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A Comparative Study on Efficacy of Potential Bio-control Agents in Dynamic Environments

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The application of the bio-control agent is on a significant rise in the current agricultural practices. Besides causing no harm to the external environment, these compounds also help in controlling the growth of pathogens. In this study, we have compared the efficiency of *Trichoderma viride* which is being used in the field over the past few years as a bio-control agent, with the novel *Streptomyces* species IF5 (GenBank Accession number FJ951435) by analysing the secondary metabolites for anti-microbial activity under various environmental conditions. Several tests were performed to quantify the effect of the metabolites produced from both the strains under varying pH, salt concentration, heavy metals and pesticide endosulfan. We found that the activity of enzymes produced by *Streptomyces* IF5 was significantly higher than that of *Trichoderma viride* and also *Streptomyces* IF5 could tolerate severe environmental stresses more efficiently as compared to *Trichoderma viride*. Our results suggest that *Streptomyces* IF5 can be used in place of *Trichoderma viride* as an effective bio-control agent. The mass production of these metabolites from *Streptomyces* IF5 and the mode of application on the field however have to be investigated further and the conditions are to be optimized, which would be the future direction of the present study.

Key words: Antifungal activity, Bio-control agent, Environmental stresses, *Streptomyces* IF5, *Trichoderma viride*.

The use of chemical fertilizers for the mass production of crops worldwide has threatened the crops cultivated on those lands by the attack of many pests (Oerke and Dehne 2004). Use of eco-friendly biopesticides is an obligation to protect the crops from microbial attack and to meet the undesirable losses of agricultural quality and yield. The losses entailed by fungal pathogens are comparatively larger than other groups. Microorganisms, when used as biopesticides, they are called microbial pesticides (USA EPA 2011). Majority of the *Trichoderma* species are potential bio-control agents against the major plant pathogens like sp, *Phythium* sp, *Rhizoctonia* sp, *Sclerotina* sp. (Laszlo Kredics *et al*., 2003).

In this scenario, other than *T.viride*, there are several reports on screening, isolation and development of bacteria, fungi and actinomycetes sp (Tahtamouni *et al.,* 2006) as bio-control agents to protect the crops. Obstacles in the commercialization remains the same despite much research progress, as bio-control agents need wide adoption to the various environmental influences. The existing biofungicides have low impact on the targets due to fluctuating environmental conditions and practically are not tolerant or less efficient in these fluctuating environments.

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The volume of study on the effects of different environmental factors on mycoparasitic *Trichoderma* strains is increasing, indicating that in order to formulate effective biological control, it is necessary to know about the ecophysiology of the genus in question, as the environmental conditions influence bio-control activity of key enzymes (Subasioglu & Cansunar 2010) like chitinase and cellulase produced by the *Trichoderma* spp that contribute to the bio control activity of the organism.

Streptomyces and other actinomycetes can also be classified as one of the best bio-control agents as they have antagonistic activity against most of the plant pathogenic fungi because of their ability to cause the lysis of the fungal hyphae by chitinase and glucanases (Kumar & Gupta, 2006).

Thus in this study, a novel *Streptomyces* species IF5, which was isolated from feather dumps of local poultry farm was evaluated for antifungal properties and was indeed found to have detrimental effect on the plant pathogen *Fusarium oxysprum.* The selected plant pathogen infects a wide range of plants like tomato, cucurbits, sweet potatoes, and banana, causing vascular wilt, corm rot, root rot, damping of (Steinkellner *et al*., 2008). Moreover, *F. oxysporum* is also the major reason for the loss of banana production worldwide by causing the Panama Wilt in Banana (Mehrotra & Aggarwal, 2003). The first report on the Panama wilt of banana caused by *F. oxysporum* was given by Bancroft (1876). As it is an established fact that the secondary metabolites produced are the reasons for the antifungal activity of the organisms, the *invitro* enzyme activities and hence the antagonistic activities of two potential bio-control agents (BCA) *T.viride* and *Streptomyces* sp IF5 under various environmental stresses like varying temperature, pH, heavy metals, sodium chloride and chemical pesticide concentrations.

MATERIALS AND METHODS

Collection of Strains

T. viride and *F. oxysporum* were collected from Microbial Type Culture Collection Centre, Chandigarh. These isolates were maintained on Potato Dextrose Agar (PDA).

Streptomyces IF5 was isolated from feather dump sites and cultured on Actinomycetes isolation agar (Jayapradha *et al.,* 2011).

Evaluation of Bio-control activity

The bio-control activity was determined by the method suggested in Jayapradha *et al*., 2009 to determine the inhibition of resistance of the test isolate by *T.viride* and *Streptomyces* sp IF5.

Effect of environmental factors on bio-control agents

The growth of any BCA are affected by several factors of which the physiological and the environmental factors play a major role (Jayaswal *et al*., 2003). All the experiments pertaining to the evaluation of the growth and production of the antagonistic metabolites; in this context, various bio polymer degrading enzymes, were performed in triplicates and the average activity with standard deviation was computed for further analysis.

Effect of temperature and pH

The effect of various temperatures on the viability, by the measurement of OD_{600} and activity of different enzymes by respective colorimetric assays, from both *T. viride* and *Streptomyces* sp IF5 were determined after growing them at different temperature ranges from 20°C to 50°C using (Scigenics Orbitek) Shaker incubator at 120 rpm for 72 h.

In a separate set of flasks, the pH of the growth medium was varied from 3 to 11 using 0.1 N NaOH and HCl. The inoculated test cultures were incubated at Room Temperature (RT) or 30°C, for 72 h to study the effect of pH on the two BCAs.

Effect of trace elements & Sodium chloride

The study was conducted to investigate the influence of the most profound soil trace elements like magnesium, manganese, zinc, cobalt and copper in the concentration of 0.01% (w/v) on the growth and enzyme production by the two BCAs.

Sodium Chloride is one of the main compounds which are involved in regulating the growth of the bio-control agent. Hence the effects of different concentrations of NaCl viz 1%, 3%, 5%, 7%, 9% were tested against the two BCAs.

Effect of pesticides

The widely used commercial pesticide endosulfan was used to study the viability and the stability of the activity of the two BCAs against the *Fusarium oxysporum*, since almost all the chemical pesticides are detrimental to the microflora. Different concentrations of endosulfan

(0.1%, 0.2% and 0.3%) were used to study the effect of pesticides.

Determination of enzyme activity at various environmental stresses

After 72 h of exposure to various environmental stresses as said above, the two BCAs were analyzed after they were spun down at 10000 rpm at 4ºC for 15 minutes. The supernatant was analyzed for chitinase, cellulase and glucanase activity, and total protein was quantified using Lowry's method. The chitinase and cellulase assay were carried on as per the protocol suggested by Ghose T.K, 1987.

RESULTS AND DISCUSSION

Evaluation of bio-control activity

The bio-control activity was determined for *T.viride* and *Streptomyces* sp IF5 by well diffusion method. The zone of inhibtion of *Strepomyces* sp IF5 against *Fusarium oxysporum* was measured to be 2.7 cm, and it was 2.1 cm for *T.viride.* The novel species *Streptomyces* IF5 isolated by Jayaprada *et al*., (2011) from the feather dumped soil site proved to be much effective than *T.viride* in acting against the plant pathogen *Fusarium oxysporium.* Several studies have shown that the anti-microbial activity was mainly due to the capacity to produce antibiotics, enzymes that have antimicrobial activity, siderophores, solubilization of phosphates and competition with plant pathogens for substratum and nutrients (Crawford *et al*.,1993). However, the production of these primary and secondary metabolities were regulated by several abiotic conditions like soil pH, temperature, salinity levels. The effect of these factors on the production of metabolites were studied and the results are given below.

Effect of pH on enzyme activity

Although *Trichoderma viride* grows at a wide range of pH, the production of the antagonistic enzymes were however maximum only at pH 3 (Table 1). This reconfirms the observation reported by Upadhyay and Rai, 1978 who concluded that the *Trichoderma* sp prefer to grow well in acidic soils. Also from the values recorded, we can illustrate that the growth of *T.viride* was not restricted to any pH. This conclusion however is ill disposed with that of Jayaswal *et al.,* 2003, concluded that *T.viride* was unable to grow above a pH of 9.

The growth of *Streptomyces* sp IF5 also was not dependent on any particular pH range. However, the utmost production of enzymes used against the potential soil borne pathogens was seen at pH 9 (Table 2), suggesting their antagonistic ability was not lost even in the alkaline soils. The growth of IF5 was also high at this pH and this result concurs with those from the studies of Sousa *et al*., 2008 who studied the growth conditions of several *Streptomyces* sp*,* and concluded that this genus grows better at pH greater than 6.5.

Effect of Trace elements

Trace elements are known to prevail in the soil at meager concentrations. However these elements are found to have major influence on the growth of the bio-control agents. *T.viride* is known to have the ability of sorption of heavy metals as

Table 1. Effect of pH on Enzymes production in *Trichoderma viride* (values given as Mean of triplicates±SD)

pH	OD measurements			
	Cellulase	Chitinase	Glucanase	Total Protein
Control	1.3840± 0.4312	0.1827 ± 0.0601	0.524±0.1285	1.636 ±0.0367
3	0.5647 ±0.0463	1.4240±0.01608	0.5180± 0.1355	0.8553±0.0053
5	0.5180± 0.1367	0.2160±0.0059	0.3017± 0.0179	0.8023±0.0450
5	0.9547 ± 0.1955	1.4976±0.0184	0.6457± 0.0645	1.1587±0.0392
7	0.5677± 0.1045	0.5733± 0.01087	0.4833±0.0123	1.3010±0.0437
7	1.1007 ± 0.1126	1.6306 ± 0.0082	0.7020 ±0.0487	1.3017±0.0024
9	0.5000± 0.0098	0.5516±0.0135	0.5243±0.0264	1.1196± 0.0298
9	1.4397 ± 0.0623	1.72± 0.0489	0.8573 ±0.0587	1.5483± 0.0582
11	0.5083 ± 0.0087	0.5313±0.0245	0.4400±0.0668	0.9113± 0.0405
11	1.327 ± 0.1000	1.676± 0.0241	0.7937 ±0.1355	1.4247± 0.03364

reported by Morley & Gadd, (1995). From the values obtained in our set of experiments (Table 3), we found that cobalt was the main element which significantly affected the growth and production of enzymes in *T. viride*.

Highest amount of the enzyme chitinase, glucanases and the proteases was observed in the samples supplemented with cobalt. Minimum production of the enzymes was observed in the media supplemented with Zinc. However, the maximum cellulase enzyme production was in the media supplemented with Iron. This is in agreement with the findings of Mandels & Resse, (1956) that put forth that the cellulase yield increases with the addition of iron. Also they had reported Iron, Manganese, Zinc and cobalt plays an important role in increasing the cellulase yield. Here, we observed that metal ions influencing the production of cellulase is of the order, Fe^{2+}> Zn^{2+}> Co^{2+} > Cu^{2+}> Ca^{2+}>Mn^{2+}.

On the other hand, in the case of *Streptomyces* IF5, calcium seemed to play a vital

Table 2. Effect of pH on Enzymes production in *Streptomyces* IF5(values given as Mean of triplicates±SD)

Heavy metals	OD Measurements			
	Cellulase	Chitinase	Glucanase	Total Protein
Copper	1.1167± 0.1327	0.4610± 0.0673	0.9933±0.1672	1.2087 ±0.1128
Cobalt	1.268 ±0.0387	0.6697±0.0812	1.4180± 0.2492	1.4120±0.0657
Manganese	0.9547 ± 0.1955	0.4393±0.1931	1.1053± 0.1998	0.8677±0.5802
Calcium	1.1007 ± 0.1126	0.3127± 0.1941	1.1093 ±0.1937	0.9493±0.0437
Iron	1.4397 ± 0.0623	0.2637± 0.0759	1.0063 ±0.1563	1.0200± 0.0908
Zinc	1.327 ± 0.1000	0.2420± 0.0283	0.9403 ±0.1742	0.8400± 0.0594

Table 3. Effect of various trace elements on Enzymes production in *Trichoderma viride* (values given as Mean of triplicates±SD)

Heavy metals	OD Measurements			
	Cellulase	Chitinase	Glucanase	Total Protein
Copper	0.4993± 0.0082	0.4840± 0.1032	0.6400±0.0355	0.7400 ±0.0216
Cobalt	0.6196 ±0.0175	0.7719±01306	0.8800± 0.02160	1.1100±0.0374
Manganese	0.8573 ± 0.0206	0.7937±0.1757	1.1067± 0.0592	1.4400±0.0294
Calcium	0.9940± 0.0302	0.8703± 0.1510	1.4000 ±0.2160	1.7500±0.0589
Iron	0.7933± 0.0096	0.9750± 0.1760	0.9833 ±0.0083	1.3500± 0.0329
Zinc	0.5247 ± 0.0091	0.6440± 0.0940	0.7637 ±0.0012	1.0200± 0.0216

Table 4. Effect of various trace elements on Enzymes production in *Streptomyces* IF5(values given as Mean of triplicates±SD)

Concentration of NaCl(g/L)	OD Measurements			
	Cellulase	Chitinase	Glucanase assay	Total Protein
Control	1.3840± 0.4312	0.1827 ± 0.0601	0.524±0.1285	1.636 ±0.0367
1	0.2260±0.0516	0.8133±0.1049	1.4293± 0.0593	1.0663±0.0603
3	0.3267± 0.1386	0.6317±0.0613	1.6093± 0.0455	1.0987±0.0748
5	0.3857± 0.1124	0.3823±0.1809	1.4647±0.2265	1.1133±0.0784
7	0.4770± 0.0978	0.4857±0.0592	1.9627±0.3466	1.0955± 0.0125
9	0.3073± 0.1263	0.9477±0.0176	1.6780±0.0462	1.7790±0.0043

role (Table 4) in regulating the production of cellulase, glucanases and other antagonistic proteases. However, Iron had an upper hand in regulating the production of chitinase. Minimum production of these enzymes was observed in the media supplemented with copper.

Effect of Sodium Chloride

From the previous reports, the production of various antagonistic primary and secondary metabolites has been found to be influenced by salinity in the soil (Kredics *et al*., 2003). While the maximum production of the enzyme cellulase and glucanases was observed at the NaCl concentration of 7 g/L, the enzyme chitinase and other proteases were produced maximally at a concentration of 9 g/L. These results are quite contradictory to those obtained by Regragui & Lahlou, (2005) who performed the experiments to test the effect of salinity on *in vitro Trichoderma harzianum* with regards to the above said enzymes that confer the organism with the antagonistic activity. They found that the salinity tends to slow down the release of antifungal metabolites, but the quantity is sufficient to inhibit the growth of pathogen. From our studies, we found that there was an increased production of antifungal metabolites at higher salinity, which contradicts their results.

Analyzing the results of *Streptomyces* sp IF5, we found that the maximal production of each enzyme was over a wide range of NaCl concentration. While we can easily understand that the production of proteases and cellulases are favored at low NaCl concentration (1 g/L),

Table 5. Effect of varying concentrations of NaCl on Enzymes production in *Trichoderma viride* (values given as Mean of triplicates±SD)

Concentration of NaCl (g/L)	OD Measurements			
	Cellulase	Chitinase	Glucanase	Total Protein
Control	0.68± 0.1268	1.4893± 0.0213	0.4857±0.0480	0.95 ±0.01633
1	0.4300±0.0408	0.7990±0.0304	1.0877± 0.1220	1.6500±0.0989
3	0.3483± 0.0157	0.6320±0.0088	1.0920± 0.0768	1.2737±0.1963
5	0.3130± 0.0171	0.3817±0.0045	1.0513±0.0767	1.2000±0.0294
7	0.3083± 0.0097	0.4847±0.0164	1.0327±0.0564	1.2057± 0.0303
9	0.3080± 0.0127	0.9420±0.01512	0.8823±0.1842	1.1120±0.0033

Table 6. Effect of varying concentrations of NaCl on Enzymes production in *Streptomyces* IF5 (values given as Mean of triplicates±SD)

Concentration of pesticide(µL)	OD Measurements			
	Cellulase	Chitinase	Glucanase	Total Protein
10	0.6143 ±0.3095	0.5163±0.0992	1.2970± 0.1372	1.2640±0.0843
20	0.9873± 0.0930	0.5023±0.0099	1.3960± 0.0187	1.066±0.4229
30	0.8543± 0.2084	0.3537±0.1651	1.4530±0.1938	1.2257±0.0495

Table 7. Effect of varying concentrations of Endosulfan on Enzymes production in *Trichoderma viride* (values given as Mean of triplicates±SD)

Concentration of pesticide(µL)	OD Measurements			
	Cellulase	Chitinase	Glucanase	Total Protein
10	1.6933 ±0.3215	1.0733±0.0366	0.9767± 0.0169	1.4560±0.0016
20	1.3400± 0.0572	0.8776±0.1089	0.8333± 0.0170	1.3400±0.0572
30	1.6900± 0.0216	0.8833±0.0931	0.8700±0.0216	1.6900±0.0216

glucanases require a slightly higher concentration (3 g/L) and chitinases were produced in maximum concentration of NaCl (9 g/L). Based on the present findings, we conclude that *Streptomyces* IF5 could act as a bio-control agent at a wide range of salt concentration. In other words, it is salt-tolerant and can withstand the salt stress (Table 5 & 6).

Effect of pesticides

The bio-control agent *Trichoderma viride* was able to resist the pesticide endosulfan at low concentrations and produce the antagonistic enzymes in its presence too (Table 7). This is because of the proteases, peroxidases, hydrolases produced by the organism which help to degrade the pesticide and rendering them harmless. The result thus obtained was congruent with those obtained in the study conducted earlier by Katayama & Matsumura, (1991) where they have indicated the role of *Trichoderma* sp in degrading several synthetic dyes, pentachlorophenol, endosulfan etc.

The production of various anti fungal enzymes by *Streptomyces* IF5 also occurs in the presence of pesticides (Table 8). It is postulated that the growth of *Streptomyces* IF5 in the presence of pesticides is (are) due to the various primary and secondary metabolites which possess a degrading action. However, investigations need to be done.

CONCLUSION

While assessing the efficacy of the conventional biocontrol agent *Trichoderma viride* and the novel *Streptomyces* IF5, we come across many evidences that prove that *Streptomyces* IF5 is comparatively a better biocontrol agent. From the several tests performed to find out the effect of pH, salt concentration, heavy metals and pesticide Endosulfan, we found that the activity of enzyme obtained from *Streptomyces* IF5 was significantly higher than that obtained from *Trichoderma viride* in all the above mentioned stress conditions. Also the novel species could tolerate severe environmental stresses, while the conventional bio control agent succumbed to it. Quantitative studies also give an indication that the novel species has an edge over the traditional biocontrol agent, reiterating that *Streptomyces* IF5 is a better biocontrol agent compared to conventional *Trichoderma viride*. The mode of application, mass cultivation of the novel species needs to be investigated further.

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REFERENCES

1. Amina Regragui, Houria Lahlou. Effect of salinity on in vitro *Trichoderma harzianum* antagonism against *Verticillium dahlia. Pakistani Journal of Biological Sciences,* 2005; **8**(6): 872-876.
2. Bancroft, J. Report of the board appointed to inquire into the cause of disease affecting livestock and plants. In: Votes and Proceedings, 1876; **3**: 1011-1038.
3. Carla da Silva Sousa, Ana Cristina Fermino Soares, Marlon da Silva Garrido. Characterization of *Streptomycetes* with potential to promote plant growth and bio-control, *Sci. Agric*. (*Piracicaba, Braz.*), 2008; **65**(1):50-55.
4. Crawford,D.L,Lynch, J.M.,Whipps, J.L. Ousley, M.A. Isolation and characterization of actinomycete antagonists of fungal root pathogens. *Applied and Environmental Microbiology*, 1993; **59**: 3899-3905.
5. Dishant Kumar and RK.Gupta; Actinomycetes as Bio-control Agents. *Indian Journal of Biotechnology,* 2006; **5**: 20-25.
6. Ghose T.K. Measurement of cellulose activities, *Pure & App. Chem*., 1987; **59**(2): 257-268.
7. Jayapradha Ramakrishnan, Hariram Balakrishnan, Selvaraj Thirupathi Kumara Raja, Natarajan Sundararamakrishnan, Sadagoban Renganathan,Venkatesh Nagarajan Radha. A novel *Streptomyces* sp for use as microbial feed and tanning industry: Mitigation of Environmental Pollution. *Brazilian journal of Microbiology* 2011; **42**(3)
8. Jayapradha Ramakrishnan, Murugesh Shunmugasundaram, Mahesh Narayanan. *Streptomyces* sp. SCBT isolated from rhizosphere soil of medicinal plants is antagonistic to pathogenic bacteria. *Iranian journal of biotechnology*, 2009; 7(2): 75-81.
9. Jayaswal.R.K, Rajesh Singh, Youn Su Lee. Influence of physiological and environmental factors on growth and sporulation of an

antagonist strain of *Trichoderma viride* RSR7. *Mycobiology*, 2003; **31**(1): 36-41.

10. Katayama, A., and F. Matsumura**.** Photochemically enhanced microbial degradation of environmental pollutants. *Environ. Sci. Technol*., 1991; **25**: 1329-1333.
11. László Kredics, Zsuzsanna Antal, László Manczinger, András Szekeres, Ferenc Kevei and Erzsébet Nagy. Influence of Environmental Parameters on *Trichoderma* Strains with Bio-control Potential. *Food Technol. Biotechnogy.*, 2003; **41**(1): 37-42.
12. Mandels, Resse. Induction of cellulase in *Trichoderma viride* as influenced by carbon sources and metals, *J.Bacteriology*, 1957; **73**: 268-278
13. Mausam Verma , Satinder K. Brar , R.D. Tyagi , R.Y. Surampalli , J.R. Val´ero. Antagonistic fungi, *Trichoderma* spp.: Panoply of biological control. *Biochemical Engineering Journal*, 2007; **37**: 1-20
14. Mehrotra R.S and Aggarwal R. Plant Pathology Second Edition, Tata McGraw Hill, 2003; India.
15. Morley GF,Gadd GM. Sorption of toxic metals by fungi and clay minerals, *Mycol. Res.*, 1995; **99**: 1429-1438
16. Oerke E. C. and Dehne H. W. (2004) Safeguarding production—losses in major crops and the role of crop protection. *Crop Protection* 2004; **23**: 275-285.
17. Siegrid Steinkellner, Roswitha Mammerler, Horst Vierheilig. Germination of *Fusarium oxysporum* in root exudates from tomato plants challenged with different *Fusarium oxysporum* strains *European Journal of plant pathology*, 2008; **122**(3): 395-401
18. Subasioglu .T. and Cansunar .E. Optimization of Culture Conditions and Environmental Factorsof Dextranase Enzyme Produced by *Paecilomyces lilacinus*Hacettepe. *J. Biol. & Chem.,* 2008.
19. Tahtamouni M. E. W. , Hameed K. M. and Saadoun I. M. Biological Control of Sclerotinia sclerotiorum Using Indigenous Chitinolytic Actinomycetes in Jordan, 2010.
20. Upadhyay, R. S. and Rai.B. A note on the distribution of *Trichoderma* in Indian soils *Acta Botanica Indica,* 1978; **6**: 196-198
21. Upadhyay, R. S and Rai, B. Ecological survey of Indian soil fungi with special reference to *Aspergilli*, *Penicillia* and *Trichoderma. Revue de Ecologie et Biologie du Sol ,* 1979; **16**: 39-49
22. USA EPA - http://www.epa.gov/oppbppd1/biopesticides/whatarebiopesticides.htm