



Bio-priming of cucurbits and okra seeds with culture filtrates of *Trichoderma harzianum* for controlling seed-borne fungi

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ABSTRACT

Culture filtrates collected from *Trichoderma harzianum* were assayed to control seed-borne fungi of selected vegetables viz. bottle gourd, wax gourd, sweet gourd, snake gourd and okra. Three fungal genera comprising of four species viz. *Aspergillus niger*, *Aspergillus flavus*, *Penicillium* spp. and *Fusarium oxysporum* were found to be associated with most of the vegetable seeds. Among the seed borne fungi the highest incidence was recorded in case of *A. niger* (49.2%) followed by *A. flavus* (40.6%) and *F. oxysporum* (40.2%). Five treatments viz. 1%, 3%, 5%, 7% aqueous solution of culture filtrates and control (without culture filtrate) were assayed against individual seed-borne fungal pathogen following poison food technique. Among the treatments, 7% aqueous solution of culture filtrate showed superior performance by suppressing mycelial growth of all fungal genera and this treatment was further considered to treat the seeds to control seed borne fungi. Prevalence of seed-borne *A. niger*, *A. flavus*, *Penicillium* spp. and *F. oxysporum* were reduced significantly by seed treatment with 7% aqueous solution of culture filtrates. The findings of the study suggested that seed priming by aqueous solution of culture filtrates of *Trichoderma* (7%) can be used for vegetable seed treatment for controlling seed-borne fungal infection.

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Introduction

Cucurbits and okra are common and economically important vegetables grown in Bangladesh. The major constraints of vegetable production in Bangladesh are availability of quality seeds, insects, diseases, favorable environment, poor marketing system and postharvest losses. Infections occurred by fungi, bacteria, nematodes or viruses are the major ones (Neergaard, 1979). Among the pathogens, the seed-borne fungi play a vital role in disease development. Common seed-borne fungal diseases occurring on vegetables are damping off, foot and root rots, phomopsis blight, fruit rots, black leg, leaf spots, *Fusarium* wilt, *Fusarium* root rot, anthracnose and downy mildews (Fakir *et al.*, 2000). High quality seed is an important pre-requisite for sustainable and profitable vegetable production. Many attempts have been made to reduce seed-borne infection by chemical treatment of the seeds (Nwachukwu and Umechuruba, 2001). Though, chemical controls of seed-borne pathogens have been found very successful, however, chemical pesticides have the additional potential disadvantages of accumulation in the ecosystem, chemical residues remain in the freshly harvested vegetables and induction of pesticide resistance to pathogens. In addition,

indiscriminate use of chemicals in agriculture causes environment pollution and health hazards, destroying the natural balance and beneficial microbes in the soil. Therefore, there is a need for development of efficient alternative measures to combat the disease in an environmental friendly way. Biological control of plant pathogens by microorganisms has been considered a more natural and environmentally acceptable alternative to the existing chemical treatment methods (Baker and Paulitz, 1996). *Trichoderma* spp. is now the most common fungal biological control agents that have been comprehensively researched and deployed throughout the world. Several fungal cell wall degrading enzymes, amongst them chitinase and glucanase, which seem to play an important role in the antagonistic action of *Trichoderma* against a wide range of fungal pathogens (Kucuk and Kivanc, 2008). The antagonistic activities of *Trichoderma harzianum* against several pathogenic fungi have been reported by many workers (Henis and Chet, 1975; Backman and Rodrigues-Kabana, 1975; Hadar *et al.*, 1979; Elad *et al.*, 1980). Therefore, this research work was undertaken to assess the *in-vitro* antagonistic ability of culture filtrates of *Trichoderma harzianum* against seed borne fungi of cucurbits and okra.

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Materials and Methods

The experiments were conducted in the Laboratory of Bioactive Compounds, Bio-formulation and Biosignaling, Plant Disease Diagnostic Clinic (PDDC), Department of Plant Pathology and Seed Pathology Centre (SPC), Bangladesh Agricultural University. Seeds of bottle gourd, sweet gourd, snake gourd, wax gourd and okra were collected from the seed traders of Notun Bazar, Mymensingh. Blotter method was followed according to ISTA rules for seed health testing (ISTA, 2010). After 7 days of incubation, individual seed was observed under stereo-binocular microscope (Zesis) at 10x and 40x magnifications in order to record the association of seed borne fungi. For proper identification of fungi, temporary slides were prepared from the fungal colony and observed under compound microscope and identified with the help of keys suggested by Neergard (1979).

Trichoderma harzianum was collected from IPM Laboratory, Department of Plant Pathology, Bangladesh Agricultural University, and Mymensingh. The effect of culture filtrate of *T. harzianum* on tested pathogens was studied by following methods, developed by Maheswari et al. (2001). Five discs (9 mm diameter) of *T. harzianum* were cut from vigorously growing margin of 5 days old cultures and inoculated separately into 100 ml sterile Potato Dextrose Broth (pH 5.6) (Singla, 2018). Flasks were incubated at 28°C with 100 rpm on a mechanical shaker for 10 days so as to fragment the hyphal mats and to maintain homogeneous growth in liquid medium. After incubation, the cultures were filtered first through Whatmann filter paper No.4 and finally through Millipore filter (0.45 µm) to obtain sterile culture filtrate. The culture filtrate was adjusted to pH 5.6 by using 0.1N HCl and / or 0.1N NaOH before use. From the stock solution 1%, 3%, 5% and 7% aqueous solutions of culture filtrate were prepared by adding required amount of double distilled water.

In vitro efficacy of aqueous solution of culture filtrates

To determine the growth inhibitory effects of the treatments against the test fungi *A. niger*, *F. oxysporum* and *Penicillium* spp were isolated from infected jute seeds. Different concentration of aqueous solution of culture filtrates viz. 1%, 3%, 5% and 7% were added to

PDA medium and stirred for 10 minutes before pouring on petridishes. Growth inhibition of *A. niger*, *F. oxysporum* and *Penicillium* spp. were measured on the culture filtrates treated PDA medium. Ten mm mycelial disc of each fungus was placed in the centre of the each petridish and incubated at 24° C. Percent inhibition was measured by using this formula (Singh et al., 2010):

$$I = [(C_2 - C_1) / C_2] \times 100$$

Here,

I = Percent inhibition of radial mycelial growth,

C₂ = Radial mycelial growth of pathogen in control plates,

C₁ = Radial mycelial growth of pathogen in treated plates.

Seed priming with aqueous solution of culture filtrates

Four hundred seeds from each seed sample were immersed in aqueous solution of culture filtrates. Then, the flask was shaken in rotary shaker for 15 minutes to give uniform coating of culture filtrates on the seeds. Afterwards, the health of seeds was examined following incubation test using blotting paper according to ISTA rules (ISTA, 2010).

Statistical Analysis

The collected data on different parameters were analyzed statistically by using MSTAT C package program. The means for all the treatments were compared by DMRT (Duncan Multiple Range Test). The differences among the means were compared by LSD test (Least Significance Difference) (Gomez and Gomez, 1984).

Results and Discussion

Three seed-borne fungi were encountered during examining the seeds of different vegetables. *Aspergillus*, *Fusarium* and *Penicillium* were predominantly associated at various intensity with the seed samples. Effects of culture filtrates of *Trichoderma harzianum* on the prevalence of fungi associated with different vegetable seeds viz. bottle gourd, sweet gourd, snake gourd, wax gourd and okra are presented in Table 1, Table 2, Table 3, Table 4, and Table 5.

Table 1. Effect of different concentrations of aqueous solution of culture filtrates on prevalence of seed-borne fungi of bottle gourd

Treatment	Prevalence of seed- borne fungi (%)			
	<i>Aspergillus niger</i>	<i>Aspergillus flavus</i>	<i>Fusarium oxysporum</i>	<i>Penicillium</i> spp.
T ₁	33 ab (35.06)	23 b (28.66)	16 b (23.57)	13 a (21.13)
T ₂	20 b (26.57)	26 b (30.66)	23 b (28.66)	0 a (0.7)
T ₃	20 b (26.57)	20 b (26.57)	13 b (21.13)	13 a (21.13)
T ₄	10 b (18.43)	10 b (18.43)	13 b (21.13)	6 a (14.18)
T ₅	40 a (39.23)	50 a (45)	50 a (45)	13 a (21.13)
LSD _{0.05}	12.12	4.688	14.51	16.66

[T₁ = 1% Aqueous solution of culture filtrate, T₂ = 3% Aqueous solution of culture filtrate, T₃ = 5% Aqueous solution of culture filtrate, T₄ = 7% Aqueous solution of culture filtrate and T₅ = control (without culture filtrates)]; Figure in the parentheses are arcsine transformed value, Column having the similar letter(s) do not differ significantly

Seed priming by culture filtrates of selected vegetables

Table 2. Effect of different concentrations of aqueous solution of culture filtrates on the prevalence of seed-borne fungi of sweet gourd

Treatment	Prevalence of seed borne fungi (%)			
	<i>Aspergillus niger</i>	<i>Aspergillus flavus</i>	<i>Fusarium oxysporum</i>	<i>Penicillium</i> spp.
T ₁	26 b (30.66)	26 a (30.66)	23 b (28.66)	13 a (21.13)
T ₂	26 b (30.66)	20 a (26.57)	16 b (23.57)	6 a (14.18)
T ₃	23 b (21.13)	20 a (21.13)	10 b (18.43)	6 a (14.18)
T ₄	13 c (21.13)	13 a (21.13)	13 b (21.13)	6 a (14.18)
T ₅	60 a (50.77)	30 a (33.21)	43 a (40.98)	13 a (21.13)
LSD _{0.05}	10.08	18.63	16.88	18.98

[T₁ = 1% Aqueous solution of culture filtrate, T₂ = 3% Aqueous solution of culture filtrate, T₃ = 5% Aqueous solution of culture filtrate, T₄ = 7% Aqueous solution of culture filtrate and T₅= control (without culture filtrates)]; Figure in the parentheses are arcsine transformed value, Column having the similar letter(s) do not differ significantly

Table 3. Effect of different concentrations of aqueous solution of culture filtrates on the prevalence of seed-borne fungi of snake gourd

Treatment	Prevalence of seed borne fungi (%)			
	<i>Aspergillus niger</i>	<i>Aspergillus flavus</i>	<i>Fusarium oxysporum</i>	<i>Penicillium</i> spp.
T ₁	20 b (26.57)	20 b (26.57)	23 b (28.66)	0 a (0.7)
T ₂	20 b (26.57)	13 b (21.13)	16 b (23.57)	6 a (14.18)
T ₃	23 b (28.66)	20 b (26.57)	23 b (28.66)	6 a (14.18)
T ₄	16 b (23.57)	13 b (21.13)	23 b (28.66)	0 a (0.7)
T ₅	56 a (48.44)	47 a (43.28)	56 a (48.44)	13 a (21.13)
LSD _{0.05}	12.01	12.55	12.16	14.66

[T₁ = 1% Aqueous solution of culture filtrate, T₂ = 3% Aqueous solution of culture filtrate, T₃ = 5% Aqueous solution of culture filtrate, T₄ = 7% Aqueous solution of culture filtrate and T₅= control (without culture filtrates)]; Figure in the parentheses are arcsine transformed value, Column having the similar letter(s) do not differ significantly

Table 4. Effect of different concentrations of aqueous solution of culture filtrates on the prevalence of seed-borne fungi of wax gourd

Treatment	Prevalence of seed borne fungi (%)			
	<i>Aspergillus niger</i>	<i>Aspergillus flavus</i>	<i>Fusarium oxysporum</i>	<i>Penicillium</i> spp.
T ₁	26 b (30.66)	23 ab (28.33)	23 a (28.33)	6 a (14.18)
T ₂	23 b (28.33)	23 ab (28.33)	26 a (23.57)	6 a (14.18)
T ₃	16 c (23.57)	20 ab (26.57)	20 ab (26.57)	6 a (14.18)
T ₄	16 c (23.57)	13 b (21.13)	13 b (21.13)	6 a (14.18)
T ₅	40 a (39.23)	26 a (30.66)	26 a (30.66)	20 a (26.57)
LSD _{0.05}	4.735	9.908	9.80	14.02

[T₁ = 1% Aqueous solution of culture filtrate, T₂ = 3% Aqueous solution of culture filtrate, T₃ = 5% Aqueous solution of culture filtrate, T₄ = 7% Aqueous solution of culture filtrate and T₅= control (without culture filtrates)]; Figure in the parentheses are arcsine transformed value, Column having the similar letter(s) do not differ significantly

Table 5. Effect of different concentrations of aqueous solution of culture filtrates on the prevalence of seed-borne fungi of okra

Treatment	Prevalence of seed borne fungi (%)			
	<i>Aspergillus niger</i>	<i>Aspergillus flavus</i>	<i>Fusarium oxysporum</i>	<i>Penicillium</i> spp.
T ₁	30 b (33.21)	26 b (30.33)	30 b (33.21)	6 b (14.18)
T ₂	20 bc (26.57)	23 b (28.33)	20 bc (26.57)	0 b (0.7)
T ₃	13 c (21.13)	23 b (28.33)	23 b (28.33)	6 b (14.18)
T ₄	13 c (21.13)	6 c (14.18)	13 c (21.13)	0 b (0.7)
T ₅	50 a (45)	50 a (45)	56 a (48.44)	23 a (28.66)
LSD _{0.05}	12.60	9.473	9.190	14.66

[T₁ = 1% Aqueous solution of culture filtrate, T₂ = 3% Aqueous solution of culture filtrate, T₃ = 5% Aqueous solution of culture filtrate, T₄ = 7% Aqueous solution of culture filtrate and T₅= control (without culture filtrates)]; Figure in the parentheses are arcsine transformed value, Column having the similar letter(s) do not differ significantly

In bottle gourd seeds, the highest prevalence of *A. flavus* (50%) and *F. oxysporum* (50%) were recorded followed by *A. niger* (40%) and *Penicillium* spp. (13%). Significantly highest reduction was recorded in T₄ (7%) aqueous solution of culture filtrates compared to control.

Up to 80% of mycelial growth reduction (T₄) was recorded against *A. flavus*. Shamsuzzaman and Hossain (2003) evaluated the substrates for *Trichoderma* conidia production and its effect in germination and seedling vigor of sweet gourd. They reported that, seed treatment

with *T. harzianum* that previously grown on blackgram bran results up to 16.66%, 263.33%, 157.14%, and 98.55% higher germination, fresh weight of shoot and root and vigor index over control respectively.

The highest prevalence of seed-borne fungi in sweet gourd, snake gourd, wax gourd and okra were recorded in untreated control treatment. Application of culture filtrates reduced fungi of all genera at various levels in different kinds of seeds. In sweet gourd seeds, the highest (60%) fungal prevalence among the all fungi was recorded and 78.33% mycelial growth reduction was recorded in T₄ (7% aqueous solution of culture filtrates) in case of *A. niger*. Lindsey and Ralph (1995) reported that 88% inhibition of *A. flavus* by *T. harzianum*. The growth suppression of different seed-borne fungi by culture filtrates of *Trichoderma* could be due to the secretions of harmful extra-cellular compounds like antibiotics i.e. gliotoxins and glioviridin and cell wall degrading enzymes such as glucanase, endochitinase and chitinase.

In snake gourd seeds, the highest prevalence of *A. flavus* (47%), *A. niger* (56%), *F. oxysporum* (56%), and *Penicillium* spp. (13%) were recorded in untreated control treatment. In case of *A. flavus* up to 72.34% mycelial growth reduction was recorded in T₄ (7% aqueous solution of culture filtrates). Kashem et al. (2011) conducted a series of experiments to assess the effect of 14 isolates of *Trichoderma* spp. (*T. harzianum* and *T. viride*) to control foot and root rot of lentil caused by *F. oxysporum* Schlecht. *Trichoderma* isolates inhibited the growth of *F. oxysporum* from 45.87 to 92.07 % at 7 days after inoculation on agar plates.

In wax gourd seeds, the highest (40%) prevalence among the all fungi was recorded and 60% reduction was recorded in T₄ (7% aqueous solution of culture filtrates) in case of *A. niger*. Choudary et al. (2007) reported 92% inhibition of *Rhizoctonia solani* with *T. harzianum*, while Amin et al. (2010) found maximum of 71.41% inhibition of *R. solani* by *T. viride*.

In okra seeds, the highest (56%) prevalence among the all fungi was recorded and 76.79% reduction was recorded in the treatment treated by 7% aqueous solution of culture filtrates in case of *F. oxysporum*. Bari et al. (2002) tested antagonistic effect of rhizosphere *Trichoderma* against foot and root rot disease (*Sclerotium rolfsii*) of barley. An isolate of *T. harzianum* (TF-24) gave 100% reduction of the disease. Seed germination and plant growth of barley were enhanced by the application of *Trichoderma harzianum*, while Yogendra and Singh (2002) found maximum of 75% inhibition of *S. rolfsii* by *T. viride*, where *T. harzianum* resulted inhibition of 64.44% at 4 DAI. Xu et al. (1993) found that mycelial growth of *F. solani* and *F. oxysporum* was inhibited with *T. harzianum*. Michalikova and Michrina (1997) reported the greatest

inhibition rate of the radial growth of *F. culmorum* (55-58%) with *T. harzianum*. Pranab et al. (2002) recorded 61.5% inhibition of *S. rolfsii* by *T. harzianum*. Yogendra and Singh (2000) recorded that *T. harzianum* exhibited strong mycoparasitism and covered 100% colony growth of *S. rolfsii* in dual culture, while Faruk et al. (2002) found that *T. harzianum* significantly reduced the radial colony growth of *S. rolfsii* in dual culture on PDA. Similar findings were obtained by Begum et al. (1997) and Kashem (2005). It is evident from the present experiment that extracellular bio-active compounds are secreted in the culture filtrates which have the antagonistic ability against different seed-borne fungi.

Table 6. Antifungal ability of different concentrations of *Trichoderma harzianum* culture filtrates against *Aspergillus flavus*

Treatment	Inhibition over control (%)				
	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
T ₁	26.40 c	55.30 d	63.23 d	77.80 c	83.87 c
T ₂	27.07 c	56.23 c	64.27 c	78.30 c	84.33 c
T ₃	32.67 b	60.10 b	66.10 b	80.60 b	85.27 b
T ₄	33.80 a	60.73 a	66.97 a	81.93 a	86.57 a
LSD _{0.05}	0.253	0.115	0.064	0.099	0.189

[T₁ = 1% Aqueous solution of culture filtrate, T₂ = 3% Aqueous solution of culture filtrate, T₃ = 5% Aqueous solution of culture filtrate, T₄ = 7% Aqueous solution of culture filtrate and T₅= control (without culture filtrates)]; Column having the similar letter(s) do not differ significantly

Table 7. Antifungal ability of different concentrations of *Trichoderma harzianum* culture filtrates against *Aspergillus niger*

Treatment	Inhibition over control (%)				
	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
T ₁	8.133 b	23.60 d	51.37 c	61.40 d	67.17 c
T ₂	8.300 b	40.90 c	60.77 b	70.53 c	73.70 b
T ₃	8.333 b	42.20 b	62.47 ab	71.90 b	74.30 b
T ₄	8.833 a	43.87 a	64.00 a	73.60 a	76.43 a
LSD _{0.05}	0.3254	1.295	1.810	0.8931	1.227

[T₁ = 1% Aqueous solution of culture filtrate, T₂ = 3% Aqueous solution of culture filtrate, T₃ = 5% Aqueous solution of culture filtrate, T₄ = 7% Aqueous solution of culture filtrate and T₅= control (without culture filtrates)]; Column having the similar letter(s) do not differ significantly

Table 8. Antifungal ability of different concentrations of *Trichoderma harzianum* culture filtrates against *Penicillium* spp.

Treatment	Inhibition over control (%)				
	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
T ₁	6.367 b	41.17 c	61.97 d	72.53 c	75.87 b
T ₂	6.233 b	41.60 c	63.07 c	72.37 c	76.63 ab
T ₃	6.467 b	43.53 b	64.10 b	73.83 b	74.53 c
T ₄	18.10 a	51.90 a	66.60 a	75.90 a	77.70 a
LSD _{0.05}	0.042	0.853	0.245	0.082	0.488

[T₁ = 1% Aqueous solution of culture filtrate, T₂ = 3% Aqueous solution of culture filtrate, T₃ = 5% Aqueous solution of culture filtrate, T₄ = 7% Aqueous solution of culture filtrate and T₅= control (without culture filtrates)]; Column having the similar letter(s) do not differ significantly

Seed priming by culture filtrates of selected vegetables

Table 9. Antifungal ability of different concentrations of *Trichoderma harzianum* culture filtrates against *Fusarium oxysporum*

Treatment	Inhibition over control (%)				
	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
T ₁	7.57 c	45.07 d	64.23 d	73.20 c	76.07 c
T ₂	7.70 c	45.60 c	65.07 c	73.23 c	77.17 b
T ₃	8.10 b	46.23 b	66.23 b	73.77 b	77.40 b
T ₄	15.17 a	50.10 a	67.73 a	75.43 a	78.33 a
LSD _{0.05}	0.011	0.059	0.033	0.082	0.090

[T₁ = 1% Aqueous solution of culture filtrate, T₂ = 3% Aqueous solution of culture filtrate, T₃ = 5% Aqueous solution of culture filtrate, T₄ = 7% Aqueous solution of culture filtrate and T₅ = control (without culture filtrates)]; Column having the similar letter(s) do not differ significantly

Conclusion

Aqueous solution of culture filtrates of *Trichoderma harzianum* is potential compounds for priming of seed to control seed-borne fungi of different vegetables. *Trichoderma* is a well-known bio-agent and also beneficial for environment and completely safe for human health. Therefore, application of *Trichoderma* culture filtrates having antagonistic metabolites can be utilized as seed treating bio-primer in place of chemical pesticides. Further intensive researches are necessary to develop effective commercial formulation using different kinds of organic solvents.

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