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EFFECT OF BACTERIAL ANTAGONISTS ON THE *IN VITRO* PRODUCTION OF CELLULOLYTIC AND PECTINOLYTIC ENZYMES BY *COLLETOTRICHUM GLOEOSPORIOIDES*

R. Udhayakumar*, S. Usharani and A. Muthukumar

Department of Plant Pathology, Faculty of Agriculture, Annamalai University, Annamalainagar–608 002 (T.N), India.

Abstract

The effect of bacterial antagonists on the *in vitro* production of cellulolytic and pectinolytic enzymes by *C. gloeosporioides* were analyzed in this study. In the present study, all the treatment significantly reduced the production of cellulolytic and pectinolytic enzymes of *C. gloeosporioides* when compared to control. Among the bacterial antagonists, the combination of *P. fluorescens* and *B. subtilis* recorded the least hydrolytic enzyme production of pathogen by 86.23, 87.74, 80.09, 80.05 and 80.72 per cent of C_1, C_x, PG, PTE and PMG activity, respectively. The fungicide Carbendazim (0.1%) recorded maximum inhibition of C_1, C_x, PG, PTE and PMG activity (86.90, 88.39, 84.00, 83.94 and 84.44 per cent, respectively).

Key words : *Colletotrichum gloeosporioides,* bacterial antagonists, cellulolytic and pectinolytic enzymes.

Introduction

Mango (*Mangifera indica* L. Anacardiaceae) is one of the most popular fruit and grown throughout the tropical and sub-tropical regions of the world. Being highly perishable, mango fruits have to be marketed immediately after harvest. The anthracnose caused by *C. gloeosporioides* is one of the most common and serious diseases of mango. Anthracnose can render the tree completely unproductive (up to 100%), as it destroys the developing or developed fruits both in field and storage conditions. It causes leaf, blossom blight and tree die-back in the orchard and can subsequently give rise to rottened fruits during storage and thus poses several problems (Arauz, 2000; Ploetz and Freeman, 2009). The disease occurs at any stage of fruit growth.

Normally fungicides are the primary means of controlling plant diseases. But the fungicides are under special scrutiny for posing potential oncogenic risks (Eckert and Ogawa, 1985). The increased consumer preference for healthy agricultural products and environmental risks associated with chemical residues in food are the major driving forces for the search of new safer control methods. Over the past few decades, biological control has emerged as an effective strategy to combat the decay of fruits. Plant growth promoting rhizobacteria (PGPR) especially *Pseudomonas fluorescens* (Ardakani *et al*., 2010) and *Bacillus subtilis* are promising candidates as bioprotectants (Ramamoorthy *et al*., 2001; Mahadtanapuk *et al*., 2007). Though remarkable success has been achieved in this direction through the use of antagonistic microorganisms, the information generated on the performance of the introduced antagonists into the ecosystem under varying field conditions still remains inadequate constituting a major obstacle in the large scale adoption of this technology. With this background, the present study has been undertaken to analyze the effect of bacterial antagonists on the *in vitro* production of cellulolytic and pectinolytic enzymes by *C. gloeosporioides*.

Materials and Methods

Isolation of *C. gloeosporioides*

The pathogen causing anthracnose disease in mango was isolated from diseased leaf and fruit samples. The infected tissue bits were separated with a sterile blade

Author of correspondence : E-mail : udhayaplantpathology@yahoo.co.in

and surface sterilized with 1 per cent sodium hypochlorite solution for 1 min. and subsequently washed three times with sterile distilled water. Then they were transferred into a sterile Petri dish containing Potato Dextrose Agar (PDA) medium (Ainsworth, 1961) amended with streptomycin. The plates were then incubated at room temperature (28±2°C) for four days. The emerging colonies were sub cultured on to PDA slants. Single hyphal tip method was followed for making pure culture and maintained on PDA slants (Aneja, 2003).

Isolation of bacterial antagonists

Antagonistic bacteria were isolated from leaf surface, fruit skin and blossom of mango collected from major mango growing areas of Tamil Nadu using leaf washing technique (Gould *et al*., 1996). A small plant material was mixed with 5 ml of sterile distilled water in a flask which was shaken on a shaker for 30 min. Then 1 ml of suspension was added to a Petri plate containing nutrient agar medium and incubated at room temperature (28±2°C) for 48 h. The growing colony was subcultured on nutrient agar (NA) using single colony isolation. The slant was kept at 10°C in refrigerator and used as stock culture.

Effect of bacterial antagonists on hydrolytic enzyme production

Preparation of enzyme source

For pectinolytic enzyme production *C. gloeosporioides* was grown in Czapek's broth, supplemented with 0.3 per cent pectin as carbon source replacing sucrose. Similarly, for cellulolytic enzymes 3 per cent cellulose and carboxy methyl cellulose were used.To 50 ml sterile Czapek's liquid media in a 250 ml Erlenmeyer conical flask, the culture filtrate of *P. fluorescens* and *B. subtilis* and combination of both were amended to the media separately. Similarly, Carbendazim 50 WP was added to the broth at the conc. of 0.1%. The 9 mm disc was cut with the help of a cork borer from the growing tip of the 7 days old culture of *C. gloeosporioides* and inoculated in each flask and incubated in the BOD incubator at 28 ± 2°C for 7 days. The control and treated flasks were maintained with three replications. After incubation, the fungal mat and the liquid media were separated by double layered Whatman No.1 filter paper placed in Buchner funnel under suction by vacuum pump. The filtrates were further centrifuged in a high speed, cooling centrifuge at 5000 rpm for 10 min. and the supernatant was used as the enzyme source.

Cellulolytic enzymes

a. Cellulase (C_1)

The reaction mixture consisted of 1.0 ml of cellulose suspension (the conc. of which was adjusted approximately to 0.85 absorbance at 620 nm), 4.0 ml of 0.2 M sodium acetate-acetic acid buffer at pH 5.6 and 5 ml of culture filtrate. The absorbance of the mixture was determined immediately at 620 nm in the calorimeter and incubated at room temperature (28±2°C). At the end of 24 h. the absorbance was again measured and the enzyme activity was expressed as units (1 unit = 0.01 absorbance at 620 nm), calculated as to difference in absorbance (Norkrans, 1950).

b. Cellulose (C_x)

The activity of cellulose (endo glucanase) was estimated by loss in viscosity of the cellulosic substrate using Ostwald-Fenske viscometer (150 size) with minimum efflux time of 20 sec. for double distilled water. Carboxy methyl cellulose of 0.5 per cent conc. was prepared in sodium acetate-acetic acid buffer at pH 4.8 and pipetted out 4 ml of CMC, 1 ml of buffer and 2 ml of enzyme substrate and transferred into Ostwald-Fenske viscometer and kept in water bath at 30±1°C. The contents were mixed and the efflux time at fixed interval (2 h.) was determined (Hussain and Diamond, 1960). The per cent loss in viscosity was calculated through the following formula.

$$\text{Per cent loss in viscosity} = \frac{T_0 - T_1}{T_0 - T_w} \times 100$$

Where,

T_0 = Flow time at zero time (sec)

T_1 = Flow time at one interval (sec)

T_w = Flow time of double distilled water (sec).

Pectinolytic enzyme

a. Polygalacturonase (PG)

The PG enzyme activity was measured by the loss in viscosity of sodium polypectate in sodium acetate acetic acid buffer at pH 5.2. One ml of buffer and two ml of culture filtrate were added and immediately transferred to Ostwald-Fenske viscometer (150 size) placed in a water bath at 30±1°C. Viscosity losses were measured and per cent loss in viscosity was calculated as detailed earlier. Culture filtrates without test compound served as control (Mahadevan and Sridhar, 1986).

b. Pectin transeliminase (PTE)

The activity of PTE was determined by viscosity loss of 1 per cent citrus pectin. The reaction mixture consisted of 4 ml of 1 per cent citrus pectin in 0.2 ml of culture filtrate. The pH of the reaction mixture was adjusted to 8.6 and immediately transferred to Ostwald-Fenske

Table 1 : Effect of bacterial antagonists on the *in vitro* production of cellulolytic enzymes by *C. gloeosporioides*.

T. No.	Treatments	C_1 activity (unit)	Per cent decrease over control	C_x activity (% loss in viscosity)	Per cent decrease over control
1	*P. fluorescens*	0.64 [a]	85.55	9.00 [a]	87.61
2	*B. subtilis*	0.67 [a]	84.87	9.30 [a]	87.19
3	*P. fluorescens* + *B. subtilis*	0.61 [a]	86.23	8.90 [a]	87.74
4	Carbendazim (0.1%)	0.58 [a]	86.90	8.43 [a]	88.39
5	Control	4.43 [b]	-	72.65 [b]	-

Values not sharing a common superscript differ significantly at $P < 0.05$ (DMRT)

Table 2 : Effect of bacterial antagonists on the *in vitro* production of pectinolytic enzymes by *C. gloeosporioides.*

T.No	Treatments	PG activity (% loss in viscosity)	Per cent decrease over control	PTE activity (% loss in viscosity)	Per cent decrease over control	PMG activity (% loss in viscosity)	Per cent decrease over control
1	*P. fluorescens*	15.41 [a]	78.09	15.00 [a]	78.11	14.64 [a]	78.47
2	*B. subtilis*	17.78 [a]	74.72	16.33 [a]	76.17	15.87 [a]	76.66
3	*P. fluorescens* + *B. subtilis*	14.00 [a]	80.09	13.67 [a]	80.05	13.11 [a]	80.72
4	Carbendazim (0.1%)	11.25 [a]	84.00	11.00 [a]	83.94	10.58 [a]	84.44
5	Control	70.34 [b]	-	68.53 [b]	-	68.00 [b]	-

Values not sharing a common superscript differ significantly at $P < 0.05$ (DMRT).

viscometer (150 size) and per cent loss in viscosity was determined as detailed above.

c. Polygalacturonase-trans-eliminase (PGTE)

The activity of the enzyme PGTE was determined by the viscosity loss of sodium polypectate. To 4 ml of freshly prepared 1.2 per cent sodium poly pectate dissolved in boric acid borax buffer at pH 8.6, 1 ml of buffer (at pH 8.6) and 2 ml of culture filtrate were added and immediately transferred to Ostwald-Fenske viscometer (150 size) and per cent loss in viscosity was determined as detailed earlier.

Results and Discussion

The results in tables 1 and 2 revealed that all the treatment significantly reduced the production of cellulolytic and pectinolytic enzymes of *C. gloeosporioides* when compared to control. The fungicide Carbendazim (0.1%) recorded maximum inhibition of C_1, C_x, PG, PTE and PMG activity, (86.90, 88.39, 84.00, 83.94 and 84.44 per cent respectively). Among the bacterial antagonists, the combination of *P. fluorescens* and *B. subtilis* recorded the least hydrolytic enzyme production of pathogen by 86.23, 87.74, 80.09, 80.05 and 80.72 per cent of C_1, C_x, PG, PTE and PMG activity, respectively. Depression in enzyme activity of the pathogen may be due to the direct effect of the antagonists, or their metabolites, on the enzymatic system of *C. gloeosporioides*. The treatments might have also inhibited the activity of lytic enzymes of the pathogen by antibiotic action. The present result substantiates the observation made by Borowitz *et al*. (1992), who reported that *P. fluorescens* and *B. subtilis* treatment degraded the lytic fungal pathogens cellulase, pectinase and xylanase mainly by the action of different antibiotics. Velazhagan *et al*. (1999) found that the extracellular chitinase and β-1,3-glucanase of *Pseudomonas* culture inhibited the chitin and glucan present in the cell wall of *R. solani.* Similar report on the activity of chitinase and â-1,3-glucanase of *Pseudomonas* participating in the inhibition of lytic enzymes of different fungal pathogens was made by Frindlender *et al*. (1993) against soil bone fungal pathogens; Lim *et al*. (1991) against *F. solani*; M'Piga *et al*. (1997) in tomato by *F. oxysporum;* Ramamoorthy and Samiyappan (2001) in *C. capsici* in chilli; Mauch *et al*. (1988) in pea plant; Chen *et al*. (2000) against the lytic enzyme activity of *Pythium* sp.

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