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Effect of chitin amendment for efficacy enhancement of *Trichoderma harzianum*

AYON ROY AND PRATEEK MADHAB BHATTACHARYA
Department of Plant Pathology Uttar Banga KRISHI Viswavidyalaya Pundibari, Cooch Behar 736 165, West Bengal

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Chitinase production by *Trichoderma* spp. is one of the major attributes of biocontrol potential and modification of the growing environment is the logical alternatives to alleviate the antagonistic ability of the isolates. Two *T. harzianum* isolates (UBT 17 and UBT 18) were activated by growing in chitin amended Czapek dox medium to test for efficacy enhancement against *Macrophomina*. The antagonistic potential of UBT 17 was found to be increased with chitin amendment. The antibiotic compound and chitinase production potential was also found to be increased with 1% chitin amendment particularly in case of UBT 18, however, increasing concentration of chitin had rather reduced the efficiency. Competitive saprophytic ability of the test isolates was increased with chitin supplementation in growing medium and that was more prominent in case of UBT 18 that made it more efficient and promising under field condition.

Key words: *Trichoderma*, chitinase, biocontrol potencial, *M. phaseolina*

INTRODUCTION

Increase in public concern about the environment has expedited the need to develop and implement effective biocontrol agents for crop protection. Exploring the potential of biocontrol microbes like *Trichoderma, Gliocladium,* fluroscent pseudomonads, etc has got a tremendous momentum during last three decades because of their unique nature in easy to deliver, improvement of plant growth, activation of resistance mechanism in the host, increase in biomass production and yield. These antagonists act through antibiosis, secretion of volatile toxic metabolites, mycolytic enzymes, parasitism and through competition for space and nutrients. Chitinolytic enzymes of *Trichoderma* play an important role in mycoparasitim, which is considered as one of the mechanism in the antagonism against a number of fungal pathogens including *Rhizoctonia, Fusarium, Alternaria, Ustilago, Venturia, Pythium, Phytophthora* and *Botrytis* (Tong and Liangltao, 2002). Presently, enhancement of efficiency of biocontrol agents either through different approach like irradiation, environmental stress, temperature shock etc. is being addressed to make these biocontrol agents more effective. However, modification of the environment (nutritional or non-nutritional) seems to be a logical alternative to alleviate efficiency of the bio-agents. *In vitro* production of chitinase by *T. harzianum* has been enhanced with chitin incorporated into the medium as a sole carbon source, and enable the antagonist capable of hydrolysing dried/ fresh mycelium of the phytopathogenic fungus *Corticium rolfsii* (El-Katatny *et al.*, 2000). The effects of *T. harzianum* against *Rhizoctonia solani* causing root rot in pepper has been significantly enhanced when it is used as a suspension with 0.5% chitin for root drenching (Sid-Ahmed *et al.*, 2003). However, being a complex substrate the effect of increasing chitin content on the antagonistic potential of *Trichoderma* is still unknown. An attempt has been, therefore, made to evaluate the biocontrol ability of *Trichoderma* sp. against *Macrophomina phaseolina* isolated from stem rot infected jute plants through change in nutritional environment with chitin amendment.

MATERIALS AND METHODS

In vitro antagonistic potential of T. harzianum under chitin amended condition against M. phaseolina

The antagonistic potential two isolates of *T. harzianum* viz., UBT17 and UBT18 was evaluated *in vitro* against *M. phaseolina* using dual culture

plate method (Dennis and Webster, 1971) on the basis of relative growth rate of the antagonist and pathogen. Both antagonist and pathogen were inoculated at opposite ends in the sterilized Petriplates (90 mm diam) containing 20 ml sterilized Czapek Dox Agar (CDA) medium and CDA amended with 1% chitin and 2% chitin. Inoculation of the pathogen was done 48 hrs before the inoculation of the antagonist and such staggering of the period of inoculation was done to get the zone of contact at middle of the Petri plate. The control plates were maintained by inoculating the pathogen at one end of Petri plate containing CDA. The inoculated plates were incubated in the BOD incubator at 28±1°C. The antagonistic potential was determined by measuring the linear growth of *M. phaseolina* in control Petri plates comparing its growth in dual inoculation at 4th and 5th day of inoculation.

Variation in antifungal metabolite production by T. harzianum in chitin amended medium

Two *Trichoderma* isolates inoculated in Czapek Dox Broth (CDB), CDB with 1% chitin and CDB with 2% chitin media were incubated at 28±1°C for five days. The broth was collected for each media and was filled in 10 ml centrifuge tube for centrifugation. The supernatant was collected and 1 ml, 2 ml and 5 ml of the supernatant was dripped into hundred ml of Potato dextrose agar medium. Then from each of the amended media twenty ml of the poisoned PDA was poured in a sterilized Petri plates. To each Petri plate, mycelial disc of *M. phaseolina* of 6 mm diameter was inoculated and incubated for 72 hr at 28±1°C. The linear growth of the pathogen was measured and compared to that in control for determination of antifungal metabolite production ability.

Assay of chitinolytic activity

The experiment was conducted to quantify the production of chitinase by the *Trichoderma* isolates when these were grown in CDB, CDB with 1% and 2% chitin or co-inoculated with *M. phaseolina* in CDB, CDB with 1% and 2% chitin. The isolates were inoculated in 250 ml conical flasks containing 100 ml media mentioned above with a disc from 9 days old culture. All the flasks were incubated at 28±1°C for 7 days with a shaking for 5 min at 125 rpm, twice daily in a shaker incubator. After incubation culture filtrate was collected using Whatman filter paper no. 1. This culture filtrate was assayed for chitinase production by the method described by Kumar and Gupta (1999).

Competitive saprophytic ability of selected isolates of T. harzianum

The competitive saprophytic ability of the test *T. harzianum* isolates after growing them on either CDB or CDB with 1% chitin was tested for their efficacy to colonize the dead organic substrate. Bioinoculated soil with *Trichoderma* population density of 10^7 g^{-1} was prepared by addition of requisite quantity of talc based formulation of the bio agent. Hundred gram of augmented soil was subsequently mixed with 10 g of sand maize meal based formulation of *M. phaseolina* and poured in plastic cups after adjusting the moisture level near 50% of moisture holding capacity. Five numbers of sterilized wheat straw pieces (2-3 cm with at least one internode) were inserted into the soil and incubated for 7 days at 28±1°C. After 7 days the straw pieces were harvested, surface sterilized and placed on *Trichoderma* specific medium. The inoculated plates were incubated for 72 hrs at 28±1°C. The per cent area of straw pieces colonized by *Trichoderma* spp. was recorded.

RESULTS

The antagonistic potential of two isolates of *T. harzianum* viz., UBT17 and UBT18 exhibited that after 48 hrs of inoculation there was not much variation in radial growth of *M. phaseolina* in comparison to the growth observed after 72 hrs of inoculation (Fig.1). At 48 hrs of inoculation both the isolates showed moderate inhibition in mycelial growth of *M. phaseolina* over control (29.12-37.52% and 13.45-23.78%, respectively). During this time the test isolates exhibited better potential when they were grown on either CDA or CDA with 1% chitin in comparison to CDA with 2% chitin. After 72 hrs of inoculation, in case of UBT17 there was no significant difference in their potentiality irrespective of the media supplementation followed but for UBT18 that was much higher when the biocontrol agent was derived from either CDA or CDA with 1% chitin (32.00 and 28.82% inhibition over control, respectively) in comparison to CDA with 2% chitin. The rate of increase in potentiality for UBT18 was also comparatively higher when derived from chitin supplemented with 1% chitin. One of the most interesting features observed for both the isolates

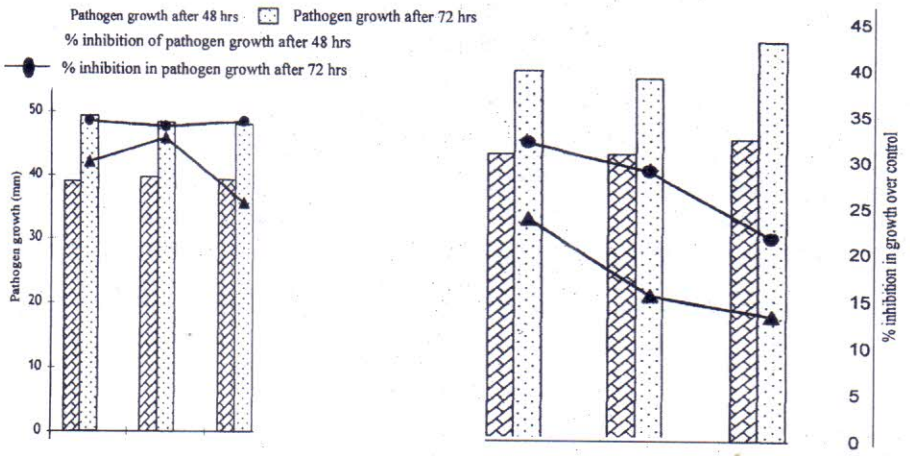

Fig.1: Antagonistic potential of *T. harzianum* isolates under activated condition / against *M. phaseolina*

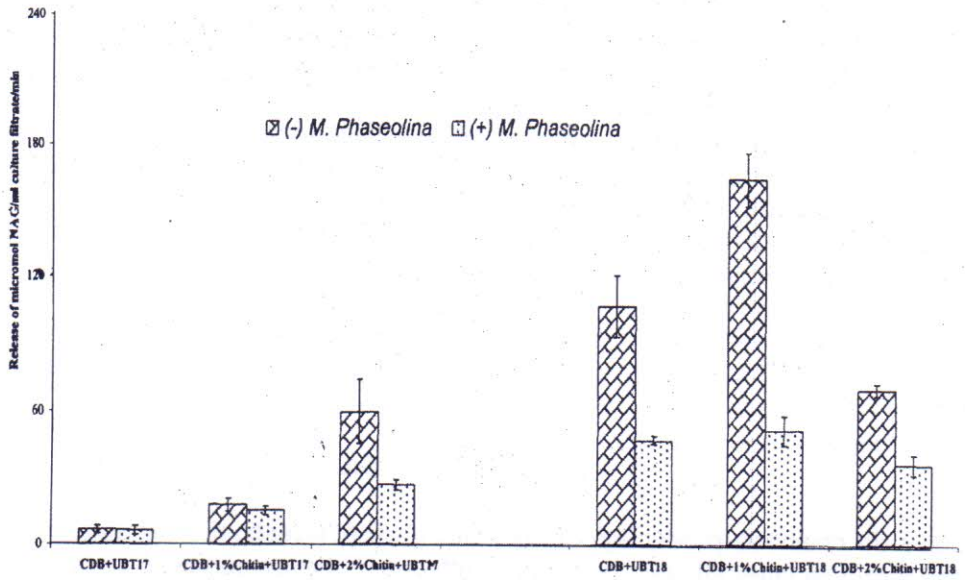

Fig. 2 : Variation in production of chitinase by *T. harzianum* isolates under activate condition

Table 1 : Production of antimicrobial compounds by *T. harzianum* isolates

Media	Per cent inhibition of *M. phaseolina* growth over control					
	Cone, of culture filtrate of UBT17			Cone, of culture filtrate of UBT18		
	1%	2%	5%	1%	2%	5%
CDB	11.81	20.56	31.46	14.03	21.60	33.54
	(19.68)*	(26.77)	(34.08)	(21.84)	(27.56)	(35.32)
CDB+ 1% chitin	18.82	22.92	39.10	18.40	29.31	39.10
	(25.36)	(28.48)	(38.70)	(25.36)	(32.76)	(38.70)
CDB+ 2% chitin	20.69	30.42	41.39	27.15	45.69	54.38
	(27.04)	(33.45)	(40.03)	(31.40)	(42.53)	(47.51)
CD (P=0.05)						
Media			3.38			1.96
Cone, of culture filtrate			3.38			1.96
Media x Cone, of culture filtrate			5.85			3.40

*Figures in parentheses are angular transformed values

Table 2 : Comperative saprophytic ability of activated *T. hazianum* with different level of population

Colony forming unit (cfu) per gof soil	Per cent colonization of straw piece by *T. harzianum* isolates			
	UBT17 grown on		UBT18 grown on	
	CDB	CDB+1% chitin	CDB	CDB+1% chitin
10^{11}	36.51(37.14)*	54.17(47.41)	65.48 (54.23)	85.00(71.41)
10^{12}	52.38 (46.37)	79.37 (67.48)	74.36 (59.97)	91.67 (80.00)
10^{13}	70.83(57.41)	84.72(71.35)	88.89(73 .93)	97.78 (85.01)
CD(P=0.05)				
Media		9.00	12.86	
Cfu/g of soil		11.02	15.75	

*Figures in parentheses are angular transformed values

was the production of some antimicrobial substances that can be identified by the change in colour of the interaction zone and that was more prominent in case of UBT18.

The antifungal metabolite production potential by UBT17 and UBT18 was found to be increased significantly with increasing concentration of culture filtrate of the test isolates irrespective of the composition of the medium used for their initial growth. The per cent inhibition in growth of the pathogen, on the other hand increased significantly with the addition of chitin in CDB irrespective of the concentrations of the culture filtrate used for the experiment (Table 1).

Quantitative assay on chitinase production ability of the test *Trichoderma* isolates showed that both of them produced less quantity of chitinase when they were co-inoculated with *M. phaseolina* (Fig. 2). In case of UBT17 chitinase production was enhanced accordingly with addition of chitin (6.56 µmol NAG/ml culture filtrate/min in CDB and 59.63 µmol NAG/ml culture filtrate/min in CDB with 2% chitin). In case of co-inoculation of the antagonist with *M. phaseolina* the enhancement was quite low. The picture was different in case of UBT18 where chitinase production was enhanced during addition of 1% chitin but decreased rapidly with addition of 2% chitin. The same trend was observed when UBT18 was co-inoculated with *M. phaseolina*. However, highest quantity of chitinase production was recorded when UBT18 was grown solely in CDB with 1% chitin (164.33 µmol NAG/ml culture filtrate/min).

Colonization percentage of wheat straw pieces by the antagonists was increased with increasing concentration of colony forming unit per g of soil as a general rule of colonization (Table 2). However, one of the most interesting features was that both the antagonist isolates when grown on CDB amended with chitin had significantly better potential to colonize wheat straw pieces. Comparison of the two isolates exhibited that UBT18 was more efficient than UBT17 in colonizing the straw pieces irrespective of the media and concentration of colony forming unit used for their growth and application to soil, respectively. Nearly 98% colonization was recorded in case of UBT18, whereas it was about 85% for UBT17 when the antagonist isolates were derived from CDB with 1% chitin and applied at the rate of $1x10^{13}$ per g of soil.

DISCUSSION

The present investigation revealed that two isolates differed in the antagonistic ability even under inducible condition and it was more important that the potentiality increased after amendment of chitin in the medium. The varying potential of different *Trichoderma* isolates against a particular pathogen has been reported earlier (Pan and Bhagat, 2007). Practically the strain specificity against a particular pathogen is one of the major deterrent factors for commercial utilization of this antagonist (Papavizas, 1985). Inductive conditions,as use of mutagens (Roy *et al,* 2005), amendments (Yang *et al,* 2002) or nutritional modification may enhance the biocontrol efficacy by virtue of increase in the production of antibiotics and/or lytic enzymes. The chitinolytic enzymes were produced when *T. harzianum* was grown on chitin containing medium and may be repressed when mycelium is provided with an easily metabolized carbon sources such as glucose (Ulhoa and Perberdy, 1993). The effects of *T. harzianum* against root pathogens significantly enhanced when used as a suspension with 0.5% chitin for root drenching (Sid-Ahmed *et al.,* 2003).

Enhanced chitinase activity in chitin amended media over non-amended one corroborated with the previous hypothesis that production of enzymes is an inducible character (Manczinger *et al*, 2001). Despite the presence of similar inducible conditions some isolates fail to produce enhanced level of lytic enzymes (Tronsmo and Harman, 1992), probably due to low biomass production of the antagonist under complex nutritional environment. Rhizosphere competence is a basic attribute of the antagonists to be effective for field application, which essentially depend on competitive saprophytic ability of the antagonist (Vannacci *et al.*, 1989). Isolates with different physiological ability of same species may differ in their efficiency to uptake and utilize nutrient from potentially growth limiting resources, may result in variation in competitive saprophytic ability among them (Smith, 1993).

The propagules of *Trichoderma* spp. produced through solid state fermentation technology are highly tolerant to adverse situation in comparison to the propagules or biomass derived from liquid fermentation, wettable powder formulations based on liquid fermentation though they are not desiccation tolerant. (Singh *et al.*, 2006). Sriram et al. (2010) observed that addition of chitin in the media resulted in enhanced initial population in the talc formulation of *T. harzianum*. Pavlyushin *et al.* (2005) immobilized *T. viride* cells on chitin, chitosan or chitin and chitosan carriers and found that these preparations were able to preserve the cells with viability for longer time in storage. The present investigation further strengthened the phenomenon that chitin amendment to a certain extent might result in improving the physiological and biochemical attributes of a *Trichoderma* isolate required for its better antagonistic potential.

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