

Original Research Article

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## Establishment of the Optimum Temperature and pH for the Growth of *Phaeoacremonium parasiticum* *in-vitro* and Standardization of the Media for its Mass Culture

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### ABSTRACT

#### Keywords

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This paper presents an *in vitro* experiment conducted to evaluate the optimum temperature and pH for growth of *Phaeoacremonium parasiticum*, responsible for oleoresin deposition in agarwood trees. In the present study, *P. parasiticum* was grown at four different levels of temperature viz.,  $25 \pm 1^\circ\text{C}$ ,  $27 \pm 1^\circ\text{C}$ ,  $30 \pm 1^\circ\text{C}$  and  $32 \pm 1^\circ\text{C}$ , and it was found to attain the highest radial growth at  $30 \pm 1^\circ\text{C}$  (5.3 cm) followed by that attained at  $32 \pm 1^\circ\text{C}$  (0.0452 m),  $27 \pm 1^\circ\text{C}$  (0.0450 m) and  $25 \pm 1^\circ\text{C}$  (0.0240 m). *P. parasiticum* was also grown at five different levels of pH viz., 5.0, 5.5, 6.0, 6.5 and 7.0, to optimize the one most favorable for its growth and it was observed that *P. parasiticum* had the highest radial growth at a pH of 6.5 (4.10 cm), followed by that attained at pH 6.0 (0.0343 m), 7.0 (0.0330 m), 5.5 (0.0168 m) and 5.0 (0.0150 m). Upon mass culturing in four different liquid media viz., potato dextrose broth (PDB), host extract broth (HEB), an equal volume of PDB and HEB (PDB + HEB), and malt extract broth (MEB), it was found that the fungus first attained 100 per cent growth in PDB after 20 days of inoculation with the biomass having the highest fresh weight (2299 mg) and dry weight (1420 mg).

### Introduction

Agarwood, *Aquilaria malaccensis* L. (Thymeleaceae), is known as “The World’s Precious Tree”, or “The Floral Wealth of India”. It is one of the most important species of commerce and valued for its fragrant resinous dark coloured wood, known in trade as “Agar”, developed as a result of fungal infection (Nath and Saikia, 2002). The tree gains commercial value only after getting infected by a fungus. In natural forests, only an estimated 7-10 per cent of the trees are infected by the fungus (Ng *et al.*, 1997). The extraction of oleoresin from the tree is a

destructive work and the whole tree has to be cut down to see if the wooden bark has been infected and turned dark in its colouration due to deposition of oleoresin. Upon burning, emission of an aromatic smoke, from this discoloured fungal infected portion confirms the presence of oleoresin in it. This destructive and difficult method of identification, of agar deposited trees has greatly added to the near-extinction of natural stands of trees. Depletion of wild trees by indiscriminate cutting for agarwood has resulted in the trees being listed and protected

as an endangered species (CITES, 2005). International Union for Conservation of Nature (IUCN) red data list of the year 2011 has mentioned it as vulnerable and at the edge of extinction from natural forest (Saikia *et al.*, 2012).

The fungus responsible for the whole process in the tree was identified as *Phaeoacremonium parasiticum* (ITCC no. 7734, IARI, New Delhi) and this study was put forth to establish the optimum temperature and pH supporting the favorable growth of the fungi *in-vitro* and to establish the media in which it could be mass cultured in order to proceed with artificial inoculation methods.

### **Materials and Methods**

The experiment was conducted at Mycology Research Section, Department of Plant Pathology, Assam Agricultural University (AAU), Jorhat, Assam, India during 2013-15

### **Survey and sample collection**

The bark portions of the tree, showing typical symptoms of fungal infection, were collected from two different host hot spots of Assam *viz.*, Sivasagar and Nazira, in Sivasagar district during, 2013-14. The samples collected in air tight zip bag separately during the roving survey were brought to the Laboratory of Mycology Research Section, AAU, Jorhat, Assam with an aim to isolate the fungi associated with oleoresin deposition in it.

### **Isolation of the agarwood associated fungi**

As many as nine different fungal species belonging to five different genera *viz.*, *Aspergillus*, *Rhizoctonia*, *Fusarium*, *Didymobotryum*, and *Phaeoacremonium* were isolated from the infected agarwood samples

collected during roving survey in agarwood hot spots of Assam (Sivasagar district), India. These nine fungi upon screening in four different media *viz.*, Media A (Host extract + PDA), B (Host extract + Agar + Dextrose), C (Host extract + Agar), and D (PDA), lead to selection of only three as the most probable pathogenic organisms responsible for causing infection and subsequent oleoresin deposition in the tree. These three fungi were thus artificially inoculated separately in the healthy host under its natural ecosystem, along with a control (plain PDA media). Symptoms of the fungal infection were reproduced in only the trees inoculated with *Phaeoacremonium* sp. and this pathogen was reisolated from those infected portions, thus confirming the Koch's postulates. The release of an aromatic smoke upon burning the *Phaeoacremonium* infected bark portion, and the absence of the same while burning the bark portions adjacent to other fungal inoculated sites in the tree, confirmed that *Phaeoacremonim* sp. is the actual pathogen responsible for oleoresin deposition in *A. malaccensis*. Hence this pathogen was later correctly identified as *Phaeoacremonim parasiticum* by Indian Type Culture collection (ITCC NO. 7734), IARI, New Delhi. The culture was thus preserved and maintained at 4°C for further studies.

### **Establishment of optimum temperature for growth of the fungus**

For establishing the optimum temperature for growth of *P. parasiticum* it was grown at four different temperatures *viz.*, 25±1°C, 27±1°C, 30±1°C and 32±1°C. Fresh fungal discs (1 cm diameter) were inoculated at the center of PDA plates and observed for its growth for the subsequent 12 days. The radial growth of the fungus was observed and recorded every 72 hours till the 12<sup>th</sup> day after inoculation. For each temperature used for the study, three replications were maintained.

### **Establishment of optimum pH for growth of the fungus**

Five different levels of pH viz., 5.0, 5.5, 6.0, 6.5 and 7.0 were tested for establishing the optimum one for growth of *P. parasiticum*. Fresh fungal discs (5mm diameter) were inoculated at the center of PDA plates having different pH with three replicates. All the inoculated plates were incubated in BOD incubator (Scigenics Biotech (Pvt.) Ltd., Model: ORBITEK<sup>R</sup>). Observation on radial growth of the fungus was recorded for the subsequent 12 days. Observations on the radial growth of fungi were recorded every 72 hours till the 12<sup>th</sup> day after inoculation.

### **Mass culture of the fungus**

To standardize the media for mass culturing of *P. parasiticum*, four different broth viz., potato dextrose broth (PDB), host extract broth (HEB), an equal volume of Potato dextrose broth and host extract broth (PDB + HEB), and malt extract broth (MEB) were tested. All the broths were inoculated aseptically with freshly cultured mycelial disc (1 cm diameter) of *P. parasiticum* with three replicates. The inoculated broths were incubated at  $27 \pm 1^\circ\text{C}$  in a BOD incubator (Scigenics Biotech (Pvt.) Ltd., Model: ORBITEK<sup>R</sup>). The fresh and dry weight of the fungal mat was noted, once the fungi attained 100 per cent growth in any one of these broth. Spore count was also taken using a haemocytometer (FEIN- OPTIK BLANKENBURG) at  $10^{-1}$  dilution to identify the media in which the fungus will have the highest sporulation.

## **Results and Discussion**

### **Establishment of optimum temperature for growth of *P. parasiticum***

Twelve days after inoculation in PDA plates, it was observed that the *P. parasiticum* had its highest growth rate at  $30 \pm 1^\circ\text{C}$  (5.3 cm,

Table 1, Fig. 1c). This was significantly higher compared to the radial growth attained by the fungus at the other three temperatures (Table 1, Fig. 1a, 1b and 1d). There wasn't much significant variation in radial growth attained by the fungus at temperature,  $27 \pm 1^\circ\text{C}$  (4.50 cm) and  $32 \pm 1^\circ\text{C}$  (4.52 cm). The least radial growth was observed at  $25 \pm 1^\circ\text{C}$  (2.40 cm). Hence, it is evident from the experiment that  $30 \pm 1^\circ\text{C}$  is the optimum temperature for the growth of *P. parasiticum*.

### **Establishment of optimum pH for growth of *P. parasiticum***

When *P. parasiticum* was grown at five different levels of pH viz., 5.0, 5.5, 6.0, 6.5 and 7.0, the maximum radial growth was observed to be attained by it, at a pH of 6.5 (Table 2, Fig. 2d), twelve days after inoculation, which was significantly higher compared to that at the other four treatments used. This was followed by the radial growth attained by the fungus at a pH of 6.0, 7.0, 5.5 and 5.0 which are 3.43 cm, 3.30 cm, 1.68 cm and 1.5 cm, respectively. There was not much significant variation in the radial growth of the fungi attained at a pH of 6.0 (3.43 cm) and 7.0 (3.30 cm) (Fig. 2c and 2e). The least radial growth was observed at a pH of 5.5. Hence, it has been proved that at a pH of 6.5 the fungus has its optimum growth.

### **Standardization of the liquid media for mass culture of *P. parasiticum***

With an aim to standardize the liquid media on which *P. parasiticum* could be mass cultured, the fungus was inoculated in four different broth viz., potato dextrose broth (PDB), host extract broth (HEB), an equal volume of PDB and HEB (PDB + HEB), and malt extract broth (MEB), and kept in the BOD incubator at a temperature of  $27 \pm 1^\circ\text{C}$ . The fungus first attained 100% growth in PDB after 20 days of inoculation (Fig. 3a).

**Table.1** Radial growth of *P. parasiticum* at different temperatures, after 12 days of inoculation

Temperature(°C)	Radial Growth (cm)
25±1	2.400
27±1	4.500
30±1	5.300
32±1	4.520
SEd (±)	0.014
CD (0.05)	0.033

Data are mean of three replications

**Table.2** Radial growth of *P. parasiticum* at different pH, after 12 days of inoculation

pH	Radial Growth (cm)
5.0	1.50
5.5	1.68
6.0	3.43
6.5	4.10
7.0	3.30
SEd (±)	0.007
CD(0.05)	0.014

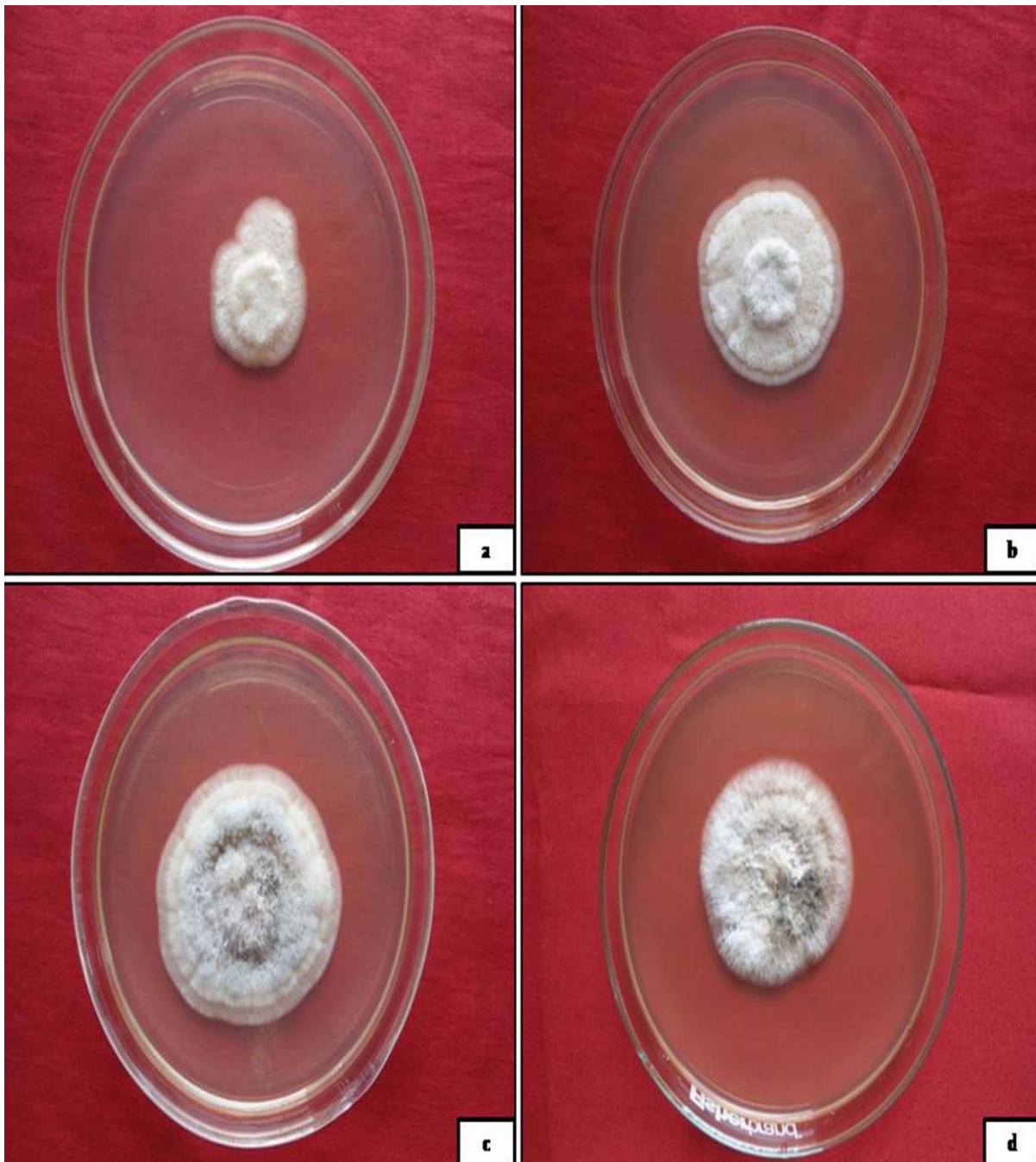
Data are mean of three replications

**Table.3** Biomass production and sporulation of *P. parasiticum* in different liquid media after 20 days of inoculation

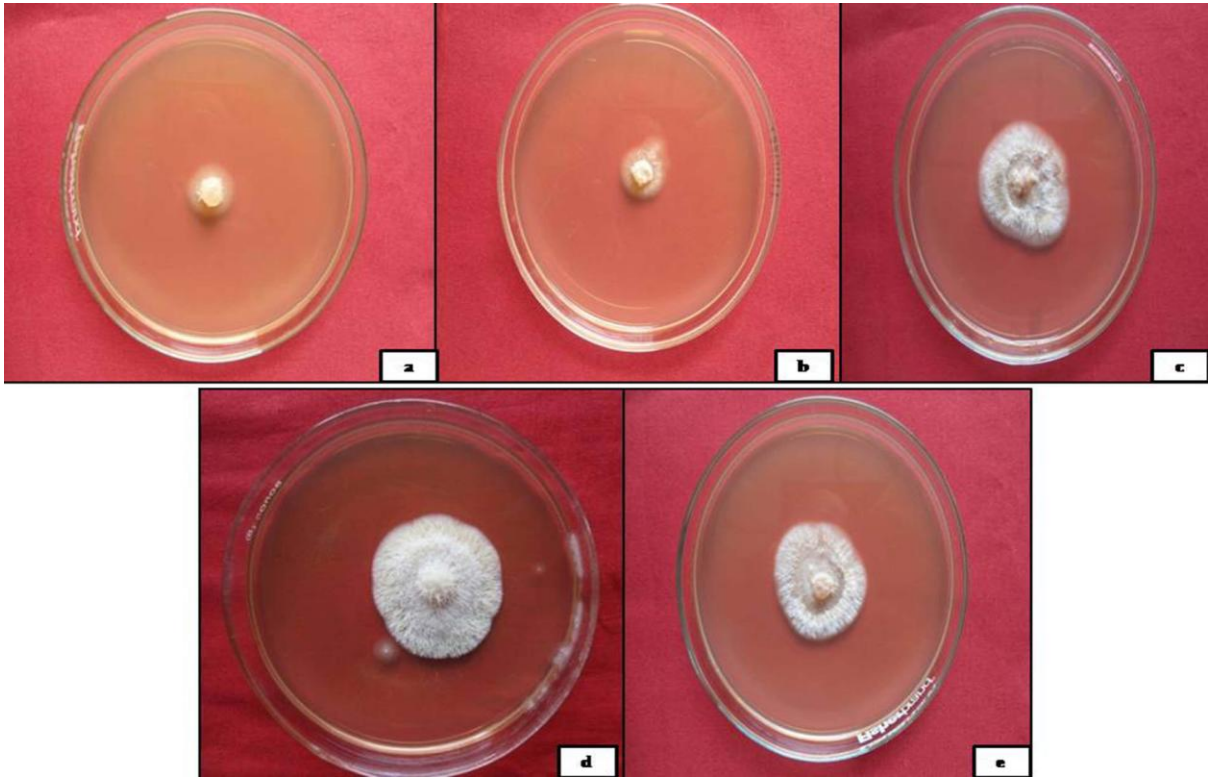
Treatments	Fresh weight (gm)*	Dry weight (gm)*	Sporulation** (log spore/ml)
Potato dextrose broth	2.299	0.142	6.24
Host extract broth	0.634	0.047	5.59
Potato dextrose broth+ Host extract broth (PDB+ HEB)	1.120	0.059	5.85
Malt extract broth(MEB)	1.616	0.088	6.01
SEd(±)	0.031	0.009	
CD(0.05)	0.073	0.020	NS

\*Data are mean of three replications, \*\* Data are mean of spore count in ten similar squares of a haemocytometer, DAI\*: Days after inoculation, NS - Not statistically analysed.

**Fig.1** a-d. Radial growth attained by *P. parasiticum* at, a.  $25\pm 1^{\circ}\text{C}$ , b.  $27\pm 1^{\circ}\text{C}$ , c.  $30\pm 1^{\circ}\text{C}$  and d.  $32\pm 1^{\circ}\text{C}$  ,after 12 days of inoculation in PDA plate



**Fig.2** a-e. Radial growth attained by *P. parasiticum* at a pH of , a. 5.0, b. 5.5, c. 6.0, d. 6.5 and e. 7.0, after 12 days of inoculation in PDA plate



**Fig.3** a-d. Fungal biomass coverage after 20 days of inoculation in, a. potato dextrose broth, b. host extract broth, c. an equal volume of Potato dextrose broth and host extract broth, and d. malt extract broth



The fresh weight (2.299 gm) and dry weight (0.142 gm) of *P. parasiticum* after 20 days of inoculation, was the highest in PDB compared to the other three liquid media (Table 3). Highest spore count (6.24 log spore/ml) was also observed in PDB (Table 3). This was followed by the fresh weight, dry weight and sporulation attained by the fungus in MEB (1.616 gm, 0.088 gm, 6.01 log spore/ml), an equal volume of PDB and HEB (1.120 gm, 0.059 gm and 5.85 log spore/ml) and HEB (0.634 gm, 0.047 gm and 5.59 log spore/ml). Thus it was evident that PDB is the best liquid media for mass culture of *P. parasiticum* than the other three broths used in the experiment and hence it can be standardized for mass culture of the fungus. It is evident from the data presented in Table 1 that *P. parasiticum* had the highest radial growth at a temperature of  $30 \pm 1^\circ\text{C}$  which is in confirmation with a similar study done by Mostert and his co-workers (2006) who established the cardinal temperatures as  $10^\circ\text{C}$ ,  $30^\circ\text{C}$  and  $40^\circ\text{C}$  for the growth of *Phaeoacremonium* sp. Data presented in Table 2 revealed that *P. parasiticum* had the highest radial growth at a pH of 6.5 (4.10 cm) followed by that at a pH of 6.0 (3.43 cm), 7.0 (3.30 cm), 5.5 (1.68) and 5.0 (1.50). In a similar study conducted by Goswami *et al.*, (2011) on “The variations in different isolates of *Rhizoctonia solani* based on temperature and pH” he found that out of the five isolates of *R. Solani viz.*, DIN-8, GAZ-9, SYL-30, JES-16 and GAZ-18 the isolate DIN-8 had the highest radial growth at a pH of 6.0 and for the other four isolates the optimum pH was 7.0. Thus it is evident that there is variation in growth of any fungi with respect to the pH of the media or the environment in which it survives.

From the data presented in Table 3, it is clear that *P. parasiticum* has the highest biomass production (fresh wt: 2.299 gm and dry wt: 0.142 gm) and sporulation (6.24 log spore/ml) in PDB followed by the fresh weight, dry

weight and sporulation attained by it in MEB (1.616 gm, 0.088 gm, 6.01 log spore/ml), an equal volume of PDB and HEB (1.120 gm, 0.059 gm and 5.85 log spore/ml) and HEB (0.634 gm, 0.047 gm and 5.59 log spore/ml). In a similar work done by Claire *et al.* (1998) on the “Effect of various liquid culture media on morphology, growth, propagule production, and pathogenic activity to *Bemisia argentifolii* of the entomopathogenic Hyphomycete, *Paecilomyces fumosoroseus*” they found that the maximal biomass was obtained with two media: Jackson and Catroux media, while high concentrations of propagules were produced in three media Jackson, Paris, and Catroux media. This in turn does mean that the fungal biomass and propagule production varies with the liquid media that has been used for its culture *in vitro*.

In conclusion, from this study, it can be concluded that for mass production of *P. parasiticum*, culturing it in PDB at a temperature of  $30 \pm 1^\circ\text{C}$  and a pH of 6.5 would give the best results.

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