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Correlation Between Cell Wall Degrading Enzyme Production and Incidence of *Colletotrichum gloeosporioides* Infecting Noni (*Morinda citrifolia*)

Manjunath Hubballi*, S. Archana, S. Nakkeeran, T. Anand and T. Raguchander
Department of Plant Pathology, Centre for Plant Protection Studies,
Tamil Nadu Agricultural University, Coimbatore - 641 003.

Different methods of inoculations were tried for proving Koch's postulates. Among them, the pin prick plus spore suspension spray was found be the best method under glasshouse. Out of the ten isolates of *Colletotrichum gloeosporioides*, isolate C1 was highly virulent and C7 was avirulent. The virulent isolate of *C. gloeosporioides* produced more cellulolytic enzymes *invitro* than the avirulent one and the activity of these enzymes increased with the increase in age of culture. Furthermore, the virulent isolate of *C. gloeosporioides* produced more pectinolytic enzymes than the avirulent one. All the pectinolytic enzymes produced by virulent isolate were highly active in 10-day-old culture and the activities decreased with the increase in age of culture. On the contrary, the activity of enzymes produced by avirulent isolate of pathogens did not decrease and these enzyme activities increased with the increase in the age of culture.

Key words: *Colletotrichum gloeosporioides*, noni, cellulases, pectinases and virulence.

Morinda citrifolia L. commonly known as *kura* or noni is known for its medicinal properties (Dixon *et al*., 1999). Noni fruit products have become quite popular for a wide range of ailments including treatment of cancer, which has encouraged laboratory and clinical research into possible efficacy. In India, recently the crop has become very popular under the umbrella of contract farming.The crop production is being challenged by an array of opportunistic pathogens. Among all, *Colletotrichum gloeosporioides* causing anthracnose disease is a major limiting factor leading to heavy losses.

Plant cells are surrounded by a rigid wall and disruption of this cell wall was evident during infection by many plant pathogens (Albersheim *et al.,* 1969). Plant cell wall degradation was due to the action of pathogen-produced enzymes that cleaved specific linkages in the wall matrix. Sequential secretion of cell wall degrading enzymes has been demonstrated during growth of several fungal pathogens on isolated host cell walls. Production of the cell wall degrading enzymes is not always constitutive. Frequently, induction by low concentrations of the major monomeric component of the enzyme substrate was also observed (Cooper *et al*., 1975) influencing the nature of cell wall degrading enzymes.

A number of cell wall degrading enzymes have been shown to be produced by plant pathogens (Chenglin *et al*., 1996) which facilitate cell wall penetration and tissue maceration in host plants.

*Corresponding author email: manjunathabtnau@gmail.com

These microorganisms produce metabolites in culture media and plant tissues which were involved in the disease syndrome (Wood, 1955). Several species of *Colletotrichum* were known to produce different types of metabolites. The role of cellulose and pectin degrading enzymes in causing cell-wall degradation is so important that it determines the virulence of many pathogens (Sinclair and Backman, 1982). In a number of systems, correlations have been established between the presence of pectinolytic enzymes, disease symptoms and virulence (Durrands and Cooper., 1988). This paper deals with importance of cell wall degrading enzymes produced by virulent and avirulent isolates of *C. gloeosporioides* and their role in disease development on noni.

Materials and Methods

Collection, isolation and establishment of isolates

The infected noni leaves showing typical symptom of anthracnose were collected from Tamil Nadu, Karnataka and Kerala. The pathogens were isolated by tissue segment method (Rangaswami, 1958). The fungus was purified by single spore isolation technique (Ricker and Ricker, 1936) and the purified isolates were maintained on PDA slants for further studies. The pathogens isolated from different locations were designated as different isolates.

Method of inoculation

Five different methods of inoculation were tried

for pathogenicity test to select the best method which may be standardized for proving pathogenicity and to study pathogen virulence. The isolate C1 of *C. gloeosporioides* was used for this study. Two months old seedlings were inoculated through different methods under glass house conditions. The seedlings were exposed to 80 per cent RH both 24 h prior and after inoculation by covering the seedlings with a polythene bag and spraying water inside the bag. Inoculation was done during cool evening hours. Three replications were maintained in each method. Fifteen days after inoculation, disease intensity was recorded in all methods. The methods tried for pathogenecity were mycelia inoculation with and without wounding (Anand, 2002 and Bobev *et al*., 2009), spore suspension spray without and with wounding (Shamim *et al*., 2008) and spore injection (Lakshmanan *et al*., 1990).

Assessment of PDI

Plants showing the symptoms of anthracnose were assessed as per the severity grade of 0 - 9 and the per cent disease index were calculated. The disease intensity of anthracnose was assessed with grade chart as proposed by TNAU (1980). The per cent disease index (PDI) was calculated using Mc Kinney's (1923) formula.

Virulence of isolates

Various isolates of *C. gloeosporioides* were inoculated on to two months old seedlings of noni maintained under glass house conditions by following pin prick plus spore suspension spray method (Lakshmanan *et al*., 1990). Three replications were maintained for each isolates with five seedlings for each replication. The symptoms were observed on 15 days after inoculation. Ten leaves per plant were randomly selected for each isolate and the PDI was calculated according to Mc Kinney (1923).

Production of cell wall degrading enzymes

To study the *invitro* production of pectinolytic and cellulolytic enzymes, the isolates namely C1 and C7 were grown on Czapek Dox broth (pH 7-7.5) wherein the carbon source was substituted with one per cent pectin (for pectic enzymes) or one per cent carboxy methyl cellulose (for cellulolytic enzymes). The media were inoculated with 9 mm diameter of nine day old actively growing culture disc of the each isolates. The culture filtrates were obtained after incubation at room temperature (27 ± 1 °C) for 5, 10, 15 and 20 days and centrifuged at 3000 g for 20 min. For the assay of pectinolytic enzymes, the culture filtrates were dialyzed for 18 h against distilled water at 40 °C. The dialysate served as enzyme source. As dialysis was found to reduce the activity of cellulolytic enzymes (Bateman, 1964), the culture filtrates as such were used for the assay of cellulases.

Assay of cellulolytic enzymes

Cellulase (C_1) activity was assayed by the method of Norkrans (1950). The enzyme activity was expressed in units (1 unit = change in absorbance of 0.01). Cellulase (C_x) activity was assayed by the viscosimetric method of Hancock *et al*. (1964) using carboxy methyl cellulose as the substrate. The results were expressed as the per cent loss in viscosity in 15 min.

Assay of pectinolytic enzymes

Macerating enzyme activity was assessed by the method described by Mahadevan (1965) using Potato discs of nine mm diameter and 30 µm thickness were obtained by using a hand microtome. The coherence of the potato discs was tested at different time intervals using a sterile glass rod and the enzyme activity was expressed as the time taken in hours for maceration of potato discs.

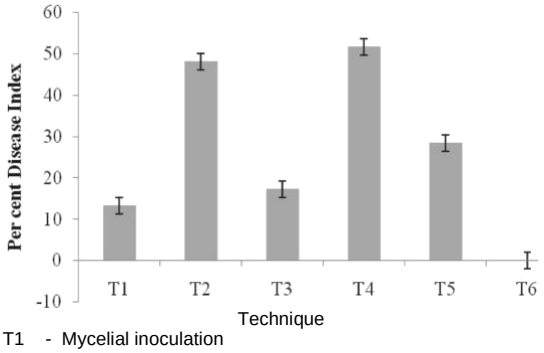
Pectin methyl esterase(PME) activity was estimated following the procedure described by Gupta (1970) and the enzymatic activity was expressed in terms of units (one unit is 0.1 ml of 0.02 N sodium hydroxide used). Endo PG activity was estimated by the standard viscosimetric method (Hancock *et al*., 1964) and the results were expressed as the per cent loss in viscosity in 15 min.

Statistical analysis

All the experiments were repeated once with similar results. The data were statistically analyzed (Gomez and Gomez, 1984) and the treatment means were compared by Dunkan's Multiple Range Test (DMRT). The package used for analysis was IRRISTAT version 92-a developed by International Rice Research Institute Biometrics Units, The Philippines.

Results and Discussion

Leaves depicting typical symptoms of anthracnose were collected from ten noni growing areas of Tamil Nadu, Karnataka and Kerala and from these leaf samples, the total of ten isolates were isolated and purified. Five different methods of inoculation were evaluated to standardize the method of inoculation for virulence studies. The results indicated that the intensity of disease symptoms varied significantly with that of different methods of inoculation. Among the different inoculation methods, pin prick with spore suspension spray method was the best, which recorded 51.60 PDI followed by pin prick with mycelial inoculation method (48.00 PDI). The lowest PDI was recorded in mycelial inoculation method (13.20 PDI) (Fig.1). Hence, for further studies pin prick plus spore suspension spray was used. The results of Rivera *et al.* (2000) revealed that wounding or puncturing of agave and Chinese rose leaves before application of spores resulted in higher per



T1 - Mycelial inoculation
T2 - Pin prick + Mycelial inoculation
T3 - Spore suspension spray
T4 - Pin prick + Spore suspension spray
T5 - Spore injection
T6 - Control

Vertical bar indicates standard deviation of three replications

Fig 1. Effect of inoculation methods on severity of anthracnose disease under glass house

cent infection of *C. gloeosporioides* when compared to spore suspension spray alone. Similar findings were also reported in case of *C. capsici* (Jeyalakshmi, 1996; Anand, 2002). The maximum infection caused in pin prick plus spore suspension spray method might be attributed to the ideal conditions like injury, ready to entry of the pathogen into the plant, ready access to the food available to the pathogen and possibly the absence of competition and antagonism by microorganisms (inside the plant). These findings suggested that wounding or injuries were the major factors for the disease initiation and further development.

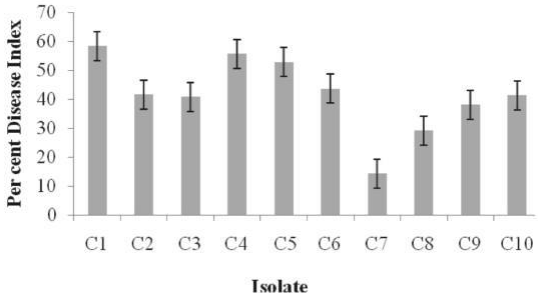
Isolates of fungal plant pathogens do exhibit a varied level of virulence even on the same host provided with same conditions. This is due to varied capacity of isolates in production of cell wall breaching enzymes and also toxins. In our study, virulence of ten isolates of *C. gloeosporioides* collected from different geographical area was tested under *in vitro*. The isolate C1 was significantly the most virulent which recorded the highest PDI of 58.30. This was followed by C4 (55.60 PDI) and C5 (52.80 PDI) while C7 was the least virulent (14.20 PDI) (Fig 2). Hence, for further studies, C1 and C7 were used. The findings are in agreement with Anand (2002) wherein among the five isolates of *C. capsici* the isolate CC1 was the most virulent followed by CC2 while CC5 was the least virulent.

Table 1. Production of cellulolytic enzymes by *C. gloeosporioides isolates*

Isolate	Incubation time (days)	Cellulase (C_1)*	Cellulase (C_x)**	Mycelial dry weight (mg)***
Virulent (C1)	5	1.60^{d}	17.00^{d}(24.35)	115.00^{d}
	10	4.50^{c}	25.57^{c} (30.37)	270.00^{c}
	15	9.00^{a}	76.87^{b} (61.25)	455.00^{b}
	20	8.00^{b}	81.90^{a} (64.82)	510.00^{a}
Avirulent (C7)	5	0.20^{c}	6.00^{d} (14.17)	45.00^{d}
	10	0.20^{c}	10.10^{c} (18.53)	62.00^{c}
	15	0.60^{b}	18.52^{b} (25.49)	73.00^{b}
	20	0.85^{a}	24.11^{a} (29.40)	85.00^{a}

* Enzyme activity in units,** Per cent loss of viscosity , *** Mean of three replications, Values in parentheses are arcsine transformed values
In a column, means followed by a common letter are not significantly different at the 5% level by DMRT

In the biotrophic phase, pathogens establish themselves in host cells, but there are many barriers against their establishment. The major barriers between the host plant and the attacking pathogen are the cuticle, cell wall and cell membrane. During biotrophic phase, pathogens invade cells by degrading cuticle, cell wall and cell membranes by producing a series of enzymes. After biotrophic phase, pathogens produce toxins, which kill cell and enter into necrotrophic phase. Thus, enzymes are important tools of pathogens for their pathogenesis. In our study, the cellulolytic enzyme production increased with increase in incubation period. The virulent isolate of *C. gloeosporioides* produced more cellulolytic enzymes (C_1 and C_x) than the avirulent one. The C_1 activity was more (8.00 units) in 20 day old culture filtrate of virulent isolate of *C. gloeosporioides* than the avirulent ones (0.85 units). Similarly, the C_x enzyme activity was found to be more (81.90 per cent loss of viscosity) in 20 day old culture filtrate of virulent isolate than the avirulent ones (24.11



Vertical bar indicates standard deviation of three replications

Fig 2. Virulence of *C. gloeosporioides* isolates on noni seedlings under glass house

units). Mycelial dry weight also increased with increase in incubation period. The dry weight of mycelium was higher in virulent isolate (510 mg) as compared to the avirulent ones (85 mg) (Table 1). The results of foregoing studies are in line with findings of Anand *et al*. (2008) who reported high cellulase activity in the culture filtrate of virulent

Table 2. Production of pectinolytic enzymes by *C. gloeosporioides*

Isolate	Incubation time (days)	Macerating enzymes*	Pectin methyl esterase**	Endopoly-galacturonase***	Mycelial dry weight (mg)****
Virulent (C1)	5	10.00 ^{c}	5.60 ^{d}	15.30 ^{d} (23.02)	240.00 ^{d}
	10	10.00 ^{c}	20.50 ^{a}	74.50 ^{a} (59.67)	360.00 ^{c}
	15	15.00 ^{b}	15.60 ^{b}	40.90 ^{b} (39.75)	555.00^{b}
	20	24.00 ^{a}	12.30 ^{c}	36.50 ^{c} (37.16)	605.00 ^{a}
Avirulent (C7)	5	No maceration	2.50 ^{d}	5.00 ^{d} (12.92)	32.00 ^{d}
	10	No maceration	6.00 ^{c}	10.12 ^{c} (18.54)	58.00 ^{c}
	15	No maceration	8.00 ^{b}	24.70 ^{b} (29.80)	80.00 ^{b}
	20	36.00^{a}	10.50 ^{a}	26.66 ^{a} (37.08)	85.00 ^{a}

* Time taken for maceration (h), ** Enzyme activity in units, *** Per cent loss of viscosity,******** Mean of three replications

Values in parentheses are arcsine transformed value, In a column, means followed by a common letter are not significantly different at the 5% level by DMRT

isolate of *C. capsici*.

After penetration through the cuticle, the invading fungus would encounter the pectinaceous barrier. Even when fungi and bacteria gain entry into plants through stomata and wounds, they would have to degrade the intercellular pectinaceous polymers to initiate infection in the plant. Plant pathogens produce multiple forms of different types of pectic enzymes. Both fungal and bacterial pathogens produce various pectic enzymes *in vitro* and *in vivo*, and many of them have been purified and characterized (Vidhyasekaran, 2002). Histological evidence has been provided to show the importance of pectic enzymes in cell wall penetration by fungal pathogens. In the study virulent and avirulent isolate were compared for pectinase production. In the virulent isolate, the production of the pectinolytic enzymes *viz*., macerating enzymes, PME and endo-PG increased up to 10 days and thereafter the activities of the enzymes decreased. Maximum enzyme activity was observed in the culture filtrate, 10 days after incubation. In the avirulent isolate, the enzyme production increased with increase in incubation period (throughout the experimental period) but it was much less than the virulent isolates. The mycelial weight increased with increase in incubation period in both virulent and avirulent isolates of *C. gloeosporioides* (Table 2). This corroborated the observation of Muthulakshmi (1990) and Anand *et al*. (2008) in the case of *C. capsici* causing fruit rot of chilli. The pectinolytic enzymes of *Colletotrichum* species have been investigated by many workers in several crops, including avocado (*C. gloeosporioides*), (Wattad *et al.,* 1997) bean (*C. lindemuthianum*), (Wijesundera *et al*., 1984) and rubber (*C. acutatum*) (Hernandez-Silva *et al*., 2007).

All these cell wall splitting enzymes are mostly adaptive, secreted by the pathogen in the presence of appropriate substrates. Pectinolytic enzymes were produced only in the presence of pectin in the medium and cellulolytic enzymes were produced only in cellulose containing medium. The production and activity of pectinolytic and cellulolytic enzymes detected *invitro* suggest their active role in disease development by the pathogen in noni leaves.

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