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R Dheepa
Ph. D. Scholar, Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

C Goplakrishnan
Professor (Plant Pathology), Agricultural College and Research Institue, Kudumiyanmalai, Pudukkottai, Tamil Nadu, India

A Kamalakannan
Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

S Nakkeeran
Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Correspondence
R Dheepa
Ph. D. Scholar, Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Influence of culture media and environmental factors on mycelial growth and sporulation of *Lasiodiplodia theobromae* in coconut

R Dheepa, C Goplakrishnan, A Kamalakannan and S Nakkeeran

Abstract
Lasiodiplodia theobromae is a cosmopolitan tropical and subtropical plant pathogen. The pathogen was isolated from infected coconut for the first time in Tamil Nadu and the pathogen did not sporulate or sporulated poorly in common media, which hinders in identification and characterization of *L. theobromae in vitro.* Hence the influence of factors such as culture media, pH, temperature and light on mycelial growth and sporulation were evaluated in the present study. Among several media tested, potato dextrose agar medium was found to be the most suitable for mycelial growth and sporulation of *L. theobromae*. The fungus grows at pH 4.5-8.5 and optimum growth was observed at pH 7.0. The fungus grows at temperatures ranging from 15º to 40ºC, with optimum growth at 28ºC and no growth was noted at 40ºC. There was no significant effect of different light period on growth of *L. theobromae*. However exposure to continuous light enhanced sporulation.

Keywords: *Lasiodiplodia theobromae*, light, growth, sporulation, pycnidial production

Introduction
Coconut crop is very important for the supply of raw materials for food industry and crafts [5] and has emerged as a major crop in South India. Recently, feasibility studies on the use of coconut fruits for biodiesel production have been done [4]. Coconut water and copra are consumed *in natura* and the consumption of the first one has increased because of the health benefits brought about by its chemical composition, nutritional value and functional properties [3], especially in tropical countries. The fungus *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl is the synonymous of *Botryodiplodia theobromae*. However [11], has adopted the name *L. theobromae* as suggested by [12]. *B. theobromae* (Pat.) is the anamorph stage of *Botryosphaeria rhodina* (Cooke) arx. The main features that distinguish this genus from other closely related genera are the presence of pycnidial paraphyses and longitudinal striations on mature conidia [11]. The fungus has been reported as mango pathogen worldwide, associated with several plant disease symptoms including decline, canker and dieback [1]. Several workers [8, 6, 9, 10] have assessed the important physiological parameters and different carbon and nitrogen sources that leads to maximum growth and sporulation of *L. theobromae* isolated from mango. However, limited attempts were made to study these requirements of isolates isolated from coconut. Therefore, experiments were conducted to explore the role of temperature, pH, carbon and nitrogen sources, lights and media on mycelial growth and pycnidial production of *L. theobromae* isolated from nut rot infected coconut.

Materials and Methods
Isolation and identification of the fungal culture
The pathogenicity of the fungus was proved by Koch's postulates using the ten numbers of 11-12 month old dehusked matured healthy coconuts. The tuft was removed and thoroughly washed in running tap water, surface sterilized with 0.1 per cent mercuric chloride solution and finally rinsed in sterile distilled water. The thin surface layer covering the pore (eye) was scraped with sharp sterile scalpel, till the meat was exposed without leakage of water from endosperm. Nuts were inoculated with 9 mm disc of pure culture of fungi. In all the cases, the inoculated area of the nut was covered with moist cotton and was placed inside perforated moist polythene bags, the mouth tied with rubber band to provide high humidity. Suitable controls were also maintained. Based on the symptoms and morphological characters of the fungus, it was identified as *Lasiodiplodia theobromae*. The culture was sent to the Indian Type Culture Collection, Indian Agricultural Research Institute, New Delhi and confirmed the pathogen as *Lasiodiplodia theobromae* (Syn: *Botryodiplodia theobromae*) (ITC No: 10,517.17) and used for the present investigation.

Effect of different culture media

The growth of *L. theobromae* on different synthetic and semisynthetic media *viz*., Potato dextrose agar medium, carrot dextrose agar medium, radish dextrose agar medium, Beet root dextrose agar medium, Potato sucrose agar and yeast mannitol agar medium was assessed. The sterilized respective warm medium @ 20 ml was poured in to 90 mm sterilized Petri dishes and medium was allowed to solidify. The pathogen was inoculated at the centre of the plate by placing a seven day's old 9 mm culture disc of the *L. theobromae*. The plates were incubated at room temperature (28±2°C) and three replications were maintained in each medium and the radial growths of the mycelium were measured at 24h interval and daily radial growth rates were calculated. After 15 days of inoculation, the numbers of pycnidia produced per plate were recorded.

Effect of different pH levels

The potato dextrose agar medium were prepared and adjusted to nine different pH levels *viz.*, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 separately with 0.1 N HCl or 0.1 N NaOH by using digital pH meter and sterilized at 15 lbs for 20 min. Twenty ml of the medium from each pH level were poured into each sterilized Petri plates separately and allowed to solidify at room temperature. A 9 mm culture disc of actively growing *L. theobromae* were cut and placed at the centre of each Petri plate separately under aseptic conditions. The plates were incubated at room temperature (28±2ºC) and three replications were maintained for each pH level. The mycelial growth and pycnidial production of the *L. theobromae* was measured 15 days after inoculation as described above.

Effect of different temperature

Effect of temperature on mycelial growth and pycnidial production was evaluated on Potato dextrose agar. The sterilized warm medium was poured in the sterilized Petri plates and allowed to solidify and this was inoculated with seven day's old 9 mm culture disc of the pathogen. The inoculated plates are incubated at 5, 10, 15, 20, 25, 30, 35 and 40°C and three replications were maintained for each temperature. Inoculated plates were kept in an incubator and temperature was adjusted to required level. The mycelial growth and pycnidial production of the *L. theobromae* was measured 15 days after inoculation described above.

Effect of different light conditions

Fluorescent lamp and black carbon paper were used to maintain different light conditions *viz*., continuous light, continuous dark, 24 hrs light and 24 hrs dark, 12 hrs light and 12 hrs dark, 16 hrs light and 8 hrs dark, 16 hrs dark and 8 hrs light. The sterilized PDA medium was poured in the sterilized Petri plate followed to solidify and inoculated with seven days old culture disc of the pathogen. Three replications were maintained for each light condition. The mycelial growth and pycnidial production of the *L. theobromae* was measured 15 days after inoculation as described above.

Results and Discussion

At the beginning, colour of the mycelia was white to light grey and became darker with the age. Among the growth media tested, potato dextrose agar supported the maximum mycelial growth (90.00 mm) followed by potato sucrose agar and yeast mannitol agar medium (89.50 mm). The mycelium growth was also found to be higher with potato dextrose agar (Table 1). The result from the present investigation showed that potato dextrose agar encouraged the maximum mycelial growth as well as pycnidial production followed by potato sucrose agar medium. These results are in close agreement with in those of [2] who recorded the highest pycnidial production and mycelium growth of *L. theobromae* on PDA. The *L. theobromae* grew well in potato dextrose medium [7]. However, the potato sucrose agar and yeast extract mannitol agar were the most favourable for fast radial growth of mycelium of *L. theobromae*. The highest number of pycnidial per plate was formed on yeast mannitol agar medium. The radial mycelial growth and pycnidial production of *L. theobromae* was medium dependent [6].

Among, the different pH levels tested, maximum growth was recorded in pH 7.0 (90.00 mm). This was followed by pH 7.5 with 87.50 mm mycelial growth. The acidic pH levels were found to be inhibitory to the growth of pathogen (Table 2). The result from the present investigation showed that the highest mycelial growth and pycnidial production were recorded at pH 7.0 and 7.5. The result indicated that slightly acidic pH was optimum for the growth of the organism. However, the maximum mycelial growth was recorded at pH 6.5 followed by pH 6.00. The excellent sporulation was recorded at pH 6.0 and 6.5 at 5.5, 7.0 and 7.5 [9].

The maximum growth was observed at both 25ºC and 30ºC (90.00 mm) followed by 35ºC (87.00 mm). Mycelial growth was drastically reduced below 25ºC. The maximum pycnidial production was also observed at 30ºC (Table 3).

The result from the present investigation, the mycelial growth of *L. theobromae* showed a variable trend in response to changes in temperature on PDA medium. There was very little or no growth at low temperatures i.e. 5ºC, 10ºC. However, mycelial growth increased as temperature increased up to 30ºC and then rapidly decreased at 40ºC and above. Optimum growth occurred at 25-30ºC. The pycnidial formation also showed same trends as mycelium growth with respect to temperature change. Highest numbers of pycnidial were recorded at 30-35ºC. At low temperatures *L. theobromae* failed to produce pycnidia. These results are in conformity with those reported by earlier investigations [2]. reported that 29ºC and 25-30ºC temperatures were optimum for the mycelial growth and pycnidial production of *L. theobromae* respectively. The maximum mycelial growth was observed at 30ºC and pycnidial production at 25ºC [9].

In the present study, there was no significant interaction between light regimes and radial mycelial growth of *L. theobromae*. Similarly, pycnidial production was also not affected by different light periods. The average mycelial growth and number of pycnidial per plate were more or less same in different light conditions. The maximum mycelial growth (90 mm) and pycnidial production was recorded in continuous light sources. The fungus also grew and sporulated well with 24 hrs light and 24 hrs dark as it recorded 86.50 mm mycelial growth and more pycnidial production (Table 4).

Similarly, [2] observed that growth of *L. theobromae* was not significantly affected by different light conditions on PDA; however sporulation was the highest in continuous light. However, the 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. 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 [10]. Overall results indicated that there was little variation in

mycelial growth under different light conditions, but light induced sporulation.

The findings of the present investigation is critically important and forms basic knowledge since the pathogen is isolated from coconuts for the first time in the region and may be utilized for inoculum production and also for better understanding biology of *L. theobromae* in coconut.

Table 1: Effect of different culture media on the growth and sporulation of *L. theobromae In vitro*

Medium	*Mycelial growth of the pathogen (mm)	Pycnidial production
Potato dextrose agar medium	90.00[a]	+++
Carrot dextrose agar medium	88.20[c]	++
Radish dextrose agar medium	75.90[d]	-
Beet root dextrose agar medium	74.50[e]	-
Potato sucrose agar medium	89.50[b]	++
Yeast mannitol agar medium	89.50[b]	++

*Values are mean of three replications

Values in parentheses are arc sine transformed values

Means followed by a common letter are not significantly different at 5% level by DMRT

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

Table 2: Effect of pH levels on the growth of *L. theobromae in vitro*

pH level	*Mycelial growth of the pathogen (mm)	Pycnidial production
4.5	23.60[i]	-
5.0	27.80[h]	-
5.5	43.50[g]	++
6.0	57.00[f]	++
6.5	60.50[e]	++
7.0	90.00[a]	+++
7.5	87.50[b]	+++
8.0	70.00[c]	+
8.5	68.90[d]	+

*Values are mean of three replications

Values in parentheses are arc sine transformed values

Means followed by a common letter are not significantly different at 5% level by DMRT

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

Table 3: Effect of temperature on the growth of *L. theobromae In vitro*

Temperature (°C)	*Mycelial growth of the pathogen (mm)	Pycnidial production
5	No growth	-
10	No growth	-
15	15.50[d]	-
20	26.00[c]	+
25	90.00[a]	++
30	90.00[a]	+++
35	87.00[b]	++
40	No growth	-

*Values are mean of three replications

Values in parentheses are arc sine transformed values

Means followed by a common letter are not significantly different at 5% level by DMRT

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

Table 4: Effect of photoperiods on the growth of *L. theobromae In vitro*

Photoperiod	*Mycelial growth of the pathogen (mm)	Pycnidial production
Continues light	90.00[a]	+++
Continues dark	75.00[d]	+
24 hrs light & 24 hrs dark	86.50[b]	++
12 hrs light & 12 hrs dark	83.00[c]	+
16 hrs light & 8 hrs dark	73.50[f]	+
8 hrs light & 16 hrs dark	74.00[e]	-

*Values are mean of three replications

Values in parentheses are sine transformed values

Means followed by a common letter are not significantly different at 5% level by DMRT

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

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