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Full Length Research Paper

Characterization of *Botrytis cinerea* isolates from chickpea: DNA polymorphisms, cultural, morphological and virulence characteristics

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Eight isolates of *Botrytis cinerea,* causal organism of chickpea from eight different locations of western and eastern Indo-Gangetic Plains (IGP) of India were analyzed for morphological, cultural, virulence and genetic variations. Characterization of virulence and genetic variations of the isolates was based on their pathogenicity against 40 selected chickpea genotypes and randomly amplified polymorphic DNA (RAPD) analysis, respectively. The isolates differed in their optimum growth, temperature, conidial formation and size of conidia on potato dextrose agar. Based on cluster analysis, the eight test isolates were separated into three pathotypes with two isolates from western and four from eastern IGP grouped together with >80% similarity. Based on cluster analysis of the RAPD banding patterns, genetic similarity of the isolates varied from 14-44%, and the isolates were separated into three groups. However, pathotypes variation detected among *B. cinerea* isolates could not be differentiated based on the RAPD markers examined.

Key words: *Botryotinia fuckeliana*, Botrytis gray mold, variability.

INTRODUCTION

Botrytis cinerea Pers. ex. Fr., the anamorph of *Botryotinia fuckeliana* (de Bary) Whetzel, is a broad host range fungus that affects more than 200 agriculturally important plant species of different families (Elad et al., 2004). The fungus causes Botrytis gray mold (BGM) disease in chickpea (*Cicer arietinum* L.) and can devastate the crop resulting in complete yield loss (Pande et al., 2005). Rapid evolution of the fungus including alterations in virulence and fungicide resistance contributes to frequent outbreaks of BGM. *B. cinerea* is known to have extreme genotypic and phenotypic variability, and adaptability to diverse environments (Pande et al., 2006). Variation in virulence of *B. cinerea* populations against one given host plant has been frequently reported (Buck and Jeffers, 2004; Derckel et al., 1999). Multinucleate and heterokaryotic nature of *B. cinerea*, and presence of transposable elements (Ma and Michailides, 2005) contribute to high levels of variation in the fungus populations, and often the clonal lineages are dissimilar (Beever and Weeds, 2004). Sexual reproduction between the two mating types of *B. cinerea*, MAT-1 and MAT-2, is a minor cause of genetic diversity as the sexual structures of the fungus are rarely observed in the field (Beever and Weeds, 2004).

The multilocus technique, randomly amplified polymorphic DNA (RAPD) that allows ready scoring of numerous polymorphic loci has been used in genetic mapping and diagnosis of several fungal species (Williams et al., 1990). Since the first report of Bergmans et al. (1993) on

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Abbreviations: BGM, *Botrytis* gray mold; **RAPD,** randomly amplified polymorphic DNA; **PDA,** potato dextrose agar; **DAI,** days after inoculation; **SDW,** sterile distilled water; **CTAB,** cetyl trimethylammonium bromide; **IGP,** Indo-Gangetic Plains.

cinerea using RAPD, this technique has the detection of high levels of genetic variation in *B.* been widely used to determine the variation in *B. cinerea* from different hosts and countries (Alfonso et al., 2000; Kerssies et al., 1997).

B. cinerea owing to its high genetic diversity and adaptability pose a risk of rapid breakdown of host resistance. Attempts for virulence characterization of *B. cinerea* from these regions are limited, inspite of the frequent BGM epidemics reported from different locations in India and adjoining Nepal (Davidson et al., 2004; Pande et al., 2005). Physiological and pathotypic specializations were observed among a collection of *B. cinerea* isolates from chickpea in India (Rewal and Grewal, 1989; Singh and Bhan, 1986). Based on microsatellite DNA markers developed specifically for the *B. cinerea* genome, genetic variation in *B. cinerea* isolates of chickpea from four regions in Bangladesh, India and Nepal has been reported recently (Isenegger et al., 2005). An understanding of the pathogenic and genetic diversity of *B. cinerea* is critical in developing disease management practices for BGM. The present study was an undertaken to characterize cultural, morphological, pathogenic and genetic variations in *B. cinerea* isolates from chickpea in BGM endemic locations of India.

MATERIALS AND METHODS

Fungal isolates

Eight *B. cinerea* isolates were obtained from BGM infected chickpea plants from eight locations of eastern and western Indo-Gangetic plains (IGP), India. The western IGP locations were Ludhiana and Pantnagar, and eastern IGP locations were Kauriala Ghat, Jarwa, Nautanwa, Bhikhna Thori, Raxaul and Jaynagar villages bordering Nepal in the states of Uttar Pradesh and Bihar in India. The isolates from these locations were numbered from IC1 to IC8, respectively. The distance between Ludhiana in the western IGP and Jaynagar in the eastern IGP was >1,100 km. BGM infected stem cuttings from each location were surface sterilized with 1% sodium hypochlorite for 2 min and plated on *Botrytis* specific medium (Burgess et al., 1997). A single spore culture of each isolate was maintained on potato dextrose agar (PDA) at 4°C.

Seed material

Seeds of 40 chickpea genotypes with varying levels of resistance to BGM were obtained from the Department of Legumes Pathology, ICRISAT, Patancheru, India.

Cultural and morphological characterization of *B. cinerea*

Variation in the radial growth and conidial formation of *B. cinerea* isolates grown on PDA was measured at different growth temperatures from 10 to 30°C. A 5 mm disc of an actively growing culture of each isolate was inoculated in the centre of a 90 mm diameter petri plate containing PDA, and incubated separately at 10, 15, 20, 25 and 30°C with 12 h photoperiod. In each treatment, the colony diameter in two perpendicular directions and color of the mycelium, were recorded 2, 4 and 6 d after inoculation (DAI).

For quantification of conidia, 1 cm diameter culture disc was cut at a distance of 1 cm from the center of a 10-d-old culture. The disc was macerated in 10 ml of sterile distilled water (SDW) and the number of conidia was quantified using a haemocytometer. In each replication, the length and width of 25 conidia was measured using an ocular lens at 200x. The experiment was conducted in three replications and repeated twice.

Virulence characterization of *B. cinerea*

Seeds of each of the 40 test genotypes were planted in 40 x 30 x 5 cm plastic trays filled with sand and vermiculite (9:1) in greenhouse maintained at 24 ± 2ºC and 12 h photoperiod. Twelve-day-old seedlings were transferred to a growth room maintained at 18 ± 2ºC with ~1500 lux light intensity for 12 h a day and allowed to acclimatize for 24 h. Isolates of *B. cinerea* were grown on autoclaved dried marigold petals for 8 d at 25ºC and 12 h photoperiod. The conidia were harvested into sterile distilled water, adjusted to 3 x 10^5 ml^{-1} using a haemocytometer and used as inoculum. Chickpea seedlings were sprayed with the inoculum using a hand-operated atomizer. The inoculum was allowed to partially dry for about 30 min after which 100% RH was maintained in the growth room. BGM severity of individual seedling was quantified 15 DAI using a 1-9 rating scale (Pande et al., 2006). The experiment was conducted with three replications (8 plants in each replication) arranged in a randomized complete block design and repeated twice. The pair wise distances in pathogenicity among the eight *B. cinerea* isolates against 40 test lines were estimated from the Euclidean distance (Excoffier et al., 1992). Pair wise comparisons of the eight isolates constituted a matrix of average distance between isolates. This matrix was used in cluster analysis with an option of UPGMA (unweighed pair group method, arithmetic average) to construct a dendrogram that depicted the hierarchical structure of pathogenic variation among *B. cinerea* isolates.

Genetic characterization of *B. cinerea*

Genetic variability among eight *B. cinerea* isolates was assessed by RAPD analysis using 26 decamer primers selected from Operon primer kits (Operon Technology, Inc., Alamdea, CA, USA). Selected primers were OPAs: 2, 8, 11 and 16; OPBs: 5, 7, 8, 9, 12 and 18; OPCs: 3, 4, 5, 11 and 12; OPDs: 2, 4, 5, 18, 19; OPEs: 11 and 15; OPGs 7, 13 and 16; and OPJ 11. Dried mycelium from four-day-old culture of each isolate grown on PDA overlaid with nitrocellulose membrane at 25°C was used for DNA extraction according to the cetyl trimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990). Extracted DNA was dissolved in TE buffer and purified by treatment with 100 µg ml^{-1} RNase (Boehringer Mannheim) for 1 h at 37°C. DNA was quantified based on the optical density at 260 nm. The extraction was repeated thrice for each isolate and the pooled DNA was used in amplification reactions.

Amplification reactions were performed in 25 µl reaction mixture containing 50 ng of template DNA, 1 mM $MgCl_2$, 5 mM of each dNTP, 10 µM of primer, 1U Taq polymerase and 2.5 µl of 10X reaction buffer. PCR reactions were performed in a programmable thermo cycler (M.J. Research, Inc., Watertown, MA) using the following conditions: 94°C for 5 min initially to denature the DNA, followed by 30 cycles of 1 min at 94°C, 1 min at 37 – 42°C and 1 min at 72°C, with a final extension at 72°C for 10 min. Amplification products were size-separated by electrophoresis on 1.4% (w/v) agarose gel in 1X TBE buffer. A 200 bp ladder was used as a size

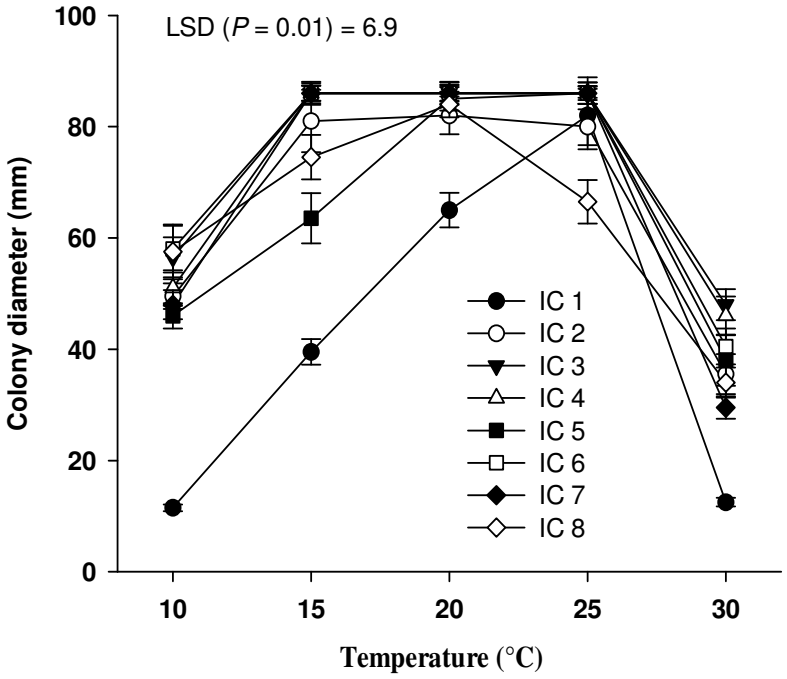

Figure 1. Effect of temperature on the radial growth of eight *Botrytis cinerea* isolates, IC1 to IC8. Values presented are the mean of nine replications in three repetitions of the experiment. The horizontal bars above each bar represent the standard error of mean.

standard. The gel was stained with ethidium bromide and photographed under UV light. All RAPD analyses were repeated thrice for each isolate and scored as 0 or 1 based on the absence or presence of PCR amplified DNA fragments on the gel. Based on this scoring, similarity among all pairs of eight *B. cinerea* isolates were calculated by Dice coefficient (Nei and Li, 1979). This similarity matrix was analyzed with NTSYS-PC 2.01 (Rohlf, 1997) and clustered with UPGMA algorithm to determine the genetic relationships among the eight isolates.

RESULTS

Cultural and morphological characterization of *B. cinerea*

The eight test *B. cinerea* isolates varied in their radial growth on PDA. Growth of five isolates, IC2, IC3, IC4, IC6 and IC7 was similar (P = 0.01) at 15, 20 and 25°C measured 4 DAI (Figure 1). Maximum growth of IC1 was at 25°C, IC8 at 20°C and IC5 both at 20 and 25°C. Radial growth of all the isolates at 10 and 30°C was significantly (P = 0.01) lower compared to 15, 20 or 25°C. The test isolates grown at 25°C varied in their color of mycelium from dirty white to dark gray (Table 1).

The isolates had a specific optimum temperature required for conidial formation - 15°C for IC4 and IC7, 20°C for IC1, IC2, IC3 and IC8, 25°C for IC5, and 30°C for IC6 (Figure 2). The maximum number of conidia formed cm^{-2} varied from 3.9 X 10^5 (IC7) to 2 X 10^3 (IC8). Conidia of different isolates varied from 12.7 x 5.6 µm (IC1) to 15.8 x 7.0 µm (IC5) in size (Table 1).

Virulence characterization of *B. cinerea*

Mean of the disease rating of 40 chickpea genotypes inoculated with individual *B. cinerea* isolate varied from 4.2 (IC7) to 7.1 (IC6) on a 1-9 rating scale (Table 2). Twenty-two of the forty chickpea genotypes tested were resistant (disease score <4.0) to IC7 while none of the lines were resistant to IC5 and IC6. Three chickpea genotypes ICC 5255, ICC 6299 and ICC 15986 showed the best differential reaction compared between the eight *B. cinerea* isolates tested. Cluster analysis based on the virulence of test isolates against 40 chickpea genotypes identified three distinct groups of *B. cinerea* isolates at a similarity level of >85% (Figure 3). Isolates IC1, IC2, IC3, IC4, IC5 and IC8 formed one group, while each of the isolate IC6 and IC7 remained as an individual group.

Genetic characterization of *B. cinerea*

Seventeen of the 26 primers tested were selected for

Table 1. Cultural and morphological characteristics of *Botrytis cinerea* isolates from different locations of Indo-Gangetic Plains (IGP) in India.

Isolate	Location[x]	Color of mycelium[Y]	Size of conidia (µm)[z]	
			Length	Width
IC1	Ludhiana	Dark gray	12.7	5.6
IC2	Pantnagar	Dark gray	15.6	5.4
IC3	Kauriala Ghat	Dark gray	12.6	5.7
IC4	Jarwa	Dark gray	13.0	6.2
IC5	Nautanwa	Light gray	15.8	7.0
IC6	Bhikhna Thori	Light gray	15.8	6.7
IC7	Raxaul	Gray	13.4	6.0
IC8	Jaynagar	Dirty white	14.5	6.2
LSD (P = 0.01)			0.11	0.46

[x] Ludhiana and Pantnagar represent the western IGP while the other six locations are in the eastern IGP in a sequential order from west to east. The distance between Ludhiana and Jaynagar is >1,100 km. [Y]Color of the mycelium was recorded 4 days after incubation at 25°C on potato dextrose agar. [z] Size of conidia was measured 10 days after incubation at 25°C on potato dextrose agar. The values presented are the mean of 225 conidia measured in nine replications.

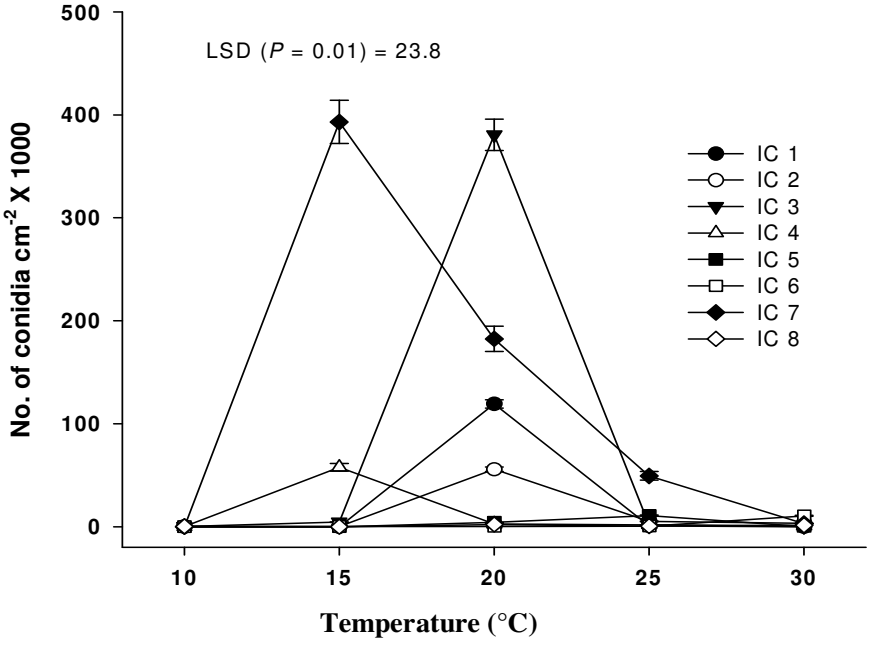

Figure 2. Effect of temperature on the conidial formation in eight *Botrytis cinerea* isolates, IC1 to IC8. Values presented are the mean of nine replications in three repetitions of the experiment. The horizontal bars above each bar represent the standard error of mean.

RAPD scoring based on amplification and repeatability of the method compared among the three repetitions. Selected primers were OPA 16; OPBs 5, 7 and 15; OPCs 5, 11 and 12; OPDs 2, 5, 18 and 19; OPEs 11 and 15; OPGs 7, 13, 16; OPJ 11, which generated 86 scorable polymorphic bands of amplified DNA fragments. Markers ranged from 500 to 2,100 base pairs. Each of the selected 17 primers amplified from two to eight (OPG 13) clear and reproducible RAPDs. None of the amplification fragments were specific to all the isolates of *B. cinerea.* Four bands produced by amplification with OPA 16, two by OPB 7, one by OPD 19 and three by OPE 11 were specific to the isolate IC2 from Pantnagar, India. Cluster analysis of the RAPD banding patterns revealed high

Table 2. Virulence of *Botrytis cinerea* isolates (IC1 to IC8) against 40 genotypes, measured on a 1-9 rating scale, in a controlled environmental facility.

Genotype	Disease rating on a 1-9 scale measured 15 days after inoculation								
	IC1	IC2	IC3	IC4	IC5	IC6	IC7	IC8	Mean
ICC 799	6.0	6.3	5.3	4.5	6.3	7.8	3.5	6.3	5.7
ICC 1894	4.8	4.8	4.8	4.3	5.5	7.9	3.3	4.5	5.0
ICC 2165	5.5	5.8	5.0	6.0	5.8	7.3	4.0	5.0	5.5
ICC 3075	5.0	7.0	5.5	5.0	5.8	8.5	4.0	6.5	5.9
ICC 3540	5.5	5.8	5.8	5.8	5.8	7.6	4.5	5.8	5.8
ICC 3538	4.8	5.3	4.8	4.3	4.8	7.0	3.8	5.0	4.9
ICC 3840	6.5	7.8	7.3	5.8	6.0	8.5	4.3	5.5	6.4
ICC 3969	7.3	7.3	6.5	5.0	5.8	8.5	4.5	6.5	6.4
ICC 3996	5.5	7.0	6.3	4.5	5.8	7.5	4.5	6.0	5.9
ICC 4055	5.3	6.0	5.3	4.3	5.3	7.0	4.0	5.5	5.3
ICC 4107	6.3	6.5	7.0	5.0	5.5	8.0	4.5	5.8	6.1
ICC 4324	6.5	7.5	6.4	5.8	5.5	7.5	5.6	5.5	6.3
ICC 5116	6.3	6.5	5.9	4.8	5.0	7.8	4.5	5.3	5.7
ICC 5255	4.0	5.3	4.3	4.5	5.5	7.3	3.8	4.0	4.8
ICC 6299	5.0	3.5	4.0	4.0	7.5	6.5	4.0	6.0	5.1
ICC 6306	6.3	5.5	4.8	4.5	4.5	7.3	4.5	4.0	5.2
ICC 6981	5.5	6.3	5.5	5.0	6.3	7.3	5.0	5.9	5.8
ICC 7322	5.5	5.3	5.9	4.8	5.0	6.0	4.0	4.5	5.1
ICC 7670	5.5	5.3	5.0	5.1	4.5	5.5	5.0	4.6	5.1
ICC 7720	5.4	5.0	5.0	4.8	4.3	5.3	4.0	4.0	4.7
ICC 8487	5.3	6.5	5.8	6.0	4.5	7.3	4.8	5.5	5.7
ICC 8502	5.0	5.0	4.0	4.0	5.5	5.0	4.0	4.5	4.6
ICC 9189	5.3	5.8	6.0	5.3	5.3	7.8	4.0	5.3	5.6
ICC 11905	5.0	4.0	4.3	5.8	6.0	6.8	4.0	5.0	5.1
ICC 13816	4.5	6.3	4.3	5.6	4.5	7.0	4.0	5.3	5.2
ICC 14912	4.3	5.0	4.0	5.0	4.5	6.1	4.5	4.9	4.8
ICC 15981	4.6	5.5	6.0	4.0	5.6	6.0	4.5	4.5	5.1
ICC 15982	3.8	4.5	3.5	5.3	5.3	4.3	3.6	4.4	4.3
ICC 15983	5.0	5.3	5.0	5.1	5.5	6.8	4.4	5.4	5.3
ICC 15985	4.8	5.8	5.5	4.3	5.0	5.8	3.9	4.5	4.9
ICC 15986	4.4	4.0	5.0	4.0	4.1	7.0	3.8	4.8	4.6
ICC 15990	5.5	6.3	4.3	4.8	5.5	8.0	4.0	4.8	5.4
ICCL 83149	5.5	6.8	6.3	5.5	5.8	9.0	4.5	6.5	6.2
ICCL 85405	5.0	5.0	5.5	5.3	6.0	8.5	3.8	5.0	5.5
ICCL 86224	5.0	5.0	5.1	5.3	5.5	8.0	3.8	4.5	5.3
ICCL 86327	5.8	6.5	4.5	5.8	6.3	4.8	6.8	9.0	6.2
ICCV 5	4.5	5.3	4.3	5.0	5.0	7.3	4.1	4.5	5.0
Pant.G 114	4.8	5.0	4.5	4.3	5.5	7.5	3.3	4.1	4.9
Pusa 256	4.5	6.0	4.8	4.4	4.5	7.5	3.8	5.5	5.1
JG 62	8.3	8.5	8.0	7.3	7.0	9.0	5.0	8.0	7.6
Mean	5.3	5.8	5.3	5.0	5.4	7.1	4.2	5.3	5.4
LSD (P = 0.01): isolate x genotype = 0.38									

level of genetic variation (56-86%) between the eight *B. cinerea* isolates. Isolates IC6 and IC8 share the maximum genetic similarity of 44%, and the eight isolates were clustered into three groups – IC1, IC3 and IC4 as group I, IC2, IC6 and IC8 as group II, and IC5 and IC7 as group III (Figure 4).

DISCUSSION

BGM resistance in cultivated chickpea is scarce and only few moderately resistant sources were identified (Davidson et al., 2004). The highly variable nature of *B. cinerea* and resulting changes in its aggressiveness pose a further threat to the deployment of available resistant sources and also the breeding programs targeted at pyramiding of BGM resistance (Pande et al., 2006). Variations in growth patterns (Martinez et al., 2003), virulence (Van der et al., 1993), fungicide sensitivity (Myresiotis et al., 2007) and production of cell wall degrading enzymes (Cotoras and Silva, 2005) of *B. cinerea* isolates are known. The *B. cinerea* isolates tested in this study varied in their growth rate as well as number and size of conidia formed. Variation in the size of conidia from different isolates of *Botrytis* sp. was not uncommon (Nielsen et al., 2001). Fungal morphological variation is highly subjected to the substrate availability and experimental conditions, and often exponentially responds to minor change in these conditions. Hence, morphological variation can be considered only as putative indication of the existence of pathogenic or genetic variability in a particular population of plant pathogenic fungi. A significant negative correlation was established between virulence of *B. cinerea* isolates on *Vitis vinifera* and *Nicotiana clevelandii* and their mycelial growth rate *in vitro* (Martinez et al., 2003).

The eight isolates of *B. cinerea* were grouped as three pathotypes based on their virulence against a set of 40 chickpea lines. The grouping is not in accordance to the geographical origin of the isolates, as two isolates from western and four from eastern locations of IGP were grouped as one pathotype. Rewal and Grewal (1989) reported that six isolates from North India varied in their virulence against a set of 40 different chickpea lines. There were no further reports on virulence characterization of *B. cinerea* isolates from the Indo-Gangetic plains of India, one major reason being the absence of a standard set of chickpea lines to differentiate between the isolates. Variation in aggressiveness of *B. cinerea* isolates from vegetable crops (Sharman and Heale, 1979), geranium (Buck and Jeffers, 2004) and grapevine (Derckel et al., 1999) is documented. In these studies there is no evidence for gene-gene interaction between host and pathogen; rather the isolates differed in their aggressiveness. Variation in aggressiveness of *B. cinerea* isolates often correlated with their variation in secretion of cell wall degrading enzymes and induction of defense responses in host plants (Derckel et al., 1999).

In the present study, five polymorphic bands were generated per each RAPD primer on an average, indicating that in *B. cinerea* populations, polymorphisms are more frequently detected. Presence of high levels of DNA polymorphisms in *B. cinerea* from different sources is evident from earlier reports (Ma and Michailides, 2005). Kerssies et al. (1997) observed 6.3 bands per each of the random primer used for amplification of genomic DNA of *B. cinerea*. In this study, 30 *B. cinerea* isolates tested were found to genetically dissimilar and also there is no relation between pathogenic and genetic variation of the isolates. Fifty-six *B. cinerea* isolates from USA subjected to RAPD analysis were observed to be distinctly separate from each other (Yourman et al., 2000). A positive correlation between the pathogenic and genetic variability of a phytopathogenic fungus has been very rarely reported (Pioli et al., 2003). Results of these different studies indicate the non-existence of different races of *B. cinerea* and the isolates were grouped into pathotypes based on the differences in their aggressiveness. However, none of the 17 primers in the present study resulted in an amplified product common to all the eight *B. cinerea* isolates.

DNA polymorphism observed between the *B. cinerea* isolates in the present study, is highly significant, and supported by the high number of polymorphic RAPD fragments. Using microsatellite DNA markers, Isenegger et al. (2005) observed a high genetic diversity within and among subpopulations of *B. cinerea* from chickpea and diversity was detected among isolates collected in a distance of 1-2 m within a field. Results of this study indicated no specific patterns of pathotypic or genetic grouping of *B. cinerea* isolates related to geographical distribution, thus indicating a high rate of gene flow among the *B. cinerea* populations.

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