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THE PHYSIOLOGY OF PARASITISM OF *PYTHIUM APHANIDERMATUM* (Edson) Fitz. INCITING SOFT-ROT OF GINGER (*ZINGIBER OFFICINALE* Rose.)

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Maceration of plant cells is an important phenomenon in the syndrome of disease caused by *Pythium aphanidermatum* (Brown, 1915). It is caused by the dissolution or middle lamella composed largely of pectic substances resulting in the loss of coherance of plant tissues. It was De Bary who established the concept that pectic enzymes may be involved in the pathological manifestation induced in plant tissues by pathogenic microorganisms. Pectic enzymes obviously play a greater role in tissue disintegration in some disease than others. Several investigators have shown that pectolytic enzyme activity of root-rot and damping-off pathogen is involved in the pathogenesis (Hancock, 1966; Bateman, 1966 and Mellano, *et al.*, 1970). The importance of pectic enzymes in soft-rot disease had been studied by many workers (Winstead and Mc Combs, 1961; Uritani and Stahman 1961). Successful penetration and establishment by the pathogen in the middle lamella is accomplished by the elaboration of pectic enzymes to produce pathogenesis in the host. Very little is understood about the physiology of parasitism of *Pythium aphanidermatum* despite the fact that it is responsible for soft-rot diseases of many crops of economic importance. Hence an investigation on the production of pectic enzymes by P. *aphanidermatum* was undertaken during the course of study of soft-rot of ginger incited by P. *aphanidermatum* (Eds.) Fitz. and the results are presented in this paper.

Materials and Methods

The *in vitro* production of pectic enzymes of the fungus was estimated in in 5 different liquid media viz., Conn's medium, Czapek's (Dox) medium, Richard's medium, Gupta's (1956) medium and host extract medium (host tissue, rhizome 200g, distilled water 1000 ml). Thirty ml of each medium was taken in conical flasks (250 ml) after adjusting the pH between 6.0 and 6.5, The fiasks were then autoclaved, inoculated with discs (5 mm dia) cut with cork borer from the growing edge of 48 h old culture of *P. aphanidermatum* on potato dextrose agar medium and incubated at room temperature. The experiment was laid out in completely randomised design with 5 treatments and 2 replications with a suitable media control. The culture filtrates were filtered through Whatman No. 1 filter paperfrom the second day onwards for four days. The culture filtrates of a particular day were centrifuged for 20 minutes at 3000 r.p.m. adopting the procedure by Bateman (1963). The clean filtrates thus obtained were dialysed against distilled water for 16 h at laboratory temperature. The samples were assayed for enzyme activities immediately after dialysis during the four different days i. e, on 2nd, 3rd, 4th and 5th day after seeding.

The pectic enzyme activity *in vivo* was estimated by preparing enzyme extracts by grinding the plant material (healthy and infected) each in one volume (w/v) of 0.25 *N* NaCl in a homogeniser for about 1 minute at room temperature. The debris was removed by squeezing the liquid fraction through cheesecloth and centrifuged and dialysed as above and used for enzyme assay.

Pectin methyl esterase (PME) activity of the fungus was assessed by the method described by Hancock *et al.* (1964). Measurement of PME activity was in terms of increase in acidity due to hydrolysis of pectin.

To assess the activity of endo-polygalacturonase (Endo-PG) the viscometric method adopted by Uritani and Stahman (1961) was employed. Percentage reduction in viscosity (A) was expressed by the equation:

$$\frac{Tcont - T}{Tcont - TH_2O} \times 100$$

where Tcont : Flow time seconds at zero time in control reaction mixture with heated enzyme.

T : Flow time of reaction mixture at flow time (time of flow after incubation).

TH_2O : Flow time of mixture in which water was substituted for the substrate,

Exo-polygalactronase was assessed by measuring the rate of increase in galacturonic acid concentration in enzyme substance reaction mixture with dinitrosalicylic acid reagent, Dinitrosalicylic acid reagent was prepared by dissolving 1 g of dinitrosalicylic acid in 20 ml of 2*N* NaOH and 50 ml of water was added. Then 30g of sodium potassium tartarate was added and the volume made upto 100 ml with water. Reaction mixture contained 2ml of culture filtrate or tissue extract, 2.5 ml of 0.25 per cent polygalacturonic acid in 0,1 per cent citrate buffer (pH 5.2) and 6 ml of water. After the reaction time of 0, 30 and 50 minutes, samples of 2.8 ml were removed from the reaction mixtures and added to 0.2 ml of 1*M* Na_2CO_3 solution in testtubes to stop reaction. Three ml of dinitrosalicylic acid was then added to each tube. The test tubes were then held in a boiling water bath for exactly 10 minutes to permit colour development. The mixtures were then cooled immediately to room temperature and colour density was measured at 575 nm in a spectrophotometer. One unit of Exo-PG was defined as that amount of enzyme which under specified assay condition changed the optical density of the solution by 0.01 per hour.

The reaction mixture for the estimation of polygalacturonase transeliminase consisted of 4 ml of 0.2 per cent sodium pectate buffered with 0.1 *M* acetate buffer at pH 5.0, 1 ml of enzyme and 1 ml of water. Measurements were taken using Ostwald viscosimeter as in Endo-PG.

The macerating enzyme activity of the pathogen was assayed by the method adopted by Mellano *et al*. (1970). The macerating activity of the enzyme produced by *P. aphanidermatum* on potato discs was determined by the method described by Brown (1915).

Results and discussion

The results of estimation to assess pectin methyl esterase activity of *P. aphanidermatum* on different media during the four days indicated that PME was not produced *in vitro* on all the four days tested in different media by the fungus. Pectin methyl esterase is the enzyme which calalyses the demethytation of pectic substances. Charkavarty and Srivastava (1987a) stated that PME has very insignificant role, if any, in the macerat ion of host cells by *P. aphanidermatum.* Winstead and McCombs (1961) and Lin Reen (1971) had also came to the same conclusion. These are in conformity with the results obtained in the present study. Muthuswamy (1972) could not detect PME activity *in vitro* but only in the affected tissues by *P. aphanidermatum.*

Exo-polygalacturonase activity was detected in culture filtrates of different media during thefour days of estimation (Table 1). The enzyme polygalacturonase (Exo-PG) causes successive terminal cleavages of galacturonic acid residues. The enzyme activity was detected in the culture filtrates of different media in varying degrees on the four days of observation. The maximum Exo-PG activity was observed on the 3rd day old growth of *P. aphanidermatum* in different media. Winstead and McCombs (1961) could not detect Exo-PG in the culture filtrates of *P. aphanidermatum.* Lin Reen (1971) detected the production of Exo-PG by *P. ultium* and *P. cucurbitacearum* and found that the maximum amount of polygalacturnonase on the sixth day by the *'cupplate'* assay and no PG was detected in neutral and basic range. This is contrary to the present observation where the maximum activity was noticed on the 3rd day growth of pathogen in different media.

The results of the production of endo-polygalacturonase in the different media by *P. aphanidermatum* are given in Table 2. The results indicated that the enzyme was produced *in vitro* in different media even within 2 days after its growth, though the quantity varied with different media and days. Statistical analysis of the data indicated that there was significant difference in the production of the enzyme in the different media. It also indicated that the hydrolysis of pectic acid due to the enzyme present in the filtrates of various media varied significantly as evidenced by the quick loss of viscosity at the four time intervals studied during all days of estimation. The results of the pooled analysis of Endo-PG activity proved that Conn's medium was the best suited for the maximum production of enzyme amongst the five employed. The efficacy of other media in this respect could be represented in the descending order as Gupta's, Bichard's, Czapek's and host extract media respectively.

The enzyme Endo-PG catalyses the hydrolysis of glycosidic bonds of pectic acid in a strictly random manner. Lin Been (1971) detected Endo-PG activity

... Table 1

Exo-polygalacturonase (Exo-PG) activity of *P. aphanidermatum* in different media on different days of estimation *(in vitro)*

Sl. No.	Media	Change in absorbance 0,01/h											
		2nd day			3rd day			4th day			5th day		
		RI	RII	Mean	RI	RII	Mean	RI	RII	Mean	RI	RII	Mean
1	Conn's	23	24	23.5	29	31	30	16	15	15.5	6	5	5.5
2	Czapek's	8	10	9,0	12	9	10,5	6	4	5.0	2	3	2.5
3	Richard's	9	10	9.5	13	15	4.0	7	6	6.5	3	3	3,0
4	Gupta's	19	17	18.0	25	24	24.5	4	13	13.5	6	4	5.0
5	Host extract	6	5	5.5	4	5	4.5	3	3	3,0	1	2	1,5

Table 2

Endo-polygalacturonase (Endo-PG) activity of P. *aphanidermatum* in different media on different days of estimation *(in vitro)*

Sl. No.	Media	Percentage reduction in viscosity *in vitro*															
		2nd day				3rd day				4th day				5th day			
		5 min	10 min.	15 min.	30 min.	5 min.	10 min.	15 min.	30 min.	5 min.	10 min.	15 min.	30 min.	5 min.	10 min.	15 min.	30 min.
1	Conn's	14.1	26.2	27.0	33.2	21.2	29.6	36.4	45.1	14.0	26.3	26.9	29.6	4.0	5.7	10.8	15.2
2	Czapek's	3.2	5.7	6.4	7.6	5.6	6.3	6.8	12.8	3.9	7.0	7.4	9.9	1.8	2.0	3.2	3.6
3	Richard's	4.7	8.2	3.4	10.4	7.2	1.8	11.0	13.0	4.9	8.6	8.9	10.2	1.9	2.1	2.2	2.9
4	Gupta's	11.4	21.7	23.2	26.4	20.4	31.1	35.1	42.8	12.6	23.5	24.0	26.0	2.2	5.5	11.2	16.4
5	Host extract	2.1	3.1	3.2	5.2	3.0	3.6	4.2	5.3	1.9	2.7	3.0	4.0	0.8	0.9	1.1	1.9

F — Highly significant

C. D. (0.05) for comparing day : 0.23

,, media : 0.33

,, reading : 0.23

Ranking: 1. Media M1 M4 M3 M2 M5

2. Day D2 D3 D1 ID4

3. Reading R4 R3 R2 R1

Table 3

Poly-galacturonase transelminase (PGTE) activity of P. *aphanidermatum* in the different media on the **four** days of estimation *in vitro*

Sl. No.	Media	Percentage reduction in viscosity *in vitro*															
		2nd day min.				3rd day min.				4th day min.				5th day min.			
		5	10	15	30	5	10	15	30	5	10	15	30	5	10	15	30
1	Conn's	16.6	18.4	25.6	34.9	16.9	18.6	27.8	38.0	14.9	20.5	21.4	22.5	15.8	18.3	18.8	19.8
2	Czapek's	5.4	6-7	7.1	9.8	6.7	7.5	9.7	11.4	5.5	6.6	6.8	8.4	4.7	5.1	6.4	6.7
3	Richard's	6.3	7.5	8.0	9.9	7.2	8.3	10.1	12.6	5.7	6.9	7.3	9,2	5.0	5.7	7.2	8.5
4	Gupta's	15.4	17.6	23.9	33.2	19.5	20.3	27.9	36.5	12.9	20.5	20.6	**21.5**	14.2	15.8	19.9	20.2
5	Host extract	2.2	3.2	3.3	4.2	2.3	2.6	3.6	4.2	2.1	2.8	3.2	3.5	2.0	2.0	2.5	2.5

F — Highly significant

C. D. (0.05): **0.662**

Table 4

Macerating enzyme activity of *P. aphanidermatum* in the different media on different days of estimation *(in vitro)*

Sl. No.	Media	Time taken to macerate potato discs in minutes							
		2nd day		3rd day		4th day		5th day	
		RI	RI	RI	RII	RI	RII	RI	RII
1	Conn's	26	29	23	22	50	55	65	70
2	Czapek's	67	00	56	67	122	118	130	135
3	Richard's	62	69	59	57	113	112	120	122
4	Gupta's	33	32	25	25	70	65	70	75
5	Host Extract	90	38	89	88	168	170	186	110

Table 5

Production of pectic enzymes by *P. aphanidermatum in vivo*

Nature of tissue	PME (equivalent of NaOH required to maintain the original pH/h/ml of tha enzyme)	Endo-PG (per cent reduction in viscosity in 30 min)	Exo-PG (change in absorbance in 0.01/h)	PGTE (per cent reduction in viscosity in 30 min)	Macerating enzyme (time taken to macerate potato discs, min)
Healthy	2.8	0.0	0.0	0.0	Not macerated
diseased	8.7	23.5	0.0	25.2	156

in the culture filtrate of fungus *P. ultimum* which macerated potato discs. In the present study, Endo-PG activity was noticed on the 4 days of estimation in the different culture filtrates and also *in vivo* which may be taken to imply the decisive role played by this enzyme in tissue maceration.

The results of the activity of polygalacturonase transeliminase (PGTE) indicated that the pathogen produced this enzyme *in vitro* in the different media in varying concentrations on the different days of estimation (Table 3). The statistical analysis of the data revealed that the reducticn of sodium polypectate as the substrate was significantly different in the various media due to enzymatic activity. At the four different times studied, the reduction of viscosity also differed significantly within the medium and between media. PGTE acts by breaking the glycosidic linkage of pectins at No. 4 carbon atom and simultaneously eliminating the hydrogen at No. 5 carbon atom resulting in oligouronides that contain an unsaturated galacturonyl unit. Bateman (1966) observed the hydrolytic and transeliminative degradation of pectic substances of beans by extracellular enzymes of *Fusarium solani* f. *phaseoli.* Lin Reen (1971) found that *P. ultimum* did not produce pectic lyase (PGTE) whereas non-pathogenic *P. spinosum* did.

The results of the macerating activity of enzyme produced by the pathogen in different media during the different days (Table 4) indicated that there is macerating activity on potato discs when dipped in the culture filtrates. In many cases, though *P.aphanidermatum* produced pectic enzymes in large quantities,it was found to be incapable of macerating susceptible tissues(Winstead and McCombs,1961;Lin Reen, 1971)But Chakravarty and Srivastava(1967b) found free maceration of potato tissues by the young culture filtrates of *P. aphanidermatum.* In the present studies, potato discs were macerated freely by the culture filtrate of different media to varying degrees but greatly by the young culture filtrates of the pathogen.

The results of the production pectic enzymes *in vivo* by the pathogen (Table 5) indicated that unlike in the *in vitro* studies, PME was detected in healthy as well as on the diseased tissues of the host. Itwas also noticed that it was present in appreciably higher levels in the diseased tissu3s than the healthy. As for the presence of other enzymes, Endo-PG and polygalscturonase transeliminase were detected only in the infected tissues. Wood and Gupta (1958) observed that the parenchyma of potato tubers was readily macerated by the culture filtrate of *P.debaryanum* but such filtrates did not degrade soluble pectates *in vivo* and on the other hand, these filtrates degraded high methoxyl pectinates in solution. This suggested either that maceration involved layers other than middle lamella or that the middie lamella contained pectin as important constituent. Bateman (1963) was of the view that the macerating activity was not the property of only one enzyme. The results obtained in the present study were also in close conformity with the above.

Summary

A study on production of pectic enzymes in five liquid media was made. *Pythium aphanidermatum* (Edson) Fitz. was found capable of elaborating various pectic enzymes *in vitro* and *in vivo,*

Estimation of pectic enzymes like pectin methyl esterase (PME), exopolygalacturonase (Exo-PG), endopolygalacturonase (Endo-PG) polygalacturonase transeliminase (PGTE) and macerating activity on potato discs were done in five liquid media on four different days and observed that the rate of production of these enzymes by *P. aphanidermatum* varied with different media and age of the culture, the maximum production being noted in Conn's medium on the 3rd day.

P.aphanidermatum(Edson)Fitz. was found to produce various pectic enzymes as judged by the *in vitro* and *in vivo* studies which play a predominant role in bringing about the tissue degradation and the eventual pathogenesis on the host.

സംഗ്രഹം

ഇഞ്ചിയുടെ മൂടുചീയൽ രോഗത്തിന്റെ പഠനാവസരത്തിൽ രോഗകാരണമായ പിതിയം അഫാനിഡെർമാറ്റത്തിന്റെ പരജീവിത സവിശേഷതകളും പഠിച്ചതിൽ ഈ കുമിളം

പലതരം പെക്ടിക് എൻസൈമുകളും ഉണ്ടാക്കുന്നതായി മനസ്സിലാക്കി. വിവിധതരം മീഡിയകളും ഉപയോഗിച്ചതിൽ കോൺസ് മീഡിയത്തിൽ ഈ കുമിളും വിവിധ എൻസൈമുകളും കൂടുതൽ ഉണ്ടാക്കുന്നതായി കണ്ടു. ഇഞ്ചിയിലും ലാബറട്ടറിയിലും ചെയ്ത പരീക്ഷണങ്ങളിൽനിന്നും ഈ എൻസൈമുകളും ഇഞ്ചിയുടെ കോശങ്ങളെ ചീയുന്നതിന് ഗണ്യമായ പങ്ക് വഹിക്കുന്നുണ്ടെന്നും തെളിഞ്ഞു.

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