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QUALITATIVE ENZYME ASSAY AND SCLEROTIA PARASITIZATION BY FUNGAL ANTAGONIST *TRICHODERMA*

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ABSTRACT
In our study morphologically and molecularly characterized thirty rhizospheric isolates of *Trichoderma* (*T. harzianum* and *T. virens*) were screened qualitatively to produce enzyme(s) and their ability to parasitize sclerotia of two phytopathogens *viz*. *Rhizoctonia solani* and *Sclerotium rolfsii*. Maximum sclerotial colonization (93.3%) of *R. solani* was recorded with isolate PB 28 which were failed to germinate again on PDA. Moreover, isolate PB 16 exhibited maximum sclerotial colonization *i.e.* 93.3 percent and 53.3 percent against *R. solani* and *S. rolfsii* respectively. Isolate PB 11 utilized maximum cellulose (69.4%) while chitin was utilized maximally by PB 2 (75.4%) as a carbon source. Our results revealed that isolate PB 16 is a potential bio-control isolate which can be used against major soil borne phytopathogens to destroy their sclerotia.

INTRODUCTION

The major soil borne sclerotial phytopathogens like *R. solani, S. rolfsii* and *Sclerotinia* spp. can survive for many years through sclerotia which play pivotal role in disease cycle and are highly resistant against fungicides or microbial attack (Metcalf *et al*. 2004, Abdulla *et al*., 2008). In contrary of chemical fungicides, use of biocontrol agents for the management of phytopathogens has no hazardous effect on the environment and it also promotes degree of disease suppression (Srivastava *et al*., 2015**)**. The idea of sclerotia parasitization or killing them using *Trichoderma* as a fungal antagonist may be a key point for successful biological control. Previous studies which were done by Blum and Kabana (2004), Liu *et al*. (2009) and Rawat and Tewari (2010) strengthen the above said where they reported that sclerotia of *Rhizoctonia solani* and *S. rolfsii* are colonized by *Trichoderma,* completely rotted and do not germinate if parasitized by *Trichoderma***.** Species of *Trichoderma* are best known for their ability to degrade cellulose and chitin (Vinale *et al*. 2009; Abd-El-Khair *et al*., 2010, Karlsson *et al*. 2010, Balakrishnan *et* al., 2012, Barakat *et al*., 2013). Biological control of soil borne phytopathogens has been the subject of extensive research in the last few decades (Ritesh Kumar et al., 2012**)** and lot of work has been done regarding extracellular enzymes of *Trichoderma***,** however, very few work have actually been done and not enough information is available so far on ability of different strains of *Trichoderma* to colonize and/or parasitize the sclerotia of soil borne phytopathogens. Keeping in view the above facts, present study was carried out to identify potential isolates of *Trichoderma* for parasitizing and killing the sclerotia of soil borne phytopathogens and their ability to produce cellulase and/or chitinase.

MATERIALS AND METHODS

Antagonistic microorganisms

Trichoderma strains were isolated from rhizospheric soils of different crops and locations of Uttarakhand (table 1) on Trichoderma Selective Medium (TSM) (Mukherjee, 1991) following serial dilution method (Krassilnikov, 1950).

Phytopathogens

The phytopathogens *viz*. *Sclerotium rolfsii* and *Rhizoctonia solani* used in the present study were isolated from infected tomato and rice fields of G.B. Pant University of Agriculture & Technology, Pantnagar (Uttarakhand) following routine procedure.

Sclerotia parasitization

Isolates were studied for their ability to colonize/parasitize the sclerotia of the pathogens *Rhizoctonia solani* and *Sclerotium rolfsii in-vitro* using a novel methodology (Deeksha Joshi, 2003, Saxena *et al*., 2014). Pre inoculated PDA plates after covered with the growth of *Trichoderma* (incubation at 26 ± 2^{o}C for approximately 72hrs) were removed from the incubator and 15 g of sterilized sandy soil (pH 6.8, 25% moisture) was then spread evenly over the surface growth of *Trichoderma* in the petri plates. Ten sclerotia of the test pathogen (*R. solani, S. rolfsii*) were then placed at equal distance from each other with three replicates for each *Trichoderma* isolate and again incubated at 26 ± 2^{o}C for 15 days. After two weeks, petri plates were visually checked

whether the sclerotia of *R. soalni* and *S. rolfsii* colonized by *Trichoderma* or not followed by sterio-binocular macroscopic examination of carefully removed sclerotia and observations were recorded on the number of sclerotia parasitized or decayed by *Trichoderma* isolates in each replication and per cent colonization was calculated. The colonized sclerotia were inoculated again on PDA to check their germination and observed whether they are parasitized or not.

Screening for enzyme production

All the cultures of *Trichoderma* were screened for production of cellulase and chitinase enzymes by plate assay on CMC-agar medium (Peciulyte, 2007, Ahmad *et al*., 2013) and chitin detection medium (Balakrishnan *et al*., 2012,) respectively and observed clear zone indicating the cellulase and chitinase production. The enzyme activity is measured by recording clear zone, the ratio of the colony diameter to clear zone diameter. The highest activity assumed by the largest clear zone ratio. Cellulase and chitinase activity of all isolates was further measured qualitatively by comparing their radial growth on modified TSM mineral medium (Cappuccino and Sherman, 1996) in which carbon source glucose was replaced by 1 per cent chitin or cellulose.

Statistical analysis

The experiments were conducted in controlled randomized design (CRD). The data obtained on sclerotial colonization, viability and percent utilization of solid media were pooled and made an average from all three replicates and subjected to one way ANOVA. The treatment means were compared using critical difference (CD) at p = 0.05.

RESULTS AND DISCUSSION

Sclerotia colonization and parasitization

Two weeks later, macroscopic observations of the plates exhibited that isolates of *Trichoderma* grew profusely, parasitized them and established close contact with the sclerotia of *R. solani* and *S. rolfsii* both the pathogens. Twenty one out of 30 isolates of *Trichoderma* colonized the sclerotia of at least one pathogen. Nineteen isolates colonized the

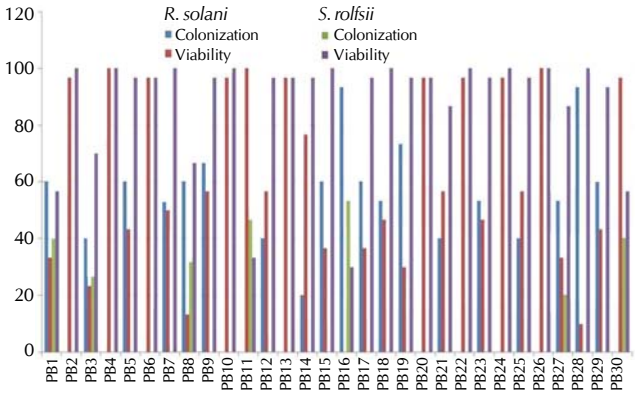

Figure 1: Comparative colonization and viability after colonization of Rhizoctonia soalani & Sclerotium rolfsii sclerotia

Table 1: Details of isolates obtained from rhizospheric soil samples from different locations of Uttarakhand.

Sl. No.	Sample Code	Crop	Location	Isolate Code	SoilpH
1	R1KG	Rice	Kathgodam-Haldwani	PB1	6.9
2	R3H	Rice	Halduchaur-Haldwani	PB2	6.8
3	R2LCb	Rice	Lamachaur-Haldwani	PB3	7.0
4	R1Da	Rice	Kherna-Almora	PB4	6.9
5	R1Db	Rice	Kherna-Almora	PB5	6.8
6	R2Da	Rice	Kherna-Almora	PB6	7.0
7	R2Db	Rice	Kherna-Almora	PB7	7.0
8	SPC1	Rice	SPC-Pantnagar	PB8	6.8
9	SPC2	Rice	SPC-Pantnagar	PB9	6.8
10	1a	Rice	Rudrapur-U.S. Nagar	PB10	7.2
11	1ab	Rice	Rudrapur-U.S. Nagar	PB11	7.2
12	1bc	Rice	Rudrapur-U.S. Nagar	PB12	7.2
13	3	Rice	Rudrapur-U.S. Nagar	PB13	7.1
14	5	Rice	Rudrapur-U.S. Nagar	PB14	7.1
15	AM	Apple	Mukteshwar-Almora	PB15	6.7
16	BM	Broccoli	Mukteshwar-Almora	PB16	6.8
17	PM1	Pea	Mukteshwar-Almora	PB17	6.8
18	PM2	Pea	Mukteshwar-Almora	PB18	6.8
19	SM	Strawberry	Mukteshwar-Almora	PB19	6.8
20	WM	Walnut	Mukteshwar-Almora	PB20	6.8
21	RP1	Rice	Premnagar-Dehradun	PB21	6.6
22	TR1	Mustard	Premnagar-Dehradun	PB22	6.7
23	A	Maize	Dhalwala-Rishikesh	PB23	6.8
24	B	Maize	Bhaniawala-Dehradun	PB24	7.0
25	B1	Rice	Bhaniawala-Dehradun	PB25	7.1
26	C1	Rice	Mazra-Ranipokhri	PB26	6.9
27	D	Maize	Geetanagar Rishikesh	PB27	6.8
28	D1	Rice	Raipur-Dehradun	PB28	6.8
29	F1	Rice	Raiwala-Hardwar	PB29	7.1
30	G1	Rice	Nagani, Tehri Garhwal	PB30	7.0

Table 2: Sclerotial colonization by different *Trichoderma* isolates

Isolate code	Colonization of sclerotia (%)		Mean
	R. solani	*S. rolfsii*	
PB1	60.0	40.0	40.0
PB2	0	0.0	0.0
PB3	40.0	26.7	31.7
PB4	0	0.0	0.0
PB5	60.0	0.0	0.0
PB6	0	0.0	0.0
PB7	53.3	0.0	0.0
PB8	60.0	33.3	28.3
PB9	66.7	0.0	0.0
PB10	0	0.0	0.0
PB11	0.0	46.7	51.7
PB12	40.0	0.0	0.0
PB13	0.0	0.0	0.0
PB14	20.0	0.0	0.0
PB15	60.0	0.0	0.0
PB16	93.3	53.3	48.3
PB17	60.0	0.0	0.0
PB18	53.3	0.0	0.0
PB19	73.3	0.0	0.0
PB20	0.0	0.0	0.0
PB21	40.0	0.0	0.0
PB22	0.0	0.0	0.0
PB23	53.3	0.0	0.0
PB24	0.0	0.0	0.0
PB25	40.0	0.0	0.0
PB26	0.0	0.0	0.0
PB27	53.3	20.0	20.0
PB28	93.3	0.0	0.0
PB29	60.0	0.0	0.0
PB30	0.0	40.0	40.0
Mean	36.0	8.7	8.7
CD (p = 0.05)	CD1 =1.70	CD 2=6.60	

sclerotia of only *R. solani* and seven isolates colonized the sclerotia of only *S. rolfsii* however five isolates (PB 1, 3, 8, 16 and 27) colonized the sclerotia of both pathogens. Nine isolates PB 2, 4, 6, 10, 13, 20, 22, 24 and PB 26 did not colonize the sclerotia of any pathogen (table 2). Sclerotia colonization ranged from 20–93.3 per cent against *Rhizoctonia solani*. Out of these 19 isolates, 14 isolates exhibited more than 50 % colonization and maximum colonization (93. %) was recorded with isolates PB 16 & 28 (plate 1. A, B & D) while minimum (20%) with PB 14 against *Rhizoctonia solani*. Against *Sclerotium rolfsii* sclerotia colonization recorded with seven (PB 1, 3, 8, 11, 16, 27 & 30) isolates and only one isolate, PB 16 (plate 1. C) resulted in more than 50% colonization and minimum (20 %) with isolate PB 27 among the sclerotia colonizing isolates. Out of these 21 sclerotia colonizing isolates, 18 belonged to *T. harzianum* and 3 were *T. virens*. Among the *T. harzianum* isolates, twelve isolates (PB 5, 7, 9, 12, 14, 15, 17, 18, 19, 21, 25 and 29) were selective for *R. solani* and two isolates (PB 11 and 30) for *S. rolfsii* while four isolates (PB 1, 3, 8 and 16) colonized the sclerotia of both pathogens. Among *T. virens* isolates, PB 23 & 28 showed selectivity for *R. solani* sclerotia while PB 27 colonized the sclerotia of both pathogens. The Isolate PB 28 was observed as a very efficient sclerotia colonizer of *R. solani* (93.3%) while isolate PB 16 was found most aggressive sclerotia colonizer against both the pathogens viz. *R. solani* (93.3%) *S. rolfsii* (53.3%). This was also recorded that parasitized sclerotia were failed to germinate again when transferred on PDA incubated at 26 ± 2°C revealed their non-viability. None of the parasitized sclerotia of *R. solani* were found viable and failed to germinate on PDA in isolate PB16. Besides isolate PB16 (0.0%) least viability of sclerotia was recoded with isolates PB28 (10.0%), PB8 (13.3%), PB3 (23.3%), PB19 (30.0%), PB1&27 (33.3%) which also showed 100 percent killing in some replications (table 3). In case of *S. rolfsii*, sclerotia parasitized by isolate PB16 (30.0%) & PB11 (33.3%) found least viable on PDA which were showed maximum killing followed by PB1&30 (56.7%). Sclerotia of both the pathogens *viz. R. solani* & *S. rolfsii* were failed to germinate on PDA showed least viability (15.0%) parasitized by PB16. Isolates PB4, 11 & 26 against sclerotia of *R. solani* whereas PB2, 4, 7, 10, 15, 18, 22, 24, 26 & 28 against *S. rolfsii* were found not effective and 100 percent germination of colonized sclerotia was recorded on PDA (Table 3 and Fig. 1).

An interesting finding of the present investigation is that isolates of *T. harzianum* colonized the sclerotia besides of *T. virens* which is general sclerotia colonizer. Moreover, in their ability to colonize sclerotia of major phytopathogens, the isolates showed considerable variability which differed significantly for parasitizing sclerotia of *R. solani* and *S. rolfsii*. These above results revealed that the presence of host selectivity at the isolate/ strain level in *Trichoderma* species. The colonized sclerotia of *Rhizoctonia solani* and *S. rolfsii,* completely rotted

Table 3: Viability of *R. solani* and *S. rolfsii* sclerotia parasitized by *Trichoderma* isolates

Treatments	Viability of Sclerotia (%)		Mean
	R. solani	*S. rolfsii*	
PB1	33.3	56.7	33.4
PB2	96.7	100.0	98.4
PB3	23.3	70.0	46.7
PB4	100.0	100.0	100.0
PB5	43.3	96.7	70.0
PB6	96.7	96.7	96.7
PB7	50.0	100.0	75.0
PB8	13.3	66.7	40.0
PB9	56.7	96.7	76.7
PB10	96.7	100.0	98.4
PB11	100.0	33.3	66.65
PB12	56.7	96.7	76.7
PB13	96.7	96.7	96.7
PB14	76.7	96.7	86.7
PB15	36.7	100.0	68.4
PB16	0.0	30.0	15.0
PB17	36.7	96.7	66.7
PB18	46.7	100.0	73.4
PB19	30.0	96.7	63.4
PB20	96.7	96.7	96.7
PB21	56.7	86.7	71.7
PB22	96.7	100.0	98.4
PB23	46.7	96.7	71.7
PB24	96.7	100.0	98.4
PB25	56.7	96.7	76.7
PB26	100.0	100.0	100.0
PB27	33.3	86.7	60.02
PB28	10.0	100.0	66.7
PB29	43.3	93.3	68.3
PB30	96.7	56.7	76.7
Mean	65.0	88.1	76.6
CD (p=0.05)	CD1= 8.25	CD2= 9.42	

Table 4: Substrate utilization by different isolates of *Trichoderma*.

Isolate code	Clear zone on media		Substrate utilization (%)		Mean
	CMC agar	Chitin	Cellulose	Chitin	
PB1	+	+	44.0	50.3	47.2
PB2	-	+	30.3	75.4	52.9
PB3	+	+	40.3	58.6	49.5
PB4	+	+	50.1	60.6	55.4
PB5	-	+	33.2	62.6	47.9
PB6	-	-	29.6	37.6	33.6
PB7	-	+	40.1	67.2	53.7
PB8	-	+	38.2	68.2	53.2
PB9	+	+	50.4	67.4	58.9
PB10	+	+	27.6	56.6	42.1
PB11	+	-	69.4	38.8	54.1
PB12	-	+	25.4	50.7	38.1
PB13	+	+	38.6	61.3	49.9
PB14	-	+	26.9	60.4	43.7
PB15	+	+	47.2	71.3	59.3
PB16	-	+	30.4	69.1	49.8
PB17	-	+	33.3	63.5	48.4
PB18	+	+	39.9	67.2	53.6
PB19	-	+	34.6	68.5	51.6
PB20	+	-	38.8	40.2	39.5
PB21	-	+	37.4	52.3	44.9
PB22	-	+	30.1	56.5	43.3
PB23	-	+	24.3	69.4	46.9
PB24	-	+	27.6	63.4	45.5
PB25	+	+	40.3	71.6	55.9
PB26	-	+	38.2	64.5	51.4
PB27	-	+	38.9	61.7	50.3
PB28	-	+	25.7	74.7	50.2
PB29	+	+	52.1	71.4	61.7
PB30	-	+	26.7	66.1	46.4
Mean			37.0	61.6	49.3
CD (p = 0.05)			CD1 = 0.05	CD 2 = 0.20	

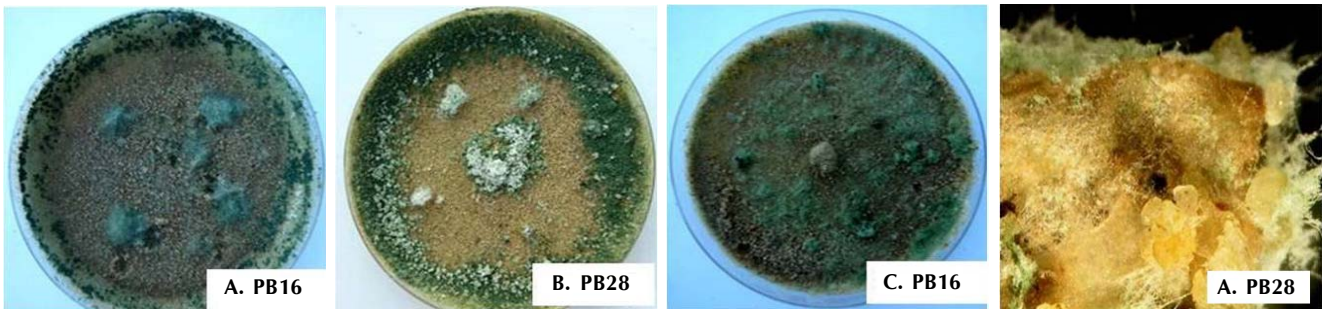

Plate 1: A. & B. Sclerotia of *Rhizoctonia solani* colonized by *Trichoderma* isolate PB 16 & 28, C. Sclerotia of *Sclerotium rolfsii* colonized by *Trichoderma* isolate PB 16, D. Stereobinocular microscopic view of colonized sclerotia of *R. solani* by *Trichoderma* isolates PB 28

and do not germinate if parasitized by *Trichoderma* (Blum and Kabana**,** 2004, Liu *et al.*, 2009 and Rawat and Tewari, 2010)**.** The present findings of sclerotial parasitization are also supported by Itamar and Jane 2000, Jones and Stewart (2000), Deeksha Joshi, 2003, Srinivasulu, 2005 and Saxena *et al.*, 2014.

Carbon source utilization

Assessment of enzymatic activities of different isolates estimated through consumption of cellulose or chitin and formation of clear zone showed differential ability for utilizing cellulose and chitin (table 4). Isolate PB11 (plate 2.B) utilized maximum cellulose (69.4%) with formation of clear zone followed by PB29 (54.4%) and PB4 (50.1%) whereas minimum cellulose was consumed by isolate PB23 (24.3%) with no clear zone formation. Among thirty isolates, only 11 isolates formed clear zone on CMC agar media and exhibited good cellulolytic activity on the basis of clear zone ratio (plate 2.B). Maximum amount of chitin (75.4%) was utilized by PB2 (plate 2.A) followed by PB28 (74.7%), PB25 (71.6%) and PB29 (71.4%) with clear zone formation. Out of 30 isolates 27 produced clear zone on chitin media but 3 isolates (PB6, 11 & 20) did not form clear zone. Isolate PB29 was most effective utilizer of

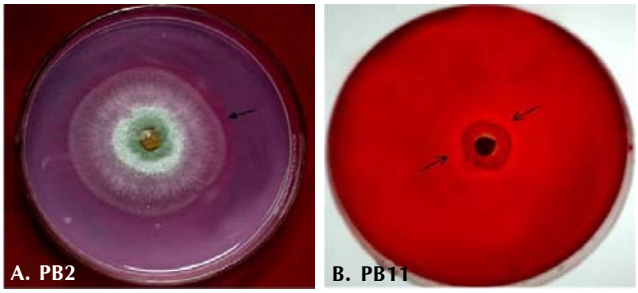

Plate 2: A. Clear zone formation by *Trichoderma* isolate PB 2 on chitin agar media, B. Clear zone formation by *Trichoderma* isolate PB 11 on CMC agar media

both cellulose and chitin. Only one isolate (PB6) failed to utilize both cellulose and chitin without clear zone formation. Isolate PB1 which was strongest cellulose utilizing isolate (69.4%) was second weakest isolate for chitin utilization and did not form clear zone on chitin media. The isolate PB2 was efficient chitin utilize but was weak for cellulose utilization. The above results revealed that 40 percent of total *Trichoderma* isolates produced clear zone on CMC-agar medium and 90 percent isolates on chitin containing medium. Regarding cellulose utilization PB3 & 25, PB8 & 26 were at par whereas isolates PB7 & 18 were at par for utilization chitin. The study revealed the fact that *Trichoderma* isolates recorded from different locations do differ in their cellulase and chitinase production activity. Tang *et al*. (2002) studied cellulose utilization by isolates of *Trichoderma* spp. and they observed *T. hamatum* utilized cellulose followed by *T. aureoviride* and *Gliocladium virens*. Above results are supported by Van Wyk and Mohulatsi (2003), Belal (2008), Devi and Kumar (2012), Florencio *et al*. (2012) and Singh *et al*. (2013) who has also been reported related findings regarding cellulose degradation using various fungi including species of *Trichoderma*. Our findings are accordance with Agrawal and Kotasthane (2012) and Pandey *et al*. (2014) who reported similar type of results with *Trichoderma* isolates screened for chitinolytic activity on chitin detection medium and Sudhakar and Nagarajan (2011) also documented the production of chitinase by solid state fermentation from *Serratia marcescens*. Kucuk and Kivanc (2003) reported the chitin as carbon source was also variably utilized by different isolates of *Trichoderma* spp.

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