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Effect of culture media and environmental factors on mycelial growth and pycnidial production of *Lasiodiplodia theobromae* in physic nut (*Jatropha curcas*)

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Abstract

Physic nut (*Jatropha curcas*) is an important commercial bio-diesel plant species and is being advocated for development of waste and dry land. The collar and root rot caused by *Lasiodiplodia theobromae* is an important soil borne disease which causes considerable yield loss in this crop. In this study, the effects of culture media, temperature, photoperiod, carbon and nitrogen sources and pH on mycelial growth and pycnidial production were evaluated. Among the growth media tested, potato dextrose agar supported the highest growth followed by potato sucrose agar and corn meal agar. Among several carbon sources tested, carboxy methyl cellulose and sucrose were found superior for growth and pycnidial production. The nitrogen sources *viz*., ammonium oxalate and ammonium dihydrogen phosphate were recorded maximum mycelial growth and pycnidial production. The fungus grows at pH 5.0-9.0 and optimum growth was observed at pH 7.0.

Key words

Physic nut, *Lasiodiplodia theobromae*, Mycelial growth, Pycnidial production

Introduction

Physic nut (*Jatropha curcas*) a non-edible oil-bearing and drought-hardy shrub with ecological advantage belonging to the Euphorbiaceae family, was found to be the most appropriate renewable alternative source of biodiesel. Among the several constraints in physic nut cultivation, diseases play a major role in the yield reduction and it is caused by many fungal and viral diseases. Among the fungal diseases, collar and root rot is caused by *Lasiodiplodia theobromae* is an economically important soil borne disease (Latha *et al.*, 2009). *Lasiodiplodia theobromae* represents the asexual state of *Botryosphaeria rhodina* (Berk. & M.A. Curtis) Arx. It has a worldwide distribution in tropical and subtropical regions and occurs on a very wide range of plants (Mohali *et al.,* 2005). The fungus has been reported as mango pathogen worldwide, associated with several plant disease symptoms including decline, canker and dieback (Khanzada *et al.,* 2004a, b; Abdollahzadeh *et al.,* 2010). The taxonomic placement of *Botryosphaeria rhodina* (anamorph *L. theobromae*) has been complicated by several names associated with this fungus. Several studies have led to the identification of cryptic species within the *L. theobromae* species complex (Burgess *et al.,* 2006; Alves *et al.,* 2008; Abdollahzadeh *et al*., 2010). Several workers (Mahmood *et al.,* 2007; Khanzada *et al.,* 2006; Patil *et al*., 2006; Saha *et al*., 2008) have recognized the important physiological parameters and different carbon and nitrogen sources that lead to maximum growth and sporulation of *L. theobromae* in other crops. But studies on the environmental and nutritional requirements of *L. theobromae* are limited and no attempts were made to study these requirements in physic nut. 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* in physic nut.

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 one year old healthy plants. The plants were inoculated with mycelia disc by making a vertical cut (3 cm) in the bark of the stem at collar region using a sterilized knife. The inoculated portion was covered with moist cotton and wrapped with parafilm and maintained at a temperature of 28±2ºC and moisture content of 40%. Plants were irrigated after inoculation and the wrapping material was removed from the stems after two weeks of inoculation. Then the inoculated plants were maintained in the glass house and observed for the development of disease symptoms and the pathogen was re-isolated from the root portion in infected plants. Based on the symptoms and morphological characters of the fungus, it was identified as *Lasiodiplodia theobromae* (Punithalingam, 1976). 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: 6969/08).

Effect of different culture media : Seven media *viz*., Basalt agar, Potato Dextrose Agar, Czapek Dox Agar, V8 Juice Agar, Cornmeal Dextrose Agar, Yeast Mannitol Agar and Potato Sucrose Agar medium were selected for screening of maximal growth supporting media. All the media were prepared, sterilized at 121^0 C for 15 min. The sterilized warm medium at the rate of 20 ml was poured in sterilized petriplates and allowed to solidify. The pathogens were inoculated at the centre of the plate by placing a 7 days old 9mm culture disc of the pathogen.

Effect of different pH levels : In solid, PDA medium was prepared and adjusted to different pH levels *viz*., 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 with 1N NaOH or 1N HCl by using pH meter and sterilized at 121 ^{0}C for 20 min. Twenty ml of the medium from each pH level were poured onto sterilized petriplate and allowed to solidify. A nine mm PDA culture disc of actively growing *L. theobromae* was placed at the centre of each petriplate under aseptic condition. In liquid, PDA broth was prepared and adjusted to different pH levels viz., 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 with 1N NaOH or 1N HCl by using pH meter and sterilized at 121º C for 20 min. A nine mm PDA culture disc of actively growing *L. theobromae* was placed onto the PDA broth under aseptic conditions.

Effect of different temperature : Effect of temperature on mycelial growth and pycnidial production was evaluated on PDA. The sterilized warm medium was poured in the sterilized petriplates and allowed to solidify and this was inoculated with seven days old 9 mm culture disc of the pathogen. The inoculated plates are incubated at 0, 5, 10, 15, 20, 25, 30, 35 and 40^0 C. Five replications were maintained in each treatment.

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 and 48 hrs dark, 8 hrs dark and 16 hrs dark. The sterilized PDA medium was poured in the sterilized petriplate plate followed to solidify and inoculated with 7 day old culture disc of the pathogen.

Utilization of carbon and nitrogen sources : The Richard's agar medium was substituted with different carbon sources *viz.,* mannitol, glucose, starch, sucrose, maltose and carboxy methyl cellulose and different nitrogen sources such as urea, ammonium oxalate, sodium nitrate, ammonium sulphate, ammonium nitrate. All the carbon and nitrogen sources were dissolved and sterilized. The medium containing without nitrogen and carbon source served as control. The sterilized warm medium was poured in the sterilized pertiplates and allowed to solidify and inoculated with 7 days old 9mm culture disc of the pathogen.

The plates were incubated at room temperature (28±2^{0}C) and five replications were maintained in each treatment. The colony diameter was measured in mm as basis of growth. Growth of the cultures was measured after three days and sporulation after fifteen days of incubation under various conditions. The observation was made on the formation of pycnidia.

Results and Discussion

Among the growth media tested, potato dextrose agar supported the highest growth (8.96) followed by potato sucrose agar and corn meal agar (8.94). The colony spreading rate was also found to be higher with the potato dextrose agar (Table 1). The result from the present investigation showed that PDA 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 Alam *et al.* (2001) who recorded highest mycelium growth of *B. theobromae* on PDA and maximum pycnidia on Czapek's Dox agar. Kumar and Singh (2000) also stated that *L. theobromae* grew well in potato dextrose medium. However, Khanzada *et al.* (2006) reported that the potato sucrose agar and yeast extract mannitol agar (YEMA) were most favourable for fast radial growth of mycelium of *L. theobromae*. The highest number of pycnidia per plate was formed on yeast mannitol agar medium. The radial mycelial growth and pycnidial production of *L. theobromae* was medium dependent.

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

Medium	*Mycelial growth of the pathogen (mm)
Basalt medium	16.0[d]
Czapeck dox agar medium	54.0[c]
Yeast mannitol agar medium	86.2[b]
V8 juice agar medium	88.6[a]
Cornmeal agar medium	89.4[a]
Potato sucrose agar medium	89.4[a]
Potato dextrose agar medium	89.6[a]
SEd	0.69

* Mean of three replications; In a column, means followed by a common letter (s) are not significantly different (P=0.05) by DMRT

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

pH level	*Mycelial growth (mm)	Pycnidial production	*Dry mycelial weight (mg)
5.0	21.40[g]	+	212.20[f]
5.5	27.00[f]	++	322.20[e]
6.0	37.00[e]	++	362.00[c]
6.5	47.00[d]	++	479.20[b]
7.0	76.40[a]	+++	766.80[a]
7.5	65.00[b]	++	570.00[c]
8.0	62.00[c]	+	542.00[d]
8.5	62.80[bc]	+	442.20[g]
9.0	63.60[bc]	+	431.40[h]
SEd	0.43		0.85

* Mean of five replications; In a column, means followed by a common letter (s) are not significantly different (P=0.05) by DMRT; - No Pycnidia Production (0) +++ Good Pycnidia Production (30-60) ; + Poor Pycnidia Production (<15) ++++ Excellent Pycnidia Production (>60); ++ Moderate Pycnidia Production (15-30)

Among, the different pH levels tested, maximum growth was recorded in pH 7.0 (76.20 mm) with highest mycelial dry weight (766.80 mg). This was followed by pH 7.5 with 65.00 mm mycelial growth and mycelial dry weight of 570.00mg. 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, dry mycelial weight and pycnidial production were recorded at pH 7.0 and 7.5. However, Patil *et al.* (2006) reported that 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, 7.5 and 8.0 it was good.

The maximum mycelial growth (89.20 mm) and pycnidial production was recorded in continuous light

Table 3 : Effect of photoperiods on the growth of *L. theobromae in vitro*

Photoperiod	*Mycelial growth (mm)	Formation of pycnidia
Continuous light	89.200[a]	++++
Continuous dark	74.800[d]	+
24 hours light & 24 hours Dark	78.000[b]	++
12 hours light & 12 hours Dark	76.400[c]	+
16 hours light & 8 hours Dark	72.800[e]	+
8 hours light & 16 hours Dark	72.000[e]	+
SEd	0.32	

* Mean of five replications; In a column, means followed by a common letter (s) are not significantly different (P=0.05) by DMRT; - No Pycnidia Production (0) +++ Good Pycnidia Production (30-60); + Poor Pycnidia Production (<15) ; ++++ Excellent Pycnidia Production (>60); ++ Moderate Pycnidia Production (15-30)

Table 4 : Effect of temperature levels on the growth of *L. theobromae in vitro*

Temperature (°C)	*Mycelial growth (mm)	Formation of pycnidia
0	No growth	-
5	No growth	-
10	No growth	-
15	12.50[f]	+
20	22.50[e]	+
25	26.40[d]	++
30	86.80[a]	++++
35	53.40[b]	++
40	38.20[c]	-
SEd	0.09	

*Mean of five replications ; In a column, means followed by a common letter (s) are not significantly different (P=0.05) by DMRT; - No Pycnidia Production (0) +++ Good Pycnidia Production (30-60) + Poor Pycnidia Production (<15); ++++ Excellent Pycnidia Production (>60); ++ Moderate Pycnidia Production (15-30)

sources. The fungus also performed well with 24 hrs light and 24 hrs dark as it recorded 78.00 mm mycelial growth and more pycnidial production (Table 3).

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 pycnidia per plate were more or less same in different light conditions. Similarly, Alam *et al.* (2001) observed that growth of *B. theobromae* was not significantly affected by different light conditions on PDA however; sporulation was highest in continuous light. Parveen *et al.* (2009) reported that the continuous light was found to be the most suitable for maximum growth of the fungi *viz., L.*

theobromae and *Fusarium solani* respectively. Saha *et al.* (2008) reported that 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. Overall results indicated that there was little variation in mycelial growth under different light conditions, but light induced sporulation.

The maximum growth was observed at 30º C (86.80mm) followed by 35ºC (53.40mm). Mycelial growth was drastically reduced below 25ºC. The maximum pycnidial production was also observed at 30ºC (Table 4).

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. 0, 5, 10. However, mycelial growth increased as temperature increased up to 35ºC and then decreased rapidly with further increase in temperature. Optimum growth occurred at 30-35ºC. The pycnidia formation also showed same trends as mycelium growth with respect to temperature change. Highest numbers of pycnidia were recorded at 30-35ºC. At low temperatures *L. theobromae* failed to produce pycnidia. These results are in confirmity with those reported by earlier investigators. Alam *et al*. (2001) reported that 29ºC and 25-30^{0}C temperatures were optimum for the mycelial growth and pycnidial production of *L. theobromae* respectively. Patil *et al.* (2006) reported that the maximum mycelial growth was observed at 30ºC and pycnidial production at 25ºC. In another study, Eng *et al.* (2003) reported similar observations when he studied the effect of temperature on growth characteristics of *Botryodiplodia theobromae.*

The maximum mycelial growth was recorded in ammonium dihydrogen phosphate and ammonium oxalate (90.00mm). The maximum pycnidial production was also recorded in ammonium dihydrogen phosphate followed by ammonium oxalate. The other nitrogen sources recorded minimal pycnidial production. In the case of carbon sources, the maximum mycelial growth was recorded in sucrose (88.80 mm) followed by carboxy methyl cellulose (88.60 mm). The maximum pycnidial production was also recorded CMC followed by sucrose and starch. The other carbon sources supported only minimal pycnidial production (Table 5 and 6).

Table 5 : Effect of carbon sources on the growth of *L. theobromae in vitro*

Carbon source	*Mycelial growth (mm)	Growth characters	Pycnidia production
Mannitol	88.20^{a}	Colonies were irregular initially white later turn grey	+
Glucose	88.40^{a}	Colonies were irregular initially white later turn black	++
Starch	87.60^{ab}	Colonies were irregular initially white later turn grey	+++
Sucrose	88.80^{a}	Colonies were irregular initially white later turn grey	+++
Carboxy Methyl Cellulose (CMC)	88.60^{a}	Colonies were irregular initially white later turn grey	++++
Control	86.40^{c}	Colonies were circular	-
SEd	0.63		

* Mean of five replications; In a column, means followed by a common letter (s) are not significantly different (P=0.05) by DMRT; - No Pycnidia Production (0) +++ Good Pycnidia Production (30-60) + Poor Pycnidia Production (<15) ++++ Excellent Pycnidia Production (>60) ++ Moderate Pycnidia Production (15-30)

Table 6 : Effect of nitrogen sources on the growth of *L. theobromae in vitro*

Nitrogen source	*Mycelial growth (mm)	Growth characters	Pycnidia production
Sodium nitrate	85.00^{b}	Colonies are irregular	+
Ammonium sulphate	88.00^{a}	Colonies are circular	+
Ammonium nitrate	88.00^{a}	Colonies are circular	+
Ammonium oxalate	90.00^{a}	Colonies are irregular	++
Ammonium dihydrogen phosphate	90.00^{a}	Colonies are circular	+++
Control	85.00^{b}	Colonies are irregular	-
SEd	0.55		

*Mean of five replications; In a column, means followed by a common letter (s) are not significantly different (P=0.05) by DMRT- No Pycnidia Production (0) +++ Good Pycnidia Production (30-60); + Poor Pycnidia Production (<15) ++++ Excellent Pycnidia Production (>60); ++ Moderate Pycnidia Production (15-30)

In the present study, mycelial growth was observed to be much higher in all the carbon sources tested when compared to control which did not have any carbon compounds. Among the carbon sources tested, sucrose, CMC and glucose were found to induce highest mycelial growth and pycnidial production. Saha *et al.* (2008) and Patil *et al.* (2006) reported that the amongst carbon sources tried for the growth of pathogen, sucrose and glucose produced higher mycelial growth. Among the five nitrogen sources studied ammonium dihydrogen phosphate recorded highest mycelial growth and pycnidial production followed by ammonium oxalate.

The findings of the present investigation is critically important for effect of culture media and environmental factors on mycelial growth and pycnidial production of *L. theobromae* that may be utilized for inoculum production and also for better understanding of their biology of *L. theobromae* in physic nut.

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