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Morphological and Molecular Diversity of *Botrytis cinerea* Infecting Chickpea in India

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ABSTRACT

Background: The Botrytis gray mold (BGM) caused by *Botrytis cinerea* Pers. ex Fr., has become a major threat for chickpea cultivation in India. In view of monetary significance of this disease and growing its spread into new regions through seed and soil (sclerotia) encouraged us to study variability of this pathogen from diverse regions.

Methods: *Botrytis cinerea* infected chickpea samples were collected from Punjab and Uttarakhand states. Pathogen was isolated using chickpea dextrose agar media. Pathogenicity was proved by inoculation of spore suspension ($3x10^5$ spores'mL^{-1}) of the fungus on susceptible cultivar. *In vitro* studies were conducted for observations of morphological and cultural variability of the fungus. The pathogenic fungus was also identified through molecular characterization using ITS primers.

Result: The isolates were categorized into different groups based on growth rate of the pathogen on PDA media. Pathogenicity test proved that *B. cinerea* isolates from Punjab were showed less disease severity scale compared to Uttarakhand state isolates. The *B. cinerea* fungus prefers PDA for efficient growth and multiplication than Chickpea Dextrose Agar (CDA) media. There was no conspicuous difference in colour of the colony on PDA and CDA media. The colour of the mycelium were initially white or dirty white or greyish and sporulated culture shown to have as grey with profuse mass on the surface of the mycelium. The fungus *B. fabae*, a different species was also responsible for causing BGM disease which was confirmed through pathogenicity and ITS sequencing. Twenty-eight isolates were studied for diversity in cultural, morphological and molecular level. The diversity in colony colour, growth type and diameter of mycelial growth, sclerotia initiation, number and pattern of sclerotia formation were observed amongst the isolates. This study would help in designing breeding strategy for development of disease resistant cultivars.

Key words: Colony, Conidia, Diversity, Morphology, Sclerotia, Virulence.

INTRODUCTION

Chickpea (*Cicer arietinum* L.), the major grain legume crop belongs to the family Leguminosae grown in at least 44 countries worldwide including India (Pande *et al*., 2006). Botrytis gray mold (BGM) is the most devastating diseases of chickpea caused by *Botrytis cinerea* Pers. ex. Fr. result in complete yield loss under extensive winter rain with high relative humidity (Davidson *et al*., 2004; Pande *et al*. 2006). It has wide host range infecting more than 200 agriculturally important plant species (Pande *et al*. 2005). BGM causes production loss in many Asian countries with80-100% yield loss in the Indo-Gangetic plains of India (Pande *et al*. 2005).

BGM symptoms appears on all aerial parts of the plant as water-soaked lesions on stem near the ground level which extend alongside the stem (Knights and Siddique, 2002). The lesions of 10-30 mm long completely girdles the stem. Affected leaves and flowers turn to rotting mass which breaks off the branches at the rotting point (Bakr, 2002; Pande, 2002). The common field diagnostic symptoms are drooping of terminal branches which later may break off at the infection point (Grewal *et al*. 1992). The fungus can form light brown to grey or brown lesions on leaflets, branches and pods covered with hairy sporophores, single celled, hyaline spore's masses (Haware and Mc Donald, 1992).

Even though, morphological and genetic diversity studies have been reported from different parts of the world on different crops but limited information is available on *B. cinerea* infecting chickpea with diverse nature of the fungus (Pande *et al*. 2010). Characterization and understanding of *B. cinerea* diversity analysis was needed for its effective management. Therefore, we aimed for (i) collection and diversity analysis of *B. cinerea* isolates from Uttarakhand and Punjab states for it's morphological, molecular and pathogenic characters and (ii) determination of phylogenetic relationships among the isolates of *B. cinerea*. This study would be a great value to expand knowledge on geographic

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and morphological diversity among *B. cinerea* for development of disease management methods through breeding for host resistance.

MATERIALS AND METHODS

Collection and isolation of *B. cinerea*

The experiment was conducted at ICAR-Indian Institute of Pulses Research, Kanpur, India. The fungal samples *B. cinerea* were collected from major chickpea growing regions of Punjab and Uttarakhand.Within a particular region, sample sites were about 3 km apart. Fungus was isolated from different plant organs such as leaves and stem portions. The small fragments (~2 cm) of infected plant tissues were surface sterilized with 1% sodium hypochlorite for 2 min followed by 70% ethanol for 3 min and then washed with sterile distilled water for 3-4 times. The isolation and plating of the plant tissue on CDA (60 g chickpea grain, 20g agar, 20 g dextrose, 1 L distilled water) were carried out under aseptic conditions. Plates were incubated at 20±1°C for 6 days and observed mycelial and conidiophores characters under the compound microscope (Olympus). The fungus purification was done through single spore isolation technique (Mian, 1995) using 2% water agar.

Pathogenicity assay

Pathogenicity test was conducted for all *B. cinerea* isolates under controlled environmental facility (CEF) consists of 20±1°C temperature with 90% relative humidity with light intensity of 1200 lux. Five seedlings of susceptible chickpea (variety JG 62) were raised in pots filled with sterilized soil: sand: vermiculite (2:1:1) mixture for inoculation of each isolate. The 14 day old seedlings were transferred to CEF and allowed to acclimatize 24 h prior to inoculum spray. The inoculum of each isolate was multiplied on sucrose aided autoclaved marigold petals in 250 mL conical flask and incubated for 12 days at 20±1°C in Precision™ Low Temperature BOD Incubator. The spore suspension (3×10^5 conidia mL^{-1}) of *B. cienrea* was sprayed on 15-day old seedlings. Plants sprayed with sterile water served as a control. Disease severity was scored after 14 days of inoculation using 1-9 disease severity scale (Gurha *et al*., 2003).

Morphological study

The fungal culture media *viz.* PDA and CDA media were used for assaying morphological characters. The Petri dishes containing sterilized media were inoculated with five (5) mm mycelium discs cut from the margin of the four-day old *B. cinerea* culture and plates were incubated at 20 ± 1°C. Data were recorded on radial growth of the fungus at five days' intervals. The period for sclerotia formation and sporulation was recorded using a haemocytometer at 12 days after incubation. The size of sclerotial bodies (Homogeneous central mass of fungal cells surrounded by transformed rind or cortex) were measured by using student scale and categorized into small (<2mm), medium (2-4mm) and large (>4mm) size. Conidia (spore) length and breadth of each fungus isolate was recorded as per Khazaeli *et al*. (2010) method. The data were analysed using OPSTAT Statistical Software (Sheoran *et al*. 1998).

Molecular characterization of *B. cinerea*

Single spore colonies of actively growing margin of *B. cinerea* were inoculated with five mm fungal disc of each isolate in 250 mL flasks containing 70 mL potato dextrose broth (PDB, Himedia) as described by Das *et al*. (2009). The fungal DNA extraction was performed using Freeze-dried fungal mycelium in powdered in liquid nitrogen as per the manufacturer's protocol of Nucleo-pore R g DNA Fungal/ Bacterial Mini Kit (Genetix Biotech Asia Pvt Ltd). DNA pellets were dissolved in 50µ of nuclease free water and stored at -20°C. Each DNA samples of the fungi were quantified in Nano Drop 1000 Spectrophotometer (Thermo Scientific Wilmington, USA) at 260 nm and diluted to 30-50 ng/µl for PCR reactions and stored at -20°C for further use.

Amplification of ITS region

The fungus was initially characterized based on morphological characteristics and consequently using the Internal Transcribed Spacers (ITS) region (3' end of the 18S rDNA, ITS1, 5.8 rDNA, ITS2 and5' end of the 28S-rDNA). The polymerase chain reaction (PCR) amplification was accomplished using ITS1 (5'-TCCGTAGGTGAACCTGCG-3') and ITS4 (5'- TCCTCCGCTTATTGATTGC -3') as defined by white *et al*. (1990). The PCR reaction mixture was prepared for 25 µL volume that contained 2.5 mM MgCl2, 10 pmol per primer, 30-40 ng DNA, 0.6 mM dNTPs and 1.5 U Taq polymerase (Genei, Bangalore, India). Thermo cycling conditions prevailing for ITS primer amplification were as follows: Initial denaturation at 94°C for 5 min with 35 cycles of denaturation at 94°C for 1 min, annealing with 58°C for 1 min, extension with 72°C for 2 min and a final extension of 72°C for 5 min. The amplified products electrophoresed on 1.5% agarose gel with 100 bp DNA ladders (Genei, Bangalore, India) in n 1x TAE buffer with bromide ethidium visualized under UV light. A specific band of 7 representative isolates of *B. cinerea* PCR products were eluted and purified using gel extraction kit (Genei, Bangalore, India). The amplicons of PCR obtained from each isolate were lyophilized and sequenced (Bio serve Biotechnologies India Pvt Ltd., Hyderabad. India.). The sequences (forward and reverse) obtained from chromatograms were processed using BioEdit version 7.2.

Phylogenetic relationship analysis

The analysis of representative isolates of ITS sequences contigs identified similarity between sequences using basic local alignment search tool (BLAST) (Altschul *et al*. 1990). BioEdit v. 7.2 software (Hall, 1999) was used to create multiple sequence alignment and pair-wise alignment. The phylogenetic tree generated based on maximum likelihood nucleotide sequences by choosing a 1000 Bootstraps value using the Clustal W2 and Neighbour Joining method by MEGA X (v 6.06) (Tamura, *et al*. 2013).

RESULTS AND DISCUSSION

The chickpea plant showing profuse/fluffy gray mass on leaves, collar portion of the stem characteristics to the symptoms of BGM disease (Fig 1). Out of 150 samples, 43 samples were proved to be pathogenic and identified as *Botrytis cinerea* and one sample was identified as *Botrytis fabae* (Table 1) on the basis of colony colour, microscopic observations of spore morphology and pathogenicity test. The major cultivars grown by the farmers were local types followed by PBG 5, PBG 7 and PG 186. The cultivar PBG 5 and PBG 7 were grown in Punjab and PG 186 was observed in Uttarkhand state. In almost all surveyed areas, we observed disease severity scale of 8 and 9 on different chickpea cultivars.

Pathogenicity

The pathogenic test was carried out for 44 isolates of Botrytis spp. in chickpea on cultivar JG 62, which showed profuse cotton growth on the leaf tips/young leaflets and brown colour lesions on the stem and leaf petioles upon fungus inoculation (Fig 1C and D). Majority isolates of *B. cinerea* were shown first symptoms after 4 days of inoculation.

Cultural and Morphological variability

Diverse types of mycelial growth were observed on potato dextrose agar and chickpea dextrose agar media. On PDA media, *B. cinerea* fungal colonies were initially white in colour which later turned to dirty white or grey as old (Fig 2). Septate and hyaline hyphae bearing pseudo-dichotomously branched conidiophores were observed 8 days old culture plates. Based on presence or absence of sclerotia, fungal isolates were categorized into mycelial (2 isolates) and sclerotial (44 isolates) types. Arrangement and distribution of sclerotia was varied among the isolates which involved sclerotia placed in centre, regularly arranged in concentric rings, towards the periphery and irregularly arranged sclerotia pattern and scattered all around the plate (Fig 2). The sclerotial initiation were varied among the isolates and majority of isolates initiated at 6 days after incubation. The size of sclerotia was medium type (2-4 mm in size). All the isolates demonstrated variation in their colony characteristics. Maximum sclerotial bodies number (331) was observed in BC 47 isolate followed by BC 31 (326.00), BC 9 (274.33), BC 32 (254.00) and BC 35 (227.00) and least sclerotial production was observed in BC 11 (11.00) BC 2 (12.00) and BC 10 (13.33) followed by BC 5 (14.00) and BC 26 (15.67).

In CDA media, fluffy, raised and irregular cottony growth, powdery or aerial mycelium with compact and radial type, profuse raised mass, Sparse and irregular in growth were observed. The colonies were initially white, dirty white or grayish white in color, or hyaline but later turn to light gray, dark gray to dark brown. Maximum growth rate of 5.63 mm was observed in the isolates *viz*, BC 15, BC 16, BC 18, BC 22, BC 25. BC 28, BC 29 and BC 37 and all were at par with each other. The minimum growth rate of 2.59 mm was observed in the isolate BC 9. Based on growth rate, *B. cinerea* isolates were grouped as slow growth, medium growth and fast growth (Fig 3).

Overall, maximum sclerotial production was observed in PDA than CDA media with sclerotia initiation started one days early than CDA. Therefore, *B. cinerea* fungus prefers PDA for appropriate growth and multiplication than CDA. The size of conidia was also varied with the isolates. The maximum conidia size was observed in the isolate BC 12 (10.74X 7.46 µm) and minimum conidial size was observed in BC 6 (6.69 X 5.70 µm) isolate.

Molecular characterization

The 7 representative isolates of *B. cinerea* ITS sequences were characterized. The test isolates were amplified at 540 bp. The six BC isolates *viz*., BC 21B, BC 26, BC 27, BC 34,

Fig 1: The Botrytis gray mold symptoms in chickpea caused by B. cinerea. A: Profuse gray mass on leaves, B: Fluffy grey mass at collar portion of the stem, C andD: Profuse grey mass on leaves of artificially inoculated chickpea plants, E: Conidiophores with grape bunch like conidia, F: Oval shaped conidia/ spore of B. cinerea under microscope (40X).

Table 1: Different isolates of *B. cinerea* of chickpea collected from Punjab and Uttarakhand.

Isolate Name	Area from which it is collected	District	Isolation from	Year of Collection	Pathogenicity test *
BC 1	PAU	Ludhiana	L-550	February 2018	8.00
BC 1A (F)	Mohi	Ludhiana (A)	PBG 7	February 2018	8.00
BC 1A(S)	Mohi	Ludhiana (A)	PBG 7	February 2018	8.00
BC 2	Nurmahal	Jalandhar	PBG 7	February 2018	8.67
BC 3	Nurmahal	Jalandhar	PBG 5	February 2018	8.00
BC 4	KVK,	Jalandhar	L-552	February 2018	8.00
BC 5	Dhani pind	Jalandhar	PBG 7	February 2018	8.00
BC 6	Landowal	Ludhiana	PBG 5	February 2018	8.00
BC 7	Landowal	Ludhiana	PBG 5	February 2018	8.67
BC 8	Mullanpur	Ludhiana	PBG-5	February 2018	8.00
BC 9	RRS	Gurdaspur	GPF 2	February 2018	9.00
BC 10	RRS	Gurdaspur	JG-62	February 2018	9.00
BC 10A	KVK	Gurdaspur	L-550	February 2018	9.00
BC 11	KVK	Jalandhar	L-552	February 2018	9.00
BC 12		Jalandhar	PBG 7	February 2018	9.00
BC 13	Khelakhera	Udam Singh Nagar	Local	February 2018	9.00
BC 14	Khelakhera	Udam Singh Nagar	Local	February 2018	9.00
BC 15	Khelakhera	Udam Singh Nagar	Local	February 2018	9.00
BC 16	——	Udam Singh Nagar	Local	February 2018	9.00
BC 17	Kaladungi	Udam Singh Nagar	Local	February 2018	9.00
BC 18	Hajeera	Udam Singh Nagar	Local	February 2018	9.00
BC 19	GBPUAT	Udam Singh Nagar	Local	February 2018	9.00
BC 20	Namuna	Udam Singh Nagar	Local	February 2018	9.00
BC 21 (F)	Tanda	Nainital	Local	February 2018	8.00
BC 21A (S)	Tanda	Nainital	Local	February 2018	8.00
BC 21B	Tanda	Nainital	Local	February 2018	8.00
BC 22	Khelakhera	Udam Singh Nagar	Local	February 2018	9.00
BC 23	Rampur	Udam Singh Nagar	Local	February 2018	9.00
BC 24	Haldwani	Nainital	PG 114,	February 2018	9.00
BC 25	Haldwani	Nainital	Local	February 2018	9.00
BC 26	Jangi Bangar	Nainital	Local	February 2018	9.00
BC 27	Lalkuan	Nainital	Local	February 2018	9.00
BC 28	Kashipur	Udam Singh Nagar	Local	February 2018	9.00
BC 29	Bichpur	Udam Singh Nagar	Local	February 2018	9.00
BC 30	Doraha, Bajpur	Udam Singh Nagar	Local	February 2018	9.00
BC 31	GBPUAT	Udam Singh Nagar	Local	February 2018	9.00
BC 32	Bajpur	Udam Singh Nagar	PG-186	February 2018	9.00
BC 33	Khanpur	Udam Singh Nagar	PG-186	February 2018	9.00
BC 34	———-	Nainital	Local	February 2018	9.00
BC 35	Durgapalpur,	Nainital	Local	February 2018	9.00
BC 36	Motiram	Nainital	Local	February 2018	9.00
BC 37	Jodha	Nainital	GPF 2	February 2018	9.00
BC 45	Jaura sangha	Gurdaspur	PBG 5	February 2017	9.00
BC 47	Kota Badal	Jalandhar	PBG 5	February 2017	9.00
BC 48	Gurdaspur	Gurdaspur	Local	March 2017	9.00
BC 49	Ramewal	Jalandhar	L-552	February 2017	9.00
CD (5%)					0.19
CV					1.39

*****Average of three replications.

BC 48 and BC 49 were more close to *B. cinerea* and 1 isolate BC 14) was matching to *B. fabae.* and sequences were submitted in the NCBI gene bank (BC-14 (MT250940),BC-21B (MT250958),BC-26 (MT50959),BC-27 (MT250960),BC-34 (MT250961),BC-48 (MT250962), BC-49 (MT250963). The isolate BC 14 misidentified as *B. cinerea* based on morphological characters showing more sequence homology with *B. fabae* was placed in *B. cinerea*.

In order to study inter-relationship between different *B. cinerea* isolates with *B. fabae*, all the sequences of 7 isolates were compared with other 20 reference sequences of *B. cinerea* available in NCBI gene bank through molecular evolutionary genetic analysis using MEGA X version 4.0. An optimal tree was generated by NJ method anddifferent taxa were clustered together in a bootstrap test with 1,000 replicates. It is clear evident from the dendrogram that all the Botrytis isolates were divided into two main clades *viz*., I and II. In clade I, *B. cinerea* isolate BC 49 (MT250963) formed separate clade (Fig 4). This clade was the smallest group accommodating one out of the 27 taxa used in the study. However, it was interesting to note that *B. cinerea* infecting chickpea clustered with clade 2 indicating its close relationship with *B. cinerea, B. fabae* and *B. pelargoni and*also *B. cinerea* and *B. fabae* isolates which were clustered together specifying two species cannot be distinguished on the basis of ITS region. If the genetic distance is lower within species of pathogenic population indicates lesser reproductive barrier between them. *B. cinerea* has superior adaptableness to survive under adverse conditions. This can be attributed to the prevalence of *B. cinerea* on various crops.

Fungi have a diverse mechanism for inducing genetic variation either during sexual reproduction or self-reliantly (Kistler and Miao, 1992). The fungus, *Botrytis cinerea* Pers. causes grey mould disease on over 230 hosts (Vallejo *et al*. 2002). For effective disease management, identification and monitoring the infected regions is the preliminary step of disease control. The *B. cinerea* isolates showed variable in

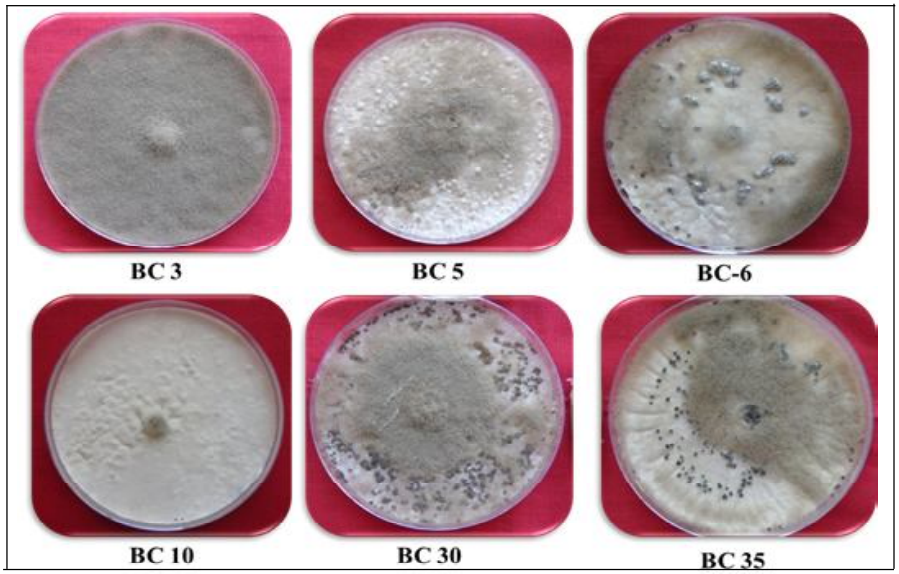

Fig 2: The mycelia and sclerotia structure of *B. cinerea* isolates on PDA medium.

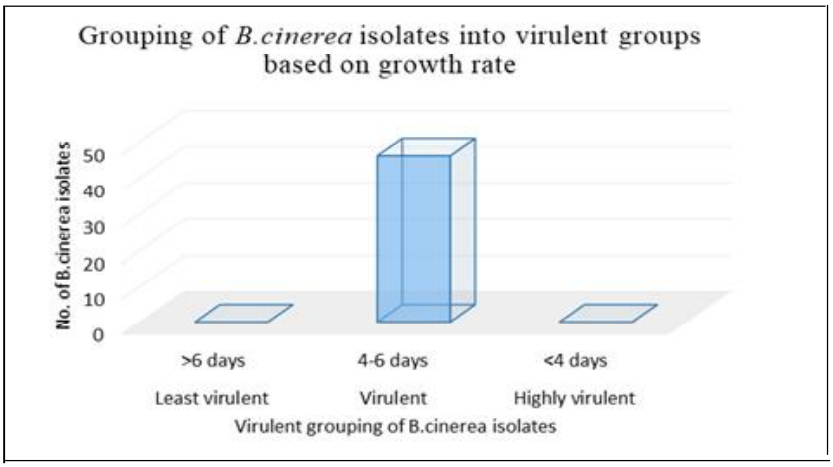

Fig 3: Grouping of *B. cinerea* isolates into different virulent groups based on growth on CDA medium.

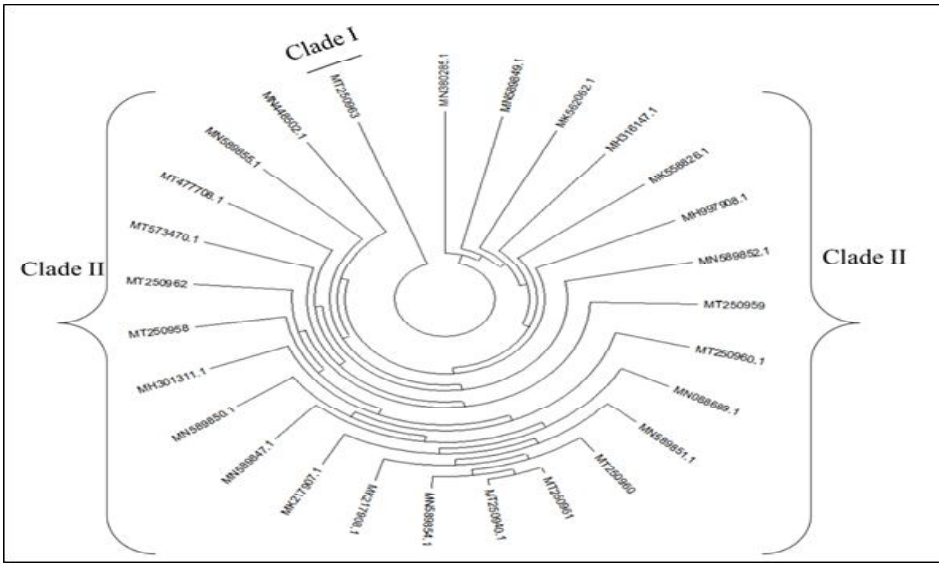

Fig 4: The Phylogenetic relationship of *B. cinerea* and *Botrytis* spp. Infecting chickpea isolates using ITS sequence alignment.

the severity of the necrotic symptoms such as brown necrotic spots with profuse cottony growth on the infected leaves, leaflets and stem portion of chickpea plants. In spite of morphological characteristics used for identification of *B. cinerea,* molecular techniques have been used for characterization of the *B. cinerea* (Pande *et al*. 2010; Kuzmanovska *et al*. 2012; Asadollahi *et al*. 2013). The *B. cinerea* is reported to have extreme diversity and adaptableness to varied environmental situations. Based on nucloetide similarity of internal transcribed spacer (ITS) regions of the *B. cinerea* indicated that lentil and chickpea fungal isolates were closely related. This is because presence of genetically diverse *B. cinerea* which may spread quickly via asexual conidia.

In the study, 44 *B. cinerea* isolates of Punjab and Uttarakhand states were characterized for morphological and molecular diversity. Minor variability was observed between the isolates collected from the same geographical region. This might be due to long distance dissemination of air borne conidia, increase gene flow and thereby restrict geographical difference within the state, as described by Mirzaei *et al*. (2009). Greater diversity was observed amongst the isolates collected from different states. These observations have been recommended to the farmers to grow different varieties in different localities in chickpea area in Punjab than Uttarakhand.

The morphological and cultural differences were determined by studying several characteristics for distinguishing the fungal isolates at the initial stage. In present study, dissimilarity in terms of colony colour, colony growth, sclerotia production, conidia production and conidial measurements were observed. Though, no particular pattern was observed to differentiate the isolates between geographical origin and morphological traits. The sclerotial pattern of arrangement, duration of initiation and their number were recorded. The time required for initiation of sclerotial bodies ranged from 5 to 7 days. We had observed sclerotia absent types in BC21A and BC21B isolates and remaining all were sclerotia producing types. This work was hold up by Mirzai *et al* (2009) identified mycelial and sclerotial morphological types in *B.cinerea* isolates. Bakr *et al* (2002) also reported morphological and cultural variation in *B.cinerea* isolates. Based on results of our study, Uttarkhand isolates of *B.cinerea* have more reproducble and high virulent as compared to Punjab isolates. Therefore, there will be more chances of development of pathoptypes/physiological races eighter through genetic mutation or recombination. Hence, gray mold disease prevalent region needs to be monitored regularly for new pathotypes and races occurrence to curtail the disease spread. Since, Uttarkhand state with high altitude and foot hills having high relative humidity and winter rains supports severe infections and spread of the pathogen. Therefore, chickpea in Uttarkhand state more prone to gray mold disease infection which results in complete crop failure. Including Punjab and Uttarkhand and adjoining staes needs to be monitored for the occurrence and spread of the disease to prevent loss caused by the BGM disease. Our study helps the breeder in development and deployment of resistant genotypes for the control of the disease.

CONCLUSION

The BGM is an emerging disease of chickpea in India due to unusual and incessant winter rains with increased humidity. Hence, morphological and cultural diversity witnessed owing to exposure of the fungus to varied climate and host range. In the present study, morphological and molecular analysis identified that BGM caused by both *B. cinerea* and *B. fabae*. For the first time, we reported that *B. fabae* is also causes gray mold in chickpea. Based on pathogenicity with early expression of symptoms,

Uttarakhand isolates (BC 30, BC 15) were more virulent and more spore producible than Punjab isolates. The results would be helpful in breeding programs to develop resistant cultivars against *B. cinerea*. Such studies can contribute in developing or devising a suitable control measure against BGM disease in India.

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Authors' contributions

L.M., U.R, perceived this work, designed the experiments, work carried out and drafted the article. L.M., B.T assisted in sample collection, analysed the data and, J.R and YK helped in manuscript correction and incubator facility provided during the study.

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