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Influence of culture media and environmental factors on mycelial growth and sporulation of *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl

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Abstract: *Lasiodiplodia theobromae, a common tea (Camellia sinensis) pathogen, usually does not sporulate or sporulates poorly in common media, which makes spore production difficult. In this study the effects of culture media, carbon source, nitrogen source, temperature, pH and light on mycelial growth and sporulation were evaluated. Among several carbon sources tested, glucose and sucrose were found superior for growth. Potassium nitrate supplemented media showed maximum growth amongst the tested inorganic nitrogen sources while peptone produced maximum growth among the tested organic nitrogen sources. Tea root extract supplemented potato dextrose agar medium was found to be the most suitable for mycelial growth and sporulation of L. theobromae. The fungus grow at temperatures ranging from 4^0 to 36^0C, with optimum growth at 28^0C and no growth was noted at 40^0C. There was no significant effect of different light period on growth of L. theobromae, but light enhanced sporulation. The fungus grow at pH 3.0 -8.0 and optimum growth was observed at pH 6.0. Tea root extract supplemented potato dextrose agar medium with pH 6.0 was the most suitable for production of conidia of L. theobromae at 28^0C. Hence this media may be recommended for inoculum production for further studies.*

Key words: *Lasiodiplodia theobromae, Camellia sinensis, Sporulation*
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Introduction

Tea occupies an important position among the plantation crops and is widely cultivated in the sub Himalayan region of northeast India. *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl is a common pathogen of tea, causing diplodia disease, which affects roots, stems and leaves of tea plants of all ages (Sarmah, 1960; Chandramouli, 1999; Singh, 2005). It particularly causes severe damage in the nurseries of tea gardens where seedlings are raised. For conducting studies on behaviour of the pathogen, it is necessary to culture the fungus in artificial media and produce inoculum in the form of spore suspension. Several workers have recognized the importance of spores as inoculum and studies have been conducted on the effects of various media components along with important physiological parameters that lead to maximum sporulation (Kim *et al.*, 2005; Saxena *et al.*, 2001). During our studies with the pathogen, it was found that the fungus sporulates very poorly in commonly used media like potato dextrose agar (PDA) and oat meal agar (OMA) making inoculum production difficult. Studies on the environmental and nutritional requirements of *L. theobromae* are limited and no such information is available on the fungus as a tea pathogen. The present study was therefore undertaken to study the effects of culture media, carbon source, nitrogen source, temperature, pH and light on mycelial growth and sporulation production of *L. theobromae*.

Materials and Methods

Fungal culture: The fungal culture, *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl was isolated from naturally infected leaves and collar regions of young tea plants in the nursery of Taipoo Tea Estate in Siliguri, West Bengal. Following verification of Koch's postulates, the organism was identified in the laboratory and was further confirmed by the Division of Plant Pathology, IARI, New Delhi (ITCC No. 4151.2K).

Influence of different culture media: Growth and sporulation of the pathogen was first studied in potato dextrose broth (PDB), oat meal broth (OMB), potato carrot broth (PCB), yeast extract glucose broth (YEGB) and malt extract broth (MEB). Media (40 ml) were prepared in 250 ml conical flasks and 3 flasks of each media were inoculated with 4 mm mycelial discs taken from advancing zones of mycelia of *L. theobromae* cultured in PDA plates. The flasks were incubated at 28 ± 1°C. Sporulation and mycelial dry weight were recorded at 5 days intervals until 25 days. Subsequently all media were supplemented with tea root extract and similar studies were conducted. For preparation of root extract, tea roots were washed thoroughly first with tap water and then with distilled water, patted dry with blotting paper and weighed. Clean fresh roots were homogenized in distilled water, centrifuged at 3000x g for 10 min and the supernatant was added to the media to get a final concentration of 5 g fresh weight of tea root in 100 ml media.

Assessment of growth and sporulation: For assessing the growth of the fungus, mycelial mat was harvested after the required incubation period, strained through cheesecloth, blotted and taken in preweighed aluminium foil. The foils were dried at 60°C for 24 hr and dry weight was recorded. For estimation of sporulation, a small portion of mycelia was mounted in lactophenol and observed under microscope.

Influence of light conditions: The fungus was allowed to grow in 40 ml tea root extract supplemented PDB medium taken in 250 ml

Table - 1: Mycelial growth and sporulation *of Lasiodiplodia theobromae* virulent isolate in different liquid media

Medium of growth	Incubation period									
	5 days		10 days		15 days		20 days		25 days	
	Mwt* (mg)	Spn**	Mwt (mg)	Spn	Mwt (mg)	Spn	Mwt (mg)	Spn	Mwt (mg)	Spn
PDB-RE	150.0 ±5.77	-	200.0 ±11.54	+	350.0 ±5.77	++	360.0 ±8.35	+++	335.0 ±9.45	++++
PDB	145.0 ±2.88	-	225.0 ±8.88	-	280.0 ±5.56	-	320.0 ±7.48	+	295.0 ±4.56	+
OMB-RE	120.0 ±5.77	-	125.0 ±2.89	+	150.0 ±5.64	+	185.0 ±6.45	++	220.0 ±5.87	++
OMB	85.00 ±3.66	-	95.00 ±3.66	-	100.0 ±4.56	-	135.0 ±4.86	-	165.0 ±7.45	-
PCB-RE	25.00 ±2.88	-	85.00 ±2.00	-	125.0 ±4.64	+	170.0 ±4.84	++	210.0 ±7.56	++
PCB	15.00 ±2.89	-	30.00 ±2.66	-	95.00 ±3.64	-	120.0 ±4.98	-	165.0 ±5.43	-
YEG-RE	140.0 ±11.4	-	170.0 ±7.54	-	225.0 ±8.76	+	295.0 ±7.27	+	320.0 ±7.43	+
YEG	120.0 ±5.77	-	135.0 ±5.27	-	270.0 ±5.77	-	305.0 ±9.45	-	295.0 ±7.88	-
MEB-RE	145.0 ±3.66	-	185.0 ±5.66	-	230.0 ±6.67	+	280.0 ±8.67	++	315.0 ±9.28	+++
MEB	122.0 ±5.92	-	165.0 ±8.67	-	210.0 ±6.56	-	285.0 ±8.56	-	324.0 ±9.41	-

*Mwt = Mycelial dry weight, Mean of 3 replicates, **Spn = Sporulation, - = Nil, + = Poor, ++ = Fair, +++ = Good, ++++ = Excellent, RE = Tea root extract supplemented, PDB = Potato dextrose broth, OMB = Oatmeal broth, PCB = Potato carrot broth, YEG = Yeast extract glucose broth, MEB = Malt extract broth, ± = Standard error values

Table - 2: Effect of different light condition on growth and sporulation of *Lasiodiplodia theobromae* virulent isolate in PDB-RE medium

Different light conditions	Incubation period									
	5 days		10 days		15 days		20 days		25 days	
	Mwt* (mg)	Spn**	Mwt (mg)	Spn	Mwt (mg)	Spn	Mwt (mg)	Spn	Mwt (mg)	Spn
Light (24 hr light)	165.0 ± 6.75	-	210.0 ± 8.56	+	265.0 ± 5.89	++	290.0 ± 7.89	+++	325.0 ± 9.46	++++
Dark (24 hr dark)	175.0 ± 4.65	-	225.0 ± 7.87	-	260.0 ± 5.34	-	320.0 ± 9.45	+	335.0 ± 8.61	++
Normal (alternate 12 hr light and 12 hr dark)	180.0 ± 6.76	-	230.0 ± 6.56	-	270.0 ± 6.73	+	340.0 ± 8.46	++	320.0 ± 9.84	++

*Mwt = Mycelial dry weight, Mean of 3 replicates, **Spn = Sporulation, - = Nil, + = Poor, ++ = Fair, +++ = Good, ++++ = Excellent
PDB-RE = Tea root extract supplemented potato dextrose broth, ± = Standard error values

Erlenmeyer flasks under three different light conditions: Alternate 12 hr light and dark, 24 hr light and 24 hr dark periods. The flasks were inoculated in triplicate with 4 mm mycelial discs and the extent of sporulation and mycelial dry weight were recorded after 15 days of incubation at 28 ± 1°C.

Influence of different pH: Tea root extract supplemented PDB medium (40 ml taken in 250 ml Ehrlenmeyer flasks) was adjusted to different pH, ranging from 2.5 to 8.5, by adding 1N HCl or 1N NaOH. Following inoculation with 4 mm mycelial discs, the flasks were incubated at 28 ± 1°C. Each treatment was replicated thrice. Mycelial dry weight and sporulation was recorded after 15 days.

Influence of temperature: Tea root extract supplemented PDB (40 ml) in 250 ml conical flasks were inoculated with 4 mm mycelial discs and incubated at different temperatures (4°C-40°C at intervals of 4°C) taking 3 flasks for each temperature condition. Mycelial dry weight was recorded after 15 days of incubation.

Effect of different carbon sources on growth and sporulation: To study the effect of different carbon sources on mycelial growth and sporulation of *L. theobromae*, a basal medium (glucose 1%; asparagine 0.2%; KH_2PO_4 0.1%; $MgSO_4$. $7H_2O$ 0.005%; Zn^{++}, Mn^{++} and Fe^{++} 2 µl/ml) was used in which the fungus was cultured and glucose was replaced individually by the different carbon sources tested in equivalent quantities of carbon as present in 1% glucose. Each flask containing 40 ml basal media substituted with different carbon sources was inoculated in triplicate with 4 mm mycelial discs and incubated at 28 ± 1°C. Control flasks did not contain any carbon compound. Sporulation and mycelial dry weight were recorded at 5 days intervals until 25 days.

Effect of different nitrogen sources on growth and sporulation: Modified Asthana and Hawker's basal medium 'A' (glucose 10 g; KNO_3 3.5 g; KH_2PO_4 1.75 g; $MgSO_4$. $7H_2O$ 0.75 g; distilled water 1l) was used for studying the effect of different nitrogen sources on the mycelial growth and sporulation of the fungus. The quantity of various nitrogen sources was adjusted by replacing KNO_3 so as to give the same amount of nitrogen as furnished by 3.5 g KNO_3 in the basal medium. The basal medium only served as control. Sterilized media were inoculated with 4 mm mycelial discs and incubated at 28 ± 1°C. Extent of sporulation and mycelial dry weight were recorded at 5 days intervals until 25 days.

Results and Discussion

From the results (Table 1) it was evident that PDB-RE (potato dextrose broth supplemented with root extract) was best for both

Table - 3: Effect of different carbon sources on growth and sporulation of *Lasiodiplodia theobromae*

Carbon source	Incubation period									
	5 days		10 days		15 days		20 days		25 days	
	Mwt* (mg)	Spn**	Mwt (mg)	Spn	Mwt (mg)	Spn	Mwt (mg)	Spn	Mwt (mg)	Spn
Glucose	220.0 ±0.81	-	280.6 ±0.72	-	302.5 ±1.04	+	288.8 ±0.43	+	266.0 ±1.00	+
Sucrose	205.2 ±0.61	-	255.4 ±0.80	+	300.0 ±0.76	+	292.5 ±0.87	+	274.0 ±0.50	+
Mannitol	70.0 ±1.15	-	125.1 ±0.58	-	156.3 ±0.65	+	186.0 ±0.58	+	175.0 ±0.87	+
Galactose	115.0 ±0.80	-	176.8 ±0.99	-	228.0 ±1.15	+	251.0 ±1.03	+	244.8 ±0.92	+
Control	8.3 ±0.43	-	10.6 ±0.30	-	13.5 ±0.76	-	17.8 ±0.61	-	20.6 ±0.30	-

*Mwt = Mycelial dry weight, Mean of 3 replicates, **Spn = Sporulation, - = Nil, + = Poor; ++ = Fair, ± = Standard error values

Table - 4: Effect of different nitrogen sources on growth and sporulation of *Lasiodiplodia theobromae*

Carbon source	Incubation period									
	5 days		10 days		15 days		20 days		25 days	
	Mwt* (mg)	Spn**	Mwt (mg)	Spn	Mwt (mg)	Spn	Mwt (mg)	Spn	Mwt (mg)	Spn
Inorganic -										
Potassium nitrate	155.0 ± 1.00	-	206.5 ± 0.76	+	261.2 ± 0.30	+	268.1 ± 0.38	+	255.0 ± 1.15	+
Sodium nitrate	135.2 ± 0.94	-	171.1 ± 0.58	+	190.4 ± 0.99	+	199.0 ± 0.82	+	188.2 ± 0.62	+
Ammonium nitrate	90.4 ± 0.67	-	128.3 ± 0.62	+	165.0 ± 0.40	+	180.0 ± 0.80	+	175.6 ± 0.76	+
Ammonium sulphate	120.0 ± 0.68	-	165.0 ± 0.96	+	190.8 ± 0.42	+	195.3 ± 0.40	+	186.5 ± 0.76	+
Organic -										
Peptone	286.0 ± 0.40	-	318.0 ± 0.80	+	347.0 ± 1.13	+	305.0 ± 0.61	+	252.0 ± 0.50	+
Yeast extract	180.0 ± 0.53	-	239.0 ± 0.50	+	272.0 ± 0.64	+	221.0 ± 0.53	+	190.6 ± 0.58	+
Beef extract	213.0 ± 0.76	-	276.6 ± 0.42	+	328.0 ± 0.58	+	310.0 ± 0.69	+	281.0 ± 0.50	+
Control	5.7 ± 0.91	-	8.2 ± 0.61	-	10.6 ± 0.87	-	11.8 ± 0.42	-	15.3 ± 0.38	-

*Mwt = Mycelial dry weight, Mean of 3 replicates, **Spn = Sporulation, - = Nil, + = Poor, ± = Standard error values

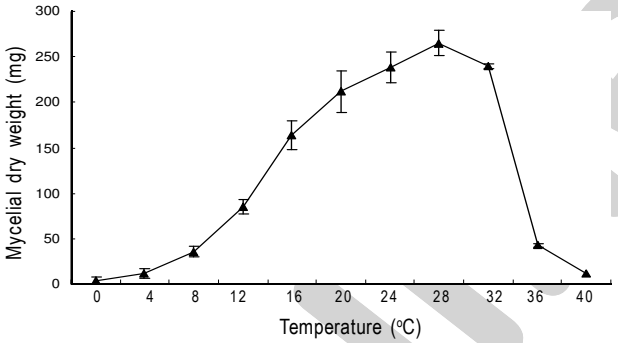

Fig.1: Effect of different temperature on mycelial growth of *Lasiodiplodia theobromae* in PDB-RE medium after 15 days of incubation

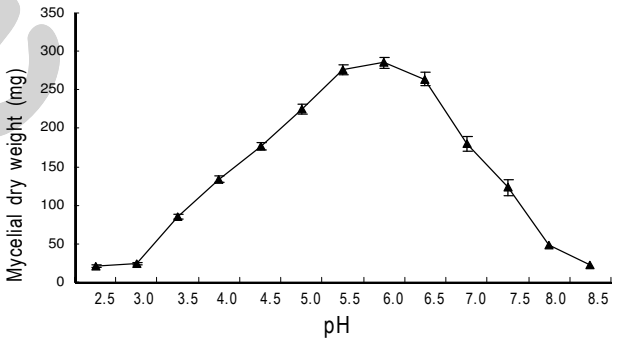

Fig. 2: Effect of different pH on mycelial growth of *Lasiodiplodia theobromae* in PDB-RE medium after 15 days of incubation

growth (360 mg after 20 days) and sporulation (excellent after 25 days). In PDB, growth was good (320 mg after 20 days) but sporulation was poor. Next to PDB-RE, MEB-RE (malt extract broth supplemented with root extract) also showed good growth and sporulation. Non-supplemented PCB recorded the least mycelial growth and no sporulation. The results revealed that in general, sporulation was remarkably high when *L. theobromae* was grown in root extract supplemented media in comparison to the non-supplemented media. Mycelial growth also increased marginally when PDB, OMB and PCB were supplemented with root extract. However, YEGB and MEB did not show any difference in mycelial growth between supplemented and non-supplemented media, but there was no sporulation in the non-supplemented media. Addition of root extract increased sporulation significantly especially in MEB-RE media. Thus the results indicated that root extract supplementation is not necessary for *in vitro* mycelial growth of *L. theobromae*, but it remarkably enhanced the sporulation. Alam *et al*. (2001) reported that highest mycelial growth and sporulation of *B. theobromae* was observed on PDA, which was in agreement to our findings. Several other workers also stated that PDA was the best media for mycelial growth (Xu *et al*., 1984; Maheshwari *et al*., 1999). Other reports (Kumar and Singh, 2000), also stated that *L. theobromae* grew well

in potato dextrose medium. Our observations also related with that reported by Karlatti and Hiremath (1989), who observed that the best mycelial growth of *Alternaria zinniae* was on leaf extract dextrose agar and potato dextrose agar media. They noticed higher sporulation on leaf extract dextrose agar medium.

Light had no significant influence on mycelial growth, which was found to be equally good under complete light, complete dark and alternate 12 hr light and dark conditions (Table 2). Sporulation was excellent and noticed after 10 days when the fungus was grown under complete light condition. However, under complete dark conditions, sporulation was poor and was delayed until 20 days. Overall results indicated that there was little variation in mycelial growth under different light conditions, but light induced sporulation. This result confirmed the findings of Alam *et al.* (2001), who showed that light was not necessary for growth of *L. theobromae*, but it enhanced the sporulation.

Results presented in figure (Fig.1) indicate that *L. theobromae* was capable of growing at temperatures that range between 8°-36°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 Alam *et al.* (2001), who observed that *L. theobromae* grew and sporulated at 10°-40°C, the optimum being 25-30°C. In another study, Eng *et al*. (2003) 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 temperatures above 40°C.

L. theobromae was able to grow within a wide range of pH, from 3.5 to 8.0 (Fig. 2). The fungus however, failed to grow in alkaline environment, beyond pH 8.0. The optimum pH for growth was recorded at the range of pH 5.5 – 6.5. The result indicated that slightly acidic pH to neutral pH was optimum for the growth of the organism.

Mycelial growth was observed to be much higher in presence of all the carbon sources tested compared to control, which did not contain any carbon compound (Table 3). Among the various carbon sources tested, glucose and sucrose containing media showed highest growth with mycelial dry weight of 302.5 mg and 300 mg after 15 days of incubation respectively. Media having mannitol as carbon source recorded minimum mycelial growth. Sporulation was found to be poor in all cases. Our result was similar to that reported by Ray (2004) who showed that lactose and glucose had similar effect on growth of *Botryodiplodia theobromae*. Jash *et al.* (2003) observed that sucrose was the best carbon source for growth of *Alternaria zinniae* followed by starch and maltose. According to them, mannitol produced least growth, which was in agreement to our observation.

Among the seven nitrogen sources tested, maximum growth was found in beef extract (310 mg) followed by peptone (305 mg) and potassium nitrate (268.1 mg) after 20 days of incubation (Table 4). Little growth was noticed in control, which was devoid of nitrogen. Poor sporulation was observed in all the nitrogen sources tested and no sporulation was found in the basal media. Holb and Chauhan (2005) observed that peptone was the best nitrogen source that produced quickest growth of *Monilia polystroma*.

The findings of the present study is critically important for further studies on the fungus as a tea pathogen and the root extract supplemented media as suggested here may be utilized for inoculum production.

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