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Understanding the role of some novel components in modulating virulence of necrotrophic fungus *Botrytis cinerea*

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ABSTRACT

***Botrytis cinerea* is the second most destructive necrotrophic phytopathogen affecting over 500 plant hosts, including several commercially valuable crop species. The infection is characterized by the presence of grey-colored mold on pre as well as post-harvest fruits and vegetables. It is a highly efficacious pathogen due to its broad host range, adaptable infection styles, and high reproductive potential. The application of fungicides is not effective in managing the disease as the pathogen rapidly develops resistance to it. Huge commercial losses and unavailability of suitable measures make it imperative to intensely comprehend the molecular mechanism of pathogenicity in *B. cinerea*. The availability of a complete errorless genome and advancement of molecular tools for genetic study has proven fundamental in elucidating the infection techniques of the pathogen. The review synopsizes the role of some novel components identified by mutational analysis that influenced the virulence of *B. cinerea*.**

Keywords: *Botrytis cinerea*, phytopathogen, virulence potential, mutant and molecular mechanism.

INTRODUCTION

Unlike, biotrophic fungi that have close communication with their host plant, necrotrophic pathogenic fungi have long been considered to have little or no factual interaction with the hosts. Necrotrophic pathogens decimate plant cells by secreting toxins and lytic enzymes, and consequently degrade the plant tissue and expend it for their development. A few necrotrophs have a limited host range as the toxins released by them display specificity to one or few plant species and is termed as host-selective toxin (HST) (Wolpert *et al*., 2002). Examples of the well-studied necrotrophs that produce HST include species of *Cochliobolus* and *Alternaria*. On the other hand, there is wide host range of necrotrophs like *Sclerotinia sclerotiorum* and *Botrytis cinerea* that are capable of infecting more than 200 plant species.

Botrytis cinerea is regarded as one of the best comprehensively reviewed necrotrophic pathogen. This pathogen is responsible for devastating losses in over 500 plant species, mainly dicots and some monocots (Williamson *et al*., 2007). Owing to its economic impact and scientific significance, *B. cinerea* has been recognized as the second major phytopathogen (Dean *et al*., 2012). It causes grey rot and is capable of inflicting disease during the pre-harvest as well as post-harvest periods (Fillinger and Elad, 2016). Amongst major post-harvest pathogens of fruits and vegetables, *B. cinerea* has been considered as the most obliterating (Zhang *et al*., 2014).

Worldwide economic losses incurred by *B. cinerea* annually surpass ten billion dollars (Weiberg *et al*., 2013). It is a highly efficacious pathogen due to its broad host range, adaptable infection styles, high reproductive potential, and ability to survive for prolonged adverse intervals. It also produces sclerotia and other infective tissues that serve as complementary inocula. The sclerotia of *B. cinerea* are extremely resilient to environmental fluctuations due to the presence of intracellular nutrient reserves of lipids, glycogen, polyphosphate, proteins and their melanized surface (Araújo *et al*., 2005; Backhouse *et al*., 1984). Nevertheless, the sexual cycle in *B. cinerea* is very rare in nature (Williamson *et al*., 2007). Sclerotia, in addition to asexual reproduction, can undertake a heterothallic sexual cycle to produce apothecia containing ascospores (Amselem *et al*., 2011; Williamson *et al*., 2007).

The application of fungicides remains the prime method to control grey mold rot, which accounts for near 8 per cent of the global demand for fungicides (Dean *et al*., 2012). Conversely, fungicides solely are not effective in managing the disease as the pathogen soon develops resistance to the applied fungicide in the field. For example, the effectiveness of dicarboximides has severely reduced after prolonged usage to control grey mould disease and is only effective when supplied in conjunction with other fungicides (Katan, 1982). Huge commercial losses and unavailability of suitable measures make it imperative to intensely comprehend the molecular premise of pathogenicity in *B. cinerea* and design novel approaches to forestall grey mold rot caused by the pathogen. Over the past few years, prodigious studies have been carried out to explore the molecular framework of pathogenesis in *B. cinerea.* However, only a few of them have been successful to guide strategies for effective control.

The advancement of molecular tools for the genetic study of *B. cinerea* (Tudzynski and Siewers, 2004) and the readiness of an EST dataset (Viaud *et al*., 2005) has proven fundamental in elucidating the infection techniques of the pathogen.The genome of *B. cinerea* was sequenced in 2005 and subsequently has been updated in 2016 with enhanced coverage and more annotation (Kan *et al*., 2016). However, the focus is on the genes of *B. cinerea* studied by examining mutants with emphasis on few genes that have been functionally analysed by us.

SYMPTOMS AND INFECTION BIOLOGY OF *BOTRYTIS CINEREA*

Symptoms of *B. cinerea* vary depending upon the host species. The development of water-soaked lesions is the typical trait of this pathogen which is later followed by the development of mouldy growth; on apple, it forms a necrotic spot and blemishes on rose flower. Colonization of host tissue by the pathogen is facilitated by the entry of the fungus through natural openings or damaged tissues (Jarvis, 1962). The infected tissue initially displays limited damage. Immediately upon infection, *B. cinerea* exists as a biotroph

within the plant (Williamson *et al*., 2007). Later on, the biochemical changes that accompany the ripening of plant tissues, such as the surge in sugar, volatile organic compounds, and nitrogen contents, induce the fungus to undergo a destructive necrotrophic phase (Neri *et al*., 2014; Prusky and Lichter, 2007). During this stage, the fungus secretes several cell wall degrading enzymes (CWDEs) and virulence factors such as oxalic acid (OA), and equivalents of phytohormones to interrupt host metabolism, cellular structure, and immune system (Gentile, 1954; Sasanuma and Suzuki, 2016). Impact of virulence associated factors on infected hosts are distinguished by rotting of fruit, leading to softening of fruit mass and formation of leathery brown fruit skin (Xiao, 2006). *B. cinerea* proliferates as mycelia and forms massive grey conidia masses on plant surfaces giving a characteristic grey moldy appearance (Williamson *et al*., 2007). Simultaneously, nearby plant tissue gets infected, permitting the coverage of grey mold across the whole plant. On killing the host tissue, *B. cinerea* persists in the form of sclerotia and mycelia, and continues to grow as a saprophyte.

GENOME SEQUENCE OF *BOTRYTIS CINEREA*

In 2005, Broad Institute and Syngenta Biotechnology applied the Sanger technique to sequence the first genome of *B. cinerea* (strain B05.10) obtained from grapes in California USA, subsequently, in 2011 the genome was assembled with 4.5 X coverage (Amselem *et al*., 2011). The latest updated version of the genome sequence obtained in 2016 generated through third-generation sequencing methodologies (Illumina and Pacific Biosciences), offers 90X coverage that is near complete and gapless (Kan *et al*., 2016). Due to its completion and more accuracy over other genome versions, it has been accepted as the standardized reference for *B. cinerea*. It also describes the sequence of mini-chromosomes 17 and 18 for the first time, containing 18 and 14 genes, respectively (Kan *et al*., 2016). Further, 40 regions with high recombination rates on chromosomes have been reported implying high recombination frequency in *B. cinerea* during sexual reproduction.

Two strains of *B. Cinerea* have been sequenced *viz.* B05.10 and T4 with a total genome size of 41.2 Mb and 41.6 Mb, containing 10,701 and 10,427 protein-coding genes, respectively, following exclusion of repetitive sequences. The genome has a GC content of an average of 42.75%, and is distributed among 18 chromosomes (Kan *et al*., 2016). Most of *B. cinerea* mutant analysis studies have focused on the strain B05.10. To generate a gapless near-finish genome assembly of the strain (B05.10), and to curtail inaccuracies during assembly, optical and linkage maps were built (Kan *et al*., 2016).

Analysis of single nucleotide polymorphisms (SNPs) in the genomes of 13 isolates of *B. cinerea* obtained from four countries (Germany, USA, United Kingdom, and South Africa) showed on an average 28 polymorphisms for every kb within the pathogen population; implying widespread complete genome recombinations among strains during meiosis (Atwell *et al*., 2015). Insertions, deletions, and point mutations were possibly the most common mutations observed. However, the studies suggest that the high genetic variation and recombination within the pathogen population, and not spontaneous mutations, account for a wide host range and rapid development of resistance against fungicides.

Initial investigations of *B. cinerea* (strain B05.10) genome reported the presence of various pathogenicity governing genes (Amselem *et al*., 2011), including crucial genes associated with the generation of reactive oxygen species (ROS), tolerance to ROS, OA biosynthesis, CWDEs production, and peptidase secretion.

VIRULENCE FACTORS REQUIRED FOR HOST PENETRATION

Fungal tetraspanins: Appressorium in *B. cinerea* is not capable of generating pressure to breach the host cell wall as it neither possesses highly melanized wall (Doss *et al*., 2003) nor contains a septum to separate the appressorium and the germ tube. *B. cinerea* appressoria secrete CWDEs for the contravention of plant surface. Nonetheless, it also necessitates a membrane-bound protein tetraspanin (*BcPLS1*) for penetration (Gourgues *et al*., 2004) that is orthologous to a protein crucial for appressorium function in *Magnaporthe grisea* (Clergeot *et al*., 2001).

Cutinase and lipases: Although the genome assembly of *B. cinerea* contains more than five cutinase coding genes and at least a dozen lipase-encoding genes, their role in the pathogenesis of the fungus continues to be unresolved. Deletion mutation of a cutinase and a lipase gene, either individually or together, does not display a measurable decrease in virulence over wild type (Reis *et al*., 2005; Van Kan *et al*., 1997). It is proposed that the penetration peg produces hydrogen peroxide in the tip that serves as a substrate for oxidases to rework on the cuticle and consequently making it amenable for decomposition (Tenberge, 2004).

Pectinases: *Botrytis cinerea* prefers host plants with high pectin content in their cell wall. Its preference towards dicots and corolliferous monocots (high pectin content) is due to the presence of effective pectinolytic machinery (ten Have *et al*., 2002). The activity of pectinases, primarily endopolygalacturonase (PG), includes the early penetration of the epidermal cell layer. *B. cinerea* genome contains more than six PG genes (Wubben *et al*., 1999) that display versatility in expression depending on the host plant species, incubation conditions, and tissue type (ten Have *et al*., 2001). While deletion mutant (Δ*Bcpg2)* of *BcPG2* in bean and tomato exhibited a discernible suspension in primary lesion formation (Tenberge, 2004), mutation in other *BcPG* genes does not show such a delay.

Pectin methylesterases: The role of pectin methyl esterases (PMEs) is divisive. While mutant of one *B. cinerea* strain deficient in Δ*Bcpme1* showed impaired virulence, other strains with either single or double mutation of Δ*Bcpme* genes including Δ*Bcpme1* showed no such phenotype (Valette-Collet *et al*., 2003). PMEs facilitate the action of endopolygalacturonases by pectin demethylation of highly methylated pectin. Hence, it is presumed that in plant tissues, such as leaves, with immensely methylated pectin as the only carbon source, PMEs serve as a virulence determinant for fungal growth. However, PMEs are dispensable for virulence on

tissues such as fruit with low pectin methylation. Nevertheless, there is evidence indicating that demethylation of pectin by PMEs is not vital for depolymerization of pectin with a high percentage of methylation (Kars *et al*., 2005a).

Cellulases and hemicellulases: Cellulases and hemicellulases secreted by *B. cinerea* have been relatively less studied. While the deletion mutants of an endo-β-1,4-xylanase gene displayed substantial delay as well as a decrease in lesion formation (Brito *et al*., 2006), no effect was observed on the deletion of the endo-β-1,4-glucanase gene (Espino *et al*., 2005).

Carbohydrate-active enzymes: Carbohydrate-active enzymes (CAZymes) annotation uncovered 229 distinct CAZymes, presumably accountable for the disintegration of cellulose, hemicellulose, and pectin present in the plant cell wall (Blanco-Ulate *et al*., 2014). In determining the diverse host spectrum of *B. cinerea* the vast variety of CAZymes plays a crucial role.

PHYTOTOXINS

Botrytis cinerea genome consists of 43 enzyme-encoding genes involved in secondary metabolism pathways, out of these 24 are specific to *B. cinerea*. Consequently, *B. cinerea* can synthesize more than 40 different secondary metabolites, including botcinic acid (polyketide) and botrydial (sesquiterpene) (Porquier *et al*., 2019).

Botrydial: Botrydial is the best-studied phytotoxic compound among the spectrum of low molecular weight toxins produced by *B. cinerea* (Colmenares *et al*., 2002). A variety of genes associated with the pathway for biosynthesis of botrydial have been identified (Siewers *et al*., 2005). Functional analysis of one of the genes involved in botrydial synthesis showed that it is a strain explicit virulence factor and not all strains exclusively depend on botrydial to destroy their host (Siewers *et al*., 2005) but can generate additional phytotoxins such as botcinolides (Reino *et al*., 2004).

Oxalic acid: Oxalic acid (dicarboxylic acid) (OA) operates as multipurpose compound for infection by *B. cinerea*. Host cell death is favourable for necrotrophs including *B. cinerea* (Govrin and Levine, 2000; Kumari *et al.,* 2014). Oxalate can make host more susceptible by directly eliciting plant cell death (Govrin and Levine, 2000; Van Baarlen *et al*., 2004; Sharma and Kapoor, 2017a), decreasing the oxidative burst and concurrent defense stimuli in host tissues (Cessna *et al*., 2000). It is a strong acid that induces synthesis as well as activity of a spectrum of enzymes such as pectinases (Kars *et al*., 2005a, b), proteinases (ten Have *et al*., 2004), and laccases (Manteau *et al*., 2003).

REACTIVE OXYGEN SPECIES (ROS)

Reactive oxygen species (ROS) have an indecisive role in pathogenesis as on one hand they cause DNA damage, oxidation of proteins, and lipid peroxidation (Heller and Tudzynski, 2011), and on the other function as secondary messengers in different signaling cascades (Heller and Tudzynski, 2011; Orozco-Cardenas *et al*., 2001). During initial stages of pathogen infection, host plants transiently produce large quantities of ROS aiming to neutralize the invasive growth of pathogen (Mellersh *et al*., 2002; Tian *et al*., 2013). However, necrotrophic fungi, including *B. cinerea* are capable of using the oxidative burst in their favour and also add to it by generating their own ROS. *B. cinerea* produces ROS either as a primary product of NADPH oxidase (NOX) or as inevitable offshoots of metabolic processes (Li *et al*., 2016). NOX is an enzyme complex containing multiple subunits, and converts oxygen to anionic radical superoxide utilising the electrons furnished by NADPH (Bedard *et al*., 2007). Pathogenicity and the development of sclerotia are the responsibility of the two catalytic subunits of NOX, namely *BcNoxA* and *BcNoxB* (Siegmund *et al*., 2015). Intriguingly, the subunits play diverse roles in *B. cinerea* pathogenesis (Marschall *et al*., 2016). While *BcNoxA* contributes to the colonization of host tissue, *BcNoxB* is required during the primary stages of infection (Segmuller *et al*., 2008). Elimination of *BcNoxR*, the regulatory subunit, displays a phenotype coherent with that of Δ*BcnoxA/B* double mutant i.e. impaired virulence, declined growth, and sporulation on tomato/French bean leaves (Li *et al*., 2016). Deletion of *BcNoxR* (Δ*BcnoxR*) changed the abundance of 49 unique downstream proteins with diverse roles in various metabolic processes such as translation, carbohydrate metabolism, intracellular signaling, and stress response. Further investigation demonstrated that 6-phosphogluconate dehydrogenase (*BcPGD)*, abundance diminished in Δ*BcnoxR* deletion mutant, plays a functional role in overall growth, sporulation ability, and virulence of the pathogen (Li *et al*., 2016).

Being a signaling molecule, ROS must transfer from the source to the site of activity. Among all the ROS radicals generated, hydrogen peroxide (H_2O_2) is the least reactive and thus appropriate for biological investigations (Waghray *et al*., 2005). It acts as an intercellular as well as an intracellular signaling molecule (Pletjushkina *et al*., 2006; Rice, 2011). However, to pass through plasma membrane requires channel proteins (Seaver and Imlay, 2001). Aquaporins, popularly referred to as membrane water channels, mediate the transport of H_2O_2 through plasma membranes (Bienert *et al*., 2007; Miller *et al*., 2010). Aquaporin protein 8 (AQP8*)* of *B. cinerea* plays a significant function in transmembrane transport and dissemination of ROS from mitochondria that are the primary source of ROS generation (An *et al*., 2016). In addition to regulating ROS transport, *BcAQP8* also influences *BcNoxR* expression. Deletion of *BcAQP8* (Δ*bcaqp8*) leads to decreased growth and sporulation, as well as reduced overall pathogenicity of the phytopathogen (An *et al*., 2016).

SOME NOVEL FACTORS THAT GOVERN VIRULENCE IN *B. CINEREA*

To identify some novel genes that govern the virulence of *B. cinerea*, monokaryotic transformants were raised using the *Agrobacterium tumefaciens* mediated transformation (ATMT) method. These transformants were subjected to pathogenicity screening and after several rounds of screening four insertion mutants that showed consistent impaired virulence were selected. Further functional characterization of these mutants led to the identification of the following

novel components modulating virulence of necrotrophic fungus *B. cinerea*.

Membrane lipids: Cell membrane component synthesis plays an inevitable role in cellular growth. Diacylglycerol O-acyl transferase 2 (DGAT2) is a rate-limiting enzyme that catalyzes the final step involving acylation of 1, 2 diacylglycerol in triacylglycerol (TAG) synthesis (Yen *et al*., 2008). TAG plays a major role in signal transduction and sustenance of lipid homeostasis in fungi. Deletion of *BcDGAT2* (Δ*Bcdgat2*) affected the ability of the mutant to form sclerotia and spores (Sharma *et al*., 2017). TAGs are the main repository of membrane lipids in fungi and constitute a high proportion of sclerotia membrane lipids. No sclerotia were found in Δ*Bcdgat2* mutant even on prolonged incubation under darkness, which is a pre-requisite for sclerotia formation **(Fig. 1)**. Re-introduction of the gene in complemented mutant restored its capacity to form sclerotia, thus confirming the putative role of *BcDGAT2* in sclerotia formation.

The aberrant hyphal growth of Δ*Bcdgat2* mutant led to impaired penetration of host tissue and decreased OA secretion. Hyphae developed from Δ*Bcdgat2* mutants had swollen tips, protecting the host tissue from penetration. Also, pH-dependent exogenous hydrolase activity decreased due to inadequate OA. The mutants also displayed incompetence to grow under membrane and cell wall stress. While TAG tends to influence the levels of OA in the pathogen.

INTRACELLULAR TRANSPORTATION

Kinesin-like protein: The cytoskeleton consists of microtubules, microfilaments, and intermediate filaments forming a complex and dynamic structure of interlinking proteins. Cell division and intracellular transportation are facilitated by some microtubules-based motor proteins. KLP7, a kinesin-like protein, corresponds to a particular subfamily of kinesin present uniquely in fungi (Tayal *et al*., 2017). It is associated with fungal growth and development. Disruption of *BcKLP7* resulted in discernible hyphal growth, spore formation, and conidia germination defects. The hyphae of mutant Δ*Bcklp7* were stunted and irregular and exhibited reduced ability to penetrate the host. KLP7 proteins may be associated with the transport of secretory vesicles bearing virulence factors such as OA, PG, and PMEs, as the absence of KLP7 proteins resulted in a severe decline in the secretion of these factors **(Fig. 1).**

Nucleopore complex protein: The nuclear envelope is

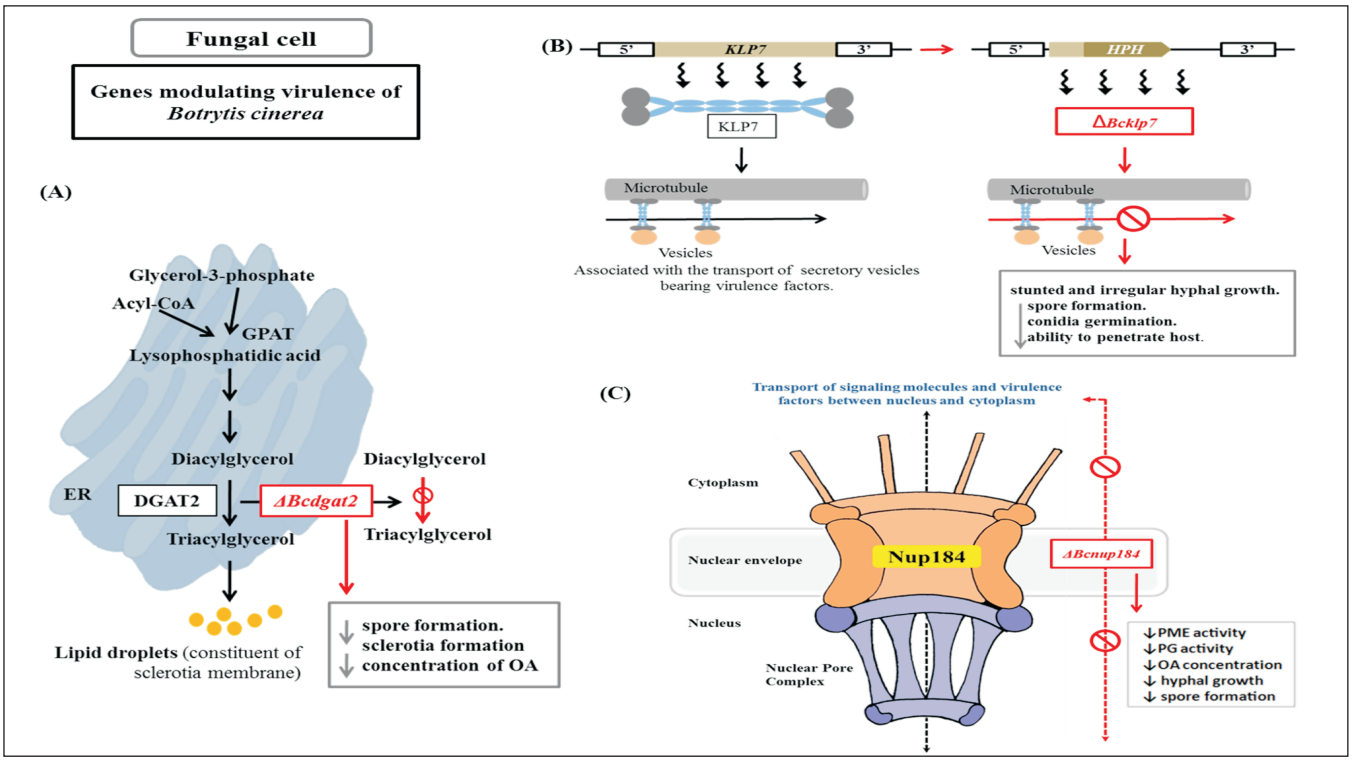


Fig. 1: Genes affecting virulence potential of *Botrytis cinerea*. (A) Diacylglycerol O-acyl transferase (DGAT2) facilitates conversion of diacylglycerol to triacylglycerol (TAG). TAGs constitute high proportion of sclerotia membrane lipids. In deletion mutant (*ΔBcdgat2*), TAG formation is inhibited. Deletion of DGAT2 affected the ability to form sclerotia and spores. The aberrant hyphal growth of mutant leads to impaired penetration of host tissue and decreased oxalic acid (OA) secretion; (B) A kinesin-like protein (KLP7) is associated with fungal growth and development. Disruption of *BcKLP7* resulted in reduced hyphal growth, spore formation, and conidia germination. The hyphae of ΔBcklp7 were stunted and irregular, and exhibited reduced ability to penetrate the host; (C) Nuclear pore complex protein 184 (Nup184) is involved in transport of virulence factors between the nucleus and cytoplasm. The mutant *ΔBcnup184* showed impaired virulence, lowered concentration of OA, and reduced activities of endopolygalacturonase (PG) and pectin methylesterases (PMEs) as compared with wild type strain. Disruption of *BcNup184* may have hampered transport of signaling molecules between the nucleus and cytoplasm.

penetrated by large aqueous channels, connecting the nuclear interior with the cytoplasm forming the nuclear pore complexes (NPC). Unlike a decade before when NPC were viewed as fixed structures that regulate trafficking of biomolecules between the cytoplasm and nucleus, NPC's are now known to be highly dynamic multi-protein assemblies associated with different cellular processes ranging from cytoskeleton organization to gene expression (D'Angelo and Hetzer, 2008). The mutant deficient (*ΔBcnpc184)* in the expression of NPC protein 184 (Nup184) also showed impaired virulence, lowered concentration of OA, and reduced activities of PG and PMEs as compared to the wild type strain. Disruption in the expression of Nup184 may have hampered the transport of signalling molecules between the nucleus and cytoplasm **(Fig. 1)**. It might have also affected the transcription of certain genes that contribute towards the virulence of *B. cinerea*. However, the exact nature of such molecules or genes is not known.

Non-pathogenic mutant deficient in a predicted protein: An ATMT raised mutant that was affected in the expression of a predicted protein showed no diseased symptoms on conidial or mycelial inoculation on the host tissues and was hence, designated as *ΔBcnpm*, *B. cinerea* non-pathogenic mutant. The predicted protein is unique to *B. cinerea* with no resemblance with any database and therefore, can also serve as a biomarker to identify this fungus (Sharma & Kapoor, 2017b).

There were no significant differences in colony diameter, morphology, or ability to form sclerotia in the *ΔBcnpm* mutant strain and it was comparable to the WT strain. The apathogenic trait of this mutant was also observed on other tested host tissues with no disease symptoms even on prolonged incubation of this strain. Interestingly, pre-inoculation of plants with Δ*Bcnpm* activated defence machinery and helped the plants to cope up with the stress in a better way. This unique predicted protein identified presumably had some role in the synthesis of oxalic acid and might be responsible for the apathogenic nature of this mutant.

CONCLUSIONS

Botrytis cinerea is a belligerent phytopathogen that causes grey mould rot in standing as well as harvested crops, resulting in enormous economic losses worldwide. It is imperative to uncover the molecular premise of pathogenicity and resistance mechanisms of *B. cinerea*. Several genes that play a role in the development and pathogenicity of *B. cinerea* have been identified and functionally analysed. Correspondingly, virulence factors and pathogenicity mechanisms have been elucidated, it is well acknowledged that *B. cinerea* subdues plant defense to colonize the host plant. The plant tissues are degenerated by the production of CWDEs, ROS, and acids.

The whole-genome assembly of *B. cinerea* is available and genetic transformation techniques such as the development of knockout or deletion mutants have been successfully employed to probe its mechanisms of pathogenesis. The speedy progress realized over the past two decades, as well as the publication of the genomes of B05.10 and T4 strains, has enthused a growing number of researchers to work with *B. cinerea*, resulting in its appearance as a model necrotrophic pathogen with a diverse host range. However, the present knowledge of pathways and biological mechanisms of this infamous fungus is yet obscure. Hitherto out of 10,500 coding genes in its genome, mutant analysis has enabled the genetic study of about 150 genes only. The advent of the CRISPR/Cas9 system has accompanied a new era of genetic manipulations. It offers a robust genome editing tool, facilitating researchers to manipulate specific genomic elements, with precision and ease and enabling the elucidation of the function of the targeted entity in biology and diseases. The application of this technology will provide impetus in the understanding of this economically important fungal pathogen.

Furthermore, the high genomic diversity among its isolates makes the deployment of fungicides for successful and durable control of the fungus more challenging. Extra teamwork amid molecular pathologists and resistance breeders will be decisive in understanding and overcoming the pathogenic mechanisms and host resistance in *B. cinerea*.

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