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SIGNALING PATHWAYS OF CONIDIAL GERMINATION AND GROWTH OF *BOTRYTIS CINEREA*: HOST DETECTION, PATHOGENESIS ON *VITIS VINIFERA* AND PREFERENCE FOR WINE GRAPES

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ABSTRACT

Botrytis cinerea is a phytopathogenic filamentous fungus that infects and causes severe damages to numerous crops. However, the European grapevine (*Vitis vinifera*) remains the major host for the pathogen. Botrytis infects mainly ripe grapes (usually over 10 degrees Baumé) unless primary infections by other fungi, or infestations by viticultural insect pests, or mechanical damages have occurred. The disease is commonly known in viticulture as "Botrytis bunch rot" or "grey mold" and can spread rapidly causing devastating losses on the field and post-harvest. Regardless of its generalist action, *B. cinerea* must develop strategies to recognise and invade its host. The fungus responds to physical and chemical stimuli, emanated from the environment to activate spore germination and growth. Once a signal from the external environment meets a target molecule of the cell, the signaling process begins. The target molecule is a protein that acts as a receptor. The physical and chemical signals that stimulate conidial germination depend on factors such as surface hardness, surface hydrophobicity, carbon sources and nutrient-rich substrates. Among sugars, fructose is the best growth inducer of *B. cinerea.* Heterotrimeric guanine nucleotide binding proteins (G-proteins) are involved in the regulation of a range of functions including germination, growth and host detection. G-proteins consist of three subunits, α, β and γ. Two of these subunits attach to the cell membrane with small lipid tails. At rest, unit α is bound to Guanosine Diphosphate (GDP) and the G-protein is inactive. When an extracellular ligand binds to the receptor, the latter interacts with the G-protein and activates it, forcing the α subunit to excrete the bound GDP and replace it with Guanosine Triphosphate (GTP). In addition to signals that trigger spore germination, pathogenesis is also influenced by several biotic and abiotic factors. In general terms, interactions between pathogen, its potential host and the environment affect level, speed and nature of the infection and must be substantially considered in integrated control strategies.

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INTRODUCTION

Botrytis cinerea (Pers.) (Ascomycota: Sclerotiniaceae) (teleomorph: *Botryotinia fuckeliana*) is a non-specific phytopathogenic fungus that has been detected in more than 200 dicotyledonous plant species and a few monocotyledonous. However, the European grapevine (*Vitis vinifera* L.) is considered as the main host of the fungus. The disease caused to grapes is commonly known in Viticulture as "Botrytis bunch rot" and has an enormous impact in wine composition and quality (Ciliberti et al., 2015; Ribéreau-Gayon et al., 1980). In general terms, Bunch rot is mainly caused by *B. cinerea*, but also by other fungi (*Aspergillus* spp., *Penicillium* spp.), and is responsible for significant losses in wine production worldwide (Steel et al., 2013). In the case of wine grapes, *Botrytis* can act alternatively by dehydrating the berries in a controlled way, causing what is known as "Noble rot" (Lovato et al., 2019; Negri et al., 2017; Roubos, 2016.). The development of Noble rot is enhanced by specific environmental conditions and preferably appears in certain wine grape varieties. Environmental factors such as temperature and humidity influence significantly spore germination, pathogenesis and the mode of infection of *B. cinerea* (Ciliberti et al., 2016). Favorable conditions for the development of *B. cinerea* are warm temperatures (around 17-28˚C) and high relative humidity (over 90%), while the disease occurs regularly during the late stages of grapes' ripening period (Broome et al., 1995; Rodríguez-Rajo et al., 2010). Except for grape berries, *B. cinerea* causes substantial economic damages (grey mold) in several more crops including fruit trees, berry producing scrubs, ornamental plants and vegetables (Elad et al., 2007; Jarvis, 1980).

There are many methods to detect *B. cinerea* in vineyards by matching the fungal DNA. Real-time PCR can detect targets of the pathogen in different samples (Alaei et al., 2009; Carisse et al., 2009). The qPCR method is suitable for the detection of *B. cinerea* because of the accuracy of the detection and the time of the processing (2-3h) that can lead to quicker results (Wahab and Younis, 2012). Aerobiological studies can indicate the presence of *B. cinerea* in vineyards by using biosensors to detect airborne spores throughout the daytime (Fernández-González et al., 2012). Once spores' concentration is higher in the vineyard, compared to the atmospheric air, conidia need about 4-6 days (depending on the phenological stage of the grapes and weather conditions) to cause infection (Carisse et al., 2008). Sclerotia of *B. cinerea* can survive during various environmental changes and can remain viable into the soil for up to 360 days.

Because of the vast economic importance, extended research has been conducted to investigate potentially effective control methods for *B. cinerea*. However, a thorough understanding of all aspects of the pathogen's physiology and decision-making is considered as crucial for long-term control strategies. In the present study, intracellular signaling pathways involved in the spore germination and growth of *B. cinerea* were analytically described. Moreover, an attempt was made to investigate the factors that influence host detection, the mechanism of infection (pathogenesis) and the reasons behind the pathogen's preference for specific grape varieties.

Life cycle of *B. cinerea* in relation to pathogenesis on *V. vinifera*

Botrytis is a filamentous ascomycete and is considered as a necrotrophic fungus because it kills host's tissues to benefit from their nutrients (Bhatia et al., 2020). Its sexual form is rarely observed and therefore, the infection in vineyards usually involves viable conidia that are able to adhere to the leaves and the inflorescences (Coertze et al., 2001; McClellan and Hewitt, 1973). Botrytis grows in ripe and over-ripe grape berries during the summer and autumn, overwinters in the form of sclerotia in a saprophytic stage and completes its life cycle on plant debris. Except for the berries, it can infect shoots, leaves and inflorescences, reducing vitality and productivity of the vines. After the infection of vines, biochemical changes appear and lead to increase of volatile organic compounds, sugar and nitrogen contents in the ripening host tissues (Neri et al., 2015; Prusky and Lichter, 2007). When these changes occur, the fungus excretes cell wall degrading enzymes, oxalic acid and other hormones to diminish host's immune system and alter its cellular structure (Gentile, 1954; Sasanuma and Suzuki, 2016). This action results in softening and darkening of the berries, as well as decay of the grapes (Xiao, 2006). In the case of Noble rot, the berries are transformed by the penetration of fungi through stomata or wounds on the skin of the grapes, the permeabilization of the skin that encourages water loss and sugar concentration, and finally by the enzymatic maceration. At the end of this process, further fungal development is prevented by the

high sugar concentration and other abiotic factors (Lovato et al., 2019; Negri et al., 2017).

Cellular communication

The communication of cells with their environment consists of the conversion of information from one form to another.This conversion process is called signal transduction.The signaling process begins when a signal from the external environment meets a target molecule belonging to the cell. In either case, the target molecule is a protein that acts as a receptor (receptor protein). This protein is usually activated by only one type of signal. The receptor performs the first step of the transfer, receives the external signal and responds by generating a new intracellular signal. This is just the beginning of a sequence of intracellular signal transduction processes. Most signal molecules are too large or hydrophilic and cannot penetrate the cell membrane. Therefore, the corresponding protein receptors must be integrated into the cell membrane in such a way that they detect a signal on the outside and transmit the message in a new form through the membrane to the inside of the cell. Most cell surface receptors belong to one of the three major families: a) receptors that cross-link with ion channels b) receptors that cross-link with G-proteins, and c) receptors that cross-link with enzymes (Alberts et al., 2003).The receptors of the three categories differ in the nature of the intracellular signals they produce once the extracellular signal molecule binds to the receptor. For receptors connected to ion channels, the intracellular signal is the flow of ions via the membrane that produces an electric current. For G-protein-binding receptors, it is the activated form of a membrane protein, which is released and diffused at the cell membrane level, triggering a sequence of other events. For enzymes that bind to enzymes, the intracellular signal is the enzymatic activity that is stimulated on the cytoplasmic side of the receptor and generates a variety of signaling molecules, including molecules that are released into the cell lysis.

Intracellular signal sequence of *B. cinerea*: Host detection and preferences

B. cinerea, as a non-specific filamentous fungal pathogen, affects numerous species of dicotyledonous plants, including several vegetables and fruits. However, the fungus must develop strategies to "recognize" its hosts, penetrate and invade plant tissues to overcome the host's defenses. To respond to these stages, the fungus is able to perceive chemical and physical stimuli of its environment from different host plants and to respond with the appropriate metabolic activities required for pathogenic growth. In general, such metabolic adaptations include attachment of conidia to the plant surface, directed growth of a germ microbial tube, differentiation of infection structures, and secretion of lytic enzymes and toxins (Knogge, 1996). This response to environmental stimuli requires a signal transduction network, such as the activation of G-proteins (Bölker, 1998), the production of cyclic AMP signaling molecule (cAMP) (Mitchell and Dean, 1995), and the MAP signal transduction system - Kinase (MAPK) (Xu, 2000) to transmit the external signal to the fungus genome so that the appropriate gene or sets of genes are activated and modulate the pathogen's functions according to the external stimuli. The physical and chemical signals that stimulate conidial germination of the plant pathogen *B. cinerea*, can be distinguished into factors that stimulate germination such as a) surface hardness, b) surface hydrophobicity, c) organic P carbon sources and (d) rich nutrient substrates, for example malt extract. It is known that conidial germination and infection through intact plant surfaces are largely stimulated by nutrient availability (Cotoras et al., 2009; Kosuge and Hewitt, 1964). In inert artificial surfaces, various amino-acids and sugars effectively induced conidia to germinate, while minerals such as ammonium and phosphate were effective only in the presence of low sugar concentrations (Blakeman, 1975). On epidermal surfaces, dry inoculated conidia can also germinate in high humidity in the absence of liquid (Prins et al., 2000). Surface hardness is the most important factor, because in the absence of a hard surface, other vegetation factors are less effective or even ineffective. Surface hydrophobicity combined with surface hardness effectively led to the germination of botrytis conidia even in the absence of nutrients. The waxes of epidermal plant cells stimulate vegetation on hard surfaces; the germination signal provided by wax layers was mainly their hydrophobicity. The initial adhesion of *B. cinerea* conidia, caused by hydrophobic interactions with the plant surface, is relatively weak. Strong adhesion release does not occur until germination occurs. Rapid germination of conidia is observed in rich nutrient substrates. The mechanism of nutrient detection by *B. cinerea* is currently not well known. As different sugars and acids induce germination with similar efficiencies, it is unlikely that nutrients from plasma membrane

proteins can be detected (Doehlemann et al., 2005).

Preferences regarding sugars and the microbial ecology of grape berries

In order for the fungus to germinate, conidia must perceive nutrients' presence, either in the plasma membrane or after being transported to the cell. Of the sugars, fructose has been identified as the best growth inducer in *B. cinerea*, it is more effective than glucose and other hexoses or disaccharides (Blakeman, 1975). This is a remarkable fact because glucose is usually the most effective hexose not only as a nutrient but also as a molecular activator of the signal sequence. Fructose is significantly more effective than glucose as a growth inducer in the wild-type strain of the fungus; on the other hand, experiments in wild-type sugar or mutant conidia revealed significantly higher affinity for glucose uptake than for fructose. This is most likely due to unknown hexose transporters present in conidia (Doehlemann et al., 2006). Microbial ecology on the skin of grape berries also affects infectivity and development of *B. cinerea*. The initial growth of the fungus is enhanced or inhibited depending on the species of yeasts, filamentous fungi and bacteria that exist on the surface of grapes. For instance, the presence of yeast species such as *Pichia membranifaciens, P. anomala and Debaryomyces hansenii* results in significant inhibitory effects against Botrytis. It is likely that yeasts can inhibit fungal pathogens through the secretion of cell wall-degrading enzymes (Santos et al., 2004). However, antagonism between yeasts and *B. cinerea* is not supported in all cases and even positive interactions can occur. In the case of Noble rot, grapes were constantly found to sustain higher microbial numbers (bacteria and yeasts) than healthy grapes, one week prior to maturation (Barata et al., 2012).

Subunits of the tripartite G-protein

Heterotrimeric guanine nucleotide binding proteins (G-proteins) are involved in the regulation of a range of cellular functions in eukaryotic cells. They interact with activated cell membrane receptors and diffuse along the cell membrane until they meet their target proteins. All G-proteins have a similar structure and they function in a similar way. They consist of three protein subunits, α, β and γ. Two of these subunits attach to the cell membrane with small lipid tails. At rest, unit α is bound to Guanosine Diphosphate (GDP) and the G-protein is inactive. When an extracellular ligand binds to the receptor, the receptor interacts with the G-protein and activates it, forcing the α subunit to excrete the bound GDP and replace it with Guanosine Triphosphate (GTP). Activation results in dissociation of the G-protein into an activated α subunit with GTP-linked and a β-complex. Thus, two separate molecules are produced and diffuse freely along the membrane. The two activated portions of a G-protein, namely the α subunit and the β-complex, interact directly with targets located in the cell membrane, which in turn transmit the signal to other destinations. The behavior of the α subunit determines how long the two activated portions of the G-protein act independently. The α subunit has inherent GTP hydrolysis activity (GTPase) and after a certain period of time hydrolyzes the bound GTP to GDP. It is then reconnected to the β complex where signal transmission stops. This sequence stops in a few seconds after the G-protein activation. Cell membrane target proteins that interact with activated forms of G-protein units may be ion channels or membrane enzymes. The various goals are influenced by different types of G-proteins and then, the different G-proteins are activated by the respective receptors on the cell membrane. Thus, the binding of an extracellular signal molecule to a receptor that binds to a G-protein only affects a subset of protein targets that are suitable for this signal and for this cell type (Alberts et al., 2003).

The Ga protein and its subunits

In *B. cinerea*, three genes have been identified in the Gα subunits of a G protein, the bcg1, bcg2 (Gronover et al., 2001) and bcg3 (Doehlemann et al., 2006). The amino-acid sequence of bcg1 has the highest rate of identification of Gα subunits with other phytopathogenic fungi. All of these Gα protein subgroups are homologous to the mammalian Gα-protein family. RT-PCR experiments clearly showed that both genes (bcg1, bcg2) are expressed in conidia at early stages of host infection (Tudzynski and Gronover, 2007). Characterization of bcg1 and bcg2 revealed that both Gα-protein subgroups affect growth and fungal pathogenesis in different ways. Bcg1 controls multiple functions, activating via the signaling pathway, growth, pigmentation, proteinolysis and pathogenesis but also plays an important role in the process of colonization in the host tissue by vegetation activation. Conidial germination and penetration stop after the formation of primary lesions. After 48 hours, rapidly expanding soft rot lesions form on leaves infected with wild-type conidia. Electron microscopy analysis of mutant conidia (Δbcg1), which contained only bcg1 genes, clearly showed that the mutant textures penetrate the

plant surface in a manner not different from the wild conidia type. These observations suggest that bcg1 protein appears to play an important role in the process of invading plant tissue. In contrast to bcg1, bcg2 exhibits Wild-Type (WT) colony morphology in axon culture and continues to produce and secrete a set of proteases visible as halogen around milk agar colonies (Gronover et al., 2001). The infection process is comparable to WT, except of lesions caused by conidia from mutant Δbcg2 where conidia spread more slowly. The bcg3 gene performs the same functions as the bcg1 gene, activating the signal transduction pathway of cyclic AMP, participating in morphogenesis, vegetation, and growth during fungal invasion (Williamson et al., 2007). In the past, suppression subtractive hybridization (SSH) has been used to identify fungal genes that are specifically expressed in the host plant (Gronover et al., 2004). Among the 22 differentially expressed genes, many were found to encode unknown proteases, some others encode enzymes involved in secondary metabolism, while others encode enzymes that degrade cells. Most of the genes controlled by the bcg1, at signal cascade, are still expressed in adenylate cyclase (bac). This fact suggests that bcg1 is involved in at least one additional signaling cascade next to the cAMP dependency.

The path of cyclic AMP

Many extracellular signals act through receptors that bind to G-proteins and affect the most active adenylate cyclase which alters the intracellular concentration of cyclic adenosine cyclic monophosphate. Usually, the activated α subunit of the G-protein activates the adenylate cyclase, thereby increasing the synthesis of AMP by ATP. The concentration of AMP can change rapidly in response to external signals. It is a water-soluble molecule that can carry the signal from the location of the membrane synthesized into proteins within the cell's nucleus or other organs. The various effects of cAMP (cyclic-AMP-dependent protein kinase) on the cell are exerted by activation of the cAMP-dependent protein kinase (A-kinase). This enzyme is inactive in a complex with another protein. Binding of cAMP causes a modulation change that releases the active enzyme. Subsequently, active protein kinase catalyzes the phosphorylation of various proteins within the cell. Some of these actions of cAMP are changes in gene expression, where A-kinase phosphorylates regulatory proteins that activate the transcription of selected genes (Alberts et al., 2003). Vegetation due to carbon sources requires cAMP signaling. Laboratory analyses have shown that mutants conidia (Δbcg3) containing only bcg3 proteins and mutant conidia (Δbac) with bac proteins, were defective in vegetation induced by carbon sources in the glass. However, when the mutant conidia were incubated on glass surfaces in fructose and added CPT-cAMP (a salt that acts as a cAMP activator), vegetation was restored to almost the level of wild conidia (Doehlemann et al., 2006). The conclusion is that only the proteins bac and bcg3, and possibly bcg1, are not sufficient to activate the germination process but a necessary element for the signal transduction pathway for germination activation is cyclic adenosine monophosphate (cAMP).

Small G-proteins and the MAP-kinase pathway

Small signaling proteins that function as natural adapters exist in the cell membrane. Specifically, these proteins form an extended aggregate, bind to the receptor and thus, they can bind to other proteins and activate them. This is how the promotion of the signal is achieved. One such small G-protein is the Ras protein, which binds to a lipid tail of the cytoplasmic membrane. The Ras protein is a small, monomeric protein that binds to GTP (monomeric GTP-blinding protein). It is referred to as monomer to distinguish it from the three-dimensional proteins that also bind to GTP, where species have been reported. The Ras protein resembles the α subunit of a G-protein and acts as a molecular switch in the same way. In Particular, it switches between two different conditions: Being active when associated with a GTP molecule and inactive when associated with a GDP molecule. Interaction with an activated protein induces the Ras protein to exchange GDP for GTP and become active. After a period of time, the Ras protein is inactivated by hydrolyzing GTP to GDP (Alberts et al., 2003). In the active state, the Ras protein leads to the activation of a series of phosphorylation, in which several kinases phosphorylate and successively activate the next sequence. This transfer sequence that carries the signal from the cell membrane to the nucleus is called the MAP-kinase cascade sequence. In this sequence, MAP-kinase (Bmp1, Bmp3, BcSak1) is phosphorylated and activated by the enzyme kinase MAP-kinase (MAPKK), which in turn is activated by the enzyme MAP kinase (MAPKKK), which is activated by the Ras protein. At the end of the signal sequence, MAP kinase phosphorylates certain gene regulatory proteins into serine and threonine residues and thus modifies

their ability to regulate the transcription of certain genes (Sumita et al., 2016). The result is a change in the pattern of genes' expression, which can stimulate cell proliferation and survival, or it can even control their differentiation. Their effectiveness depends on other genes that are activated in the cell and the various molecules that affect the cell.

Brief description of control approaches for *B. cinerea*

Conventional methods to control *B. cinerea*, in a worldwide scale, include the use of synthetic chemical fungicides. Chemical control of Botrytis based diseases, currently counts for about 8% of the world's fungicide market (Nishimoto, 2019). However, the use of chemical fungicide products is harmful for human health and the environment (Droby et al., 2009). Moreover, the fungus is highly likely to develop resistance at most of those drastic chemical compounds. Biological control methods can be applied in combination with fungicides, at high rates of infection, to reduce the use of fungicides. In cases of less severe infections, biological control methods can be effective against *B. cinerea* even at single treatment applications. Biological control of *B. cinerea* includes the use plant extracts, essential oils and certainly the use of natural enemies of the pathogen. Natural enemies include bio-pesticides based on the Bacterium *Bacillus subtilis* (Bu et al., 2021; De Simone et al., 2020) and the use of antagonistic fungi such as *Gliocladium roseum*, *Trichoderma harzianum* and *Muscodor albus*. Even the presence of the entomopathogenic fungus *Metarhizium anisopliae* has been reported to have an effect on the growth and development of *B. cinerea* (Sammaritano et al., 2018). The use of antagonistic yeasts in order to inhibit growth of grey mold in post-harvested grapes has not shown promising results yet. However, a few studies indicate potential efficacy of yeasts for the control of *B. cinerea* (Calvo-Garrido et al., 2013; Santos et al., 2004). The removal of excessive leaves and shoots as well as the successful management of plant debris can reduce the conidial transmission of *B. cinerea* (Gubler et al., 1987). In general terms, all viticultural practices that assist in reducing high humidity levels within vineyard's micro-environment have a significant impact in reducing fungal infection. The use of resistant to Botrytis bunch rot *Vitis vinifera* cultivars is also considered as a preventing mean of great importance.

CONCLUSIONS

B. cinerea is considered as a non-specific plant pathogen, infecting a vast number of hosts. However, it still presents specificity and selecting action regarding host detection, conidial germination and pathogenesis. As most generalist organisms, *B. cinerea* shows particular preferences for specific plant species and cultivars. For instance, *B. cinerea* evidently prefers to host on any cultivar of *Vitis vinifera* grapes in contrast to other species of the *Vitis* genus that are considered as resistant (e.g., *V. labrusca*, *V. rotundifolia*, and *V. aestivalis*). Regarding *V. vinifera* varieties, wine grapes suffer more from Botrytis infections as compared to table grapes and this fact is based on multiple factors. Moreover, specific wine grape varieties are considered as more susceptible to Botrytis than others (e.g. Pinot noir, Chardonnay, Semillon, Gamay). Those preferences are reflected on the response of the fungus to chemical and physical stimuli emanated from its micro-environment. Specificity is based on a combination of biotic and abiotic factors such as grape skin's micro-fauna, bunch density, thickness and color of the skin, must composition, sugar-acid ratio, sugar and phenolic ripeness levels and certainly, the environmental conditions. Overall, Bunch rot causes severe losses and damages to grapes and has a substantial impact on the quality, chemical composition and organoleptic characteristics of wines. Although extensive research has been conducted in the field of control strategies against *B. cinerea*, further study regarding the physiology of the fungus must be held.

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AUTHORS' CONTRIBUTION

All the authors equally participated in collecting, organizing, writing and editing the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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