RESEARCH PAPER Asian Journal of Bio Science, Vol. 4 Issue 1 : 29-32 (April to September, 2009)

Screening of *Trichoderma* isolates for chitinolytic activity using glycol chitin plate assay

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(Accepted : January, 2009)

Thirty three isolates of *Trichoderma* belonging to different species which were previously screened against *Sclerotium rolfsii*, *Fusarium oxysporum* and *Rhizactonia solani* were used for checking chitinolytic activity using agarose plate containing glycol chitin. Differences were observed among species and isolates of the same species with respect to chitinase production after induction with colloidal chitin and chitin degradation. Among all the isolates, *T. virens* IABT 1010, *T. koningii* IABT1016, *T. polysporum* IABT1018 were found to be more efficient chitinase producers. In all the isolates, chitinase production started within 24 hours of induction, but maximum production reached in 48 hours. Therefore, culture filtrates taken after 48 hours of induction can be used for routine screening of the *Trichoderma* isolates**.**

Key words : *Trichoderma*, Chitinolytic activity, Glycol chitin, Colloidal chitin

INTRODUCTION

Biological control of plant pathogens is an attractive way to decrease heavy dependence of modern agriculture on costly chemical fungicides, which not only cause environmental pollution but also lead to the development of resistant strains (Harjono and Widyastuti, 2001). Biological control of plant disease is defined as the involvement and the use of beneficial microorganisms, such as specialized fungi and bacteria, to attack and control plant pathogens and the diseases they cause (Lewis and Papavizas, 1991). Different biological control agents (BCAs) can be used for the control of diseases. These include bacteria, fungi and actinomycetes. The most important BCAs belong to the genus *Trichoderma*. *Trichoderma* controls plant pathogens through several mechanisms including mycoparasitism, antibiosis, production of hydrolytic enzymes such as chitinases and glucanases, inactivation of pathogen's enzyme etc (Chet *et al.*, 1997; Weindling, 1941; Harman *et al.*, 2004). These mechanisms can act synergistically to combat pathogen. However, which mechanism to use at a particular time or pathogen is left to *Trichoderma*. *Trichoderma* adopted best mechanism either alone or in combination through evolution.

There are about 41 species in the genus *Trichoderma*. The species and the isolates within the same species differ in their biocontrol potential (Goes *et al.*, 2002; Umamaheswari and Sankaralingam, 2005; Upendra, 2006). Therefore, isolation and characterization of *Trichoderma* from different geographical locations is likely to provide an array of diverse isolates within desired biocontrol potential against plant diseases. Further, in recent years with the advancement in molecular biology and plant biotechnology, genes encoding chitinases and glucanases from *Trichoderma* are being exploited to develop plant varieties resistant to diseases. In this regard identification of *Trichoderma* isolates which are efficient producer of chitinases are important to use them as a source of chitinase genes. Different methods such as substrate hydrolysis in the medium, substrate degradation and estimation of reducing sugars, glycol chitin plate assay (Luis and Ray, 2004) etc., can be used to detect the chitinolytic activity of any organism. Luis and Ray (2004) used glycol chitin plate assay for chitinase activity in plants. This assay is very efficient, simple and less time consuming. The assay is based on the affinity of calcofluor white M2R for chitin (Maeda and Ishida, 1967) using glycol chitin as a substrate for endochitinase (Pan *et al.*, 1991). Glycol chitin embedded in an agarose gel provides a homogeneous substrate for the reaction to take place (Zou *et al.*, 2002). Glycol chitin serves as substrate for chitinase (Koga and J., 1983) binds to fluorescent brightener 28 by affinity (Maeda and Ishida, 1967). After proper incubation and enzymatic activity, the brightener is bound only to undigested glycol chitin (Trudel and Asselin, 1989). The result is easily visualized as well defined dark area on a fluorescent background when

viewed under UV Trans illuminator. The gel based glycol chitin assay was used to analyze the chitinase activity in *Pantoea dispera* (Gohel *et al.*, 2004). The polyacrylamide gel electrophoresis was carried out using 15 per cent gel as described by Sambrook *et al.* (1989) and gel was run at 4^0 C . After SDS electrophoresis, the gel was incubated at 37^0C for four hours in sodium acetate buffer (0.2 M, pH 5.0) containing 1 per cent (V/V) Triton X-100 to remove SDS and the gel was washed with distilled water. This gel was then transferred to each chitin plate containing a different dye. Thin layer of acetate buffer (0.2 M, pH 5.0) was added to hasten the diffusion of chitinase from polyacrylamide gel to agar plate. These plates were incubated at 37^0 C under dark condition. The dark bands appear against the fluorescein background on chitin agar plate with calcofluor white M2R and fluorescein isothiocynate after 7 hours. These bands were observed under UV- transilluminator (Gohel *et al.*, 2005). In this study glycol chitin plate assay was used to check chitinolytic activity in 33 isolates of *Trichoderma* belonging to different species.

MATERIALS AND METHODS

Thirty three isolates of *Trichoderma* belonging to different species [*T. virens* (8), *T. harzianum* (6), *T. viride* (11), *T. koningii* (2), *T. pseudokoningii* (2), and *T. polysporum* (4)] which were previously isolated and screened against *Sclerotium rolfsii*, *Fusarium oxysporum* and *Rhizactonia solani* (Upendra, 2006) were used for checking chitinolytic activity.

Preparation of colloidal chitin:

Colloidal chitin was prepared by the method of Roberts and Selintrenikoff (1988) with certain modifications. 5 g of chitin powder (HiMedia Laboratories Pvt. Ltd., Mumbai) was added slowly into 60 ml of concentrated HCl (Sd. Fine Chemicals Ltd., Mumbai) and left for vigorous shaking overnight at 4^0C. The mixture was added to two liters of ice-cold 95 per cent ethanol with rapid stirring and kept overnight at room temperature (25^0C). The precipitate was collected by centrifugation at 5,000g for 20 minutes at 4^0C and then washed with sterile distilled water until the pH of the colloidal chitin turned neutral (pH 7.0). Later, colloidal chitin solution (5 per cent) was prepared and stored at 4^0C for further use.

Preparation of culture filtrate for plate assay:

Trichoderma spores were inoculated on 25 ml of 0.5 x Potato Dextrose Agar (PDA) and incubated at 28^0C for 3-4 days or until the full growth of mycelium. After incubation, mycelium was washed several times with distilled water. Clean mycelium was then transferred to Mandels and Reese (1965) broth containing 0.3 per cent colloidal chitin as sole carbon source and incubated at 28^0C under shaking condition at 100 rpm. One ml of culture supernatant was taken at the intervals of 24, 48, 72 and 96 hours, in clean micro centrifuge tube. This was centrifuged at 13,000 rpm for one minute at 4^0C and supernatant was transferred to micro centrifuge tube and stored at -20^0C for further use.

Preparation of the substrate and assay for chitinase activity:

The chitinase specific plate assay was done as described by Luis and Ray (2004) with some modification. One per cent (w/v) agarose solution was prepared in sodium phosphate (0.01 M pH 5.5) and heated to boiling point. One ml of 1 per cent glycol chitin solution was added to 100 ml of agarose solution. The resulting suspension was stirred to ensure homogeneous distribution of the substrate, and 30 ml aliquot was poured into polypropylene Petri dishes (90 cm diameter). The agarose was allowed to cool and solidify for 20-25 minutes. Small wells (3 mm diameter) were created using cork borer in agarose gels at 1.5 cm from each other to form a grid. About 30 µl of crude enzyme solution (culture filtrate prepared in the previous section) was loaded into each well. The plate was incubated at 7 hours at 37^0C. After incubation 50ml of 0.5M solution of Tris-Cl (pH 8.9) with 0.01 per cent calcofluor white M2R (Sigma) (Appendix IX) was added to the Petri dish to stop the reaction and stain the plate. The gel was rinsed twice with water followed by overnight color development in the dark.

RESULTS AND DISCUSSION

One of the ways to screen *Trichoderma* species for their biocontrol potential is to screen for their chitinolytic activity. Among the various tests, estimation of reducing sugar released and fluorescence assay has widely been used for screening *Trichoderma* for chitinolytic activity. Luis and Ray (2004) used the glycol chitin plate assay to detect the chitinase produced after induction with acibenzolar-S-methyl at the rate of 20 ppm in cucumber. In this study, this technique was used for thirty three isolates. The results of this experiment are presented in Table 1 and typical plate with hydrolysis zone is shown in Plate 1.

Differences were observed among species and isolates of the same species with respect to chitinase

Table 1: Chitinolytic activity of *Trichoderma* isolates belonging to different species

Sr. No.	Isolate no.	*Trichoderma* species	Duration of induction (hr) 24	48	72	96
1.	IABT1001	*T. virens*	+	++	+++	+++
2.	IABT1002	*T. virens*	++	+++	+++	++
3.	IABT1003	*T. reesei*	++	+++	+++	+++
4.	IABT1004	*T. polysporum*	+	+++	+++	+++
5.	IABT1005	*T. virens*	-	-	-	-
6.	IABT1006	*T. virens*	+	++	++	+
7.	IABT1007	*T. virens*	++	++	++	++
8.	IABT1008	*T. pseudokoningii*	+	+	+	+
9.	IABT1009	*T. polysporum*	+	+	+	++
10.	IABT1010	*T. virens*	+++	+++	++	++
11.	IABT1011	*T. polysporum*	-	++	++	+++
12.	IABT1012	*T. viride*	+	++	++	++
13.	IABT1013	*T. viride*	++	+++	+++	+++
14.	IABT1014	*T. viride*	++	+++	+++	+++
15.	IABT1015	*T. harzianum*	++	+++	+++	+++
16.	IABT1016	*T. koningii*	+++	+++	+++	+++
17.	IABT1017	*T. virens*	-	-	-	-
18.	IABT1018	*T. polysporum*	+++	+++	+++	+++
19.	IABT1019	*T. harzianum*	++	+++	+++	+++
20.	IABT1020	*T. viride*	+	+	+	+
21.	IABT1021	*T. viride*	-	+	+	+
22.	IABT1022	*T. viride*	++	+++	+++	+++
23.	IABT1023	*T. viride*	-	-	-	-
24.	IABT1024	*T. pseudokoningii*	++	+++	+++	+++
25.	IABT1025	*T. harzianum*	++	+++	+++	+++
26.	IABT1026	*T. viride*	+	++	++	++
27.	IABT1027	*T. viride*	++	+++	++	+
28.	IABT1028	*T. viride*	+	+	++	+++
29.	IABT1029	*T. viride*	++	++	++	++
30.	IABT1030	*T. koningii*	+	++	++	++
31.	IABT1031	*T. harzianum*	+	++	++	++
32.	IABT1032	*T. harzianum*	+	++	++	++
33.	IABT1033	*T. harzianum*	+	+	+	+

Legend
+++: Good zone of hydrolysis - : No hydrolysis
++ : Moderate zone of hydrolysis, +: Very small zone of hydrolysis

production after induction with colloidal chitin and chitin degradation. Among all the isolates, *T. virens* IABT 1010, *T. koningii* IABT1016, *T. polysporum* IABT1018 were found to be more efficient chitinase producers. Chitinase production was observed within 24 hours of induction with colloidal chitin containing Mandels and Reese (1965) medium. For screening, culture filtrate was taken 24, 48, 72 and 96 hours after induction with colloidal chitin. In all

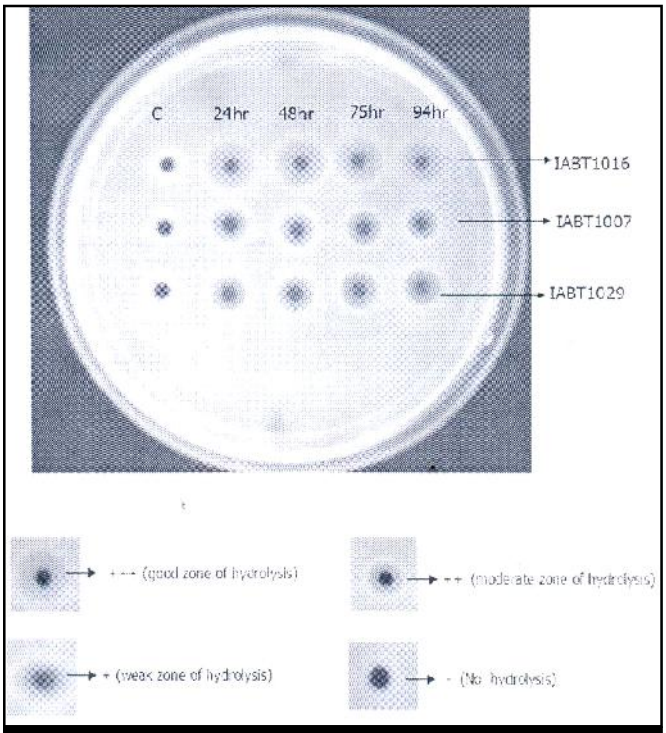

Plate 1 : Glycol chitin plate assay to asses the chitinase activity

the isolates chitinase production started within 24 hours of induction, but maximum production reached within 48 hours. Therefore, culture filtrate taken after 48 hours of induction can be used for routine screening of the *Trichoderma* isolates using glycol chitin plate assay. In most of the isolates, it remained stable up to 96 hours of induction. However, in few isolates (IABT1010, IABT1027, IABT1002, IABT1006) chitinase production decreased after 72 hours. Of the eight *T. virens* isolates, three gave good dark zone (IABT1001, IABT1002, IABT1010) and the other two (IABT1006, IABT1007) were found to be moderate chitin degraders. Of the four *T. polysporum* isolates tested, two isolates (IABT1004, IABT1018) formed good dark zone, one isolate (IABT1011) formed moderate dark zone and other isolate produced a minor zone of hydrolysis. Among the 11 *T. viride* isolates, three isolates (IABT1013, IABT1014, IABT1022) gave good dark zones, five isolates gave moderate zone of hydrolysis (IABT1012, IABT1026, IABT1027, IABT1028, IABT1029), two isolates (IABT1020, IABT1021) gave very small dark zone in glycol chitin plate and one isolate (IABT1023) failed to form hydrolysis zone on glycol chitin plate. Of the two *T. pseudokoningii*, isolates tested, one isolate (IABT1024) gave good dark zone and the other isolate (IABT1008) gave very small dark zone. *T. koningii* included two isolates; one isolate (IABT1016) showed good dark zone and the other isolate (IABT1030) showed moderate dark

zone. Of the six *T. harzianum* isolates tested three isolates (IABT1015, IABT1025, IABT1019) gave good dark zone, two isolates (IABT1031, IABT1032) showed moderate dark zone and one isolate (IABT1033) formed the very small hydrolysis zone. In isolates where chitinase production was not observed, growth of the isolates was very slow. In this study glycol chitin plate assay was compared with the dual culture assay. In general, isolates which produced good zone of hydrolysis on glycol chitin plate assay also inhibited growth of the pathogens completely in dual culture. However, in some of the isolates though complete growth inhibition of the pathogen was observed, in glycol chitin plate assay they were found to be moderate chitin degraders. This may be because, *Trichoderma* uses different mechanisms for the control of the plant pathogens In addition, three isolates (IABT1005, IABT1017 and IABT1023) did not show any hydrolysis zone on glycol chitin plate and they were also very slow in their growth.

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