J. Mycopathol. Res. **48(1)** : 57—63 (2010)

Organic based bioformulation of *Trichoderma* spp. suitable for organic farming

SOMESHWAR BHAGAT AND SITANSU PAN
Department of Plant Pathology, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur 741 252, Nadia, West Bengal

The experiment was conducted to screen out the best combination of cow dung (partially decomposed) and different augmenters viz., leaf mould, vermicompost and oilcakes at different concentrations for the growth and sporulation of antagonistic fungi, *T. harzianum*. When cow dung alone was used as growth substrate yielded low population (10.8 and 6.8 cfu/g of substrate) in sterilized and non-sterilized condition, respectively. The sterilized cow dung alone as well as with different augmenters appeared to be better substrates than the unsterilized one for mass multiplication of *T. harzianum*. Irrespective of the nature of oil cakes used as augmenter either at 10% or 100% concentration (sterilized or unsterilized) produced lower population of cfu and low conidia: chlamydospore ratio than at 20% or 40% concentrations. Similarly, either vermicompost or leaf mould (sterilized or unsterilized) when added with cow dung at 50% concentration appeared to produce higher cfu (114.6 x 10^8 or 85 x 10^8) than other concentrations used. Among the four oil cakes neem cake gave better results.with respect to conidia: chlamydospore ratio (at 40%) and cfu development (at 20%) followed by mustard cake, groundnut cake and least effect was observed with linseed cake. The highest population of *T. harzainum* in leaf moul and vermicompost amended substrate was recorded at 50% concentration than other concentrations used The findings appeared to be of significance with respect to organic based bioformulation of Trichoderma spp. with special reference to organic farming.

Key words: Bioformulation, organic substrates, oilcakes, vermicompost, leaf mould, *T. harzianum*

INTRODUCTION

Biological control of soil borne plant pathogens by antagonistic microorganisms is a potential, ecofriendly and sustainable approach for management of plant and a vital component of integrated disease management. Biocontrol is an essential part of organic farming and to achieve the biocontrol of soil borne plant pathogens in microbial community, different species of *Trichoderma* have been considered as potent antagonist in plant disease management (Papavizas, 1985). *Trichoderma* spp. are known to proliferate abundantly in various natural soils when the organism was added in intimate contact with a suitable organic food base (Lewis and Papavizas, 1984). The solid substrates promoted better growth and biocontrol mechanism of *Trichoderma* spp. (Singh, 2006) over the liquid substrates. For large scale production of antagonistic microorganism is mostly made by solid-state fermentation which is locally available organic materials like farm yard manure, different oil cakes, cereal brans, compost are recommended as suitable food base and delivery materials. Therefore, present investigation has been carried out to find out a best combination of organic substrates with enhanced production and development of cfu/g of formulated product along with highest conidia and chlamydospores of *T. harzianum* through solid state-fermentation under controlled condition.

MATERIALS AND METHODS

T. harzianum was isolated from the rhizosphere soil of potato by soil dilution technique (Dhingra and Sinclair, 1995) and plated on modified *Trichoderma* selective medium (Elad and Chet, 1983) and incubated at 28 ± 1°C for 7 days. The isolate was purified by repeated subculture and identified by following taxonomic keys and monograph of Rifai (1969). The pure culture of *T. harzianum* was maintained and preserved on PDA slants at 4°C for subsequent use.

Preparation of conidial and chlamydospores inocula

An aliquot of 100 ml potato dextrose broth medium into Erlenmeyer flasks (250 ml) was sterilized at 121°C for 15 min., inoculated with young growing mycelial (4 days old) plug (6 mm dia) of *T. harzianum* and incubated at 28 ± 1°C for 10 days. The conidia along with mycelial mat of *T. harzianum* harvested, separated the conidia from the mycelial mat by moderate shaking with wrist action shaker and centrifuged at 6000 rpm for 15 min. The pellet formed in the bottom of centrifuged tube was suspended into sterilized distilled water to make desired concentration and this inoculum was immediately used.

Preparation of different combinations of cow dung and various organic wastes

Different oil cakes viz., mustard cake, groundnut cake, linseed cake and neem cakes were mixed with main bulking material, cow dung (sterilized and non-sterilized) to make the variable concentrations (10, 20, 40 and 100%) of oil cakes and vermicompost / leaf mould (25, 50, 75 and 100%). This mixture was added with distilled water to maintain 40% MHC. filled into polypropylenge bag (200 g) and sterilized at 121°C for 30 min for two consecutive days. The sterilized substrate was inoculated with spore suspension of *T. harzianum* (1 x 10^8 conidia/ml) by injecting into the substrate in polypropylene bags through a disposable syringe and incubated at 28 ± 1°C for 21 days with periodical shaking.

Counting of conidia, chlamydospores and total population (c.f.u.) of T. harzianum

For counting total population of antagonist, 10 g substrate (cow dung + oilcakes/leaf mould/ vermicompost) was randomly taken from polypropylene bag after 21 days of incubation and mixed thoroughly with 100 ml of distilled water. This suspension was diluted serially, plated on modified TSM medium and incubated at 28 ± 1°C for one week. The number of colony forming unit (cfu/g of substrate) was counted by simple eye observation. The conidia and chlamydoses present in the substrates were counted by similar method as stated above, but the number of conidia and chlamydospores were counted directly from the suspension under Haemocytometer along with compound microscope. Counting of total population (c.f.u.)y conidia and chlamydospores from the sterilized and non-sterilized substrates on which *T. harzianum* was multiplied, was counted separately and the conidia: chlamydospores ratio with reference to increasing concentration of oil cakes/ leaf mould / vermicompost was calculated. The experiments were laid out in completely randomized block design (CRD) and the treatments were replicated four times.

RESULTS AND DISCUSSION

The results presented in Table 1 revealed that the total population of *T. harzianum* increased with increasing concentration of oilcakes up to 20% (neem cake and linseed cake) and 40 % (mustard cake and groundnut cake) concentration and thereafter declined, whereas the population was increased with increasing concentration of leaf mould and vermicompost up to 50.0% and thereafter starts declining, in both sterilized and unsterilized condition. Irrespective of substrate combinations, the sterilized substrates supported better growth of the *T. harzianum* than unsterilized substrates and lowest population (mycelial fragments + conidia + chlamydospores) was low at both 10%/25 % and 100 % concentration of different augmenters. The mustard cake + cow dung was found most efficient in growth and development of *T. harzianum* in both sterilized and unsterilized condition, producing 154.2 and 32.8 cfu/g of substrate followed by neem cake (121.2 and 25.0 cfu/g substrate), groundnut cake (115.5; and 29.5 cfu/g substrate and linseed cake

Table 1 Total population of *T. harzianum* on cow dung augmented with oilcakes after 21 days

Substrates	Population of *T. harzianum** (x 10^8c.f.u./g of substrate)									
	Sterilized					Unsterilized				
	10%	20%	40%	100%	Control	10%	20%	40%	100%	Control
Cow dung+Neem cake	82.2	121.2	116.0	16.8	10.8	11.8	25.0	16.4	1-.0	6.8
	(1.914)	(2.083)	(2.064)	(1.225)	(1.170)	(1.071)	(1.397)	(1.214)	(1.000)	(0.832)
Cow dung+Mustard	42.4	98.2	154.2	53.8	10.8	18.2	21.0	32.8	8.4	6.8
cake	(1.627)	(1.992)	(2.188)	(1.709)	(1.170)	(1.260)	(1.322)	(1.515)	(0.924)	(0.832)
Cow dung+Groundnut	51.2	59.8	115.5	34.4	10.8	15.3	17.2	29.5	8.8	6.8
cake	(1.709)	(1.776)	(2.062)	(1.536)	(1.170)	(1.184)	(1.235)	91.469)	(0.944)	(0.832)
Cow dung+Linseed	20.8	46.2	34.8	15.4	10.8	6.4	12.8	8.6	6.6	6.8
cake	(1.318)	(1.664)	(1.541)	(1.187)	(1.170)	(0.806)	91.107)	(0.934)	(0.819)	(0.832)
	25%	50%	75%	100%	Control	25%	50%	75%	100%	Control
Cow dung +	14.8	114.6	78.4	58.4	10.8	12.0	20.5	15.8	6.8	6.8
Vermicompost	(1.170)	(2.059)	(1.584)	(1.766)	(1.170)	(1.079)	(1.311)	(1.198)	(0.832)	(0.832)
Cow dung+Leaf mould	16.2	85.2	70.4	38.4	10.8	14.6	9.9	12.9	5.6	6.8
	(1.209)	(1.930)	(1.017)	(1.453)	(1.170)	(1.164)	(0.995)	(1.1100	(0.748)	(0.832)
SEm(±m)	4.52	5.83	6.31	5.12	–	3.57	3.89	4.56	2.34	–
CD (0.05)	10.5	10.69	11.32	9.35	–	7.43	6.21	8.50	5.21	–

*Means of five replications

Table 2 Effect of variable concentrations of neem cake on sporulation of *T. harzianum**

Substrates	Neem cake (%)	Number of spores (x 10^8 c.f.u./g of substrate)					
		Sterilized			Unsterilized		
		Conidia	Chlamydospores	Conidia:Chlamydospores ratio	Conidia	Chlamydospores	Conidia:Chlamydospores ratio
Cow dung +	10	4.5	0.21	1 : 0466	2.5	0.15	1 : 0.06
Neem cake		(0.653)	(–0.677)		(0.397)	(–0.823)	
Cow dung +	20	15.7	0.56	1 : 0.0523	5.0	0.21	1 : 0.042
Neem cake		(1.195)	(–0.251)		(1.301)	(–0.677)	
Cow dung +	40	6.7	1.10	1 : 0.1639	4.2	0.49	1 : 0.1166
Neem cake		(0.826)	(0.414)		(0.623)	(–0.309)	
Cow dung +	100	3.0	1.44	1 : 480	3.0	0.82	1 : 0.2386
Neem cake		(0.522)	(0.158)		(1.522)	(–0.086)	
Cow dung	0.0	3.5	0.16	1 : 0457	1.8	0.11	1 : 0611
only		(0.544)	(–0.795)		(0.255)	(–0.958)	
SEm(±)	–	1.24	0.22	–	0.65	0.15	
CD (0.05)	–	5.475	0.554	–	2.249	0.523	–

*Means of five replications

(46.2; and 12.8 cfu/g substrate) in both sterilized and unsterilized condition, respectively. The maximum population of *T. harzianum* was recorded at 50.0% concentration in both vermicompost (114.6 cfu/g) and leaf mould (85.2 cfu/g) under sterilized condition.

The results on effect of variable concentrations of neem cake in neem cake + cow dung mixture is presented in Table 2, which indicated that with increase in concentration of neem cake in the substrate, the number of conidia increased up to 20% (15.7 conidia/ml) and thereafter declined to 3.0

Table 3 : Effect of variable concentrations of neem cake on sporulation of *T. harzianum**

Substrates	Groundnut cake (%)	Number of spores (x 10^8 c.f.u./g of substrate)					
		Sterilized			Unsterilized		
		Conidia	Chlamydospores	Conidia:Chlamydospores ratio	Conidia	Chlamydospores	Conidia:Chlamydospores ratio
Cow dung + Groundnut cake Cow	10	6.3 (0.799)	0.21 (–0.677)	1 : 0333	4.1 (0.612)	0.11 (–0.958)	1 : 0.0268
Cow dung + Groundnut cake	20	16.5 (1.217)	0.28 (–0.552)	1 : 0.0169	8.1 (0.908)	0.11 (–0.958)	1 : 0.0135
Cow dung + Groundnut cake	40	10.3 (1.012)	0.33 (–0.481)	1 : 0.0320	5.5 (0.740)	0.11 (–0.958)	1 : 0.02
Cow dung + Groundnut cake	100	5.5 (0.740)	0.35 (–0.455)	1 : 0636	1.7 (0.230)	0.11 (–0.958)	1 : 0.0647
Cow dung only	0.0	3.5 (0.544)	0.16 (–0.795)	1 : 0457	1.8 (0.255)	0.11 (–0.958)	1 : 0611
SEm(±)	–	0.81	0.14	–	0.69	0.21	–
CD (0.05)	–	2.51	0.394	–	1.89	0.264	–

*Means of five replications

Table 4 : Effect of variable concentrations of linseed cake on sporulation of *T. harzianum**

Substrates	Linseed Cake (%)	Number of spores (x 10^8 c.f.u./g of substrate)					
		Sterilized			Unsterilized		
		Conidia	Chlamydospores	Conidia : Chlamydospores ratio	Conidia	Chlamydospores	Conidia : Chlamydospores ratio
Cow dung + Linseed cake	10	3.9 (0.591)	0.18 (-0.744)	1 :0461	2.6 (0.414)	0.11 (-0.958)	1 :0.0423
Cow dung + Linseed cake	20	9.2 (0.963)	0.30 (-0.5222)	1 : 0.0326	7.2 (0.857)	0.11 (-0.958)	1 : 0.0347
Cow dung + Linseed cake	40	5.1 (0.707)	0.35 (-0.455)	1 : 0.0686	4.4 (0.643)	0.11 (-0.958)	1 : 0.681
Cow dung + Lincseed cake	100	2.5 (0.397)	0.45 (-0.346)	1 : 0.1800	1.9 (0.278)	0.11 (-0.958)	1 : 0.200
Cow dung only	0.0	3.5 (0.544)	0.16 (-0.795)	1 : 0457	1.8 (0.255)	0.11 (-0.958)	1 : 0611
S Em (±)	-	0.81	0.29	-	1.5	0.31	
CD (0.05)	-	3.51	0.894	-	4.25	1.26	

*Means of five replications

conidia/ml at 100 %, under sterilized condition. Similar trend was also observed in unsterlized condition with comparatively low conidia and chlamydospores population. The conidia: chlamydospore ratio decreased with increasing concentration of neem cake in the substrates under both sterilized and unsterilized condition which indicated that the number of chlamydospore was increased continuously up to 100 % concentration. The highest conidia (16.5) and chlamydospore (0.48) was recorded at 20 % and 100 % concentration of groundnut cake substrate, under sterilized condition (Table 3). Irrespective of sterilized or unsterilized substrates, the number of conidia was increased with increasing concentration of groundnut cake up to 20 %, whereas the number of chlamydospores was continuously increased. Linseed cake at 20% of substrate produced 9.2; 7.2 conidia and 0.30; 0.25 chlamydospores / ml) and thereafter declined in both sterilized and unsterilized condition (Table 4). Similarly, the conidial number of *T. harzianum* was recorded highest at 20% concentration of mustard cake (19.9; conidia/ml) and starts decline to 1.5 conidia/ml at 100%, whereas reverse trend was noticed in the chlamydospore population, where it increased with increasing concentration (Table 5). Among the oilcakes, mustard cake produced highest number of conidia (19.9/ml) whereas highest number of chlamydospores was produced by neem cake (1.44/ ml) augmenter as compare to others.

The results presented in Table 6 and 7 revealed that irrespective of sterilized and unsterilized substrate the conidial population of *T. harzianum* increased with increasing concentration of the vermicompost and leaf mould up to 50 % and beyond this it declined to a minimum of 4 conidia in both cases. The conidia chlamydospores ratio increased with increasing concentration of both leaf moulds and vermicompost in the substrates. The highest number of conidia (11.5 and 10.9/ml) of *T. harzianum* was recorded with cow dung i vermicompost and cow dung + leaf mould, respectively

The direct introduction of antagonistic microorganisms into soil were strikingly effective against certain soil borne plant pathogens in sterilized soil but not in natural soil due to soil fungistasis. Studies on growth and sporulation of *Trichoderma* and *Gliocladium* spp. *in vitro* can be useful since these information helps to plan and execute the suitable technology for large scale production for field application. Most of the commercial formulations of *Trichoderma* contained mainly conidia and chlamydospores and rarely

Table 5 : Effect of variable concentrations of mustard cake on sporulation of *T. harzianum**

Substrates	Mustard Cake (%)	Number of spores (x 10^8 c.f.u./g of substrate)					
		Sterilized			Unsterilized		
		Conidia	Chlamydospores	Conidia : Chlamydospores ratio	Conidia	Chlamydospores	Conidia : Chlamydospores ratio
Cow dung + Mustard cake	10	5.3 (0.724)	0.21 (-0.667)	1 :0396	4.0 (0.602)	0.23 (-0.638)	1 :0.0575
Cow dung + Mustard cake	20	19.9 (1.298)	0.54 (-0.267)	1 : 0.0545	5.1 (0.707)	0.33 (-0.481)	1 : 0.0402
Cow dung + Mustard cake	40	7.8 (0.892)	0.72 (-0.143)	1 : 0.0923	6.4 (0.806)	0.41 (-0.387)	1 : 0.0640
Cow dung + Mustard cake	100	1.5 (0.176)	0.92 (-0.136)	1 : 0.613	1.0 (0.0)	0.63 (-0.200)	1 : 0.630
Cow dung only	0.0	3.5 (0.544)	0.16 (-0.795)	1 : 0457	1.8 (0.255)	0.11 (0.958)	1 : 0611
S Em (±)	-	0.63	0.44	-	1.5	0.31	
CD (0.05)	-	3.29	3.89	-	4.25	1.26	

*Means of five replications

Table 6. Effect of variable concentrations of vermi compost on sporulation of *T. harzianum**

Substrates	Vermi compost (%)	Number of spores (x 10^8 c.f.u./g of substrate)					
		Sterilized			Unsterilized		
		Conidia	Chlamydospores	Conidia : Chlamydospores ratio	Conidia	Chlamydospores	Conidia : Chlamydospores ratio
Cow dung + Vermi compost	25	4.2 (0.623)	0.19 (0.721)	1 :0396	3.9 (0.591)	0.23 (-0.638)	1 :0.0575
Cow dung + vermi compost	50	11.5 (1.060)	0.53 (-0.275)	1 : 0.0545	6.4 (0.806)	0.33 (-0.481)	1 : 0.0402
Cow dung +	75	5.9 (0.770)	0.68 (-0.167)	1 : 0.0923	5.1 (0.707)	0.41 (-0.387)	1 : 0.0640
Cow dung + vermi compost	100	5.5 (0.740)	0.90 (-0.0.045)	1 : 0.613	1.0 (0.0)	0.63 (-0.200)	1 : 0.630
Cow dung	0.0	3.5 (0.544)	0.16 (-0.795)	1 : 0457	1.8 (0.255)	0.11 (-0.958)	1 : 0611
S Em (±)	-	0.63	0.44	-	1.5	0.31	
CD (0.05)	-	3.29	3.89	-	4.25	1.26	

*Means of five replications

Table 7 : Effect of variable concentrations of leaf mould on sporulation of *T. harzianum**

Substrates	Leaf mould (%)	Number of spores (x 10^8 c.f.u./g of substrate)					
		Sterilized			Unsterilized		
		Conidia	Chlamydospores	Conidia : Chlamydospores ratio	Conidia	Chlamydospores	Conidia : Chlamydospores ratio
Cow dung + Leaf mould	25	4.5 (0.653)	0.22 (-0.657)	1 : 0422	2.2 (0.342)	0.14 (-0.853)	1 :0.0636
Cow dung + Leaf mould	50	10.9 (1.037)	0.49 (-0.309)	1 : 0.0552	6.9 (0.838)	0.39 (-0.408)	1 : 0.0402
Cow dung + Leaf mould	75	6.1 (0.785)	0.65 (-0.187)	1 : 0.0131	3.8 (0.579)	0.46 (-0.337)	1 : 0.0640
Cow dung + Leaf mould	100	5.7 (0.755)	0.86 (-0.0655)	1 : 0.22	2.7 (0.431)	0.59 (-0.229)	1 : 0.630
Cow dung only	0.0	3.5 (0.544)	0.16 (-0.795)	1 : 0457	1.8 (0.255)	0.11 (-0.958)	1 : 0611
S Em (±)	-	0.91	0.12	-	0.87	0.29	
CD (0.05)	-	2.88	1.23	-	1.79	2.37	

*Means of five replications

mycelia as it was highly sensitive to moisture stress (Papavizas, 1985). This biocontrol agent had the potentiality and aggressiveness to colonize and establish themselves in organic substrates in natural environments. Our present findings suggested that the four oilcakes, viz., neem cake, mustard cake, linseed cake and groundnut cake as well as vermicompost / leafmould in combination main bulking material cow dung had supported significantly higher growth and sporulation of *T. harzainum* than the cow dung alone in both sterilized and non-sterilized conditions. Similar findings were reported by several researchers (Saju *et al.*,2002; Tiwari and Bhanu, 2003; Pan and Bhagat, 2007). The increases in population of *T. harzianum* with increase in concentration of oilcakes / vermicompost / leaf mould up to certain concentrations in partially decomposed cow dung manure was mainly due to synergistic effect of both components. Oilcakes being rich in nitrogen supported initial establishment and subsequent proliferation of biocontrol agent throughout the medium. The studies on production of chlamydospores (Papavizas *et al.*, 1984) and conidia by liquid fermentation had been made earlier, but not through solid-state fermentation. However, the conidia produced on solid-state fermentation had better shelf life than liquid fermentation due to thicker wall (Minoz *et al.*, 1995). Present findings indicated, that the initial increase in number of conidia up to certain concentration (20% of neem cake and linseed cake; 40% in case of groundnut cake and mustard cake and 50 % of vermicompost and leaf mould) and thereafter declined, but the number of chlamydospores was increased continuously. The possible reasons may be due to the stress condition developed in the substrates at higher concentration oilcakes and lowering down the concentration of cow dung in vermicompost and leafmould combination which leads to stimulation of production of more chlamydospores and comparatively low conidia (Pan and Bhagat, 2007). Thus these findings may be used for developing suitable organic substrate by combining the main bulking materials with various augmenters and can be an integral component of organic farming practices.

REFERENCES

Dhingra, O.P. and Sinclair, J.B. 1995. *Basic Plant Pathology Methods*, 2nd edn. CRC press. Bocca Raton, America.

Elad, Y. and Chet, 1. 1983. Improved selective media for isolation of *Trichoderma* spp. and *Fusarium* spp. *Phytoparasitica*, **11**: 55-58.

Lewis, J.A. and Papavizas, G.C. 1984. A new approach to stimulate population proliferation of *Trichoderma* species and other potential biocontrol fungi introduced into natural soils. *Phytopathology* **74**: 1240-1244

Minoz, G.A.; Agosin, E.'; Cotoras, M.; Mortin, R.; San and Volpe, D. 1995. Comparison of aerial and submerged spore properties of *Trichoderma harzianum*. *FEMS Microbiol Lett.* **125**: 63-70.

Pan, S. and Bhagat, S. 2007. Effect of substarte's physical factors on mass multiplication of *T. harzianum* in management of seedling blight of Jute. *J. Biolog. Control* **21**:127-136.

Papavizas, G.C. 1985. *Trichoderma* and *Gliocladium* : Biology, Ecology and Potential for biocontrol. *Ann. Rev. Phytopathol.* **23** : 23 - 54.

Papavizas, G.C.; Dunn, M.T.; Lewis, J.A. and Beagle-Ristaino, J. 1984. Liquid fermentation technology for experimental production of biocontrol fungi. *Phytopatholoy* **74**: 1171-1175.

Rifai, M.A. 1969. A revision of the genus *Trichoderma*. *Mycological Papers*, **116**:1-56.

Saju. K.A.; Anandraj, M; and Sarma, Y.R. 2002. On farm production of *Trichoderma harzianum* using organic matter. *Ind. Phytopath.* **55:** 277-281.

Singh, H.B. 2006. *Trichoderma*: a boon for biopesticides industry, *J. Mycol. Pl. Pathol.* **36**: 373-384.

Tiwari, L. and Bhanu, C. 2003. Screening of various substrates for sporulation and mass multiplication of biocontrol agent *T. harzianum* through solid-state fermentation. *Ind. Phytopath.* **56**: 476-478.

(*Accepted for publication November 20, 2009*)