NBU Journal of Plant Sciences
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Vol. 9, No. 1 March 2015 p. 87-93
ISSN No. 09746927

Influence of culture media and environmental factors on mycelial growth and sporulation of *Alternaria alternata* (Fr.) Keissler causing leaf blight disease of niger (*Guizotia abyssinica* (L.f.) cass)

Aniruddha Saha[1*], Chandrani Choudhuri[1] and Dipanwita Saha[2]

[1]*Molecular Plant Pathology and Fungal biotechnology laboratory, Department of Botany, University of North Bengal, Siliguri-734013, India*

[2]*Department of Biotechnology, University of North Bengal, Siliguri-734013, India*

ABSTRACT

Alternaria alternata is isolated from naturally infected niger leaf for their morphological characteristics, mycelia growth and sporulation, spore germination in different culture media and environmental conditions. RMA was best for both growth and sporulation. Excellent sporulation was observed ion PCA. PDB supported best growth among the liquid media tested. Highest mycelia dry weight was recorded at 28°C and pH 6.5. Among several carbon sources tested, Mannitol showed optimum growth and sporulation while peptone produced maximum growth among the tested organic nitrogen sources. The present study will help to maintain the fungus in the laboratory condition for preparation of inoculums for different studies related to the control measures of the pathogen.

Key words: *Alternaria alternata*, Niger, growth, sporulation

Introduction

Niger [*Guizotia abyssinica* (L.f.) cass] is an oil seed crop and its seeds contain clear, excellent, vegetable edible oil. India is one of the important niger producing countries in the world. The oil is slow drying and it contributes about 3% of India's total oil seed production (Getinet and Sharma, 1996). In India, niger oil is frequently used as a substitute for sesame oil (Weiss, 1983). Leaf blight of niger caused by *Alternaria* sp. is the most serious disease of niger (Gebre-Medhin and Mulatu, 1992; Getinet and Sharma, 1996). The pathogen attacks the leaves. In later stage leaves of whole plant are blighted and become dark brown in colour. Fungi get food and energy from the substrate where they grow. to know their food requirements it is necessary to culture the fungus in artificial media components at optimum physical para meters that lead to maximum sporulation (Kim *et al.*, 2005; Saxena *et al.*, 2001; Saha *et al.*, 2008). The present study was under taken to assess the morphological characteristics of the fungi, the effect of different culture media, incubation periods, carbon sources, nitrogen sources, temperature and pH on the mycelia growth and sporulation of fungus *A. alternanta*, a pathogen of Niger.

*Corresponding Author:
Email: asahanbu@yahoo.co.in

Materials and Methods

Fungal culture

The fungal culture, *Alternaria alternata* (Kr.) Keissler was isolated from naturally infected leaves of niger plants from the Barobisha region of Jalpaiguri district in West Bengal. Following verification of Koch's postulates, the organism was identified in the laboratory and our identification was further confirmed by the division of plant pathology, IARI, New Delhi (Identification no. 6250.05).

Observation of morphology

The pathogen was cultured in Potato carrot agar (PCA) and Potato dextrose agar (PDA) for ten days. A bit of fungal mycelia were taken from PDA slant and was placed on a grease free slide and stained with lactophenol and cotton blue. For study of spores, brownish mass of spores produced on the surface of the PCA slant were carefully taken out, placed on a slide and stained with lactophenol and cotton blue. Length and breadth of the spores, breath of mycelia

were measured by stage micrometer under light microscope. The detailed morphology of the fungus was noted.

Influence of different culture media

Growth and sporulation of the pathogen was first studied in nine different solid media *viz.* Richard's agar medium (RMA), Corn meal agar (CMA), Potato dextrose agar (PDA), malt extract agar (MEA), Oat meal agar (OMA), Potato carrot agar (PCA), Yeast extract mannitol agar (YEMA) and Czapak Dox Agar (CDA). Sterile petriplates (70 mm diameter) containing 10 ml of the different sterile medium were inoculated with 4 mm mycelial block. The mycelial blocks were taken from advancing zones of a PDA plate of *A. alternata* culture. The petriplates were incubated at 28±1°C for 10 days. Radial growth of mycelia was measured at 2 days intervals until 10 days. To assess the mycelia growth in liquid medium on mycelial agar block (4 mm) was transferred to a conical flask of 250 ml, containing 50 ml of liquid medium. Then Potato dextrose broth (PDB), Potato carrot broth (PCB) and Richard's medium (RM) were incubated at 28 ± 1°C. The fungal mycelia were strained through double layered cheese cloth after 5, 10, 15, 20 and 25 days of incubation and then blotted by a blotting paper and dried in hot air oven at 60°C for 24 hours. Finally, mycelial mats were cooled and dry weight was noted.

Assessment of spore germination

For assessing the spore germination the fungus was initially cultured in PCA medium for 15 days at 28 ± 1°C. Distilled water was added aseptically in the fungal culture tube, shaken and stained through a muslin cloth. The filtrate was used as spore suspension. Concentration of the spores was measured by haemocytometer count and spore concentration was adjusted by adding sterile distilled water. Thirty microliter spore suspension (1×10^6 ml) was placed on slides and allowed to incubate for 2, 4, 6, 8, 10 and 12 hours in a humid chamber at 28 ± 1°C. The slides were stained with cotton blue-lacto phenol and observed under light microscope. Finally the percent spore germination [(no. of germination spores/ no. of spores counted) ×100)], average germ tube length in each case were calculated.

Influence of different pH

Potato dextrose broth (PDB) was adjusted to pH 5, 5.5, 6, 6.5, 7 and 8 respectively by adding 1(N) NaOH or 1(N) HCL drop-wise into the medium before sterilization. After adjusting the pH in PDB the media (50 ml in 250 ml Erlenmeyer flask) was sterilized. Media of different pH were inoculated separately by 4 mm mycelial discs of *A. alternata,* cut from advancing zone of petriplate and incubated at 28 ± 1°C. Mycelial dry weights were noted after 5,10,15,20 and 25 days of incubation.

Influence of different temperature

To assess the growth of *A. alternata* at different temperatures, the fungus was inoculated in sterile PDB media (50 ml in 250ml Erlenmeyer flask) and was inoculated at different temperatures *viz.* 10°C, 15°C, 20°C, 25°C, 28°C, 30°C, 35°C, and 40°C. After 5, 10, 15, 20 and 25 days of incubation, mycelia were harvested, strained through muslin cloth, blotted and finally dried at 60°C. Mycelial dry weights were noted. Similarly, to study the influence of different temperature on spore germination, spore suspension of *A. alternata* was prepared. Sterile distilled water was added to attain optimum concentration (1×10^{-6} ml) of spores. Spore suspension drops (30 µl) were placed in different slides in triplicates and incubated at different temperatures (10, 15, 20, 25, 28, 30, 35 and 40°C) for 2, 4, 6, 8, 10 and 12 hours.

Effect of different carbon sources on growth and sporulation

To study different carbon sources for the optimum growth and sporulation of *A. alternata,* a basal medium (Glucose 1%; Asparagine 0.2%; $MgSO_4$, $7H_2O$ 0.05%; Zn^{++}, Mn^{++} and Fe^{+++} 2µg/ml) was used for the purpose. The different carbon sources tested were glucose, sorbitol, sucrose, fructose, and mannitol. The equivalent amount of carbon present in 1% glucose was used as standard and added separately to the basal medium. The medium (50ml) was taken in

250 ml Erlenmeyer flasks and sterilized at 15 lb. p.s.i. for 15 minutes. The media was inoculated by the pathogen using 4mm mycelial discs in PDA and incubated at 28 ± 1°C for 5, 10, 15, 20 and 25 days. In control sets, no carbon sources were used in the basal medium. After incubation for the specified time periods, the mycelia were harvested, dried at 60°C and weighed. After each incubation period before harvest of mycelia, sporulation of the fungus was also recorded.

Effect of different nitrogen sources on growth and sporulation

To assess the mycelia growth and sporulation of *A. alternata* on different nitrogen sources (both organic and inorganic), modified Asthana and Hawker's medium 'A' (Glucose 10 g; KNO_3 3.5; KH_2PO_4 1.75g; $MgSO_4$, $7H_2O$ 0.75; Agar agar 20 g and distilled water 1L) without agar was used as basal medium. The quantity of various nitrogen sources was so adjusted to give the same amount of nitrogen as furnished by 3.5 g KNO_3 in the basal medium. The quantity of various nitrogen sources was prepared and dispersed separately in 50 ml medium and was taken in 250 ml Erlenmeyer flasks. The flasks were sterilized and the flasks were inoculated by test fungi using 4 mm mycelia discs in PDA and were incubated at 28±1°C for 5, 10, 15, 20 and 25 days. In control set no nitrogen source was provided in the basal medium. After specified incubation periods, sporulations were checked and were recorded. Harvested mycelia were dried at 60°C and weighed.

Statistical Analysis

Statistical analysis was done with the help of statistical package for the Social Sciences (SPSS), version 11.0, SPSS Inc. Illinois.

Result and Discussion

Microscopic study of the fungus revealed that mycelia were hyaline in colour but on maturity it becomes gray in colour. Conidia of the fungus were obclavate to beaked and brownish in colour having transverse and longitudinal septa (Fig.1a & b). Conidia were produced from simple

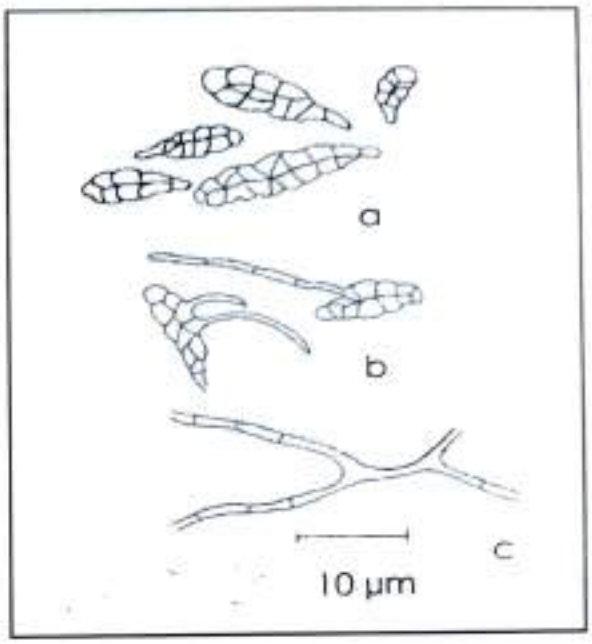

Fig. 1: (a) Spores of *A. alternata*. (b) Germinating spores with germ tubes. (c) *A. alternata* with septa and branching.

septate conidiophores in simple or branched acropetal chains. The length and breadth of mature hyphae ranged between 3-5 µm (Fig. 1c). Similar observations regarding conidial size and shape have been reported by Maiti *et al.* (2006).

From the result presented in Table 1, it was evident that RMA (Richard's agar) was best for both growth and sporulation of *A. alternata*. After 10 days of incubation on RMA, radial growth of mycelia was 90 mm in diameter and sporulation was also good. Saha *et al* (2009) reported that best growth and sporulation of *C. gloeosporioides* was observed on PDA and RMA, which is in agreement with our findings. In PDA sporulation was comparatively less, although good growth of mycelia was evident. Our observations were also related with that Karlatti and Hiremath (1983), who found best mycelia growth of *A. zinniae* on leaf extract and potato dextrose agar media whereas *A. helianthi* showed less sporulation on potato dextrose agar (Allen *et al.* 1983; Mukewar *et al.* 1974). Excellent sporulation of *A. alternata* was observed in PCA with moderate growth of mycelia (69.33 mm in diameter). Similar results

Table 1: Mycelia growth and sporulation of *Alternaria alternata* in different solid media.

Medium for growth	Radial growth (mm)* and sporulation									
	2		4		6		8		10	
	Growth	Sp**	Growth	Sp**	Growth	Sp**	Growth	Sp**	Growth	Sp**
RMA	16.66±0.34	–	36.66±0.92	–	57.50±0.76	+	76.83±0.55	++	90.00±0.58	+++
CMA	28.16±0.57	–	14.83±0.17	–	56.16±0.74	–	70.16±0.45	++	84.16±0.45	++
PDA	13.83±0.50	–	32.40±0.95	–	50.66±0.61	–	65.83±0.44	–	82.16±0.84	+++
MEA	12.00±0.29	–	27.66±0.88	–	48.16±0.93	–	64.33±0.67	++	80.33±0.60	++
OMA	11.50±0.50	–	26.33±0.67	–	42.83±0.44	–	60.00±0.85	++	76.16±0.72	++
PCA	11.83±0.44	–	26.16±0.72	–	41.66±0.73	+	55.50±0.36	++	69.33±0.12	++++
YEMA	12.33±0.34	–	26.33±0.67	–	40.33±0.57	+	52.00±0.87	++	69.83±1.83	+++
CDA	10.33±0.17	–	15.33±0.67	–	32.16±1.09	–	31.00±1.00	+	83.33±0.88	++

*Mean of three replications, **Sp=Sporulation, –=Nil, +=poor, ++=fair, +++=good, ++++=excellent. Data after ± represent standard error values. Incubation temperature=28±1°C, CD= critical difference. PDA= Potatto dextrose agar, OMA= Oat meal agar, CDA= Czapeak Dox Agar, RMA= Richard's agar medium, YEMA= Yeast extract manitol agar, MEA= Malt extract agar, PCA= Potato carrot agar, CMA= Corn meal agar

were obtained by Prasad *et al.* (2008), who reported that growth and sporulation of *A. helianthi*, a pathogen causes leaf blight disease in sunflower, was maximum in sunflower leaf extract medium followed by carrot agar medium.

Maximum growth of *A. alternata* was recorded at 480 mg after 20 days of inoculation in PDB medium (Fig. 2). In PCB, mycelia dry weight was found 240 mg after 20 days of incubation but in RM mycelia growth was poor. After 20 days, mycelia dry weight declined due to autolysis and depletion of media.

It was found that *A. alternata* was able to grow within a wide range of pH, from 5.0 to 8.0 (Table 2). The fungus however, failed to grow in

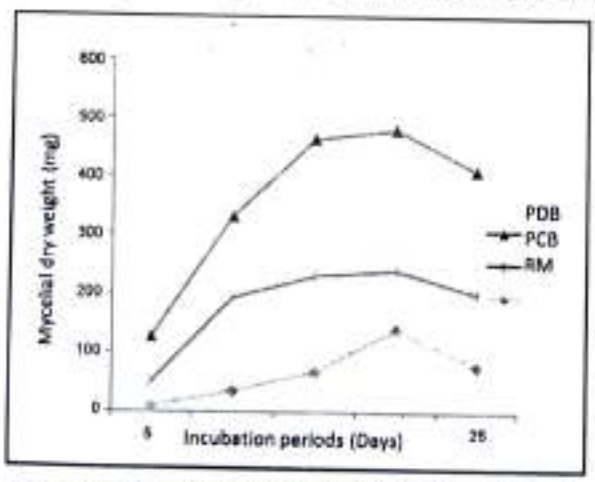

Fig. 2: Growth of *Alternaria alternata* after different incubation periods in three different liquid media. PDB= Potato dextrose broth; PCB= Potato carrot broth; RM= Richard's medium.

alkaline environment, beyond pH 8.0. Highest growth (473.4 mg) was recorded at pH 6.5. Poor growth was observed at pH 5.0 and at pH 8.0. The result indicated that slightly acidic pH to neutral pH was optimum for the growth of *A. alternata*. In a similar study, Thompson-Eagle (1989) also reported similar results where optimum pH for growth and sporulation of *A. zinnia* was found in the range of pH 6.0-6.5.

Results presented in figure (Table 3) indicate that *A. alternata* was capable of growing at temperatures that range between 10-40^0 C. Best growth was recorded at 28°C while no growth was observed at temperatures 40°C and above. These results were in agreement to those reported by Saha *et al.* (2008) who observed that optimum growth of *L. theobromae* at 28°C and no growth were noted at above 40°C. They were also in agreement with Alam *et al* (2001). Eng *et al* (2003) also reported similar observations when he studied the effect of temperature on growth characteristics of *Botryodiplodia theobromae*. He stated that the growth density and radial velocity was affected at temperature above 40°C.

During the present study, germination of spores began after 2 hours of incubation and all the spores were germinated within 12 hours (Fig. 3). Percent of germination of spores and germ tube length were recorded as 100% and 340.1 µm respectively after 12 hours of incubation. Similar results were observed by Saha *et al.* (2009), who studied the conidial

Table 2: Effect of different pH on growth of *Alternaria alternata*

pH	Mycelia dry weight (mg)				
	5 days	10 days	15 days	20 days	25 days
5.0	52.6±4.32	109.3±4.08	252.6±3.06	306.4±4.94	287.1±3.52
5.5	64.5±2.06	165.8±2.83	286.3±2.00	335.6±2.40	298.4±3.66
6.0	111.0±3.55	225.3±2.64	361.1±4.50	466.3±2.04	415.7±2.86
6.5	125.7±2.80	308.6±3.43	445.5±2.74	473.4±3.22	422.6±2.00
7.0	103.2±4.09	195.7±4.72	296.8±1.90	413.3±4.56	392.2±2.49
8.0	24.4±4.08	55.2±2.40	92.4±1.01	102.1±3.13	73.3±2.54
CD (5%)	7.12	8.53	7.63	9.50	2.60

*Mean of 3 replications: Data after ± represent standard error values. Dry weight of inoculating mycelia block was 10mg

Table 3: Effect of different temperature on mycelia growth of *Alternaria alternata*

Temperature (°C)	Mycelial dry weight (mg)*				
	5 days	10 days	15 days	20 days	25 days
10	11.2±3.24	15.1±3.33	24.8±2.36	29.5±1.53	19.7±3.47
15	16.6±2.77	22.1±3.86	32.4±3.95	38.3±1.27	29.2±4.87
20	49.0±4.60	98.7±1.17	152.3±4.41	208.4±3.84	191.3±2.27
25	89.8±4.03	182.3±3.19	241.7±3.92	369.5±4.46	345.2±4.38
28	120.3±2.51	325.1±2.36	462.7±3.79	483.4±3.88	419.5±1.40
30	70.6±3.48	151.0±4.35	189.4±2.04	224.2±3.80	213.0±4.97
35	20.2±3.56	44.7±2.55	82.4±3.39	165.6±1.54	110.5±3.48
40	15.3±3.55	34.9±2.77	55.8±3.65	64.2±2.95	51.0±3.14
CD (5%)	1.74	4.11	3.97	4.64	4.26

*Mean of 3 replications: Data after ± represent standard error values. Dry weight of inoculating mycelia block was 10mg

Table 4: Effect of different carbon sources on the growth and sporulation of *A. alternata*

Carbon source	Incubation period (Days)									
	5D		10D		15D		20D		25D	
	Mwt. (mg)	Sp**	Mwt. (mg)	Sp**	Mwt. (mg)	Sp**	Mwt. (mg)	Sp**	Mwt. (mg)	Sp**
Glucose	50.00±0.89	–	100.00±1.17	–	110.00±0.80	+	125.00±0.90	++	100.00±1.29	+++
Sucrose	110.60±0.99	–	200.00±1.15	+	340.40±1.14	++	410.60±0.66	+++	385.00±0.68	++++
Fructose	100.00±1.04	–	190.10±0.95	–	310.2±1.25	+	420.40±0.76	++	390.00±1.46	+++
Sorbitol	80.00±0.81	–	170.60±0.59	–	280.3±0.85	+	300.40±0.83	++	310.00±0.87	++
Mannitol	110.50±0.45	–	240.80±0.85	+	380.90±0.87	++	500.00±1.29	++++	495.80±0.31	+++
Control***	10.30±1.08	–	12.60±0.95	–	13.90±0.03	–	14.10±0.80	–	13.11±0.49	–
CD (5%)	2.03		2.01		12.55		1.66		2.58	

*Mean of three replications. Sp=Sporulation, –=Nil, +=poor, ++=fair, +++=good, ++++=excellent. ***Control Basal medium without any carbon source. Mwt. (mg)= Mycelial dry weight in mg; Data after ± represent standard error values.

germination and appresoria formation in the brinjal anthracnose causing fungus *C. gloeosporoides*. They reported germination and appresoria formation within a period of 8 hours. Spore germination of *Bipolaris carbonum* was also observed to begin within 2-4 hours *in vitro* by Saha and Chakraborty (1990). Effect of temperature on spore germination was studied and results are presented in Fig. 4. Result revealed that spore germination was optimum at

28°C (100% after 12 hours) whereas 2.45% spore germination occurred at 10°C after 12 hours but no germination occurred at 40°C or above.

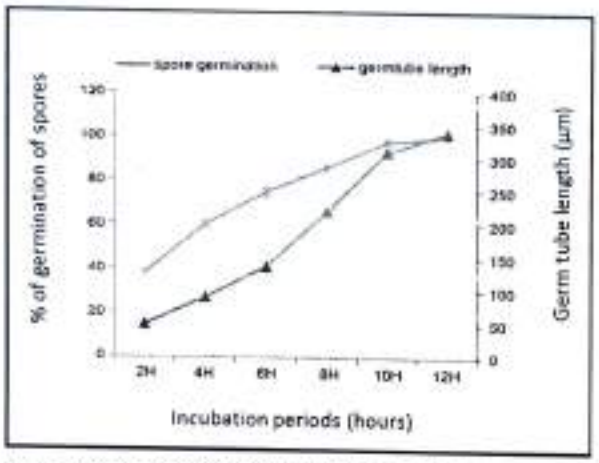

Fig. 3: Effect of different incubation periods on spore germination and germ tube elongation.

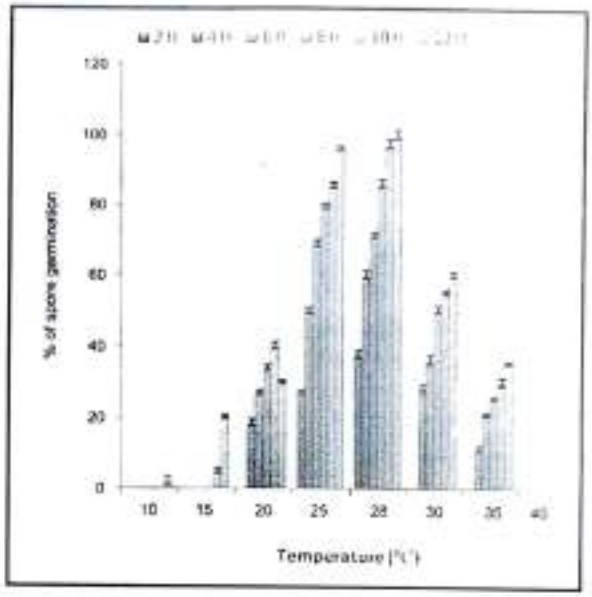

Fig. 4: Effect of different temperatures on spore germination of *A. alternate*.

Nutritional requirements of the pathogen were studied and it was concluded that mannitol was the best carbon source for optimum growth and sporulation of *A. alternata* (Table 4). Next to mannitol, good growth was observed in fructose and sucrose respectively. When nitrogen sources were tested, peptone produced best growth and sporulations, while potassium nitrate showed best growth among the tested inorganic nitrogen sources (Table. 5). Several workers (Devdath *et al.*, 1977; Jadhav *et al.*, 2002; Jash *et al.*, 2003) studied the influence of various carbon and nitrogen sources on fungal metabolism. Jadhav *et al.*, (2003) observed that highest mycelia growth and sporulation was recorded when mannitol was used as carbon source and peptone was used as nitrogen source. Wu and Wu (2003) observed that *Alternaria protenta,* a pathogen of sunflower showed abundance of sporulation on glucose

Table 5: Effect of different nitrogen source on the growth and sporulation of *A. alternata*

Carbon source	Incubation period (Days)									
	5D		10D		15D		20D		25D	
	Mwt. (mg)	Sp**	Mwt. (mg)	Sp**	Mwt. (mg)	Sp**	Mwt. (mg)	Sp**	Mwt. (mg)	Sp**
					Inorganic					
Potassium nitrate	150.00±1.00	-	220.50±0.66	+	265.2±0.70	+++	286.00±0.98	+++	250.00±1.32	+++
Sodium nitrate	130.60±0.74	-	186.20±0.76	+	210.40±0.90	++	275.00±0.92	++	225.00±0.76	++
Ammonium sulphate	120.80±0.53	-	163.40±0.83	+	198.00±0.81	++	245.10±0.72	++	215.00±0.72	++
					Organic					
Peptone	230.10±1.27	-	298.60±0.61	+	340.00±1.53	++	360.00±1.25	++	310.00±1.15	++
Yeast extract	210.40±1.00	-	250.00±1.26	+	290.30±1.12	++	340.00±1.44	+++	300.00±1.15	+++
Beef extract	115.00±0.81		165.00±1.63	-	200.60±0.70	++	235.40±0.72	++	215.00±0.72	++
Control***	5.90±0.32	-	910±0.92	-	10.60±0.31	-	11.50±0.55	-	14.80±0.67	-
CD (5%)	1.58		2.05		1.32				0.77	

Mwt. (mg)= Mycelial dry weight in mg; *Mean of three replicates. Data after ± represent standard error values. Sp=Sporulation, -=Nil, +=poor, ++=fair, +++=good, ++++=excellent. ***Control Basal medium without any carbon source.

peptone agar but not on dextrose nitrate agar.

The result of the present study is crucial for further studies of the fungus as a pathogen of niger and supplemented media as suggested here may be utilized for inoculum production.

Acknowledgements

Authors are thankful to Dr. Prameela Devi , Dr. Nita Mathur and Dr. P. N. Chowdhury, ITCC, IARI, Pusa, New Delhi for identifying the organism and also thankful to Dr. Mehbub Isha for his assistance in collection of naturally infected plant materials.

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