CHAPTER III

Crop plant pathogenecity of *Fusarium moniliforme* NCIM 1276.
Summary

Healthy tomato (*Lycopersicon esculentum*) and cauliflower (*Brassica oleracea botrytis*) plants inoculated with *Fusarium moniliforme* NCIM 1276 showed yellowing and crinkling of leaves after 8 to 10 days. Therefore this estuarine isolate is a pathogen on at least these two crops.

Transverse sections of tomato and cauliflower hypocotyls examined under the light microscope showed that the fungus penetrates in about 4 days through the epidermal layer to the cortical tissue. Fluorescence labeled antibodies also showed that fluorescence of infected hypocotyls of cauliflower and tomato plants increase by 5 to 10 fold as compared to control plants.

At physiological pH both enzymes are produced by this pathogen although the ratios between the enzymes varies between tomato and cauliflower systems. Tomato has an acidic cell sap of 6.6 whereas cauliflower has a cell sap pH of 7.6. In the roots of both plants the ratio between PG:PL production in tomato is 3.6 as compared to 1 in cauliflower clearly indicating that pectate lyase production is enhanced in the latter species. Therefore in acidic environment such as in *Lycopersicon esculentum* (Tomato) there is greater production of the hydrolytic polygalacturonase (EC 3.2.1.15) as compared to the β-eliminative cleaver pectate lyase (EC 4.2.2.2) and conversely, in the neutral to alkaline cell sap host environment of *Brassica oleracea botrytis* (Cauliflower) pectate lyase production is enhanced.

The hypocotyl regions of both plants showed lower activity of enzymes.

*Fusarium* (Deuteromycotina, Moniliales) is a widely occurring plant-pathogen. The species is also known to exhibit a saprophytic mode of nutrition. The data presented here suggest that *Fusarium moniliforme* NCIM 1276 has adapted to estuarine conditions but has retained its virulence against crop plants through the production of pectin-degrading enzymes.
Introduction

The role of pectinases in pathogenesis has been well established. Early studies (Karr and Albersheim, 1970) established what the authors referred to as Cell Wall Modifying Enzymes which were shown to be closely related to a polygalacturonase contained in one fraction: R-10. More recently polygalacturonase has been shown to determine virulence by Erwinia caratovora (Lei et al., 1985) and cause necrosis in Vigna unguiculata by Aspergillus niger (Cervone et al., 1987). A pectate lyase has been implicated in pathogenicity of Fusarium solani var pisi (Crawford and Kolattukudy, 1987) and pectinolytic enzymes are produced by the plant pathogen Sclerotina sclerotium (Riou et al., 1992). Polygalacturonase has been reported to be a virulence factor in Agrobacterium tumefaciens Biovar 3 (Rodriguez-Palenzuela et al., 1991) and pectin-degrading enzymes have been isolated from the culture of Sclerotina borealis (Takasawa et al., 1997). Although cutinases have also been reported to be produced during penetration by Fusarium solani f pisi into its host Pism sativum (Shaykh et al., 1977) and it has been shown that specific inhibition of cutinase prevents infection (Maiti and Kolattukudy, 1979), lack of pectic enzymes for example in species like Verticillium makes strains of this species non pathogen (Leal and Villanueva, 1962). Thus, pectinases are important in pathogenecity of organisms.

Studies on Erwinia have shown that organisms have more than one set of genes responsible for the production of important enzymes like polygalacturonase (Reid and Collmer, 1986 and Kelemu and Collmer, 1993). It was therefore desirable to study whether the enzymes produced by Fusarium in submerged culture are antigenically similar to the enzymes produced in the host tissue.

The present strain of Fusarium moniliforme has been isolated from an unusual environment. Mangroves are tidal estuaries with diurnally changing conditions of salinity and pH. It was therefore desirable to determine whether the isolate was pathogenic to crop plants, and whether it produces polygalacturonase or pectate lyase when invading host tissue depending on cell sap pH of the host tissue.

The objective of the work reported in this chapter were to determine whether the isolate is a crop pathogen, whether the same genes are expressed in submerged culture and on the host tissue, and whether there is a difference in the ratios between the two enzymes.
polygalacturonase (EC 3.2.1.15) and pectate lyase (EC 4.2.2.2) when the organism is grown on plants with different cell sap pH. For this study we used tomato (*Lycopersicon esculentum*) and cauliflower (*Brassica oleracea botrytis*).
Materials

Pectin (Citrus fruit), polygalacturonic acid, horseradish peroxidase, anti-rabbits IgG (whole molecule), sulforhodamine (Texas Red) were purchased from Sigma Chemical Company USA. Tetramethyl benzidine/ hydrogen peroxide (TMB/H₂O₂) was purchased from Bangalore Genei Pvt. Ltd. ELISA plates were purchased from Greiner Laborteknik Pvt Ltd Chandigharh, India. Tomato (Lycopersicon esculentum) and cauliflower (Brassica oleracea botrytis) seeds were purchased from local seed store. All chemicals and reagents used were of analytical grade.

Methods

*Microorganism and culture conditions*

Growth, maintenance and production of polygalacturonase and pectate lyase from *Fusarium moniliforme* NCIM 1276 were described in Chapter 2.

*Enzyme assay*

Polygalacturonase and pectate lyase activity was determined using standard assay conditions as described in Chapter 2.

*Protein assay*

Protein concentration was determined in accordance with Lowry *et al.* (1951) with BSA standard.

*Labeling of anti rabbit IgG (whole molecule) with Texas Red*

Labeling of anti rabbit antibodies with Texas Red was carried out according to Goldman (1968). 2 mg/ml of antibodies were incubated in carbonate buffer (0.5 M) pH 9.0 and mixed with 10% solution of Texas Red. The reaction mixture was then passed through Sephadex G-20 (1.5 x 20 cm) column which was already equilibrated in phosphate buffer pH 7.2. Labeled antibody fractions were pooled and concentrated using ultrafiltration. Labeled antibodies were stored at 4°C until required.
Pathogenicity tests

Pathogenicity of *F. moniliforme* was tested on healthy 30 days old tomato and
cauliflower plants grown under field conditions. *Fusarium* culture grown in pectin medium
for two days was harvested and used as an inoculum. 50 ml of inoculum was applied to
exposed root around the base of each plant after making slight injury to the roots with the
help of sterile needle. Control plants were inoculated with sterile distilled water. A moist,
sterile cotton pad was placed over the site of inoculation to reduce desiccation. The
inoculation was repeated after 5 days. Inoculated and control plants were observed
periodically for the appearance of disease symptoms.

Additionally pathogenicity of the *Fusarium moniliforme* was tested on healthy
tomato and cauliflower plants grown in tissue culture. 30 days old seedlings were raised in
sterile Whites basal tissue culture medium. 1 ml of (10^6/ml) spore suspension of *Fusarium*
was injected to the hypocotyls and roots region of the plants without disturbing the plants
themselves using sterile syringe. Samples of infected plant part were collected after 8-10
days. The media composition of Whites medium was as published by Whites (1943).

Isolation and identification of the pathogen from field grown infected tomato and
cauliflower plants

Infected plants and their roots were collected from the field, washed thoroughly in
running tap water and examined under the microscope.

The samples were cut to 2 cm small size, washed with sterile distilled water, surface
sterilized using 0.01 % mercuric chloride solution for 1 min, washed again with sterile
distilled water and blotted on filter paper. Then the roots and hypocotyls were placed on
Czapec-Dox agar plate modified with 1% pectin. The plates were incubated at 30°C for 4
days and colony emerging around the infected hypocotyls and roots were purified by single
spore isolation method. The pathogen was identified using morphological characteristics as
*Fusarium moniliforme* NCIM 1276 and it was maintained on Czapec-Dox agar at 10 °C.
Isolation polygalacturonase and pectate lyase from infected tissue of tomato and cauliflower plants grown in Whites basal medium

Cauliflower and tomato were grown under sterile conditions in Whites basal medium for one month at 25°C under constant light intensity of 2.5 W m⁻². Then 1 ml spore suspension Fusarium moniliforme (10⁶ spores) was injected using sterile syringe around the roots and hypocotyls region of the plants. Care was taken that the plants were not damaged. The infected and control plants were incubated for 4 to 8 days at 25°C under constant light intensity 2.5 W m⁻². The infected plants roots and hypocotyls were collected, washed with sterile distilled water, and 1 gram of infected tissues of roots and hypocotyls were taken for the isolation of polygalacturonase and pectate lyase enzymes. The infected tissues were frozen in liquid nitrogen and ground in a mortar and pestle. The powder of the frozen tissue was dissolved in 0.25 M NaCl, and after centrifugation, the supernatant solution was used for the determination of polygalacturonase and pectate lyase activity. The antibody specific protein in the supernatant was measured using sandwich ELISA as described in chapter 2. Control plants were grown under the same conditions without infection by Fusarium moniliforme spores.

Localization of the pathogen in host tissue

Transverse sections of hypocotyls of infected and control tomato and cauliflower plants were stained with cotton blue. The sections were washed with distilled water two to three times and observed under the light microscope.

Localization of polygalacturonase and pectate lyase in Whites medium grown tomato and cauliflower plants

Transverse sections of the hypocotyls of tomato and cauliflower infected and control plants were equilibrated with PBS/T at pH 7.0. The sections were blocked with 1% BSA for 1 h. Then sections were washed three times with PBS. Thereafter they were incubated with 1:300 diluted polygalacturonase and pectate lyase antibodies overnight at 4°C. Then sections were again washed with two to three times with PBS and incubated with 1:100 diluted second antibody labeled with Texas Red at 37°C for 3 hrs. The sections were
washed repeatedly with PBS and observed under Leitz Laborlux S fluorescent microscope using N₂ filter (excitation wavelength between 540-600 nm).

100 µm thin sections of infected and control hypocotyls were cut using Leica ultramicrotome Model RM 2155. Both sections were blocked with 1% BSA and after washing with PBS/T, the sections were incubated with 1:300 diluted primary antibody for overnight at 4°C. The sections were washed with two to three times with PBS/T and incubated with 1:100 diluted second antibody labeled with Texas Red at 37°C for 3 hrs. After washing with PBS/T, a single section each of infected and control hypocotyls was crushed in 2 ml 10 mM phosphate buffer at pH 7.0. The cell debris was removed by centrifugation. The supernatant solution was excited at 596 nm and fluorescence intensity of the Texas Red (I₀) was measured using a Perkin Elmer Spectrofluorimeter LS 5B at 25°C.
Results and discussion

When healthy tomato (*Lycopersicon esculentum*) and cauliflower (*Brassica oleracea botrytis*) plants grown under field conditions were inoculated with *Fusarium moniliforme* NCIM 1276 they showed yellowing and crinkling of leaves after 8 to 10 days. In the last stage of infection growth was arrested (Fig 3.1, Fig 3.2). At this stage root tips and hypocotyls showed decay and cortical tissue sloughed off. These are symptoms typical of vascular wilt. Infected material incubated on Czapec-Dox agar medium containing 1% pectin at 30°C, showed fungal colonies morphologically identified as *Fusarium moniliforme* NCIM 1276.

Bacon and Hinton (1996) and Yetes *et al.* (1997) have shown that some species of *Fusarium* cause endophytic symptomless infection in *Zea mays* but the present isolate apparently belongs to the main group of *Fusaria* which are plant pathogens. These data confirm the hypothesis that this species has adapted to the environment from which it was isolated, and is not in itself a new species.

Pectic enzymes are the first polysaccharidases produced by fungal pathogen in their attack on plants (Collmer and Keen, 1986). Bateman (1966) has reported the production of hydrolytic and trans-eliminative enzymes at different pH by *Rhizoctonia solani*. Young cultures of *Rhizoctonia* grown at acidic pH contained primarily polygalacturonase whereas older, alkaline cultures contains mostly pectate lyase. In the case of the present organism varying the pH of the medium in which the organism was cultured (Chapter 2) resulted in a similar shift in the type of enzyme produced showing that virulent strains express a variety of enzymes to suit the local pH conditions.
Figure 3.1
Pathogenicity testing of *Fusarium moniliforme* NCIM 1276 on field grown tomato plant
A) 8 days after infection B) Control tomato plant.
Figure 3.2
Pathogenicity testing of *Fusarium moniliforme* NCIM 1276 in Whites basal medium grown tomato and cauliflower plants
A) Tomato plant 1) Control 2) 4 days after infection 3) 8 days after infection
B) Cauliflower plant 1) Control 2) 4 days after infection 3) 8 days after infection.
Transverse section of infected *in vitro* tomato and cauliflower hypocotyls showed that the fungus penetrates in about 4 days through the epidermal layer to the cortical tissue (Fig 3.3). Sporulation occurs around the 6th day after infection. The hypocotyls were sectioned 4 days after inoculation with fungal spores and the section challenged with Texas Red labeled second antibodies. Fluorescence intensity in the vascular bundles of both infected tomato and cauliflower plants clearly shows that both enzymes were produced in both host tissues (Fig 3.4, Fig 3.5). The fluorescence intensity differed by 5 to 10 fold between control and infected plants (Fig.3.6). Crawford and Kolattukudy (1987) and De Lorenzo et al. (1987) have shown that *F. solani* f.sp.pisi and *F. moniliforme* use pectate lyase and polygalacturonase for penetration into host tissue.

During optimizations studies (Chapter 2) it has been shown that only one polygalacturonase and only one pectate lyase is produced in liquid culture by the organism at acidic and alkaline pH respectively. Although it is not known whether different enzymes may be produced if these two genes are knocked out, for example in mutated forms of the organism, the present data shows that the polygalacturonase produced in culture and on host are antigenically similar (as is also the case for the pectate lyase) and therefore likely to be produces of the same gene whether the organism is grown on submerged culture or in host.
Figure 3.3

Transverse section of tomato hypocotyl.

A) Control transverse section  B) Infected transverse section of tomato hypocotyl.
Figure 3.4
Transverse section of tomato hypocotyl incubated with Texas Red labeled second antibodies. (Staining procedure was described in Methods Section)
A) Control  B) Infected
Figure 3.5
Transverse section of cauliflower hypocotyls incubated with Texas Red labeled second antibodies. (Staining procedure was described in Methods Section)
A) Control B) Infected
Figure 3.6
Fluorescence intensity spectrum of tomato and cauliflower control and infected hypocotyls transverse section

PG 1\textsuperscript{st} antibodies used A) Tomato hypocotyls: Test (---), Control (-----).
B) Cauliflower hypocotyls: Test (-----), Control (---).

PL 1\textsuperscript{st} antibodies used C) Tomato hypocotyls: Test (-----), Control (---).
D) Cauliflower hypocotyls: Test (---), Control (-----)
As seen in Table 3.1 extracts from the control plants showed very low amount of polygalacturonase and pectate lyase activity. Furthermore these enzymes did not cross-react with antibodies raised against *Fusarium* proteins indicating that the plants themselves produce antigenically different polygalacturonases and pectate lyases. In fact *Fusarium* polygalacturonase and pectate lyase antibodies do not cross react with the polygalacturonase or pectate lyase produced by either *Aspergillus ustus* or *A. niger*. On infection of tomato and cauliflower plants with *Fusarium* polygalacturonase and pectate lyase activity increase and the proteins produced cross reacted with the purified antibodies.

Tomato which has an acidic cell sap of 6.6 showed an 77 fold increase in polygalacturonase activity between infected roots and control. In comparison pectate lyase activity increased only 30 fold from 0.04 units to 1.2 units per gram of infected tissue (Table 3.1). Infected cauliflower roots which have a cell sap pH of 7.5 shows a 125 fold increase in polygalacturonase production and a 167 fold increase in pectate lyase over control plant. The ratio between PG:PL production in tomato is 3.6 as compared to 0.93 in cauliflower (calculated from Table 3.1) clearly indicating that pectate lyase production is enhanced in the roots of the latter species.

In general the hypocotyls showed lower activity of enzymes both in control and after infection (Table 3.1). However the increase in polygalacturonase activity in infected tomato was over 130 fold as compared to the control whereas pectate lyase activity increased only 3 fold. In cauliflower on the other hand the increase in polygalacturonase in infected hypocotyls was only 19 fold as compared to a 40 fold increase in pectate lyase.
Table 3.1

Distribution polygalacturonase and pectate lyase in control and infected tomato and cauliflower plants

<table>
<thead>
<tr>
<th>Item</th>
<th>PG</th>
<th>PL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(U)</td>
<td>(μg)</td>
</tr>
<tr>
<td>Control tomato hypocotyls</td>
<td>0.0262</td>
<td>ND</td>
</tr>
<tr>
<td>Control tomato roots</td>
<td>0.056</td>
<td>ND</td>
</tr>
<tr>
<td>Infected tomato hypocotyls</td>
<td>2.6</td>
<td>35</td>
</tr>
<tr>
<td>Infected tomato roots</td>
<td>4.32</td>
<td>90</td>
</tr>
<tr>
<td>Control cauliflower hypocotyls</td>
<td>0.051</td>
<td>ND</td>
</tr>
<tr>
<td>Control cauliflower roots</td>
<td>0.021</td>
<td>ND</td>
</tr>
<tr>
<td>Infected cauliflower hypocotyls</td>
<td>0.992</td>
<td>18</td>
</tr>
<tr>
<td>Infected cauliflower roots</td>
<td>2.63</td>
<td>45</td>
</tr>
</tbody>
</table>

ND - Not Detectable.

Protein biomass was determined using sandwich ELISA as described in chapter 2.

In conclusion *Fusarium moniliforme* NCIM 1276 produces only two enzymes, possibly representing two gene products, whether in submerged culture or in host tissue. In host tissue there is a difference in the ratio of the two enzymes produced which appears to depend on the internal cell sap pH. In acidic environments there is greater production of the hydrolytic polygalacturonase (EC 3.2.1.15) over the β-eliminative cleaver pectate lyase (EC 4.2.2.2) and conversely in a neutral to alkaline cell sap host environment, pectate lyase is produced in larger quantity. These results duplicate the behavior of the organism in submerged culture.