Disease cycle and infection strategies of systemic plant pathogen *Botrytis cinerea*

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Abstract

*Botrytis cinerea* is a ubiquitous filamentous fungal pathogen of a wide range of plant species. This fungus is able to infect all aerial parts of its host plants; where infection may cause enormous damage both during plant growth and in the post-harvest stage (during cold storage or transport). *B. cinerea* is a major cause of economic losses in the production chain of cut flowers, bulb flowers and pot plants. Molecular-genetic studies performed over the past decade have provided a wealth of novel insights into the infection mechanisms utilized by this pathogen. Fungal genes important for successful infection by *B. cinerea* were identified. Such knowledge provided perspectives for designing novel, and rational plant protection strategies that could effectively counteract important pathogen virulence factors. In this study; the infection process will be divided into different stages; moreover, the role of various fungal enzymes and metabolites in the different stages will be discussed. The aim of the current study was to address perspectives for novel control strategies that may reduce and/or delay the damage incited by *B. cinerea* infection.

Keywords: *Botrytis cinerea*, Grey mold, post-harvest damage, molecular genetics, virulence genes

1. Introduction

*Botrytis cinerea* Persoon: Fries (teleomorph *Botryotinia fuckeliana*, commonly known as grey mold fungus) is a member of the phylum Ascomycetes, family Sclerotiniaceae. It causes devastating diseases on about 500 plant species worldwide, such as fresh fruits and vegetables, resulting in great economic losses ranging from $10-$100 billion (Hua *et al*., 2018). Williamson *et al*., (2007) considered *B. cinerea* as the most widely distributed causal agent of diseases of vegetables, ornamental fruits and field crops throughout the world. It triggers clear disease symptoms in the pre-harvest stage or remain dormant until post-harvest stage (Fillinger and Elad, 2016). Vegetables stored for weeks or months at temperatures ranging from 0-10°C, transported fruit, ornamental flowers and bulbs, as well as forest seedlings were all attacked by this pathogen (Govrin and Levine, 2000; Elad *et
al. 2004; Zhao et al., 2009). Depending on the climatic conditions, losses may be complete in some areas such that continued crop production becomes impossible. The pathogen is a necrotroph, inducing host cell death resulting in serious damage to plant tissues culminating in rot of the plant or the harvested product. Previous researchers have focused on the infection mechanisms of B. cinerea; with special emphasis on molecular genetics (Prins et al., 2000b), microscopic and biochemical studies (Williamson et al., 2007). The applications of molecular-genetic tools such as; transformation (Elad et al., 2004; Williamson et al., 2007; Rajaguru, 2008), differential gene expression analysis (Elad et al., 2004), gene cloning (Agrios, 2005) and targeted mutagenesis (Beever and Weeds, 2004; Van Kan, 2005), have led to novel insight on the structure of B. cinerea genes and their role in the infection process. However, current study focused only on the disease cycle and the mechanisms employed by B. cinerea to infect its host plants.

2. Disease caused by B. cinerea

B. cinerea has two stages in its life cycle; the asexual stage or anamorph named as B. cinerea, and sexual stage or teleomorph Sclerotinia fuckeliana (Agrios, 2005). According to Holz et al., (2004), the pathogen exists as mycelia, conidiophores, conidia, ascospores, spermatia, germ-tubes, apressoria, chlamydo sporces and apothecia. Earlier, Elad et al., (2004); Holz et al., (2004) reported that mycelia, ascospores, conidia and sclerotia were important forms of disposal propagules; although mycelia and sclerotia in host residues were the primary survival structures of B. cinerea. Conidia were easily dispersed by humid air currents, splashing water, tools and clothing to healthy plants where they initiate new infections (Holz et al., 2004). These conidia can infect seedlings, flowers, stems, or leaves (Agrios, 2005). Once tissues have been infected and colonized by the fungus; spores are readily produced during periods of high humidity (Agrios, 2005), and cool damp weather (15-25°C). Spores were released when infected leaves or fruit clusters were disturbed by wind, insects or human activities. Agrios, (2005) added that the infection hyphae release enzymes that cause the host tissue to senesce in advance of the fungus; then the tissues become soft and start to rot, as a result more conidia were produced which could infect new plants. The sexual cycle of B. cinerea involves the fertilization of the female gamete (sclerotia) by the male gamete (microconidia), resulting in the production of apothecia and asci with eight binucleated spores (Beever and Weeds, 2004). The sexual stage was rare in most crops attacked by B. cinerea (Williamson et al., 2007). However, Elad et al., (2004) reported that B. cinerea can enter the host tissue and remain dormant for a varying period. During this dormancy; fungal growth and colonization were halted, and signs of the pathogen were not visible (Cadle-Davidson 2008).

Elias et al., (2010) found that B. cinerea could grow systemically and endophytically without showing symptoms in lettuce plants. Such systemic infection was caused by an isolate of B. cinerea which grew throughout the body of healthy plant without showing symptoms. This was attributed to the fact that B. cinerea did not produce toxic compounds or produced such compounds at a low level which might allowed it to continue to grow without being detected by the plant defensive mechanisms.

3. Life cycle of B. cinerea

3.1. Attachment of conidia

Shafia, (2009) reported that surface physical interactions on the plant cuticle mediated the attachment of B. cinerea with the host plant. However, two steps were involved in the attachment to host tissue. The first step preceding the hydration of conidia typically involved weak adhesive forces resulting from hydrophobic interactions between host and conidial surfaces. Stronger binding however occurred in the second step several hours after inoculation, when conidia have germinated.
The tips of germ tubes were covered with fibrillar-like extracellular matrix material (Sowley, 2006), consisting of carbohydrates and proteins (Shafia, 2009). This matrix contained fungal enzymes (Gil-Ad et al., 2001). Moreover, it might acted as an adhesive on the host surface (Shafia, 2009), that protected the hyphae from dehydration and from defense mechanisms of the host.

3.2. Germination of conidia of *B. cinerea*

Germination of conidia of *B. cinerea* (Fig. 1) on the plant surface was influenced by many factors. High relative humidity of free surface water (>93% RH) was essential for germination and penetration of the host epidermis (Williamson et al., 2007). When dried conidia were inoculated on plant surfaces and incubated in the absence of free surface water, the emerging germ tube usually remained short before it could penetrate the surface. Moreover, Williamson et al., (2007) added that inoculation of conidia in an aqueous suspension required the addition of nutrients, which might mimic the situation in a wound on the plant epidermis from which nutrients leach. According to Sowley, (2006), a highly efficient germination and synchronous infection of tomato leaves were obtained, when conidia were pre-incubated for 2-4 h in liquid medium supplemented with phosphate and sugar prior to inoculation.

![Fig. 1: Conidia and conidiophores of *B. cinerea*](image)

Gaseous compounds may also stimulate germination. Elad, (2004) reported a correlation between the level of ethylene production by rose cultivars and the severity of grey mold symptoms. Stimulation of grey mold development by ethylene was also recorded in strawberry, tomato, cucumber and pepper. This observation was usually ascribed to host tissue senescence that coincided with ethylene production. Germination of conidia on a hydrophobic surface was stimulated by exogenous ethylene; however, the germ tube length was unaffected (Williamson et al., 2007). Barnes and Shaw, (2003) reported earlier that application of 2,5-norbornadiene; a competitive inhibitor of ethylene perception in plants, inhibited conidia germination in a reversible manner.

Ethylene produced by the plant during tissue senescence or fruit ripening might function as a signal for the conidia on the hydrophobic plant surface to germinate and initiate the infection (Elad, 2004). Germ tube elongation might subsequently be stimulated by ethylene present in the more hydrophilic environment of the invaded plant tissue. Thus, ethylene might favor grey mold development by weakening the host, as well as by stimulating germination of *B. cinerea* conidia and subsequent outgrowth of hyphae. Molecular and biochemical approaches are required to further elucidate the effects of ethylene on conidia of *B. cinerea*.

4. Differentiation of infection structures on the host surface

During penetration, *B. cinerea* forms appressoria which is typical for many plant pathogenic fungi (Agrios, 2005). Several authors observed the swelling of hyphal tips of germ tubes and interpreted these as an appressorium-like structure (Elad, 2004). The microscopic, histochemical studies and gene function analysis of Tenberge et al., (2002) showed that these structures act as functional appressoria. Swelling of the hyphal tip might be the consequence of a rise in osmotic pressure resulting in more water absorption. However, Skamnioti, and Gurr (2007), reported that in the absence of a rigid layer in the outer germ tube wall, swelling cannot result in an equally high turgor as in the appressoria of *Magnaporthe grisea*. The extracellular matrix might contribute to the swelling
by retaining water, as its major polysaccharide component (cinerean) was extremely hygroscopic.

5. Host penetration by *B. cinerea*

Invasion of host tissue can be achieved by active penetration or passive ingress. *B. cinerea* is an opportunist that can initiate infection at wound sites, or at sites previously infected by other pathogens. *B. cinerea* can also enter the substomatal cavity via an open stomata. Nevertheless, this pathogen is also perfectly able to penetrate intact host surfaces. However, only direct penetration of the epidermal surface was discussed in this review.

Plant cell wall is the first line of defence that an invading pathogen encounters. It is a heterogeneous structure composed of polysaccharides and proteins (Kubicek et al., 2014). The cuticle present externally on plant cell wall consists of cutin; a polyester of hydroxylated and epoxidized C16- and C18-fatty acids, covered with wax. Physical damage or brute mechanical penetration of the cuticle by *B. cinerea* was not usually observed (Williamson et al., 2007). Accordingly, Sowley, (2006) considered that enzymatic (cutinolytic) activity was required for penetrating intact host surfaces. The gene encoding this cutinase enzyme was cloned, and a gene mutant isolate was made which was entirely devoid of this cutinase activity (Elad, 2004). This mutant was equally virulent as the wild type isolate both on gerbera flowers and tomato fruits; moreover, the fungus retained its ability to penetrate intact cuticle surfaces.

A different enzyme that might mediated host penetration was a 60 kDa lipase (Van Kan, 2005); inducible by apple cutin (Elad, 2004; Agrios, 2005), as well as grape berry cuticle components (Agrios, 2005). Van Kan, (2005) reported that this lipase enzyme possessed cutinolytic activity although with clearly distinct kinetic properties than the typical cutinase mentioned above. When polyclonal antibodies raised against this lipase enzyme were applied prior to inoculation with *B. cinerea* conidia, germ tubes were unable to penetrate the cuticle. These antibodies however did not affect germination of conidia (Elad, 2004; Shafia, 2009). Therefore, constructing a targeted lipase-deficient mutant of *B. cinerea* and determining its virulence should assess the role of the lipase enzyme in host tissue penetration.

6. Killing of the host by *B. cinerea*

According to Williamson *et al.* (2007), *B. cinerea* kills host cells before they were invaded by hyphae. Invasion of plant tissue by *B. cinerea* triggered nuclear condensation and plant membrane damage, indicating programmed cell death in a ring of cells around the fungal hyphae (Govrin and Levine, 2000). These results implied that diffusible factors have direct or indirect phytotoxic activities. These factors might be proteins or low molecular weight compounds secreted by the fungus into its environment. This induction of programmed cell death facilitated invasion of *B. cinerea* and might be essential for successful infection. This was achieved through the followings:

6.1. Mycotoxins

Mycotoxins are toxic secondary metabolites produced by several mold fungi, whose occurrence in food and feed cause deleterious health effects on humans and poultry. Yeast cell walls, clay binders, and antioxidant additives are the most widely used products to eliminate mycotoxin and to reduce their harmful effect (Horky *et al*., 2018). *B. cinerea* culture filtrates may induce toxic effects when applied to plant tissue (Elad, 2004). Phytotoxic compounds were identified as botcinolide; a highly substituted lactone (Agrios, 2005), and botrydial; a tricyclic sesquiterpene (Williamson *et al*., 2007). The observation that both secondary metabolites were only secreted by *B. cinerea* in medium with high glucose levels initially raised doubts about their physiological relevance in planta. Analytical chemistry studies have demonstrated that botrydial
accumulated in infected tissue at physiologically relevant concentrations (Deighton et al., 2001; Muckenschnabel et al., 2003). Production of botrydial might be an important factor in the infection of some host plants, but this needs to be evaluated by constructing mutants in the botrydial synthetic pathway.

6.2. Oxalic Acid

Fungi usually cause changes in pH of the surrounding environment through secreting number of acids or alkalis. Organic acids produced by fungi include; oxalic acid, citric acids, gluconic acid, butyric acid, succinate and malate, whereas alkalis secreted mainly refers to ammonia (Vylkova, 2017). Secretion of oxalic acid (OA) occurred in fungi from various taxonomic classes (Agrios, 2005). A key role for OA in pathogenesis has been reported in Sclerotinia sclerotiorum (Govrine and Levine, 2000), a close relative of B. cinerea. Mutants of S. sclerotiorum deficient in OA production; were unable to infect Arabidopsis plants (Deighton et al., 2001); however, this deficiency could be reversed by supplementing the inoculum with OA. B. cinerea produced OA both in vitro (Agrios, 2005), and in plant (Sowley, 2006). The sizes of lesions induced by several strains of B. cinerea on grapevine and bean leaves correlated with the amount of OA that these strains secreted in vitro (Williamson et al., 2007). OA might be in fact a co-factor in pathogenesis rather than the primary phytotoxic agent. Van Kan, (2005) postulated that OA might act in synergy with endo-Polygalacturonases (PGs) during tissue maceration. Fungal endo-PGs have an optimum activity at low pH, and might therefore be stimulated by the simultaneous secretion of OA. Moreover, OA may stimulate pectin degradation resulting from endo-PG action by sequestering the Ca^{2+} ions produced from intact or partially hydrolyzed Ca-pectates in the host cell walls. Removal of Ca^{2+} ions disturbed the intermolecular interactions between pectic polymers, and disrupted the integrity of the pectic backbone structure. Consequently, the pectic structure absorbed water and swelled as described earlier by Elad, (2004).

6.3. Induction of Reactive oxygen species

Previous researches have focused on production of reactive oxygen species (ROS) in relation to B. cinerea pathogenicity. ROS is the joint term for the superoxide anion, hydroxyl radical and hydrogen peroxide. ROS can be generated in B. cinerea either as unavoidable metabolic byproducts or as major products of NADPH oxidase (Li et al., 2016). The level of H_{2}O_{2} released from bean leaf discs (Phaseolus vulgaris) inoculated with different B. cinerea isolates; correlated with the aggressiveness of this isolate on such leaf tissue (Deighton et al., 2001). Moreover, the sensitivity of different genotypes of bean to oxidative stress was correlated to their susceptibility to B. cinerea. However, in Arabidopsis thaliana leaves infected with B. cinerea; H_{2}O_{2} was detected in the apoplastic space in many cell layers away from the fungal hyphae (Govrin and Levine, 2000). Elad, (2004) added that lipid peroxidation was observed, and also antioxidants were found to reduce grey mold disease development. Furthermore, the generation of ROS occurred at the host-fungal interface, these processes were enhanced by both the fungal and host enzymes (Prins et al., 2000b; Schouten et al., 2002a). Sowley, (2006) reported that oligogalacturonides released from the plant cell wall by pectinases of B. cinerea were potential elicitors of an oxidative burst; presumably mediated by a plasma membrane-bound NADPH oxidase induced by fungal elicitors, and requiring extracellular Ca^{2+}. Infiltration of the NADPH oxidase inhibitor into leaves of Arabidopsis thaliana prior to inoculation with B. cinerea, resulted in reduction of ROS production and slower colonization of host tissue by this fungal pathogen (Govrin and Levine, 2000). The fungal extracellular sugar oxidases (Deighton et al., 2001); or superoxide dismutase enzyme (Elad, 2004), might be responsible for generating the H_{2}O_{2}. 
7. Induction of primary lesions, defence responses in the host

Host surface penetration and rupture of plant cell walls by enzymes of *B. cinerea* triggered a cascade of processes in the fungus as well as in the host. This was achieved through the following stages:

7.1. Induction of necrosis

The initial establishment of primary necrotic lesions coincided with and was in fact the result of activation of the host defense in the neighboring tissue in response to the death of an invaded cell. However, it was not clear whether cell death caused by a necrotroph such as *B. cinerea*, was equivalent to cell death due to a hypersensitive response (HR) to a biotrophic pathogen (Sowley 2006; Shafia 2009). Govrin and Levine, (2000) showed that an oxidative burst occurred in plant tissue several cell layers away from the fungal hyphae. Agrios, (2005) added that cytological staining provided evidence for rapid nuclear condensation and irreversible membrane damage, indicative of a programmed cell death process. Largely the same defense responses were activated during an infection by *B. cinerea* as caused by a hypersensitive response to avirulent races of a biotrophic pathogen. The total spectrum of defense responses resulted in a primary necrotic lesion in which the fungus was effectively restricted. Therefore, depending on the type of host tissue and on unidentified physiological aspects of the host, the lesions entered a lag phase in which they did not expand. However according to Diaz et al., (2002); Agrios, (2005), a proportion of the primary lesions eventually developed into aggressive expanding ones. Thus, an active defence contributed temporarily to restricting the fungus within the primary lesions, giving rise to a period of quiescence.

7.2. Quiescence

Usually *B. cinerea* is quiescent in the host tissues for long periods of time, thus disease symptoms appear later than host infection. Therefore, several apparently healthy tomato fruits rot during their storage or during their transport to the stock markets. Accordingly, gray mold disease causes rotting to tomato fruits before and after harvesting (Gao et al., 2018). Prominent examples were previously described in other soft fruits such as; strawberry, raspberry and grape. In these hosts; *B. cinerea* predominantly infected the host flowers and resided quiescent in the developing fruit tissues, often for several weeks (Sowley, 2006). Fungal growth however resumed at the onset of fruit ripening. It has been postulated that high levels of fungitoxic or fungistatic compounds (i.e. phytoalexins) in immature fruits contributed to grey mold quiescence. The level of these compounds decreased during the ripening process concomitant with fungal outgrowth. Many attempts have been undertaken to increase the levels of these antifungal compounds or to prevent their degradation during ripening. The level of the stilbene phytoalexin in grapes was correlated with grey mold resistance (Deighton et al., 2001). A significant partial resistance was recorded in tobacco (Williamson et al., 2007), but not in tomato (Deighton et al., 2001).

De Lorenzo et al., (2001) pointed that besides phytoalexins, immature fruits contained high levels of proteinaceous inhibitors of fungal cell wall degrading enzymes such as the Polygalacturonase inhibiting proteins (PGIPs); however, their level decreased during ripening. Due to the important role played by polygalacturonases in the infection process, efforts to produce transgenic plants with over expressed PGIPs have been undertaken to obtain resistance towards *B. cinerea*. According to Powell et al., (2000); Ferrari et al., (2003), high constitutive expression of a heterologous PGIP gene in tomato and in *Arabidopsis* resulted in an increased resistance to *B. cinerea*. One of the important considerations in this strategy was that PGIP had differential activities towards individual fungal endo-PGs (De Lorenzo et al., 2001). This made it relevant to choose PGIP that were potent
against *B. cinerea* endo-PG isozymes, which in turn were vital for virulence (Ten Have *et al*., 2002).

8. Evasion of chemical defence

Plants have evolved several defense mechanisms to protect themselves against microbial colonization including; constitutive and inducible resistance methods. Potential pathogens are prevented from entrance into the plant by preformed physical and chemical barriers; moreover, pathogens induce a complex array of defense responses in plants after infection (Yang *et al*., 2018). Pathogenic fungi have developed mechanisms to overcome deleterious effects of plants preformed phytoanticipins, or induced phytoalexins defence compounds. One of such mechanisms involved an energy-dependent secretion by ABC-transporters that conferred some degree of tolerance to the fungitoxic effects of such compounds (Agrios, 2005). The major strategy that phytopathogenic fungi used to deal with plant antifungal compounds was enzymatic detoxification (Agrios, 2005). *B. cinerea* produced a substrate-specific laccase (stilbene oxidase) that was able to oxidize antifungal compounds to non-toxic derivatives (Agrios, 2005). *B. cinerea* possessed a set of cell wall degrading enzymes (CWDE) including pectin lyase (Deighton *et al*., 2001), pectin methylesterase (Shafia, 2009), exo- and endo-polygalacturonase (Deighton *et al*., 2001) and cellulases (Elad, 2004). BcGs1 was a CWDE produced by *B. cinerea*; which induced defense response and improved disease resistance to this pathogen in tomato (Zhang *et al*., 2015).

9. Maceration of tissue and disease expansion

*B. cinerea* must be able to macerate plant tissue and convert it into fungal biomass, to be able to enter it. To overcome the barrier of the plant cell wall, most pathogenic fungi produce different cell wall degrading enzymes including; cellulases, pectinase, hemicellulases, proteases and cutinase. Most of these enzymes not only degrade plant cell wall components to get pathogen carbon sources necessary for its growth, but also they trigger plant defense responses (Yang *et al*., 2018). Cell wall degradation facilitates the entry of the pathogen and it provides nutrients for growth (Ten Have *et al*., 2002).

Microscopic studies showed that after penetration of the cuticle, hyphae of *B. cinerea* frequently invade the wall between two epidermal cells. The concomitant swelling of the epidermal cell wall (Barnes and Shaw, 2003); was indicative of the degradation of pectin in the matrix of the epidermal wall, resulting thus in water absorption. *B. cinerea* possesses a set of cell wall degrading enzymes (CWDE) including pectin lyase (Deighton *et al*., 2001), pectin methylesterase (Shafia, 2009), exo- and endo-polygalacturonase (Deighton *et al*., 2001) and cellulases (Elad, 2004). BcGs1 was a CWDE produced by *B. cinerea*; which induced defense response and improved disease resistance to this pathogen in tomato (Zhang *et al*., 2015). *B. cinerea* genes have been cloned that encode CWDE: pectin and pectate lyase (Mulder, unpublished), rhamnogalacturonan-hydrolase (Agrios, 2005), six endo-PGs (Valette-Collet *et al*., 2003), pectin methylesterase (Valette-Collet *et al*., 2003) and cellulases (van Kan *et al*., unpublished). The expression patterns of the individual endo-PG genes in plants depend on the host species infected, on the stage of the infection, as well as on the external conditions during which infection took place (Ten Have *et al*., 2001).

Targeted deletion mutants were made in Bcpg1 by gene replacement. Lesion expansion rate of such
mutants was reduced by about 25% as compared to the wild type (Shafia, 2009). The Bcpg1 protein might be important in facilitating intercellular growth at the periphery of the invading hyphae. Another explanation for the reduction in virulence might be purely nutritional. The absence of BcPG1 reduced the release of pectin degradation products that served as nutrients, hence resulting in slower growth of the fungus throughout the plant tissue.

10. Control of *B. cinerea* infection

The control of losses caused by *B. cinerea* required the use of fungicides; modifying the techniques of cultivation, and the development of resistant or tolerant varieties. However, continuous use of fungicides to control this fungus resulted in the appearance of resistant strains (Leroux *et al.*, 2002). Moreover, the gray mold disease caused by *B. cinerea* is difficult to control using fungicides. Accordingly, manipulation of alternative methods such as biocontrol might be the best choice to control this pathogen and reduce damages caused by synthetic fungicides (Gao *et al.*, 2018). Therefore, extensive research efforts were focused on management of this fungal pathogen using ecofriendly strategies such as biocontrol through beneficial insects (Pscheidt, 2007).

Conclusion

Microscopic and biochemical observations of the infection strategy of *B. cinerea* are presently validated by the availability of molecular-genetic tools. Therefore, several genes have been cloned and their expression *in vitro* or in plants has been studied. The functions of different individual genes in the infection process can be analyzed by targeted mutagenesis, and by studying the behavior of mutants on various hosts. Current finding showed that the pathogen was versatile and used different combination of factors during the process of pathogenesis. Therefore, understanding the roles of various factors in the different stages of the disease cycle, and the ways in which some of these factors interact is of paramount importance. It is clear that there was equilibrium between the different attack mechanisms of the fungus and the defence of the host, therefore, knowledge of such mechanisms will immensely assist in disease control strategies. Finally, our future challenge lies in the design of methods that can change the balance in the host\-pathogen interaction in favor of the host. Such future type of strategy most likely involves combination of biocontrol agents, appropriate/partially resistant plant genotypes, and chemicals that either improve the plant defence response or interfere with the critical steps in the infection process.

Conflict of interests

No potential conflict of interest was reported by the authors.

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11. References


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