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Cultural, physiological comparison and fungicidal sensitivity between two isolates of *Botrytis cinerea* and *Stemphylium botryosum*

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Abstract: Cultural and physiological comparison between *Botrytis cinerea* and *Stemphylium botryosum* causing gray mold in chickpea and stemphylium blight in lentil were studied. Both isolates differed significantly on the basis of their colony diameter, color, texture, shape, margin, conidia dimension, spore production and sensitivity to fungicides. The maximal colony diameter (80.16 mm) and spore (2.7×10^4) mL^{-1} was found in chickpea dextrose agar (CDA) and lentil dextrose agar (LDA) respectively, for *Botrytis cinerea*. *Stemphylium botryosum* maximal colony diameter (57.00 mm) and spore (1.7×10^4) mL^{-1} was obtained from LDA. The optimum temperature and pH for *Botrytis cinerea* and *Stemphylium botryosum* growth was 20°C and 25°C; pH 4.5 and 5.5, respectively. The conidia size were found to be 11.74×8.12 µm for *B. cinerea* and 16.27×9.07 µm for *S. botryosum*. Rovral 50WP from the iprodione group was found to be the most effective chemical fungicide to inhibit the radial mycelial growth of both isolates. *Trichoderma harzianum* was found to be an effective antagonist against both pathogens.

Keywords: Chickpea, Fungicides, Lentil, Variability and *Trichoderma harzianum*.

مقارنة الصفات البيئة والفسيولوجية والحساسية من الفطريات بين عزلتين من *Stemphylium btoyosum* and *Botrytis cinerea*

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مختبر التنوع البيئى والبيوجرافي ، معهد كونيمنج للنبات، المعهد الاكاديمي للعلوم ، الصين كونيمتج 650204 ، يونيمان ، الصين

الملخص:تمت دراسة مقارنة الصفات البيئية والفسيولوجية والحساسية من الفطريات بين مستخلصى نوعين من *Stemphylium botryosum* and *botrytis cinerea* التى تسبب العفن الرمادي فى الحمص و مرض *Stemphylium* فى العدس . اختلفت كل العزلات الى حد كبير على اساس فطر مستعمراتهم ، اللون والملمس والشكل والهامش والبعد وانتاج الجراثيم الحساسة للمبيدات الفطرية. وكان أقصى قطر للمستعمرة 80.16مم وعدد خلايا فى 1 مل 10^4 * 2.7 موجود فى اجار دكستروز الحمص والعدس، على التوالي،لل *Botrytis cinerea*. اما فطر*Stemphylium botryosum* فكان أقصى قطر مستعمرة العضوي 57.00مم وعدد الخلايا فى 1 مل 10^4 * 106 تم الحصول عليها فى 1 مل من LDA. وكانت درجة الحرارة المثلى والرقم الهيدورجينى لنمو *Botrytis cinerea* *Stemphylium* ***ea*** *btoyosum* هو 20 م و 25م و درجة الحموضة 4.5 و 5.5 على التوالي . كان حجم الجراثيمµm 8.12×11.74 ل *B. cinerea*و 16.27×9.07 µm *S. botryosum* . كانRovral 50WP من مجموعة iprodirne اكثرمضادات الفطريات فعالية لكبح نمو الفطريات لكل من العزلات. وكان *Trichoderma harzianum* ذو فعالية كمضادلكلا مسببي الامراض.

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Introduction

Botrytis cinerea and *Stemphylium botryosum* both are known to causes enormous yield loss in several crops they also have a wide range of host. Seed-borne *B. cinerea* is associated with a huge number of plant species including *Stylosanthes* spp., a forage crop in sub-Saharan Africa (Nan et al., 1998), chickpea (Burgess et al., 1997), flax and lettuce (Legard et al., 2000) and lentil (Morral, 1997). This pathogen either reduces marketability of the plant or renders them unmarketable and the inoculums for the disease generally is thought to be spread by wind or rain splash dispersal of conidia onto above ground plant parts (Mwakutuya, 2006). However, *B. cinerea* causes botrytis gray mold (BGM) in chickpea and rare cases in lentil and *S. botryosum* causes stemphylium blight in lentil. The climatic conditions of Bangladesh favor disease development and rapid growth of plant pathogen. *B. cinerea* threatened the chickpea cropped area so much that come down to 16,000 ha from area than 10,00,000 ha with a span of 10 years (BBS, 1999). The disease is of serious concern in Bangladesh, India, Nepal, Pakistan, Australia and Argentina where 100% yield losses have been reported under conduciveness conditions (Bakr et al., 1993; Davison et al., 2004; Dhar et al., 1993; Pande et al., 2002). *S. botryosum* is a defoliating fungal pathogen of lentil causes lentil blight and yield losses of up 62% have been reported in Bangladesh (Bakr, 1991; Erskine and Sarker, 1997; Mwakutuya, 2006). *S. botryosum* causing up to 100% yield loss have been reported under epidemic condition from Bangladesh, northeast India and Nepal. Thus, due to the importance of them more concentrate needs to pay for knowing the information regarding the variability for its management. This investigation was carried out to compare *B. cinerea* and *S. botryosum* isolates and screen out an effective chemical with least concentration that will to inhibit the radial mycelial growth of *B. cinerea* and *S. botryosum*.

Materials and methods

Collection, isolation and purification

The research work was undertaken at the Laboratory of Plant Pathology Division, Bangladesh Agricultural Research Institute, Gazipur, Bangladesh from January to May, 2008. Samples of chickpea and lentil plants showing BGM and stemphylium blight infection were collected and isolated. Purification was carried out using single spore isolation technique then transferred into the potato dextrose agar (PDA) medium and incubated at 20 ± 0.5°C with alternating dark and light phase then kept it in refrigerator at 4 ± 0.5°C for further consecutive studies.

Cultural comparison

Cultural features were noted on PDA medium after 4 days of incubation. Cultural features of colony color, shape, texture and conidial size of *B. cinerea* and *S. botryosum* isolates were observed on PDA medium after 4 days of incubation both naked eyes and microscopically. Dimensions of conidia were measured using calibrated ocular micrometer.

Physiological comparison

Temperature and pH

The effect of temperature and pH on the colony diameter of *B. cinerea* and *S. botryosum* were studied using 4 days old fungal cultures. Five (5) mm diameter discs were cut from the edge of colony growth of both pathogens and placed on the sterilized PDA into Petri-dishes. Inoculated plates were shifted in an incubator at different degrees of temperature and pH viz, 15, 20, 25, 30, 35 and 40°C; 4.5, 5.0, 5.5, 6.0 and 6.5 for 4 days having five replications and then examined. The different levels of pH were maintained using pH meter adding 0.1 N HCl and NaOH.

Nutrient media

Five different nutrient media (Table 1) were also evaluated for their impact on the mycelial radial growth of *B. cinerea* and *S. botryosum*. Fungal inoculation and other protocol were carried out as mention earlier. Inoculated plates were incubated at 20 ± 0.5°C for 5 days and then examined.

Table 1. Composition of five different growth media for 1 L.

Media[1]	Potato	Chickpea grain	Barley grain	Lentil	Agar	Dextrose
PDA	200 g	-	-	-	15 g	20 g
CDA	-	200 g	-	-	15 g	20 g
BDA	-	-	200 g	-	15 g	20 g
LDA	-	-	-	200 g	15 g	20 g
WA	-	-	-	-	15 g	-

[1]PDA= Potato Dextrose agar, CDA=Chickpea Dextrose Agar, BDA=Barley Dextrose Agar, LDA=Lentil Dextrose Agar and WA=Water Wager

All media were sterilized at 121°C temperature for 30 minutes and 15 psi

Antagonist effect

The antagonist effect of *Trichoderma harzianum* was evaluated against *B. cinerea* and *S. botryosum* in dual culture technique. Five (5) mm diameter discs were taken from four days old culture of *B. cinerea*, *S. botryosum* and antagonist of *T. harzianum* and placed apart on solidified PDA medium in an equal distance comprising five replications and then incubated as stated earlier. Observations were made after 24 hrs of incubation.

Fungicidal sensitivity

Three fungicides namely, Rovral 50WP (Iprodione), CP-Zim 50WP (Carbendazim) and Agromil 72WP (Metalaxyl + Mancozeb) were evaluated on the radial colony diameter of *B. cinerea* and *S. botryosum* against different concentrations viz., 500, 1000 and 1500 mg/L. Different quantities of tested fungicides were added to conical flasks containing double strength (For 1 L: 400 g peeled potato, 30 g agar and 40 g dextrose) PDA medium before its solidification to achieve the proposed concentrations, then shaken gently to ensure equal distribution of the fungicidal concentration and poured into sterilized glass Petri-dishes. The plates were inoculated and incubated as stated previously with five replications in alternating 12 hrs of light and dark phase. After 4 days of incubation, diameter of the colony was measured and per cent of growth inhibition was calculated (Hosen et al., 2010).

Statistical analysis

The data were analyzed statistically using MSTAT-C (Russell, 1994) package programme and means were compared with Duncan's Multiple Range Test (DMRT) with F values at 5% level of probability.

Results and discussion

Comparison of cultural characteristics

Marked variations were observed in colony characteristics i.e. colony color, texture, shape and margin (Figure 1 and Table 2). Light ash colony color was observed in *B. cinerea* and greenish brown in *S. botryosum*. Distinguished comparison was noted on colony texture, velvet colony texture was found in *B. cinerea* whereas effuse type was found in *S. botryosum* isolates. Regular with sector colony shape noted in *B. cinerea* and regular without sector was found in *S. botryosum*. Entire and regular colony margin were found in *B. cinerea* and *S. botryosum*, respectively. *S. botryosum* colonies varied from velvety to cottony in texture with a gray brown or brownish black color, greenish brown to dirty white, regular to irregular shape (Latorre et al., 2002; Hosen et al., 2009). Variation exists in morphology and cultural characteristics of different isolates of *B. cinerea* (Bakr et al., 2002; Hosen et al., 2010b).

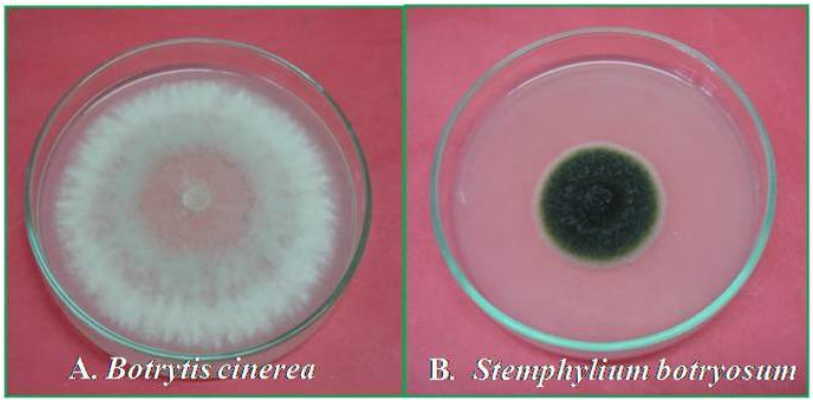

Figure 1. Cultural characteristics of *B. cinerea* (A) and *S. botryosum* (B) at 20°C after 4 days of incubation.

Table 2. Cultural comparison of *B. cinerea* and *S. botryosum* isolates.

Isolates	Cultural characteristics[1]			
	Colony color	**Colony texture**	**Colony shape**	**Colony margin**
B. cinerea	Light ash	Velvet	Regular with sector	Entire
S. botryosum	Greenish brown	Effuse	Regular without sector	Regular

[1] Cultural characteristics were taken on PDA medium after 4 days of incubation at 20°C

Comparison of Conidia

The conidia length of *B. cinerea* range from 7.75 to 15.35 µm and *S. botryosum* from 12.35 to 23.45 µm was observed and shown in Table 3 and Figure 2. The breadth of conidia varied from 5.25 to 11.00 µm in *B. cinerea* and 9.35 to 15.00 µm in *S. botryosum.* It appears that the conidial dimension (16.27 × 9.07 µm) of *S. botryosum* was comparatively larger than *B. cinerea* (11.74 × 8.12 µm). *B. cinerea* conidia size was reported to range from 7.50-12.00 × 6.00-8.25 µm (Hosen et al., 2010a) and *S. botryosum* ranged from 10.00 to 25.00 µm (Hosen et al., 2009, Hosen et al., 2010).

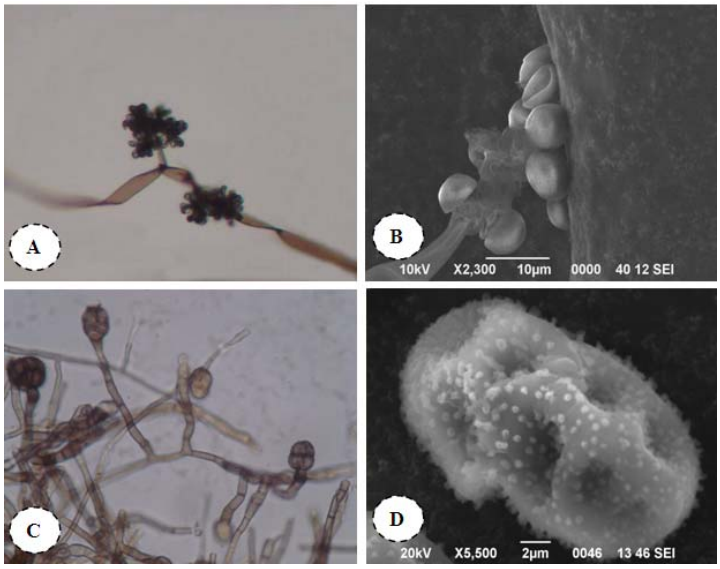

Figure 2. Microscopic view of *B. cinerea* (A&B) and *S. botryosum* (C&D) under light microscope (A &C) and scanning electron microscope (B&D).

Table 3. Conidia dimension of *B. cinerea* and *S. botryosum* isolates after 16 days of incubation at 20°C growth on PDA medium.

Isolates	Conidial dimension (µm)			
	Conidia length[1] (µm)		Conidia breadth[1] (µm)	
	Range	Mean	Range	Mean
B. cinerea	7.75-15.35	11.74	5.25-11.00	8.12
S. botryosum	12.35-23.45	16.27	9.35-15.00	9.07

[1] Means number of 20 times observations for each isolate

Physiological comparison

Effect of temperature

The effect of different temperature viz. 15, 20, 25, 30, 35 and 40°C on the radial mycelial growth of *B. cinerea* and *S. botryosum* are presented in Figure 3. Both pathogens grew well in a wide range of temperature (°C). The highest (84.30 mm) mycelial radial growth of *B. cinerea* was found at 20°C followed by 15°C (64.27 mm) whereas *S. botryosum* at 25°C (43.56 mm) followed by 30°C (37.00 mm). Increasing of temperature levels the radial mycelial growth increases up to 20°C for the *B. cinerea* but *S. botryosum* up to 25°C. No growth was observed for *B. cinerea* at 35°C and 40°C. On the other hand *S. botryosum* growth recorded up to 35°C and no growth was observed at 40°C. A similar result was found by Hosen et al. (2009) for the *S. botryosum*. Temperature 20°C was the optimum for maximum colony diameter of *B. cinerea* and fungal growth completely restricted at 5°C and 35°C (Ahmed et al., 2007; Hosen et al., 2010, 2010a). It is appearing that suitable temperature for *B. cinerea* and *S. botryosum* 20°C and 25°C, respectively.

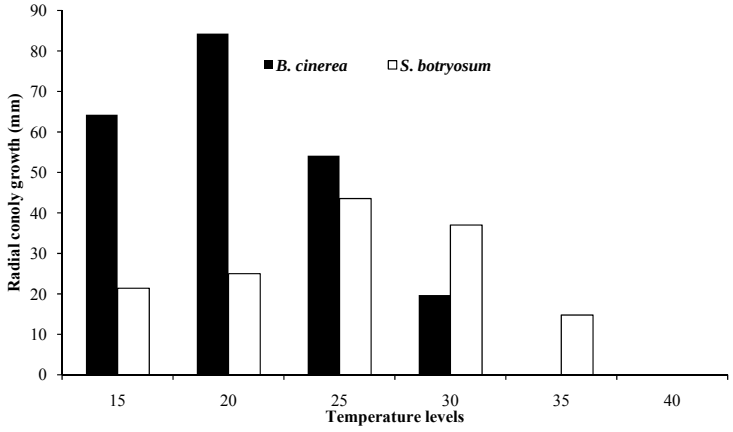

Figure 3. Effect of temperature (°C) on the mycelial radial growth of *B. cinerea* and *S. botryosum*.

Effect of pH

The pH of PDA medium had a significant effect on the radial mycelial growth of two isolates and shown in Figure 4. Both pathogens grew well with a wide range of pH. Luxuriant (77.73 mm) mycelial radial growth of *B. cinerea* was recorded at pH 4.5 followed by 59.33 mm (pH 5.0) whereas *S. botryosum* 40.37 mm (pH 5.5) followed by 38.85 mm (pH 6.0). Increasing pH levels after 4.5 mycelial colony growth of *B. cinerea* decreases but *S. botryosum* mycelial colony growth increases

up to 5.5 and then its mycelial colony growth gradually decreases. The pH 5.5 was an optimum level for the highest mycelial colony growth when worked on *S. lycopersici* (Rajani et al., 1991). The maximum radial colony growth of *B. cinerea* was recoded same pH level (Hosen et al., 2010a). From the findings it is concluded that *B. cinerea* was an extremely acid loving fungus than *S. botryosum*.

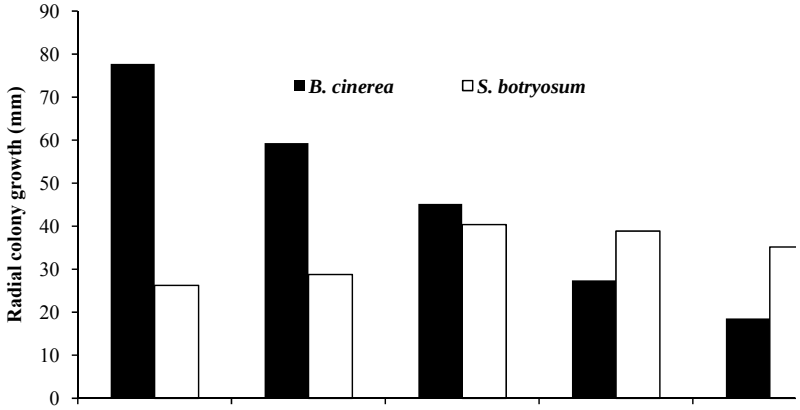

Figure 4. Effect of pH levels on the mycelial radial growth of *B. cinerea* and *S. botryosum*.

Nutrient media effect

The effect of growth media on the radial colony growth of *B. cinerea* and *S. botryosum* is presented in Table 4. The growth media significantly play an important role on the mycelial radial growth and spore production. Both pathogens grew well in a varied culture media. The highest (80.16 mm) colony diameter was observed on CDA medium followed by LDA (79.44 mm) medium and this were statistically similar for *B. cinerea* isolate and the maximal number of spore 2.7×10^4) mL^{-1} was recorded on LDA followed by 2.1×10^4 mL^{-1} on CDA. For *S. botryosum* maximal (57.00 mm) colony diameter and the highest (1.7×10^4) number of spore mL^{-1} was recorded in LDA. This finding is well defined by other researcher. Seven growth media were evaluated by Hosen et al. (2010a) and found that CDA medium was the best for enhancing the radial colony growth and LDA for sporulation of *B. cinerea*. *S. botryosum* grew rapidly on different culture media. It appears that LDA medium is good one for growth and sporulation of both isolates.

Table 4. Comparison of radial mycelial growth and sporulation of *B. cinerea* and *S. botryosum* isolates on different growth media.

Culture Media	*B. cinerea*		*S. botryosum*	
	Colony diameter (mm)[1]	Spores (mL^{-1})*	Colony diameter (mm)[1]	Spores (mL^{-1})*
PDA	77.48 b	1.8×10^4	49.00 b	1.1×10^4
CDA	80.16 a	2.1×10^4	42.87 c	1.3×10^4
BDA	77.08 b	2.0×10^4	40.98 d	1.2×10^4
LDA	79.44 a	2.7×10^4	57.00 a	1.7×10^4
WA	33.36 c	-	26.07 e	-
CV (%)	0.67		1.42	

[1] Means of five replications for each culture medium

* Means number of 20 times observations for each isolate

Antagonist effect on *B. cinerea* and *S. botryosum*

T. harzianum revealed to be an effective antagonist against *B. cinerea* and *S. botryosum* in dual culture technique shown in Figure 5. Incase of *B. cinerea* after 24 hrs radial mycelial growth came in contact with *T. harzianum* hyphae but *S. botryosum* later on and takes time 72 hrs. The hyphal tips of both pathogens become swelled and curved at the point of contact. A thick band of over growing antagonist mycelia was observed within 144 hrs of incubation. The growth of the antagonist become dark green and could not be re-isolated from any part of the over grown petriplates. *T. harzianum* is highly antagonist against *B. cinerea* and parasitized mycelia at the point of contact (Pande et al., 2006; Hosen et al., 2010).

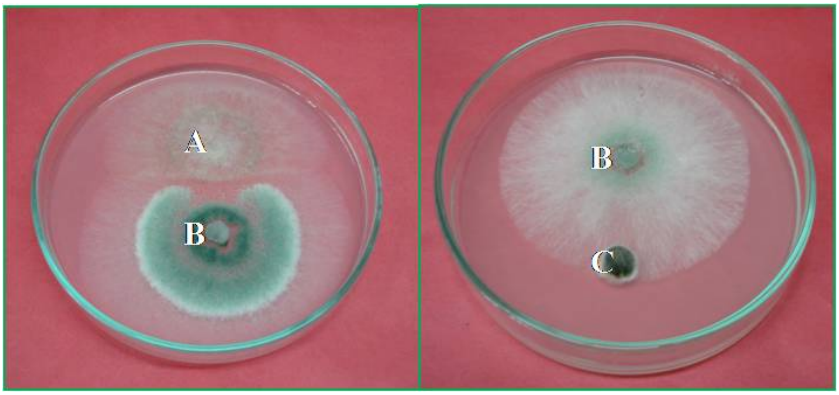

Figure 5. Dual culture technique: A) *B. cinerea*, B) *T. harzianum* and C) *Stemphylium botryosum* after 3 days of incubation at 20°C temperature.

Fungicidal sensitivity

The sensitivity to fungicides of *B. cinerea* and *S. botryosum* were also studied on the basis of their inhibition to mycelial radial growth as shown in Table 5. Both pathogens showed sensitivity to Rovral 50WP and *B. cinerea* also sensitivity to CP-Zim 50WP and 100% inhibition was observed. Agromil 72WP also inhibited the colony diameter over control. Agromil is more sensitive to *S. botryosum* than *B. cinerea*. CP-Zim and Rovral both are effective to control mycelial radial growth of *B. cinerea* at lower concentration (500 mgL^{-1}) than Agromil at higher concentration (1500 mgL^{-1}). Rovral 50WP is similar effective fungicide to control *S. botryosum* at least concentration (500 mgL^{-1}) followed by CP-Zim (9.61 mm) and Agromil (10.00 mm) at the higher concentration (1500 mgL^{-1}) with statistically similar. Six fungicides evaluated and found that Rovral, CP Zim and Bavistin were the best fungicides to inhibit the colony diameter of *B. cinerea* at a lower concentration (500 mgL^{-1}) (Pande et al., 2002). From the *in vitro* test of different fungicides the best control of radial colony diameter of *S. botryosum* was achieved by using Rovral 50WP at 2000 mgL^{-1} concentration (Huq, 2003).

Conclusion

From the present study it is concluded that both isolates of *B. cinerea* and *S. botryosum* showed variations in cultural and morphological aspects. Rovral from the iprodione group and antagonist *T. harzianum* have the potentials to suppress the radial colony growth of *B. cinerea* and *S. botryosum* which are causes of botrytis gray mold in chickpea and stemphylium blight in lentil. Further studies will be needed for consecutive years to validate the activity of Rovral 50WP in farmers' field.

Table 5. Sensitivity to fungicides at different concentrations of *B. cinerea* and *S. botryosum* isolates.

Fungicides	Concentration (mgL^{-1})	*B. cinerea*		*S. botryosum*	
		Colony diameter (mm)[1]	Per cent (%) inhibition of colony diameter	Colony diameter (mm)[1]	Per cent (%) inhibition of colony diameter
Control (sterile water)	0	80.00 a (8.98)	-	62.56 a (7.91)	-
Agromil 72WP (Metalaxyl+Mancozeb)	500	30.12 b (5.48)	62.35	15.05 c (3.88)	75.94
	1000	25.43 c (5.04)	68.21	13.69 d (3.70)	78.12
	1500	19.71 d (4.44)	60.29	10.00 e (3.17)	84.01
Rovral 50WP (Iprodione)	500	0.00 e (0.71)	100	00.00 f (0.71)	100
	1000	0.00 e (0.71)	100	00.00 f (0.71)	100
	1500	0.00 e (0.71	100	00.00 f (0.71)	100
CP-Zim 50WP (Carbendazim)	500	0.00 e (0.71)	100	20.01 b (4.49)	68.01
	1000	0.00 e (0.71)	100	14.66 c (3.83)	76.57
	1500	0.00 e (0.71	100	9.61 e (3.10)	84.64
CV (%)	-	0.64	-	1.00	-

[1]Means of five replications for each concentration

Figures within the parenthesis are square root transformed values

Numbers with dissimilar letters differ significantly at 1% level according to Duncan's Multiple Range Test (DMRT)

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