JBiopest 7(Sup):33-37 (2014)

Mycolytic effect of extracellular enzymes of entomopathogenic fungi to *Colletotrichum falcatum*, red rot pathogen of sugarcane

Santosh Kumar Sanivada*and Muralimohan Challa

ABSTRACT

Twenty three strains of selected entomopathogenic fungi were tested for the production of chitinolytic enzymes and their involvement in the suppression of *Colletotrichum falcatum*, red rot pathogen of sugarcane. Among twenty three strains tested for chitinolytic activity, 9 strains showed a clearing zone on chitin-amended agar medium. Among these, entomopathogenic fungal strains ARSEF-6646, ARSEF-6647, ARSEF-6648, ARSEF-6650 and ARSEF-2417*Beauveria bassiana* strains produced clearing zones of a size larger than 10 mm. The antifungal activity of these strains increased when chitin was incorporated into the medium. When mycelial discs of the pathogen were treated with the secondary fungal mycelia, the results indicated that antagonistic *B. bassiana* caused a higher level of lysis of the pathogen mycelium, and the inhibitory effect was more pronounced when the lytic enzymes were produced using chitin as carbon source.

MS History: 19.09.2013 (Received)-16.03.2014 (Revised)-25.03.2014 (Accepted)

Key words: Entomopathogenic fungi, *Colletotrichum falcatum*, red rot disease, sugarcane.

INTRODUCTION

Sugarcane is an important crop in India because of its high commercial value. *Colletotrichum falcatum*, phytopathogenic fungus causes the red rot disease triggering severe loss of yield in many parts of sugar cane growing states in India (Alexander and Viswnathan, 1996). Fungicides have commonly been used for the management of this disease; however, resistance of pathogens to conventional fungicides is becoming a major problem and in turn fungicides affect environment and human health. Recently, the use of biological control has increased. Production of chitinase from microorganisms has been suggested for the control of red rot disease (Viswanathan and Samiyappan, 2001). Entomopathogenic fungi were well known to control agricultural pests. Suppression of several plant pathogens using entomopathogenic fungi was well documented. *In vitro* bioassay studies reported that mycelial growth inhibition of various plant disease causing fungi such as *Gaeumannomyles graminis* (Renwick *et al.,* 1991), *Fusarium oxysporum, Armillaria mellea, Rosellinia necatrix* (Reisenzein and Tiefenbrunner, 1997), *Fusarium oxysporum, Botrytis cinerea* (Bark *et al.,* 1996), *Pythium ultimum*, *Rhizoctonia solani* (Vesely and Kobava, 1994; Lee *et al.,* 1999), *Pythium* sp.(Clark *et al*., 2006; Ownley *et al.,* 2008) and *Rhizoctonia* (Ownley *et al.,* 2000; Ownley *et al.,* 2004) using entomopathogenic fungus *Beauveria bassiana*. Another entomopathogenic fungus *Lecanicillium* sp is also known to show inhibition of plant pathogens such as Powdery mildew (Verhaar *et al.,* 1996) and rust fungi (Whipps, 1993). Sahab (2012) studied the phytopathogenic efficiency of secondary metabolites of *Beauveria bassiana*. Recently, Sasan and Bidochka (2013) reported antagonism of *Metarhizium robertsii* against *Fusarium solani*. In the present work, *in vitro* investigation of extracellular enzymes were carried out from twenty three isolates of entomopathogenic fungi and their relation to inhibition of *C. falcatum*.

MATERIALS AND METHODS

Culture maintenance of fungi

Twenty three isolates of entomopathogenic fungi (Table 1) used in the present study were collected from US Department of Agriculture – Agricultural Research Service (USDA-ARS, Ithaca, NY, USA)). Pure cultures of *Beauveria* sp., *Metarhizium* sp. were maintained on Sabouraud Dextrose Agar (SDA) and *Nomuraea rileyi* on Sabouraud Maltose Agar (SMA) slants respectively at 4 C until further use. Virulent isolate of *C. falcatum* was obtained from plant pathology section of Regional Sugarcane Agricultural Research Station, Anakapalli, Andhra radesh, India and maintained on oatmeal agar slants.

Table 1. Source of Entomopathogenic fungi collection.

ARSEF No.	Entomopathogenic fungus	Host insect	Location
6646	*Beauveria bassiana*	*Spodoptera litura*	India
6647	*B. bassiana*	*S. litura*	India
6648	*B. bassiana*	*S. litura*	India
6650	*B. bassiana*	*S. litura*	India
2412	*Beauveria* sp	*Xylorycetes jamaicensis*	India
1886	*Beauveria* sp	*Chilo infuscatellus*	India
8250	*Beauveria* sp	*Basilepta fulvicornis*	India
2417	*B. bassiana*	*Emmalocera depressella*	India
2597	*B. bassiana*	*Hyblaea puer*	India
2660	*B. brongniartii*	Adult [Coleoptera]	India
1059	*Metarhizium anisopliae*	*Chlosyne lacinia saundersii*	Brazil
2596	*M. globosum*	*Pyrausta machaeralis*	India
703	*M. guizhouense*	*Bombyx mori*	PR China
8736	*M. anisopliae*	*Spodoptera* sp.	Malaysia
1727	*M. anisopliae*	*Nilaparvata lugens*	India
1728	*M. anisopliae*	*N. lugens*	India
1744	*M. anisopliae*	*N. lugens*	India
1745	*M. anisopliae*	*N. lugens*	India
539	*Nomuraea rileyi*	*S. exigua*	Thailand
6645	*N. rileyi*	*S. litura*	India
711	*N. rileyi*	*B. mori*	PR China
6239	*N. rileyi*	*Helicoverpa armigera*	PR China
9490	*N. rileyi*	larvae [Lepidoptera: Noctuidae]	Russian federation

Conidial suspensions of all the twenty three strains of entomopathogenic fungi at a concentration of 3.5×10^8condia/ml and a volume of 200 ml of minimal media (0.003% NaCl, 0.03% $MgSO_4$ and 0.015% K_2HPO_4)was taken in Erlenmeyer glass flasks. Suspensions of twenty three strains of entomopathogenic fungal conidia without addition of chitin constituted the control. one per cent colloidal chitin was added to minimal media. The flasks-both the control and those containing chitin were put on an orbital shaker at 25 C for 4 days.

Mycelial growth of *C. falcatum*

200 µl conidial suspensions of all the twenty three strains of entomopathogenic fungi at a concentration of 3.5×10^8condia/ml were prepared by diluting conidia obtained from the stored culture slants using sterile 0.01% tween 80 solution and inoculated in 200 ml of minimal media (0.003% NaCl, 0.03% $MgSO_4$ and 0.015% K_2HPO_4) was taken in Erlenmeyer flasks at 25 C for 6 days. Addition of chitin constituted as positive control. 100 µl conidial suspensions of twenty three cultures were incubated in their respective SDA and SMA agar media at 25 C for 12 days. A 8mm mycelia disc of entomopathogenic fungi obtained from the fully grown plates using sterile cork borer and placed at the centre of oatmeal agar Petri plate inoculated with the 100µl of *C. falcatum* at a concentration of 1×10^8 conidia/ml and incubated at 25 C and zone of inhibitions were recorded after three days. Each experiment was repeated three times. Mean zone of inhibition was calculated.

Extracellular enzyme studies

After the incubation period culture broths of twenty-three isolates of entomopathogenic fungi were evaluated for extracellular secretion patterns of the five enzymes *viz.,* protease, amylase, lipase, chitinase and caseinase. The use of solid media for the detection of a wide array of extracellular enzymes produced by fungi was carried out as per

the method described by Hankin and Anagnostakis (1975).

Disc preparation
A 100 µl of 1×10^6 spores/ml spores of four day old cultures of chitin embedded and control media were spread plated on SDA and SMA medium respectively and the plates were incubated for 3 days at 28 °C. At the end of 3 days, 5 mm mycelial disc with agar was retrieved with the help of cork borer and placed in the middle of fresh test substrate containing plates and incubated at 28 °C for 10 days. Enzyme activities were calculated as an index of the total diameter of the colony plus halo divided by the diameter of the colony (St. Leger *et al.,* 1997). Enzymatic index value of >1.0 indicates enzymatic activity.

***In vitro* extracellular enzyme production**
Protease : A 1% Gelatin extract in minimal media (0.003% NaCl, 0.03% $MgSO_4$ and 0.015% K_2HPO_4) in conjunction with 2% agar was used. The pH of the medium was adjusted to 7.0 just before autoclaving. The plates were inoculated with 5 mm agar disc with mycelia (as described under 'Disc preparation') and incubated at 28 °C for 10 d (5 replicates/isolate were maintained). At the end of the incubation period, the plates were flooded with 15% Mercuric chloride in 2 N HCl. A clear transparent zone of clearance could be seen around the colony while the rest of the plate appeared translucent white in color (Hankin and Anagnostakis, 1975).
Amylase: A 1% soluble starch in minimal media (0.003% NaCl, 0.03% $MgSO_4$ and 0.015% K_2HPO_4) in conjunction with 2% agar was used. The pH of the medium was adjusted to 7.0 just before autoclaving. The plates were inoculated with 5 mm agar disc with mycelia (as described under 'Disc preparation') and incubated at 28 °C for 10 d (5 replicates/isolate were maintained). At the end of the incubation period, the plates were flooded with Lugol's iodine solution and a yellow colored halo around the colony could be seen in an otherwise blue medium indicating amylolytic activity.
Caseinase : A 1% milk powder in minimal media (0.003% NaCl, 0.03% $MgSO_4$ and 0.015% K_2HPO_4) in conjunction with 2% agar was used. The pH of the medium was adjusted to 7.0 just before autoclaving. The plates were inoculated with 5 mm agar disc with mycelia (as described under 'Disc preparation') and incubated at 28 °C for 10 d (5 replicates/isolate were maintained). At the end of the incubation period, a clear transparent halo could be seen around the colony while the rest of the plate appeared opaque white in color.
Lipase
A 1% Tween 20 in minimal media (0.003% NaCl, 0.03% $MgSO_4$ and 0.015% K_2HPO_4) in conjunction with 2% agar was used. pH of the medium was adjusted to 7.0 just before autoclaving. The plates were inoculated with 5 mm agar disc with mycelia (as described under 'Disc preparation') and incubated at 28 °C for 10 d (5 replicates/isolate were maintained). On the tenth day, formation of lipolytic enzymes by a colony was seen as either a visible precipitate due to the formation of crystals of the calcium salt of the lauric acid liberated by the enzyme, or as a clearing of such a precipitate around a colony due to complete degradation of the salt of the fatty acid.

RESULTS AND DISCUSSION
The enzymatic index values of twenty three entomopathogenic isolates for the five extracellular enzymes showed varied quantitative differences among the isolates (Table 2). Enzymatic index value of 3.8 was recorded as the highest (ARSEF. 2417) isolate ARSEF.9490 was recorded lowest of 1.5 for the extracellular protease production. The lowest Amylase value recorded as 1.4 and the highest recorded as 4.3 for cultures of ARSEF.6645 and ARSEF.703 respectively. Production of the highest value of 3.9 was recorded for ARSEF6646, 6650 and lowest of 1.1 for ARSEF.2596. The extra cellular caseinase enzymatic index value recorded the highest of 3.6 for culture ARSEF.711 and 1.5 the lowest recorded for ARSEF6645. Among twenty three strains tested for chitinolytic activity, 9 strains showed a clearing zone on chitin-amended agar medium. Among these, entomopathogenic fungal strains ARSEF -6646, ARSEF- 6647, ARSEF-6648, ARSEF-6650 and ARSEF-2417 *B. bassiana* strains produced clearing zones of a size larger than 10 mm. The results showed a positive relation between production of chitinase and antimycolytic activity against pathogen *C. falcatum.* These studies revealed the potential of entomopathogenic fungi as biocontrol agents for controlling red rot disease in sugarcane.

Table 2. Zone of inhibition of entomopathogenic fungi against *C. falcatum* and enzymatic index

ARSEF Culture No.	Zone of Inhibition (mm)	Enzymatic Index				
		Protease	Amylase	Chitinase	Caseinase	Lipase
6646	10.5	2.2	2.6	3.9	2.3	2.1
6647	11	2.6	2.5	2.4	3.2	2.5
6648	10.2	1.8	2.3	3.8	2.4	1.9
6650	10.2	2.7	3.4	3.9	2.6	1.9
2412	6.2	2.7	2.2	2.3	2.6	1.7
1886	7.3	2.6	2.3	1.8	2.3	1.9
8250	6.2	2.1	2.5	1.6	2.1	1.9
2417	10.4	3.8	2.4	3.8	2.9	2.1
2597	5.2	1.8	1.5	1.4	1.7	1.1
2660	8.6	3.3	2.3	2.2	2.1	1.5
1059	8.2	2.4	1.6	1.3	1.9	1.4
2596	7.3	1.7	1.8	1.1	2.1	1.4
703	8.7	1.9	4.3	1.7	2.2	1.9
8736	7.8	2.3	2.2	2.1	2.3	2.6
1727	7.6	2.6	3.4	2.8	2.6	2.9
1728	5.7	3.4	2.8	2.3	2.8	2.5
1744	3.6	1.6	1.6	1.3	1.8	1.2
1745	7.2	2.6	2.3	2.6	2.9	1.3
539	5.2	2.2	3.6	2.9	2.8	2.8
6645	9.8	3.8	1.4	2.4	1.5	1.8
711	7.9	2.2	2.5	3.5	3.6	3.2
6239	7.9	2.4	2.9	1.6	2.8	2.4
9490	5.3	1.5	3.1	3.2	3.2	1.2

The results showed a positive relation between production of chitinase and antimycolytic activity against pathogen *C. falcatum.* Indeed previous studies have demonstrated the role of antifungal activity of chitinase produced from fluorescent pseudomonas against *C. falcatum* (Viswanathan and Samiyappan, 2001). Viswanathan *et al,* 2003 reported mycolytic effect of extracellular enzymes of antagonistic bacterial cultures to *C. falcatum*. Recently, Ghosh and Chakraborty (2012) reported control of *Colletotrichum* sp. using biocontrol agents like *Trichoderma* and *Beauveria bassiana*. However present studies revealed the potential of entomopathogenic fungi as biocontrol agents for controlling red rot disease in sugarcane.

ACKNOWLEDGMENT

We are thankful to R.A. Humber, Curator, ARSEF Collection, Itchaca, NY for providing the cultures of entomopathogenic fungi. The authors are also thankful to the Department of Plant Pathology, RARS, Anakapalli, Andhrapradesh, India for providing us with the virulent strain of *C. falcatum*.

REFERENCE

Alexander, K. C. and Viswanathan, R. 1996. Major diseases affecting sugarcane production in India and recent experiences in quarantine. In*: Sugarcane Germplasm Conservation and Exchange* (Croft, B. J., Piggin, C. M., Wallis, E. S., Hogarth, D. M. ed.) ACIAR Proceedings No. 67, Canberra, 46-48PP.

Bark, Y. G., Lee, D. G, Kim, Y. H. and Kang, S. C. 1996. Antibiotic properties of an entomopathogenic fungus, *Beauveria bassiana*, on *Fusarium oxysporum* and *Botrytis cinerea*. *Korean Journal Plant Pathololoogy*, **12**:245–250.

Clark, M. M., Gwinn, K. D. and Ownley, B. H. 2006. Biological control of *Pythiummy riotylum*. *Phytopathology*, **96**:S25.

JBiopest 7(Sup):33-37 (2014)

Ghosh, S.K and Chakrborty, N. 2012. In vitro biological control of *Colletotrichum gloeosporioides*, causal organism of anthracnose of sarpagandha (Roulvolfia serpentine). *Agriculture and Biology Journal of North America*, **3**(8): 306-310.

Hankin, L. and Anagnostakis, S.L. 1975. The use of solid media for detection of enzyme production by fungi. *Mycologia*, **67**: 597-607.

Lee, S. M., Yeo, W. H., Jee, H. J., Shin, S. C. and Moon, Y. S. 1999. Effect of entomopathogenic fungi on growth of cucumber and *Rhizoctonia solani*.*FRI Journal of Forest Science*, **62**:118–125.

Ownley, B. H., Bishop, D. G. and Pereira, R. M. 2000. Biocontrol of *Rhizoctonia* damping-off of tomato with *Beauveria bassiana*. *Phytopathology*, **90**:S58.

Ownley, B. H., Griffin, M. R., Klingeman, W. E, Gwinn, K. D., Moulton, J. K. and Pereira, R. M. 2008. *Beauveria bassiana*: endophytic colonization and plant disease control. *Journal of Invertebrate Pathology*, **3**:267–270.

Ownley, B. H., Pereira, R. M., Klingeman, W. E., Quigley N. B. and Leckie, B. M. 2004. *Beauveria bassiana*, a dual purpose biocontrol organism, with activity against insect pests and plant pathogens. In: Lartey RT, Caesar A (eds) Emerging concepts in plant health management. Research Signpost, Kerala. 256–269.

Renwick, A., Campbell, R. and Coe, S. 1991. Assessment of in vivo screening systems for potential biocontrol agents of *Gaeumannomyces graminis*. *Plant Patholology*, **40**:524–532.

Reisenzein, H. and Tiefenbrunner, W. 1997. Growth inhibiting effect of different isolates of the entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuill.to the plant parasitic fungi of the genera *Fusarium*, *Armillaria* and *Rosellinia*. *Pflanzenschutz Berichte*, **57**:15–24.

Sasan, R. K. and Bidochka, M. J. 2013. Antagonism of the endophytic insect pathogenic fungus *Metarhizium robertsii* against the bean plant pathogen *Fusarium solani* f. sp. phaseoli, *Canadian Journal of Plant .Pathology*, **35**(3): 288-293.

Sahab, A. F. 2012. Antimicrobial Efficacy of Secondary Metabolites of *Beauveria bassiana* against selected bacteria and phytopathogenic fungi. *Journal of Applied Sciences Research*, **8**(3): 1441-1444.

St. Leger, R.J., Joshi, L. and Roberts, D.W., 1997. Adaptation of proteases and carbohydrases of saprophytic, phytopathogenic and entomopathogenic fungi to the requirements of their ecological niches. *Microbiology*, **143**:1983-1992.

Vesely, D. and Koubova, D. 1994.In vitro effect of the entomopathogenic fungi *Beauveria bassiana* (Bals.-Criv.)Vuill. And *B. brongniartii* (Sacc.)Petch on phytopathogenic fungi.*Ochrana Rostlin*, **30**(2): 113-120.

Verhaar, M. A., Hijwegen, T. and Zadoks. J. C. 1996. Glasshouse experiments on biocontrol of cucumber powdery mildew (*Sphaerotheca fuliginea*) by the myco parasites *Verticillium lecanii*and *Sporothrix rugulosa*. *Biological Control*, **6**:353-360.

Viswanathan, R. and Samiyappan, R. 2001. Antifungal activity of chitinases produced by some fluorescent pseudomonads against *Colletotrichum falcatum* went causing red rot disease in sugarcane. *Microbiological Research*, **155**: 309-314.

Viswanathan, R., Ramesh Sundar and Merina Premkumari, S. 2003. Mycolytic effect of extracellular enzymes of antagonistic microbes to *Colletotrichum falcatum*, red rot pathogen of sugar cane.*World Journal of Microbiology and Biotechnology*, **19:** 953-959.

Whipps, J. M. 1993. A review of white rust (*Puccinia horiana* Henn.) disease on chrysanthemum and the potential for its biological control with *Verticillium lecanii* (Zimm.) Vie´- gas. *Annals of Applied Biology*, **122**:173–187.

Santosh Kumar Sanivada*and Muralimohan Challa**

*Department of Microbiology and Food Science and Technology, GITAM Institute of Science, GITAM University, Gandhi Nagar, Rushikonda, Visakhapatnam-530 045, Andhra Pradesh, India. Email: santoo.sanivada9@gmail.com; Phone: +08790607539.

**Department of Biotechnology , GITAM Institute of Technology, GITAM University, Gandhi nagar, Rushikonda, Visakhapatnam-530 045, Andhrapradesh, India.