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Prevalence of capsicum grey mould and characterization of the pathogen associated under protected cultivation in Himachal Pradesh

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Abstract

Gray mould of capsicum (*Capsicum annum* var. *grossum*) caused by *Botrytis cinerea* Pers. is one of the most destructive diseases of winter season capsicum under polyhouse conditions in Himachal Pradesh. During 2013-14, different polyhouses of Bilaspur, Hamirpur, Kangra and Kullu districts of Himachal Pradesh were surveyed and disease severity was observed between 18.0-52.0 per cent in all the polyhouses. Maximum disease (52.0%) was recorded at Palampur in Kangra district whereas, minimum (18.0%) at Ghumarwin in Bilaspur. Studies on symptomological, morphological and cultural characteristics identified the associated pathogen as *Botrytis cinerea*. The identity of pathogen was also confirmed through rDNA analysis by targeting ITS1 and region using primer pair ITS1 and 4 which generated an amplicon of ~500bp and phylogenetic analysis showed 99 per cent homology of the isolate with *Botrytis cinerea*.

Keywords: capsicum, grey mould, *botrytis cinerea*, ITS

Introduction

Capsicum is an important vegetable crop because of its high nutritional value and delicacy. It is a rich source of ascorbic acid and 100g fruit contains 1.2 g of protein, 11 mg of calcium, 870 I.U. of vitamin A, 175 mg of ascorbic acid, 0.06 mg of thiamine, 0.03 mg of riboflavin and 0.55 mg of niacin (McGillivray, 1953) [18]. It also contains zinc which is vital for strong and healthy immune system. In India capsicum is cultivated over an area of about 29.72 thousand ha with production of 166.88 thousand MT (Anonymous, 2014) [1]. In field cultivation, bell pepper growers are facing several challenges like biotic stresses and vagaries of weather viz., diseases & insect-pests, fluctuating temperature, unprecedented rains and frequent hailstorms, which affect the yield and quality of the produce, thereby reducing the profit margin of producers (Ochigbo and Harris, 1989) [16]. Protected cultivation is getting preference over open field cultivation for off-season quality production of the crop in the hill state. Though, proteit provides appropriate conditions for off-season and quality production of capsicum, it also provides inviting conditions suitable for the development of various diseases. Among these, gray mould of capsicum caused by *Botrytis cinerea* Pers. has emerged as an important disease under polyhouse during cool, wet and humid conditions especially in winter season in Himachal Pradesh. Gray mould is probably the most common disease of polyhouse grown crops especially in the spring and fall, when the vents of polyhouse are closed at night to prevent heat loss. Gray mould pathogen infects leaves, fruits, flowers, petioles and other parts of the plant (Sutton, 1998). Initial symptoms of gray mould has been reported by Chang *et al.*, (2001) [2] as irregular water-soaked lesions which gradually enlarge, coalesce, turn brown and ultimately results in death of infected plant parts. Gray to grayish brown, velvety mouldy growth consisting of several conidiophores with numerous conidia often appears on the lesions under moist conditions. On ripe fruits, brown firm areas, spreading from calyx to other parts of the fruit were observed by Maas, (1998) [13]. On the affected fruits a velvet-like mycelium and clusters of conidiophores grows, as a result infected fruit becomes mummified (Hancock 1999) [5]. Stem rotting has been reported as the most devastating symptom of gray mould on tomato by Shtienberg *et al.*, (1998) [20] which may result in death of plant. Losses up to 42 per cent have been reported in *Capsicum annum* from vinyl housed fields (Kim *et al.* 1996) and 72 per cent in tomato due to gray mould (Shtienberg and Elad, 1997) [19]. *Botrytis cinerea* is an economically important pathogen that can infect more than 200 plant species in the field,

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greenhouse and storage (Jarvis 1980; Holz *et al.* 2004) [7, 6], which is attributed to its great flexibility in adapting to various environmental conditions. The status and identification of the pathogen associated with the disease under protected cultivation was utmost importance for further management strategies. Hence, the present study was taken up to know the status of the disease in the state and exact identification of the pathogen associated with.

Material and Methods

Status and distribution of gray mould of capsicum

Survey of different capsicum growing districts of Himachal Pradesh i.e. Bilaspur, Hamirpur, Kangra and Kullu were undertaken in the months of December to February during 2013-14 to assess the status and distribution of gray mould of capsicum caused by *Botrytis cinerea* Pers. In each polyhouse at least three spots of 10 m² were selected randomly and data on disease severity were recorded on 10 plants in each selected areas.

Identification of pathogen

Symptomatology

Different capsicum growing polyhouses were visited during the crop season and plants infected with disease were collected and brought to the laboratory for further analysis. Observations were recorded on the diseased plants and characteristic symptoms on infected leaves, stems and fruits were recorded.

Isolation, purification, maintenance and multiplication of pathogen

Fungal cultures of each isolate were raised from the diseased samples using standard methodology on Potato Dextrose Agar (PDA). Small bits of infected tissues were surface sterilized by dipping in mercuric chloride (0.1%) solution for 10-15 seconds followed by washing thrice in sterilized water under laminar air flow hood. The bits were dried and then transferred to PDA medium and incubated in BOD incubator at 25±1°C for 5 days. Fungal cultures were purified by hyphal tip method. Fungal colony arising from single hypha of each isolate was multiplied on PDA medium and used for further studies. The pathogen was transferred to live host after 3 sub-culturing to avoid loss of virulence.

On the basis of morphological and cultural characteristics

To identify the species associated, the cultural characteristics of the pathogen were studied on petri dishes containing PDA medium. Mycelial bits of 5 mm in diameter were cut with the help of cork borer from the margin of an actively growing colony and placed in the centre of media plates. Cultures were incubated at 25±1°C in a BOD incubator. Three replications having three Petri plates each were kept for each isolate of the pathogen. Observations were recorded on colony characteristics (colour, shape and type of growth of colony and size, shape and colour of conidia).

On the basis of molecular markers

Molecular identification of isolate was done using Internal Transcribed Spacer (ITS) region of nuclear ribosomal DNA (rDNA). Initially disease samples were subjected to microscopic examination after purification of isolate, the

identity species level was also confirmed by molecular analysis of rRNA.

PCR amplification and rDNA sequencing

Total genomic DNA of fungal isolates was extracted using CTAB method (Murray and Thomson 1980) [15] with minor modifications.

PCR was carried out in 0.2 ml PCR tubes volume using ITS1 (5'-TCCGTAGGTGAACCTGCG-3') and ITS4 (5'-TCCTCCGCTTATTGATATATGC-3') primer pairs. The reaction was performed in 25 µl volume containing 2.5 µl of 10x buffer (20 mM Tris HCl, pH 8.0, 50 mM KCl), 3 µl of 1.5 mM (MgCl₂), 2µl of dNTP mix (0.2 mM each) (Bangalore Genei, India), 0.2 µl of Taq polymerase (Bangalore Genei, India, 5U/µl), 1-2 µl of DNA template (~ 1µg), 0.5 µl of each primer (10 µM). Final volume was adjusted using nuclease free water (15.3 µl). Amplifications were performed using thermal cycler (GeneAmp PCR system 9700, Applied Biosystems, USA) programmed for initial denaturation of 94°C for 5 min followed by 30 cycles of 94°C for 1min, 47°C for 1 min, 72 °C for 2 min and a final extension at 72 °C for 5 min. Remaining PCR products were lyophilized and sent for custom sequencing. The sequences were analysed using BLASTN program from the website <http://www.ncbi.nih.gov/blast> and alignment was performed using Clustal W program using website <http://www.ebi.ac/clustalw/> (Higgins *et al.*, 1994). Phylogenetic and molecular evolutionary analysis was conducted using MEGA version 7 (Kumar *et al.*, 2016) [10].

Pathogenicity

Purified culture of the test pathogen (*Botrytis cinerea*) isolated from capsicum was inoculated on capsicum seedlings as well as on the fruits and typical symptoms of the disease were produced after 4 days of inoculation. The pathogen was re-isolated from the infected parts and fruits of capsicum and the pathogenicity of the test pathogen was confirmed.

Results and Discussion

Status and distribution of gray mould of capsicum in Himachal Pradesh

To study the status of gray mould of capsicum surveys were conducted at different locations in capsicum growing districts of Himachal Pradesh during December 2013 to January 2014. The data presented in table I revealed that disease severity of gray mould of capsicum ranged between 18.0 to 52.0 per cent with an overall average of 31.1 per cent in different polyhouses in Bilaspur, Hamirpur, Kullu and Kangra districts of Himachal Pradesh.

Maximum disease severity was observed at Palampur (52.0%) in district Kangra and minimum at Ghumarwin (18.0%) in Bilaspur district. The average disease severity was observed highest in Kangra (37.7%) followed by Kullu (32.3%), Hamirpur (29.7%) and Bilaspur (24.8%) districts. The disease severity varied from 22.0 to 52.0 per cent at various locations of district Kangra with an average of 37.7 per cent, being maximum at Palampur (52.0%) and minimum at Malan (22.0%). In district Kullu, disease severity varied from 30.0 to 35.0 per cent with an average of 32.3 per cent. Maximum disease severity was recorded at Bhunter (35.0%) and minimum (30.0%) at Shamshi.

Table 1: Status and distribution of gray mould of capsicum caused by *Botrytis cinerea* in Himachal Pradesh during 2013-14

Districts	Locations	No. of polyhouses surveyed	Disease severity (%)
Bilaspur	Berthin	4	24.0
	Bhrari	3	29.0
	Narwal	2	28.0
	Ghumarwin	2	18.0
	Average		24.8
Hamirpur	Barsar	2	34.0
	Kangoo	2	29.0
	Harmandir	3	26.0
	Average		29.7
Kangra	Palampur	4	52.0
	Nagri	2	43.0
	Malan	3	22.0
	Nagrota	3	39.0
	Samloti	2	28.0
	Tanda	4	42.0
	Average		37.7
Kullu	Shamshi	2	30.0
	Bhunter	3	35.0
	Bajaura (Jhiri)	4	32.0
	Average		32.3
	Overall Average		31.1

Identification of the pathogen

Symptomatology

The pathogen produced symptoms on all the above ground plant parts. The disease initially began from flower petals and the fungus grows from fading flower petals into developing fruit. On fruits, symptoms appeared as water-soaked spots that expanded into large yellowish-green to grayish brown irregular lesions of soft and spongy texture. On leaves gray coloured irregular water soaked areas appeared which enlarged coalesced and resulted in withering of leaves. On stem, symptoms appeared in form of small orange-brown lesions which on expanding covered entire stem and led to

girdling of stem. Disease symptoms were also observed on young pepper seedlings as small water soaked lesions which ultimately resulted in sudden collapse and death of affected seedling. (Fig 1).

The symptoms of gray mould on capsicum as described above were similar to the symptoms observed by different workers. *Chang et al.*, (2001) [2] described the symptoms of gray mould caused by *B. cinerea* as irregular water-soaked lesions which enlarged, coalesced and ultimately resulted in death of infected plant parts. They also observed, gray to grayish brown, velvety growth on the lesions. Similar symptoms of gray mould on flowers, fruits, leaves and stems of tomato plants were also observed by *Elad et al.*, (2007) [3]. *Vagelas et al.*, (2009) [22] and *Gupta and Bharat* (2013) [4] also noticed gray mould on infected fruits and stem of capsicum plants which later on encircled the whole stem and infected portions of the stem led to death of entire plant.

Morpho-cultural characteristics

The morpho-cultural characteristics of the pathogen were studied under *in vitro* and data were recorded. The results showed that on Potato Dextrose Agar at 25°C test pathogen produced white, dense, cottony mycelium, which after sporulation turned to greyish brown after 5 days of incubation. The conidia were single celled with 7.0-14.0 x 5.25-10.5 µm size, hyaline to pale brown in colour, ovate, ellipsoidal, globose to sub-globose and pyriform in shape. The sclerotia were observed small to larger in size with smooth surface after 20 days of incubation (Fig 2). *Botrytis cinerea* had been traditionally identified on the basis of morphological and cultural characteristics and almost similar characteristics as observed in our study were also reported by other workers. *Khazaeli et al.* (2010) [8] observed that mycelium of *B. cinerea* was branched, septate, hayline to brown and conidiophores were septate, more or less straight branched at the apex, often dichotomously or trichotomously with conidial dimensions in the range of 8(4)-13(16) x 4(2)-7(10) µm.

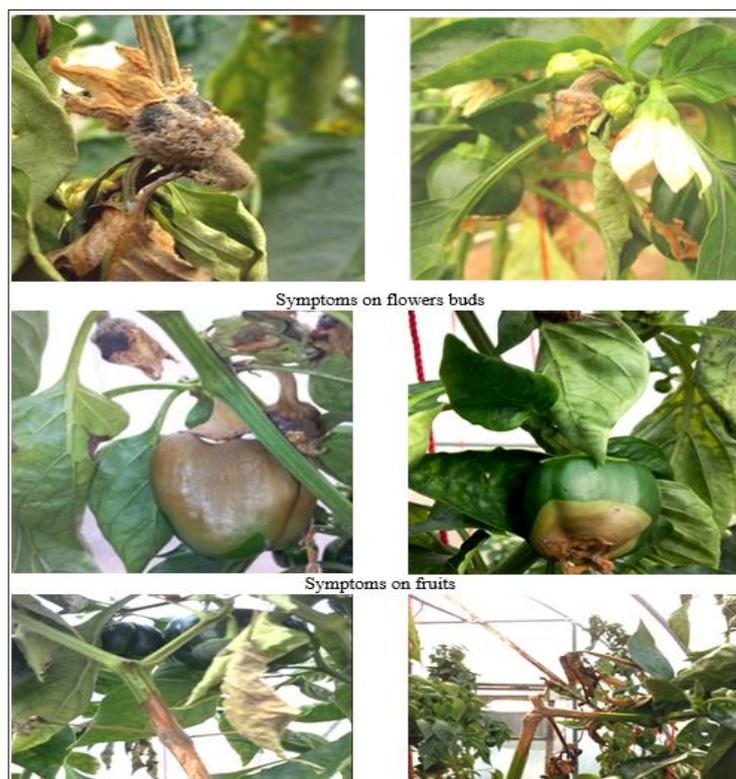


Fig 1: Symptoms of gray mould caused by *Botrytis cinerea* on capsicum

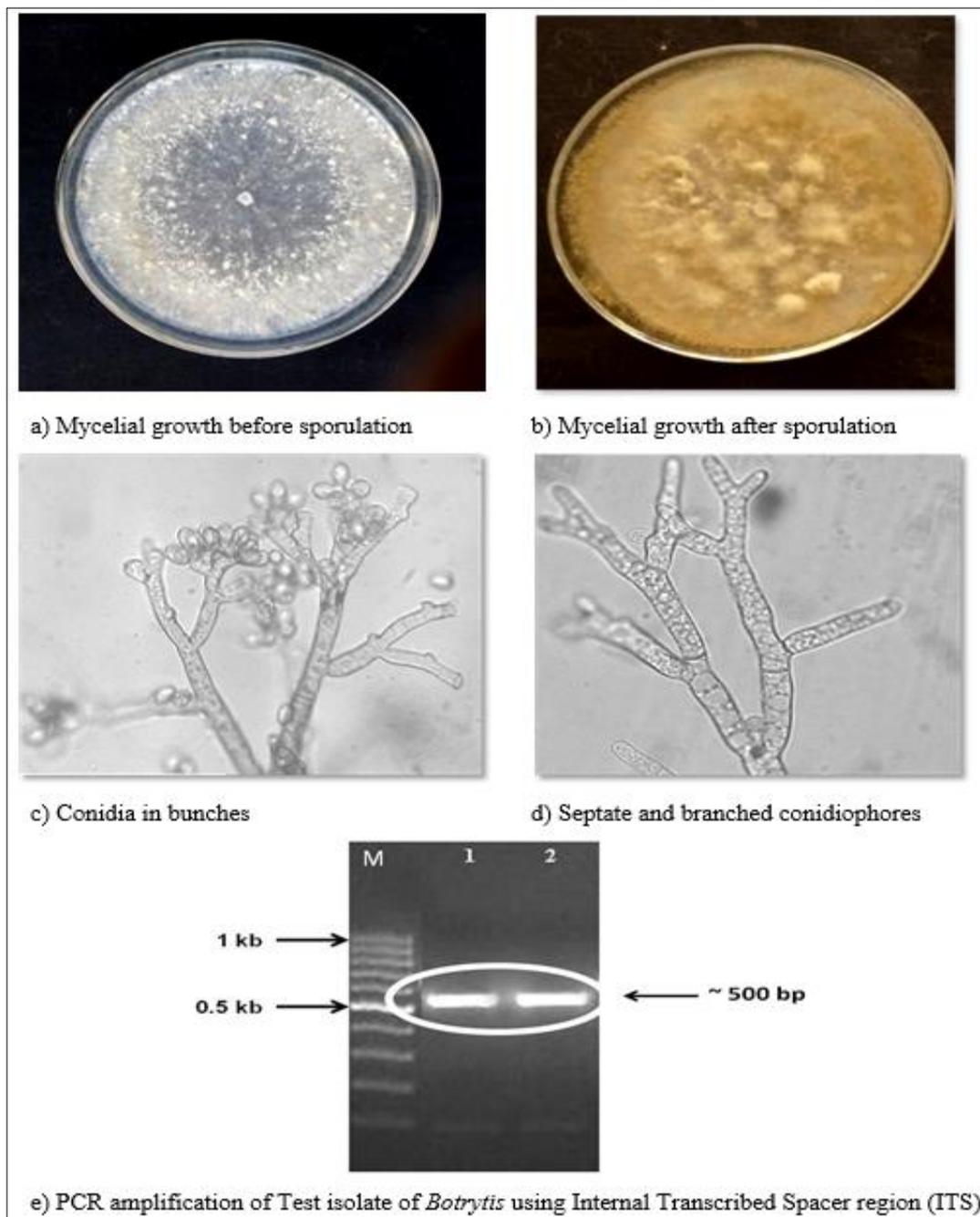


Fig 2: Identification of the pathogen causing gray mould in capsicum on morphological (a-d) and molecular (e) basis

Ellipsoidal, globose to sub-globose and smooth, measuring 14.08-9.10 μm in (length) \times 10.28-7.60 μm (width) conidia was observed by Miclea and Puia (2012) [14]. Gupta and Bharat (2013) [4] observed that conidia of *B. cinerea* were hyaline to brown, ovate, ellipsoidal and pyriform in shape measuring 7.5-13.3 \times 7.0-10.5 μm . Conidiophores were more or less straight, septate, branched towards the apex often dichotomously. Ozer and Bayraktar (2014) [17] observed that *B. cinerea* initially formed white coloured colony which turned to gray-brown and observed that conidia were single-celled, ovoid to ellipsoid, colourless to pale brown, smooth and measured 6.8-10 \times 8.1-11.9 μm in size. Conidiophores were brown, slender and branched with enlarged apical cells bearing clusters of conidia. The morpho-cultural characteristics of the test pathogen were observed similar to that of *B. cinerea*. Hence, the test pathogen on the basis of morpho-cultural characteristics was identified as *B. cinerea* causing gray mould of capsicum.

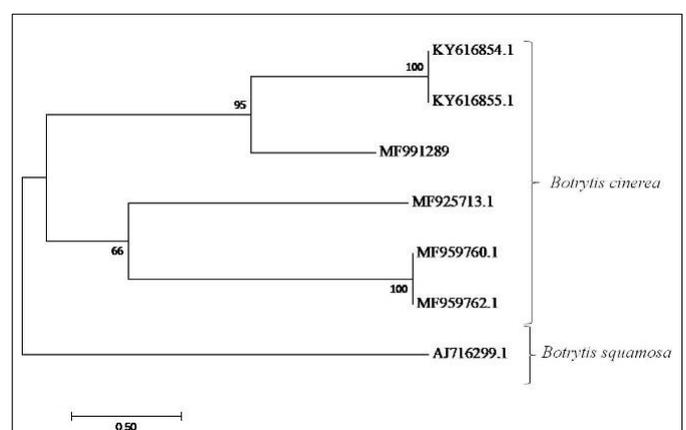


Fig 3: Comparative phylogenetic analysis using MEGA-7 along with reference species of the pathogen. *Botrytis squamosa* used as out group

Molecular characterization

The identity of test isolate was established by characterization of the 'Internal transcribed spacer' (ITS) region using universal primer pair ITS1 and ITS4. PCR amplification revealed an amplicon of ~500bp, which was further gel purified and lyophilized before sequencing (Fig 2). The purified amplicon was custom sequenced using ITS1 and ITS4 primers. BLASTn analysis of the sequence showed 99 per cent homology with *B. cinerea*, confirming the findings of morphological characterization (Fig 3).

Botrytis cinerea was also identified through molecular techniques by Khazaeli *et al.*, (2010) [8] in which a single band of 0.7 kb specific to *B. cinerea* was amplified in all isolates tested. However, no band was amplified in the negative control. Kwon *et al.*, (2011) confirmed the identity of causal agent as *B. cinerea* on ITS-r DNA region analysis. They used ITS1 and ITS4 for PCR amplification of Internal Transcribed region and found 613 bp sequences. Kumari *et al.*, (2014) [11] confirmed the identity of 79 isolates of *B. cinerea* by using specific primer pair C_{729/729} which amplified amplicon of 700 bp in all the isolates tested.

Hence, in the present studies morphological as well as molecular identification conclusively proved, that gray mould of capsicum is caused by *Botrytis cinerea* under protected cultivation in India.

Conclusion

Survey conducted during 2013-14 at Bilaspur, Hamirpur, Kangra and Kullu districts of Himachal Pradesh revealed that disease severity ranged between 18.0-52.0 per cent. Maximum disease (52.0%) was recorded at Palampur in Kangra district whereas, minimum (18.0%) at Ghumarwin in Bilaspur. Studies on symptomological, morphological and cultural characteristics identified the associated pathogen as *Botrytis cinerea* and the identity of pathogen was also confirmed through rDNA analysis by targeting ITS region which showed 99 per cent homology of the isolate with *Botrytis cinerea*.

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