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Characterization of a mutant of a unique predicted protein essential for virulence in *Botrytis cinerea*

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ABSTRACT

A nonpathogenic mutant of *Botrytis cinerea* was obtained by insertional mutagenesis using *Agrobacterium tumefaciens* mediated transformation. The mutant (Bcnpm) was deficient in expression of a predicted protein unique to *B. cinerea.* No disease symptoms were observed on tomato plants inoculated with Bcnpm. Bcnpm exhibited reduced ability to penetrate and colonize leaf tissue as compared to its wild type (WT). The concentration of oxalic acid was considerably reduced in Bcnpm. The tomato plants inoculated with Bcnpm provoked lesser oxidative damage in terms of H_2O_2 production and lipid peroxidation. The magnitude of defence response elicited by Bcnpm was far lesser than that from WT. Bcnpm inoculated plants possessed lower concentration of phenols and reduced activities of defence and antioxidative enzymes. Additionally, qRT-PCR analysis revealed lesser transcript levels of some defence related genes in Bcnpm as compared to WT. It was further observed that pre-inoculation of tomato plants with Bcnpm offered protection to plants challenged with biotic stress. The mechanism behind this protective ability could be induced resistance which might have resulted in priming the plant defence response.

Keywords: *Botrytis cinerea*, predicted protein, non-pathogenic mutant, virulence, Oxalic acid, protective ability

INTRODUCTION

Botrytis cinerea causes gray mold disease in more than 200 plant species and inflicts substantial losses worldwide (Nakajima and Akutsu, 2014; Dean *et al*., 2012). Disease control of *B. cinerea* is extremely difficult as the fungus is capable of attacking crops at all stages of their growth and under storage (Droby and Lichter, 2004). Although, combination of fungicides (Leroux, 2004; Couderchet, 2003) have been tried to control the devastating pathogen but the approach has been only partially successful (Hahn, 2014). Due to growing apprehensions about adverse environmental effects of chemical control, adoption of biological control method is one of the environmental friendly approaches to avoid the menace of rapid manifestation of resistance in the target population.

The use of saprophytic or nonpathogenic isolates of *Fusarium* spp. for biological control of pathogenic strains of *Fusarium* spp. in various crops has been extensively studied and applied (Aime´ *et al*., 2008; Nahalkova *et al*., 2008; Panina *et al*., 2007; Olivain *et al*., 2006; Bao and Lazarovitz, 2001). However, basic problem with the use of nonpathogenic isolate is that other than inoculation of the plant, there is no other means that enable characterization of a fungal strain's pathogenicity. The molecular methods do not facilitate differentiation between pathogenic and nonpathogenic strains (Lievens *et al*., 2008). For this reason it seems preferable to focus on non-pathogenic mutants of the fungus deficient in expression of a known gene. Use of nonpathogenic mutants to elicit defence response in host plant against wild type infection has been reported by some mycologists (Pareja-Jaime *et al*., 2010; Freeman *et al*., 2001). Although *B. cinerea* ranks second among the ten most important fungi based on its scientific and economic relevance (Dean *et al*., 2012), such studies have not been performed on this pathogen.

Targeted knock out of candidate genes and random insertional mutagenesis without prior knowledge of gene has helped in identification of new virulence determinants and novel factors (Liu *et al*., 2016; Weld *et al*., 2006; Mullins *et al*., 2001). Nevertheless, a limited number of attempts have been made to study response of identified mutant towards plant defence (Pareja-Jaime *et al*., 2010; Di Pietro *et al*., 2001; Freeman *et al*., 2001; Redmann *et al*., 1999b).

In pursuit of identifying novel factors contributing to virulence, several *B. cinerea* mutants were developed *via Agrobacterium tumefaciens* mediated transformation. A non-pathogenic mutant (Bcnpm) deficient in expression of a predicted protein was chosen for this study. The protein showed no similarities with known gene in any of the fungal databases. The objectives of this study were to (i) improve our knowledge on the identified predicted protein in virulence of *B. cinerea*; (ii) compare the defence response of tomato to wild type and non-pathogenic mutant; and (iii) to examine the hypothesis that inoculation with non-pathogenic mutant (Bcnpm) would equip tomato plants to deal with subsequent biotic stress in a better way.

MATERIALS AND METHODS

Fungal strains

Botrytis cinerea strain BO5.10 used in this study is provided by Dr. Paul Tudzynski (Department of Phytopathology, Germany). The wild type (WT) strain was used as a recipient in transformation experiments. *Agrobacterium tumefaciens* strain LBA 4404 carrying the plasmid pBIF (Nizam *et al.*, 2010) was used to carry out random insertional mutagenesis in *B. cinerea* following Rolland *et al*. (2003) with slight modifications. In total, 800 monokaryotic transformants were obtained using this approach. All the transformants were screened for any pathogenicity defect using chickpea and tomato plants. The whole plant screening method was performed under controlled environmental conditions at National Phytotron Facility, Indian Agricultural Research Institute (IARI), New Delhi. A mutant that showed complete loss of virulence after several rounds of screening was chosen for this study. The non pathogenic mutant (Bcnpm) was assessed for T-DNA integration by using selectable marker (hygromycin resistance) gene specific primers. Mitotic stability of the mutant was tested by inoculating it on potato dextrose agar medium (PDA, Himedia) without hygromycin for five generations (Soares *et al*., 2005). Stability was

ascertained by the expression of hygromycin resistance after transformants had been serially transferred in the absence of selection. The tagged gene in mutant was identified by isolation of flanking regions using Thermal Asymmetric Interlaced PCR followed by bioinformatics analysis.

Inoculum production

Both wild type strain and the mutant were separately grown on PDA in petri plates and incubated at 21°C under continuous light for conidiation. Conidia were harvested from the sporulating plates by washing with 3 ml sterile water containing 0.05% Tween 80. The conidia were filtered, washed by three rounds of centrifugation and resuspended in Gamborg's B5 medium (Duchefa Biochemie, Netherlands) with 2% glucose and 10 mM phosphate buffer, pH 6.4. The concentration of conidia was adjusted to 10^5 spores/ml for inoculation purpose using a haemocytometer.

Test Plant

Seeds of tomato (*Solanum lycopersicum*) var. Pusa Ruby were procured from IARI, New Delhi. Plants were grown in plastic pots containing sterile soil and sand (3:1) under greenhouse conditions with a photoperiod of 16 h at a temperature of 24±2° C. Four week old tomato plants were used for future experiments unless stated otherwise.

Characterization of mutant for virulence

Tomato leaves were harvested and sterilized using 70% ethanol followed by washing in 1% sodium hypochlorite. The leaves were washed in sterile water, and mycelial discs (2 mm) collected from freshly grown cultures of both wild type and mutant were placed on the abaxial surface of leaves kept in moistened petri plates in order to maintain humidity. Disease symptoms were analyzed until 96 hour post inoculation (hpi) and the size of the lesion was determined using Axovision software (version 4.6.3, 2007).

In order to quantify cell wall degrading enzymes, strains were grown for 10 days at 20°C in induction medium containing pectin as a substrate. Induction medium without fungal inoculation served as control. Culture filtrates were used as enzyme extracts. The procedure for the estimation of pectin methylesterase and polygalacturonase activity is same as that described in Kumari *et al*. (2014).

Oxalic acid was quantified in culture filtrates of fungal strains grown in potato dextrose broth for 15 days following the protocol of Durman *et al.* (2005). Concentration was calculated by extrapolating the absorbance obtained against a standard curve and was expressed as µg/ml. All the assays were performed in triplicates and the experiment was repeated twice.

Characterization of mutant for penetration and colonization of plant tissue

For penetration assay, onion epidermis was peeled and surface sterilized as described by Doehlemann *et al*. (2006) and inoculated with equal spore density (10^5 spores/ml) of both strains. The samples were incubated in moistened petri plates. Epidermal layer was stained with lactophenol cotton blue and visualized at 10X magnification under a Carl Zeiss microscope equipped with an AxioCamMRc camera and the Axiovision Rel 4.8 software package (Zeiss, Germany) at different time points (0, 16, 24, 48 and 72 hpi).

Plant response to wild type and Bcnpm strains

***Sampling procedure*:** Tomato plants were inoculated with equal number of spores of wild type *B. cinerea* and its non-pathogenic mutant. The inoculated plants were covered with polythene bags to avoid dislodging of spores and to maintain 100% relative humidity. Control plants were treated with sterile distilled water. Thus, there were three treatments with ten pots and four plants in each pot. All the biochemical assays were performed at two time points i.e. 24 and 72 hpi of fungal strains.

***Quantification of fungus development in tomato plants*:** In order to quantify development of fungus in host tissue, genomic DNA was extracted from infected tomato leaves using DNeasy Plant Mini Kit (QIAGEN, Germany) at four different time points (0, 24, 48 and 72 hpi). The relative amount of fungal DNA in tomato leaves was determined with real-time PCR run in triplicates using the Brilliant SYBR Green qPCR reagents (Stratagene, USA) on a Mx 219 5000P qPCR equipment (Stratagene, USA) following Gachon and Saindrenan (2004) based on the amplification of *B. cinerea cutinase A* gene and DNA levels were normalized using tomato actin gene.

***Evaluation of disease severity*:** Ten plants from each treatment were randomly analysed for any disease symptoms. Leaves were monitored regularly and the size of lesion was recorded using a qualitative scale of 0 5 where 0 indicates no lesion development and 5 indicates completely rotten plants (Elad *et al*., 1994) at 24, 48, 72 and 96 hpi. The experiment was performed in triplicates.

Assesssment of damage by fungal strains

***Chlorophyll concentrations*:** Concentration of photosynthetic pigments was estimated by following the protocol of Hiscox and Israeltam (1979). Leaves (1 g) were chopped into small pieces and placed in glass tubes containing dimethyl sulfoxide (DMSO). The entire mixture was incubated at 65°C till the leaf tissue decolorized. Chlorophyll concentration was measured by reading the absorbance at 645 nm and 663 nm and expressed as mg/g fresh weight. The values were calculated following Lichtenthaler and Wellburn (1983).

***H_2O_2 production*:** Leaf sample (0.1 g) was homogenized in an ice bath with 2.0 ml of 0.1% (w/v) of trichloroacetic acid (TCA). The homogenate was centrifuged at 12, 000 g for 15 min. To 0.5 ml of the supernatant, 10 mM of potassium phosphate buffer (pH 7.0) and 1 ml of potassium iodide solution was added and incubated for 5 min. The absorbance of resulting mixture was measured at 390 nm (Velikova, 2000). The amount of H_2O_2 formed was determined from the standard curve made with known concentrations of H_2O_2 and expressed as n mol/g fresh weight.

***Lipid Peroxidation*:** Oxidative damage was evaluated by assessing malondialdehyde (MDA) concentration using thiobarbituric acid (TBA) as the substrate (Heath and Packer, 1968). The specific absorbance of supernatant was recorded

at 532 nm and non-specific absorbance at 600 nm. Concentration of MDA was calculated using extinction coefficient 155 mM^{-1} cm^{-1} and expressed as n mol/g fresh weight.

Biochemical estimation of plant defence response

***Antioxidant enzyme activities*:** Tomato leaves (1 g) were ground in 50 mM sodium phosphate buffer (pH 7.8) containing 2% polyvinyl pyrrolidone. The mixture was centrifuged at 13,000 *g* for 20 min at 4 °C and the supernatant obtained was used as an enzyme extract. Protein concentration in the supernatant was determined according to Bradford (1976) using bovine serum albumin as standard.

The activity of superoxide dismutase (E.C. 1.15.1.1) was estimated by following the protocol of Elavarthi and Martin (2010). Activity was measured spectrophotometrically at 560 nm. One unit of enzyme activity indicates 50% inhibition in NBT reduction and was expressed as Unit activity.

Ascorbate peroxidase (E.C. 1.11.1.11) activity was measured according to the method of Nakano and Asada (1981). Decrease in absorbance was recorded 1 min after addition of enzyme extract. The oxidation of ascorbic acid was measured at 290 nm and the enzyme activity was expressed as n mol ascorbate oxidized/min/mg protein.

The estimation of glutathione reductase (E.C. 1.6.4.2) was performed following the method of Foyer and Halliwell (1976). One unit of enzyme activity indicates decomposition of 1 µmol of NADPH per min at 25°C. Enzyme activity was expressed as µmol NADPH oxidase/min/mg protein.

Catalase (E.C. 1.11.1.6) activity was determined by following the protocol of Aebi *et al.,* (1984). Decay in absorbance after addition of H_2O_2 was measured at 240 nm for 3 min. One unit of enzyme activity is that which catalyzes the oxidation of 1 µmol of H_2O_2 per minute at 25°C. Activity of CAT was expressed as n mol min/mg/protein.

***Concentration of total phenols*:** Total phenols were assayed according to Bray and Thorpe (1954). Leaves (1 g) were chopped and boiled in 100% ethanol. After 5 min, the ethanolic extract obtained was mixed with Folin-Ciocalteu's phenol reagent and 20% Na_2CO_3. This mixture was incubated at room temperature for 1 min and absorbance was read at 650 nm. The concentration of total phenols was determined by using standard curve of pyro-catechol.

***Defence enzyme activities*:** The activity of phenylalanine ammonia lyase (PAL, E.C. 4.3.1.5) was assessed following the protocol of Nadernejad *et al.* (2013) with few modifications. PAL activity was determined spectrophotometrically at 290 nm. One unit of PAL activity corresponds to 1 µmol of trans-cinnamic acid produced per min. The specific activity was expressed as trans-cinnamic acid µg/ml/h/mgprotein.

Activity of β-1, 3-glucanase (E.C. 3.2.1.6) was assayed according to Ji and Kuc (1995) using the laminarin dinitrosalicylic method. The absorbance of resulting solution was recorded at 560 nm. One unit of enzyme activity was defined as the amount of enzyme that produced 1 µmol reducing sugar/h under standard assay conditions.

In order to estimate chitinase (E.C. 3.2.1.14) activity leaf samples (1 g) were homogenized in 5 ml of 0.1M sodium citrate buffer (pH 5.0). The homogenate was centrifuged for 10 min at 10, 000g at 4 ^{O}C and the supernatant was used to estimate chitinase activity. The activity was determined by estimating concentration of N-acetyl glucosamine in the reaction mixture (Reissig *et al*., 1959). One unit of chitinase activity was defined as the amount of enzyme required to liberate 1 µmol of N- acetyl-D glucosamine.

Transcription analysis of some defence genes

RNA extraction and RT-PCR analysis: Leaves from five different plants for each treatment were harvested 48 hpi, pooled and immediately frozen in liquid nitrogen. Non-inoculated tomato leaves sprayed with sterile water served as control. Total leaf RNA was isolated using RNeasy Plant Mini kit (QIAGEN, Germany). RNA was quantified by spectrophotometry (NanoDrop, Eppendorf) and quality was analysed on 1.5% formaldehyde agarose gel. For RT-PCR, cDNA was synthesized using Superscript III (Invitrogen, Carlsbad, CA, USA) using equal amounts of total RNA (2 µg). The cDNA was further diluted and the transcript level of seven pathogenesis related genes were quantified using real time quantitative PCR. The PCR reactions were performed on Mx 5000P qPCR equipment (Stratagene, USA). The tomato actin gene was amplified as a control to demonstrate relative quantity of the cDNA. The reactions were run in technical triplicate for each biological replicate, and the average values were used for quantification. MxPro analysis software (Agilent technologies, USA) was used to calculate cycle threshold (CT) values. The relative quantification of target genes was determined using the ΔΔCt method. Primer pairs were designed using Primer 3 software based on mRNA sequences of *S. lycopersicum* available in NCBI database; the primer sequences used in the study are listed in **Table 1**.

Experiment to assess protective role of Bcnpm

Table 1. List of gene specific primers used in qRT-PCR analysis

Name	Left primer (5'-3')	Right primer (5'-3')
ACC	TTAAAAGGGAAGAATTTAATT	TAACAATATAATCGAGAAAG
PR-1	CTCAGTCCGACTAGGTTGTGGTC	AAGGACGTTGTCCGATCCAG
HSR203J	GGAAACGTCGTCCATCAAGT	AGGGGGTTTGTTCCTGTTCT
CHI3	GAACAATGACGCATGTCCTG	CGTGAGATGTTTGACCGAAA
cel5	CAAGTAGGAGACCCCAACCA	TCAGCAGCAACATCAGAACC
AOS-2	CGATTACCTCCGATTCTGGT	AAATCTTCATCCCACCGAAG
PI-II	TGATGAACCCAAGGCAAATA	ACACAACTTGATGCCCACAT

An experiment was set to test the protective ability of Bcnpm against its wild type strain and a biotrophic pathogen, *Pseudomonas syringae*. pv. tomato. For this, tomato plants were grown in 50 earthen pots each containing four plants under greenhouse conditions. Out of these, 20 pots were inoculated with apathogenic mutant of *B. cinerea* (Bcnpm). The remaining 30 pots that served as control were sprayed with water. Following 48 hpi, both control and Bcnpm-inoculated plants were challenged with either virulent strain of *B. cinerea* (WT) or *P. syringae pv. tomato*. Thus there were five treatments viz. (i) Control; (ii) Control + WT (iii) Bcnpm pre-inoculated + WT (iv) Control + *P. syringae*; and (v) Bcnpm pre-inoculated + *P. syringae*.

Conidial suspension (10^5spores/ml) of wild type was drop inoculated on Bcnpm treated plants. *P. syringae* was inoculated in plants by infiltrating bacterial spore suspension (0.01 OD) made in 10 mM $MgCl_2$with a syringe on the abaxial surface of leaves. Plants were covered with polythene to maintain humidity.

At 96 hpi with pathogens (WT or *P. syringae*) disease severity was assessed in the form of lesions. The bacterial growth was determined by randomly collecting ten leaves from each treatment and homogenizing in 10 mM $MgCl_2$. After a series of gradient dilutions, the bacterial suspensions were plated onto 100 µg/ml rifampicin plates, incubated at 28° C and colony forming units were counted.

Statistical analysis

The data were analyzed using the Statistical Package for Social Sciences version 16 (SPSS Inc., Wacker Drive, Chicago, IL) for windows. All results are given as mean ± standard deviation. One way ANOVA was performed for the biochemical parameters studied. Differences between the individual mean were compared using Duncan's test.

RESULTS

Successful random insertional mutagenesis of WT was achieved by using LBA 4404 strain of *A. tumefaciens*. A mutant (Bcnpm) was selected for its lack of pathogenicity towards different plant hosts. Plants inoculated with WT showed disease symptoms typical of *Botrytis* rot such as tissue necrosis, maceration and mouldy growth on aerial parts of the plants. However, mutant caused no infection on chickpea and tomato plants. Integration of hygromycin-resistance cassette in the mutant conveyed by the vector *pBIF* was checked by using *hph* specific primers which resulted in a 1.5 kb gene amplicon (**Fig. 1**). Identification of T-DNA tagged gene was achieved by TAIL-PCR. The recovered sequence corresponded to right border junction. The sequence on BLAST analysis was found to be similar to BC1G_03602 that highlighted a sequence from *B. cinerea* genome (**Table 2**). Further analysis indicated its similarity to a predicted protein that was unique to *B. cinerea*.

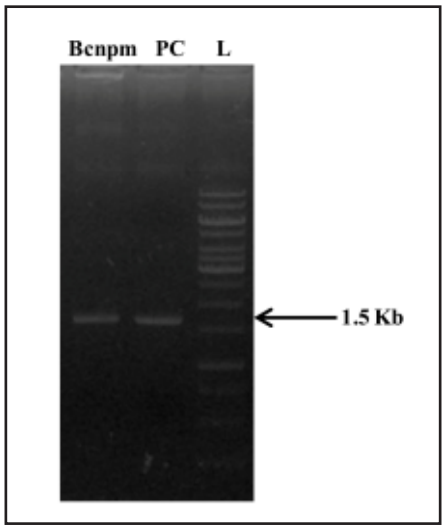

Fig. 1. Confirmation of T-DNA integration in Bcnpm using *hph* gene specific primer pair. PC indicates positive control and L stands for 1 Kb ladder.

In vitro growth of mutant on potato dextrose agar was identical to that of the wild type strain (**Fig. 2**). The mutant was capable of producing conidiophores, conidia as well as sclerotia identical to the wild type. This indicated that the mutation did not affect fungal growth and would have affected any gene whose product is essential for *B. cinerea* virulence.

Table 2. NCBI blast analysis of tagged gene in Bcnpm

Tagged sequence in Bcnpm (5'-3')	Accession no. in NCBI database	Sequence similarity
ATGCAATCCCAAGCACCAAAACCCGCGTATGCAA ATCAAATTCCGCATTCCGCCTTCGCACCTCCCATC CCAAAGCCCCAAGGCCTGAGACGAGACGCCCCCC AAAAAAAAAGAGAGACAAAGGGGAGGAGGGAA AAAAAAACACAGAAACGGGAAATCCCGACCTTCT CCGCAACAGCCTCAGCAACCCTCTGCGCAAAACA GGAGTTTTCAAAGCCCACTCCACCCCACGCCATCC CGATTCCATCCCGAAACCATCCACCCATCCATCCA TCCATTCTCGCTACCTCGCTACCTCGCTCTCTCCCC CTACTCACCTCAACGCAGGAATTCCCCGTTTCAAC ACCCGGATACCAAACCACCAAGCCCCAAGT AGAC CCGGCCGGGACGAGGACCCCCCACCCCCTTCCCA AGTCTCCAGCCACGGGAATGAAAACCTGCAGACA CGAACCCCCATCCACCCAGCATTTATTTCACCATT CGCTCCCCCAAGTGCGGAAAGAAGGACGGAATTA TACTCCAAGCCACTTCATCCATCCATCACCTACCG ACCTAGGTATGCGTATGCAGCTCTCCCTTCCCCCC AAGCCCAAGTGTCAAATTCTCAATGCAAAATGCA AAAACATGAACCTTGA	Similarity to XM_001557289.1	BC1G 03602 that encodes for a predicted protein

Characterization of mutant for virulence

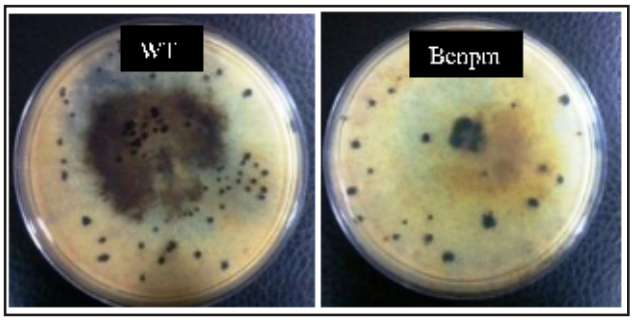

Fig. 2. Morphological characterization of WT and Bcnpm in terms of their growth pattern on potato dextrose agar medium. Strains were incubated at 21° C for seven days and observations were taken.

In vitro pathogenicity assay was performed on detached tomato leaves to assess the differences in pattern of virulence in WT and Bcnpm. Water-soaked necrotic lesions accompanied with significant maceration of tomato leaf was observed in case of wild type at 96 hpi. However, no disease symptom was observed on tomato leaves inoculated with Bcnpm even after prolonged exposure (**Fig. 3A**).

B. cinerea produces many hydrolytic enzymes such as pectin methylesterases (PME) that deesterifies pectin into methanol and polygalacturonic acid (PGA), allowing consequent action of polygalacturonases (PG) that cleave glycosidic bonds between d-galacturonic acid residues in non-methylated homogalacturonan and cause tissue maceration, resulting in soft-rot symptoms (Manfredini *et al*., 2005; Valette-Collet *et al*., 2003). It was observed that PME and PG activity though reduced slightly in Bcnpm, the differences were not statistically significant (**Fig. 3B, C**).

The early stage of *B. cinerea* infection involves the production and accumulation of a large amount of oxalic acid (OA) which appears to be one of the essential determinants of the pathogenicity (Dutton and Evans, 1996; Guimaraes and Stotz, 2004). The concentration of OA in cultural filtrate of

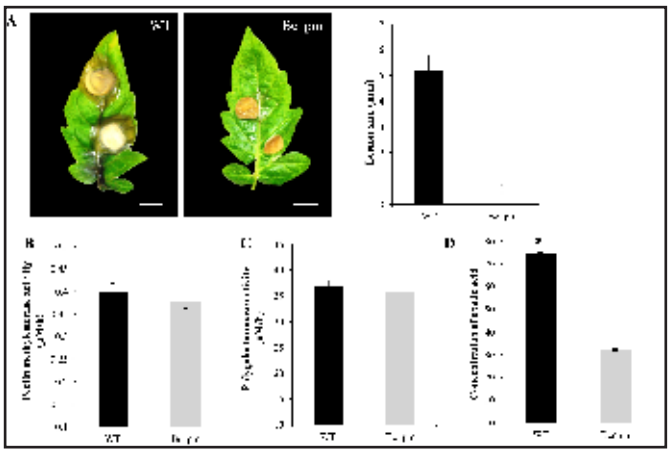

Fig. 3. Characterization of wild-type strain BO5.10 and nonpathogenic mutant (Bcnpm) of *Botrytis cinerea* for virulence. (A) Virulence assay on tomato leaves. Mycelium plugs of WT and Bcnpm were applied to the upper face of detached leaf. Observations were made 96 hours post inoculation. (B-D). Production of virulence factors in the cultural filtrates. (B) Activity of Pectin methylesterase, (C) Activity of Polygalacturonase, and (D) Concentration of oxalic acid. *Means are statistically different at $P< 0.05$.

Bcnpm was considerably less as compared to its concentration in WT (**Fig. 3D**).

Characterization of mutant for penetration and colonization of plant tissue

It was of interest to determine if the variation in disease progression was due to differences in penetration ability. Infection related morphogenesis of WT and Bcnpm was studied on onion epidermis as the translucid cells combined with cotton blue staining allows easy visualization of fungal hyphae penetration. Epidermal strips were observed under a microscope at different time intervals (**Fig. 4**). A slight delay in conidial germination was observed in Bcnpm compared to WT. Percent spore germination at 16 h was less in Bcnpm (80%) compared to WT (95%). While nearly all spores germinated in WT at 16 h, maximum spore germination were observed at 20 hpi in Bcnpm. Hyphae were long and slender in WT; while Bcnpm formed thick and distorted hyphae. At 24 hpi, very few hyphae were formed in case of mutant, while WT grew abundantly and formed secondary infectious hyphae to invade the underlying tissue at many locations. Dense colonisation was observed in the onion epidermis at 48 hpi by WT that further increased at 72 hpi. While in case of Bcnpm tissue colonization rate at 72 hpi was similar as that at 48 hpi.

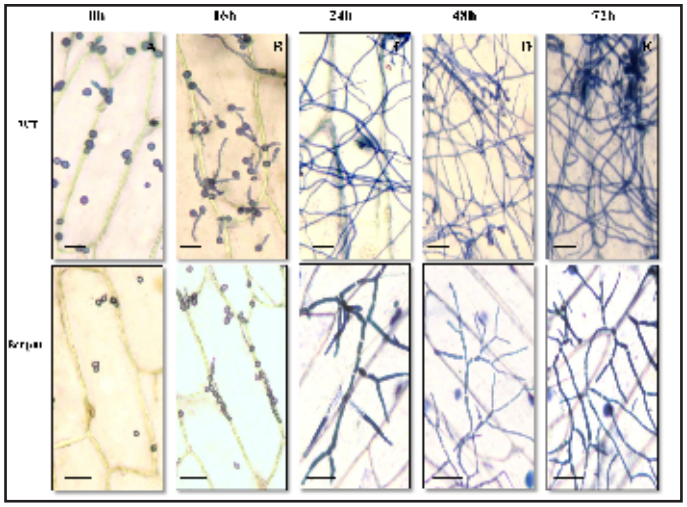

Fig. 4. Penetration and colonization assay on onion epidermal tissue. (A) Spores on the leaf surface at the time of inoculation (B) Spore germination at 16 hours post inoculation. (C) WT formed long and slender hyphae while Bcnpm formed long and distorted hyphae; (D &E) Dense colonization by WT in comparison to Bcnpm at 48 and 72 hours post inoculation. Bar =10µm.

Quantification of fungal progression and disease severity in tomato plants

Relative abundance of cutinase A gene was analysed using real-time PCR to follow the fungal progression in tomato plants during the time course of infection. There were no significant differences in both the strains at the time of inoculation (0 hpi) (**Fig. 5A**). However, at 24 hpi the growth of WT was significantly higher than that in Bcnpm. The progression pattern was found to deviate in both the strains after 24 hpi, while WT strain continued to proliferate from 48 to 72 hpi, on the contrary Bcnpm showed very less increase in fungal load upto 48 hpi and remained same thereafter.

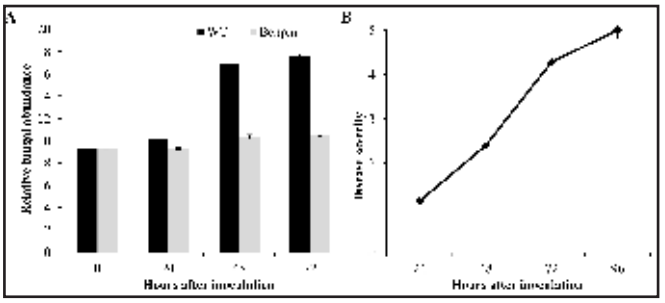

Fig. 5. Quantification of fungal growth and disease development in tomato plants by WT and Bcnpm over time course of infection. (A) Growth of fungal strains was quantified in terms of abundance of cutinase A gene (*cutA*) in plant DNA samples and normalized with actin gene of tomato. (B) Development of disease in terms of disease severity. Bars represent mean data and standard deviation of triplicates.

Disease severity at different time point was determined by using a disease rating scale. Wild type strain started showing necrosis symptoms earliest by 24 h and continued to expand till 96 hpi after which the whole plant collapsed (**Fig. 5B**). However, mutant showed no lesions or tissue necrosis.

Assessment of damage by the fungal strains

Inoculation with WT decreased the concentration of chlorophyll-a over control at 24 and 72 hpi, however no significant decrease was observed in plants inoculated with Bcnpm (**Fig. 6A**). There were no significant differences in concentration of chlorophyll-b among the treatments at 24 hpi. However, at 72 hpi the reduction was significant in WT inoculated plants (56.6%) as compared to un-inoculated control (**Fig. 6B**).

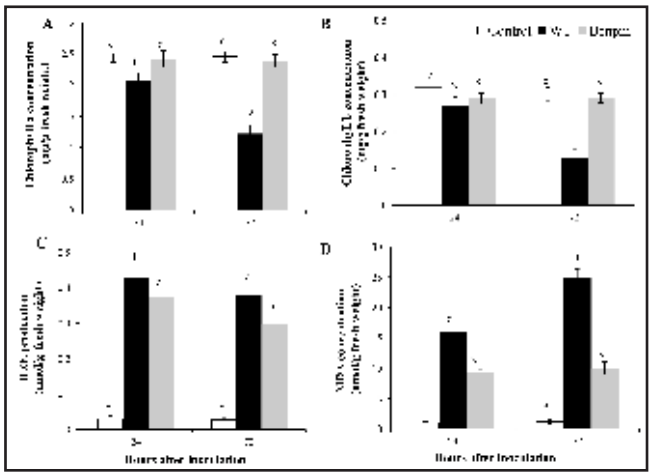

Fig. 6. Assessment of damage in tomato plants inoculated with WT and Bcnpm at 24 and 72 hour post inoculation. (A) Concentration of Chlorophyll-a, (B) Concentration of Chlorophyll-b, (C) Production of hydrogen peroxide; and (D) Lipid peroxidation in terms of MDA concentration. Significant differences are indicated by different letters above bars.

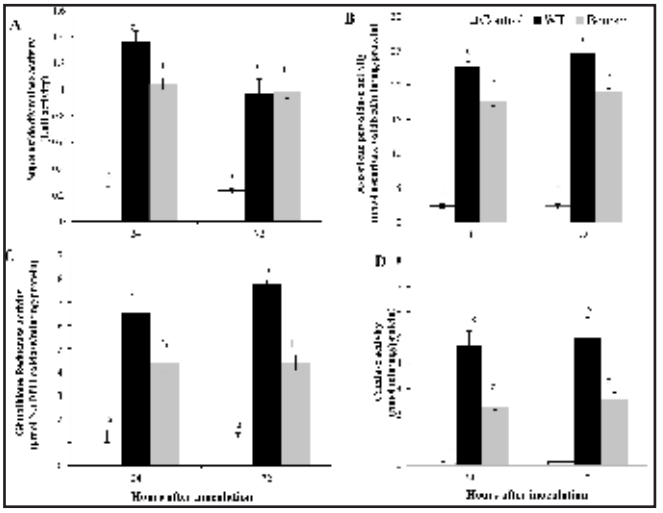

Fig. 7. Effect of inoculation with WT and Bcnpm on antioxidant enzyme activities in tomato leaves at 24 and 72 hour post inoculation. (A) Superoxide dismutase, (B) Ascorbate peroxidase, (C) Glutathione reductase; and (D) Catalase. Significant differences are indicated by different letters above bars.

Both WT and Bcnpm were capable of inducing oxidative stress in tomato plants. WT inoculated plants produced highest levels of H_2O_2(**Fig. 6C**)and accumulated higher levels of MDA (**Fig. 6D**). The differences between the three treatments were statistically significant. There was a fall in concentration of H_2O_2 at 72 hpi while lipid peroxidation increased with time in the WT-inoculated plants.

Plant response to inoculation with fungal strains

Antioxidant enzyme activities: Increased SOD activity in leaf tissues was observed after inoculation with WT and mutant strains, but this increase was significantly higher in the WT-inoculated plants. The activity was highest at 24 hpi and was found to decrease with time. There was an increase of 6.18 fold in activity of WT inoculated plants while Bcnpm inoculated plants showed 4.72 fold increase at 24 hpi compared to that of control (**Fig. 7A**). The activity of ascorbate peroxidase increased with time and was highest in WT inoculated tomato plants. The enzyme activity of WT inoculated plants was 1.3 times higher as compared to Bcnpm inoculated plants at 72 hpi (**Fig. 7B**).

Induction levels of glutathione reductase activity were higher in pathogen inoculated plants with maximum activity observed in WT inoculated plants followed by Bcnpm inoculated plants (**Fig. 7C**). The activity was 1.50 times higher in WT inoculated plants as compared to Bcnpm inoculated plants at 24 h. There was no significant difference with time course in the activity of enzyme in Bcnpm inoculated plants. Activity of catalase was also highest in WT inoculated plants (41.6 fold) as compared to 21.3 fold in Bcnpm inoculated plants over control at 72h (**Fig. 7D**). However, no significant differences in catalase activity were observed over the time interval.

Concentration of total phenols and defence enzyme activities: Accumulation of phenolic compounds is indicative of stress in plants (Lattanzio *et al*., 2006). There was an upsurge observed in concentration of total phenols in pathogen inoculated plants. Maximum increase was observed at 72 hpi and was 2.4 fold higher in WT inoculated plants with respect to Bcnpm inoculated plants (**Fig. 8A**). Induction in PAL activity was also observed in WT-inoculated plants and corresponded with the similar increase observed for concentration of phenols (**Fig. 8B**). However, there was no significant effect of Bcnpm inoculation on activity of this enzyme in tomato plants.

Activity of chitinase and β-1,3 glucanase also increased following pathogen inoculation. Maximum increase was observed in WT inoculated plants followed by Bcnpm inoculated plants. At 72 hpi chitinase activity increased 2.93

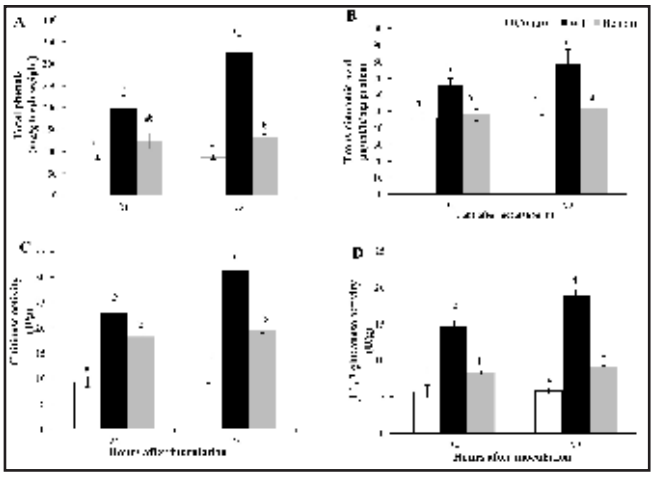

Fig. 8. Biochemical estimation of defense in tomato plants in response to inoculation with wild type strain and Bcnpm at 24 and 72 hours post inoculation. (A) Concentration of total phenols, (B) phenylammonia lyase activity, (C) Chitinase, activity; and (D) β-1, 3 glucanase activity. Significant differences are indicated by different letters above bars.

fold and β-1,3 glucanase activity increased 3.24 fold in WT inoculated plants over control. Though, the enzyme activities increased in Bcnpm inoculated plants but induction was less as compared to WT. No significant differences in the enzyme activities were observed in Bcnpm inoculated plants over the time interval (**Fig.8C and 8D**).

Expression analysis of plant defence genes

Transcript level of *ACC* gene encoding aminocyclopropane-1-carboxylic acid synthase (ACC synthase) enzyme that catalyses the synthesis of ethylene precursor (**Fig. 9A**) were higher in WT inoculated plants as compared to Bcnpm inoculated plants. The expression levels of *PR-1*, a salicylic acid dependent gene (**Fig. 9B**) *CHI-3*, a gene encoding chitinase and *cel-5*, a gene encoding β-1, 4 endoglucanase showed similar pattern of response. Their transcript levels were higher in wild type inoculated plants in contrast to Bcnpm inoculated plants (**Fig. 9C, D**). Prominent induction of *HSR203J* was also observed in WT-inoculated plants over that of Bcnpm inoculated plants (**Fig. 9E**). *HSR203J* gene is activated rapidly and specifically during pathogen-triggered hypersensitive response (HR). Allene oxide synthase catalyzes the first committed step at the branching point of octadecanoid pathways in the biosynthesis of jasmonates (Lu *et al*., 2012; Howe *et al*., 2000). Real-time PCR revealed an induction of *AOS-2* in WT and Bcnpm inoculated plants over that of controls (**Fig. 9F**). The expression of *AOS-2* gene was similar in both WT and Bcnpm inoculated plants. Wound induced and jasmonic acid responsive gene, *PI-II* increased only 2-fold in WT infected plants, while there was 3-fold downregulation in Bcnpm inoculated plants (**Fig. 9G**).

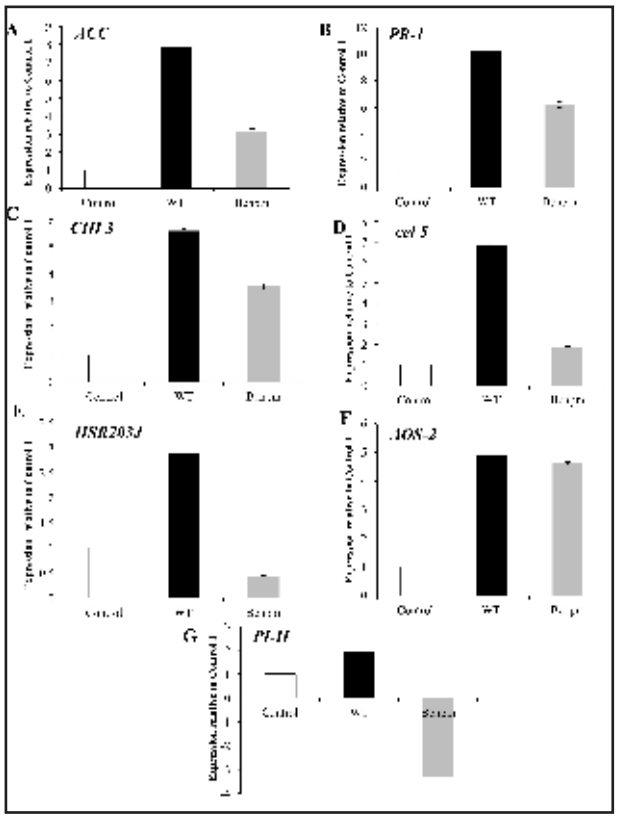

Fig. 9. Transcript analysis of defense-related genes in tomato plants in response to inoculation with WT and Bcnpm. (A) *ACC*, (B) *PR-1*, (C) *CHI-3*, (D) *cel-5*, (E) *HSR203J*, (F) *AOS-2* and (G) *PI-II*. Data bars indicate average ± SD of three biological replicates.

Protective role of Bcnpm against plant pathogen attack

Bcnpm enhanced tomato resistance to its wild type and *Pseudomonas syringae*. In order to test the efficacy of protection conferred by Bcnpm pretreated plants against its wild type, disease severity in the form of necrotic lesions was observed at 96 hpi. Necrotic spot induced by WT was limited to the inoculation point in Bcnpm pre-inoculated plants till 96 hpi and did not expand even after its prolonged exposure (**Fig. 10A**). However, there was a considerable increase in lesion size in control plants that finally resulted in tissue collapse.

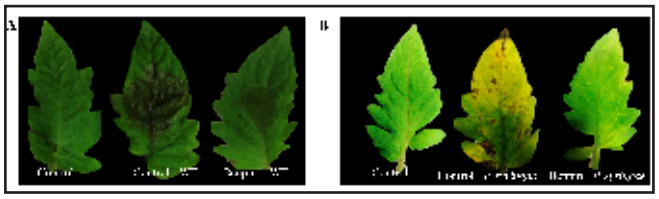

Fig. 10. Assessment of protective role of Bcnpm against infection after 96 hours post inoculation by (A) *Botrytis cinerea* wild type strain BO5.10, and (B) *Pseudomonas syringae* pv. tomato. Control+WT control plants challenged with BO5.10; Bcnpm+ WT Bcnpm preinoculated plants challenged with BO5.10; Control+ *P. syringae* Control plants challenged with *P. syringae*; Bcnpm+ *P. syringae* Bcnpm pre-inoculated plants challenged with *P. syringae*.

Bcnpm was also effective against the control of *P. syringae*. In the control plants that were infiltrated with *P. syringae* alone typical bacterial specks were observed. The bacterial growth at 96 hpi in control plants was $4X10^7$ CFU/ml. While in Bcnpm pre-inoculated plants it drastically reduced to 10^2 CFU/ml and no bacterial specks were observed on leaves (**Fig. 10B**).

DISCUSSION

In this study, ATMT was used to generate pathogenicity mutants of *B. cinerea* to identify novel factors required during pathogenesis. An insertional mutant (Bcnpm) that displayed complete loss of pathogenicity was identified. Identification of tagged gene was successfully fulfilled by TAIL-PCR. The sequence was identified as a predicted protein specific to *B. cinerea* as it did not show sequence similarity to the close relative *Sclerotinia sclerotiorum* or any other published fungal sequence in the NCBI database.

In vitro pathogenicity assay carried out on tomato leaves revealed that Bcnpm was impaired in virulence as it caused no necrotizing lesions even after prolonged exposure to the plant. Interestingly, on PDA medium Bcnpm did not show any growth abnormality and was similar to WT. It suggests that the tagged gene was not required for the saprophytic growth of the fungus. Therefore, it is unlikely that apathogenic behaviour of the mutant was dependent on the growth rate. Thus, it may be speculated that T-DNA integration primarily occurred in the gene that plays an important role during the infection stages and is required for disease progression.

Our understanding on the non-pathogenic behaviour of Bcnpm was aided by the cytological observations on onion epidermis. Conidia germination, penetration of host cell wall and colonization of the plant constitute distinct successive stages of *B. cinerea* infection process (Tenberge, 2004). The microscopic studies revealed that the mutant was affected at the infection stages i.e. spore germination and penetration of intact host cell wall. Bcnpm conidia showed decreased and delayed germination with low penetration and colonization abilities. This observation is further substantiated by cutinase A gene abundance that showed no change in fungal load in Bcnpm inoculated plants between 48 and 72 hpi while it continued to increase for WT inoculated plants.

Discrepancy in aggressiveness of *B. cinerea* has been often associated with variation in the production of extracellular enzyme activities and secretion of a potential pathogenicity factor oxalic acid (Derckel *et al*., 1999). The activity of pectin methylesterases and polygalacturonases were reduced in Bcnpm but the differences were not significant with respect to wild type. On the other hand, there was remarkable difference between the strains in terms of oxalic acid production. Concentration of oxalic acid in Bcnpm was significantly less as compared to that in wild type. Oxalic acid can degrade or weaken the plant cell wall via acidity or chelation of cell wall Ca^{2+} (Bateman and Beer, 1965). Oxalic acid is directly toxic, functioning as a nonspecific phytotoxin. Oxalic acid mutants of *S. sclerotiorum* are nonpathogenic even though the fungus still maintains its full arsenal of cell-wall degrading enzymes (Godoy *et al*., 1990). It can be presumed that the deleted predicted protein plays an important role in the synthesis of oxalic acid, and absence of necrotic lesions characteristic of *Botrytis* rot can be explained by substantial decrease in the production of oxalic acid by the mutant strain.

To further characterize the mutant, we explored the varied response of tomato plants towards WT and Bcnpm. Damage to photosynthetic machinery was analyzed by learning the differences in concentration of chlorophyll-a and chlorophyll-b. The concentration of photosynthetic pigments was significantly reduced in WT inoculated plants in contrast to Bcnpm, which showed non-significant differences when compared to control. Oxidative burst and transient production of ROS is considered to be the fastest response of a plant towards microbial infection (Lubaina and Murugan, 2013). *B. cinerea* also modulates the redox environment during pathogenesis (Unger *et al*., 2005; van Baarlen *et al*., 2004). In our study, production of H_2O_2 was found to be significantly higher in WT inoculated plants in contrast to Bcnpm inoculated plants. The oxalate-induced ROS production and programmed cell death (PCD) activities are correlated with pH (Kim *et al*., 2008). In the beginning of pathogen infection, low level of oxalic acid at relatively high pH (>5), triggers ROS and PCD. As oxalic acid buildup, the pH is lowered and the interaction becomes necrotic in nature, and is accompanied by suppression of ROS and PCD, enabling further ingress of the pathogen into plant tissue. Significant decrease in the production of H_2O_2 at 72 hpi observed by us is in accordance with the above trend. Oxalic acid is reported to chelate Ca^{2+} ions and inhibit ROS production in host plant (Beneloujaephajri *et al*., 2013). Higher production of H_2O_2 in response to a virulent strain with more oxalic acid production abilities is contrary to the above.

Concentration of MDA is the marker for extent of lipid peroxidation and formed by the reaction of ROS with lipid molecules (Shimizu *et al*., 2006). The increased concentration of MDA correlated with increased levels of H_2O_2 in WT inoculated plants. Correspondingly higher activities of SOD, APX, GR and CAT were observed in WT inoculated plants as compared to Bcnpm inoculated plants. It indicated that these enzymes played an important role in maintaining ROS levels in plants and tried to limit cellular damage caused by the pathogen.

Phenylalanine ammonia-lyase (PAL), the first enzyme of the phenylpropanoid pathway, is involved in the biosynthesis of various defence-related compounds (phenolics, lignin, and salicylic acid). Increased PAL activity and concentration of phenols was observed in WT inoculated plants. It points out that plant channelizes its energy in production of secondary metabolites only when it perceives attack of a virulent pathogen that stimulates higher defence response.

Bcnpm varied conspicuously in its ability to produce oxalic acid as compared to wild type. Depending on the concentration, oxalic acid has been shown to play two distinct roles (Lehner *et al*., 2008). At high concentration, oxalic acid induces ROS and PCD and contributes to the progression of the fungi. However, at low concentrations it is able to induce the resistance in plant against the fungi. Ethylene is shown to be implicated in oxalic acid-induced cell death (Errakhi *et al*., 2008). Furthermore, *B. cinerea* infection has been associated with ethylene accumulation as the hormone accelerates senescence which predisposes the plant to disease development by the necrotroph (Diaz *et al*., 2002). We therefore assessed the effects of WT and Bcnpm on ethylene synthesis gene, and hypersensitive response related gene transcripts. The increased transcript levels of *ACC*, an ethylene synthesis gene in WT inoculated plants suggested its importance in promoting disease development. Transcript analysis showed higher expression of classical SA responsive genes (such as *PR-1*, *CHI3*, *cel5* and *HSR203J*) in WT as well as Bcnpm infected plants. The biochemical estimation of chitinase and β-1, 3 glucanase activities further supported transcription analysis. Increased level of SA promotes disease development by *B. cinerea* (El Rahman *et al*., 2012). The necrotroph has been found to manipulate the antagonistic effect between SA and JA to promote disease development (El Oirdi *et al*., 2011). A more virulent strain of *B. cinerea* elicits enhanced SA accumulation and induces higher accumulation of mRNA of TGA1.a a transcription factor that interacts with NPR1 leading to activation of SA dependent responses (El Rahman *et al*., 2012). Lower display of SA dependent response in plants infected by Bcnpm as compared to WT further corroborates impaired virulence of the mutant strain. There were non-significant differences in the transcript level of *AOS-2* in the wild type and mutant inoculated plants. In spite of induction of JA synthesis gene by both the strains, the magnitude of induction was far less for *PI-II* and it was down-regulated in Bcnpm inoculated plants.

Oxalic acid in non-lethal level has been reported to induce

expression of defence genes such as *PAL1, PR1* (Lehner *et al*., 2008). This is further corroborated by our results, wherein mutant expressing low levels of oxalic acid elicited expression of *PR1, CHI3 cel5* and *HSR203J*. Pre-treatment with non-lethal dose of oxalic acid is able to induce defence mechanisms and considerably limit the development of *S. rolfsii* in *Arabidopsia thaliana* (Lehner *et al*., 2008). This led us to hypothesize that Bcnpm can be used as a priming agent.

Pre-inoculation of Bcnpm protected plants from subsequent infection by virulent WT or *P. syringae* pv. tomato. The use of nonvirulent mutants as biocontrol agents for fungal pathogenesis has been reported in earlier studies (Pareja-Jaime *et al*., 2010; Redman *et al*., 1999a; Freeman and Rodriguez, 1993). Use of avirulent mutant due to loss of function mutation in a single gene, has the advantage over nonpathogenic isolate as it provides enough information about the biochemical and genetic bases that differentiate it from its virulent wild type. A nonvirulent mutant *Colletotrichum magna* deficient in the synthesis and secretion of pectate lyase and endopolygalacturonase (Wattad *et al*., 1995), was able to colonize cucurbit plants without eliciting disease symptoms, and prevented plants from attack of *C. magna*, *Fusarium oxysporum* (Freeman and Rodriguez, 1993) and *Colletotrichum orbiculare* (Redman *et al*., 1999a). Similarly, *C. magna* R1 nonvirulent mutant conferred protection to watermelon plants against the wild-type strain (Redman *et al*., 1999b). *Fusarium oxysporum* f. sp. *lycopersici*, deficient in class V chitin synthase protected tomato plants against wild type infection (Pareja-Jaime *et al*., 2010).

The possibility of inhibition of direct growth was eliminated by *in vitro* inhibition assay. The direct competition between two strains of *F. oxysporum* (nonvirulent and virulent) within the *Dianthus caryophyllus* stem has been proposed as the cause of decrease in disease severity (Postma and Luttikholt, 1996). In our study Bcnpm had competitive advantage as the plants were pre-inoculated with the mutant strain. Decrease in disease severity in Bcnpm pre-inoculated plants is primarily due to activation of defence response in the tomato plants that primes the plant to subsequent attack either by a necrotroph or a biotroph.

CONCLUSIONS

The study identified a unique predicted protein that is essential for the pathogenicity of *Botrytis cinerea*. The mutant strain deficient in the expression of this protein could grow inside the plant cells without causing apparent damage. The mutant exhibited reduced ability to penetrate and colonize leaf tissue. While the activities of cell wall degrading enzymes such as pectin methyl esterase and polygalacturonase were not affected, the concentration of oxalic acid was considerably reduced in the mutant. Since the biochemical and expression analysis of plant response were performed on non-inoculated leaves of the inoculated plants, it suggested systemic effect of the mutant strain on the plant defence. Presumably, during early stages of inoculation, nonpathogenic mutant is perceived as potential invader. As the ability of the mutant to penetrate and colonize the plant issue was impaired, less abundance of elicitors exerted a weaker response in comparison to the virulent WT strain. The induction of some hypersensitive responsive genes may be due to low levels of oxalic acid produced by nonpathogenic mutant. This also attributed a protective role to the mutant. Nonpathogenic mutant pre-inoculated plants when challenged with virulent strain of *B. cinerea* or *Pseudomonas syringae* pv. tomato showed a significant reduction in disease severity.

The study emphasizes on the need to identify new factors that contributes to the virulence of *Botrytis cinerea.* It would help in better understanding of the plant-fungus interactions and allow development of biocontrol strategies for a very challenging broad-spectrum and economically important pathogen like *B. cinerea*.

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