

PECTIN METHYLESTERASE ACTIVITY IN CAULIFLOWER LEAVES INFECTED BY *ALTERNARIA BRASSICICOLA*

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The role of pectin methylesterase (PME) activity in the development of leaf blight disease of cauliflower caused by *Alternaria brassicicola* was studied. Enzyme activity was found in the culture filtrate of pathogen when grown in a medium containing glucose and sodium polypectate. Enzyme activity increased in the infected leaves of cauliflower. About 90 per cent inhibition was noticed both in the healthy and infected leaves and 53 per cent in the mycelial extracts of the pathogen when 1.5 per cent sodium laurylsulphate was added. The optimum pH for the PME activity was 5.5 and Ca and catechol were found to have remarkable inhibitory effects on the PME. The results reveals that increased PME activity in infected leaves is mostly due to increase in host enzymes and only 10 per cent of the total activity is due to fungal enzymes.

INTRODUCTION

The most important barrier the pathogen has to encounter initially is the host cell wall. It is now well-known that both pectin, cellulose and other carbohydrates of the host cell walls are broken down subsequently by a team of enzymes of the pathogen during the course of pathogenesis (Wood, 1960 ; 1961).

Much of the early work on the activity of pectin enzymes have been reviewed by Kertesz (1951), Demain and Phaff (1957) and Wood (1967). Bateman and Bear (1965) and Hancock and Miller (1965) have shown that pectin methylesterase activity increased more in diseased tissue than in healthy tissue of many plant species. The present investigation has been undertaken to determine the role of pectin methylesterase enzyme in the leaf blight disease of cauliflower (*Brassica oleracea* var. *botrytis*) caused by *Alternaria brassicicola*.

MATERIALS AND METHODS

Cauliflower plants (*Brassica oleracea* L. var *botrytis*, susceptible variety 'Snow ball') were grown in 25 cm diameter earthen pots. Pots were filled with sundried field soil. The soil in the pots was sealed with 1% formaldehyde solution and covered with polythene sheets for 24h. After this period, the covers were withdrawn and the soil was kept to allow the formaldehyde to

escape. After 7 days, when no trace of formaldehyde could be detected, the soil was ready for sowing of seeds.

Seeds previously treated with agrosan (a mixture of phenyl mercuric urea and ethyl mercuric chloride with total mercury content 1%) were sown in pots. The seeds germinated and seedlings came up in 2-3 days. Ammonium nitrate was applied usually 12 days after germination at the rate of 40 kg of nitrogen per hectars.

A virulent isolate of *Alternaria brassicicola* (Schew.) Wiltshire was used in the study. A single spore culture of this pathogen was obtained from infected cauliflower leaves collected from the agricultural field at the Baruiপুর Farm, Calcutta, West Bengal. Stock cultures of this isolate were maintained on *potato-dextrose* agar at 10°C. Subculturing was made at an interval of 2 months.

Cauliflower seedlings were inoculated artificially with a suspension of conidia of *A. brassicicola*. Conidial suspension in sterile distilled water was prepared from 15 days old mycelial cultures by gently shaking and followed by filtering through a thin layer of muslin to get rid of mycelial fragments and agar medium. Then the suspension was centrifuged at 5000 g for 10 min to get rid of suspended material which was decanted off. The conidial suspension was standardised to have 10^6 conidia/ml. The pots were well watered to maintain the optimum relative humidity and were covered with a polythene tent one day before inoculation. After 24 h 1.5 ml conidial suspension of *A. brassicicola* was injected into the mid-rib of each leaf of intact cauliflower plants with a hypodermic syringe. Leaves injected similarly with sterile distilled water served as controls.

Enzymes preparation was done following the method of Hancock et al. (1964). The pathogen was grown on Richard's medium in conical flasks inoculated with 1 ml conidial suspension for 8-10 days at 27°C. The mycelium was removed by filtering through a Buchner funnel and the culture filtrate was centrifuged at 5,000 g for 15 min at 4°C. The supernatant fluid was dialysed in a large volume of distilled deionised water for 24 h at 4°C. Following dialysis, the culture filtrate was ready for enzymes analysis. The enzyme preparation was kept at -20°C.

Thirty g of the infected and control cauliflower leaves were collected after 7 days of inoculation and homogenized in 50 ml of 0.25 M NaCl solution. The weighed plants were stored at 20°C for 3 h for thawing. After this the plants were minced, pressed through muslin and the supernatant was centrifuged at 30,000 g for 15 min at 4°C. The residue was extracted in a chilled mortar with buffered saline containing 0.025M-Tris-HCL, 0.85% NaCl and 0.01% merthio-late at pH 7.5. The buffer solution was added at the ratio of 1 ml/g of plant.

The extract was then centrifuged at 30,000 g for 15 min at 4°C. The combined supernatants were dialysed overnight at 4°C as before. For the DEAE-cellulose fractionation experiments 20 days inoculated plants were used.

The activity of pectin methylesterase was measured by a modification of the method of Kertesz (1951). The substrate solution contained 1.5 per cent pectin (Pectin, 1.5 g ; 15 ml, 1.0 M-NaCl added to 75 ml water, adjusted to pH 5.5 and made upto 100 ml.). The reaction mixture was buffered with citratephosphate buffer at the same pH as the substrate and titrated at intervals of 10, 20 and 40 min of incubation so that the pH did not shift more than 0.5 units from the original pH. To 30 ml of the substrate, 6 ml of enzymes preparation and 6 ml of buffer were added in a 25 ml beaker and the pH was noted as zero time. After incubation in room temperature, the mixture was titrated with 0.025 M NaOH to the pH at zero time. Data are expressed as 10^2 enzyme activity units per mg of protein.

One ml sodium lauryl sulphate solution (1.5 percent) was added to the normal assay mixture and the assay was carried out as above. Similarly, 1 ml (specific concentration) of Ca^{++} , oxalate, catechol and diethyldithiocarbamate were added to the mixture and the assay was carried out.

The fractionation of PME on DEAE-cellulose was conducted following the methods of Lee and Macmillan (1968) and Langcake *et al.* (1973).

Ten g DEAE-cellulose (Whatman DE22) was regenerated with 0.5 M HCL and 0.5 M-NaOH. This was then equilibrated with 0.01 M, pH 8.2, Tris-HCL buffer and stored in 150 ml of the same buffer at 4°C. Ten ml DEAE-cellulose suspension and 1.0 ml enzyme preparation were shaken vigorously at 25°C for 20 min and then filtered. The filtrate was collected as Fraction 1. The residue was then successively treated with 2 x 5 ml aliquots of each of the following different molarity of buffer: 0.01 M ; pH 8.2, Tris-HCL, 0.005M pH 8.2, Tris-HCL containing 0.025M-NaCl ; 0.005M, pH 8.2, Tris-HCL containing 0.5 M-NaCl. In all cases the same procedure was followed as in Fraction 1. The filtrates thus obtained were collected as Fraction 2-7 stored at 20°C and finally assayed.

RESULT AND DISCUSSION

The assay of PME activity revealed that there was no activity in the culture medium supplemented with glucose but with sodium polypectate it showed enzyme activity (Table 1). It revealed that PME activity was induced by polypectate only. The enzyme activity was increased in the infected leaves compared to those of healthy ones. In the culture filtrate maximum activity was noticed after 7 days of growth while it gradually increased in the case of healthy and infected leaves.

TABLE 1. *Data (mean) showing the pectin methylesterase activity in culture filtrates of Alternaria brassicicola and in healthy and infected leaves of B. oleracea var. botrytis*

Days after inoculation	Specific activity ^a				
	Culture filtrates ^b			Leaves	
	with 1.5% glucose	with 0.5% glucose & 1.0% pectin	With 0.5% glucose & 1.5% sodium polypectate	Healthy (control)	Infected ^c
1	No activity	Ne activity	0.34±0.03	3.00±0.02	3.30±0.04
2	"	"	0.88±0.07	3.20±0.04	3.66±0.04
7	"	"	2.16±0.02	3.36±0.06	5.26±0.08
10	"	"	1.87±0.07	3.50±0.05	6.12±0.04
13	"	"	1.67±0.08	4.40±0.05	8.86±0.10

^a Specific activity has been expressed as 10² enzyme activity units per mg protein. Each value represents an average of five separate determinations from different preparations±S.E.M.

^b Culture grown on Richard's medium with different carbon sources.

^c Tissue from well developed symptoms were taken for analysis.

The data in Table 2 indicates that PME activity was noticed in a wide range of pH 4.0—7.0 though the optimum pH for the PME activity in healthy, infected leaves and culture filtrate was 5.5.

These findings suggest that the increase in PME activity might be due to enzymatic activity of the pathogen or the host.

TABLE 2. *Data (mean) showing the effect of pH on PME activity of A. brassicicola*

pH	Relative activity ^a of pectin methylesterase from		
	Culture filtrate	Infected leaves	Healthy (control)
4.0	0.50±0.03	0.84±0.02	0.62±0.02
4.5	0.70±0.03	3.80±0.04	1.16±0.06
5.0	1.50±0.04	4.60±0.04	2.00±0.05
5.5	2.27±0.02	8.20±0.03	4.10±0.06
6.0	1.86±0.03	4.62±0.05	3.16±0.07
6.5	1.00±0.06	2.85±0.04	2.26±0.05
7.0	0.40±0.05	0.80±0.06	1.00±0.04

^a Activity / activity at pH 7.0 each value represents an average of separate determinations from different preparations ± S.E.M.

TABLE 3. Data (mean) showing the effect of sodium lauryl sulphate (.5%) on the activity of PME after 7 days of inoculation

Sources of pectin methylesterase	Enzyme activity		Inhibition %
	With NaLS	Without NaLS	
Heathy leaves	0.31±0.14 ^b	3.40±0.05	90.8
Infected leaves	0.57±0.04 ^b	5.30±0.04	89.2
Fungal culture filtrate	1.01±0.03 ^b	2.16±0.02	53.2

^a One ml of 1.5% sodium lauryl sulphate solution was added to mixture.

^b Specific activity has been expressed as 10² enzyme activity units per mg of protein. Each value represents an average of five separate determinations from different preparations.

The data in Table 3 revealed that sodium lauryl sulphate inhibited the PME activity in all cases. But the inhibition was almost complete in case of healthy and infected leaves where as it was 53 per cent in case of culture filtrates. This indicates that the increase in PME activity in the infected leaves was possibly not due to the pathogen's PME activity.

The PME activity might undergo changes under the influence of factors present in the host tissues. The data in Table 4 indicated that Ca⁺⁺ had an inhibitory effect on PME activity of the pathogen which could be partially recovered by oxalate ions probably by precipitating with Ca⁺⁺ as calcium oxalate. The PME activity of the pathogen was also inhibited by catechol and this inhibition could be recovered by diethyldithiocarbamate. It indicates that catechol being

TABLE 4. Effect of ions and agents on fungal pectin methylsterase activity

Ions and agents	Concentrations of ions and agents	Activity (%)
None	—	100
Ca ⁺⁺	1X10 ⁻³ M	43
Oxalate ion	"	100
Ca ⁺⁺ and oxalate ion	"	76
Catechol	"	31
Diethyl dithiocarbamate	1X10 ⁻⁴ M	100
Catechol and	1X10 ⁻³ M	100
Diethyl dithiocarbamate	1X10 ⁻⁴ M	100

oxidized by pathogen polyphenoloxidase becomes inhibitory to the PME activity of the pathogen. This finding indicates that the increased PME activity in the infected leaves was possibly due to activity of the host.

The data of the adsorption and elution from DEAE-cellulose of pectin methyl-esterase from culture filtrate and healthy and infected Brassica leaves are given in Table 5.

TABLE 5. Fractionation of pectin methylesterase of culture filtrate of *A. brassicicola* and healthy and infected leaves of *B. oleracea* var *botrytis*.

Pectin methylesterase from	Pectin methylesterase units in fractions from DEAE-cellulose					Input	Recovery (%)
	3	4	5	6	7		
Fungal Culture filtrate	10	17	0	0	0	14.1	76
Infected leaves	26	43	0	217	55	392 ^a	80
Healthy leaves	0	0	0	37	26	63	86

^a Include 63 units not absorbed and recovered in Fractions 1 and 2.

The data show all three enzymes were adsorbed to DEAE-cellulose. The adsorbed enzymes from fungal culture filtrate was eluted with 0.025 M-sodium chloride and the same of leaves was eluted with 0.5 M-sodium chloride. The recovery percentage was higher in case of healthy and infected leaves than the fungal one.

Pectin enzymes are regarded as important in pathogenesis and their role in pathogenesis, nature, amount, origin and occurrences in infected and healthy tissue have been worked out by several investigators (Wood 1967; Deese and Stahman 1962; Mc Donnell 1962; Patil and Dimond 1968; Langcake and Drysdale 1975; Langcake *et al.* 1973). In the present study the role of pectin methylesterase enzyme in the blight disease of cauliflower caused by *Alternaria brassicicola* has been evaluated.

The PME activity of *A. brassicicola* was found to be inducible by sodium polypectate. A similar observation was reported by Deverall and Wood (1961) in chocolate spot of bean. The activity of PME in the infected cauliflower leaves was increased compared to that of healthy ones and it was almost completely inhibited by sodium lauryl sulphate. Sodium lauryl sulphate was able to inhibit the PME activity in the culture filtrate by about 53 per cent only. Though the experimental data revealed that the optimum pH for PME activity was the same (5.5) in infected leaves and culture filtrate and healthy leaves

yet the nature of inhibition of PME activity in the healthy and infected leaves indicate that most of the increase in PME activity is due to the host enzyme (Table 2 and 3). Further more it is indicative from experiment that plant phenols beings oxidised by pathogen's enzyme might have acted as an inhibitor of PME activity of pathogen in diseased leaves (Table 4). However it appears from these findings that the increase in PME activity in the infected leaves is mostly due to host enzyme and fungal enzyme activity accounts for about 10 per cent of the total activity only (Table 3 and 5).

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