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Effect of media, temperature and pH on the growth and Pycnidial production of *Lasiodiplodia theobromae* (Pat.), causative of black root rot in mulberry

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Abstract
Mulberry (*Morus* spp.,) is an important crop for rearing silkworm and sole food to the mulberry silkworm. The black root rot caused by *Lasiodiplodia theobromae* is an important soil borne disease results in major yield loss in mulberry production. The present study investigates the effect of media, temperature and pH on the growth of *L. theobromae* under *in vitro.* Among the five media tested, potato dextrose medium recorded highest mycelial growth (90 mm) and with good Pycnidial production (52 numbers) in Petri plate. Maximum mycelial growth (85.16 to 90 mm) of *L. theobromae* was observed at temperature range from 25 to 35ºC and no growth (0 mm) was observed at 10 and 40 ºC. The mycelial growth of *L. theobromae* was maximum at pH 7 (90 mm) with 54 numbers of Pycnidial production.

Keywords: *Lasiodiplodia theobromae*, mulberry, mycelial growth, Pycnidial production

Introduction
Mulberry (*Morus* spp.,) is a sole food plant for the domesticated mulberry silkworm (*Bombyx mori* L.). It is a fast growing, deciduous and deep-rooted perennial tree cultivated as monocrop. Mulberry cultivation accounts more than 40% of total cost of production during rearing of silkworms. The mulberry is affected by many diseases *viz.*, Dry root rot, black root rot, charcoal root rot, violet root rot, white root rot, bacterial root rot and *Armillaria* root rot in which black root rot and charcoal root rot are serious problem due to soil borne nature. Black root rot caused by *Lasiodiplodia theobromae* was reported in Tamil Nadu and Karnataka by Radhakrishnan *et al.*, (1995) [16]. *Lasiodiplodia theobromae* (Pat). is an opportunistic fungus that causes various diseases within the tropical and subtropical conditions with wide hosts range (Domsch *et al.*, 2007) [8]. The fungus has been reported as mango pathogen worldwide, associated with various disease symptoms like canker, decline and dieback (Abdollahzadeh *et al.*, 2010) [1]. Several workers have reported the physiological parameters and different nutrition sources to increases the growth and sporulation of *L. theobromae* in other crops (Burgess *et al.*, 2006; Alves *et al.*, 2008) [5, 4]. The studies on the nutritional requirements of *L. theobromae* are less and no such study was done for black root rot pathogen in mulberry. Therefore, the study was conducted to test the effect of temperature, pH, media on the mycelial growth and pycnidial production of *L. theobromae* infecting mulberry.

Materials and Methods
Isolation and identification of the pathogen
The root rot infected mulberry samples were collected from farmers field of western zone of Tamil Nadu and root bits of 5 mm length were surface sterilized with 1% sodium hypochlorite and washed with sterile water and inoculated into PDA plates. The pure culture of the pathogen was obtained by single hyphal tip method. The pathogenicity of the fungus was tested using 25 numbers of three-month-old mulberry saplings. The plants were artificially inoculated with 20 g of sorghum inoculum of *L. theobromae* and observed the disease development, symptoms as wilting and rotting with disease incidence (60%) and pathogen was reisolated from the infected plants to prove Koch postulates. Based on the symptoms, cultural and morphological characters of the fungus, the pathogen involved was identified as *L. theobromae*. Molecular confirmation through PCR was done and sequences were deposited in NCBI. The Sirumugai isolate of *L. theobromae* (LTSM- MW590682) was used for the present study.

Effect of different media on the growth of *L. theobromae*

To check the growth of *L. theobromae* on different media *viz*., Potato Dextrose Agar (PDA), Potato Sucrose Agar (PSA), Carrot Dextrose Agar (CDA), Beetroot Dextrose Agar (BDA) and Nutrient Agar (NA) were selected. The medium was prepared and autoclaved at 15 lbs for one hour. The sterilized warm media were poured into 90 mm Petri plates at the rate of 20 ml and allowed to solidify. The *L. theobromae* was inoculated in center of the plate by placing 9 mm seven-day old pure culture and incubated at room temperature. The radial growth of the pathogen was measured at 24 hr intervals. After 15 days of incubation, the number of pycnidia on Petri plate was calculated by counting the numbers under light microscope from each 1.6 cm sample disk to 8 cm diameter, which corresponded to the whole colonies growing on the plates.

Pycnidial numbers were observed based on the range given by Dheepa *et al*., (2018) [7]

- No Pycnidia Production (0)
+ Poor Pycnidia Production (<15)
++ Moderate Pycnidia Production (15-30)
+++ Good Pycnidia Production (30-60)
++++ Excellent Pycnidia Production (>60)

Effect of different temperature on the growth of *L. theobromae*

Effect of temperature on mycelial growth of *L. theobromae* was evaluated on PDA. The sterilized warm medium was poured into plates and placed 9 mm disc of seven days old culture under aseptic condition. The inoculated plates were incubated at different temperature like 10ºC, 15 ºC, 20ºC, 25 ºC, 30 ºC, 35 ºC and 40 ºC using BOD incubator to adjust the required temperature. The mycelial growth was observed in daily basis and pycnidial production was recorded after 15 days, as given by Dheepa *et al*., (2018) [7].

Influence of different pH on the growth of *L. theobromae*

PDA medium was prepared and adjusted to different pH *viz*., 4.0, 5.0, 6.0, 7.0, 8.0. and 9.0 using 0.1N NaOH or 0.1N HCL using pH meter and autoclaved at 121ºC for one hour. Medium with different pH were poured (20 ml) in Petri plate and allowed to solidify. The 9 mm disc of 7 days old pure culture of *L. theobromae* was placed in centre of the Petri plate and kept for room temperature for 5 to 7 days. Mycelial growth was recorded after of 24 hrs of inoculation, pycnidial production was recorded after 15 days of incubation.

Results and Discussion

To check the suitable media for growth of *L. theobromae* (LTSM), five media with different composition were selected and tested. Among the growth media tested, Potato Dextrose Agar medium supported maximum mycelial growth (90 mm) with in 48hr of inoculation followed by potato sucrose agar and carrot dextrose agar medium showing mycelial growth of 88.50 mm and 87.25 mm respectively (Table.1). The same result was recorded by Alam *et al.* (2001) [3] for *B. theobromae of* banana crown rot disease with the highest mycelial growth (76-90 mm) on PDA. Kumar and Singh (2000) [12] reported that the foliar disease of parthenium caused by *L. theobromae* shows fastest mycelial growth (90 mm) on PDA, whereas lowest mycelial growth of 13.25 mm was recorded in nutrient agar medium. Initially the mycelial colour was white and later it turned to grey and finally changed to black colour. Philips (2007) [15] reported best mycelia growth of apple fruit rot pathogen (*L. theobromae*) in PDA. The pycnidial formation was good (52 numbers) in potato dextrose agar medium and moderate (28 numbers and 19 numbers) in potato sucrose agar medium and carrot sucrose medium. Dheepa *et al.,* (2018) [7] also recorded similar result with that of *L. theobromae* isolated from coconut with highest mycelial growth (90 mm) and good (30-60) pycnidial production on PDA. The radial growth and pycnidial production of *L. theobromae* was medium dependent. Khanzada *et al*., 2006 [11] found that the potato dextrose agar (9 cm) was most suitable to the growth of *L. theobromae* isolated from mango. Chukunda and Onyeizu (2019) [6] reported that the potato dextrose agar medium (18.4 mm to 32.5 mm) was suitable for mycelial growth of *L. theobromae* causal agent of gummosis in African mahogany compared to potato dextrose agar stem exudates medium (15.6 mm to 22.0 mm) after 24 hrs incubation.

Temperature plays crucial role in growth and development of the pathogen. In this study, *L. theobromae* was incubated at different temperature *viz.*, 10, 15, 20, 25, 30, 35 and 40 ºC and mycelial growth was recorded in 24 and 48 hrs. The results showed that the pathogen grew well at 30 ºC (90 mm), whereas no mycelial growth was observed at 10 ºC and 40ºC (Table.2). Adnan *et al*, (2018) [18] recorded nil growth of *B. theobromae* isolated from guava at 10ºC under *in vitro*. In the present study, the growth was varying in the temperature of 25 ºC and 35ºC with 88.50 mm and 85.10 mm after 48 hrs of inoculation. These results are strongly supported with the findings of Patil *et al.,* 2006 [14], where maximum mycelial growth (88.60 mm) was observed at 30ºC and pycnidial production at 25 ºC for *B. theobromae* isolated from mango. Rehman *et al.* (2011) [17] recorded maximum growth of 87.20 and 70.10 mm of *B. theobromae* infecting mango, when incubated at 30 ºC and 25ºC respectively, whereas no growth was obtained under 15ºC. The mycelial growth was drastically reduced below 25ºC. Pycnidial formation was good at 30 ºC (52 numbers) followed by 25ºC (29 numbers) and at 35 ºC (26 numbers) with moderate production. Similarly, Saha *et al.,* (2008) [18] reported good (30-60) spore production between 25ºC to 35ºC in the common tea pathogen (*L. theobromae*). Fovo *et al.,* (2017) [9] observed excellent spore production (>60) of *L. theobromae* isolated from African oil nut at 28ºC.

The influence of different pH on mycelial growth of *L. theobromae* revealed that the maximum mycelial growth (90 mm) was at pH 7 followed by pH 6 with 80.25 mm after 48hrs of inoculation. The minimum growth was observed in pH 4 and 5 (67.00 and 73.50 mm) followed by alkaline pH 8 and 9 (65.60 and 51.00 mm) and Pycnidial formation was good (54 numbers) in pH 7, moderate (22 and 27 numbers) in pH 5 and 6, poor (13 numbers) in pH 8(Table. 3). Similar results were reported by Patil *et al*., (2006) [14] for dieback causing pathogen of mango *B. theobromae* with maximum mycelial growth (88.00 mm) at pH 7. Longitudinal splitting of bark and wood disease of acid lime caused by *Botryodiplodia theobromae* showed the highest mycelial growth (87.50) at pH 6.0 and Pycnidial formation was good (30-60) at pH 7 (Gouri Sankar *et al.,* 2016) [10]. Latha *et al*., 2012 also reported the same trend in collar and root rot pathogen L. *theobromae* in physic nut with maximum mycelial growth (76.50 mm) and excellent (>60) sporulation at pH 6.5.

Table 1: Effect of different media on the growth and pycnidial production of LTSM isolate of *L. theobromae*

S. No.	Name of the Medium	Mycelial growth (24 h)	Mycelial growth (48 h)	Pycnidial production
1.	Potato Dextrose agar (PDA)	43.50[a]	90.00[a]	+++ (52)
2.	Potato sucrose agar (PSA)	38.50[b]	88.50[b]	++ (28)
3.	Carrot Dextrose agar (CDA)	36.50[b]	87.25[b]	++(19)
4.	Beet root Dextrose agar (BDA)	30.75[c]	71.50[c]	-
5.	Nutrient agar (NA)	7.50[d]	13.25[d]	-
S. Ed		0.72	0.61	
CD (0.05)		1.54	1.30	

Values are the means of five replications. Means followed by a common letter are not significantly different at 5% level by DMRT. - No Pycnidia Production (0); + Poor Pycnidia Production (<15); ++ Moderate Pycnidia Production (15-30); +++ Good Pycnidia Production (30-60); ++++ Excellent Pycnidia Production (>60).

Table 2: Effect of different temperature on the growth and pycnidial production of LTSM isolate of *L. theobromae*

S. No.	Temperature	Mycelial growth (24h) *	Mycelial growth (48h) *	Pycnidial production
1.	10ºC	0.00[f] (0.71)	0.00[f] (0.71)	-
2.	15ºC	5.33[e] (2.41)	13.77[e] (3.78)	-
3.	20ºC	16.16[d] (4.08)	37.83[d] (6.19)	-
4.	25ºC	41.77[b] (6.50)	88.50[b] (9.43)	++ (29)
5..	30ºC	44.50[a] (6.67)	90.00[a] (9.51)	+++ (52)
6.	35ºC	38.53[c] (6.24)	85.16[c] (9.25)	++ (26)
7.	40ºC	0.00 [f] (0.71)	0.00 [f] (0.71)	-
S. Ed		0.02	0.02	
CD (0.05)		0.05	0.58	

Values are the means of five replications. *Values in parenthesis are square root transformed values. Means followed by a common letter are not significantly different at 5% level by DMRT. - No Pycnidia Production (0); + Poor Pycnidia Production (<15); ++ Moderate Pycnidia Production (15-30); +++ Good Pycnidia Production (30-60); ++++ Excellent Pycnidia Production (>60).

Table 3: Effect of different pH on growth and pycnidial production of LTSM isolate of *L. theobromae*

S. No.	pH	Mycelial growth (24h)	Mycelial growth (48h)	Pycnidial production
1.	4.00	29.25	67.00 [cd]	-
2.	5.00	31.25	73.50 [c]	++(22)
3.	6.00	38.30	80.25 [b]	++ (27)
4.	7.00	42.75	90.00[a]	+++ (54)
5.	8.00	28.00	65.60 [de]	+ (13)
6.	9.00	26.25	51.00 [e]	-
S. Ed		0.99	0.78	
CD (0.05)		2.16	1.70	

Values are the means of five replications. Means followed by a common letter are not significantly different at 5% level by DMRT. - No Pycnidia Production (0); + Poor Pycnidia Production (<15); ++ Moderate Pycnidia Production (15-30); +++ Good Pycnidia Production (30-60); ++++ Excellent Pycnidia Production (>60).

Conclusion

Mulberry is the only food source for silkworm rearing in Tamil Nadu. Root rot diseases in mulberry is a great menace which in turn drastically reduce the plant stand and biomass production. Recently the black root rot pathogen emerged as a serious disease due to changes in climatic conditions and soil parameters. The present study revealed that the pathogen *L. theobromae* grows well on PDA medium with good pycnidial production at the pH of 7 and temperature range of 25 to 35ºC. This indicates that if the soil temperature increases between 25 to 30ºC, the pathogen will easily attack mulberry root and establish in short notice resulting in death of the plant. These optimization studies will definitely help to multiply *L. theobromae* for effective implementation of screening protocols with bioagents, fungicides against black root rot pathogen and resistance screening of varieties/ genotypes by artificial inoculation.

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