J. Mycopathol. Res. 39(1):21–27 (2001) *Printed in India*

Spoilage of sweetpotato tubers in tropics. V. Java black rot by *Botryodiplodia theobromae* Pat : Phenol accumulation in culture and in tubers during spoilage

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The fungus *Botryodiplodia theobromae* Pat. causes Java black rot of sweetpotato in storage, produced phenolics in culture and in infected sweetpotato tubers. The optimum temperature which favoured phenol accumulation in culture was around 30°C while the optimum pH for phenol accumulation was between 5.0–6.0. There was very little polyphenol oxidase (PPO) activity in the culture filtrate (CF). The maximum phenol synthesis was observed in mineral medium (Czapek Dox) using fructose as carbon source followed by xylose and dextrose, while organic nitrogen (tryptone, beef extract and yeast extract) yielded more phenol than the inorganic nitrogen sources. Further, *Botryodiplodia* infected tubers exhibited between 2-13 times more phenol accumulation as compared to the uninfected tubers and incidentally, white coloured varieties (Pusa Safed and P-13-10-2) showed more phenol accumulation than the red coloured variety ('Gouri'). The phenol synthesis by *B. theobromae* in culture suggests that the fungus contributed partly to the phenol pool of the infected tubers.

Key words : *Botryodiplodia theobromae*, Java black rot, phenol, spoilage, sweetpotato

INTRODUCTION

Botryodiplodia theobromae Pat., an active pathogenic fungus causes a dry rot (Java black rot) in sweetpotato tubers in tropics and subtropics (Arinze and Smith, 1979, 1982; Ray and Misra, 1995; Ray and Punithalingam, 1996; Somner, 1986). The characteristics of Java black rot have been studied in our previous papers (Ray *et al.*, 1994; Ray and Punithalingam, 1996) and elsewhere (Punithalingam, 1980; Snowdon, 1991). The affected roots show externally dark patches within which develop innumerable pycnidia and internally the tissues turn yellow and later coal black. Ultimately, the roots become shriveled, brittle and mummified (Ray *et al.*, 1994).

Little is known on the biochemical aspects of spoilage in sweetpotatoes during *B. theobromae* infection. There are reports which show an increase in total phenolics and decrease in carotene and ascorbic acid content in roots infected with *Rhizopus stolonifer* (Thomson, 1979) and *R. oryzae* (Ray *et al.*, 1997). Uritani *et al.* (1984) reported enhanced deposition of phenol in sweetpotato tissues attacked by the black rot fungus *Ceratocystis fimbriata* Ell. Halst. Arinze and Smith (1982) studied the distribution pattern of polygalacturonase, total phenolics, polyphenoloxidase (PPO) and peroxidase in sweetpotato tissues infected with *Botryodiplodia theobromae*, *Botrytis cinerea* and *Fusarium solani*.

There are few reports to show that certain plant pathogenic fungi secrete phenols in culture (Turner and Aldridge, 1983) and in host tissues (Reddy and Rao, 1975). However, a definite explanation is not available regarding the role played by such phenols in host-pathogen interactions. In this paper, we report the extracellular accumulation of phenol by *Botryodiplodia theobromae* in culture and in tubers of sweetpotato infected with the fungus.

MATERIALS AND METHODS

Isolate and sweetpotato provenance

Botryodiplodia theobromae Pat. used in this study was

isolated from the post-harvest spoilage of sweetpotatoes. Spore suspensions of *B. theobromae* was prepared by washing fungal culture grown on PDA slants in 100 ml sterile distilled water so as to retain 6 x 10^6 spores per ml suspension. The same concentration was used in all experiments.

Freshly harvested sweetpotatoes (cv. Pusa Safed, Gouri and P-13-10) were collected from the farm of the Regional Centre for Central Tuber Crops Research.

Effect of sucrose concentration and carbon sources

Czapek Dox (CD) medium (100 ml) in Erlenmeyer flasks with variable concentrations of sucrose (2–10%) were inoculated with one ml of *B. theobromae* spore suspension and incubated at room temperature (30 ± 2°C) for 10 days. At 5 day intervals, triplicate flasks were removed and the culture fillrate (CF) was analyzed for cell mass growth and total phenolics.

To study the effect of carbon sources on phenolic production, different carbon compounds were incorporated into CD medium in place of 3% sucrose. Cell mass and total phenolics were estimated in CF at 5 day intervals.

Effect of nitrogen and sulphur sources

Different nitrogen and sulphur sources (0.005%) were used in medium to study the cell mass, total phenolics and PPO activity. The other conditions of the experiment were essentially same as in the previous experiments.

Phenol and PPO accumulation

Freshly harvested and healthy tubers (cv. Pusa safed, Gour and P-13-10) were surface sterilized with 70% ethanol. These tubers were inoculated with mycelial discs of *B. theobromae* by the method of Ray and Punithalingam (1996). Ten tubers were inoculated for each test and three replicates were maintained at room temperature (30 ± 2°C) for 18 days. Tubers (three replicates) inoculated with agar disc (in place of mycelium) were kept as control. Both inoculated and control tubers were analyzed for total phrnolics at 6 days interval. Total phenolics were quantified as mg phenols per gm dry wt of tubers.

Total phenolics and O-dihydroxy phenolics in culture and sweetpotato tissues were assayed by Folin-Ciocelteu method and Arnow's method respectively (Mahadevan and Sridhar, 1998). PPO activity in culture and tissues was assayed following Kar and Misra (1976) using pyrogallol as the substrate.

Samples of tuber of fungal mass (about 200 mg) were homogenized with 10 ml of 0.1 M phosphate buffers (pH 6.8) and divided into two equal 5 ml portions. One 5 ml portion was used for phenol assay. The other 5 ml was centrifuged at 2°C for 15 min at 17,000 g in a refrigerated centrifuge. The clear supernatent was directly assayed for phenol content and PPO activity.

One ml of the extracts was mixed with 1 ml of Folin-Ciocalteu reagent and 2 ml of 20% Na_2CO_3 solution in glass tubes. The tubes were shaken throughly and heated in a boiling water bath for 1 min and then cooled under a running tap. The content was diluted to 25 ml with water when blue colour developed and the absorbances were recorded at 650 nm. A blank containing the reagents minus the tissue extract was used to adjust the absorbance to zero. The unknown phenol quantity was estimated from a standard curve of pyrocatechol.

One ml of the extract was mixed with 1 ml of 0.05 N HCL, 1 ml of Arnow's reagent (1 g of $NaNO_2$ + 1 g of Na_4MoO_2 + 10 ml of distilled water), 10 ml of distilled water and 2 ml of 1 N NaOH. The pink colour developed showed the presence of ortho-dihydroxy phenols. The absorbance was measured at 515 nm and the amount present in the samples were calculated from a standard curve prepared using catechol.

The assay mixture for polyphenol oxidase comprised : 125 moles of phosphate buffer pH 6.8, 50 moles of pyrogallol and 1 ml of enzyme extract. This was incubated at 25°C for 5 min and the reaction was stopped by adding 0.5 ml of 5% (v/v) H_2SO_4. The amount of purpurogallin formed was determined by measearing the absorbance at 420 nm. The enzyme activity was expressed in absorbance units.

Analysis of variance (ANOVA)

ANOVA was performed using MICROSTAT software following completely randomized block design. Mean comparison within the treatments was performed by Least Significant Differences (LSD) test at p = 0.05 level (Panse and Sukhatme, 1967).

RESULTS AND DISCUSSION

Preliminary results revealed that the CF of *B. theobromae* in CD medium developed a blue colour when tested with Folin-Ciocalteu (FC) reagent. To confirm whether the blue colouration was due to the

Table 1. Cell mass, total phenolics and PPO activity in tissues of *Botryodiplodia theobromae* in PD and CD medium

Media	Days of incubation	Cell mass (mg)	Total Phenolics (mg/g)	PPO Activity (O.D.)
PD	3	290	193	0.201
	6	810	307	0.229
	9	1040	311	0.248
CD	3	305	98	0.189
	6	775	225	0.205
	9	990	278	0.218

LSD between treatments at p = 0.05 level are 51.2 (cell mass), 36.2 (total phenolics) and 0.9 (PPO activity), respectively.

Table 2. Effect of sucrose concentrations on cell mass and phenolic content of *Botryodiplodia theobromae* in CD medium

	Cell mass (mg)		Total Phenolics (mg/ml)	
Sucrose (%)	5 D	10 D	5 D	10 D
2	405	645	16.1	50.89
4	445	1045	49.4	80.7
6	520	1120	54.4	82.59
8	705	1255	59.2	82.79
10	815	1325	66.0	85.35

LSD between treatments at p = 0.05 level are 11.9 (cell mass) and 10.6 (total phenolics), respectively.

reaction between FC reagent and extracellular phenolics and/or extracellular soluble proteins, the CF was treated with 20% trichloroacetic acid to precipitate any soluble protein. The resulting supernatans were decanted and reacted with FC reagent to confirm the accumulation of total phenolics (Mahadevan and Sridhar, 1998). The results obtained from three different media (PD, CD and CD + YE) for growth and phenol accumulation are shown in Fig. 1.

The results show that *B. theobromae* showed better growth in CD + YE than on CD or PD media after 12 days incubation. The peak growth was obtained on 9th day but thereafter, the cell masses constantly decreased. This was probably due to either autolysis (Lahoz *et al.*, 1976) or nutrient limitation.

There was no definite trend in phenol accumulation with respect to the type of media. After 3 days, maximum phenol accumulated in PD medium followed by CD + YE and CD media in discending order while on 6th day, CD + YE gave maximum

Table 3. Effect of carbon, nitrogen and sulpur sources on cell mass and phenolic content of *Botryodiplodia theobromae* in CD medium

Sources	Incubation			
	Cell mass (mg)		Phenolics (mg/ml)	
(C, N and S)	5 D	10 D	5 D	10 D
*C source**				
Sucrose	355	575	12.6	34.6
Lactose	280	470	29.8	32.9
Dextrose	280	750	62.9	78.8
Fructose	415	1000	134.5	145.8
Xylose	200	670	80.4	130.6
Maltose	550	1200	60.8	50.4
Soluble starch	515	940	24.0	34.6
Raw strach	510	760	15.6	6.7
*N source***				
Pot. nitrate	480	820	18.4	14.1
Ammon. nitrate	270	885	22.4	36.0
Sod. nitrite	240	720	44.7	27.3
Pot. nitrite	215	245	46.8	46.7
Peptone	215	935	63.5	55.6
Beef extract	285	950	81.7	92.5
Tryptone	375	1020	88.0	99.4
Yeast extract	695	1140	66.2	47.0
Ammon. oxalate	990	1980	58.8	50.6
Ammon. acetate	270	1150	45.6	47.0
Sod. nitrate	380	810	25.0	20.4
Ammon. chloride	235	560	24.2	11.5
Ammon. sulphate	300	835	15.6	18.4
*S source****				
Copper sulphate	225	60	5.5	0.0
Sod. sulphite	185	180	10.8	20.5
Sod. thiosulphate	340	245	18.8	34.6
Sod. sulphate	205	485	20.8	17.3
Magn. sulphate	820	990	30.2	39.7
Ammon. persulphate	125	165	20.3	24.0
Zinc sulphate	465	175	11.8	13.2
Pot. sulphate	200	430	10.8	10.3

* LSD at p = 0.05 level between treatments are 41.2 (cell mass) and 29.1 (phenolics).

** LSD at p = 0.05 level between treatments are 33.9 (cell mass) and 18.8 (phenolics).

*** LSD at p = 0.05 level between treatments are 36.9 (cell mass) and 26.1 (phenolics).

phenolic content followed by PD and CD media. However, on 9 and 12 days of incubation, phenols accumulated more in CD than in CD + YE or PD media. There was no sign of accumulation of O-dihydroxy phenols in CF in any of the samples. There are a few reports on extracellular accumulation of phenolics in microbial culture (Turner and Aldridge,

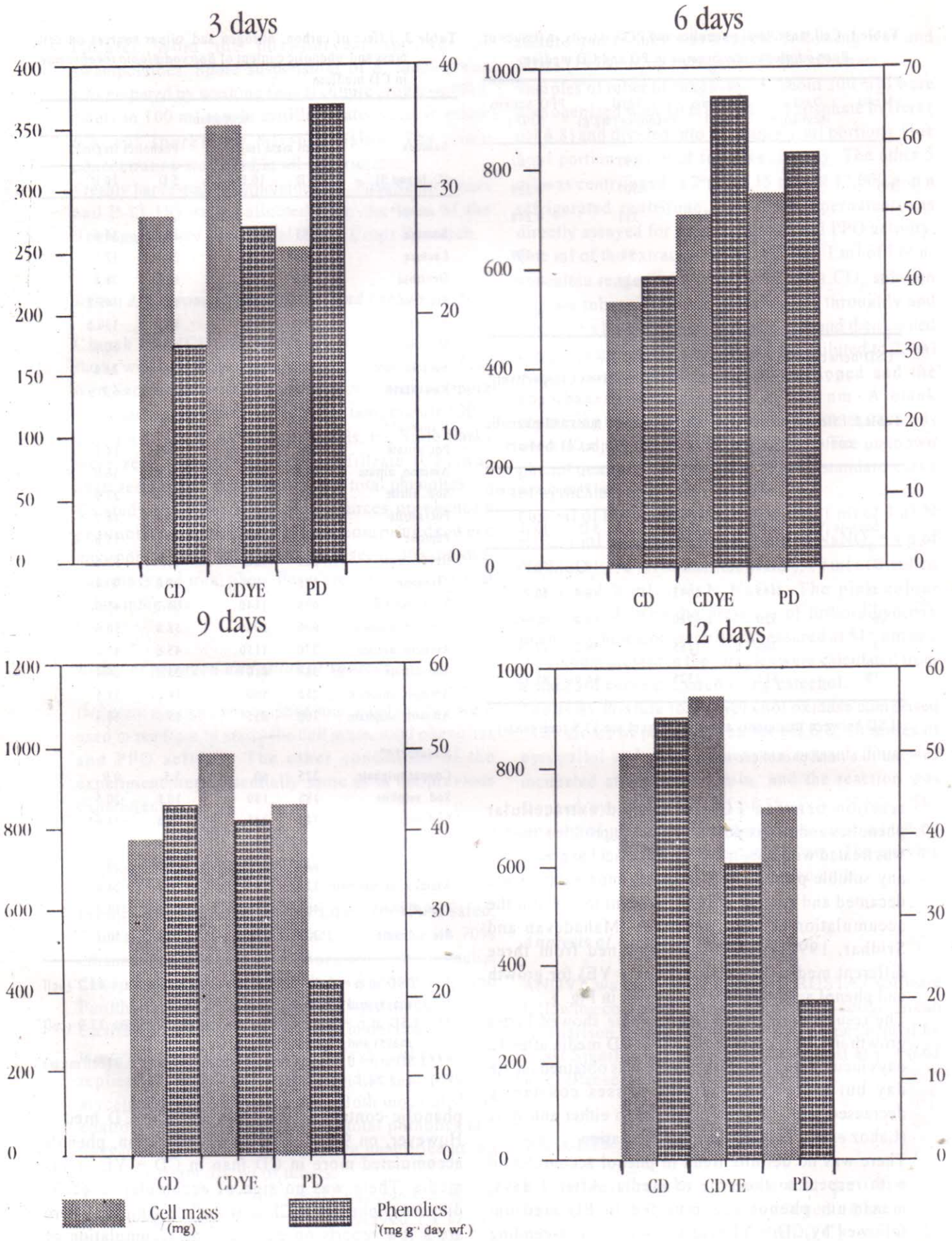

Fig. 1. Growth (cell mass) and phenolic accumulation of B. theobromae grown in different media during 12 days

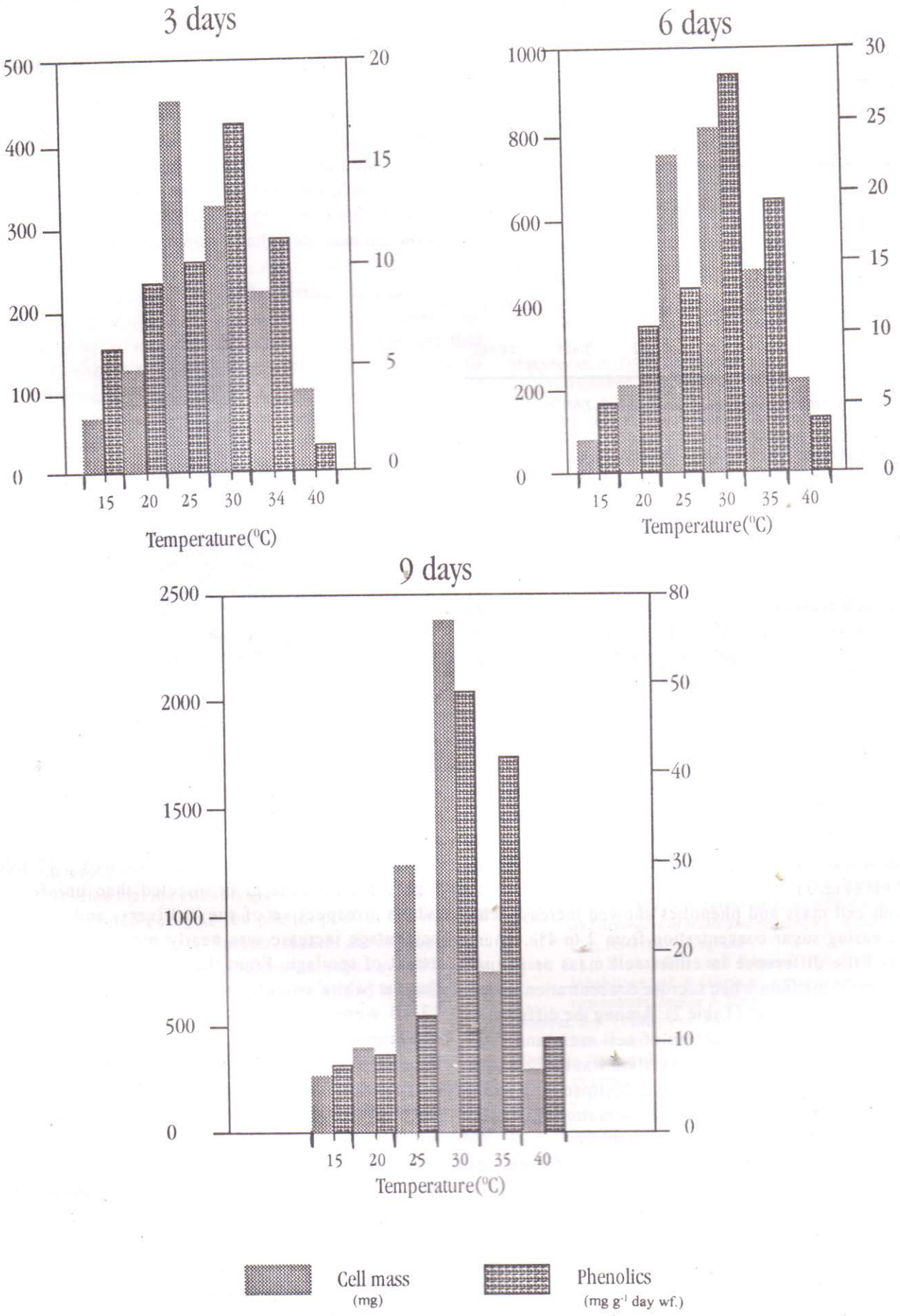

Fig. 2. Effect of temperature on the cell mass and total phenolics in the CF of B. theobromae in CD medium during 9 days

Table 4. Total phenolics accumulated in *B.theobromae* infected and uninfected tissues of sweetpotato tubers

Cultivars		Total phenolics (mg/g dry wt. tissue)		
	Days	6	12	18
Pusa Safed	Uninfected	0.451	0.748	0.949
	Infected	0.809	3.119	12.821
8516	Uninfected	0.854	2.775	1.778
	Infected	4.537	10.097	12.956
P-13-10	Uninfected	3.34	1.066	1.671
	Infected	5.632	3.025	18.047

LSD at p = 0.05 level between treatments is 2.63.

1993). *Rhizoctonia solani,* the pathgoen of damping off disease in groundnut, was found to produce a variety of phenols i.e. transcaffeic acid, protocatechuic acid etc., in culture. Similar type of phenols was also observerd which were probably produced by the fungus (Reddy and Rao, 1978).

The cell mass accumulation was highest at 30°C and lowest at 15°C and 40°C. Ray and Punithalingam (1996) also reported optimum growth of *B. theobromae* at 25-30°C. This also corroborated the findings of Somner (1986) that the post-harvest pathogens grew best between 25°C and 30°C depending on the species. (Fig. 2).

The maxmimum phenol accumulation was observed between pH 5.0 and 6.0. Above and bellow this pH range it decreased significantly whereas the cell mass was less affected particularly at the higher tested levels of pH (Fig. 3).

Both cell mass and phenolics showed increase with increasing sugar concentration from 2 to 4%. There was little difference in either cell mass or phenol content in medium when sucrose concentration varied from 4 to 10 percent (Table 2). Among the different C sources tried for production of cell mass and total penolics in CF, phenol accumulation was highest with fructose followed by xylose and dextrose in contrast to cell mass which was highest with maltose followed by fructose and soluble strach (Table 3).

Organic N sources were better suited for phenol synthesis than inorganic N sources (Table 3). In contrast, growth of the fungus was greater in medium supplemented with inorganic N rather than organic N. Soni *et al.* (1992) observed that *Fusarium oxysporum* grew well in culture medium containing inorganic N but inhibited in medium with organic N. Incorporation of $MgSO_4$ among the S sources gave minimum change in cell mass as well as phenol accumulation.

Very little PPO activity (O. D. = 0.008 – 0.090) was noticed in the CF of *B. theobromae* with either pyrogallol or pyrocatechol as the substrate in all the above experiments. This may be one of the reasons for accumulation of phenolic in CF. However, there was significant PPO activity in the tissues of *B. theobroame* (Table 1) and enzyme activity increased with cell mass and phenol concentration with time.

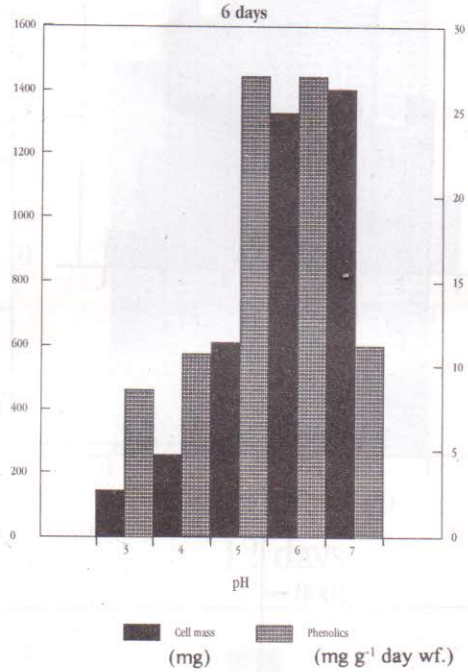

Fig. 3. Effect of pH on the cell mass and total phenolics in the CF of B. theobromae in CD medium after 6 days

Phenolic contents in *Botryodiplodia* infected and uninfected tissues of sweetpotato showed 2-13 fold higher phenol content in infected than uninfected tubers irrespective of the cultivars, and that the percentage increase was nearly proportional to the extent of spoilage. From the results, it was evident that the 'white' coloured varieties, Pusa Safed and P-13-10 were incidentally more susceptible to *B. theobromae* than the 'red' coloured variety Gouri where the phenol content in infected tubers was higher as compared to infected former varieties.

There are several reports on phenol concentration increasing several fold in plant tissues infected with pathogens. For instance, Arinze and Smith (1970) reported that *B. theobromae* (sweetpotato isolate) produces polygalacturonase (PG) isoenzymes i.e. PG 1, 2, 3 and 4 in liquid culture whereas only PG 3 was recovered from extracts of sweet potato rotted by *B. theobromae*. PG activity was highest at the advancing edge of the lesion, less at the unrotted periphery and still less at the centre (oldest part) of the lesion. Total

phenol content was highest at the periphery of the lesion and progressively decreased towards its centre. Both phenol oxidase and peroxidase were distributed in the same pattern as the total phenols. Arinze and Smith (1982) corroborated that total phenolic content was generally higher in and around the lesions on sweetpotato infected by *B. theobromae*, *Botrytis cinerea* or *Cladosporium cucumerinum*. Further, increase in phenolic content in the infected tissue was associated with accumulation of ipomeamarone and ipomoeamaronol, the phytoalexins of sweetpotato. Kim and Kim (1989) reported in higher total phenolics in infected apples than healthy ones, suggesting that phenols were involved in disease resistance to *Botryosphaeria dothidea*. Likewise, concentration of total phenols was greatly increased in sweetpotato roots infected with *Rhizopus stolonifer* (Thomson, 1979). Woolfe (1992) reported that the majority of phenolics in sweetpotato are esters formed between quinic acid and caffeic acid. These phenolics esters are chlorogenic acid, isochlorogenic acid and related compounds. They accumulate in sweetpotato tissues when the tuber is either mechanically wounded (McClure, 1960) or attacked by the black rot fungus *Ceratocystis fimbriata* (Uritani, 1984).

Mahadevan (1979) outlined four possible routes for phenol accumulation in plants at the site of infection : i). release of phenols from glycosides, ii) translocation of phenols from neighbouring cells to infection zone, iii) *de novo* synthesis of phenol by the infected tissues and iv) synthesis of phenols by the infecting parasites. In the present studies, it was observed that the Java black rot fungus *B. theobromae* synthesized phenols in culture. It was, therefore, quite reasonable to presume that the phenols synthesized by *B. theobromae* contributed partly to the phenol pool of the infected sweetpotato tubers. Further, studies are in progress to explain critically the biochemical pathways of *in vitro* phenol production by *B. theobrmae* and its role *vis-a-vis* host pathogen interactions.

ACKNOWLEDGEMENT

The authors thank Director of the Institute. Technical assistance of Mr. B. B. Das is duly acknowledged. The work is funded by an ad-hoc grant of Indian Council of Agricultural Research, New Delhi (Code No. 10(3)/ 94-Hort.II)

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(Accepted for publication October 24, 2000)