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Hydrolytic enzymes in relation to the fusarial wilt of brinjal

M.R. CHAKRABORTY AND N.C. CHATTERJEE

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See end of the article for authors' affiliations

Correspondence to:

N.C. CHATTERJEE
Department of Botany,
University of Burdwan
BURDWAN (W.B.) INDIA

ABSTRACT

Wilt of eggplant caused by *Fusarium solani* occurs in almost all the fields under cultivation that causes a great loss in yield of the vegetable. *In vitro* and *in vivo* cellulolytic and pectinolytic enzyme activities were assayed during pathogenesis. The β-glucosidase activity, among the all the unit components of cellulolytic enzyme was recorded to be the highest, which was followed in accordance with their activities by endoglucanase and exoglucanase. Pathogen was capable of producing all the unit components of the pectolytic enzymes and showed that the higher activity of both PG and PTE in *in vivo* and *in vitro* conditions than PMG and PGTE which indicated their greater role in pathogenesis and in disease development.

Key words : Brinjal, *Fusarium solani*, cellulase, pectinase.

Eggplant (*Solanum melongena* Linn.) is an important commercial vegetable which enjoys a high demand of premium price through out the year. Wilt caused by *Fusarium solani* (Mart.) Appel and Wollenw is a dreadful disease posing a threat towards the cultivation of brinjal. Cell wall degrading enzymes play a vital role in the development of the disease. Several enzymes are known to degrade pectic and cellulose substances of plant cell walls and as a result of which the pathogen gets an entry into the host tissues (Mesaphy and Levavon, 1992, Chakraborty, 2005). The present paper deals with the studies on the activities of cell wall degrading enzymes like cellulases and pectinases secreted by *Fusarium solani* and their relationship to the development of wilt of brinjal.

MATERIALS AND METHODS

Fusarium solani was isolated from wilted brinjal plants and maintained in pure line on potato dextrose agar (PDA) slants at 4^0C till used. The identification of the pathogen was confirmed by Indian Agricultural Research Institute (IARI), New Delhi (ITCC No.4124.2K).

Cellulolytic enzymes:

In order to obtain the enzyme source, Czapek's medium was used as the basal medium (Mahadevan and Sridhar, 1982) serving as substrate for *in vitro* production of the enzyme.

For *in vivo* study, 25g of tissues from *F. solani* infected plant was cut into 1-2 cm pieces, homogenized with chilled double distilled water. The slurry was passed through two layers of cheese cloth and centrifuged at 2,000 rpm for 30 min, at 4°C. The dialysed supernatants were used as enzyme source. Both *in vitro* and *in vivo* enzyme activities were studied at intervals of after 7, 14, 21 and 28 days of inoculation.

The cellulase enzymes were assayed following mainly the method of Mahadevan and Sridhar (1982). Exoglucanase activity was determined by incubating 0.25 ml of culture filtrate with 0.75 ml of sodium acetate buffer (0.2M) at pH 5.0, along with 50 mg Whatman No.1 filter paper discs at 50^0C for 60 minutes.

Endoglucanase or carboxymethyl cellulase (CMCase) activity was determined by incubating 0.5 ml of culture filtrate with 0.5 ml of sodium acetate buffer (0.2M) at pH 5.2 and 1 ml of carboxymethyl cellulose (1%) dissolved in acetate buffer at pH 5.2. The reaction mixture was then incubated at 50^0C for 30 minutes.

For determination of? b-glucosidase activity, 0.5 ml of sodium acetate buffer (0.2M) at pH 5.0 were incubated with 1.0 ml of 0.01% cellobiose (Sigma) in sodium acetate buffer (pH 5.0) at 50^0C for 30 minutes.

In all the cases after a specific period of incubation, reactions were terminated by adding 3 ml of dinitrosalicylic acid (DNS) reagent, boiled for 5 minutes, cooled at room temperature and the absorbance read at 540 nm in a Spectrophotometer (Sicospec 100). Enzyme activity was expressed in terms of unit (U) defined as the amount of enzyme required to liberate 1.0 m mole of reducing sugar (glucose) per minute under conditions of assay.

Pectolytic enzyme:

For studying the *in vivo* enzyme activity, the same method was followed as done for cellulolytic enzyme activity.

To study *in vivo* enzyme activity 20g of tissue was cut into pieces of 1 to 2 cm, homogenized with 0.15M-chilled sodium chloride at a rate of 3 ml per gm. The slurry was passed through two layers of cheese cloth and centrifuged at 10,000 rpm for 2 min at 4°C. The

supernatants were used as enzyme source for measuring the *in vivo* enzyme activity. Both *in vitro* and *in vivo* enzyme activities were studied at intervals of after 7, 14 and 21 days of inoculation.

Different unit components of pectolytic enzymes *viz.*, PG, PMG, PTE and PGTE require different types of substrates for assaying their activities.

Preparation of 0.75% sodium polypectate-acetic acid substrate for assaying PG activity: To prepare this substrate, 750 mg of sodium polypectate (Sigma) was dissolved in 100ml of sodium acetate acetic acid buffer (0.2M) at pH 5.2 by heating it at 50°-60°C and blended for 2-3 min. at low speed following by a high speed for another 3 min. The blended material was then passed through two layers of cheese cloth and the pH was verified with a digital pH meter.

Preparation of 1% pectin acetate substrate for assaying PMG activity: To prepare this substrate, 1 gm of pectin was dissolved in 100ml of sodium acetate acetic acid buffer (0.2M) at pH 5.2 by heating it at 50°-60°C and blended for 2 to 3 min at low speed and at high speed for another 3 min. It was then passed through two layers of cheese cloth and the pH was verified.

Preparation of 1% pectin-boric acid-borax substrate for determining PTE activity: This substrate was prepared by dissolving 1gm pectin in 100ml of boric acid-borax buffer (0.2M) at pH 8.7 with heating at 50°-60°C and blended for 2 to 3 min. at low speed and at high speed for another 3 min. It was then passed through two layers of cheese cloth and the pH was verified.

Preparation of 1.2% sodium polypectate-boric acid-borax substrate for determining PGTE activity: To prepare this substrate, 1.2gm of sodium polypectate was dissolved in 100ml boric acid-borax buffer (0.2M) at pH 8.7 by heating at 50°-60°C and then blended in a blender for 2 to 3 min. at a relatively low speed and at high speed for another 3 min. It was then passed through double layers of cheese cloth and the pH was verified.

Enzyme activity was determined as per the methods described by Mahadevan and Sridhar (1982). The enzyme reaction mixture consisted of 6ml of enzyme extract, 12ml of respective substrate and 3ml of specific buffer in which the substrate dissolves. The mixture was taken into an Ostwald Viscosimeter. The contents were mixed thoroughly and the efflux time of mixture was noted. This was taken as zero time. After this, the reaction mixture was incubated at 37°C for different intervals (30 min, 60 min and 120 min) and again the efflux time of the mixture was noted. The blank used contained a mixture of 6ml of distilled water, 12 ml of respective substrate and 3 ml of specific buffer which was treated in an identical manner.

The per cent loss in viscosity of the substrate (V) was interpreted as being proportional to the enzyme activity. The per cent loss in viscosity was calculated from the following formula:

$$V = T_0 - \frac{T_t}{t_0} - T_{H_2O} \times 100$$

where, V = Per cent loss in viscosity of the substrate,
T_0 = Flow time at seconds in zero time,
T_t = Flow time of the reaction mixture at time, t and
T_{H_2O} = Flow time of distilled water.

RESULTS AND DISCUSSION

The result reveals that the pathogen possesses the capacity of producing all the components of cellulolytic enzymes both under *in vivo* and *in vitro* conditions. The activity of b-glucosidase was recorded to be the highest in both *in vivo* and *in vitro* conditions, which was followed in accordance with their activities by endoglucanase and exoglucanase (Table 1). *F. solani* showed highest activities of β-glucosidase, endoglucanase and exoglucanase on 21st day of incubation under *in vivo* condition. With time of incubation, the activities of all the units of cellulolytic enzymes showed a general tendency of the formation of a peak in between 14th and 21st day or incubation with highest peak on 21st day under *in vivo* condition and 14th day under *in vitro* condition. The activities were observed to be descended more or less after 21st day of incubation

Table 1: *In vitro* and *in vivo* cellulolytic enzyme activity of *F. solani* at different incubation periods (in terms of μ/ml.)

Enzymes	Days of incubation	*In vitro* activity*	*In vivo* activity*
Endoglucanase	7	0.022 ± 0.001	0.12 ± 0.001
	14	0.044 ± 0.002	0.396 ± 0.002
	21	0.038 ± 0.001	0.506 ± 0.002
	28	0.016 ± 0.002	0.414 ± 0.006
Exoglucanase	7	0.028 ± 0.001	0.106 ± 0.004
	14	0.033 ± 0.002	0.322 ± 0.002
	21	0.023 ± 0.001	0.364 ± 0.002
	28	0.012 ± 0.002	0.272 ± 0.002
β-glucosidase	7	0.037 ± 0.003	0.176 ± 0.002
	14	0.051 ± 0.001	0.454 ± 0.002
	21	0.032 ± 0.002	0.564 ± 0.002
	28	0.022 ± 0.001	0.496 ± 0.002

	S.E.±	C.D. (P=0.05)
Endoglucanase	± 0.031	0.06603
Exoglucanase	± 0.022	0.04686
β-glucosidase	±0.035	0.07455

*Data are the mean values of five replicates.

Table 2: *In vitro* and *in vivo* pectolytic enzyme activity of *F. solani*

Enzyme	Days of incubation	*In vitro activity*G		*In vivo* activity*	
		Percentage reduction in viscosity of reaction mixture after 1 hr of incubation			
		Treated (with pectin)	Control (without pectin)	Treated (Infected plant)	Control (Healthy plant)
PMG	7	17.74 ± 0.024	3.26 ± 0.024	13.22 ± 0.02	2.12 ± 0.02
	14	28.1 ± 0.04	6.38 ± 0.02	24.42 ± 0.04	2.66 ± 0.02
	21	36.5 ± 0.02	9.14 ± 0.02	34.42 ± 0.04	2.94 ± 0.02
PG	7	32.8 ± 0.02	5.44 ± 0.02	30.36 ± 0.02	2.42 ± 0.02
	14	48.5 ± 0.04	6.34 ± 0.02	44.24 ± 0.02	2.6 ± 0.03
	21	64.3 ± 0.02	6.84 ± 0.02	58.76 ± 0.02	2.96 ± 0.02
PTE	7	24.4 ± 0.02	3.86 ± 0.02	16.14 ± 0.02	3.04 ± 0.02
	14	61.2 ± 0.02	2.14 ± 0.02	32.66 ± 0.04	3.94 ± 0.02
	21	74.4 ± 0.02	1.68 ± 0.02	46.78 ± 0.05	4.4 ± 0.05
PGTE	7	16.1 ± 0.02	4.68 ± 0.02	12.68 ± 0.02	3.72 ± 0.02
	14	25.3 ± 0.05	5.28 ± 0.02	17.38 ± 0.02	4.18 ± 0.02
	21	38.5 ± 0.05	4.26 ± 0.02	32.12 ± 0.02	4.64 ± 0.02

(PMG = Polymethyl galacturonase; PG= Polygalacturonase ; PTE = Pectin trans eliminase ; PGTE = Polygalacturonase trans eliminase)

	In vitro activity		*in vivo* activity	
	S.E.±	C.D. (P=0.05)	S.E.±	C.D. (P=0.05)
PMG	±2.24	4.7712	±2.65	5.6445
PG	±4.28	9.1164	±4.17	8.8821
PTE	±5.47	11.6511	±3.08	6.5604
PGTE	±2.37	5.0481	±1.88	4.0044

* Data are the mean values of five replicates.

under *in vivo* and 14th day under *in vitro* condition.

β-glucosidase is the specific enzyme for the terminal production of glucose through the conversion of cellobiose, the product made through the activity of the enzyme exoglucanase using smaller cellulase chains as the substrate produced by the enzymatic action of endoglucanase on native cellulose. The greater activity of β-glucosidase, as is evident from the result, taking the greater role in breaking the macromolecules of cellulose microfibrills down to glucose, might be accounted for the essential need of getting energy for the pathogen facilitating the development of the disease. Although higher b-glucosidase activity has been reported to have no direct action on cellulose (Sternberg *et al.*, 1977), its high level in the enzymatic system prevents accumulation of cellulobiose to inhibitory level for exoglucanase activity and as such higher level of β-glucosidase activity as is evident in the present experiment is considered as an attractive feature for breaking down cellulose microfibrills by a system of cellulose enzyme of pathogenic origin (Mukhopadhyay and Nandi, 1997, Chakraborty, 2005). Cellulolytic enzyme activities showing their constant presence and rise in the present findings might be due to synergistic action of the enzymes. Lowering of cellulase activities in the latter stages of incubation may probably be due to catabolic repression of cellulase as soluble reducing sugar gradually increased in the medium.

In the present experiment, the activity of both PTE and PG remained higher in infected plant even after 21st day of infection whereas in healthy plant, the activity was somewhat lesser than the former (Table 2). It is evident from the result, that comparatively the higher activity of both PG and PTE in *in vivo* and *in vitro* conditions than the other two components like PMG and PGTE indicates their greater role in pathogenesis. Increase in the activities of PGTE and PMG at the 21st days of infection may be due the involvement of these enzymes in the later stages of pathogenesis. PG is probably the chief instigator of breakdown of the tissues by action on the pectic substances in the middle lamella. While PTE is not a pectin-splitting enzyme, its demethylating action may speed up the action of PG. It may thus be concluded that the greater activity of PG and PTE at early stages of infection facilitates the hastening up of breaking cell wall fragments. Role of pectic enzymes is of great importance in causing wilt in which parenchymatous tissues of host plants are being invaded rapidly followed by dissolution of the middle-lamella resulting in the reduction of the water flow through the xylem and thereby destroying the water balance (Subramaniam, 1970). The role of pectolytic enzyme to degrade plant cell wall substances, cleavage of substances results in the appearance of smaller

fragments, which are further broken down to still smaller molecules before being utilized by the pathogen as nutrient is well established (Mahadevan, 1970; Gupta and Kaul, 1998).

Authors' affiliations:
M.R. CHAKRABORTY, Mycology and Plant Pathology Laboratory, Department of Botany, University of Kalyani, Kalyani, NADIA (W.B.) INDIA

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