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INDUCTION OF BENLATE RESISTANCE FOR IMPROVED CELLULASE PRODUCTION IN *TRICHODERMA HARZIANUM RIFAI*

by

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ABSTRACT

The quality and quantity of cellulase enzymes of *Trichodermaa* spp. can be improved by induced mutagenesis. In this study UV light was used to induce such mutations in *T. harzianum* isolated from Hingyou, Ifugao which was found to be naturally resistant to fungicide cupravit. Spores from one week old cultures at 10^1 dilution were plated in sterile coconut water agar and then exposed to UV light for 50 minutes. Spores of surviving colonies were isolated and further exposed to UV for 100 minutes. Survivors were reexposed for another 100 minutes. The best growing isolate that survived this treatment (a total of 250 minutes of UV exposure) was screened for resistance to benomyl, the active ingredient in the fungicide benlate. A highly resistant strain, HUV 250, was obtained. It survived up to 562 ug benomyl/ml, a concentration three times that of the full dosage recommended for this fungicide. The parental strain, isolate from 50 minutes exposure (HUV 50), and isolate from 150 minutes exposure (HUV 150) were found to grow only in a medium with benomyl at a concentration not higher than 1 ug/ml. Tests indicated the resistance in HUV 250 was maintained even if it was kept in benlate free medium for seven months.

A comparison of the cellulases from crude enzyme extract obtained through ammonium sulfate precipitation of the parental strain and those of HUV 250 was done. Avicelase and B-glucosidase activities of the new strain were significantly higher than those of the parental. On the otherhand protein contents of the extract from parental strain were significantly higher than those of the new strain. CMC-ase and filter paper activities of the parental strain were higher than those of the benlate resistant strain though differences were not significant. HUV 250 grown in coconut water medium produced spores within 24 hours and sporulated more heavily than the parental strain.

Introduction

The cellulase producing fungus, *T. harzianum* Rifai is the subject for genetic improvement schemes because of its use as activator in compost making (Cuevas, 1987), as a biocontrol agent of several soilborne pathogens (Baker, 1989 and Papavizas, et. al., 1982) and as a potential alternative source of et. al., 1982) and as a potential alternative source of cellulase in countries where *T. reesei*, the more active cellulase producer of the genus, is not indigenous. Strategies for improvement include selection, induced mutagenesis, protoplast fusion and gene manipulation (Papavizas, 1982).

New biotypes of *T. harzianum* tolerant to benomyl and with enhanced biocontrol capabilities of *R. solani*, *P. ultimum* and *S. ceprivorum* have been produced by Papavizas et al. (1982) using UV light mutagenesis. Ahmad and Baker (1987) also generated benomyl resistant strains in this species using the chemical mutagen N- methyl-N'-nitrosoguanidine (NTG). These strains had significantly higher cellulase activities and increased competitive saprophytic ability on cellulose rich substrates. Prolonged exposure to increasing concentrations of fungicide generated resistance of *T. harzianum* to chlorothalonil, procymidone, iprodione vinclozolin (Abd-el-Moity et al. 1982) and cupravit (Tancongco, et al. 1990). In the former an iprodione-tolerant strain reduced *S. cepivorum* infection while in the latter increased cellulase activity was reported (Tancongco et al., 1990, Cuevas et. al., 1991). It appears then that induction of fungicide resistance is linked to increased cellulase activity and biocontrol ability of *T. harzianum*.

This study was undertaken primarily to improve the cellulase activity of *Trichoderma harzianum* strains in the Philippines to produce potential compost activators for the National Program for Rapid Composting. Nonetheless, induction of fungicide resistance is useful not only to serve as a marker for enhanced cellulase activity but also to provide genetically marked strains for future protoplast fusion work.

Materials and Methods

Mutagenesis

A cupravit (a fungicide) resistant wild strain of *Trichoderma harzianum* from soil collected from Hingyon, Ifugao was chosen as parental strain. One ml of spore suspension at 10^1 dilution from a one week old culture was plated into coconut water-agar medium (CWA). This medium is cheap, easy to prepare, readily available and gave twice as much biomass as that of GAE (Glucose-asparagine-yeast extract), a chemically defined medium for *Trichoderma* (Benitez et al. 1975). Plates were exposed to UV light (15 watts Panasonic Germicidal UV lamp G15T8) at a distance of 43 cm for 50, 100 and another 100 min successively. Five surviving colonies were randomly picked from each exposure and plated for the next UV treatment.

Screening for Benomyl Resistance

Isolates from the final UV exposure (total of 250 min) were screened for resistance to benomyl [(methyl-butylcarbamoyl) 2 benzimidazole carbamate] by plating 1 ml of 10^6 dilution of spores to CWA medium. The benlate used (Du Pont, 50% benomyl/weight) has a recommended dosage of 187.50 µg benomyl/ml. Initial screening was at 2.5% (4.685 µg/ml benomyl) and 5% (9.375 µg/ml benomyl) of recommended dosage. The best growing isolate (HUV 250) of the 3 surviving colonies at 5% was further tested for resistance at increasing benlate concentrations. One ml of 10^6 dilution of spores from one week old cultures of HUV 250 was plated into each of 3 replicates per benlate concentration tested.

Parental strain, isolate from 50 min UV exposure (HUV 50) and isolate from 150 min UV exposure (HUV 150) were also tested for benomyl resistance at 0.5% (1.0 µg/ml benomyl), 1% (1.87 µg/ml benomyl) and 5% (18.75 µg/ml benomyl) concentration.

Test for Stability of Resistance Trait

HUV 250 was cultured in CWA slants. At regular intervals five culture slants were tested for resistance to benlate at the recommended dosage. One ml of a 10^6 dilution of spore suspension from each slant was plated into three replicates. Colonies were counted after eight days.

Cellulase assays

The two organisms, parental and HUV 250 were grown separately in sterile rice straw-rice bran medium at slightly acid pH for 14 days. After incubation, mycelia of the organisms were removed and extracellular enzymes secreted in the medium were then concentrated by ammonium sulfate precipitation. The prepared crude enzyme extracts were lyophilized and evaluated in Sephadex G-100-50 column following the methodology previously described by our group (Cuevas, et al., 1991). Each fraction was assayed for different cellulase enzymes.

Four different assays were conducted, a) avicelase using 2%, avicel microcrystalline cellulose as substrate, b) CMC-ase using 1% carboxy methyl cellulose as substrate, c) β-glucosidase using 1% salicin as substrate, and d) filter paper activity using Whatman filter paper as substrate. Enzymatic activities were measured in terms of International Units (I.U.). The procedures followed in these assays were those that were previously described by our group (Cuevas, et al., 1991).

Results and Discussion

Mutagenesis

The number of colonies that survived per UV exposure treatment is presented in Table 1. From the data, it may appear that many spores survived UV exposure as reflected in the number of colonies that developed in the plates. However, since platings were done at 10^1 dilution, the number of spores exposed ran into millions. The parental species is fast growing and in seven days cultures were heavily sporulated, displaying a dark green color. The first 50 min UV exposure yielded only nine colonies indicating the lethal effects of irradiation. Normally the exposed strain would have 257 x 10^6 colonies/plate in CWA medium. The number of viable spores were 130 and 56 respectively for the two 100 min UV exposure.

Table 1. Number of colonies that grew per exposure to UV light

Length of exposure to UV (mins)		*Number of isolates from previous treatment used for plating*	*Total number of colonies developed per unit time of UV exposure (5 reps/isolate)*
I	50	-	9
II	100	5	130
III	100	5	56
Total	250		
Control (unexposed to UV)			2.57 x 10^8

Growth Rate

Microscopic examination revealed no morphological distinctions between HUV 250 and the wild type *T. harzianum* Hingyon strain. Rate of growth (Table 2A) was also the same for both organisms when cultured in CWA. However, HUV 250 started spore formation within 24 hours and sporulated more heavily than the parental strain. On the other hand, the parental strain accumulated more biomass in liquid medium after 5 days incubation (Table 2B).

Table 2. A. Growth of *T. harzianum* wild type and HUV 250 in coconut water medium

	Colony diameter (cm)					
	24 hours		*48 hours*		*72 hours*	
	Parental	*HUV 250*	*Parental*	*HUV 250*	*Parental*	*HUV 250*
R1	2.1	1.9	6.2	6.5	9.0	9.0
R2	2.0	2.4	6.4	7.3	9.0	9.0
R3	1.7	2.0	6.1	7.0	9.0	9.0
R4	1.8	1.9	6.0	6.6	9.0	9.0
R5	2.2	1.7	6.5	6.5	9.0	9.0
mean	1.96	1.98	6.25	6.72	9.00	9.00

B. Mycelial dry weight in coconut water

	Dry weight after 5 days incubation (mg)	
	Parental	*HUV 250*
R1	213.7	150.5
R2	262.9	146.3
R3	192.8	135.4
R4	181.2	170.0
mean	182.2	150.6

Screening for Benomyl Resistance

HUV 250, was chosen as the test organism since it could grow in CWA with 9.3 µg benomyl/ml when its spores were plated in this medium. The parental strain, isolate from 50 min UV exposure (HUV 50), and isolate from 150 min UV exposure (HUV 150) grew only in CWA with 1.0 µg benomyl/ml (0.5% conc). Growth of the three organisms in coconut water with 1.0 µg benomyl/ml was almost equal with their growths in plain coconut water (Fig. 1).

It is interesting to note that the wildtype strain can tolerate 1.0 µg benomyl. This is the first report of such tolerance to benomyl of a wild strain of *Trichoderma*. It was mentioned earlier that this wildtype has also a natural resistance to the fungicide cupravit. The tolerance to different fungicides of the wildtype strain in this study may be attributed to wide and indiscriminate use of pesticides in the vegetable-growing region of the Cordilleras where this strain was isolated. The presence of the fungicides in the soil has exerted a natural selection pressure for the development of fungicide resistance among the fungi naturally occurring in the area. This phenomenon is quite alarming. This tolerance to fungicides by saprophytes like *Trichoderma* can also be true for fungal pathogens. One consequence of such developed tolerance of pathogens would be the ineffectiveness of these fungicides as crop protection agents.

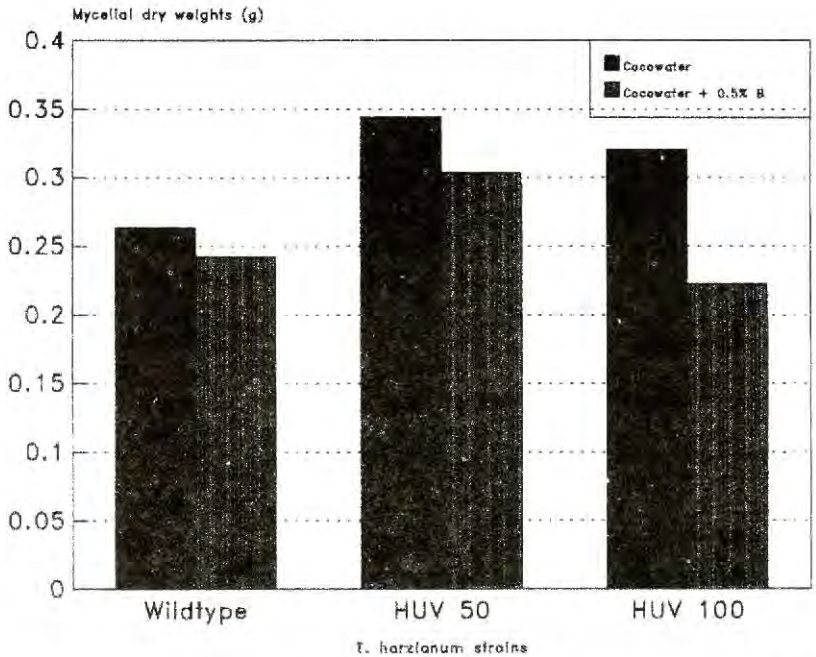

Figure 1. Growth of three strains of T. harzianum in coconut water medium with benomyl (B).

The results of plating of HUV 250 at various concentrations of the fungicide are presented in Table 3. Statistical analysis based on Duncan's Multiple Range Test (DMRT) indicated that the number of colonies of HUV 250 growing at 1.87, 9.37 and 18.75 µg benomyl/ml concentrations are not statistically different from its growth in the absence of benomyl control treatment (Table 4). These results showed that these low concentrations of benomyl had no effect on HUV 250. A slight decrease in the number of colonies growing in benomyl was observed, starting at concentrations from 23.40 µg/ml up to 187.50 µg/ml (recommended full dose of the fungicide). The number of colonies growing at this range of concentration of benomyl were not statistically significant. Thus the full dose of the fungicide can only slightly inhibit the growth of HUV 250. These results showed that this strain is truly resistant to the fungicide.

A drastic decrease in the number of viable spores began when this isolate was plated at 206.25 to 375.00 µg/ml (LD50). The latter concentrations were twice the full dose of the fungicide. It therefore takes twice the recommended dose of benomyl to effect severe inhibition of growth. At 458.75 and 562.5 µg/ml growth was even more reduced at these concentrations which are three times the recommended dosage. There was still growth of the fungus in every replicate but at an average of 4-6 colonies/plate.

Table 3. Number of colonies growing in increasing benomyl concentration of one ml spore suspension at 10^6 dilution of strain HUV 250.

	DMRT grouping		*ug benomyl/ml*	*Ave. no. of colonies x 10^6 (3 replicates)*
I.	Concentrations where differences in growth were not statistically different from the control		0	314
			1.87	458
			9.37	465
			18.75	297
II.	Growth not statistically different from full dosage		23.40	183
			28.10	220
			32.80	168
			37.50	140
			46.90	131
			56.25	143
			65.60	139
			75.00	121
			93.75	174
			112.75	153
			131.25	187
			150.00	186
			168.75	139
		*	187.50	145
III.	Growth not statistically different from growth in 2x full dosage		206.25	39
			225.00	61
			243.75	55
			262.50	32
			282.25	31
		**	375.00	22
IV.	Growth not statistically different from growth in 3x dosage		468.75	6
		***	562.50	4

* recommended full dosage of fungicide
** 2x full dosage
*** 3x full dosage

Table 4. Number of colonies growing in 187.50 µg benomyl/ml concentration of 1 ml spore suspension at 10^6 dilution of HUV cultured in coconut water agar medium (CWA).

Age of culture in CWA before exposure to benomyl (months)	*Ave. number of colonies x 10^6 (3 replicates)*
0	145
1	101
2	118
7	68

The resistance of HUV to benomyl is very high since the parental strain never grows at 1 µg/ml benomyl. This level of resistance is comparable to, if not even better than that in Papavizas' review (1985) wherein he reported that *T. harzianum* induced mutants (by UV or NTG) tolerated between 50-500 µg benomyl/ml. It is noteworthy that no benomyl resistant strain was ever obtained by prolonged exposure to increasing fungicide concentrations. In Papaviza's report it was mentioned that no benomyl-resistant strain was ever obtained by prolonged exposure to increasing fungicide concentrations, but this was disproved by the present study. Papavizas pointed out that UV-induced mutants are either selections of spores resistant to irradiation or chromosomal mutations. The fact, however, that the parental strain exhibit very minimal resistance to benomyl and that very high resistance was developed only after irradiation strongly argue for these strains being mutants and not merely selections.

Stability of Resistant Trait

Although fungicide resistance can be attributed to non-genetic adaptation (Abd-El-Moity et al. 1988), this is usually lost if the strain is cultured in a fungicide free medium. HUV 250 still retains resistance to benomyl after culturing in benomyl free medium for seven months (Table 4). An average of 101 colonies/plate grew in full dosage of the fungicide after the first month and 118 colonies after two months of culture in benlate-free medium. However, 68 colonies were monitored after the seventh month of incubation. The lower number of viable spores after seven months of incubation can be attributed to drying out of the culture medium rather than to the loss of resistance of the organism to benlate. The culture slants were incubated at room temperature, 28-30°C and drying of agar resulted after six months exposure to these temperatures. These results point out that the resistant character of HUV 250 is stable.

Cellulase of HUV 250 and the Parental Strain

Benomyl resistance is closely linked to high cellulase activity. Ahmad and Baker (1988), found that 95% of their NTG induced benomyl resistant strains had significantly higher cellulase activity than the wild type, suggesting a possible close linkage of regulatory genes for benomyl tolerance and cellulase production. High cellulase activities were also observed in another *T. harzianum* strain resistant to cupravit, another fungicide (Cuevas, et al., 1991; Tancongco, et al., 1990).

Catalytic activities measured in terms of International Units (I.U.) of the components of the cellulase enzyme complex of the parental strain and those of HUV 250 are presented in Tables 5 and 6. The activities of B-glucosidase and avicelase enzymes of HUV 250 were much higher than the activities of the corresponding enzymes in the parental strain (Figs. 2a and 2b). The observed differences were statistically significant (Appendix Table 2). However, the CMC-ase and filter, paper activities of the parental strain were higher than the activities of the corresponding enzymes of HUV 250. The protein contents of the parental strain were also higher (Figs. 2c and 2d). The observed differences though were not statistically significant. These results on the analysis of the cellulase enzyme components of HUV 250 and the wild type further proved that these two organisms are distinct.

Table 5. CMCase, B-glucosidase, avicelase, and filter paper activities (I.U.) of fractions collected by gel chromatography on Sephadex G-100 of freeze dried samples of *T. harzianum* from Hingyon, Ifugao naturally resistant to fungicide cupravit.

Fraction No.	*Protein*	*CMCase*	*B-glucosidase*	*Avicelase*	*FP-activity*
1	0.012	0.0056	0.0286	0.0043	0
2	0	0	0	0	0
3	0.058	0.0654	0	0.0086	0
4	0.522	0	0.0344	0	0.0144
5	0.522	0.4265	0.1268	0	0.0144
6	0.461	0.4558	0.0979	0	0
7	0.594	0.5424	0.0806	0	0.0245
8	0.705	0.4154	0.0848	0	0.1096
9	0.862	0.4038	0	0	0
10	0.799	0.4420	0	0	0.0086
11	0.767	0.0691	0	0	0.0086
12	0.468	0	0	0	0
13	0.257	0	0	0	0.0072
14	0.156	0	0	0.0101	0.0014
15	0.081	0	0	0	0

Table 6. CMCase, B-glucosidase, avicelase, and filter paper activities (I.U.) of fractions collected by gel chromatography on Sephadex G-100 of freeze dried samples of *T. harzianum* with code HUV 250 grown in rice straw-rice bran medium

Fraction No.	*Protein*	*CMCase*	*B-glucosidase*	*Avicelase*	*FP-activity*
1	0.007	0.0748	0	0	0.0011
2	0.027	0.0402	0	0.0158	0.0057
3	0.006	0.0056	0.0864	0	0.0086
4	0.203	0.0157	0.1209	0	0.0231
5	0.189	0.0964	0.0848	0	0.0375
6	0.147	0.2595	0.1152	0.0288	0.0043
7	0.309	0.2133	0.1094	0.0216	0.0216
8	0.210	0.2076	0.2134	0.0418	0.0101
9	0.289	0.1499	0.1787	0.0274	0
10	0.339	0.1383	0.1614	0.0331	0
11	0.376	0.1152	0.5759	0.0173	0.0144
12	0.241	0.0806	0.1672	0.0129	0.0043
13	0.068	0.0633	0.1614	0.0361	0
14	0.118	0.0023	0.0691	0.0259	0
15	0.037	0.0229	0.2076	0.0562	0.0014

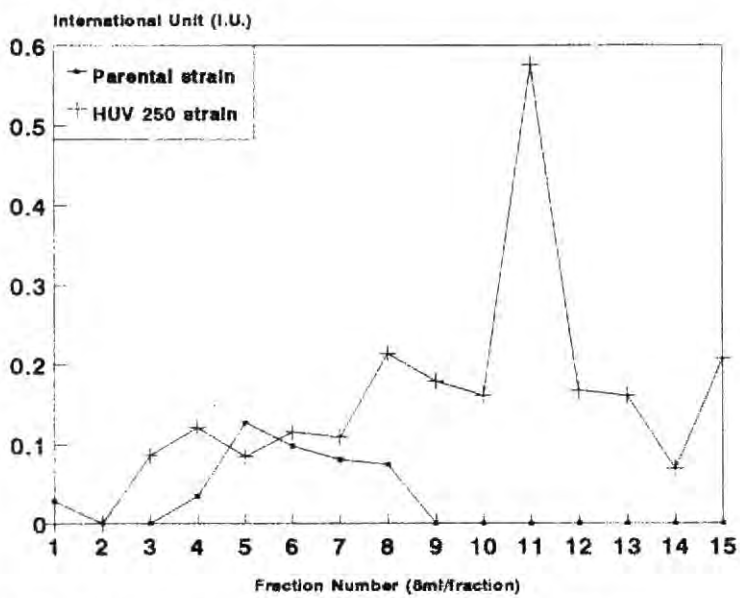

Figure 2A. B-glucosidase activities of HUV 250 and parental strain.

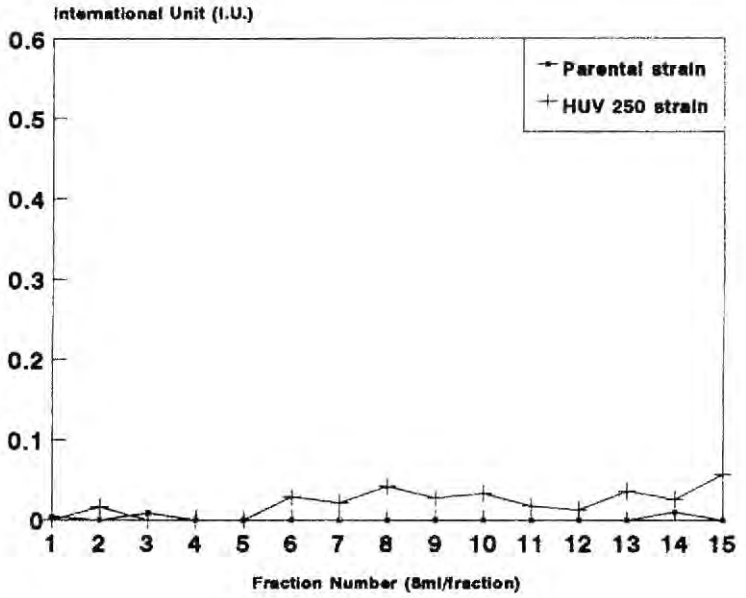

Figure 2B. Avicelase activities of HUV 250 and parental strain.

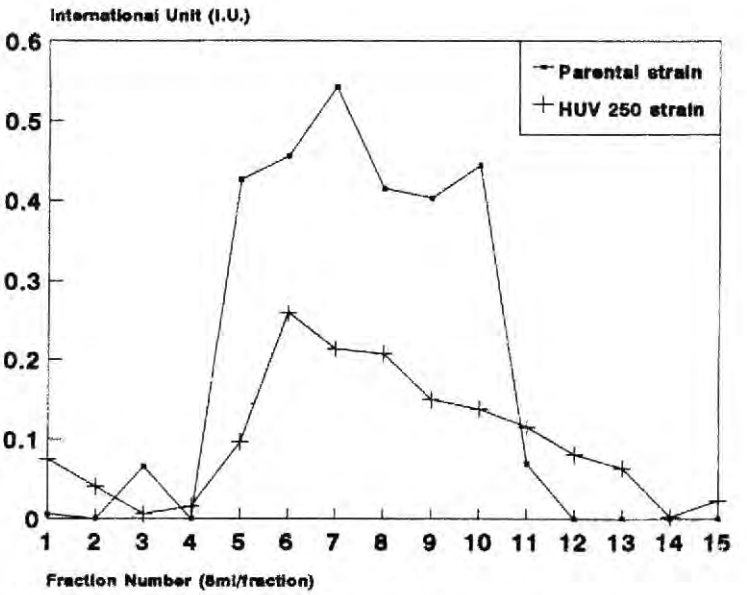

Figure 2C. CMC-ase activities of HUV 250 and parental strain.

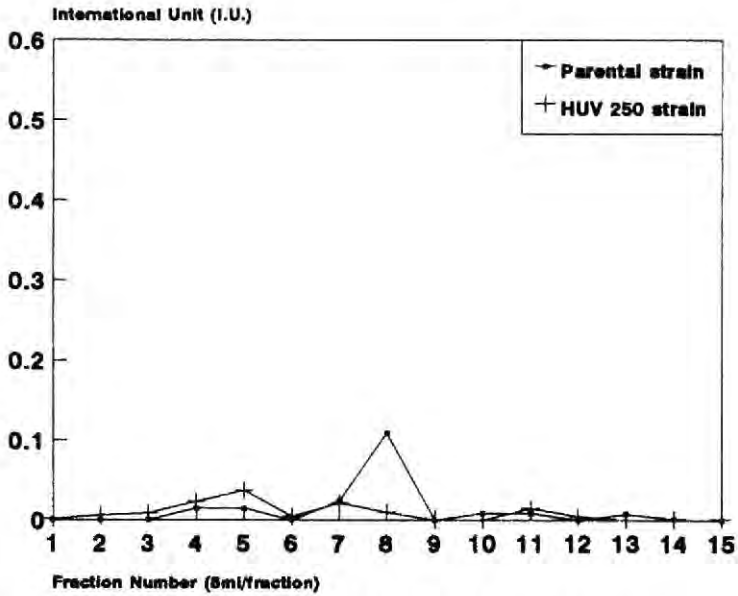

Figure 2D. Filter paper activities of HUV 250 and parental strain.

Appendix Table 1. Duncan's Multiple Range Test for number of colonies of *T. harzianum* that developed in plates with CWA medium with different concentrations of benomyl. Spores were diluted 10^6 times during the plating. Means with the same letters are not statistically significant.

Duncan Grouping	*Transformed values to log of mean number of colonies of the fungus growing at different concentrations of benomyl*	*N*	*Concentrations of benomyl - µg/ml*
A	6.141	3	9.375
A A			
A	6.116	3	1.875
A			
B A	5.721	3	0
B A			
B A	5.688	3	1.875
B			
B C	5.391	3	28.125

B	C				
B	C		5.230	3	131.25
B	C				
B	C		5.224	3	150
B	C				
B	C		5.193	3	23.4375
B	C				
B	C		5.157	3	93.75
B	C				
B	C		5.121	3	32.8125
	C				
	C		5.012	3	112.5
	C				
	C		4.967	3	187.5
	C				
	C		4.962	3	56.25
	C				
	C		4.938	3	37.5
	C				
	C		4.936	3	65.625
	C				
	C		4.933	3	168.75
	C				
	C		4.876	3	46.875
	C				
	C		4.788	3	75
	D		4.051	3	225
	D				
E	D		3.842	3	243.75
E	D				
E	D	F	3.611	3	206.25
E		F			
E		F	3.434	3	281.25
E		F			
E		F	3.311	3	262.5
E		F			
		F	3.070	3	375
	G		1.561	3	468.75
	G				
	G		1.290	3	562.5

Analysis of Variance

Source	*DF*	*Sum of Squares*	*Mean Square*	*F value*	*Prob>f*
Model	1	101.89909	101.89909	449.374	0.0001
Error	76	17.23358	0.22676		
C Total	77	119.13267			

Root MSE	0.47619	R-square	0.8553
Dep Mean	4.56008	Adj R-sq	0.8534
C.V.	10.44260		

Appendix Table 2. Analysis of variance for B-glucosidase, Avicelase, CMC-ase, protein, and filter paper activities of HUV 250 and parental strain.

Class	Levels	Values
S	2	250, Parental

Number of observations in data set = 30

Dependent variable: Protein

Source	*DF*	*Sum of Squares*	*Mean Square*	*F value*	*Pr >f*
Model	1	0.4502	0.4502	8.40	0.0072*
Error	28	1.4999	0.0536		
Corrected Total	29	1.9501			

R square	CV	Root MSE	PR mean
0.2308	78.4839	0.2314	0.2949

Source	*DF*	*Anova SS*	*MS*	*F value*	*Pr > F*
S	1	0.4502	0.4502	8.40	0.0072

Dependent variable: CMC-ase

Source	*DF*	*SS*	*MS*	*F value*	*Pr > F*
Model	1	5.5987	5.5987	2.02	0.01662[ns]
Error	28	77.5841	2.7708		
Corrected Total	29	83.1828			

R square	CV	Root MSE	PR mean
0.0673	114.5255	1.6646	1.4538

Source	*DF*	*Anova SS*	*MS*	*F value*	*Pr > F*
S	1	5.5987	5.5987	2.02	0.1662

Dependent variable: B-glucosidase

Source	*DF*	*SS*	*MS*	*F value*	*Pr > F*
Model	1	9.8350	9.8350	9.73	0.0042*
Error	28	28.3021	1.0108		
Corrected Total	29	38.1371			

R square	CV	Root MSE	PR mean
0.2579	109.0789	1.0054	0.9217

Source	*DF*	*Anova SS*	*MS*	*F value*	*Pr > F*
S	1	9.8350	9.8350	9.73	0.0042

Dependent variable: Avicelase

Source	*DF*	*SS*	*MS*	*F value*	*Pr > F*
Model	1	0.2879	0.2988	19.21	0.0001*
Error	28	0.4198	0.0420		
Corrected Total	29	0.7076			

R square	CV	Root MSE	PR mean
0.4069	108.1209	0.1224	0.1132

Source	*DF*	*Anova SS*	*MS*	*F value*	*Pr > F*
S	1	0.2879	0.2879	19.21	0.0001

Dependent variable: FP-activity

Source	*DF*	*SS*	*MS*	*F value*	*Pr > F*
Model	1	0.0076	0.0076	0.17	0.6829^{ns}
Error	28	1.2488	0.0446		
Corrected Total	29	1.2256			

R square	CV	Root MSE	PR mean
0.0061	192.2790	0.2112	0.1093

Source	*DF*	*Anova SS*	*MS*	*F value*	*Pr > F*
S	1	0.0076	0.0076	0.17	0.6820

Conclusion

Various studies are being done to improve genetically cellulase production of wild type strains of *Tichoderma* spp. One method is by induction of fungicide resistance since there are evidences to show that regulatory genes for fungicide resistance are linked with cellulase enzymes. In this study, we were able to show that UV irradiation is a very simple and non-hazardous way of inducting benlate resistance in *T. harzianum*. The resulting benlate resistant strain had improved B-glucosidase and avicelase activities, two enzymes which *Trichoderma* species are known to have very minimal activities. Although we have not produced a hyper cellulase producer strain this new strain can be further improved by other methods i.e. protoplast fusion with the parental strain which has good CMC-ase and filter paper activities.

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