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Physiological variability and *in vitro* antifungal activity against *Botrytis cinerea* causing botrytis gray mold of chickpea (*Cicer arietinum* L.)

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Abstract

Physiological variability was studied in 10 isolates of *Botrytis cinerea* causing botrytis gray mold of chickpea, collected from diverse agro climatic areas in Bangladesh. The optimum temperature and pH for the best mycelial radial growth of *B. cinerea* were 20°C and 4.5, respectively. The mycelial radial growth increased with the temperature up to 20°C thereafter it decreased gradually up to 30°C and no growth was observed at 35°C. Chickpea dextrose agar (CDA) medium supported the highest mycelial radial growth (79.17 mm). The quickest (in 5 days) sclerotia initiation was recorded on chickpea destrose agar and lentil dextrose agar (LDA) culture media while the highest number of spores (2.510^4 mL^{-1}) were recorded on LDA medium. The antagonist *Trichoderma harzianum* was found to be a good bio-control agent against *B. cinerea*. Among the seven fungicides Bavistin® 50 WP (Carbendazim), CP-Zim 50 WP (Carbendazim), Sunphanate 70 WP (Thiophanate methyl) and Rovral 50 WP (Iprodione) were the most effective to inhibit the mycelial radial growth of *B. cinerea* at 500 mg L^{-1} concentration.

Additional key words: antagonist, culture media, fungicide, pH, temperature.

Resumen

Variabilidad fisiológica y actividad antifúngica in vitro contra *Botrytis cinerea* causante del moho gris en garbanzo (*Cicer arietinum* L.)

Se estudió la variabilidad fisiológica de *Botrytis cinerea*, causante del moho gris de la botrytis de garbanzo, en 10 aislados recolectados en diversas zonas agro climáticas en Bangladesh. La temperatura y el pH óptimos para el mejor crecimiento radial del micelio de *B. cinerea* fueron de 20°C y 4,5, respectivamente. El crecimiento radial del micelio se incrementó con la temperatura hasta los 20°C; a partir de esta temperatura se redujo gradualmente hasta los 30°C y a 35°C no se observó crecimiento. El mayor crecimiento radial (79,17 mm) se obtuvo en el medio garbanzo-dextrosa agar (CDA). La iniciación más rápida de esclerocios (en 5 días) se produjo en los medios de cultivo CDA y lenteja-dextrosa agar (LDA), mientras que el mayor número de esporas ($2,510^4$ mL^{-1}) se registró en el medio LDA. El antagonista *Trichoderma harzianum* se reveló como un buen agente de control biológico contra *B. cinerea*. Entre los siete fungicidas, los más efectivos para inhibir el crecimiento micelial radial de *B. cinerea* fueron Bavistin® 50 WP (Carbendazim), CP-Zim 50 WP (Carbendazim), Sunphanate 70 WP (metil tiofanato) y Rovral 50 WP (Iprodione), a una concentración de 500 mg L^{-1}.

Palabras clave adicionales: antagonista, fungicida, medios de cultivo, moho gris de Botrytis, pH, temperatura.

Introduction

Chickpea (*Cicer arietinum* L.) is the most important food legume crop grown globally in at least 44 countries (Bakr *et al*., 2002; Pande *et al*., 2006) covering an area of about 11.12 million hectare, with 8.62 million tons of grain production (FAO, 2005). Chickpea suffers from various abiotic and biotic stresses. Among

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Abbreviations used: BDA (barley dextrose agar), BGM (botrytis gray mold), CBDA (chickpea barley dextrose agar), CDA (chickpea dextrose agar), CRD (completely randomized design), CV (coefficient of variation), DMRT (Duncan's multiple range test), LDA (lentil dextrose agar), PDA (potato dextrose agar), V-8A (V-8 juice agar), WA (water agar).

them, botrytis gray mold (BGM), caused by *Botrytis cinerea* Pers. Ex. Fr., is an economically detrimental disease in areas with cool, cloudy and humid weather (Pande *et al*., 2006). The disease has taken a heavy toll on chickpea resulting in reduction in Bangladesh to 16,000 ha from more than 1,000,000 ha in 10 years (Bangladesh Bureau of Statistics, 1999). BGM can result in complete yield loss in years of extensive winter rain and high humidity (Pande *et al*., 2006). The disease is of serious concern not only in Bangladesh but also in countries viz. India, Nepal, Pakistan, Australia and Argentina (Dhar *et al*., 1993; Davidson *et al*., 2004; Pande *et al*., 2006). Limited studies have been done on the influence of temperature, pH and culture media on *B. cinerea*. Hence, present work was aimed to study the influence of these physical factors on various growth parameters of *B. cinerea* isolates collected from seven major chickpea growing districts of Bangladesh. In addition, *in vitro* efficacy of some fungicides and biocontrol agent was tested against the most prevalent and virulent isolate AHI-9.

Material and methods

Collection, isolation, purification and preservation of *B. cinerea*

The experiment was conducted in Bangladesh Agricultural Research Institute, Joydebpur, from June to December, 2007. *B. cinerea* isolates were collected from seven major chickpea growing districts of Bangladesh namely, Chuadanga (AHI-1), Meherpur (AHI-2), Faridpur (AHI-5), Rajbari (AHI-6), Rajshahi (AHI-10), Pabna (AHI-3, AHI-4 and AHI-9) and Kushtia (AHI-7 and AHI-8). The *B. cinerea* was isolated using standard isolation techniques and purified following single spore isolation technique (Mian, 1995). The stock culture of the isolates were maintained on potato dextrose agar (PDA) at 4 ± 0.5°C.

Physiological variability

Four day old Cultures of *B. cinerea* were used in this study. Five (5) mm mycelium discs was cut from the periphery of the four day old culture of *B. cinerea* and then transferred into the centre of the Petri dishes containing solidified PDA medium. Inoculated plates were incubated in incubators at 5, 10, 15, 20, 25, 30 and 35 ± 0.5°C following completely randomized design (CRD) comprising of three replications. Data were recorded on mycelial radial growth after 4 days of incubation.

The isolates were inoculated onto PDA medium having five pH levels viz., 4.5, 5.0, 5.5, 6.0 and 6.5 in 90 mm diameter glass Petri dishes and incubated at 20 ± 0.5°C. The design of experiment and data recording protocol were the same as mentioned under temperature studies.

Culture media

Seven non-synthetic culture media viz. PDA (200 g pealed potato slice, 17 g agar, 20 g dextrose, 1 L distilled water), chickpea dextrose agar (CDA) (200 g chickpea grain, 17g agar, 20 g dextrose, 1 L distilled water), barley dextrose agar (BDA) (200 g barley grain, 17 g agar, 20 g dextrose, 1 L distilled water), chickpea barley dextrose agar (CBDA) (100 g chickpea grain, 100 g barley grain, 17 g agar, 20 g dextrose, 1 L distilled water), lentil dextrose agar (LDA) (200 g lentil grain, 17 g agar, 20 g dextrose, 1 L distilled water), V-8 juice agar (V-8A) (100 mL V-8 juice, 17 g agar, 20 g dextrose, 3 g $CaCO_3$, 1 L distilled water) and water agar (WA) (17 g agar, 1 L distilled water), were used. The prereplicated Petri dishes containing sterilized media were inoculated with culture of *B. cinerea* and the plates were incubated at 20 ± 0.5°C. Data were recorded on the mycelial radial growth of *B. cinerea* when mycelial growth completely covered the glass Petri dishes. The sporulation was recorded using a haemocytometer after 15 days of incubation and the period for sclerotia formation were also noted up to 7 days of incubation.

In vitro antifungal activity

The anti fungal activity of *T. harzianum* was evaluated against isolate AHI-9. Five mm diameter discs of 4-days old cultures of test pathogen and antagonist was cut separately from the periphery of the culture dishes (PDA media) with the help of a sterilized cork borer. These discs were placed apart on solidified PDA in an equal distance having 3 replications and were incubated in an incubator at 20 ± 0.5°C. Petri dishes were observed regularly for the growth of the antagonist and the test pathogen.

The antifungal activity of seven fungicides (viz. Bavistin® DF 50 WP (Carbendazim), CP-Zim 50 WP

(Carbendazim), Sunphanate 70 WP (Thiophanate methyl), Rovral 50 WP (Iprodione), Zhetalux 25 WP (Metalaxyl 25 WP), Kafa 80 WP (Mancozeb) and Agromil 72 WP (Metalaxyl + Mancozeb) were observed on mycelial growth of isolates AHI-9. Desired quantity of test fungicides (concentrations 500, 1,000, 1,500 and 2,000 mg L^{-1}) were added to flasks containing double strength PDA medium before its solidification to achieve the proposed concentrations. The PDA medium was poured into sterilized glass Petri dishes. The Petri dishes were inoculated in 3 replications and incubated at 20 ± 0.5°C; alternating by 12 h light and 12 h dark phase. The mycelial radial growth was measured 4 days after incubation and percentage of mycelial radial growth inhibition (*I*) was calculated using the following formula:

$$I = \frac{C - T}{C} \times 100,$$

where *C* and *T* are average fungal colony diameter (mm) in control and in antifungal treated PDA medium, respectively.

Statistical analyses

The data were analyzed using MSTAT computer package program and means were compared with Duncan's Multiple Range test (DMRT) where F values indicated significant differences at 5% level of probability.

Results

Physiological variability

The effect of temperature on the mycelial radial growth of *B. cinerea* on PDA is presented in Table 1. The mycelia of *B. cinerea* increased with the time at each temperature. The growth gradually increased up to 20°C and at 35°C no growth was recorded. The colony diameter was highest for all ten isolates at 20°C and it decreased gradually thereafter. At 20°C maximum colony diameter (86.00 mm) was obtained in isolate AHI-9 followed by AHI-10 (85.33 mm).

The pH of the culture medium had a significant effect on colony growth of *B. cinerea*. The results on development of fungus on PDA medium at pH ranging from 4.5 to 6.5 are given in Table 2. Profound growth was recorded at pH 4.5 than the other pH levels. The optimum pH for radial growth of all the isolates was pH 4.5 followed by 5.0 and it decreased at other pHs (Table 2). The highest colony diameter was noted in the isolate AHI-6 (81.00 mm) followed by AHI-9 (80.00 mm) and AHI-8 (79.33 mm) at pH 4.5. The lowest radial growth was recorded in isolate AHI-4 (7.50 mm) preceded by AHI-1 (9.33 mm) at pH 6.5. The highest radial growth (81.00, 66.33, 53.8 and 26.00 mm) were recorded at pH 4.5, 5.0, 5.5 and 6.0 levels respectively for the isolate AHI-6, which grew well at all pH levels except pH 6.5 (16.17 mm).

Table 1. Mycelial radial growth of *B. cinerea* at different temperatures

Isolate	Radial colony growth[1] (mm)							Average growth (mm)
	5°C	10°C	15°C	20°C	25°C	30°C	35°C	
AHI-1	11.33^{d}	32.50^{f}	52.17^{e}	81.17^{c}	40.50^{d}	5.83^{g}	0.00	37.25
AHI-2	18.10^{a}	55.00^{a}	63.67^{bc}	83.83^{ab}	63.83^{b}	16.33^{d}	0.00	50.13
AHI-3	14.17^{b}	53.50^{b}	62.00^{d}	81.00^{c}	64.33^{b}	17.83^{c}	0.00	48.81
AHI-4	11.17^{d}	37.67^{d}	51.83^{e}	78.00^{d}	35.00^{e}	8.50^{f}	0.00	37.03
AHI-5	12.00^{cd}	46.33^{d}	64.50^{b}	84.50^{ab}	58.17^{c}	19.00^{b}	0.00	47.42
AHI-6	19.50^{a}	55.83^{a}	65.00^{b}	84.17^{ab}	63.50^{b}	9.17^{f}	0.00	49.53
AHI-7	11.17^{d}	37.83^{e}	44.76^{f}	83.83^{ab}	25.00^{f}	14.17^{e}	0.00	36.13
AHI-8	12.00^{cd}	38.50^{e}	64.83^{b}	83.50^{b}	63.33^{b}	18.50^{bc}	0.00	46.78
AHI-9	18.90^{a}	56.17^{a}	66.50^{a}	86.00^{a}	66.00^{a}	20.33^{a}	0.00	52.32
AHI-10	13.17^{bc}	51.50^{c}	62.50^{cd}	85.33^{ab}	65.83^{a}	18.83^{b}	0.00	49.53
CV[2] (%)	6.09	1.62	1.26	1.42	1.47	3.89	—	

[1] Means of three replications for each isolate. Numbers with similar letter do not differ significantly at 5% level according to Duncan's multiple range test (DMRT). [2] CV: coefficient of variation.

Table 2. Mycelial radial growth of *B. cinerea* at different pH values

Isolate	Radial colony growth[1] (mm)					Average growth (mm)
	4.5	5.0	5.5	6.0	6.5	
AHI-1	38.83^{f}	33.83^{f}	21.50^{g}	16.00^{d}	9.33^{d}	23.90
AHI-2	75.33^{b}	54.17^{c}	39.20^{e}	24.33^{b}	19.83^{a}	42.57
AHI-3	54.67^{e}	43.50^{e}	40.80^{d}	26.17^{a}	18.50^{b}	36.73
AHI-4	31.33^{g}	43.83^{e}	24.30^{f}	13.50^{e}	7.50^{e}	24.09
AHI-5	63.17^{d}	50.83^{d}	41.70^{d}	20.83^{c}	18.67^{b}	39.04
AHI-6	81.00^{a}	66.33^{a}	53.80^{a}	26.00^{a}	16.17^{c}	48.66
AHI-7	22.00^{h}	21.83^{g}	16.20^{h}	12.17^{e}	9.50^{d}	16.34
AHI-8	79.33^{a}	59.50^{b}	46.20^{b}	27.33^{a}	17.83^{b}	46.04
AHI-9	80.00^{a}	60.50^{b}	43.00^{c}	26.83^{a}	18.17^{b}	45.70
AHI-10	71.50^{c}	51.67^{cd}	38.70^{e}	20.33^{c}	15.83^{c}	39.61
CV[2] (%)	1.52	3.07	1.92	3.87	4.22	

[1] Means of three replications for each isolate. Numbers with similar letter do not differ significantly at 5% level according to Duncan's Multiple Range Test (DMRT). [2] CV: coefficient of variation.

Culture media

The effect of different culture media on mycelial growth of *B. cinerea* is given in Figure 1 and Table 3. After 24 h of incubation, maximum increase in colony diameter was observed on CDA (25.00 mm) followed by CBDA, PDA (21.00 mm) and LDA (19.00 mm) and it was statistically at par. The lowest increase in colony diameter was recorded on WA (8.00 mm) and V-8A (9.00 mm) preceded by BDA (18.00 mm). The highest increase in mycelial colony diameter was recorded on CBDA (28.00 mm) followed by PDA (27.00 mm) and BDA (27.00 mm) after 48h of incubation. At 72 h of incubation, maximum incremental rate of mycelial radial growth was recorded on LDA (39.00 mm) followed by BDA (31.00 mm). The highest growth was recorded 3 days after incubation on CDA (79.17 mm) followed by LDA (78.83 mm) which were statistically at par and the lowest radial growth that was obtained on WA (34.83 mm) preceded by V-8A (44.17 mm).

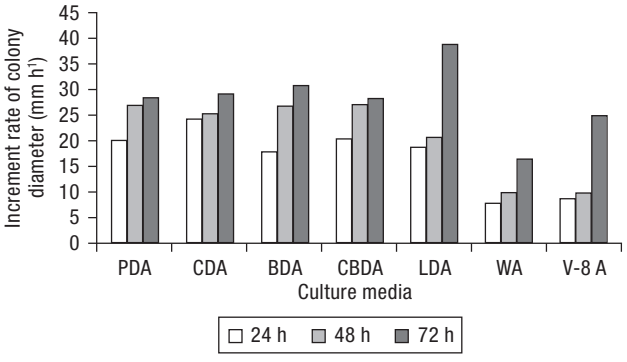

Figure 1. Incremental rate of colony diameter of *B. cinerea* at 24, 48 and 72 h of incubation on different culture media.

Sporulation and sclerotia formation of *B. cinerea* on different culture media

The sporulation of *B. cinerea* on different media is given in Table 3. The maximum sporulation was observed on LDA (2.5×10^4 mL^{-1}) followed by CDA (2.3×10^4 mL^{-1}) and BDA (2.2×10^4 mL^{-1}) media. The lowest sporulation (1.9×10^4 mL^{-1}) was recorded on PDA followed by CBDA (2.0×10^4 mL^{-1}) medium. No sporulation was observed on WA and V-8A media. The

Table 3. Effect of culture media on colony growth, sporulation and sclerotia formation of *B. cinerea*

Culture media	Colony growth (mm)[1]	Sporulation (mL^{-1})[2]	Sclerotia initiation[3]
PDA	76.00^{b}	1.9 × 104	7 days +++
CDA	79.17^{a}	2.3 × 104	5 days ++++
BDA	76.17^{b}	2.2 × 104	6 days ++
CBDA	76.67^{b}	2.0 × 104	6 days +++
LDA	78.83^{a}	2.5 × 104	5 days ++++
WA	34.83^{d}	—	Absent
V-8A	44.17^{c}	—	Absent
CV (%)	1.14		

[1] Numbers with similar letter do not differ significantly at 1% level according to Duncan's Multiple Range Test (DMRT). [2] —: no sporulation. [3] Grade: + = poor, ++ = fair, +++ = good and ++++ = excellent.

sclerotia formation was earliest (5 days) both on CDA and LDA. Both the media developed excellent sclerotia. No sclerotia production was found on WA and V-8A media.

Antifungal activity against *B. cinerea*

The effect of bio-control agent on *B. cinerea* is depicted in Figure 2. The hyphal growth of *B. cinerea* was inhibited to some extent at the zone of contact with *T. harzianum*. The microscopic observations showed that hyphal tips of *B. cinerea* swelled and curved at the point of contact. A thick band of over growing antagonistic mycelia was observed within 6 days of incubation and the advancing *T. harzianum* hyphae covered the entire Petri dishes suppressing the growth of *B. cinerea*. The growth of test pathogen become dark green after 6 days of incubation and it could not be re-isolated from any part of the over grown Petri dishes.

The seven fungicides were bio-assayed for *in vitro* antifungal activity against of *B. cinerea* and the results are given in Table 4 and Figure 3. The four fungicides Bavistin, CP-Zim, Sunphanate and Rovral were highly effective and completely inhibited the mycelial growth at all concentrations. Fungicide Zhetalux completely inhibited the mycelial growth at 1,000, 1,500 and 2,000 mg L^{-1} and an inhibition of 32.40% radial mycelial growth was recorded at 500 mg L^{-1}. Fungicide Kafa, resulted 55.09, 58.50, 62.88 and 64.63% inhibition in colony growth at 500, 1,000, 1,500 and 2,000 mg L^{-1}, respectively. Agromil resulted 31.5, 26.2, 20.00 and 12.7 mm radial mycelial growth at 500, 1000, 1500 and 2000 mg L^{-1}, respectively causing 61.35, 67.85,

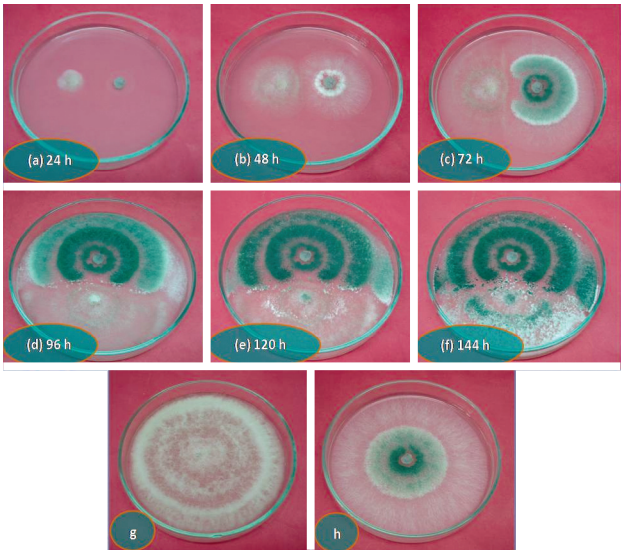

Figure 2. Antagonistic activity of *T. harzianum* (dual culture technique) against *B. cinerea* after (a) 24 h, (b) 48 h, (c) 72 h, (d) 96 h, (e) 120 h, (f) 144 h of incubation and (g) pure culture of *Botrytis cinerea* (4 days old) and (h) *T. harzianum* (3 days old) on PDA medium.

Table 4. Antifungal activity of seven fungicides at different concentrations on radial colony growth of *B. cinerea*

Conc. (mg L^{-1})	Bavistin® DF 50 WP	Sunphanate 70 WP (Thiophanate methyl)	Agromil 72 WP	CP-Zim 50 WP (Carbedazim)	Kafa 80 WP (Mancozeb)	Rovral 50 WP	Zhetalux 25 WP (Metalaxyl 25 WP)
Radial growth of B. cinerea *(mm)*[1]							
0	75.30^{b} (8.68)	75.30^{b} (8.68)	81.50^{a} (9.03)	75.20^{b} (8.67)	80.00^{a} (8.94)	75.30^{b} (8.68)	74.70^{b} (8.62)
500	0.00^{j} (0.71)	0.00^{j} (0.71)	31.50^{e} (5.61)	0.00^{j} (0.71)	35.93^{d} (5.99)	0.00^{j} (0.71)	50.50^{c} (7.12)
1,000	0.00^{j} (0.71)	0.00^{j} (0.71)	26.20^{g} (5.12)	0.00^{j} (0.71)	33.20^{e} (5.76)	0.00^{j} (0.71)	0.00^{j} (0.71)
1,500	0.00^{j} (0.71)	0.00^{j} (0.71)	20.00^{h} (4.47)	0.00^{j} (0.71)	29.70^{f} (1.5.45)	0.00^{j} (0.71)	0.00^{j} (0.71)
2,000	0.00^{j} (0.71)	0.00^{j} (0.71)	12.70^{i} (3.56)	0.00^{j} (0.71)	28.30^{f} (5.32)	0.00^{j} (0.71)	0.00^{j} (0.71)
Inhibition of colony growth (%)							
0	0	0	0	0	0	0	0
500	100	100	61.35	100	55.09	100	32.40
1,000	100	100	67.85	100	58.50	100	100
1,500	100	100	75.46	100	62.88	100	100
2,000	100	100	84.42	100	64.63	100	100
CV (%)				1.63			

[1] Mean of three replications for each concentration. Figures within the parenthesis are square root transformed values. Numbers with similar letter do not differ significantly at 5% level according to Duncan's multiple range test (DMRT).

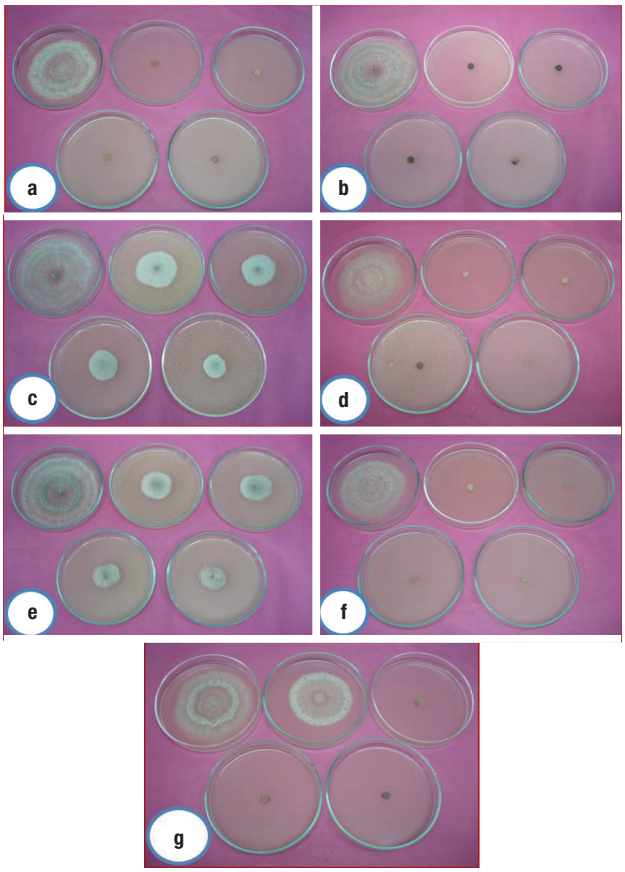

Figure 3. Antifungal activity of seven fungicides (a) Bavistin, (b) Sunphanate, (c) Agromil, (d) CP-Zim, (e) Kafa, (f) Rovral and (g) Zhetalux at 0, 500, 1,000, 1,500 and 2,000 mg L^{-1} concentrations (upper row to lower row) against colony growth of *B. cinerea*.

75.46 and 84.42% inhibition of radial growth over control.

Discussion

The pathogen *B. cinerea* showed good growth at a wide range of temperature (5-30°C). No growth was recorded at 35°C and comparatively the least growth was observed at 5 and 30°C. The optimum temperature for the growth of the pathogen (all isolates) was 20°C and the maximum (86.00 mm) colony diameter was recorded in the isolate AHI-9. The present findings are supported by earlier studies (Bakr and Ahmed, 1992; Bakr *et al*., 1997; Ahmed *et al*., 2007) that found that 20°C was the optimum temperature for mycelial development of *B. cinerea* and the growth was completely inhibited at 5°C and 35°C respectively.

All the isolates grew well at pH 4.5 but growth was reduced gradually with increased pH up to 6.5. Highest colony diameter was observed in the isolate AHI-6 at pH 4.5 followed by AHI-9 at pH 4.5. At pH 6.5 colony diameter was the lowest in AHI-4 (7.50 mm) followed by AHI-1 (9.33 mm) at pH 6.5. Similarly, Ahmed *et al*. (2007) reported that *B. cinerea* is an acid loving fungus and pH 5.5 was suitable for its growth and sporulation. They also observed that different isolates of *B. cinerea* behaved differently in response to varied pH levels. It is evident from the study that *B. cinerea* was an acid loving fungus and this isolates showed variation in mycelial radial colony growth with a varied pH levels.

B. cinerea showed variation in mycelial growth on different culture media as well. Mycelial growth increases with the increase of incubation period. The highest colony growth (79.17 mm) was recorded on CDA followed by LDA (78.83 mm) and the lowest growth (34.83 mm) was on WA and preceded by V-8A (44.17 mm). The highest sporulation (2.5×10^4 mL^{-1}) was found on LDA followed by CDA (2.3×10^4 mL^{-1}). The quickest sclerotia formation was observed on CDA and LDA and it was slowest on PDA. Probably it was happen due to chickpea and lentil as because both the crop is the major host of *B. cinerea*. Mirzaei *et al*. (2007) also recorded similar data on sclerotial characteristics after 7 days of incubation.

The result from dual culture assay indicated that *T. harzianum* had inhibitory effect on *B. cinerea* and this antagonist could be used in controlling BGM of chickpea. The results are in accordance with findings of Pande *et al*. (2006) who found that *T. harzianum* is highly antagonistic to *B. cinerea*.

In vitro studies on fungicidal activity showed that Bavistin, CP-Zim, Sunphanate and Rovral were highly effective against *B. cinerea* and had the potential to inhibit the mycelial growth of *B. cinerea* at a concentration as 500 mg L^{-1}. Similar results are reported by Madhu Meeta *et al*. (1986) and Agarwal and Tripathi (1999) who reported that carbendazim at 10 µg mL^{-1} completely inhibited the growth of *B. cinerea*. It is revealed from the test of chemicals that *B. cinerea* could be managed effectively using Bavistin, CP-Zim, Sunphanate and Rovral.

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