Indian J.Sci.Res. 7(2) : 7-13, 2017 ISSN : 0976-2876 (Print)
ISSN : 2250-0138 (Online)

INDUCTION OF MUTANT STRAINS OF *Trichoderma viride* 1433 FOR BIOCONTROL OF *Fusarium oxysporum f.* sp. *lycopersici*

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ABSTRACT

Twenty two mutants of *Trichoderma viride* 1433 were obtained after treatment with NTG which were further screened against *Fusarium oxysporum f. sp. lycopersici* by dual culture method. All the mutants indicated high sporulation count and better growth patterns as compared to wild type but only four mutants showed enhanced antagonistic activity against *Fusarium oxysporum f. sp. lycopersici* in colony plate assay as compared with the wild type and control. Since mycoparasitism plays important role in antagonism mechanism of *Trichoderma* species, extracellular enzymatic activity of mutant strains was also assayed. The result of extracellular enzymatic activity showed that cellulase and β 1, 3-glucanase significantly increased in the mutant strains while protease activity was found suppressed in case of two mutant strains as compared to wild type. Among the mutant strains Tv m9 was found as most potent strain against *Fusarium oxysporum f. sp. lycopersici*, therefore, this strain can be studied in glasshouse experiment against the above pathogen.

KEYWORDS : *Trichoderma viride* 1433, Mutant Strains, *Fusarium oxysporum f.* sp. *lycopersici*

Fusarium oxysporum [(Schlecht.)*f. sp. lycopersici* (Sacc.)] W.C. Synder and H.N. Hans, is the most prevalent serious diseases of tomato (Reis et al., 2005; Sudhamoy et al., 2009). The pathogen occurs throughout most tomato-growing worldwide causing a vascular wilt that can severely affect the crop (Moretti et al., 2008), and the disease is considered as one of the main soil-born systemic diseases (Schwarz and Grosch 2003). It causes significant losses in tomato production both in greenhouse and field (Nusret and Steven 2004; Asha et. al., 2011). The most common means to check the disease in plants is by using fungicides. Frequent use of these chemicals leads to environmental pollution. The increasing awareness of fungicide-related hazards has emphasized the need of adopting biological methods as an alternative disease control method. Species of the genus *Trichoderma* are well documented fungal biocontrol agents (Lewis and Papavizas, 1991; Haran et al., 1996a; Haran et al., 1996b; Elad, 2000; Howell, 2002; Joshi et al., 2010; Hermosa et al., 2012). Extracellular enzymes produced by *Trichoderma* species are considered important determinants of the antagonistic ability of these fungi (Thrane et al., 2000). Although wild strains of various *Trichoderma* spp. have shown considerable effect in the biocontrol of plant diseases, efforts have been made in the last few decades to develop more effective strains through mutagenesis (Kredics et al., 2003; Papavizas et al., 1982; Mandels et al., 1971; Zaldivar et al., 2001). In view of the above, the present study was carried out to develop potent mutant strains of *Trichoderma* viride 1433 for enhanced antagonism against *Fusarium oxysporum f. sp. lycopersici*.

MATERIALS AND METHODS

Fungal Isolates

The pure culture of *Trichoderma* viride 1433 was obtained from the culture collection of Institute of Microbial Technology (IMTECH), Chandigarh. A virulent strain of *Fusarium oxysporum f. sp. lycopersici* was obtained from the Department of Mycology and Plant Pathology, Institute of Agriculture Science, Banaras Hindu University (BHU), Varanasi. The pathogenic and antagonistic strains were maintained on Potato Dextrose Agar medium (PDA; Merk) at 25 ± 2°C by regular subculturings.

Induction of mutant through N'-methyl-N'-nitro-N'-nitrosoguanindine (NTG) treatment

Mutagenesis of *T. viride* 1433 by the treatment with N'-methyl-N'-nitro-N'-nitrosoguanidine (NTG) was followed by the method of Chadegani and Ahmajian (1991) with some modifications. Conidial suspension of four-day old culture of *T. viride* 1433 was prepared in 5.0 ml sterile 0.1M sodium citrate buffer (pH 5.6), centrifuged twice at 10,000 rpm and subsequently washed with the same buffer. The spore concentration was adjusted to $1x10^5$spores/ml in sodium citrate buffer. A stock solution of NTG (1mg/ml)

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was prepared in sodium citrate buffer and the final concentration used was 50µg/ml of spore suspension. The NTG treated spore suspensions were incubated at 37^0C in a shaking water bath in cool light for 10-90 min in order to achieve 5-10% viability. At selected intervals mutagenesis were stopped by passing entire sample through a 0.45µm Millipore filter, washing the spores with 0.1M phosphate buffer (pH 6.0). The few pinhead colonies of treated spores that developed were picked-up and inoculated on minimal medium for colony forming units.

Selection of Mutants

The mutants obtained after the treatment with NTG was selected on PDA medium on the basis of colony growth, screening against *Fusarium oxysporum f. sp. lycopersici* and sporulation count. These mutants were compared with the parental isolate for the next seven to eight generations. About twenty two mutants showed increased growth rate and high sporulation count in comparison to wild type but only four mutants showed enhanced antagonistic activity against *Fusarium oxysporum f. sp. lycopersici* in colony plate assay as compared with the wild type and control. The stable desired colonies of mutant *Trichoderma* strains were transferred on PDA slants and maintained at 25°C.

Colony growth inhibition assay

In vitro antagonistic activity of wild type and mutant strains of *Trichoderma viride* 1433 against *Fusarium oxysporum f. sp. lycopersici* was studied in dual culture by followed the method described by Upadhyay and Rai (1987). The colony interactions were assayed as percent inhibition of the radial growth by the following formula (Fokkema, 1976); R1-R2 / R1x100, where, R1 denotes diameter of the radial growth of the pathogen towards opposite side and R2 denotes the radial growth of the pathogen towards the antagonist. The experiment was conducted in three replicates.

Comparative sporulation count of wild type and selected mutant strains

The estimation of spore production of the wild type and mutant strains of *T. viride* 1433 was done using the method described by El-Abyad et al. (1983). After incubating the inoculated Petri dishes at 25 ± 2°C for 7 days, one square cm block which was cut from the margin of the actively growing colony of isolates of wild type and mutant strains of T. viride 1433 was transferred to a test tube containing 2.5 ml of sterile distilled water. The tube was shaken continuously for 5 minutes. The density of spores was then counted using a haemocytometer under high power (x450) in a Nikon Trinocular Microscope (Model E-600).

Assay of enzyme activity of wild type and mutant strains of *T. viride* 1433

For assay of enzyme activity, *Trichoderma* species were grown on minimal synthetic medium (MSM) contained the following components (in grams per litre); $MgSO_4.7H_2O$, 0.2; KH_2PO_4, 0.9; KCl, 0.2; NH_4NO_3,1.0 ; $FeSO_4.7H_2O$, 0.002; $MnSO_4$, 0.002 and $ZnSO_4$, 0.002. The medium was supplemented with the appropriate carbon source (Cellulase assay Cellulose; 20g, β-1, 3 glucanase assay Glucose; 20g, Protease assay Peptone, 10g, Glucose; 20g). The pH was set to 6.3 with 50 ml phosphate buffer and autoclaved at 15 psi for 20 minutes. The medium was inoculated with a spore suspension to give a final concentration of $5x10^6$ conidia per millilitre and placed on a rotator shaker at 150 rpm at 25°C for different time intervals. The cultures were harvested at fourth day of incubation and were filtered through Whatman No. 44 filter paper and finally centrifuged at 12000 rpm for 10 min at 4 to get cell-free culture filtrate which were then used as enzyme source.

Cellulase Assay

Cellulase activity was assayed following the method of Miller (1959). The assay mixture contained 1 mi of 0.5% pure cellulose (Sigma Company) suspended in 50 mM phosphate buffer (pH 5.0) and 1 mi of culture filtrates of different *Trichoderma* strains. The reaction mixture was incubated for 30 min at 50°C. The blanks were made in the same way using distilled water in place of culture filtrate. The absorbance was measured at 540 nm and the amount of reducing sugar released was calculated from the standard curve of glucose. One unit cellulase activity is defined as the amount of enzyme that catalyzed 1.0 mol of glucose per minute during the hydrolysis reaction.

β- 1, 3 Glucanase Assay

β- 1, 3 glucanase was assayed similarly by incubating 1ml 0.2% laminarin (w/v) in 50mM sodium

acetate buffer (pH 4.8) with 1ml enzyme solution at 50 for 1h and by determining the reducing sugars with DNS (Nelson, 1944). The amount of reducing sugars released was calculated from standard curve for glucose. One unit of β-1, 3 glucanase activity was defined as the amount of enzyme that catalyzed the release of 1µmol of glucose equivalents per minute.

Protease Assay

Protease activity was determined by a modified Anson's Method (Yang and Huang, 1994). The substrate used (1% casein in 50mM phosphate buffer, pH 7.0) was denatured at 100 for 15 min in a water bath and cooled at room temperature. The reaction mixture containing 1ml of substrate and 1ml of enzyme solution were incubated at 37 for 20 min with shaking and was stopped with 3ml of 10% trichloroacetic acid (TCA). The absorbance of the liberated tyrosine in the filtrate was measured at 280 nm. One unit of protease activity was defined as the amount of enzyme that produced an absorbance at 280nm equivalent to 1µmol of tyrosine in one minute under the assay condition.

RESULTS

Generation of mutants through NTG treatments

The mutants of Trichoderma viride 1433 were generated to enhance biocontrol efficacy of antagonism against *Fusarium oxysporum f. sp. lycopersici*. Treatment of spores with NTG was much stable and generated the isolates which had high significant levels of production in spores, colony growth antagonistic ability. Finally, four isolates were obtained after a series of 7 to 8 serial transfer on PDA media. Twenty two mutants that were obtained after treatment of NTG with better colony growth and higher sporulation count were given the name as Tv m1, Tv m2, Tv m3, Tv m4……Tv m22.

In *vitro* screening of the wild type and mutant strains against *Fusarium oxysporum f. sp. lycopersici*

All the four mutants showed significantly ($P<0.05$) enhanced antagonistic activity against *Fusarium oxysporum f. sp. lycopersici*. In comparison to wild type (Table 1). The mutant Tv m9 exhibited maximum inhibition of the pathogen (93.6%) which was followed by Tv m13 (87.6%), Tv m21 (79.3%) and Tv m6 (78.4%). The least inhibition in the growth of the pathogen was depicted by the wild type (72.0%).

Phenotype variation and sporulating ability of wild type and mutant strains of *T. viride* 1433

Table 2 reveals the phenotypic variation and sporulating ability of the wild type and mutant strains of *T. viride* 1433. Pigmentation on reverse side of the culture medium was recorded in case of wild type and two mutant strains namely Tv m9 and Tv m13. The other mutant strains did not show any pigmentation in the reverse. Maximum spores (362.7×10^5) were produced in case of the mutant Tv m9. This was followed by mutant Tv m13 (309.8×10^5), Tv m21 (289.4×10^5) and Tv m6 (267.4×10^5). The least number of spores (258.2×10^5) was produced by wild type. It is clear from the table that there is a great degree of variation among the isolates in their sporulating ability.

Extracellular enzymatic activities of the wild type and mutant strains of *T. viride* 1433

The results of extracellular enzymatic activities of the wild type and mutant strains of *T. viride* 1433 have been shown in Table 3. The highest cellulase activity was produced by the mutant Tv m9 (3.7U/ml) followed by Tv m13 (2.7U/ml), Tv m21 (2.4U/ml) and Tv m6 (1.8U/ml).

Table 1 : In *vitro* Screening of the Wild Type and Mutant Strains of *T. viride* 1433 Against *Fusarium Oxysporum f. sp. lycopersici*

***Trichoderma* strains**	**Per cent Inhibition (*Fusarium oxysporum f. sp. lycopersici*)**
Tv m6	78.4
Tv m9	**93.6**
Tv m13	87.6
Tv m21	79.3
Tv 1433	72.0

Average of three replicates

Table 2 : Phenotypic Variation and Sporulating Ability of the Parent and Mutant Strains of *T. viride* 1433

Strains	Colony Morphology	Pigmentation	10^5 spores per ml
Tv m6	Colony-green, compact mycelium. Sporulation is dark green at the centre and light green towards periphery and white coloured conidiogenous pustules appear in a scattered manner	None	267.4
Tv m9	Colony-dark green, highly ramified, compact tufted mycelium with dark green conidiogenous pustules	Yellow	362.7
Tv m13	Colony-dark green, mycelium is sparsed with white and pinhead like conidiogenous pustules at the centre and periphery	Yellow	309.8
Tv m21	Colony light-green, mycelium is sparsed with dark green coloured conidiogenous fascicles	None	289.4
Tv 1433	Colony-light green, dense compact mycelium with dark green conidia	Yellow	258.2

Values are average of three replicates

Table 3 : Assay of Extracellular Enzyme Activities of the Wild Type and Mutant Strains of *T. viride* 1433

Trichoderma strains	Cellulose activity (U/ml)	β - 1, 3 glucanase activity (µg/ml)	Protease Activity (U/ml)
Tv m6	1.8ab±0.1	35.8b±0.3	293a±3.1
Tv m9	**3.7d±0.2**	**42.6e±0.2**	**348d±3.5**
Tv m13	2.7cd±0.3	40.6d±0.3	373e±2.4
Tv m21	2.4bc±0.2	37.6c±0.2	314b±2.0
Tv m1433	1.5a±0.1	33.6a±0.1	327c±2.9

Average of three replicates
Means in the column followed by same letter are not significantly different (P=0.05)

The rate of enzyme production in case of wild type was recorded minimum (1.5U/ml) as compared to other mutant strains.

All the mutants significantly produced the higher β- 1, 3 glucanase activity than the wild type. The maximum enzymatic activity was produced by Tv m9 (42.6µg/ml) followed by Tv m13 (40.6 µg/ml), Tv m21 (37.6 µg/ml) and Tv m6 (35.8 µg/ml). The least activity of β- 1, 3 glucanase (33.6 µg/ml) was measured in case of wild type.

The maximum protease activity was produced by the mutant Tv m13 (373U/ml) followed by Tv m9 (348U/ml). The other mutant strains Tv m21 (314U/ml) and Tv m6 (293U/ml) showed lower enzymatic activity as compared to the wild type (327U/ml).

DISCUSSION

Development of *Trichoderma* mutants toward suppression of fungal plant pathogen is an important method in strain improvement, which yields effective and reliable strains for biological control. After development of mutants, assessing the efficacy through various techniques is equally important for the suppression of the pathogen (Nakkeeran et al., 2005). Four mutants (designated as Tv m6, Tv m9, Tv m13 and Tv m21) were obtained that grew faster and sporulated better as compared to the wild type. These strains were generated as a result of mutation and not by selection due to fact that the mutant isolates were capable of growing and sporulating better even after 7 to 8 generations.

The result of the dual culture (Table 1) revealed that the highest mean inhibition values, above 90% were obtained due to Tv m9 followed by Tv m13. Antagonism of *Trichoderma* species against several pathogens has been reported (Chet and Baker, 1980; Papavizas, 1985; Elade, 2000; Howell, 2002). Formation of inhibition zone at the contact between *Trichoderma* and *Fusarium oxysporum f. sp. lycopersici* in dual cultures could be explained on the basis of production of volatile and non volatile metabolites as well as the production of extracellular enzymes by *Trichoderma* (El-Katatny, et al., 2001). In the present study mycoparasitic activity by coiling of hyphae was observed. The mycoparasitic activity of the *Trichoderma* species might be as a result of production of cell wall degrading enzymes (Di Pietro, et al., 1993; Schirmbock, et al., 1994).

The extracellular enzymes play a vital role in mycoparasitism and degrade the pathogen cell wall. The mycolytic activity of *Trichoderma* species is an important mechanism proposed to explain their antagonistic activity against soil-borne plant pathogenic fungi (Ridout, et al., 1986). The results (Table 3) showed that Tv m9 was efficient producer of extracellular enzymes such as cellulases, β-1, 3 glucanases and proteases. This might be one of the reasons for its biocontrol potentially. Hyper cellulase producing mutants were also developed by Hao, et al., (2006). The production of extracellular enzymes such as chitinases, β-1, 3 glucanases and proteases by Trichoderma species to degrade phytopathogen cell wall has been established (Elad, et al., 1982; Haran, et al., 1996a; Hjeljord and Tronsmo, 1998). As the cell wall of Fusarium species are composed of cellulose and β-1, 3 glucan (Bartinicki-Garcia, 1968), the enzymes, cellulase and β-1, 3 glucanases produced by Trichoderma might be involved in hydrolysis of *Fusarium oxysporum f. sp. lycopersici* cell wall during antagonism (Thrane, et al., 1997). The involvement of glucanases in mycoparasitism has been demonstrated by Lorito et al., (1994). Fungal cell wall also contains lipids and proteins (Hunsley and Burnett, 1970), therefore, antagonistic fungi synthesized proteases which might act on the host cell wall. The role of proteases in mycoparasitism has been reinforced with the isolation of new protease-overproducing strains of *T. harzianum* (Szekeres, et al., 2004). Although, Tv m9 produced lesser protease than Tv m13 (Table 3), the biocontrol potential of Tv m9 is maximum. The result is consistent with Rey, et al., (2001) who reported that *T. harzianum* mutant with low levels of protease activity was best biocontrol agent rather than the transformants with highest protease level. The reason was attributed to the high amounts of protein provoking toxicity in the transformants (Flores, et al., 1997). Papavizas (1985) correlated the lytic activity of *Trichoderma* on cell walls of phytopathogenic fungi to the degree of biological control of these pathogens in vivo. These enzymes lysed the host cell wall and leads to leakage of protoplasmic contents which are in turn used as food material for the proliferation of antagonists (Tronsmo and Harman, 1993).

In conclusion, mutant Tv m9 was more effective biocontrol agents as compared to the other strain. Tv m9 produced higher extracellular enzymatic activities and possessed high competitive saprophytic ability (CSA). Therefore, the mutant Tv m9 may be a promising biocontrol agent against *Fusarium oxysporum f. sp. lycopersici*.

ACKNOWLEDGMENT

Author is thankful to Ex Professor and Head, Dr. Bharat Rai, Department of Botany, Banaras Hindu University for providing necessary facilities and valuable supervision during the course of study.

REFERENCES

Asha B.B., Chandra N., Udaya Shankar A.C., Srinivas C., Niranjana S.R. 2011. Biological control of *F. oxysporum f. sp. lycopersici* causing wilt of tomato by *Pseudomonas fluorescens*. IJMR **3**(2): 79-84.

Bartinicki-Garcia S. 1968. Cell wall chemistry, morphogenesis and taxonomy of fungi. Annu. Rev. Microbiol. 22: 87-109.

Chadegani M. and Ahmadjian, V. 1991. Protoplast fusion between mutant strains of Microsporum gypseum. Mycologia, **83**(6): 779-786.

Chet I. and Baker, R. 1980. Induction of suppressiveness to Rhizoctonia solani in soil. Phytopathology, **70**: 994-998.

Di Pietro A., Lorito, M., Hayes, C., Broadway, K. and Harman, G. E. 1993. Endochitinase from Gliocladium virens; Isolation, characterization, synergistic antifungal activity in combination with gliotoxin. Phytopathology, **83**: 308-313.

El-Abyad M. S., Ismail, I. K. and Al. Mashhadani, S. 1983. Effects of some biocides on Fusarium oxysporum formae specials causing cotton and tomato wilt in Egypt. Trans. Br. Mycol. Soc., 80: 283-287.

Elad Y. 2000. Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. Crop Protect., **19**: 709-714.

Elad Y., Chet, I. and Henis, Y.1982. Degradation of plant pathogenic fungi by *Trichoderma harzianum*. Can. J. Microbio., **128**: 719-725.

El-Katatny M. H., Gudelj M., Robra, K. H., Elnaghy M.A. and Gubitz, G. M.2001. Characterization of a chitinase and an endo β-1, 3 glucanase from *Trichoderma harzianum* Rifai T24 involved in control of the phytopathogen *Sclerotium rolfsii*. Appl. Microbiol Biotechnol. **56**: 137-143.

Flores A., Chet, I. and Herrera-Estrella, A. 1997. Improved biocontrol activity of *Trichoderma harzianum* by overexpression of the proteinase-encoding gene prb1. Curr. Genet. **31**: 30-37.

Fokkema N. J. 1976. Antagonism between fungal saprophytes and pathogens on aerial plant surfaces. In: Microbiology of Aerial Plant Surfaces. Dickinson, C. H. and Preece, T. F. (Eds.), pp. 487-506, Academic Press, London.

Hao X. C., Yu, X. B. and Yan, Z. L. 2006. Optimization of the medium for the production of cellulase by the mutant *Trichoderma reesei* WX-112 using Response Surface Methodology. Food Technol. Biotechnol. **44** (1): 89-94.

Haran S., Schikler, H., Chet, I. 1996a. Molecular mechanisms of lytic enzymes involved in the biocontrol activity of *Trichoderma harzianum*. Microbiology, **142**:2321-2331.

Haran S., Schikler, H., Oppenheim, A., Chet, I. 1996b. Differential Expression of *Trichoderma harzianum* chitinases during mycoparasitism. Phytopathology, **86**:981-985.

Hermosa M.R., Viterbo, A., Chet, I., and Monte, E. 2012. Plant beneficial effects of *Trichoderma* and of its genes. Microbiol., **58** : 17-25.

Hjeljord L. and Tronsmo, A. 1998. *Trichoderma* and *Gliocladium* in biological control: An overview. In: Harman GE and Kubicek CP (eds.) Trichoderma and Gliocladium, 2: Enzymes, Biological Control and Commercial Applications, pp. 131-151, Taylor and Francis, London.

Howell C. R., 2002. Cotton seedling preemergence damping-off incited by Rhizopus oryzae and Pythium spp. and its biological control with Trichoderma spp. Phytopathology, **92**: 177-180.

Hunsley D. and Burnett, J. H. 1970. The ultrastructural architecture of the walls of some fungi. J. Gen Microbiol. **62**: 203-218.

Joshi B. B., Bhatt, R. P. and Bahukhandi, D. 2010. Antagonistic and plant growth activity of *Trichoderma* isolates of Western Himalayas. Environmental Biology. **31** : 921-928.

Kredics L., Antal, Z., Do´czi, I., Manczinger, L., Kevei, F. and Nagy, E. 2003. Clinical importance of the genus Trichoderma. A review. Acta Microbiol Immunol Hung. **50**: 105-117.

Lewis J. A. and Papavizas, G. C. 1991. Biocontrol of plant diseases: the approach for tomorrow. Crop Prot., **10**: 95-105.

Lorito M., Hayes, A., Di Pietro, A., Woo, S.L., Harman, G.E. 1994. Purification, characterization, and synergistic activity of a glucan β-1, 3-glucosidase and an N-acetyl-β-glucosaminidase from *Trichoderma harzianum.* Phytopathology, **84**:398-405.

Mandels M., Weber, J. and Parizek, R. 1971. Enhanced cellulase production by a mutant of *Trichoderma* viride. Appl. Microbiol., **21**: 152-154.

Miller G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chem., 31: 426-428.

Moretti M., Gilardi G., Gullino M. L., Garibaldi A., 2008. Biological control potential of Achromobacter xylosoxydans for suppressing Fusarium wilt of tomato. Int. J. Bot. **4**: 369-375.

Nakkeeran S., Renukadevi, P. and Marimuthu, T. 2005. Antagonistic potentiality of *Trichoderma viride* and assessment of its efficacy for the management of cotton root rot. Archives of Phytopathology and Plant Protection, 38 (3): 209-225.

Nelson N. 1994. A photometric adaption on the Somogyi method for the determination of glucose. J. Biol. Chem. 153: 375-380.

Nusret O., Steven, E.N. 2004. Fusarium crown and root rot of tomato and control methods. Plant Pathol. J. 3: 9-18.

Papavizas G. C., 1985. *Trichoderma* and *Gliocladium*: biology, ecology and potential for biocontrol. Annu. Rev. Phytopathol. **23**: 23-54.

Papavizas G. C., Lewis, J. A. and Abd-ElMoity, T. H. 1982. Evaluation of new biotypes of *Trichoderma harzianum* for tolerance of benomyl and enhanced biocontrol capabilities. Phytopathology, **72**: 126-132.

Reis A., Costa, H., Boiteux, L.S., Lopes, C.A. 2005. First report of *Fusarium oxysporum* f. sp. *lycopersici* race 3 on potato in Brazil. *Fitopathologia Brasileira* **30**: 426-428.

Rey M., Delgado-Jarana, J. and Benitez, T. 2001. Improved antifungal activity of a mutant of *Trichoderma harzianum* CECT 2413 which produces more extracellular proteins. Appl. Microbiol. Biotechnol., **55**: 604-608.

Ridout C. J., Coley-Smith, J. R. and Lynch, J. M. 1986. Enzyme activity and electrophoretic profile of extracellular protein induced in *Trichoderma* spp. by cell walls of *Rhizoctonia solani*. J. Gen Microbiol., **132**: 2345-2352.

Schirmbock M., Lorito M., Wang Y. L., Hayes C. K., Arisan-Atac, I., Scala, F., Harman, G. E. and Kubicek, C. P. 1994. Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. Appl. Environ. Microbiol. **60**: 4364-4370.

Schwarz D. and Grosch R. 2003. Influence of nutrient solution concentration and a root pathogen on tomato root growth and morphology. Sci. Hortic. **97**: 109-120.

Sudhamoy M., Nitupama, M., and Adinpunya, M. 2009. Salicylic acid-induced resistance of *Fusarium oxysporum* f. sp. *lycopersici* in tomato. Plant Physiol. Biochem. **47**: 642-649.

Szekeres A., Kredics, L., Antal, Z., Kevei, F. and Manczinger, L. 2004. Isolation and characterization of protease overproducing mutants of *Trichoderma harzianum* FEMS Microbiol Let., **233**: 215-222.

Thrane C., Jenson, D. F. and Tronsmo, A. 2000. Substrate colonization, strain competition, enzyme production in vitro, and biocontrol of Pythium ultimum by *Trichoderma* spp. isolates P1 and T3. Eur. J. Plant Pathology, **106**: 215-225.

Thrane C., Tronsmo, A. and Jenson, D. F.1997. Endo-1, 3 β-glucanase and cellulose from *Trichoderma harzianum*: purification and partial characterization, induction of and biological activity against plant pathogenic Pythium spp. Europ. J. Plant Pathol. **103** (4): 331-344.

Tronsmo A. and Harman, G. E. 1993. Detection and quantification of N-acetyl-a-D-glucosaminidase, chitobiosidase and endochitinase in solutions and on gels. Analytical Biochemistry, **208**: 74-79.

Upadhyay R. S. and Rai B. 1987. Studies on antagonism between F. udum Butler and root region micro flora of pigeon pea. Plant and Soil, **101**:79-93.

Yang S. S. and Huang, C. I., 1994. Protease production by amylolytic fungi in solid state fermentation. J. Chin. Agric. Chem. Soc., **32**: 589-601.

Zaldivar M., Velasquez, J. C., Contreras, I. and Perez, L. M. 2001. *Trichoderma atroviride* 7-121, a mutant with enhanced production of lytic enzymes: its potential use in waste cellulose degradation and /or biocontrol. EJB, 4(3): 1-9.