

Original Research Article

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**Growth of *Botryodiplodia theobromae* an Incitant of Longitudinal Splitting of Bark and Wood Disease in Acidlime (*Citrus aurantifolia* Swingle) as Influenced by pH Levels, Temperature and Growth Media**

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Studies on effect of pH levels, temperature and growth media on growth of *Botryodiplodia theobromae* causing longitudinal splitting of bark and wood disease on acid lime revealed that higher dry mycelial weight of the fungus was obtained at 25 to 30°C which was considered as optimum for the vegetative growth. Among the different media tested Potato Dextrose Broth (PDB) supported for maximum dry mycelial weight followed by Richards Broth (RB), Czapek Dox Broth (CPB) and Czapek Dox Broth (CDB) medium. Among the different media used PDB gave maximum dry mycelial weight at pH ranged from 5.0 to 6.0 followed by CPB, CDB and RB media.

**Introduction**

Citrus is one of the most important fruit crop grown throughout the world. Citrus is grown in more than 50 countries of the world and it is one of the choicest fruits having highest consumer's preference both as fresh fruits as well as for its refreshing processed juice. Citrus is the third most important fruit crop of India after banana and mango. The most important citrus cultivars in India are the mandarins (*Citrus reticulata* Blanco), sweet orange (*Citrus sinensis* Osbeck) and acidlime (*Citrus*

*aurantifolia* Swingle). Collectively citrus fruit in India have an estimated production of 3.79 million tonnes from an area of 0.45million hectares.

Acid lime is one of the important citrus fruit grown over an area of 0.90m ha and constitute nearly 20 per cent of the total citrus production of 90.75mt in India. Andhra Pradesh, Maharashtra, Karnataka, Punjab, and Assam are the leading acid lime growing states. A.P occupies the first place

both in area (2.02 lakhs ha) and production (13.50 MT) of limes followed by Maharashtra.

Among the diseases responsible for acid lime decline, the bark and wood splitting disease is one of the major factor affecting both life and production under field conditions (Gopal *et al.*, 2000). The disease is seen on almost all the acid lime clones/varieties both in farmers orchards and in the Research station. The proportion of the disease is reported to be up to 15 per cent in farmer's orchards (Gopal *et al.*, 2005).

The infected plant shows internal discoloration and darkening of the bark of infected twigs. Ash coloured discoloration of vascular tissues are also seen on splitting the twigs/branches. In early stages, epidermal and sub-epidermal cells of twigs and branches appear slightly shriveled (Gopal *et al.*, 2005). Keeping the importance and the severity of the disease, in present studies, emphasis was given to investigate the growth requirements to the pathogen.

## **Materials and Methods**

### **Collection and isolation of pathogen from diseased acidlime plants**

The infected bark and wood samples were collected from the acidlime trees at Citrus Research Station, Tirupati, Chittoor District, A.P. For isolating the pathogen, 2 to 3 mm long bits were cut from the bark portion of the affected acidlime plants, which showed apparently dark brown discoloration of the bark longitudinally rectangular, starting from base of the trunk. The bits were treated with 0.1% HgCl<sub>2</sub> for 30 seconds followed by three washings with sterile distilled water. The bits were transferred to another Petri plate containing sterile discs of

blotting paper. The dried bits were subsequently transferred to the slants of PDA under aseptic condition and incubated at 25±1°C. Subculturing was done on medium whenever required.

### **Growth of *B. theobromae* on different solid media**

Four solid media viz., Potato Dextrose Agar, Richards Agar (RA), Carrot Potato Agar (CPA) and Czapek's Dox Agar (CDA) media were used to assess the growth etc. for the isolates of the pathogen (Fig.1).

Mycelial disc of 20 mm diameter was cut with sterilized cork borer from the periphery of an actively growing three days old culture of the fungus grown on PDA, RA, CPA and CDA medium and transferred aseptically to the centre of each plate. Each isolate was replicated twice and the plates were incubated at 28 ± 2°C.

For determining the variation among different isolates of *Botryodiplodia theobromae* in terms of colony diameter, colony growth of the fungus in each Petri plate was measured 3 days after inoculation. The colony growth was measured along two diameters at right angles and averaged. For growth rate, colony growth in mm per day was measured.

### **Effect of different temperatures on growth and sporulation of *B. theobromae* in liquid media**

To determine optimum temperature for growth and sporulation of the test fungus, the fungus was distributed in different flasks containing 25 ml of medium in each. Then the flasks were incubated at different temperatures viz., 10, 20, 30 and 40°C for recording observations up to 14 days. The data in terms of dry weight of the fungal

vegetative growth as well as extent of sporulation were recorded.

Effect of different hydrogen ion concentration on the growth and sporulation of *B. theobromae* in liquid media

In order to study the effect of different hydrogen ion concentrations on growth, production of conidia, pH levels viz., 3.0, 4.0, 5.0, 6.0 and 9.0 were adjusted in basal medium with the help of BDH narrow range pH paper using N/10 Na OH or HCl. The flasks containing 25 ml of the basal medium adjusted to different pH levels were autoclaved, incubated at  $25 \pm 1^\circ\text{C}$ . The data in terms of mycelial dry weight and sporulation were recorded after 14 days.

## Results and Discussion

Twelve isolates of the pathogen namely LBWP-1, LBWP-2, LBWP-3, LBWP-4, LBWP-5, LBWP-6, LBWP-7, LBWP-8, LBWP-9, LBWP-10, LBWP-11, and LBWP-12 were obtained from infected samples of acidlime plants with longitudinal splitting of bark and wood disease (LBWSP) and named accordingly. These isolates were used in the present investigation.

## Growth on solid media

The isolates of *B. theobromae* were grown on the four solid media at  $28 \pm 2^\circ\text{C}$  for 2 days. A significant variation was noticed among the isolates of *B. theobromae* with respect to colony growth, mycelia characters and growth rate and chlamyospores production on different solid media. The colony growth of 12 isolates on different solid media are presented in Tables 1. The growth rate of *B. theobromae* depending on the growth rate on solid medium, all isolates of *B. theobromae* were categorized into three groups viz., fast, medium and slow growing isolates. The colonies of isolates

which grew 40-82 mm in diameter within 72 h of incubation were categorized as slow growing isolates and the isolates whose colonies grew 40 to 82 mm in diameter with in 48 h of incubation were categorized as medium growing, where as the colonies which grew 40 to 82 mm in diameter within 24 h of incubation were categorized as fast growing isolates.

The isolates LBWP-1, LBWP-6, LBWP-7, LBWP-8, LBWP-10 and LBWP-12 are medium growing where as LBWP-2, LBWP-3, LBWP-4, LBWP-5, LBWP-9 and LBWP-11 are slow growing, whereas on CDA medium all the isolates are showing slow growth rate (Table 1).

In case of RA medium, the isolates LBWP-1, LBWP-2, LBWP-5 and LBWP-7 are medium growing where as LBWP-3, LBWP-4, LBWP-6, LBWP-8, LBWP-9, LBWP-10, LBWP-11 and LBWP-12 are slow growing isolates (Table 1). The isolates LBWP-1, LBWP-5, LBWP-7, LBWP-10, LBWP-11, and LBWP-12, are medium growing where as LBWP-2, LBWP-3, LBWP-4, LBWP-7, LBWP-9, are slow growing isolates in case of CPA medium (Table 1). In all the four media used none of the isolate has shown fast growth.

In present investigation most of the isolates have showed slow to medium growth rate. However, no isolate showed fast growth which confirmed that the pathogen is slow growing organism. Among the different media tested for their growth rate, CPA medium was observed to be the best which supported for maximum growth of different isolates of pathogen followed by PDA. However, on CDA and RA media, all the isolates have showed least growth rate.

Similar studies were carried out by Ram (1993) and observed variation in the size of conidia, vegetative growth and sporulation.

Spore size was generally similar in all isolates, but spores were smaller in isolates from mango. Vegetative growth rate, mycelial dry weight and sporulation in coconut isolates were lower on all the media tested compared with isolates from other hosts. Chowdhury *et al.*, (1987) studied the morphology of different *B. theobromae* isolates in terms of growth on PDA, aerial mycelium, conidial size and pycnidial status in 6 crops from 5 countries. Almeida *et al.*, (2001) studied morphological characteristics of a strain 715 of *B. theobromae*, a causal agent of plant and fruit rots, and observed production of jasmonic acid, by using CPA and PDA.

#### **Effect of different temperatures on growth of *Botryodiplodia theobromae***

It is evident from table 2 that the maximum mycelial dry weight of 680 mg was at 30°C followed by mycelial dry weight (560 mg) at 25°C and (450 mg) at 20°C. The least dry weight of 370 mg was recorded at 40°C in PDB medium. In CDB, the maximum mycelial dry weight of 370 mg was at 30°C followed by (350 mg) at 40°C and (220 mg) at 20°C. The least mycelial dry weight (200 mg) was recorded at 25°C.

In RB the maximum mycelial dry weight was (640mg) at 30°C followed by 40°C (420 mg) and 25°C (400 mg). The least mycelial dry weight was observed at 20°C (390 mg). In CPB the maximum mycelial dry weight of 510 mg at 30°C followed by 25°C (460 mg) and (450 mg) in 20°C. The least mycelial dry weight was observed at 40°C (330 mg) (Table 2, Fig.2).

In present study, higher dry mycelial weight of the fungus was obtained at 25 to 30°C which was considered as optimum for the vegetative growth. Least dry weight was obtained at 40°C. Among the different

media tested PDB supported for maximum dry mycelial weight followed by RB, CPB and CDB medium.

#### **Effect of different pH levels on growth of *Botryodiplodia theobromae***

The maximum mycelial dry weight (510mg) was at pH 6 followed by pH 5 (440 mg) and pH 7 (410 mg) and they were on par with each other. The fungus did not grow at pH 3 and pH 4 in PDB. In CDB the maximum mycelial dry weight of 400 mg was at pH 6 followed by pH 5 (310 mg) and pH 7 (310 mg). Further, there was no growth at pH 3 and 4 (Table 3, Fig.3).

In RB the maximum mycelial dry weight of 350 mg was at pH 6 followed by pH 7(320 mg). The least mycelial dry weight was recorded at pH 5 (210 mg). There was no growth at pH 3 and 4. In CPB the maximum mycelial dry weight of 460 mg was at pH 7 followed by pH 6 (440 mg). The least mycelial dry weight of 400 mg was at pH 5. No growth of isolates was observed in the broth's pH 3 and pH 4.

The hydrogen ion concentration had marked influence on growth of the fungus. The fungus grew best at pH 6.0 followed by pH 5.0. Hence, the optimum pH for the growth of this fungus was 5.0 to 6.0. No growth was observed at pH 3.0 and 4.0.

More or less similar opinion was expressed by Pati *et al.*, (2001) and who studied the effect of temperature and pH on growth of *Botryodiplodia theobromae* causing Java black rot of sweet potato tubers. The optimum temperature which favoured the growth of *B. theobromae* was between 25 and 30°C, while the optimum pH required for growth was in the range of 5.0-6.0.

**Table.1** Growth of *B. theobromae* isolates on different growth media

Isolate	PDA medium		CDA medium		RA medium		CPA medium	
	Colony diameter (mm)	Growth rate	Colony diameter (mm)	Growth rate	Colony diameter (mm)	Growth rate	Colony diameter (mm)	Growth rate
<b>LBWP-1</b>	82.0	Medium	81.6	Slow	82.0	Medium	82.0	Medium
<b>LBWP-2</b>	80.0	Slow	79.2	Slow	79.8	Medium	82.0	Slow
<b>LBWP-3</b>	80.6	Slow	80.6	Slow	79.8	Slow	80.6	Slow
<b>LBWP-4</b>	81.6	Slow	80.4	Slow	78.8	Slow	81.8	Slow
<b>LBWP-5</b>	81.4	Slow	81.6	Slow	81.0	Medium	82.0	Medium
<b>LBWP-6</b>	82.0	Medium	82.0	Slow	82.0	Slow	82.0	Slow
<b>LBWP-7</b>	82.0	Medium	82.0	Slow	82.0	Medium	82.0	Medium
<b>LBWP-8</b>	82.0	Medium	82.0	Slow	82.0	Slow	82.0	Slow
<b>LBWP-9</b>	82.0	Slow	82.0	Slow	81.4	Slow	82.0	Slow
<b>LBWP-10</b>	82.0	Medium	82.0	Slow	82.0	Slow	82.0	Medium
<b>LBWP-11</b>	80.7	Slow	82.0	Slow	81.8	Slow	82.0	Medium
<b>LBWP-12</b>	82.0	Medium	82.0	Slow	81.6	Slow	82.0	Medium
<b>CD (0.05)</b>	0.5209	--	0.6230	--	0.6230		0.2176	

**Table.2** Effect of different temperature levels on *B. theobromae* growth on different growth media (in mg)

<b>Temperature</b>	<b>PDB</b>	<b>CDB</b>	<b>RB</b>	<b>CPB</b>
<b>20°C</b>	450	220	390	450
<b>25°C</b>	560	200	400	460
<b>30°C</b>	680	370	640	510
<b>40°C</b>	370	350	420	330
<b>CD (.05)</b>	2.03	1.88	2.88	1.88

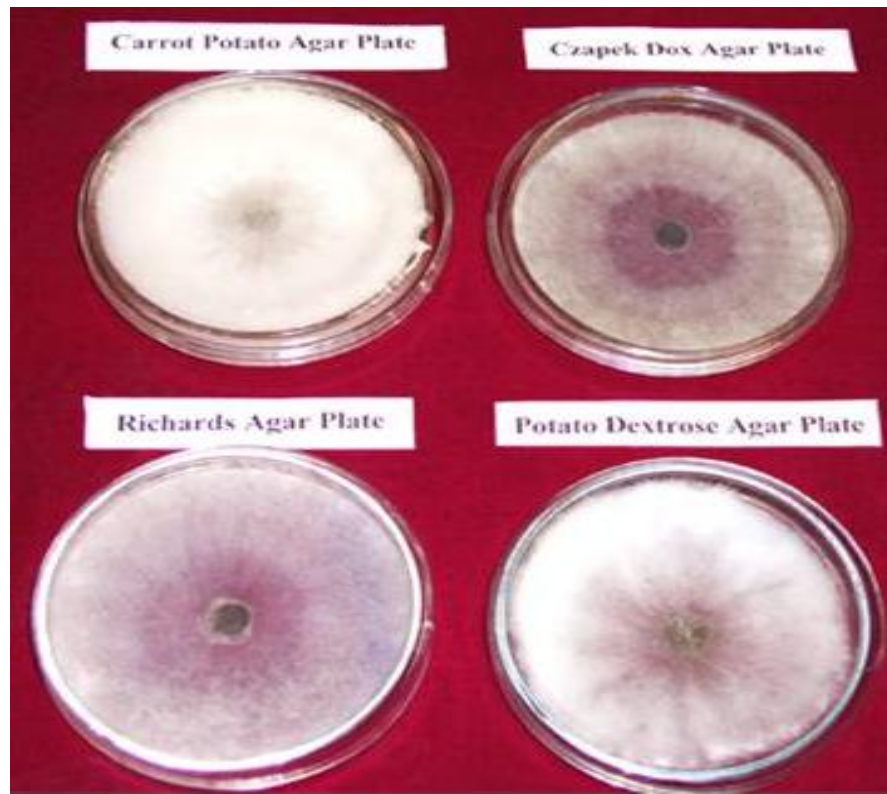
PDB = Potato Dextrose Broth  
CDB = Czapek Dox Broth  
RB = Richards Broth  
CPB = Carrot Potato Broth

**Table.3** Effect of different pH levels on *B. theobromae* grown on different growth

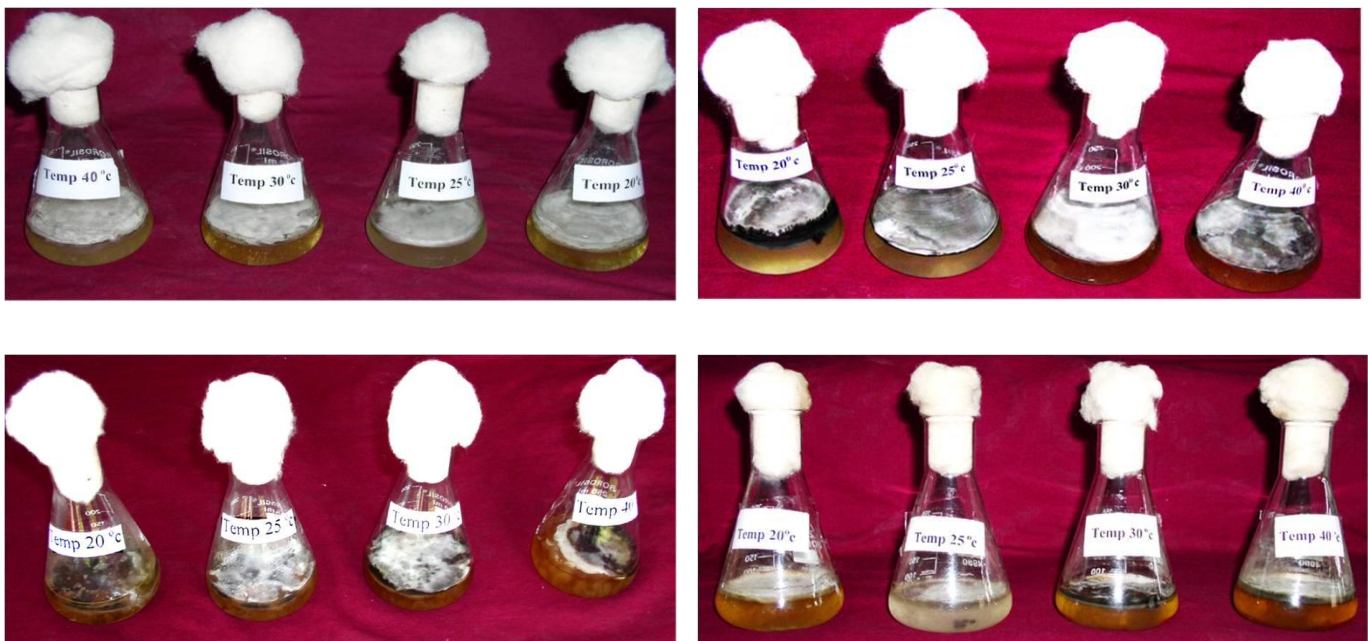
<b>pH</b>	<b>PDB</b>	<b>CDB</b>	<b>RB</b>	<b>CPB</b>
<b>7.0</b>	410	310	320	460
<b>6.0</b>	510	400	350	440
<b>5.0</b>	440	310	210	400
<b>4.0</b>	0	0	0	0
<b>3.0</b>	0	0	0	0
<b>CD (.05)</b>	2.82	2.44	1.56	3.13

PDB = Potato Dextrose Broth  
CDB = Czapek Dox Broth  
RB = Richards Broth  
CPB = Carrot Potato Broth

**Fig.1** Colony morphology of *Botryodiplodia theobromae* on different solid media



**Fig.2** Effect of different temperature levels on growth of *B.theobromae* in (A) Richards broth (B) Carrot potato broth (C) Potato dextrose broth (D) Czapek dox broth



**Fig.3** Effect of different pH levels on growth of *B.theobromae* in (A) Potato dextrose broth (B) Czapek dox broth (C) Richards broth (D) Carrot potato broth



Eng *et al.*, (2003) studied effect of temperature and pH on colony growth of *Botryodiplodia theobromae* both temperature and culture medium influenced the growth density, but radial velocity of growth was affected by temperature above 40°C. In addition, initial pH of culture media did not affect either parameter.

Sabet *et al.*, (1995) observed the factors affecting severity of *B. theobromae* on date palm cv. Zaghlol off-shoot and its control. The optimum temperature for infection by the fungus was ranged from 30 to 35°C at 50% RH. Propiconazole was the best fungicide to inhibit the linear growth of the pathogen and to eliminate new infections in the field. Mali *et al.*, (1987) has given the methods of inoculation, temperature and humidity in relation to rotting of corm of elephant foot yam (*Amorphophallus campanulatus* Blume) by *Botryodiplodia*

*theobromae*. In inoculation experiments, corm developed rotting only when inoculum placed within the pulp and surface inoculated corms remained healthy. Rotting was initially slow but increased after 1 month. Maximum rotting occurred at 30°C and none occurred at 10 or 40°C. High RH (85-90%) has contributed to maximum rotting where as none occurred at low temperature with high RH or at room temperature with low RH.

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