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Role of hydrolytic enzymes in leaf spot and stem anthracnose diseases of betelvine

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No PGTE secretion was recorded *in vitro.* Moderate PG and negligible C_x (cellulase) was recorded. Isolates varied in their ability to secrete these enzymes but not in consonance to their virulence. Optimum *p*H for PG production was found to be 6.0 and optimum temperature ranged between 28-30°C.

Pattern of enzyme production *in vivo* was quite different than that observed *in vitro.* In the leaves PG secretion was poor but cellulase secretion increased with time. In stem significant PG production was recorded in infected tissues upto 7 days after which the enzyme activity decreased. But cellulase secretion, low during early stages of infection, increased as infection progressed indicating a sequential secretion of enzymes. PG degrades pectin and exposes the cellulose for being degraded as infection progresses.

Key words : Betelvine, Enzyme, Pathogenesis, *Colletotrichum capsici*

The pathogen *C. capsici*, appears to have the ability to produce polygalacturonase (PG), polygalacturonase transeliminase (PGTE) and cellulase (C_x) under special conditions. Thirupathiah and Subramanian (1972, 1978 a) demonstrated that (i) macerating enzyme is produced in diseased tissue, being most active between pH 5.5-7.0 ; (ii) exo-PG is produced in media containing isolated cell walls of ripened chilli fruits only ; (iii) both endo- and exo-PG are not produced in media containing unripe fruit ; (iv) endo-PGTE is produced during spore production and germination only and is active at pH 9.1 and above and temperature of 26° C. Solanki *et al.*, (1974) claimed that *C. capsici* isolated from chilli fruits produce protopectinase, PG and polymethylesterase in culture.

Cellulase production has been demonstrated in most of the species of *Colletotrichum* tested. *C. capsici* was shown to produce cellulase in medium containing isolated cell walls from ripened but not green fruits of *Capsicum annuum* (Thirupapathiah and Subramanian, 1978b).

Moreover, PG and C_X are known to play an important role in degradation of cell wall in diseased tissue. In order to know their role in cell wall breakdown in leaf spot and stem anthracnose affected tissue of betelvine caused by *C. capsici* PG and C_X production were studied both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Extracellular enzyme preparation

For *in vitro* studies, the isolates of *C. capsici* were grown in 250 ml Erlenmeyer flasks containing 50 ml PD broth fortified with pectin (Sunskist Growmers Inc., Calif, USA) for pectic enzymes and carboxyl methyl cellulose sodium salt (CMC-Na ; BDH) for cellulase. The flasks were inoculated with 6 mm mycelial discs cut with a sterile disc cutter from the growing margins of 7 days old culture on PDA medium and incubated at 28 ± 1°C for 10 days. After requisite period of growth the culture medium was filtered and the dry weight determined. The filtrate was centrifuged at 5000 rpm at 5°C for 15 min. The supernatant was dialysed against distilled water for 20-24 hr at 5°C. The dialysate was used immediately as enzyme source.

Enzyme preparation from infected leaf and stem

Healthy leaf and stem were collected from field and inoculated with isolate virulent on leaf (col.14) and isolate virulent on stem (col.3) respectivaly as described by Dasgupta (1981).

Infected leaf tissues were collected after 3, 7, 14 and 21 days and stem tissues after 7 and 14 days of inoculation washed with sterile distilled water and blended thoroughly in pre-chilled glass vessels in appropriate buffer of requisite *p*H in the ratio of 1 : 1 (W/V). The blending and freezing was repeated thrice and the homogenate filtered through folds of cheese cloth. The filtrate was centrifuged and supernatant collected and dialysed against distilled water. The dialysate was used immediately as enzyme source. Suitable checks were also maintained with uninoculated leaves and stem.

Buffers used for enzyme assay :

For assay of enzyme buffers were prepared as described by Gomori (1955).

The final *p*H was adjusted using photoelectric *p*H meter. The following buffers were used :

Name of the buffer	Constituents	Stock molarity	*p*H range	Final molarity
Citrate	Citrate acid and sodium citrate	0.1	3.0-6.2	0.01
Acetate	Acetic acid and sodium acetate	0.2	3.6-5.6	0.02
Citrate phosphate	Citrate acid and dibasic sodium phosphate	0.1 and 0.2	3.2-7.0	0.02
Phosphate	Monobasic sodium phosphate and dibasic sodium phosphate	0.2	5.7-8.0	0.02
Tris amino methane	Tris (hydroxy methyl) aminomethane and hydrochloric acid	0.2	7.2-9.0	0.02

Viscometric assay of polygalacturonase and cellulase

Viscometric assay of PG and C_X was done following the method described by Bell *et al.* (1959) with modifications suggested by Hancock *et al.* (1964). One ml enzyme was added to 9 ml buffered substrate in an Ostwald's Viscometer for *in vitro* studies and for *in vivo* 2 ml of enzyme preparation and 1 ml of 0.001 M Calcium chloride were added to a 8 ml of buffered substrate. The flow time was recorded at specific time intervals. The flow time of water and the flow time of buffered substrate and inactivated enzyme (autoclaved for 10 min. at 15 psi) mixture was also determined. The per cent reduction in viscosity was calculated from the formula.

$$PDFT_t = (E - E_t) / (E - E_w) \times 100$$

E = Flow time of substrate and inactivated enzyme

E_t = Flow time of substrate + enzyme preparation after time t

E_w = Flow time of distilled water.

Per cent decrease in flow time (PDFT) was plotted against incubation time and the time required for 50% reduction in viscosity was calculated. Specific enzyme activity was calculated as 1/t x 1000 where, t is the time required in min. for 50% reduction in viscosity by an enzyme preparation. (Hancock *et al.* 1964). The results are expressed as specific activity per 100 mg dry weight of mycelium produced for *in vitro* studies.

The effect of *p*H was studied by adjusting *p*H of the substrate using suitable buffer.

The effect of temperature was studied by incubating the enzyme-substrate mixture at different temperatures in a thermostatic water bath.

RESULTS

PG activity as a function of pH and temperature

The activity of PG in culture filtrates was studied over a range of *p*H 3.5-8.0 at room temperature 30±3°C. Production of PG was induced by adding 1% sodium polypectate (NAPP) in PD broth. Isolate col. 14 of *C. capsici* was used for its high relative virulence towards leaf.

The results (Table 1) showed that the PG activity of isolate col. 14 was recorded over a wide range of *p*H 3.5-6.0. The maximum PG activity was recorded at *p*H 6.0 and the activity rapidly decreased above *p*H 6.0.

The effect of temperature on extracellular PG was studied over a temperature range of 20° 40° C with the same isolate at *p*H 6.0. The maximum PG activity was recorded at temperature 28° to 30° C. The activity rapidly fell above 30° C and below 28°C (Table 2).

The PG activity of other test isolates of *C. capsici* were studied at *p*H 6.0 and at temperature 28° C and 30° C respectively. Highest PG activity was recorded in isolate col.2 at both the temperature though less than that of isolate col. 14. Very little reduction in viscosity was recorded in isolates cols. 5 and 13 (Tables 2 and 3).

Cx activity and pH

In vitro Cx activity of most virulent isolate on leaf (col. 14) was studied over a range of *p*H 3.0-8.5 at room temperature 30°±3°C and negligible Cx activity was recorded over *p*H 3.0-7.0 (Table 4) and none above *p*H 7.0.

PG and Cx activity in betelvine leaf infected with C. capsici

Betelvine leaves were inoculated with spore suspension of isolate col. 14 of *C. capsici* ($5x10^5$ spores/ml). The infection progressed rapidly and PG and Cx activity was studied 3, 7, 14 and 21 days after inoculation at *p*H 6.0 and at room temperature 28±3° C. Negligible PG activity was recorded in inoculated betelvine leaves (Table 5).

Cx activity was significantly high at 14 and 21 days after inoculation but negligible after 3 and 7 days (Table 5). No PG and Cx activities were recorded in healthy leaves of betelvine.

PG and Cx activity in betelvine stem infected with C. capsici

PG activity was high after 7 days of inoculation but became insignificantly low

Table 1. *In vitro* polygalacturanase activity of isolate col. 14 of *C. capsici* at different *p*H

Time (min)	Percent decrease in flow time *									
	*p*H 3.5	*p*H 4.0	*p*H 4.5	*p*H 5.0	*p*H 5.5	*p*H 6.0	*p*H 6.5	*p*H 7.0	*p*H 7.5	*p*H 8.0
5	7.75	6.79	10.24	2.13	0.58	12.76	1.12	1.70	1.05	3.70
10	10.50	13.62	14.62	7.44	1.16	22.22	1.74	4.54	3.35	9.26
20	14.50	22.75	23.45	9.57	6.97	34.90	4.49	5.68	8.43	9.26
30	16.42	28.22	30.35	12.76	15.11	43.15	5.62	7.95	12.31	9.26
40	21.13	31.95	33.28	17.02	19.78	52.76	7.86	8.52	12.31	9.26
50	25.31	34.00	38.82	19.15	23.25	62.15	10.11	8.52	12.31	9.26
60	27.10	36.21	43.45	23.40	25.58	64.62	10.11	10.22	—	9.26
70	30.64	43.15	48.00	27.66	30.23	70.75	10.67	10.22		—
80	34.65	46.65	54.23	30.65	34.88	—	12.37	12.50		
90	36.72	48.79	57.64	34.04	38.37		15.73	12.50		
100	38.62	53.62	59.45	36.16	41.86		17.10	12.50		
110	39.84	53.76	—	39.36	45.35		17.10	—		
120	41.65	—		42.55	46.51		17.10			
130	43.50			43.61	52.32		—			
140	45.68			46.80	52.32					
150	46.61			48.93	52.32					
160	48.48			51.06	—					
170	50.31			51.06						
180	—			52.02						
190				54.25						
200				54.25						
specific activity/ 100 mg dry wt	97.04	173.38	225.16	105.40	126.93	442.95				

*Flow time of distilled water-68 secs.

Flow time of substrate and inactivated enzyme-162 secs.

Table 2. *In vitro* polygalacturonase activity of isolate col. 14 of *C. capsici* at different temperature

Time (min)	Percent decrease in flow time at different temperature (°C)					
	20	25	28	30	35	40
5	3.20	9.09	11.00	11.82	11.23	8.64
10	4.40	12.39	22.01	21.67	17.97	14 81
20	10.40	14.04	31.19	32.51	21.34	16.05
30	10.40	22.31	41.28	41.37	26.96	24.69
40	10.40	24.79	50.41	49.26	29.21	27.16
50	12.00	28.09	56.88	56.15	35.95	27.16
60	14.40	31 81	—	—	37.07	38.27
70	20.00	32.23			39.92	39 50
80	20.80	33 05			41.57	39.50
90	27.20	34.71			46.06	43.21
100	27.20	40.49			46.06	45.68
110	27.20	42.15			46 06	45.68
120	27.20	46.28			46.06	45 68
130	—	46.28			46.06	—
140	—	46.28			—	
Specific activity/ 100 mg dry wt			408 22	404.75		

after 14 days. C_X activity of infected betelvine stem was high both after 7 and 14 days of inoculation (Table 6).

DISCUSSION

In the present studies significant extra-cellular activity of pectic enzymes was recorded at *p*H 6.0 and temperature between 28°-30° C. In general, it has been found by various workers that the *p*H optima for pectic hydrolase are generally acidic, whereas the lyases are most active at alkaline conditions (Albersheim *et al.*, 1960 ; Bateman, 1966 ; Hall and Wood, 1970). This gives some indication therefore, that pectic enzymes primarily produced here extracellularly is of a hydrolase type and no extracellular lyase activity was demonstrated. For this pathogen Thirupathiah and Subramonian (1972, 1978a) demonstrated production of extra-cellular exo-PG and endo PGTE when *p*H of media reaches 9.1. For other species of *Colletotrichum* also it has been shown that PG is produced at acid *p*H (Hancock, 1966 ; English *et al.*, 1971 ; Romano and Nicholson. 1973 ; Porter, 1969) and PGTE is produced only when *p*H of the medium becomes highly alkaline (Hancock and Millar, 1965). Further, production of endo PGTE appears to be linked with sporulation even when grown on autoclaved host tissue. Apparently PGTE was not detected in the present isolates because unlike studies of other workers, in these isolates the *p*H of the medium did not shift towards alkalinity with time but shifted towards neutrality.

Table 3. *In vitro* polygalacturonase activity of isolates of *C. capsici* at *p*H 6.0 and 20°C and 30°C

Time (min)	Percent decrease in flow time									
	Col. 2		Col. 3		Col. 4		Col. 5		Col. 13	
	28°C	30°C	28°C	30°C	28°C	30°C	28°C	30°C	28°C	30°C
5	12.45	16 54	19.16	8.32	0.75	7.35	2.15	4.50	1.50	7.12
10	20.69	21.59	24.22	16.45	11.15	14.22	3.62	5.64	7.22	9.85
20	28.19	36.74	33.98	25.10	19.24	14.22	9.00	7.45	9.15	21.00
30	41.00	40.95	39.12	31 50	32.10	28.86	9.00	9.15	11.25	22.35
40	48.95	48.40	42.63	31.50	36.12	35.75	9.00	10.68	17.62	25.80
50	52.15	56.62	45.25	40.64	40.40	38.64	—	13.25	17.62	25.80
60	—	—	48.65	49.95	45.00	46.15	—	17.00	—	25.80
70	—	—	52.32	50.96	52.18	50.54	—	18.43	—	—
80	—	—	—	—	—	51.34	—	19.78	—	—
90	—	—	—	—	—	—	—	19.78	—	—
specific activity/ 100 mg dry wt	290 88	299.12	273.62	286 25	170.16	169.06	—	—	—	—

Table 4. *In vitro* cellulase activity of isolate col. 14 of *C. capsici* at different *p*H

Time (min)	Percent decrease in flow time at different *p*H							
	3.0	3.5	4.0	4.5	5.0	5.5	6.0	7.0
10	2.91	4.91	3.80	1.36	1.52	2.02	2.84	3.50
20	5.10	8.19	4.28	2.73	4.57	6.06	7.38	3.50
30	8.75	8.19	4.76	3.83	7.62	6 06	7.38	3.50
40	8.75	8 19	4.76	4.93	9.14	6 06	7.38	5.70
50	8.75	8.19	4 76	5.75	10.06	6.06	7.38	5.70
60	8.75	11.47	4 76	6 84	11.58	6.06	7.38	7 45
70	—	11.47	4.76	7.94	12.50	—	7 95	7.45
80	—	11.47	4.76	7.94	12.50	—	11.36	7 45
90	—	11.47	5.71	7.94	12.50	—	11.36	7.45
100	—	11.47	6.19	7.94	12.50	—	11.36	—
110	—	—	7.14	—	12.50	—	11.36	—
120	—	—	7.14	—	—	—	—	—
130	—	—	7.14	—	—	—	—	—
140	—	—	7.14	—	—	—	—	—

Table 5. Polygalacturonase and cellulase (C_x) activity in betelvine leaves infected with isolate col. 14 of *C. capsici*

Time (min)	Percent decrease in flow time							
	Days after inoculation							
	3		7		14		21	
	PG	C_x	PG	C_x	PG	Cx	PG	Cx
5	7.95	2.29	7.79	2.35	2.63	2.68	3.84	2.11
10	10.22	2.87	10.38	4.28	3.94	—	5.12	—
20	11.36	3.44	11.68	5.06	5.26	36.97	5.12	39.18
30	12.50	3.44	11.68	6.61	5.26	—	5.12	—
40	12.50	5 74	11 68	6.61	6.57	53.73	5.12	63.23
50	12.50	5.74	11.68	7.39	6.57	—	5.12	—
60	13.63	5.74	11.68	7.39	6.57	61.78	5.12	74.29
70	13.63	—	11.68	8.17	6.57	—	5.12	—
80	—	—	—	9.73	—	—	—	—
Specific activity	—	—	—	—	—	28.17	—	34.48

However, such shifts in semi-synthetic medium may not be typical of what actually happens when plant tissues are infected.

Table 6. Polygalacturonase and cellulase (C_x) activity in betelvine stem infected with isolate col. 3 of *C. capsici*

Time (min)	Percent decrease in flow time			
	Days after inoculation			
	7		14	
	PG	Cx	PG	C_x
5	14.03	2.01	4.10	5.39
10	28.07	—	4.10	—
20	43.86	41.16	5.48	27.50
30	52.63	49.21	6.85	39.33
40	57.89	55.48	6.85	47.04
50	61.04	59.73	8.22	52.18
60	—	—	8.22	56.04
70	—	—	8.22	—
Specific activity	37.69	32.00	—	21.70

Cellulase production in *C. capsici* has been demonstrated when isolated cell walls were used as inducers (Thirupathiah and Subramonian, 1978b) and for *C. trifolii* in different media (Hancock and Miller, 1963), In the present studies when CMC-Na was used as inducer only negligible quantities of Cx was detected.

When the PG and Cx were investigated in infected leaves and stem as the disease progressed a different picture emerged. In leaf infections negligible PG secretion was noted. However, Cx which was not produced extracellularly in defined medium, appeared in infected leaf tissues and the quantity which was negligible for first 7 days increased considerably at 14 and 21 days. In stem, high pectic hydrolase activity was noted upto 7 days which decreased subsequently but Cx activity continued to persist till and beyond 14 days. It would appear therefore, that some pectic enzymes acts on the pectic wall component first exposing the cellulosic substrate to be acted upon by cellulases. In leaves, in the present studies, the nature of pectic enzyme could not be identified but in stem apparently a pectic hydrolase (possibly PG) acted on pectate and accumulation of end products lead to its reduction in activity after 7 days cellulose was slowly decomposed and hence cellulase activity continued over a much longer period. In other host pathogen combinations it has been demonstrated that PG was secreted earlier than cellulase (English *et al.*, 1971 ; Lisker *et al.*, 1975).

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