Trends in
Plant Science

CellPress
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Review

Killing softly: a roadmap of *Botrytis cinerea* pathogenicity

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***Botrytis cinerea*, a widespread plant pathogen with a necrotrophic lifestyle, causes gray mold disease in many crops. Massive secretion of enzymes and toxins was long considered to be the main driver of infection, but recent studies have uncovered a rich toolbox for *B. cinerea* pathogenicity. The emerging picture is of a multilayered infection process governed by the exchange of factors that collectively contribute to disease development. No plant shows complete resistance against *B. cinerea*, but pattern-triggered plant immune responses have the potential to significantly reduce disease progression, opening new possibilities for producing *B. cinerea*-tolerant plants. We examine current *B. cinerea* infection models, highlight knowledge gaps, and suggest directions for future studies.**

Highlights

Botrytis cinerea infection can proceed by a number of different routes which vary according to the plant species, tissue type, and external conditions.

There is no single silver (virulence) bullet; disease development is multilayered and regulated by multiple factors, with subtle contributions from each virulence factor.

Infection cushions have emerged as a factory for the production of virulence factors. These structures are likely essential for disease development in tissues with relatively low susceptibility to infection and possibly under suboptimal conditions.

A morphogenetic program is essential for pathogenicity; disrupting proper fungal morphogenesis can be detrimental for successful infection.

Plant defense is activated early on; despite the lack of complete resistance against *B. cinerea*, PAMP-triggered immunity (PTI) has the potential to reduce and even prevent disease development.

B. cinerea: past and present

The genus *Botrytis* (family Sclerotiniaceae) is one of the oldest and most well studied fungal taxa. According to recent taxonomical analysis, there are over 35 *Botrytis* species, of which *B. cinerea* is the most well known and well studied [1,2]. Unlike the majority of *Botrytis* species, which are necrotrophic plant pathogens with a rather narrow host range, *B. cinerea* is a generalist pathogen that is capable of infecting a wide range of plant species, including leading agricultural crops [3]. In this respect, *B. cinerea* resembles the phylogenetically closely related *Sclerotinia sclerotiorum,* an aggressive pathogen that causes white mold disease in hundreds of plant species [4]. *B. cinerea* is less aggressive than *S. sclerotiorum,* and while it can infect intact leaves and stems under optimal conditions, it more frequently attacks soft tissues such as fruits, vegetables, and flowers. Nevertheless, the fungus is widespread worldwide and is considered to be among the most economically important plant pathogens, causing massive crop losses both pre- and post-harvest [5]. It has long been assumed that *B. cinerea* infection is primarily promoted by the massive secretion of plant-cell-wall-degrading enzymes (PCWDEs) and nonselective toxins [6–9]. However, more recent studies have revealed that this fungus has a much richer toolbox than previously thought. The emerging picture (although incomplete) is that *B. cinerea* infection is a multilayered process governed by the exchange of a wide range of factors that collectively determine disease development and severity [10]. In this review, we examine current *B. cinerea* infection models in light of recent findings, highlight knowledge gaps, and suggest potential directions for future research that could aid in the development of new approaches for disease management.

B. cinerea infection stages and models

B. cinerea infection is predominantly initiated by 50–75 μm^3 oval **conidia** (see Glossary) that attach to and germinate on the plant surface. Germ tubes or more elongated hyphae differentiate simple **appressoria** and **infection cushions** (**ICs**). Both are specialized structures that assist in host penetration, but *B. cinerea* has also been shown to enter the host through stomata or directly penetrate the cuticle via short conidial germ tubes [11,12]. Following the initial contact with the host, two distinct phases have been described: an early phase characterized by the formation of local infection foci without spreading, and a late stage characterized by the production of

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abundant fungal biomass and lesion spread. A recent study of disease dynamics uncovered a previously unrecognized intermediate stage between the initial and late phases (Box 1), which is critical for disease progression [13].

Establishment of infection

According to a model suggested by Shlezinger *et al*. [14], the early infection stage must culminate in the killing of a sufficient number of host cells to create a region of dead tissue in which fungal biomass accumulates prior to the transition to intermediate and then late infection phases. This model predicts that compounds that facilitate the rapid killing of host cells play a major role in the successful completion of the early infection phase. In addition to producing PCWDEs and toxins, *B. cinerea* produces an array of **cell-death-inducing proteins** (**CDIPs**), and there is evidence that it also manipulates the plant **regulated cell death** (**RCD**) machinery to facilitate local host cell death [15]. Conversely, towards the end of the early phase and into the intermediate phase of infection, the fungus undergoes massive RCD that is induced by antimicrobial plant metabolites [14]. Fungal survival at this stage depends in part on antiapoptotic machinery that prevents killing of the entire fungal biomass. The balance between plant and fungal cell death likely determines whether the fungus will be blocked or will manage to progress to the next phase.

Studies of the *S. sclerotiorum* infection process suggested an alternative model in which the fungus first maintains plant cell viability [16]; a similar scenario has been proposed for *B. cinerea* [10]. According to this model, following initial fungal invasion, the host cells remain viable due to the suppression of autophagic cell death that prevents activation of self killing. When the fungus establishes within the host tissues and accumulates sufficient biomass, the secretion of **autophagy**-suppressing molecules is replaced with production of RCD-promoting compounds, which leads to killing of the plant tissue and disease spreading (Box 2).

Lesion spread

Whether the early stages of disease establishment include a short 'biotrophic' phase [10] or the immediate induction of cell death [14] is unclear, but there is consensus between the two models that the initial processes result in the formation of an infection court that enables the accumulation

Box 1. A three-stage infection process

The methods used to assess plant infection usually provide a snapshot rather than a dynamic picture of disease development. To address the need for continuous, quantitative disease measurement, Eizner *et al*. [13] developed the PathTrack© system, which captures images during the entire infection process, analyzes the images, and provides numerical values that describe disease progression. Analysis of *B. cinerea*-infected leaves using PathTrack© uncovered a previously unrecognized intermediate stage marked by the spread of necrosis out of the infection site and ending when lesion expansion reaches a maximum spreading rate. When a leaf is infected under controlled conditions, the intermediate stage begins at a predictable time, after the coalescence of the microlesions into a single local necrotic spot, and ends when the lesion starts to spread, concomitant with the development of radiating hyphae. This scenario resembles the development of the hemibiotrophic rice blast pathogen *Magnaporthe oryzae,* which is also characterized by predictable phases with constant variables in which the fungus moves from one cell to another [77]. However, whereas *M. oryzae* kills the host cell after moving to the next cell, it appears that *B. cinerea* first kills the host cells and only then moves into the dead tissue. In order to increase the sensitivity of the analysis, we used the Eulerian motion magnification algorithm [78], which amplifies subtle differences in motion and color, enabling the identification of temporal changes that are not recognizable by the human eye. This analysis revealed that fungal activity begins at ~18 h post-inoculation in small sporadic spots within the inoculation droplet. The activity then increases as the sporadic spots begin to merge, first into larger regions and ultimately forming a single region of activity; this activity drops before intensifying again towards the transition to the intermediate stage (A. Sharon, unpublished). During lesion spread, most of the activity is restricted to a progressing ring at the edge of the lesion, with limited or no activity at the center of the lesion. The drop in fungal activity towards the end of the first stage coincides with intense fungal RCD at this stage [14], supporting the model that predicts parallel plant and fungal cell death during the early infection phase. The type of plant cell death is yet to be determined, and similar to the 'biotrophy' model, it is unclear if killing of the host cells occurs before or after cell invasion.

Glossary

Appressoria: specialized infection structures of fungal pathogens that are used to breach and penetrate the outer surface of the host. They vary in shape and size, depending on the species. The *B. cinerea* appressorium is a relatively small, unmelanized swelling at the tip of a germ tube.
Autophagy: a conserved eukaryotic degradative process used to remove and recycle unnecessary or dysfunctional cellular components through a lysosome-dependent regulated mechanism. It is involved in stress adaptation as well as the elimination of intracellular pathogens.
Cell-death-inducing protein (CDIP): any type of secreted phytotoxic protein produced by a plant pathogen. Previously called necrosis-inducing protein (NIP).
Conidium (plural conidia): an asexual spore. It is the main mode of *B. cinerea* dissemination and source of inoculum.
Hypersensitive response (HR): rapid, localized, regulated plant cell death at the point of pathogen invasion. HR is a defense response that efficiently prevents the spread of biotrophic and hemibiotrophic pathogens but is inefficient in controlling necrotrophic pathogens. HR involves the activation of regulated cell death processes.
Infection cushion (IC): a complex, multicellular structure formed on a host surface and used by certain plant pathogenic filamentous fungi to produce virulence factors and for host penetration. *B. cinerea* generates ICs in some, but not all, types of interaction.
Necrotic cell death: an unregulated cell death that occurs in response to overwhelming internal or external stresses such as mechanistic injury, environmental stresses, and chemical agents.
Regulated cell death (RCD): an ubiquitous active cell-death process in living organisms that refers to all types of non-necrotic cell death, including apoptosis, necroptosis, and ferroptosis, which differ from unregulated (also called necrotic) cell death that does not involve regulatory cellular processes. In the context of this review, we do not include autophagy and when suing RCD, we mainly refer to apoptotic-like programmed cell death.
Small RNAs (sRNAs): short, noncoding regulatory RNAs that silence genes via base complementarity. There

Box 2. Do broad host range necrotrophs have an early biotrophic phase?

Despite nearly 200 years of study, important details about the initial developmental events in *B. cinerea* following spore germination are not completely clear. Most importantly, it is unclear if the fungus penetrates into living plant cells or if it kills cells with the aid of secreted molecules without cell invasion. An intriguing model suggests that *S. sclerotiorum* has a short biotrophic phase before switching to necrotrophy [16]. According to this model, following initial penetration into the host tissue, the host cells remain viable due to the suppression of autophagic cell death. Upon the transition to necrotrophy, the fungus switches to the induction of RCD; according to this model, both the inhibition of autophagy during the 'biotrophic' phase and induced RCD during necrotrophy are mediated by oxalic acid [16,79]. In accordance with this model, work with onion epidermis showed that cells adjacent to intercellular hyphae remained viable [80], but with no evidence for intracellular, host-membrane-wrapped fungal organs, which is the hallmark of biotrophy. Therefore, care must be taken, and at least at this point, it might be more accurate to refer to this stage as a 'non-destructive' rather than a true biotrophic phase. It is also unclear if the fungus would survive within living host cells, induce cell death without invasion, or kill the cells immediately upon cell invasion. Additionally, earlier studies showed that *S. sclerotiorum* induces RCD in plants as early as 4 h post-inoculation and that plants expressing antiapoptotic genes showed reduced sensitivity to infection [21]. These seemingly contradicting observations might simply indicate that the suppression of autophagy and induced RCD occur simultaneously and that both might be important for successful plant colonization. In this case, the role of autophagy in protecting plants from necrotrophic pathogens should be reconsidered, as it might be associated with processes other than RCD. A similar scenario that includes the suppression of autophagy and a short 'biotrophic' phase before the transition to necrotrophy has been suggested during *B. cinerea* infection [10]. Unlike for *S. sclerotiorum*, there is no evidence to support the suppression of autophagy by oxalic acid during *B. cinerea* infection, and so far, no other candidate molecules that suppress autophagy have been proposed. Additionally, the mechanism underlying the killing of the host cells at this stage, particularly whether it occurs before or after cell invasion, and the exact role of autophagy remain unclear.

are two major types of sRNA in plants and fungi: (i) siRNAs generated from double-stranded RNAs; and (ii) microRNAs (miRNAs) generated from single-stranded RNAs with a stem–loop structure. sRNAs induce mRNA degradation and cleavage, translational inhibition, or transcriptional gene silencing in a sequence-specific manner. *B. cinerea* produces hundreds of sRNAs, some specifically for infection.

of fungal biomass. Both models also share the notion that lesion spread is facilitated by RCD-inducing molecules. It is possible that CDIPs and toxins induce both necrosis as well as RCD at the early infection phase; however, they are not involved in spreading cell death. Apart from oxalic acid – which can induce RCD and possibly contributes to spreading cell death – no other RCD-inducing molecules have been identified, even though several lines of evidence support their existence. First and foremost, spreading lesions are surrounded by a ring of dead plant cells that precedes fungal spreading [14,17], and it is tempting to speculate that this type of cell death is facilitated by diffusible fungal agents (Figure 1). It is also possible that spreading cell death represents a phenomenon known as 'runway cell death', in which plants exhibit uncontrolled spreading of cell death following activation of the **hypersensitive response** (**HR**) [18]. In this case, cell death induced by CDIPs, toxins, or as yet undiscovered activators of the otherwise local HR, keeps propagating due to the manipulation of HR-regulatory systems by putative fungal effectors. This possibility is supported (to some extent) by the finding that the HR, which is used to protect plants from biotrophic pathogens, is necessary for *B. cinerea* infection [15] and that expressing antiapoptotic genes in plants blocked RCD and prevented infection [16,19,20]. At least in the case of transgenic tobacco [21], infection by *S. sclerotiorum* or *B. cinerea* was blocked after initial necrosis was observed but before lesion expansion. Taken together, these findings suggest that lesion spread is promoted by compounds that induce RCD, whereas the formation of local lesions likely involves **necrotic cell death** and possibly also RCD (Figures 1 and 2).

Plant defense

After breaching the external layers of the plant, including the cuticle and cell wall, the fungus must cope with preformed as well as pathogen-induced (phytoalexins) plant antimicrobial compounds, which significantly affect disease development [22]. *B. cinerea* has evolved various mechanisms to tolerate mycotoxic plant metabolites, such as the degradation of α-tomatine [23] and the export of toxic glucosinolate degradation products [24] or the *Arabidopsis thaliana* phytoalexin camalexin [25]. *B. cinerea* strains that are defective in the ability to cope with such plant defense compounds are hypovirulent, which demonstrates the importance of these compounds in attenuating disease severity. NEP like proteins (NLPs), which are a class of noncatalytic CDIPs, interact with the glycosylinositol phosphorylceramide (GIPC) sphingolipids in the plant cell membrane and

Table 1. *B. cinerea* CDIPs[a]

CDIPs	Domain	Activity	Phenotype[b]	CDI epitope[a]	Defense	BAK1/SOBIR1
BcCrh1[c] [33] Bcin01g06010	GH16	Trans-glycosidase	No	35 aa	Yes	No
BcXyn11A [81] Bcin03g00480	GH11	β-1,4 xylanase	Yes	25 aa	Yes	NT
BcXYG1 [82] Bcin03g03630	GH12	Xylo-glucanase	No	NT	Yes	Yes
BcGs1 [83] Bcin04g04190	GH15/ CBM20	α-1,4 glucanase	No	NT	Yes	NT
BcPG1-2 [44] Bcin14g00850/Bcin14g00610	GH28	Polygalact-uronase	Yes	NT	Yes	SOBIR1 only
BcSSP2 [84,85] Bcin05g03680	ND	No	No	NT	Yes	No
BcNep1 [86] Bcin06g06720	NLP	No	No	20 aa	Yes	Yes
BcNep2 [86] Bcin02g07770	NLP	No	No	20 aa	Yes	Yes
BcSpl1 [87] Bcin03g00500	Cerato-platanin	No	Yes	40 aa	Yes	BAK1 only
BcIEB1 [31] Bcin15g00100	ND	No	OE	35 aa	Yes	BAK1 only
BcCFEM1 [88] Bcin10g02180	CFEM	No	Yes	NT	NT	NT
BcHip1 [89] Bcin14g01200	ND	No	OE	NT	NT	NT
BcPLP1 [90] Bcin10g01020	VmE02 Homolog	No	No	NT	NT	Yes
BcSGP1 [91] Bcin01g05310	UvSGP1 Homolog	No	NT	NT	NT	BAK1, SOBIR1 NT

[a]Abbreviations: CDI, cell death induction; ND, not detected; NT, not tested; OE, overexpression (strain shows enhanced virulence, but the deletion strain shows no obvious phenotype).
[b]Phenotype, reduced virulence of deletion strains.
[c]BcCrh1 is localized to the plant cytoplasm, all other proteins are localized to the apoplast.

induce cell death in dicot plants. It was found that differences in the lengths of the GIPC head groups prevent interaction of NLPs with the GIPC in monocots, making them insensitive to NLPs [26]. This difference might also account for the lack of gray mold disease in cereals, although it should be noted that *B. cinerea* can infect and cause spreading lesions in maize and wheat under artificial conditions.

Once inside the plant, the fungus must cope with plant immune responses. The *B. cinerea* toolbox does not include host-specific toxins or Avr effectors. Therefore, no single gene confers plant resistance against *B. cinerea,* and effector-triggered immunity (ETI), which is mediated by specific interaction of a pathogen effector with a plant receptor (*R* gene), is largely irrelevant. In the absence of complete resistance, the quantitative virulence of *B. cinerea* is paralleled by a quantitative plant defense response based on the recognition of conserved self and non-self pathogen associated molecular patterns (PAMPs) by plant pattern-recognition receptors (PRRs) (for a recent review on PAMP triggered immunity (PTI) see Liao *et al.*, 2022 [96]). Common PAMPs include molecules that are recognized as a fungal signature, such as chitin oligomers, as well as molecules that are vital for virulence, such as CDIPs, which are recognized by plant receptor-like kinases (RLKs) and receptor-like proteins (RLPs) that activate an immune response.

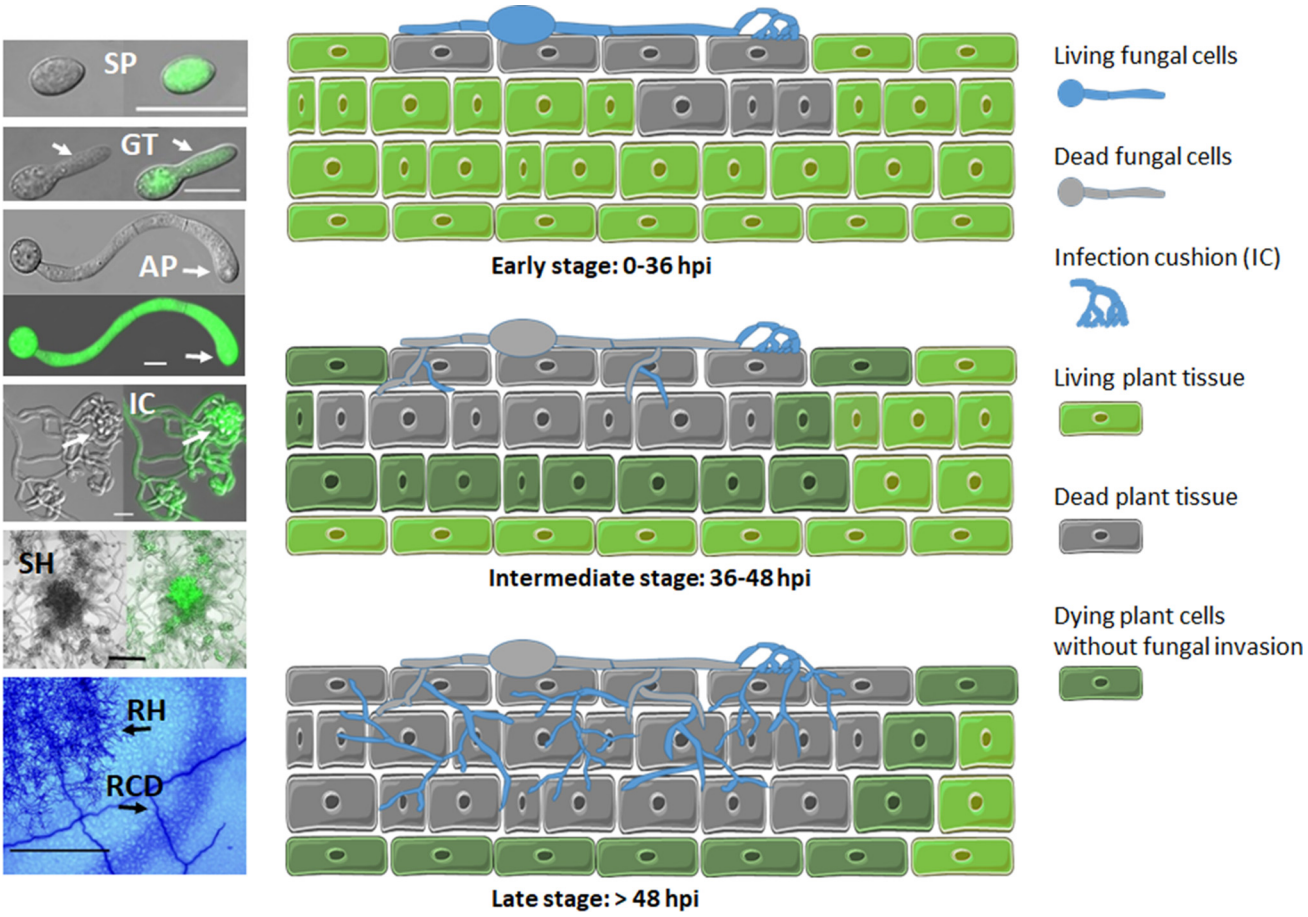

Figure 1. Main steps of the infectious process of *Botrytis cinerea*. Left: images of the main infection structures produced by a green fluorescent protein (GFP)-expressing *B. cinerea* strain. Bright field and fluorescent images are shown. Spores (SP) attach to the plant surface and produce a germ tube (GT) 4–6 hours post-infection (hpi). The germinated spores produce appressoria (AP, 12–18 hpi) and infection cushions (IC, 18–24 hpi) that assist in host penetration. Along with penetration of the host tissue, superficial hyphae (SH) keep developing on the surface. After establishment of infection (completion of Phase 1, 32–48 hpi) radiating hyphae (RH) differentiate and facilitate lesion spreading. This stage (Phase 2) is associated with the formation of a layer of cells that probably undergo regulated cell death (RCD) at the infection front, as can be seen by trypan blue staining (credit Liang Ma). Right: the cartoon shows developmental events during the three main infection stages. It should be noted that this schematic presentation may vary considerably depending on conditions (see Figure 2 for details). During the first 0–24 hpi, spores germinate and form initial infection sites with the aid of appressoria and ICs. At this stage, the fungus remains mainly on the surface of the host and facilitate killing of a limited number of host cells with the aid of cell-death-inducing proteins (CDIPs). In the following stage (24–48 hpi, time varies according to host and conditions), hyphae penetrate the host tissues, more plant cells are killed, but the fungus is also under attack by plant compounds that lead to massive fungal cell death that is primarily regulated (RCD) [14]. Cells that survive the attack proliferate within the dead plant tissue, marking the transition from local to spreading lesion. The figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 Unported Licence (https://smart.servier.com/).

The perception of pathogens by receptors leads to calcium influx and initiates a phosphorylation cascade that activates receptor-like cytoplasmic kinases (RLCKs), calcium-dependent protein kinases (CDPKs), and mitogen-activated protein kinases (MAPKs). The Arabidopsis RLCK gene *BOTRYTIS INDUCED KINASE 1* (*BIK1*) is one of the earliest genes to be induced following *B. cinerea* infection [27]. BIK1 integrates PTI signals downstream of multiple PRRs independently of MAPKs, connecting plant growth to immune responses through its function in ethylene signaling [28,29]. The immune responses downstream of these signal cascades include production of reactive oxygen species (ROS), callose deposition, cell wall reinforcement, and the synthesis of the defense compounds, phytoalexins [27,30]. These processes limit local infection and systemically increase immunity in uninfected parts of the plant, a phenomenon known as systemic

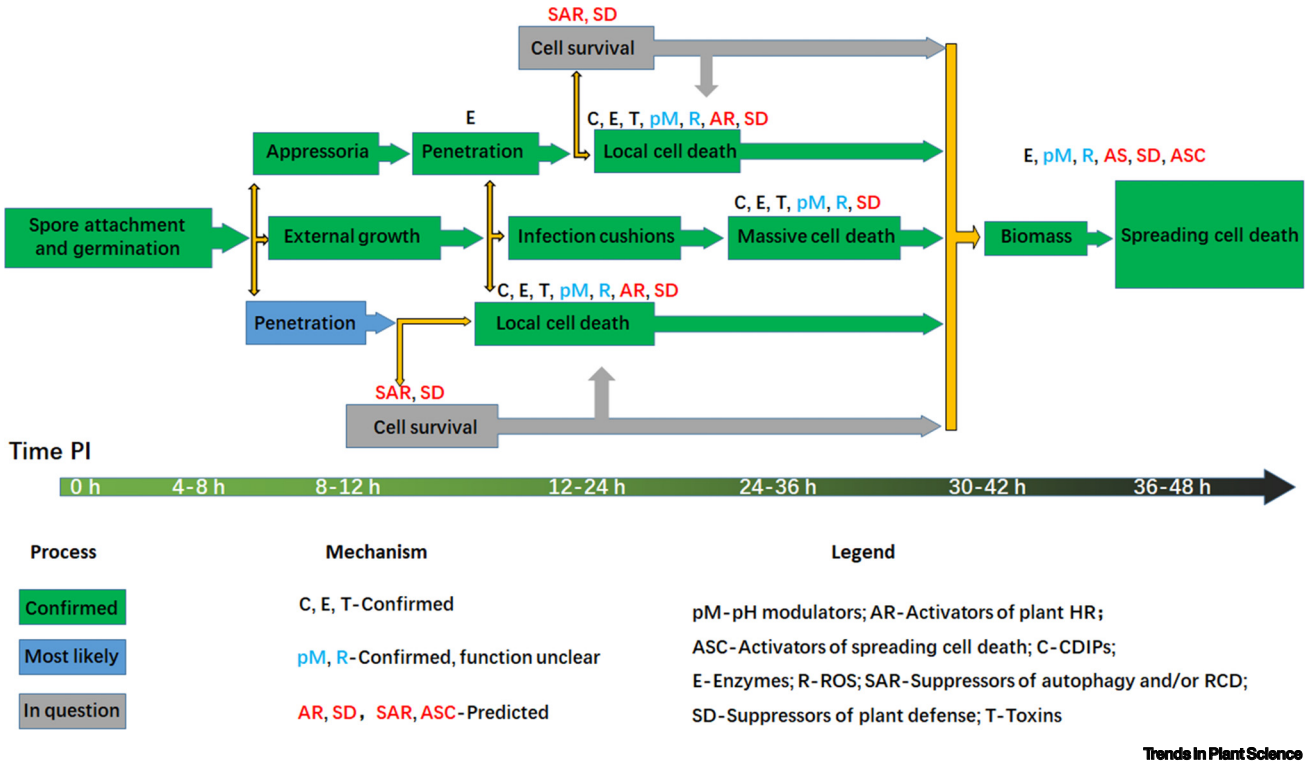

Figure 2. *Botrytis cinerea* infection roadmap. Under conditions favorable for disease development (susceptible host and tissue, freely available water, and optimum temperatures), germ tubes may penetrate the external layers, either directly or with the aid of appressoria. Following host penetration, the fungus induces local cell death with the aid of enzymes (E), cell-death-inducing proteins (C), and toxins (T), and change the internal environment to its advantage by modulation of the pH (pM) and the production of reactive oxygen species (R). Alternatively, immediately after host penetration, the fungus might suppress autophagy and/or regulated cell death (SAR) to prevent local cell death. There is also evidence for the activation of the hypersensitive response (AR) and the suppression of plant defense (SD), but these concepts require further study. Under less favorable conditions, the fungus may develop superficial hyphae and mycelia and differentiate infection cushions. It is assumed that these massive structures produce abundant amounts of virulence factors that aid in host penetration and toxification. All routes lead to the local accumulation of fungal biomass at the infection site, which supports the transition from local infection to expanding lesions (vertical yellow bar with a horizontal arrow on the right) that represent the late infection stage.

acquired resistance (SAR). The potential impact of plant immune responses on the susceptibility to *B. cinerea* has been demonstrated by treating or engineering plants with immunity-inducing epitopes. For example, treating tobacco plants with purified *B. cinerea* CDIP BcIEB1 increased systemic resistance to *B. cinerea*; a BcIEB1-derived 35-amino-acid peptide was almost as active in this process [31,32]. Arabidopsis plants expressing *B. cinerea bccrch1*, encoding a CDIP that is translocated into plant cells, showed reduced disease levels [33]. Finally, pretreatment of tomato, bean, and tobacco (but not maize) plants with a BcCrh1-derived 35-amino-acid peptide reduced local and systemic susceptibility to *B. cinerea* infection (our unpublished results).

The *B. cinerea* toolbox

In the following section, we examine the *B. cinerea* toolbox and how its components are used at each stage of infection. We also highlight gaps in our knowledge of the functions of these components (Figures 1 and 2).

Confirmed virulence factors

Bona fide classes of virulence factors include toxins, degradative enzymes (PCWDEs, cutinases, proteases, and lipases), and CDIPs. Other verified factors include the detoxification of mycotoxic plant metabolites, such as α-tomatine and camalexin, by enzymatic modification or via efflux transporters [23,25,34].

Toxins

B. cinerea produces two major phytotoxic metabolites that are required for full virulence: the sesquiterpene botrydial and the polyketide botcinin [35,36]. Additional phytotoxic metabolites have been described, including oxalic acid (which is required for virulence) and several other phytotoxic molecules with unclear effects on virulence [8].

Degradative enzymes

There are 275 predicted secreted CAZymes (carbohydrate-active enzymes) in *B. cinerea*, many of which are capable of degrading different types of sugar polymers in the plant cell wall [37,38]. These secreted PCWDEs help the fungus to overcome the cell wall barrier and utilize cell wall materials for nutrition [6,39–41]. There is high redundancy among PCWDEs, which complicates functional analysis [42], but in a few cases, the deletion of a single gene had a clear effect on pathogenicity. For example, deletion of either of two endo-polygalacturonase (PG) genes, *bcpg1* and *bcpg2* (but not the four other PGs) reduced fungal virulence [43,44]. Notably, BcPG1 and BcPG2 were the only PGs identified in the early secretome [45], and both are among the most highly expressed genes and proteins during infection [39,46]. A general reduction in pathogenicity was also observed following the deletion of a cellobiohydrolase (*bccbh*) and an endoglucanase (*bceg*) gene [47], while deletion of the endo-arabinanase gene *bcara1* reduced infection in Arabidopsis but not in other plant species [48]. The hallmark of many nonpathogenic strains of *B. cinerea* in a T-DNA mutant library are defects in the secretion of degradative enzymes, including PCDWEs [49]. The Snf1 kinase and the vesicular trafficking protein clathrin, both affect *B. cinerea* virulence through regulation of activation and secretion of degradative enzymes, respectively [50,51]. The transcription factor XyrR1 regulates the expression of genes encoding PCWDEs, particularly xylanolytic and cellulolytic enzymes [42,52]. A recent analysis indicated that BcXyr1 positively regulates the expression of CAZyme-encoding genes, and the deletion of *bcxyr1* significantly reduced fungal virulence (L. Ma, unpublished). In addition to PCWDEs, several other classes of secreted degradative enzymes might promote infection, including cutinases, proteases, and lipases. Proteins in these categories also share highly redundant functions, and therefore it is difficult to confirm their contributions to virulence. The recent development of a CRISPR/Cas9-based method that enables the generation of strains with deletions of multiple genes [53] is expected to provide more conclusive evidence for the roles of specific functional groups of degradative enzymes in disease development.

CDIPs

CDIPs are generally regarded as virulence factors [54]. However, like PCWDEs, CDIPs share high functional redundancy, and therefore their contribution to fungal virulence has been demonstrated in only a few cases (Box 3). To overcome this high functional redundancy, Leisen *et al.* [55] used CRISPR-Cas9-mediated gene editing to generate *B. cinerea* mutant strains with deletions of up to 10 CDIP and two toxin genes. The effect on virulence varied depending on plant species and tissue, from no effect in tomatoes to up to a 40% reduction in virulence in apples, suggesting that the effect of CDIPs is host-specific. Culture filtrates of the multiple deletion strains retained cell-death-inducing activity, pointing to the existence of yet undefined CDIPs and/or toxins. Further work, including generation of strains with deletions of even a larger number of CDIPs, might provide more conclusive evidence on the role of these proteins in *B. cinerea* virulence and pathogenicity.

ROS metabolism system

Another group of potential virulence factors are proteins that contribute to ROS metabolism and affect the cellular oxidative state, such as oxidoreductases [38]. *B. cinerea* also produces

Box 3. CDIPs

CDIPs are secreted phytotoxic proteins that are regarded as virulence factors in hemibiotrophic and necrotrophic fungal and Oomycete plant pathogens [54]. Similar to PCWDEs, CDIPs have high functional redundancy, and a clear contribution to fungal virulence has been shown in only a small number of cases. Broadly, all CDIPs can be divided into two groups: proteins that lack a recognizable domain (noncatalytic), and enzymes that, in addition to their catalytic activity, also induce plant cell death. Over 15 CDIPs have been characterized in *B. cinerea* (see Table 1 in main text) [31,33,44,81–91]. Six of these are noncatalytic CDIPs and the rest are PCWD hydrolases, except for the transglycosylase BcCrh1, which catalyzes the crosslinking of chitin and glucan polymers in the fungal cell wall [33]. The plant cell-death-inducing activity of a number of catalytic CDIPs was found to be independent of their enzymatic activity, and in several cases, 20–40-amino-acid epitopes were found to be sufficient for the induction of cell death [33,82,92]. Deletion mutants of *bcxyn11a* [81], and *bcspl1* [87] showed reduced virulence, supporting a role for these CDIPs in pathogenicity. Deletion of any of the other CDIPs had no clear effect on virulence, but overexpressing *hip1* [89] or *bcieb1* [31] slightly increased virulence, and overexpressing *bxyg1* stimulated the appearance of early necrosis [82].

In addition to cell death, most CDIPs are recognized by plant receptors and elicit defense responses [31,82]. A few protein derivatives that induce defense without inducing cell death have been produced [33,82,93], while to our knowledge, there are no examples of the induction of cell death without the induction of defense. In several cases, the immune response was shown to be mediated by the BAK1 and/or SOBIR1 co-receptors, and a receptor-like protein (RLP23) that recognizes a 20-amino-acid epitope derived from BcNEP proteins was identified [26,94,95]. The contradicting activities and functional redundancy of CDIPs complicate their analysis; nevertheless, accumulating data strongly support their contribution to *B. cinerea* pathogenicity.

hydrogen peroxide, which accumulates in hyphal tips and ICs, possibly facilitating host penetration by promoting the oxidation of cuticle polymers [56,57]. Deletion of the *bcsod1* (superoxide dismutase) and *bcnoxb* (NADPH oxidase) genes reduced fungal virulence, supporting a role for H_2O_2 and other types of oxidizing agents in pathogenicity [58,59]. However, no change in ROS production was observed in *bcnoxa* or *bcnoxb* single or double mutants. These findings suggest that NADPH oxidase might not contribute significantly to the oxidative burst in *B. cinerea* and that reduced pathogenicity might be associated with changes in fungal development rather than a direct effect of ROS on the plant. ROS are also produced by plants as part of the defense response, and while there is strong evidence for their involvement in fungal–plant interactions, the precise roles of ROS and how they affect pathogenic development in *B. cinerea* require further investigation [60]. Recent research revealed that *B. cinerea* secretes a cytochrome *c*-peroxidase that facilitates plant invasion by detoxification of host-derived ROS [61].

Modulation of pH

Another pathogenicity mechanism is the local modulation of pH of the host tissue [62]. Modulation of pH, including both acidification and alkalinization of the host tissue, is necessary for virulence and is achieved by the temporally and spatially controlled secretion of organic acids, primarily citric acid that acidifies the tissue, followed by accumulation of ammonia that alkalinizes it [46,63]. It was also reported that oxalic acid is produced at the late colonization stage; however, unlike *S. sclerotiorum*, in which oxalic acid is essential for pathogenicity, the role of oxalic acid in *B. cinerea* pathogenicity is unclear [63,64]. Li *et al.* [65] found that the content of the *B. cinerea* secretome is significantly affected by pH: at pH 4, most of the identified proteins were associated with proteolysis, whereas the major proteins detected at pH 6 were PCWDEs. A hypovirulent VELVET mutant was impaired in the ability to acidify the host tissue; artificially reducing the pH partially restored the virulence of the mutant strain [46]. Based on these and other studies, it is clear that *B. cinerea* acidifies the host tissue and that this acidification is required for the proper progression of infection. However, a causal connection between impaired acidification and modified acid secretion and gene expression remains unclear and warrants further investigation.

Predicted virulence mechanisms and factors

As outlined in Figure 2, certain factors and mechanisms appear to be necessary for infection but lack sufficient scientific evidence. These include the activation of HR and the spread of cell death, the suppression of plant defense, and the suppression of autophagy/RCD. Here we examine the existing evidence for each of these processes.

Activators of the HR

Govrin and Levine [15] demonstrated for the first time that HR promotes *B. cinerea* pathogenicity. Later studies in Arabidopsis showed that various proteins contribute to pathogenicity, including proteins that mediate ROS production (such as RbohD) [18], regulators of cell death (such as the transcription factors Lsd1 and Lol1 [66]), and regulators of effector-triggered HR (such as Sgt1 [67]). These and similar studies suggest that the manipulation of host cell death by *B. cinerea* is an important element in plant colonization. In addition, these studies highlight potential molecular targets through which HR might be affected [68]. Unlike the rich information on the plant side, the only fungal molecule that has been demonstrated to activate plant RCD is oxalic acid, which might activate RCD via the induction of a ROS burst during the late infection phase [16]. However, it has been suggested that oxalic acid has multiple additional effects, including pH modulation, calcium chelating, suppression of the oxidative burst, regulation of stomata opening, and activation of PCWDEs, all of which can affect pathogenicity [63,64]. Moreover, unlike *S. sclerotiorum,* there is no evidence for the impact of oxalic acid on RCD or autophagy during *B. cinerea* infection [10]. Therefore, how *B. cinerea* activates the HR and RCD in the plant and uses them to its advantage remains an unsolved mystery.

Activators of spreading cell death

The transition from local to spreading lesions is associated with a developmental switch from the production of small amounts of unoriented hyphae to the massive production of radiating mycelia that can spread as quickly as 0.5 mm/h [13]. Remarkably, the hyphae always remain behind the lesion edge, which is preceded by a halo of dead cells [14,17] (Figure 1). This process entails the activity of RCD-inducing diffusible molecules, but candidate molecules have not yet been identified, and while they are assumed to originate in the fungus, a plant origin cannot be ruled out. Prime suspects are metabolites or small proteins, such as the secreted *S. sclerotiorum* SsSSVP1 virulence protein, which is internalized and moves to adjacent cells [69], and possibly also **small RNA species** (**sRNAs**) that might interfere with the regulation of plant RCD.

Suppressors of plant defense

Despite intensive research, *B. cinerea*-specific effector proteins that target the plant's defense system have not been discovered. The lack of candidate effectors might be due to technical difficulties in isolating them, but it is also possible that, in the case of a broad host range necrotrophic pathogen, 'classical' effectors are inefficient or that their contributions are masked by other factors. Another possibility is that *B. cinerea* produces other types of immune-suppressing molecules. One such molecule is sRNAs. Both pathogens and plants exchange sRNAs during their interaction, some of which alter disease progression [70,71]. *B. cinerea* contains two dicer homologs, *bccdl1* and *bccdl2*, both of which are required for virulence, and certain *B. cinerea* sRNAs were shown to suppress defense-related Arabidopsis gene expression and affect disease levels [72]. Further research is needed, including detailed studies of the effects of sRNAs on infection in plant species other than Arabidopsis, to obtain more concrete evidence for the true impact of sRNAs and their significance in affecting disease development.

Suppressors of autophagy and/or RCD

Biotrophic and hemibiotrophic pathogens secrete RCD-suppressing effectors that block the manifestation of the plant HR [73]. Such activity is counter-intuitive in the case of necrotrophic pathogens, which induce cell death and require an active HR system for pathogenicity, but could be relevant if *B. cinerea* has a short 'biotrophic' phase (Box 2). This aspect requires careful investigation, since so far there is no evidence for *B. cinerea*-derived RCD or autophagy-suppressing molecules. More research is needed to clarify the role of plant autophagy during infection and to determine whether *B. cinerea* indeed has a short nondestructive phase and whether it produces and utilizes autophagy/RCD-suppressing molecules during plant colonization.

Concluding remarks and future perspectives

Research in the past decade has yielded rich data revealing new details about the events and genes that control pathogenic development in *B. cinerea* [13,34,74,75]. While the picture is far from complete, these discoveries led to a paradigm shift for *B. cinerea* pathogenesis: from a brutal attack governed mainly by the massive secretion of enzymes and toxins, to a complex, multifactorial and multilayered process, including subtle mechanisms that can follow different infection routes (Figure 2). Similarly, it has become apparent that disease resistance is quantitative rather than complete [76]. Clearly, the balance between the fungus and plant is delicate, and even subtle changes can significantly affect disease progression, for example, changes in the fungal inoculum, timing of defense activation, or external conditions. These findings imply that effective control might be achieved using an integrative approach combining the utilization of the plant defense systems, altering the plant environment, and impairing fungal development and pathogenic processes. In the coming years, new technologies, such as advanced imaging, microfluidics, and genome editing, are expected to provide detailed information on specific processes and molecules that affect disease development. Alongside laboratory studies, special attention should be given to studies of the interaction under field conditions and how it is affected by environmental factors (see also Outstanding questions).

Outstanding questions

Does infection include a short nondestructive phase, or is it mediated by the immediate induction of cell death to generate a 'sterile' zone?

After penetrating the cuticle, do hyphae invade and then kill plant cells, or do they induce cell death before cell invasion?

Which molecules activate RCD and induce the spreading of cell death during the second infection phase? So far, only CDIPs and phytotoxins have been identified; it is expected that effectors that target the plant RCD machinery are also involved in various stages of disease progression.

At what stage is the fungus first exposed to, and affected by, the plant immune system?

Does *B. cinerea* produce protein effectors that suppress plant defense responses?

To what extent does the exchange of sRNAs between *B. cinerea* and the host plant affect disease development?

Acknowledgments

The work was supported by BARD grant #5261-20C to A.S. and T.M. Y.L. (202108280009) was funded by the China Scholarship Council (2021). K.B. was supported by Open Fund (#2022ZTSJJ6) from Key Laboratory of Integrated Pests Management on Crops in Central China/Hubei Key Laboratory of Crop Diseases, Insect Pests and Weeds Control.

Declaration of interests

No interests are declared.

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