Effect of Amendments on Biocontrol Efficiency of *Trichoderma* spp. and its Subsequent Effect on Seedling Growth

Anamika Debnath*, Prateek Madhob Bhattacharya and Ayon Roy

Department of Plant Pathology, Uttar Banga Krishi Viswavidyalaya, Pundibari, Cooch Behar, West Bengal (736 165), India

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Correspondence to

**E-mail*: dr.anamikadebnath@rediffmail.com

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Abstract

Use of microbes for environmentally safe plant disease management largely depends on antagonistic ability of the isolate, and *Trichoderma* spp. occupies a major domain in biocontrol research. An attempt was made to improve the bioefficacy of the *Trichoderma* isolate through chitin (0.5% and 1%) and ammonium sulphate (0.5%, 1% and 1.5%) as amendments in the synthetic medium at UBKV, Coochbehar, India during 2007-2009. Competitive saprophytic ability of the *Trichoderma* isolate recorded highest with 1% ammonium sulphate amendment. *Trichoderma* isolate showed higher chitinase activity with ammonium sulphate addition irrespective of dose. Ammonium sulphate addition also increased the plant growth promoting ability of the *Trichoderma* isolate. Chitin enhanced the bioefficacy of the *Trichoderma* isolate but it is not at par with ammonium sulphate and highest bioefficacy was found when the *Trichoderma* isolate was grown with 1% ammonium sulphate as reflected in the antagonism index value. *In vivo* trial was conducted with *Trichoderma* grown on amended and non-amended media with cowpea as test plant and *Rhizoctonia solani* as test pathogen. Reduction in pre-emergence damping off was recorded with addition of *Trichoderma*. Highest reduction of the disease was observed in chitin amendment. Vigor index was also further increased with addition of amendments

1. Introduction

Among various ways employed for efficacy enhancement of *Trichoderma* such as modification of the substrate, UV radiation, use of chemical mutagen and protoplast fusion to name a few, and most commonly changes in nutritional composition of growth substrate seems to be a simple procedure with profound effects as available in the literatures. Increase in the chitinase activity was noticed after amending the media (Krishnamurthy et al., 1999; Manczinger et al., 2001; Yang et al., 2002). The bioefficacy of *Trichoderma harzianum* was enhanced with chitin incorporated into the medium as a sole carbon source, (El-Katatny et al., 2000).El-Katatny et al. (2000) reported that chitin incorporation into the medium as a sole carbon source enhances the bioefficacy of *T. harzianum* Rifai, and made the antagonist capable of hydrolyzing dried/fresh mycelium of the phytopathogenic fungus *Corticium rolfsii* Cuzi. According to Jayaraj and Ramabardran (1996) ammonium sulphate enhances colonization and propagule number of *T. harzianum*. Some recent works indicate that use of nitrogen in different form such as urea, ammonium sulfate and potassium nitrate, influenced the bio-efficiency of the antagonist, where ammonium sulfate was the most favored form by *Trichoderma* and it also enhanced the efficiency of the antagonist against *Sclerotium rolfsii* anamorth Sacc. (Khattabi et al., 2004). An increase in saprophytic colonization was observed with amendments as found by many workers (Jayaraj and Ramabadran, 1998).One *T. harzianum* isolate (UBT 18) was screened out with high potentiality against some important soil borne pathogens on the basis of antagonism index based screening technique (Debnath et al., 2011), and in the present study an attempt was made to enhance the biocontrol potential as well as plant growth promotion potential, ultimately yielding higher plant health management potential, so that the isolate can be used to protect a wide range of crops against a wide range of pathogens

2. Materials and Methods

T. harzianum isolate was grown in a synthetic media with chitin (0.5 and 1%) and ammonium sulfate (0.5, 1 and 1.5%) at different rates as amendments. The effects of amendments on antagonistic potential of the isolate was evaluated against *Rhizoctonia solani* after sufficient growth on non-amended and amended media following the method of computation of an-

tagonism index with cowpea (*Vigna radiate* (L) R. Wilczek) as a test plant at UBKV, Coochbehar, India during 2007-2009.

2.1. Competitive saprophytic ability (CSA) of Trichoderma isolate

The *Trichoderma* isolate after growing through different amended and non-amended media were mass-multiplied separately in vermicompost and cfu of the product was determined on *Trichoderma* specific medium. The pathogen *R. solani* was mass-multiplied in sterilized chopped rice straw and sclerotia were harvested after 20 days of incubation. Sterilized soil was used for evaluation and mass-multiplied *Trichoderma* isolates in desired amount were mixed separately with 300 g of soil to maintain the antagonistic population at 10^7, 10^8, 10^9 cfu g^{-1} soil. Fifteen sclerotia were added to soil mixture to provide competitive environment. This augmented soil was distributed in three replicates and poured in plastic cups (100 g soil capacity) after adding sufficient water to maintain the water holding capacity to nearly 50%. In every cup, 5 sterilized wheat straw pieces of 4 cm length and with at least one internode were inserted. These cups were incubated at 28±1°C for 5-7 days. Then, the straw pieces were recovered, surface sterilized with 0.1% $HgCl_2$, washed in sterilized distilled water, soaked by sterilized blotting paper and placed on *Trichoderma* Specific Medium. Petri-plates were incubated at 28±1°C. The per cent area of the straw pieces colonized by *Trichoderma* sp. was recorded and potentiality graded as 1 and 2 according to less or more than the mean value, respectively.

2.2. Colonization behavior (CB) of Trichoderma isolate

Colonization behavior of *Trichoderma* isolate produced through different amendment treatments was enumerated by dual culture plate method (Dennis and Webster, 1971b) where both the antagonist and *R. solani* were inoculated at opposite ends in petri-plates containing PDA medium. The plates were then incubated at 28±°C. Control plates were maintained by inoculating *R. solani* only on PDA. Both the pathogen and the antagonist grew towards each other, after contact the extent of invasion by the antagonist over the pathogen was recorded following modified Bell's scale (Saha and Pan, 1997). The hyperparasitic potentiality of the *Trichoderma* isolate was scored as 1=antagonist overlapped total growth of pathogen; 2=antagonist overlapped two-third the growth of pathogen; 3=antagonist overlapped one-half the growth of pathogen; 4=pathogen growth restricted at the point of contact and 5=pathogen overlapped antagonist.

2.3. Volatile activity (VA)

Volatile metabolite production ability by the *Trichoderma* isolate produced through growing in different amended and non-amended media was measured following the method of Dennis and Webster (1971b) and potentiality was graded as 1 and 2 according to less or more than the mean value, respectively.

2.4. Assay of chitinase production

Quantitative assay of lytic activity was estimated spectrophotometrically to identify the production potential of chitinase enzyme by the test *Trichoderma* isolate after growing in amended and non-amended media following the method described by Kumar and Gupta (1999). Quantity of chitinase enzyme produced by the *Trichoderma* isolates was expressed as µmol N-acetyl glucosamine (NAG) ml^{-1} of culture filtrate min^{-1} and graded as 1 and 2 according to less or more than the mean value, respectively.

2.5. Pathogen growth inhibition (PI) by Trichoderma isolates

Percent inhibition was calculated by 'Bangle method' (Singh et al., 2004) and graded as 1 and 2 according to less or more than the mean value, respectively.

2.6. Speed of growth of Trichoderma isolates over the growth of pathogen

Speed of overgrowth was measured by the technique of dual culture through Bangle method. The speed of overgrowth was measured after 24 and 48 h. *Trichoderma* isolates with respect to this particular property were graded as 1 when pathogen overgrows *Trichoderma*, 2 when pathogen does not overgrows *Trichoderma*, 3 when *Trichoderma* overgrows pathogen after 48 h, and 4 when *Trichoderma* overgrows pathogen between 24-48 h.

2.7. Inhibition zone (IZ) production ability of Trichoderma isolates

Inhibition zone was recorded at the point of contact of pathogen and *Trichoderma* isolate which was measured 72 h after inoculation in dual culture plate method (Denis and Webstar, 1971a) and rated as 1=no inhibition, 2=1-2.5 mm, 3=2.5-5 mm and 4=>5 mm inhibition zone.

2.8. Determination of plant growth promotion ability (vigor index)

The plant growth promotion ability was determined for *Trichoderma* isolate using roll paper towel method and calculating vigor index (VI) for the candidate host plant. The suspension of the *Trichoderma* isolate after growing it in different amended and non-amended media was mixed separately with talc based carrier in a certain proportion to maintain the population level of the antagonist at 10^7 cfu g^{-1} of talc. The plant growth promotion ability was determined for *Trichoderma* isolate using roll paper towel method and calculating VI for the candidate host plant, i.e. cowpea. Cowpea seeds were surface sterilized with 0.1% mercuric chloride solution. Then the cowpea seeds were treated with the talc formulations. The treated seeds were then placed on a wet paper towel, covered with another and rolled

and placed in plastic bags after putting rubber bands with both ends. These sets were incubated at 25±1°C in seed germinator with 12 h light and dark rotation. After 10 days of incubation, germination percentage, root and shoot lengths were measured and VI was calculated by the following formula:

VI=[(Root length+shoot length) × % germination]/100.

VI or plant growth promotion potential was classified as 1 and 2 according to less or greater than the mean population, respectively. Finally the antagonism index (AI) of a particular *Trichoderma* isolate was calculated by taking the product of the above mentioned biocontrol properties.

AI= CSA × SOOP × PI × CB × IZ × VA × LE × VI

Where AI=Antagonism index, CSA=Competitive saprophytic ability, PI=per cent inhibition, IZ=Inhibition zone, VA=Volatile activity, VI=Vigor index, SOOP=Speed of overgrowth on pathogen, CB=Colonizing behavior, LE=Lytic enzyme

2.9. Efficacy enhancement of selected Trichoderma isolate

Efficacy enhancement through nutritional enrichment is one of the most promising approaches to make the biocontrol agent more effective. In view of this an attempt was made by supplementation of growth media with different concentrations of chitin (0.5 and 1%) and ammonim sulphate (0.5, 1 and 1.5%) to observe the extent of efficacy enhancement of selected *Trichoderma* isolate. *Trichoderma* isolate was grown on the chitin and ammonium sulphate amended media and used for study of different antagonistic properties against different pathogens and AI was calculated using the above mentioned procedure using *R. solani* as a test pathogen.

2.10. In vivo efficacy of Trichoderma isolate

Trichoderma isolate was further grown on amended and non-amended media and formulated in talc and applied as seed treatment to check the efficacy of the amended and non-amended *Trichoderma* isolate under *in vivo* condition also. Soil was sterilized and amended with organic matter and vermicompost at 2.5 g kg^{-1} of soil. The soil was poured in 4 kg pot and 20 sclerotia of *R. solani* were added to each pot to make the soil sick. The moisture holding capacity of the soil was maintained at 50% and the inoculated pots were incubated at room temperature for 7 days. Talc formulation of *Trichoderma* was prepared after growing the isolate in Capek-Dox Broth (CDB) supplemented with 1% chitin, 0.5% ammonium sulphate and 1% ammonium sulphate. Selected *Trichoderma* isolate UBT 18 grown separately in CDB was also talc formulated for further use in this experiment. Cowpea seeds were soaked overnight, surface sterilized and two-third of the seed lot was dressed with different talc formulations. Rest of the seeds was kept as untreated check after dressing with talc only. The seeds were sown @ 5 seeds pot^{-1}. The pots were then kept at glass house and watered frequently to maintain the moisture holding capacity at optimum level. Vigor index was determined at 21 days after planting. Germination percentage was recorded to calculate the occurrence of pre-emergence damping off. Root and shoot length was measured to enumerate the growth promoting efficacy of the isolate. The root and shoot portion were separately oven dried in hot air oven at 60°C and kept for the time until the weight became constant, and measured the dry weight of the plant.

3. Results and Discussion

The *Trichoderma* isolate UBT 18 was found to be the most effective against the pathogen *R. solani* following the AI procedure (Debnath et al., 2011) so this isolate was taken for further study that is for efficacy enhancement. Different biocontrol attributes of the *Trichoderma* isolate were evaluated after growing it in different amended and non-amended treatments and the result is presented in Table 1.

The results indicate that CSA of the isolate was recorded highest when it was grown with 0.5% ammonium sulfate as amendment followed by chitin. Ammonium sulfate as amendment also increased volatile activity as well as plant growth promotion activity. Chitinase activity (LE) also enhanced with ammonium sulfate addition irrespective of dose. A significant increase in chitinase production was noticed with chitin amendment at higher dose (1%) with respect to control, but it was never found to be at par with ammonium sulfate. The isolate did not produce any inhibition zone. VI was measured to determine the enhancement in plant growth promotion activity; here also a clear cut advantage of 7% increase in efficiency due to 1% ammonium sulfate amendment was recorded along with other concentrations. Addition of 1% ammonium sulfate recorded a 3% higher VI over 1% chitin amendment mainly due to higher root growth and germination. Even an enhancement of AI was also noticed with addition of 1% chitin in media. Higher efficacy of ammonium sulfate in inducing bioefficacy of *Trichoderma* seems to be due to higher plant growth promotion activity in general. When AI was calculated (Table 2) it was found that *Trichoderma* isolate showed highest AI value (256) when grown with 1% ammonium sulfate followed by 0.5% ammonium sulfate and 1% chitin. Efficacy of *Trichoderma* under amended and non-amended also tested in the *in vivo* condition and the result is presented in Table 3.

Seed treatment with *Trichoderma* not only protected the plant against pathogen but also increased the plant initial vigor and growth (Hassan et al., 2011). *Trichoderma* grown without any amendment reduced the disease from 33 to 20% showing the disease reducing efficacy of about 40%. Under amended condition 0.5% ammonium sulfate did not show any improvement in biocontrol efficacy. However, 1% ammonium sulfate, 1% chitin improved the efficacy to about 60 and 70%, respectively. The

Table 1: Biocontrol and growth promoting attributes of *Trichoderma* isolate under amended and non-amended condition

Treatment	CSA (%)	% inhibition	Volatile activity (% inhibition)	LE (µmol NAG ml-1)	% germination	Root length (cm)	Shoot length (cm)	VI (%)	CB
Without amendment	64 (53.17)	60.00 (50.77)	2.35 (8.913)	2.820	88	7.38	9.71	15.05 (22.83)	4
0.5% chitin	69 (56.32)	60.00 (50.77)	4.12 (11.52)	3.044	92	9.70	9.76	17.90 (25.02)	3
1% chitin	69 (56.32)	61.11 (51.42)	15.88 (23.48)	2.956	92	9.07	11.17	19.43 (26.15)	3
0.5% Amonium sulphate	77 (62.36)	62.7 (52.40)	2.94 (9.547)	3.59	92	9.70	12.50	20.42 (26.86)	3
1% Amonium sulphate	54 (45.00)	61.6 (51.94)	21.76 (27.80)	3.612	96	12.37	13.00	24.35 (29.57)	4
1.5% Amonium sulphate	50 (45.00)	58.33 (50.23)	8.82 (17.27)	3.614	92	10.94	12.30	21.40 (27.56)	4
Average	63.83	60.65	3.274	9.31				19.76	
SEm±	1.926	0.447	1.042					0.4355	
CD (*p*=0.05)	6.069	1.216	3.283					1.372	

CSA=Competitive saprophytic ability; LE=Lytic enzyme; VI=Vigor index CB= Colonization Behaviour

plants under treatments did not show any significant difference in shoot length but the treatment effect was highly significant in root length at the time of harvesting the plants. However, the plants treated with *Trichoderma* grown with 0.5% ammonium sulfate exhibited the highest root length (8.22 cm), but it was not significantly more than other amendments. VI was calculated at an age of 20 days and indicated that application of bio-agent improved the vigor of the plants which recorded the highest level under *Trichiderma* grown with 1% chitin (18.85). Shoot and root dry weight was taken as an indicator of plant growth. However, the treatments did not have any significant effect on shoot dry weight at the initial stage. The treatments recorded about 4-7 mg increase in the dry weight at initial stage. On the other hand, effect of biological agent either amended or non-amended condition on root dry weight was profound similar to their effect on the root length. *Trichoderma*

Table 2: Antagonism index (AI) of *Trichoderma* isolate under activated and non-activated condition

Treatment	CSA	PI	IZ	LE	VA	VI	SOOP	CB	AI
1	2	1	1	1	1	1	4	4	32
2	2	1	1	1	1	1	4	3	24
3	2	2	1	1	2	1	4	3	96
4	2	2	1	2	1	2	4	3	192
5	1	2	1	2	2	2	4	4	256
6	1	1	1	2	1	2	4	4	64

CSA=Competitive saprophytic ability; PI=Per cent inhibition; IZ=Inhibition zone; LE= Lytic enzyme; VA=Volatile activity; VI=Vigor index; SOOP=Speed of overgrowth on pathogen; CB=Colonization behavior

Table 3: *In vivo* biocontrol and growth promoting efficacy of *Trichoderma* isolate UBT 18 under activated and non-activated condition

Treatment	Germination %	Shoot length (cm)	Root length (cm)	Vigor index	Dry weight of shoot (g)	Dry weight of root (g)
No treatment	66.67	11.82	5.65	11.64	0.1046	0.0263
Without amendment	80.00	13.14	6.84	15.98	0.1136	0.0304
0.5% Amonium sulphate	80.00	11.97	8.22	16.15	0.1266	0.0396
1% Amonium sulphate	86.67	12.55	7.75	17.59	0.1259	0.0380
1% chitin	90.00	13.24	7.70	18.85	0.1269	0.0385
SEm±		0.753	0.5434		0.03808	0.004082
CD (*p*=0.05)		2.222	1.603		0.0129	0.01204

grown in 0.5% ammonium sulfate resulted in the highest root dry weight which was about 50% more than that of the plants without any *Trichoderma* inoculation. Thus the results of the *in vivo* experiment indicate that bio-inoculation improves rooting behavior of the plants that may be pivotal in increasing the fitness of plants under stress condition.

4. Conclusion

Addition of 1% ammonium sulfate in the medium may be useful by virtue of increased volatile activity and vigor index. Thus it implies that although addition of chitin enhanced the biological antagonism index of the isolate, it never resulted at par with ammonium sulfate due to higher plant growth promotion activity of the isolate under the influence of ammonium sulfate. Apart from these, there was a marked increase in chitinase activity and saprophytic colonization on amending the medium Reduction in pre emergence damping off was recorded along with vigor index of the seedlings and plant dry matter as growth parameter. The results indicated that use of *Trichoderma* without any amendment reduced the disease as well as it increased the vigor index. A positive increment was noticed in biomass production also. However, the disease was reduced further by amendments and the highest reduction was observed in chitin amendment. Vigor index was also further increased with addition of amendments.

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