition (Bhadauria et al. 2011). There are notable differences in the lifestyles of each *Colletotrichum* species, including the location and timing of necrotrophic switches, the presence of a biotrophic stage, or a latency period between infection and necrotrophy (De Silva et al. 2017). The modulation of the transition is less likely to be controlled by environmental signals and instead is most likely linked to metabolite levels and nutrient availability within host cells. For example, the depletion of glycerol in plant tissues due to *Colletotrichum* infection may help signal the switch to necrotrophy so the pathogen can access nutrients for continued growth (Wei et al. 2004). During postharvest conditions, where fruits are transported and kept in climate-controlled warehouses, damage to the fruit can occur. Mechanical damage, insects, or the development of physiological disorders in postharvest conditions can create a breach point for the pathogen to enter more vulnerable host tissues (Prusky et al. 2013). Further cues for the necrotrophic switch in postharvest conditions may include fruit ripening, which often correlates to a weakening of host defences. As the fruit softens during the ripening process in cold storage, the fruit breaks down cell walls and weakens key defence mechanisms for pathogen recognition (Cantu, Vicente, Greve, et al. 2008; Cantu, Vicente, Labavitch, et al. 2008). These developments in postharvest conditions allow *Colletotrichum* to exit the biotrophic phase and develop necrotrophic hyphae that penetrate deep into the fruit and cause typical bitter rot symptoms (Prusky et al. 2013).

### 3 | Geographic Distribution and Optimal Conditions for *Colletotrichum* Growth

The major species complexes that cause apple bitter rot are *C. gloeosporioides* and *C. acutatum*, with the former being preferential to warmer climates and the latter favouring cooler

climates. A majority of *Colletotrichum* spp. exhibit growth ranging from 10°C to 35°C, while some species in the *C. gloeosporioides* clade can grow at temperatures exceeding 35°C. Sporulation typically occurs around 25°C for most *C. gloeosporioides* spp. while *C. acutatum* complex members sporulate around 19°C (Salotti, Ji, and Rossi 2022). Conidial germination temperatures are much higher in *C. gloeosporioides* clades, with an optimal temperature range of 25°C–31°C while *C. acutatum* spp. germinate at 21°C–26°C. We suspect that some species of *C. acutatum* and *C. gloeosporioides* may also be better suited to differences in elevation (Khodadadi, Santander, et al. 2023). For example, the distribution of *Colletotrichum* spp. in the Mid-Atlantic shows that *C. fiorinae* is the most prevalent and widely distributed species across the region, especially in Pennsylvania. Isolates from southern New York exhibit a higher prevalence of *C. gloeosporioides* spp. compared to Pennsylvania. At the time of collection, the average summer temperatures between the two regions differed by only 0.4°C (Martin et al. 2021; Khodadadi, Santander, et al. 2023). This difference could be explained by cooler temperatures in higher elevations in Pennsylvania, while the lower lying areas in New York exhibit a higher prevalence of *C. gloeosporioides* spp. (Martin et al. 2021).

Global distribution highlights that the *C. gloeosporioides* complex is present in most of Asia including Japan and Indonesia, Australia and New Zealand, most of the Americas and Europe. Observations at a global level highlight that changes in elevation may account for some differences in geographic distribution. The species in *C. acutatum* complex are present across Australia, most of Europe and South America, the United Kingdom, the United States and some areas of southern Africa (Talhinhas, Baroncelli, and Baroncelli 2021). A study conducted in South Tyrol (a high-elevation apple-producing region in Italy) assessed the presence of various *Colletotrichum* species in packing houses in the region. They found 19 total isolates that were identified by multilocus sequencing to all be members of the *C. acutatum* species complex (Carneiro and Baric 2021). An additional 58 isolates were found in Belgium that were linked to the *C. acutatum* species complex (Grammen et al. 2019). In Argentina, all 31 isolates assessed in a study of low-chill apple cultivars were found to be members of the *C. gloeosporioides* complex. In a 2005 report, *C. gloeosporioides* and *C. boninense* were identified as the most common species in New Zealand. However, recent developments in *Colletotrichum* taxonomy may help distinguish the specific species present in that region. This is because both *C. gloeosporioides* and *C. boninense* have been reclassified into new species complexes (Damm et al. 2012; Johnston, Pennycook, and Manning 2005). In light of global climate change increasing surface temperatures across the globe, it is not unreasonable to assume that *C. acutatum* species might be decreasing in prevalence across increasing warming temperate zones because those regions no longer support *C. acutatum* optimal growth conditions (Dmuchowski, Baczewska-Dąbrowska, and Gworek 2022).

Beyond temperature, bitter rot infection is highly dependent on plant surface wetness, which has an inverse relation to the average temperature. Higher cumulative wetness hours lead to an increased likelihood of infection. When taken together, temperature and surface wetness can predict the infection pressure that correlates positively with observed bitter rot incidence.

From surface wetness and temperature data, models have been developed to predict infection and may give growers early warnings when conditions are favourable for *Colletotrichum* growth (Carraro et al. 2022; Crusius et al. 2002). Some advanced models may also account for plant nutrition, soil conditions, sun exposure and physiological aspects, among others (Moreira et al. 2020).

## 4 | *Colletotrichum* Omics

The omics field (genomics, transcriptomics, metabolomics, proteomics) has fuelled the discovery of fungal virulence factors. As it stands, we know more now about the signalling pathways, regulators and small molecules, in part due to the powerful systems-based omics modality. Integrating different omics technologies with functional studies further helps to elucidate gene, protein and molecule function in host–parasite interactions. For *Colletotrichum* spp. foundational achievements in genome and transcriptome work will be discussed.

### 4.1 | Advances in *Colletotrichum* Classification and Detection Through Genomic Approaches

The *Colletotrichum* genus is represented by over 200 known species, whose genetic variability is diverse and at times confusing from a taxonomic perspective. The first genome of *Colletotrichum* was completed in 1991 for the maize pathogen *C. graminicola*, which now serves as one of the model pathogens for genetic studies of the genus, alongside *C. higginsianum*, which was sequenced later in 2004 (O'Connell et al. 2004; Vaillancourt and Hanau 1991). Genome sizes in the first set of sequenced *Colletotrichum* spp. ranged from 53 Mb to 58 Mb, with later sequences of *C. orbiculare* reaching up to 88 Mb (Gan et al. 2013; O'Connell et al. 2012). Early reports cited the confounding nature of the genus's genetics and attempted to develop common tactics for the correct taxonomic classification of *Colletotrichum* species (Hyde et al. 2009). Original attempts at taxonomic classification were based loosely on morphological characteristics, which saw issues due to a lack of standardised cultivation and ambiguous characteristics that were inadequate to differentiate many species (Denoyes and Baudry 1995; Peres et al. 2002). Further attempts at characterising *Colletotrichum* spp. included analysis of secondary metabolite profiles, pathogenicity testing, cross mating, physiology, carbon source utilisation and molecular phylogeny (Cai et al. 2009). Later studies leveraged a polyphasic approach to differentiating the numerous *Colletotrichum* spp. to correct the issues of using one singular approach to determine similarities between the species. The hope was eventually to develop a single DNA barcode that could be used to differentiate *Colletotrichum* spp., which was found commonly across all species. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), actin (*ACT*), chitin synthase (*CHS*), histone 3 (*HIS3*) and  $\beta$ -tubulin (*TUB2*) were all original contenders (Cai et al. 2009). Later studies, however, would find that no single DNA barcode marker would be suitable for correctly identifying and sorting all *Colletotrichum* species. A successful use of multilocus identification for an apple bitter rot pathogen occurred in 2014, which identified *C. godetiae* in the United Kingdom using the rDNA internal transcribed spacer (ITS), *TUB2* and *GAPDH*

(Baroncelli et al. 2014). The bitter rot causal agents, however, are more elusive than identification by multilocus sequencing suggests. For example, a 2020 study found that the *C. gloeosporioides* complex was best differentiated by an intergenic spacer between DNA lyase and the mating-type locus (*APN2/MAT-IGS*), intergenic spacer between *GAPDH* and a hypothetical protein (*GAP2-IGS*) and actin DNA lyase (*APN2*) markers while *C. acutatum* species were best differentiated by *HIS3*, *GAPDH* and *TUB2* (Khodadadi et al. 2020). This suggests that different complexes and perhaps even individual species may need a personalised suite of markers for reliable identification.

While both species complexes are major causal agents of bitter rot on apples, their differences in determining classification highlight a challenge in the rapid identification of the pathogen for the further application of species-specific treatment approaches (Vieira et al. 2020). Hence, translating genomics information can help aid a multilocus sequencing approach of *Colletotrichum* spp. to better differentiate the complex genus. This approach leverages a collection of conserved genes across *Colletotrichum* spp. including *GAPDH*, ITS and *TUB2*, among other loci by mining the genomes for diagnostic and phylogenetically informative loci. Combining analysis of these conserved molecular markers alongside morphological studies revealed a novel *Colletotrichum* species causing apple bitter rot, *C. noveboracense* and for the first time reported a previously described *C. chrysophilum* as a causal agent for bitter rot on apples (Khodadadi, Santander, et al. 2023). Current efforts are directed towards using genome sequencing to further refine the *Colletotrichum* spp. taxonomy, secondary metabolites, carbohydrate enzymes and effector proteins (Khodadadi, Giroux, et al. 2023).

With the development of taxonomic breakthroughs, highly sensitive detection methods can be leveraged to determine inoculum levels of rot-causing pathogens. Detection measures for *Colletotrichum* are critical for the development of bitter rot resistant apple trees and for minimising the risk of single-site fungicide resistance (McHenry and Aćimović 2024). Recently the use of a filter disc DNA extraction loop-mediated isothermal amplification (FDE-LAMP) has been shown to detect low levels (as low as 1 pg) of *C. siamense* from infected tea plants in the field in approximately 20 minutes without the use of specialised instruments (Zou et al. 2023). A real-time PCR assay was developed for species-specific detection of *Colletotrichum* species targeting major causal agents of apple bitter rot (*C. chrysophilum*, *C. fiorinae*, *C. fruticola*, *C. gloeosporioides sensu stricto* *C. henanense*, *C. noveboracense*, *C. nymphaeae*, *C. siamense* and *C. theobromicola*) for faster species-specific detection than traditional multilocus sequence typing). Using five genes across the nine species, the assay could detect differences among some species with as little as 0.5 pg of sample. The least efficient primer-probes set required only 5 pg of sample for high-efficiency amplification (McHenry and Aćimović 2024). Approaches like these can provide timely information for growers about the level of inoculum in their orchards, allowing managers to make more informed decisions about the timing and rates of treatments they apply. Further research into year-round inoculum levels in the orchard may allow growers to adopt a more dynamic spray programme that targets *Colletotrichum* species when they are most abundant. Beyond PCR-based detection methods, a 2018 study showed that optical coherence tomography could accurately

determine disease development from the initial symptom to the infected region (Wijesinghe et al. 2018). However, we suspect that this method could not differentiate between *Colletotrichum* infections and infections developed from other rot-causing pathogens (Johnson et al. 2024).

## 4.2 | Transcriptomics

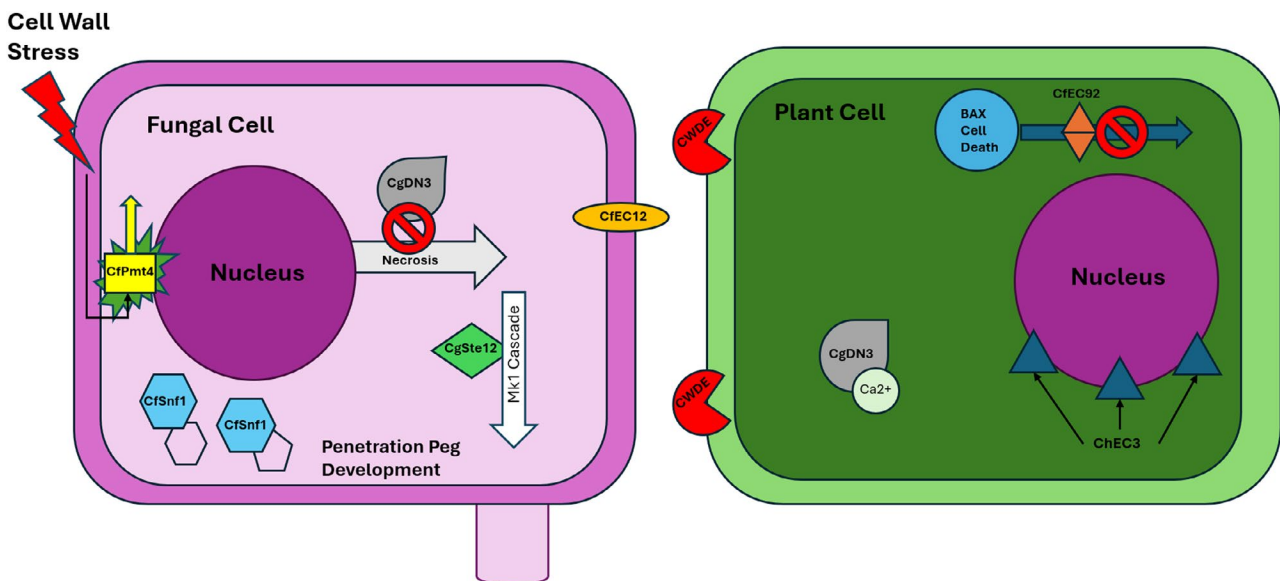
Transcriptomic analyses of *Colletotrichum* spp. focus on the discovery of unknown genes encoding putative effector proteins that are expressed during infection. Thus, the function would be ascertained by targeted gene deletion experiments to define their roles and if promising, can be the subject of novel management strategies. *C. fruticola* was chosen for transcriptomic analysis of candidate virulence factors during infection in a 2018 study that defined 88 secondary metabolite synthases (SM) and 52 clusters, 552 small secreted proteins (SSPs), 1129 carbohydrate-active enzymes (CAZs) and 96 secretory proteases. Of the genes identified, 180 were upregulated, with a majority being SSPs (3.2-fold enrichment  $p=1.2e-5$ ), SMs (6.8-fold enrichment  $p=2.6e-4$ ) and CAZs (1.6-fold enrichment  $p=0.03$ ). Of the SSPs isolated, 30 members showed specific in planta expression or had similarities to known fungal effectors, including CECs 11, 28, 31 and 33 (ChEC11, ChEC28, ChEC31 and ChEC33) (Liang et al. 2018). The expression of specific factors within those groups varies between species and infection types. *Colletotrichum* spp. are also causing GLS and often, the same species of pathogen can be a causal agent for GLS and bitter rot in apples (Andrello et al. 2024). Transcriptomic analysis, however, highlights the difference in expression levels of single genes during the infection process of *C. aenigma* isolates

from a GLS infection and *C. gloeosporioides*-derived bitter rot infections. Compared to two bitter rot isolates, the GLS infection exhibited upwards of 95% differential, single gene expression (Jiang et al. 2022). Although it is difficult to discount the possibility of differential expression due to species differences, both belong to the *C. gloeosporioides* species complex and share similar characteristics (Gan et al. 2013).

RNA-seq and comparative transcriptome profiling have become routine in host-parasite interactions. However, the field still lacks the tools to analyse gene function at a systems level. Thus, reliance on a reductionist, single-gene deletion strategy remains a contemporary strategy. Albeit a powerful tool, in vivo gene deletion has defined limitations for analysing the function of genes within families and multicopy orthologues thus necessitating innovation and development of breakthrough techniques and technologies to advance the science forward that can exceed the current pace of gene discovery.

## 5 | Virulence Factors

Elucidation of fungal virulence factors has been accomplished using a variety of methods that include classical biochemistry, cell biology and functional genetics to ascertain the function of specific loci in the host-plant interaction. In the case of the hemibiotrophic lifestyle, *Colletotrichum* spp. rely on the production of virulence factors that target host plant systems to establish successful infections (Figure 2). Across hosts and species, the effectors that *Colletotrichum* uses are largely conserved or have orthologues, with some species containing as little as 4% unique effector genes (Lu et al. 2022). Throughout the lifecycle,



**FIGURE 2** | Effectors present during *Colletotrichum* infection. The hypothesised localization and interaction targets of effectors that are upregulated during infection are shown above. Cell wall-degrading enzymes (CWDE) like PGTE are leveraged by *Colletotrichum* to break down host cell walls. ChEC3 has a hypothesised role in nuclear expansion and plant cell death. It has been found to localise to host cell nuclei. Conversely, CFC92 is an effector used to interfere with BAX related plant cell death pathways. CFC12 has suspected role for full virulence and contains a domain found in many extracellular membrane fungal proteins. CgSTE12 is a significant protein used in penetration peg development but not appressorium formation. CgDN3 has suppressive effects on calcium signalling in plants by recruiting calcium to binding sites and plays a role in control of the necrotrophic switch. Cfpmt4 is a virulence factor in *Colletotrichum* species that may play a role in regulating cell wall stress responses and appressorium formation and has a role in protein modification. Cfsnf1 allows the fungus to utilise more diverse carbon sources for energy.

the fungus will deploy a variety of factors to facilitate plant infection. The pathogen relies on toxin production and effector proteins as a primary way to halt host immune responses, cause plant cell death, or incite hormonal imbalances in the host. The following selection of effectors relies mostly on *in vitro* or *in vivo* studies of other plant hosts because very little research has been conducted on effectors present specifically in the development of apple bitter rot.

## 5.1 | Cell Wall-Degrading Enzymes

Enzymes targeting plant cell walls are a key step in the successful growth of a necrotrophic pathogen. Therefore, *Colletotrichum* spp. deploy cell wall-degrading enzymes (CWDEs) to break down plant cell walls and provide a point of entry for necrotrophic hyphae and cell-death-inducing effector molecules (Figure 2). Enzymes such as polygalacturonase *trans* eliminase (PGTE), pectin *trans* eliminase (PTE), polygalacturonase (PG) and pectin methyl esterase (PME) have all been identified as part of the *C. gloeosporioides* complex during infection of banana (Jat, Sharma, and Gour 2013). PGs act by cleaving polygalacturonase acid chains, which help degrade cell wall polymers. Pectin lyases (PL) are also present during infection and attack cell walls by cleaving polysaccharide chains. PG is most active at 30°C at pH 5.0 while PL is most active at 45°C at pH 8.5. Furthermore, these enzymes vary in their timing, with PLs highly active at fruit maturity and PGs active at semi-maturity, suggesting that *Colletotrichum* may be using signals from the plant to secrete appropriate CWDEs (Martínez-González, Higuera-Mancipe, and Martínez-Peralta 2018).

## 5.2 | CECs—*Colletotrichum* Effector Candidates

The *Colletotrichum* effector candidates (CECs) are a group of effector proteins found exclusively among *Colletotrichum* spp. with some variation in which species use which effectors. These effectors are typically separated into four stages based on expression from unpenetrated appressoria, penetrated appressoria with nascent biotrophic hyphae, biotrophy to necrotrophy switch and late necrotrophy. Over 90 of these effectors have been identified in *C. higginsianum*, a pathogen of cruciferous plants including *Arabidopsis*, but their conservation across pathogens involved in apple bitter rot infections is still unknown. For example, *Colletotrichum higginsianum* effector candidate 90 (ChEC90) was isolated from model *Colletotrichum* pathogen *C. higginsianum* during transcriptomic sequencing aimed at unveiling in planta expressed effectors. The protein does contain two homologues in *C. graminicola* and has homology to a known effector of *C. lindemuthianum* called *Colletotrichum* Intracellular Hyphae 1 (CIH1), but further homology in causal apple bitter rot species is unsolved (Kleemann et al. 2012).

Some *C. higginsianum* effectors are conserved across more species of interest including *Colletotrichum higginsianum* effector candidate 3 (ChEC3). ChEC3 is a secreted effector protein conserved in four major *Colletotrichum* species including *C. fiorinae*. It has activity that induces plant cell death as well as nuclear expansion in host plant cells (Tsushima et al. 2021). Transgenic expression of ChEC3 indicates that the protein will

localise to mobile punctate structures and nuclear envelopes (Figure 2). It is hypothesized that the association with the nuclear envelope may be playing some role in nuclear expansion. The role in the infection process is not exactly clear, as the absence of this effector does not seem to decrease pathogenicity (Tsushima et al. 2021).

*Colletotrichum fruticola* Effector Candidate 92 (CfEC92) was isolated from *C. fruticola* and only contains homologues found within the *Colletotrichum* genus (Liang et al. 2018). Co-infiltration of CfEC92 and BLC2-associated X protein (BAX) in *Nicotiana benthamiana* showed widespread suppression of plant cell death, indicating that CfEC92 may play some role in the suppression of BAX-induced cell death. The highest expression of this effector is probably 36 hpi. Furthermore, deletion of this effector from *C. fruticola* showed a significantly reduced virulence in apple fruit and leaves. Only 56% of the knockout mutants could penetrate host epidermal cells compared to the 82% in the wild type, although appressoria formation was similar (Shang et al. 2020).

An additional effector candidate *C. fruticola* Effector Candidate 12 (CfEC12) was identified as a CFEM (common in several fungal extracellular membrane proteins) domain-containing protein. Some evidence suggests these domains play a role in fungal pathogenicity and has been demonstrated in other virulence factors of other plant-pathogenic fungi (Kou et al. 2017; Kulkarni, Kelkar, and Dean 2003). Transcriptomic analyses noted an upregulation of *CfEC12*, measured by transcript accumulation, from 8 to 24 hpi with peak expression lining up with appressorium formation at 24 hpi. Deletion strains of *CfEC12* reduced lesion diameter by up to 60% when apples (Gala cultivar) were infected, while colony morphology and growth rate were unaffected demonstrating that CfEC12 is required for full virulence of *C. fruticola*. The effector also interacts with *Malus domestica* non-inducible immunity (nim1) interacting—2 (MdnIMIN2) in yeast two-hybrid screens and confirmed by point-to-point identification in the nucleus of host plant cells. MdnIMIN2 is suspected to be involved in host immune responses as shown by upregulation of the protein at 8–24 hpi during *C. fruticola* infection. This interaction suggests that CfEC12 suppresses non-expressor of pathogenesis related-1 (NPR1) pathways by preventing the binding of MdnIMIN2 to NPR1 (Shang et al. 2024).

## 5.3 | CgSte12—STERile Protein 12 in *C. gloeosporioides*

The STERile protein was first isolated from *Saccharomyces cerevisiae* and noted for its function in yeast mating, conferring sterility in some mutations. Further examination of the STE family of proteins found that some are involved in the mitogen-activated protein kinase cascade (Wang, Lu, and Tian 2021). Mitogen-activated protein kinase 1 (Mk1) is in part responsible for the development of appressorium in *Colletotrichum* spp. and other plant-pathogenic fungi (Jiang et al. 2018; Li et al. 2024). CgSTE12 is a *C. gloeosporioides*-specific homologue of STE12 and has been found to be a key regulator of appressorium development in *C. gloeosporioides*. Gene deletion studies found that CgSTE12 does not play a

significant role in the formation and melanization; more than 90% of wild-type and deletion strains produced appressoria with a 98.79% melanization rate. Instead, electron microscopy highlights a lack of penetration peg and pore development and appressorium cone development was abnormal. CgSTE12 knockout strains of *C. gloeosporioides* failed to break Gelbond membranes used to test for appressorium strength. These were confirmed by transcriptomic studies that highlighted the upregulation of appressorium development genes when CgSTE12 was functional. Data in this study further indicated that CgSTE12 was involved in the expression of genes related to cell composition, which showed changes in gene expression of integral membrane components at 12, 24 and 48 hpi (Li et al. 2024).

#### 5.4 | CgDN3

The CgDN3 protein encoded by the *CgDN3* gene was originally isolated in 2000 from *C. gloeosporioides* as an early expressed protein during infection and was further characterised as a suppressor of necrosis-inducing secreted protein 1 (NIS1) in 2012 (Stephenson et al. 2000; Yoshino et al. 2012). The early action of CgDN3, in combination with its suppressive activities of NIS1, suggests a role in the control of the necrotrophic switch during infection. Mutant studies highlighted the necessity of CgDN3 calmodulin (CaM)-binding domain for the suppression of plant cell death shown by the reduced lesion formation among CaM-binding domain mutants. This interaction may be interfering with plant calcium signalling by recruiting calcium to the site of binding. As calcium is involved in immune signalling in plants, this effector may be interfering with calcium signalling cascades (Isozumi et al. 2019).

#### 5.5 | CfPmt4—Protein O-Mannosyltransferase 4 in *C. fruticola*

Protein O-Mannosyltransferase is a fundamental protein involved in protein modification pathways in eukaryotes. Seven Pmt proteins have been discovered in *S. cerevisiae* and Pmt4 has been shown to be important for full virulence and cell wall integrity in *Cryptococcus neoformans* (Olson et al. 2007). Recent studies have examined the role of Pmt4 in *C. fruticola* growth, development and pathogenicity. Deletion studies of CfPmt4 in *C. fruticola* have shown a significant decrease in lesion diameter of around 1.25 cm on apple (cultivar unspecified) compared to wild-type controls. Deletion of *CfPmt4* also showed an approximately 25% decrease in appressorium formation rates when compared to wild-type controls. Under cell wall stress conditions, deletion strains of *CfPmt4* showed >60% inhibition rates in Congo red medium when compared to the wild type in the same conditions, highlighting the role of CfPmt4 in cell wall stress responses (Yang et al. 2024). Though a potentially interesting target for single-site fungicides or other management strategies (including future RNAi-based fungicides), targeting a key protein in protein modification pathways that is conserved in eukaryotes could be a potentially damaging treatment and put the environment, animal and plants at risk.

#### 5.6 | CfSnf1—Sucrose Non-fermenting 1

Originally identified in *S. cerevisiae*, Sucrose non-fermenting 1 (Snf1) allows the utilisation of specific carbon sources through the AMP-activated protein kinase pathway (AMPK), which is promoted by low glucose levels (Hong and Carlson 2007). A homologue of Snf1 was found in apple pathogens *C. fioriniae* and *C. gloeosporioides* during a study of tea-oil trees and designated as *Colletotrichum fioriniae* Sucrose non-fermenting 1 (CfSnf1). Targeted deletion of *Snf1* in *C. fioriniae* led to impairment in specific carbon source utilisation, asexual development, osmotic stress response and appressorium formation. This highlights the broad-acting role of CfSnf1 in *C. fioriniae*, which is likely to be occurring in other pathogenic *Colletotrichum* species. The presence of CfSnf1 is critical for successful infections and could probably be a target for antifungal inhibitors (Zhang, Guo, et al. 2019).

#### 5.7 | Mycotoxins

Mycotoxins, small molecules and secondary metabolites are commonly produced by fungi during host–parasite interactions. They often facilitate pathogen ingress, serve to increase virulence and result in plant cell death. Lately, research has shown additional roles of toxins in host–fungal and fungal–fungal interactions (Bartholomew et al. 2021, 2022). While significant strides have been made to detect secondary metabolic gene clusters, the encoded proteins and products, the *Colletotrichum* genus has lagged in comparison to *Aspergillus*, *Penicillium*, *Fusarium* and other famous toxin producers. However, the omics field has fuelled discoveries of previously unknown toxin clusters and their role in disease and the discovery of additional toxins in *Colletotrichum* spp. is envisioned to rapidly increase in the future. We highlight cercosporin below as a toxin leveraged by *Colletotrichum* species. Other toxins like ferricrocin, colletotrichins and colletotric acid are present during infection periods, but their role in pathogenesis is not as well characterised.

##### 5.7.1 | Cercosporin

Originally isolated from the *Cercospora* genus of fungi, cercosporin is the only light-activated non-host-specific toxin that affects mammals, plants, bacteria and other fungal species. It is used by several members of the *Cercospora* genus that cause leaf spot and blight diseases on crops such as maize, soybean, coffee, tobacco and sugar beet but has recently been found to be produced by several *Colletotrichum* spp. including *C. fioriniae*, probably accumulated by horizontal gene transfer (Daub and Ehrenshaft 2000; de Jonge et al. 2018). Classified as a photosensitizer, cercosporin is activated by visual wavelengths of light that produce damaging reactive oxygen species. Light is the most critical factor for the action of cercosporin, making it a likely candidate at the beginning of preharvest rot development where light activates the toxin. The compound causes damage to membrane lipids that can cause cell death and allow for fungal uptake of cellular nutrients and lethal levels of cercosporin can be in concentrations as low as 1 µM in plant cells and 0.3 mg per 15 g in mice (Daub and Kuang-Ren 2007; Yamazaki et al. 1975).

Synthesis of cercosporin is managed by the cercosporin toxin biosynthesis cluster (CTB). This cluster was previously thought to contain eight genes that include zinc finger transcription factors, transporters and a polyketide synthase. Recently, however, the gene cluster has been expanded to include at least four more genes located in the same region that share similarity with evolutionary-related CTB gene clusters that are expressed under the same conditions as the original cluster (Jonge et al. 2018). Jonge et al. (2018) showed that the CTB cluster is launched during *C. fioriniae* infection of apple fruit, but the toxin is not detectable in planta. Exogenous application of cercosporin to apple fruit tissues recreated part of bitter rot symptoms caused by *C. fioriniae* on apples. However, the toxin could not be reisolated from apple tissue, suggesting its chemical lability and/or metabolism by the host cells. Hence, the role of cercosporin in *C. fioriniae*–apple fruit interactions remains to be determined and would need to be conducted using genetically defined cercosporin mutants in the CTB cluster. *Cercospora*'s innate resistance to cercosporin is of interest from a translative standpoint, for the development of resistance to cercosporin in host plants. Thus far, two genes (*ATR1* and *71cR*) derived from *C. nicotianae* have been identified as key resistance genes that allow transgenic plants to resist applications of cercosporin. Transgenic host silencing of *CTB1* has also been shown to be an effective measure of providing disease resistance to otherwise susceptible host plants (Thomas et al. 2020).

## 6 | Management Strategies

### 6.1 | Chemical and Biological Controls

There is an increasing interest in the development of alternative approaches to manage pre- and postharvest rots and other fungal infections to reduce the pressure of fungicide resistance selection, alleviate pesticide inputs into the environment and remove dependency on products that are increasing in cost but are steadily being removed or restricted from the market (Romanazzi et al. 2016). Historically, calcium and copper salt applications have been effective in the prevention of bitter rot infections in apples and mangoes and play a role in the suppression of growth during preharvest management (Biggs 1999; Uddin et al. 2018). Since those early developments, the widespread use of synthetic fungicides such as triazole, prochloraz, benzimidazoles, benomyl and carbendazim and strobilurins, has become common. In recent years, however, more studies have shown that there is an increase in fungicide resistance among *Colletotrichum* spp. especially to QoI (FRAC 11 also known as strobilurins) and MBC (FRAC 3) fungicides that are commonly used to treat bitter rot in apples. Furthermore, increasing restrictions on fungicide usage due to carcinogenic or toxic effects are becoming more prevalent, leading to a pressing need for effective alternative treatments to existing products (Chechi et al. 2019; Ciofini et al. 2022).

The use of alternative chemicals such as fludioxonil (FRAC 12), fluazinam (FRAC 29) and benzovindiflupyr (FRAC 7) is becoming of interest to control bitter rot and other diseases caused by *Colletotrichum* spp. and in some instances have been shown to be more effective than current fungicides (Aćimović et al. 2020;

Martin et al. 2022). Fludioxonil was effective at reducing postharvest decay in mango by around 55% when dissolved in cold water and applied as a postharvest dip compared to untreated controls and around 35% more effective than prochloraz. The exact impact on *Colletotrichum* spp. is yet unknown in this context, but fludioxonil's usefulness as a general purpose, postharvest rot treatment is promising (Diskin et al. 2019). Benzovindiflupyr may reduce bitter rot incidence, providing up to 90.1% control of the *C. acutatum* species complex, while captan provides a 97.8% reduction in bitter rot. Current recommendations suggest benzovindiflupyr may be useful when applied once or twice combined with captan or ziram in July or early August (Aćimović et al. 2020).

The application of essential oils has also been shown to exhibit antifungal properties against *C. gloeosporioides*. At a MIC of 80  $\mu$ L/mL essential oils were extracted at high concentrations from the peels of Sanh orange and were effective at visually reducing lesion size during *C. gloeosporioides* infection while having a negligible effect on mango quality. Additionally, disease inhibition was increased at 16% vol/vol of Sanh essential oils by 42.3% at 4 days after inoculation and 39.5% after 8 days after inoculation (Duong et al. 2023). Some biological control agents are also useful in the inhibition of *C. gloeosporioides* infection by acting as a competitor, or through the production of volatile organic compounds dubbed 'mycofumigants' in the case of a fungal biocontrol agent (Holkar et al. 2023; Morath, Hung, and Bennett 2012). In a study of 51 leaf endophytes isolated from grape leaves, the *Aspergillus* genus (a prominent grape leaf endophyte) could inhibit the growth of *C. gloeosporioides* by more than 80%, especially in the case of *A. flavus* (88.42%–86.31%), *A. niger* (83.15%) and *A. oryzae* (88.42%). *A. oryzae* is especially promising as it is nontoxic and nonpathogenic, although its suppression ability and growth capabilities on apple are still uncertain (Holkar et al. 2023).

### 6.2 | Fungicide Resistance Management

The consistent monitoring of fungicide resistance across *Colletotrichum* spp. is essential to appropriately balance the application of traditional fungicides with alternative approaches. These resistant strains can often be traced back to single point mutations that are selected after repeated application of common single-site fungicides. For example, high levels of QoI fungicide resistance can be attributed to a glycine-to-alanine mutation in the mitochondrial cytochrome b gene (Cortaga et al. 2023). A recent screen of symptomatic apples showed resistance to several FRAC 7 and 11 fungicides with some isolates from *C. siamense* showing 100% growth under 100 ppm applications of kresoxim-methyl (Sovran) (Martin et al. 2022). FRAC 29 fungicide fluazinam (Omega) showed high effectiveness at controlling *Colletotrichum* growth, averaging just over a 75% reduction in growth when compared to untreated controls. FRAC 12 fungicide fludioxonil (Scholar) varied in effectiveness across species, with *C. chrysophilum* standing out as a more resistant species, although its growth was reduced by around 50% when exposed to 0.1 ppm of fludioxonil. Furthermore, the study concluded that EC<sub>25</sub> values acted as better predictors of bitter rot control than EC<sub>50</sub> when comparing FRAC 1, 7, 11 and 12 fungicides and during normal conditions (Martin et al. 2022).

### 6.3 | Cultural Practices and Sanitation

Traditional approaches for the management of pre- and postharvest rots are implemented to help reduce the use of synthetic fungicides and reduce the inoculum of rot-causing pathogens like *Colletotrichum*. Good cultural practices include canopy pruning for adequate ventilation and fungicide coverage during growth, appropriate harvest time (not immediately after rain), removal of decayed or mummified fruits, cool chain handling to slow down ripening and hot water treatments (Bordoh et al. 2020; Rosenberger 2012; Zhang, Liu, et al. 2019). Postharvest conditions should also be tightly controlled to reduce pathogen incidence.

Controlling temperature, relative humidity, atmospheric gases (especially CO<sub>2</sub> and O<sub>2</sub>), air circulation and light are all critical for minimising optimal pathogen growth conditions (Gk, Varma, and Shilpa 2023). These control methods are often effective against a large group of pathogenic agents and are not specific to *Colletotrichum* species. Additionally, some postharvest treatments may disrupt pathogen growth. Recently, butylated hydroxyanisole (BHA) has been shown to be effective at inhibiting *C. gloeosporioides* growth. BHA has been used as an antioxidant that extends food shelf life and may be useful as a postharvest treatment for tree fruits at risk of *Colletotrichum* infection. On citrus, at a minimum fungicidal concentration of 5 g/L BHA caused loss of virulence in *C. gloeosporioides* and completely inhibited mycelial growth in vitro at 0.25 g/L (Wang et al. 2024).

### 6.4 | Host Susceptibility to Bitter Rot

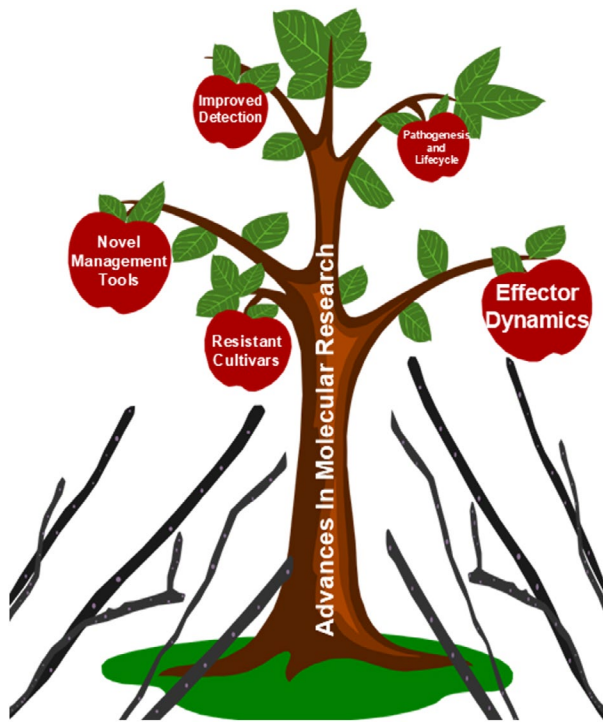
The current suite of apple cultivars grown in the United States are susceptible to the species of *Colletotrichum* present in the country, indicating a need for the development of rot-resistant cultivars (Jurick II et al. 2011; Khodadadi, Santander, et al. 2023; Martin et al. 2021). A recent study indicated that when apple fruit of 10 apple cultivars were inoculated by skin wounding, all cultivars were susceptible to both species' complexes, with the Honeycrisp fruit being the most susceptible and *M. sylvestris*, accession PI 369855, being the most resistant (Jurick II et al. 2011; Khodadadi, Santander, et al. 2023). Across the Mid-Atlantic, five cultivars make up 75% of the reported acreage of cultivated area, namely Honeycrisp, Fuji, Gala, Golden Delicious and Red Delicious. The Honeycrisp cultivar, which was recently introduced to the Mid-Atlantic region, is highly susceptible to bitter rot infection and has incurred losses of up to 80% in some cases with an average loss of 30% (Martin et al. 2021). Other cultivars such as Delicious are less susceptible to infection, seeing between 0%–5% losses. Cultivars like Ginger Gold, Red Delicious and Pink Lady were found to have lower percent loss to bitter rot when compared to cultivars like Honeycrisp and Empire in a survey of 34 apple growers across three states and 33 cultivars (Martin et al. 2021). A Kentucky study showed that there was a significant difference in susceptibility between Red Stayman Winesap apples and Golden Delicious with isolated spore concentration from *C. fruticola* infected fruit reaching as high as 4 × 10<sup>5</sup> spores per mm<sup>2</sup> (Munir et al. 2016). Fruit

of some wild apple accessions have demonstrated resistance to both *C. acutatum*-derived bitter rot and blue mould caused by *Penicillium expansum*. Among hundreds of samples analysed, only six were resistant to both diseases, while more were susceptible to one of the two diseases, with a majority being susceptible to both diseases. The development of resistance among these accessions is due to genetics of a specific wild apple accession. Ongoing omics-based and breeding efforts are underway to transfer resistance to existing apple fruit cultivars (Jurick II et al. 2011).

Additional efforts have assessed the qualities of 92 cultivars including resistance to *C. gloeosporioides* and *P. expansum*. Lesion size in *C. gloeosporioides* infection is loosely, but significantly, negatively correlated to harvest time among early-ripening cultivars but not in late-ripening cultivars ( $R^2=0.24$  and  $R^2=0.003$ , respectively). Firmness and softening had little correlation with lesion size during *C. gloeosporioides* infection, although there was a positive and significant effect of fruit softening in early maturing cultivars. It seems that overall quality traits (fruit firmness, acidity, etc.) do not often impact resistance to postharvest *C. gloeosporioides* infections. Although harvest time played a more significant role than either softening or firmness. Overall, the study concluded that there were significant differences between various cultivars for resistance to both *C. gloeosporioides* and *P. expansum*, further solidifying the presence of host genetics responsible for the high levels of resistance demonstrated in some cultivars (Ahmadi-Afzadi, Tahir, and Nybom 2013).

## 7 | Future Directions

As bitter rot infections among apple and other tree fruit grow in prevalence, new molecular approaches will be required to tackle the threat (Figure 3). Although great strides have been made in recent years to understand more fundamental details about *Colletotrichum*, more studies that target the application of those fundamental breakthroughs should be conducted in the future. Further research into effector–host relationships in the apple bitter rot pathosystem may be of increasing usefulness in the development of resistant cultivars by allowing molecular biologists and breeders to identify resistance (R) genes or quantitative trait loci (QTLs) within resistance wild apple germplasm (Janisiewicz et al. 2008; Jurick II et al. 2011). New approaches are in development for the treatment of active bitter rot infections such as topical RNAi applications which aim to alleviate the burden of increasingly expensive and scarce fungicides (Niño-Sánchez et al. 2022). Understanding the molecular signals involved in the *Colletotrichum* necrotrophic switch may also prove useful in limiting the damage caused by the pathogen as a pre- and postharvest rot through the development of inhibitor molecules or host-induced silencing (Islam and Sherif 2020). The identification of rot-resistant wild apple accessions has been a major leap forward in providing hope for generating truly resistant commercial apple cultivars, given the future of limited chemical and biological controls for apple pathogens. Understanding the complex pathogen *Colletotrichum* in apple bitter rot infections is a monumental multidisciplinary undertaking that will likely provide answers in the pest management arena.



**FIGURE 3** | Molecular research is the foundation of our future advances in bitter rot management, distribution and life cycle dynamics. Improvements in detection research can provide early warning signs or confirmation to growers of a bitter rot infection allowing for effective management tools to be leveraged. By uncovering key molecular details in the life cycle and effector dynamics of bitter rot, new fungicides can be developed to exploit critical effectors and life cycle steps to reduce or prevent destructive growth.

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### Conflicts of Interest

The authors declare no conflicts of interest.

### Data Availability Statement

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

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