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RESEARCH ARTICLE

Production of cell wall degrading enzymes by *Fusarium oxysporum* f.sp. *cepae* causing basal rot of onion and its histopathological changes

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ABSTRACT

Basal rot is a devastating disease of onion caused by *Fusarium oxysporum* f. sp. *cepae. Fusarium oxysporum* f. sp. *cepae* excreted several extracellular pectinolytic and cellulolytic enzymes in the onion plants after infection. The production of polygalacturanase, pectin methyl esterase and pectin *trans*-eliminase increased in the onion plants infected with the pathogen. Histopathological changes occurred in onion due to infection of *Fusarium oxysporum* f. sp. *cepae*. Large numbers of small vacuoles were also observed in the cytoplasm. The xylem vessels were thickened and both proto and meta xylem plugged with mycelium. Infected onion bulbs showed disintegration of epidermal layer, cortex tissue and vascular bundle cells. Compared to healthy tissues of bulb, epidermal layer of infected bulbs were disrupted at several points.

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INTRODUCTION

Onion (*Allium cepa* var. *aggregatum* G.Don) is a major vegetable crop grown in India which is called as "Queen of kitchen". Basal rot is the most serious and destructive disease of onion which causes severe yield loss (Coskuntuna and Ozer, 2008).The fungus causes pre and post-emergence damping off, root rot of older plants and stem plate discoloration and basal rot of bulbs in the field and in storage (Abawi and Lorbeer, 1971, 1972). In adverse environmental conditions, *F. oxysporum* f. sp. *cepae* has the ability to significantly reduce the crop yield approximately 45 t/ha in USA (Schwartz *et al*., 1991). In Japan, during summer period more than 50 per cent loss occurs in welsh onion due to Fusarium basal rot (Dissanayake *et al*., 2009).

Awuah *et al*. (2008) reported that in the field, diseased plants were chlorotic and the disease progressed, blighting and toppling over occurred. The foul smell observed in the field which was found to emanate from water-soaked organs of decayed portions was ascribed to secondary infection. Pectic enzymes have been frequently associated with wilt disease on various crops (Cooper and Wood, 1980). The fungal endo- polygalacturonase has been implicated in pathogenesis in plant disease. Holz and Knox- Davies (1986) reported that *F. oxysporum* f. sp. *cepae*, the onion bulb rot pathogen produced endo-pectatelyase.

Niemann *et al*. (1991) found that the early demethylation of pectic acid and degradation of pectin are the first signs of colonization by *F. oxysporum* f. sp. *dianthi* in carnation cultivars. Patino Alvarez *et al*. (1999) reported that *F. oxysporum* f. sp. *radicis lycopersici* produced more of exo-polygalacturonase in the infected roots of tomato. Turco *et al*. (1999) found that the nine isolates of *F. oxysporum* f. sp. *vasinfectum* produced the pectinolytic (pectate lyase and polygalacturonase) enzyme activity in roots of cotton.

Dimond (1970) reported that vascular wilt pathogens produced metabolites into vessels and these being carried

with the transpiration system. Among the products released were compounds of low molecular weight, such as growth regulants and large molecules consisting of polysaccharides, glycopeptides and hydrolytic enzymes.

Histological studies are helpful for better understanding of tissue damage caused to plants by pathogen attack and host pathogen interaction. However, less work has been done related to cellular changes in onion bulb. Thus, the present investigation was carried out to study the production of extracellular pectinolytic and cellulolytic enzymes and histopathological changes in onion bulbs.

MATERIAL AND METHODS

Isolation of pathogen :

The pathogen was isolated from the diseased tissues of onion by tissue segment method (Rangaswami, 1958). The infected portions of diseased plants were cut into small pieces using sterilized scalpel and these were surface sterilized with 0.1 per cent mercuric chloride for one minute and washed in three changes of sterile distilled water and then placed on Petri dish containing potato dextrose agar (PDA) medium. These plates were incubated at room temperature (28 2^0C) for five days and observed for the growth of the fungus. Totally fifteen *Fusarium* isolates were isolated from infected onion bulbs collected from different places of Tamil Nadu, India.

Production of cell wall degrading enzymes :

The bio-chemical changes taking place in the onion bulbs after inoculation with *Fusarium oxysporum* f.sp. *cepae* was studied as described below. Onion bulb was cut into small pieces (8 mm).Ten pieces were placed in a sterile Petri dish and 10 ml of culture filterate was added and macerate the solution which was used as enzyme source for the following assay. Pieces without inoculation were kept as control (Mahadevan, 1965).

Assay of pectin methyl esterase (PME) activity :

The enzyme activity was assayed as per the procedure given by Mahadevan and Sridhar (1982). Twenty ml of pectin solution was pipetted in a 50 ml beaker and pH was adjusted to 7.0. Ten ml of the enzyme solution was then added and the pH was adjusted immediately to 7.0 by adding 1N NaOH. At every 15 min, the pH was checked and alkali was added from the burette when the pH falls below the reference point, while stirring. To adjust the pH, 0.02 N NaOH was used and the volume of alkali consumed was noted, which was equal to the enzyme activity. The enzyme activity was expressed as m mole of hydrogenion per min per ml of the enzyme preparation.

Assay of polygalacturonase (PG) activity :

The activity of PG was assayed as per the method described by Mahadevan and Sridhar (1982). One g of bulb tissue was transferred to a wearing blender and five ml of 0.1M chilled phosphate buffer (pH 6.6) was added. The material was blended for five min, filtered through two layers of cheese cloth and centrifuged at 2000 rpm for 30 min at 4ºC. The supernatant was decanted and the clear extract was taken as enzyme source. Four ml of the substrate, one ml of acetate buffer (pH 5.2) and two ml of enzyme source were taken in a viscometer and the contents were mixed gently by drawing air rapidly through the large arm of the viscometer by suction. The efflux time of the mixture was determined by suction through small arm (zero time). The efflux time of the mixture after 30 min was measured. From this, the enzyme activity was calculated as per cent reduction in viscosity of the substrate from the following formula :

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

where,

T_o - Flow time in seconds at zero time

T - Flow time of reaction mixture at time T

T_{H_2O} - Flow time of distilled water.

Assay of pectin trans eliminase (PTE) activity :

PTE activity was estimated by the viscometric method as described by Mahadevan and Sridhar (1982). One ml of the enzyme solution and four ml of the substrate were pipetted into the viscometer. The loss in viscosity of the pectin solution was determined by using of Vinsell Viscometer of size 300. The activity was expressed as per cent reduction in viscosity :

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

where,

V - Per cent loss in viscosity

T_o - Flow time in seconds at zero time

T - Flow time of reaction mixture at time T

T_{H_2O} - Flow time of distilled water

Histopathological studies :

Onion bulbs were collected from basal rot infected and healthy plants. Healthy and disease infected onion bulbs were washed and fixed with five parts of 30 per cent formalin, five parts of glacial acetic acid (Formalin Acetic Acid (FAA)) and 90 parts of 70 % ethanol for 29 h at 4^0C. The samples were washed in running tap water for 10-15 min and in different concentrations of ethanol, *viz.*, 10, 50, 70 and 100 per cent for five min. and embedded in paraffin wax with a melting point of 56 to 58ºC. Embedded material was sectioned (10 mm) transversely in a Spencers Rotary microtome (USA) and stained with safranin and fast green (Johansen, 1940). The sections were observed under image analyzer and photomicrographed (CETI, Antwerp, Belgium).

Statistical analysis :

The statistical analysis of the experiment data was carried out by adopting the standard method as described by Gomez and Gomez (1984).

RESULTS AND DISCUSSION

Pectinases were important for phytopathogenic fungi during infection in plants. Pectic enzymes produce modification of cell wall structure, increasing accessibility of cell wall components for degradation by other enzymes, cell lysis and plant tissue maceration. Pectic enzymes are the first polysaccharidases to be induced when fungi are cultured on isolated plant cell walls and the first produced in infected tissue. Pectic enzymes were present both in culture filtrates and in diseased plants (Waggoner and Diamond, 1954). Generally they were found in relatively large amount in succulent soft tissues. The pectin methyl esterase secreted by the fungus to increase the activity of pectin depolymerase produced pectic and cellulolytic enzymes. Pectin depolymerase degraded the pectin in the primary cell wall and pectate in the middle lamellae. These enzymatic reactions may lead to vessel plugging.

In this present investigation fifteen isolates of *F. oxysporum* f. sp. *cepae* were assessed for their ability to produce PME activity in onion. The accumulation of PME increased in three days after inoculation and attained a peak at seven days after inoculation and slowly declined thereafter in all the inoculated onion plants. The PME activity at seven days after inoculation ranged from 0.31 to 0.42µ mole hydrogen ion per min per ml in the isolates. Enhanced activity of PME was recorded in the bulbs inoculated with DNSFOC1 isolate (0.42 µ mole hydrogen ion per min per ml) after seven days of inoculation followed by DNOFOC2 (0.38 µ mole hydrogen ion per min per ml). The isolate THBFOC2 exhibited the least amount of PME activity (0.31 µ mole hydrogen ion per min per ml) (Table 1).

The PG activity after seven days of inoculation was ranged from 12.56 to 15.76 (per cent reduction in viscosity). The isolate DNSFOC1 showed higher activity of polygalacturonase (15.76 per cent reduction in viscosity) on seventh day after inoculation followed by MDPFOC2 (14.52 per cent reduction in viscosity). The PG activity was less in the isolate ERVFOC2 (12.56 per cent reduction in viscosity) (Table 2).

Pectin trans eliminase activity increased after inoculation with the pathogen. The PTE activity in seven days after inoculation ranged from 48.21 to 55.21 per cent reduction in viscosity. The plants inoculated with DNSFOC1 showed higher activity of PTE (55.21 per cent reduction in viscosity) followed by COPFOC2 (54.12 per cent reduction in viscosity) on seventh day after inoculation. The PTE activity was less in the isolate DNTFOC3 (48.21 per cent reduction in viscosity) (Table 3).

The present investigation revealed the production of polygalacturanase, pectin methyl esterase and pectin trans-

Table 1 : Production of pectin methyl esterase (PME) by different isolates of *Fusarium oxsporum* f. sp. *cepae* in onion plants

Isolates	PME (µ mole hydrogen ion min^{-1} ml^{-1})			
	Days after inoculation*			
	3	5	7	9
ERKFOC1	0.15	0.24	0.33	0.32
ERVFOC2	0.14	0.26	0.34	0.33
ERPFOC3	0.06	0.21	0.32	0.31
MDSFOC1	0.15	0.25	0.37	0.37
MDPFOC2	0.13	0.22	0.35	0.34
MDUFOC3	0.14	0.25	0.32	0.32
DNSFOC1	0.18	0.29	0.42	0.41
DNOFOC2	0.13	0.23	0.39	0.38
DNTFOC3	0.17	0.25	0.38	0.37
COUFOC1	0.15	0.23	0.35	0.35
COPFOC2	0.13	0.21	0.32	0.31
COAFOC3	0.15	0.24	0.33	0.33
THMFOC1	0.08	0.26	0.34	0.33
THBFOC2	0.10	0.21	0.31	0.31
THPFOC3	0.14	0.25	0.33	0.32
Control	0.02	0.03	0.04	0.04
CD (P=0.05)	Isolates = 0.012	Days = 0.006	Isolates x Days = 0.024	

* Mean of three replications

eliminase increased in the onion plants inoculated with *F. oxysporum* f. sp. *cepae*. In this study, the most virulent isolate DNSFOC1 showed the highest PME, PG and PTE activity. Saravanan *et al.* (2004) indicated pectin methyl esterase activity of *Fusarium oxysporum* f. sp. *cubense* in banana plants. Endo polygalacturanase activity of *Colletotrichum capsici* and *Alternaria alternata* in chilli was reported by Anand *et al.* (2008).

Table 2 : Production of poly galacturonase (PG) by different isolates of *Fusarium oxsporum* f. sp. *cepae* in onion plants

Isolates	Polygalacturonase (per cent reduction in viscosity)			
	Days after inoculation*			
	3	5	7	9
ERKFOC1	8.04	9.76	13.45	13.12
ERVFOC2	8.45	9.54	12.56	12.09
ERPFOC3	9.23	9.25	13.67	13.11
MDSFOC1	8.18	10.45	13.72	13.02
MDPFOC2	9.46	10.42	14.52	14.12
MDUFOC3	8.34	10.87	13.26	13.01
DNSFOC1	9.45	12.76	15.76	15.21
DNOFOC2	8.16	9.98	13.87	13.23
DNTFOC3	8.34	10.65	13.56	13.15
COUFOC1	8.57	10.47	12.73	12.17
COPFOC2	8.02	9.65	13.72	13.21
COAFOC3	8.42	9.32	13.89	13.16
THMFOC1	8.87	9.43	13.82	13.05
THBFOC2	8.95	10.57	14.21	14.11
THPFOC3	8.45	9.21	12.68	12.17
Control	0.15	0.34	0.37	0.33
CD (P=0.05)	Isolates = 0.454	Days = 0.227	Isolates x Days = 0.909	

*Mean of three replications

Table 3 : Production of pectin trans eliminase (PTE) by different isolates of *Fusarium oxsporum* f. sp. *cepae* in onion plants

Isolates	Pectin trans - eliminase (per cent reduction in viscosity)			
	Days after inoculation*			
	3	5	7	9
ERKFOC1	34.23	42.45	48.45	48.21
ERVFOC2	43.15	41.56	49.54	49.01
ERPFOC3	34.31	46.23	51.98	51.11
MDSFOC1	38.47	41.91	50.32	50.15
MDPFOC2	36.65	47.34	50.12	50.05
MDUFOC3	32.99	46.23	50.02	49.67
DNSFOC1	46.11	51.23	55.21	55.13
DNOFOC2	35.40	45.76	49.65	49.15
DNTFOC3	36.23	50.31	48.21	48.02
COUFOC1	35.25	49.67	52.56	52.25
COPFOC2	36.24	48.97	54.12	54.04
COAFOC3	32.87	49.21	52.76	52.37
THMFOC1	34.28	47.34	50.34	50.14
THBFOC2	33.49	42.15	50.11	50.03
THPFOC3	37.14	41.56	50.76	50.25
Control	12.78	13.09	13.98	15.83
CD (P=0.05)	Isolates = 1.82	Days = 0.91	Isolates x Days = 3.65	

* Mean of three replications

The results also revealed that the toxins of all the isolates exhibited wilting symptoms in tomato stem cuttings. Vascular browning and vessel plugging were the characteristic symptoms of *Fusarium* infected tomato plants and inability of vessels in petioles to conduct fluid was correlated with wilting (Scheffer and Walker, 1953).

Fusarium oxysporum f. sp. *cepae* invaded in roots of onion by both direct penetration and/or wounds and grew in the intercellular spaces of the root or stem plates. Formation of tyloses and occlusion of xylem vessels were observed in onion stem plate (Abawi and Lorbeer, 1971). In the present study, microtome sections of healthy onion bulbs showed regular arrangement of epidermal cells, cortical parenchyma cells, conducting vessels and pith cells. Abundant conidia and mycelia were observed in the infected tissues of onion bulb. The epidermal cells and cortical parenchyma were collapsed and larger cavities were also seen. Large numbers of small vacuoles were also observed in the cytoplasm. The xylem vessels were thickened and both proto and meta xylem plugged with mycelium. Infected onion bulbs showed disintegration of epidermal layer, cortex tissue and vascular bundle cells. When compared the healthy tissues of onion bulb with infected tissues, the epidermal layer of infected bulbs were fragile and disrupted at various points. Enzymes or toxic metabolites were produced by the pathogen which resulted in disintegration of epidermal layers and vascular bundles.

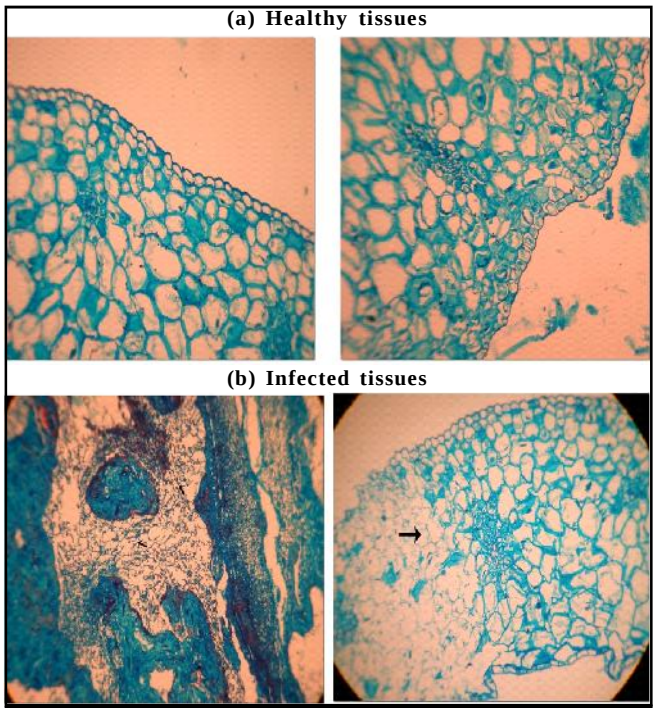

Fig. 1 : Histopathological changes in onion tissues
(a) Healthy tissues showing regular arrangement of epidermal cells
(b) Infected tissues disintegration of epidermal layer, cortex tissue and vascular bundle cells

Hyphae of *F. oxysporum* f.sp. *chrysanthemi* were visible in the xylem vessel elements and breakdown of xylem parenchyma tissues was observed in chrysanthemum leaf sections. In highly infected leaf tissues, the xylem vessels become hypertrophied (Barbara and Paul, 1981). Rey *et al.* (1998) found the deposition of phenolics and thickening of cell wall in tomato plants infected by *Pythium oligandrum*. They also observed the thickening of xylem vessels, cytoplasmic disorganization and large number of small vacuoles.

Pectin degradation results in liquefaction of the pectic substances that hold plant cells together and in the weakening of cell walls. This leads to tissue maceration, softening and loss of coherence of plant tissues and separation of individual cells, which eventually die. The weakening of cell walls and tissue maceration undoubtedly facilitate the inter- or intracellular invasion of the tissues by the pathogen.

Chattopadhyay and Bhattacharjya (1968) also reported that the cortical regions of the stem and root showed distinct discolouration and damage and light brown discolouration was noticed in vascular tissues. Histopathology in naturally wilted and artificially inoculated plants revealed the presence of *F. solani* and *F. oxysporum* in vascular tissues (Sohi, 1983).

Belanger *et al.* (2003) observed the external hyphal growth of *Blumeria graminis* f.sp. *tritici*, the powdery mildew pathogen of wheat, on the epidermal surface and penetrated the cells by producing haustoria. They also observed thickening of plant cells in the infected tissue due to suberization. Sahayarani (2003) stated that *Phyllanthus niruri* plants exhibited several histological changes due to infection by powdery mildew pathogen. Kamalakannan (2004) observed that, *Macrophomina phaseolina* infected coleus root and stem showed disruption of epidermal layers and cortical parenchyma cells, the presence of inter and intra cellular mycelium and thickening and plugging of xylem vessels.

Oruade-Dimaro *et al.* (2010) reported that *Raphia hookeri* leaf Infected with *Glomerella cingulata.* Their intact cells were darkened as a result of disease development which resulted in complete disintegration of the palisade tissues and disappearance of transfusion cells. The uninoculated seedling leaf remained healthy with glossy appearance. Prominent hyperplasia and hypertrophy occurred in *Rhus typina* plants infected with *Fusarium oxysporum* f.sp. *callistephi* (Guillemond, *et al*., 2006). Salah Eddin Khabbaz (2006) reported that the microtomy section of roots tissue of cotton plants showed, root rot infection which led to several histological changes in the infected root tissue of cotton plant. In the infected root thickening of cell wall was pronounced and cytoplasm was dense and granular.

Mycelium of *F.oxysporum* f.sp. *glycines* colonized profusely

on the upper surface of soybean seeds and seed coat tissues became distorted in infected seeds (Begum *et al.*, 2007). Singh *et al.* (2007) reported that abundant conidia were observed in root cells of cowpea inoculated with *Fusarium oxysporum.* Guava root infected with *Fusarium oxysporum* f.sp. *psidii* showed disintegration of the epidermal tissues and vascular bundle cells. Due to necrosis in vascular bundle restricts the movement of water and nutrients (Gupta *et al*., 2012).

Conclusion :

The production of polygalacturanase, pectin methyl esterase and pectin *trans*-eliminase increased in the onion infected with *F. oxysporum* f. sp. *cepae*. Histopathological studies conducted in the present study revealed that necrosis and disintegration of tissue occurred in infected onion which may be due to these pectinolytic and cellulolytic enzymes released by the pathogen.

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