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Full Length Research Paper

Studies on pectinase activities of isolates of *Erwinia carotovora* and *Rhizopus sp.* causing soft rot in cabbage (*Brassica oleracea* var *capitata* L.)

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Four isolates of *Erwinia carotovora*, isolated from cabbage, potato and carrot and one isolate of soft rot causing *Rhizopus* sp., were evaluated for their ability to cause soft rot and to produce pectinases. The results showed that all isolates varied in macerating ability and carrot isolate of *E. carotovora* showed the highest activity of pectinases. All the *Erwinia* isolates and Rhizopus isolate exhibited significant differences in pectinase activities as confirmed by cup-plate and viscometric assays. *Rhizopus* isolate showed the least pectinase activity as compared to bacterial isolates.

Key words: Pectinase activity, *Erwinia carotovora*, *Rhizopus sp.*, Macerating ability.

INTRODUCTION

Tissue degrading enzymes play a prominent role in diseases like bacterial and fungal soft rot of vegetables. Pectinases are enzymes that hydrolyze the pectin material of host tissue and play an important role in the development of soft rot. These enzymes produced by soft rot causing bacteria like *Erwinia carotovora* and fungi like *Rhizopus* dissolve middle lamella, which serves to cement the adjacent cell walls together, and thus, loosen the cells from one another. In infections like bacterial soft rot, the pathogenic action is actually brought about by the diffusion of enzymes in advance of bacteria, which later on thrive as saprophytes upon the hydrolyzed lamella and ex-osmoses material.

E. carotovora has been found to be most common bacterial pathogen associated with the soft rot disease (Larka, 2004).The bacterial soft rot pathogen has a broad host spectrum including genera from all the plant families of vegetables, fruits and ornamentals having fleshy tissues (Anonymous, 1990).

According to most of the workers, pectic enzymes which are currently known are pectinestrases (PE) or pectin methylestraces (PME) and polygalacturonases (PG). Pectin methylestraces catalyze the hydrolysis of methyl ester groups of pectinic acid to methyl alcohol and the rate of cleavage of alpha 1, 4 bond of pectic substances is enhanced by removal of methoxy group because chain splitting endopolygalacturonase prefers de-methylated pectin (Bateman and Miller, 1966; Mehrotra and Aggarwal, 2003).

Polygalacturonases or pectic glycosidase are chain splitting enzymes which break the links between adjacent galacturonic acid units in pectic substances due to hydrolytic mechanism (hydrolases) or an eliminative mechanism (lyases) (Mehrotra and Aggarwal, 2003). Phytopathogenic bacteria capable of producing cell wall degrading enzymes during host pathogen interaction are usually capable of producing such enzymes in cultures as well.

There have been several reports in the past suggesting pathogenic superiority of strains *E. carotovora* associated with the production of greater quantity of pecteolytic enzymes (Gregg, 1952; El-Shaieb and Milibari, 1995). Certain other reports of high level of aggressiveness associated with strains of *E. carotovora* have been associated with their abilities to secrete higher amounts of macerating enzymes (Phokum et al., 2006; Smith and Bartz, 1990). Present investigations were

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carried with the objectives to detect differences in aggressiveness or macerating ability of different soft rot causing isolates of *E. carotovora* and differences in their abilities to produce pectic enzymes like Pectin methylestraces (PME) and chain splitting enzymes that is, hydrolases and lyases in cell free culture filtrates of different *Erwinia* isolates. Attempts were also made to compare soft rot causing ability and pactinase activities of soft rot causing *Rhizopus* sp. with those of strains of *Erwinia* isolates.

MATERIALS AND METHODS

Isolates of casual bacteria and *Rhizopus* sp.

Isolation was carried out from the diseased samples of cabbage (*Brassica oleracea* var *capitata* L.), carrot (*Dacus carota*) and potato (*Solanum tuberosum*) collected from various locations of Kashmir valley. Diseased vegetable samples bearing young developing, water soaked slimy lesions without much discoloration were selected for isolation of the pathogen. Bacteria were obtained from these samples in pure culture. Pathogenecity test on respective hosts produced symptoms of soft rot identical to those seen under natural conditions. This artificially induced disease yielded same bacterium upon resolution. The causal bacterial isolates were short rod measuring 0.7 to 1.0 µm in width, 1 to 2.5 µm in length, Gram negative, facultatively anaerobic, produced acid from D lactose and trehalose and maltose did not hydrolyze starch but reduced nitrates, liquefied gelatin and pectin, produced H_2S from cystine, was catalase positive, oxidase negative, urease negative and not sensitive to erythromycin. All these morphological, cultural and biochemical tests of the causal bacterium were suggestive to *E. carotovora*, which was also confirmed by bacteriologists at the Division of Plant Pathology, IARI, New Delhi. Soft rot causing *Rhizopus* sp. was also isolated from cabbage showing soft rot lesions and having fungal growth. Diseased portion of cabbage tissue was surface sterilized in 0.1% mercuric chloride for 0.5 to 1 min followed by three rinses with sterilized distilled water. The pieces were then aseptically placed on potato dextrose agar (PDA) medium in 90 mm petri dish and incubated at 25 ± 2°C for 72 h. The fungal growth was examined and cultured on PDA. Purification was done by single hyphal tip method (Rangaswami, 1972) .The fungal isolate was characterized as *Rhizopus* sp. by pathogenicity on cabbage, cultural and morphological characters. A total of four *E. carotovora* isolates were raised and two cabbage isolates showing different virulence during pathogenicity were named “C-1” and “C-2” respectively, the isolate from potato and carrot was labeled “Pt” and “Iso”, respectively and the *Rhizopus* sp. isolated from cabbage was labeled as “R”

Maceration test

The test was conducted on fresh cabbage (*B. oleracea* var *capitata* L.) parts which were cut to fit in 90 mm Petri dishes using the technique of DeBoer et al. (1978) with slight modification. The plant parts to be inoculated were surface sterilized by immersing them into 0.1% sodium hypochlorite and then washed twice with sterile distilled water followed by air drying under a laminar flow hood. All the test isolates of *E. caratovora* were grown for 48 h in YEP broth containing yeast extract 10 g/L and pectin 2.5 g/L. The cell suspensions were adjusted to uniform optical density at 600 nm showing percent absorbance of 250.5 mm diameter culture discs from 48 h old *Rhizopus* sp. culture grown on PDA were mixed with sterile distilled water and the suspension containing a CFU above 2 × 10^6 also prepared.A drop of an aqueous cell suspension of the test bacterium and *Rhizopus* sp. from each suspension was applied with the help of a sterile pipette on each vegetable part and a flamed straight pin was inserted through the drop into the centre of the vegetable part and then withdrawn. In the case of control, sterile water drop was applied instead of aqueous cell suspension. Five replications were maintained in each case. The inoculated vegetable parts were put in sterile Petri plates, and were incubated at 30 ± 1°C for 48 h. The Maceration test and amount of macerated tissue was measured as described by Phokim et al. (2006) with modification. To calculate percent tissue macerated, cabbage tissue was weighed before incubation and again after removing decayed tissue after incubation. The final mass was subtracted from initial mass to give total amount of decayed tissue or loss in weight. Loss in weight due to drying was deduced with the help of un-inoculated controls.

Preparation of cell free culture filtrates

A loopful of an overnight grown culture of test isolates of pathogen suspensions which were adjusted to uniform optical density at 600 nm showing percent absorbance 250 was inoculated in 50 ml of sterile YEP broth and incubated at 30°C for 48 h. Uninoculated control of YEP broth was also maintained. The absorbance was recorded at 600 nm before and after incubation to check the growth of the bacterium. After the incubation, the culture was centrifuged at 7000 × g for 10 min. The cell free supernatant was checked for pectinase activities. The enzyme assay was carried out by cup plate assay and standard viscometric assay.

Cup-plate assay

The assay for hydrolases and lyases was based on the procedure as described by Dingle et al. (1953) for polygalacturonases. Hydrolase activity was assayed at pH 5.2 in a medium containing 1% (w/v) pectin, 0.5% (w/v) ammonium oxalate, 1.5% agar and 0.2 M sodium acetate buffer pH 5.2. In the assay for lyase activity, the pH was fixed at 8.6 and the gel contained 1.5% pectin, 0.002% (w/v) $CaCl_2.6H_2O$, 1.5% agar and 0.1 M Tris (hydroxymethyl) aminomethane hydrochloride buffer, pH 8.6. For pectinesterase activity, the method described by McComb and MeCready (1958) was used with slight modification. The gel contained 1% (w/v) pectin, 1.5% agar, and 0.2M Tris (hydroxymethyl) aminomethane hydrochloride buffer, pH 8.6 was prepared. To avoid non-enzymatic de-esterification of pectin, the crude enzyme preparation was adjusted to pH 7 with a few drops of 0.1 M NaOH before inoculation of cups. All the above assay media were sterilized by heating at 100°C for 40 min and dispensed in 15 ml amounts in 9 cm Petri dishes. After solidification, the open plates were dried in oven at 50°C for 45 min and cups 8 mm in diameter were made in the gel with the help of a cork borer. Four cups were made in each plate and each cup was filled with 200 µl of the enzyme preparation. After 24 h of incubation at 30 ± 1°C, the plates were developed by flooding with HCl (1: 2 dilution of concentrated HCl). Observations on diameter of clear un-precipitated zones around the cups were recorded as the diameter of the clearing zone.

Viscometric assay

This method was used to check hydrolases and lyase activities which are both chain splitting enzymes. Two different substrate reaction mixtures were prepared to test pectin hydrolases and pectin lyase activity. For hydrolase activities, the substrate mixture containing 0.3% Pectin (w/v), 0.1 M NaCl and 0.025 M sodium acetate buffer, pH 5.2 was used. Similarly, the substrate mixture for

Table 1. Assay mixtures prepared for viscometric assay for pectin hydrolases and lyase.

Reactant	Proportion (ml) in tube		
	A (water control)	B(substrate control)	C(Test)
Substrate	-	11	11
Enzyme	-	-	1
Water	12	1	-

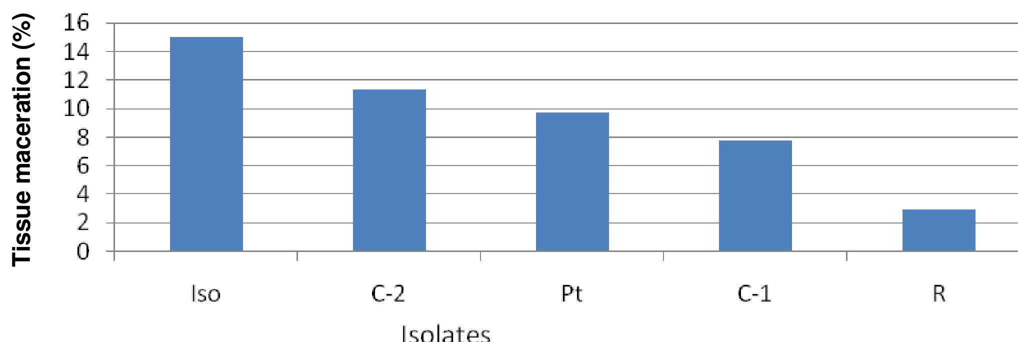

Figure 1. Percent tissue maceration of cabbage tissues by isolates of *Erwinia carotovora* and *Rhizopus* sp. Isolated from different vegetables. C-1 = *E. carotovora* isolated from cabbage, C-2 = 2nd isolate isolated from cabbage, Pt = *E. carotovora* isolated from potato, Iso = *E. carotovora* isolated from carrot, R = *Rhizopus* sp. Isolated from cabbage.

lyase assay containing 0.25% pectin (w/v), 0.25 mM $CaCl_2.2H_2O$ and 0.005 M Tris (hydroxymethyl) aminomethane hydrochloride buffer at pH 8.6 was prepared (Nasuno and Starr, 1966).Each assay mixture was prepared as shown in Table 1. The tubes were inoculated at 30 ± 1°C and viscometric runs were made at 30, 60, and 120 min starting with tube C. Enzyme activity was expressed as the percent decrease in viscosity (D) and calculated with the formula:

$$D= 100 \times (Ts-Ti)/ (Ts-Tw).$$

Where; Ts =Flow time of the substrate control, Ti = flow time of test and Tw =Flow time of water control.

RESULTS AND DISCUSSION

Results on maceration of cabbage tissue are depicted in Figure 1. Results revealed that carrot isolate (ISO) of *E. caratovora* caused highest amount of maceration of about 15.03% followed by one of the two isolates of cabbage (C_2). These were followed by potato isolate (Pt) and another cabbage isolate (C-1), respectively. *Rhizopus* isolate (R) caused least maceration of cabbage tissue upon 48 h of inoculation (Figure 2).

Results clearly indicate that there is considerable variation among isolates of E. *carotovora* with respect to macerating ability and it is also evident that the soft rot caused by bacteria is more rapid than that caused by the fungal species used in the study. Studies depicting the variability among soft rot causing isolates of *E. carotovora* with respect to their disease inducing ability are in conformity with those of Phokum et al. (2006) who also reported that *Erwinia* isolate from carrot was most severe when compared with isolates of ten different varieties of vegetables. Smith and Bartz (1990) also reported significant differences among the strains of *E. carotovora* for their aggressiveness on tubers and fruits of some vegetables. Quantitative pathogenic superiority of a strain of *E. carotovora* over certain other strains in potato tubers was also reported by Gregg (1952). Besides this, the present study also indicates the variability among the strains of *E. carotovora* present in the region. Although, there is no previous study for comparison of bacterial soft rot with that caused by fungi especially, *Rhizopus* sp. but the present study clearly indicates the rapid infectivity and development of bacterial soft rot then that caused by fungi, probably, this is the reason that bacterial soft rot has been considered most serious disease of vegetables causing highest total loses of produce than any other post harvest disease of vegetables (Agrios, 2007).

Crude pectinase enzymes obtained in the form of culture filtrates from different isolates were assayed by cup-plate method (Table 2). Results indicated that the culture filtrate of the carrot isolate showed highest activity of pectin hydrolase, pectin lyase and pectin methyl esterase exhibiting a clear zone of 4.60, 1.95 and 2.15

Figure 2. Tissue maceration on cabbage tissue by *Erwinia carotovora* isolates isolated from different vegetables (C-1 = *E. carotovora* isolated from cabbage, C-2 = 2nd isolate isolated from cabbage, Pt = *E. carotovora* isolated from potato, Iso = *E. carotovora* isolated from carrot, R = soft rot causing *Rhizopus sp* in cabbage).

cm in diameter, respectively. This was followed by C_2 isolate of cabbage which gave the clear zone of 3.70, 1.40 and 1.60 cm in diameter in case of three enzymes, respectively. Others which followed in the decreasing order of activity were Potato isolate (Pt), C-1 isolate of cabbage and culture filtrates of *Rhizopus* sp. (Figure 3).

Pectin lyase and hydrolase activity of *Erwinia* isolates was also carried out with viscometric assay where the percent decrease of viscosity gives the measure of chain splitting enzymes in the test samples. Since splitting of chains will reduce viscosity, therefore, only chain splitting enzymes like Pectin lyase and hydrolase activities were determined by this method. The results depicted in Tables 3 and 4 which revealed that pectin lyase and hydrolase activities of isolates of *E. caratovora* and one isolate of *Rhizopus* sp. differ significantly from each other. Results further confirmed that crude enzyme (culture filtrate) of carrot isolate (ISO) has highest hydrolase as well as, lyase activities (Table 4), followed by C_2 isolate of cabbage and isolate of potato (Pt), respectively. *Rhizopus* isolate (R) showed least enzyme activities. Reduction of viscosities of pectin substrates by crude enzymes was enhanced with the passage of time. However, in the case of C-1 and R isolate, there was no reduction in viscosity in 60 to 120 min in case of pectin hydrolase activity. Further 'R' isolate did not show reduction in viscosity even in the case of pectin lyase activity.

Table 2. Pectinase activity of *E. carotovora* isolates by cup-plate method.

Isolate	Pectin Hydrolase activity (diameter of clearing zone) (cm)	Pectin Lyase activity (diameter of clearing zone) (cm)	Pectin methyl esterase activity (diameter of clearing zone) (cm)
C-2	3.70	1.40	1.60
Pt	2.50	1.25	1.45
Iso	4.60	1.95	2.15
C-1	2.30	0.85	1.30
R	0.50	0.60	0.60
Control	0.0	0. 0	0.00

C-1 = *E. carotovora* isolated from cabbage, C-2 = 2nd isolate isolated from cabbage, Pt = *E. carotovora* isolated from potato, Iso = *E. carotovora* isolated from carrot, R = soft rot causing *Rhizopus* sp. in cabbage.

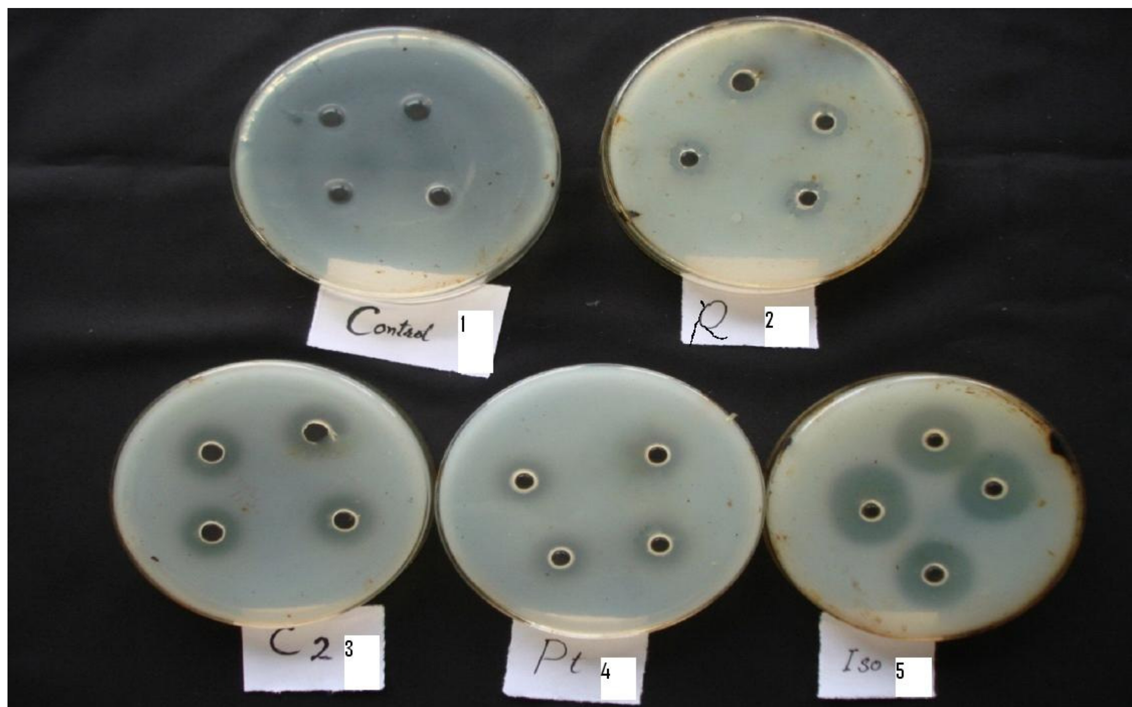

Figure 3. Pectinase activity of *E. caratovora* isolates by cup-plate method showing enzyme activity as clearing zones; 1= control (from uninoculated broth), 2= soft rot causing *Rhizopus sp* in cabbage, 3= C-2 isolate of *E. carotovora* from cabbage, 4= isolate of *E. carotovora* from potato and 5= Isolate of *E. carotovora* isolated from carrot.

Table 3. Pectin hydrolase activity of *E. caratovora* isolates by viscometric method.

Isolate	Percent reduction in viscosity by enzyme after (min)		
	30	60	120
C-2	34.44	40.25	43.50
Pt	27.92	33.76	35.71
I so	42.85	46.75	50.00
C-1	26.66	27.27	27.92
R	7.99	9.74	9.74

C-1 = *E. carotovora* isolated from cabbage, C-2 = 2nd isolate isolated from cabbage, Pt = *E. carotovora* isolated from potato, Iso = *E. carotovora* isolated from carrot, R = soft rot causing *Rhizopus* sp. in cabbage.

Table 4. *P*ectin lyase activity of *E. caratovora* isolates by viscometric method.

Isolate	Percent reduction in viscosity by enzyme after (min)		
	30	60	120
C-2	22.89	26.50	33.13
Pt	9.63	11.44	17.46
Iso	33.13	43.37	49.39
C-1	7.22	8.43	9.03
R	3.61	7.22	7.22

C-1 = *E. carotovora* isolated from cabbage, C-2 = 2nd isolate isolated from cabbage, Pt = *E. carotovora* isolated from potato, Iso = *E. carotovora* isolated from carrot, R = soft rot causing *Rhizopus* sp. in cabbage.

Phytopathogenic bacteria capable of producing cell wall degrading enzymes during host pathogen interaction are usually capable to produce such enzymes in cultures as well. Therefore, investigations were carried out to detect pectic enzymes in cell free culture filtrates of different *Erwinia* isolates and soft rot causing *Rhizopus* sp. The results indicated that there was considerable variability among isolates with respect to pectin hydrolase, pectin lyase (but by cup-plate and viscometric methods) and pectin methyl esterase (PME) activity (by cup-plate method) which are more or less as per the relative macerating ability of the bacterial isolates and *Rhizopus* sp. The results on enzyme activities by all the methods are similar and hence confirming their accuracy with each other and that of maceration test with only the exception that assay for pectin methyl esterase (PME) cannot be carried out with viscometric assay as pectin methyl esterase (PME) cannot reduce viscosity. Phokum et al. (2006) also reported variations in pectin lyase and polygalacturonases (PG) by spectrometer and found that *Erwinia* isolate from carrot was more severe as it produced higher amount of enzymes and macerated more tissue than the ten other isolates of pathogen. El-Shaieb and Milibari (1995) also reported variation among isolates of *E. caratovora* with respect to their enzyme activities of PME and PG (polygalacturonase). Zaki et al. (1978) also showed variation in enzyme activities in *E. caratovora* isolates, among which pepper isolate showed higher enzyme activities. Others reports of significant differences among the strains of soft rot causing *Erwinia* for aggressiveness and their association with variation of production quantities of pecteolytic enzymes are of Gregg (1952) and Smith and Bartz (1990).

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