PURIFICATION, CHARACTERISATION AND CLONING OF β-1,3 GLUCANASE GENE FROM *Trichoderma harzinum*

NOHA F. EL-BADAWY[1] AND R. S. YEHIA[2]

1. *Plant Pathology Research institute, ARC, Giza, Egypt*
2. *Department of Botany and Microbiology, Faculty of Science, Cairo University, Giza 12613, Egypt*

Fusarium wilt, a vascular disease caused by several soil borne fungal pathogens are wide spread and serious in many crops cultivated in different soil types. Wilt pathogens such as *Fusarium solani* and *Fusarium oxysporium* are reported to attack plant roots causing serious losses in seed germination and plant stand as well (Srivastava *et al.*, 2014). Traditionally, this disease is controlled by the application of synthetic fungicides. But the indiscriminate use of fungicides resulted in the accumulation of residual toxicity, environmental pollution and altered the biological balance in the soil by over killing the non-targeted microorganisms. Increase awareness of fungicide-related hazards has emphasized the need of adapting biological methods as an alternative disease control method. Fungal species belonging to the genus *Trichoderma* (*Ascomycota*, *Hypocreales*) are widely spread in the soil and plant root ecosystems. Many *Trichoderma* species are effective biological control agents against a range of crop diseases (Abo-Elyousr *et al*., 2014). The capability of *Trichoderma* spp. to suppress plant diseases usually is attributed to their direct antagonistic effects on the fungal pathogen, and especially their ability to produce lytic enzymes (Benítez *et al*., 2004). As chitinase and β-1, 3-glucanase are two main hydrolytic enzymes associated with fungal cell wall lysis (Pandey *et al*., 2014). In the last few years, commercially available *Trichoderma* products (biopesticides, biofertilizers and soil amendments) have been used as part of environmental friendly protocols to defend crops against plant pathogenic organisms and to increase yields (Mukherjee *et al*., 2012). The aim of the present study was to amplify and clone the β 1,3 glucanase gene (*βgn13.1*) from *Trichoderma harzianum* and investigate its physical characteristics.

MATERIALS AND METHODS

Fungal isolates

Three isolates of *Trichoderma* sp. (*T. harzianum, T. viridi* and *T. longibrachiatum),* which were kindly provided by Microorganisms Identification and Evaluating Pesticides Unit, Agriculture Research Centre, Giza, Egypt, were used in this study as bioagents. Pathogenic fungal isolates were obtained from disease of legumes crops Department, Agriculture Research Centre, Giza, Egypt. All of the fungal isolates were maintained on potato

dextrose agar (PDA) medium for further use.

Dual culture tests (Antagonistic activity)

The antagonistic potentialities of the *Trichoderma* isolates were determined against *Fusarium sp* by dual culture technique described by Morton and Stroube (1955). About 7-days old culture, mycelial disc (5 mm) from each of the *Trichoderma* isolates and test pathogens were placed on the same plate opposite to each other equidistant from the periphery and then incubated at 25°C. During 5 days of the incubation period, radial growth of pathogen was recorded and inhibition percentage was calculated in relation with control according to the following formula:

$$L= (C-T)/C*100$$

L = Percentage of inhibition

C = radius of the radial growth of the pathogen towards opposite side in control plate

T = radius of the radial growth of the pathogen towards the opponent antagonist in test plate

β 1-3 glucanase assay

For enzyme production, *T. harzianum* isolate was grown in 100 ml liquid mineral synthetic medium (MSM) containing: $MgSO_4 \cdot 7H_2O$, 0.2; K_2HPO_4, 0.9; KCl, 0.2; NH_4NO_3, 1.0; $FeSO_4 \cdot 7H_2O$, 0.002; $MnSO_4$, 0.002 and $ZnSO_4$, 0.002 (in g l^{-1}), and supplemented with 0.1% cell walls of FOL to induce cell wall enzymes production, or 0.1% glucose as control (Mondejar *et al.*, 2011). The cultures were grown at 150 rpm on a rotary shaker for 6 days at 25°C. The mycelia were harvested by filtration through Whatman No. l filter paper and the filtrate centrifuged at 4°C for 10 min at 5000x*g*. The supernatant was decanted and stored at −20°C until used to assay enzyme activity (El-Katatny *et al.*, 2000).

β-1,3 Glucanase activity was determined by a colorimetric method (Burner, 1964)**.** The amount of reducing sugar released from laminarin (Sigma Chemical Co. St. Louis, MO) was measured. The standard assay contained 10 µl of the crude enzyme solution and 90 µl of 5mg/ml laminarin in 0.1M sodium acetate buffer pH 5.0. After incubation at 40°C with gentle shaking for 10 min, the reaction was stopped by boiling for 5min and 0.2 ml of 1% dinitro salicylate (DNS) and 0.2 ml of sodium acetate buffer were added and boiled for another 5min, then placed in an ice bath and 0.9 ml distilled H_2O was added. The optical absorption was measured at 540 nm. The amount of reducing sugar released was calculated from a standard curve prepared with glucose and the glucanase activity was expressed in units (µmol glucose equivalent/min). Proteins concentrations were determined by the method of Bradford (1976) and bovine serum albumin was used as standard.

Purification of β 1-3 glucanase

A supernatant of the *T. harzianum* isolate was obtained after low speed cen-

trifugation. All purification steps were carried out at 4°C. Proteins were precipitated from the supernatant with 80%saturation of ammonium sulphate and centrifuged at 9000xg for 15 min. The pellet was dissolved in 50 mM Tris–HCl buffer, pH 7.5 and dialyzed in the same buffer. The enzyme solution was loaded onto a (2.5 cm × 25 cm) column with DEAE-Sephacel (Pharmacia) equilibrated with 50 mM Tris–HCl buffer, pH 7.5 at a flow rate of 18ml/h. The column was washed with 390ml of the same buffer and the proteins were eluted with 420ml of a linear 0-0.5M NaCl gradient collecting 3 ml fractions. Fractions containing glucanase were pooled, dialyzed against the same buffer and concentrated. The concentrated enzyme was applied to a Sephadex G-100 (Pharmacia, 1.3 cm × 60 cm) column equilibrated with 50mM Tris–HCl buffer, pH 7.5 and eluted with the same buffer at a flow rate of 6 ml/h collecting 1ml fractions. The active fractions were pooled, concentrated and then stored at 4°C.

Characterization of β 1-3 glucanase

The effect of temperature and pH on enzyme activity was determined. The standard assay conditions were used for 30 min at temperatures between 20 and 90°C. The pH of the reaction mixture was varied between 3 and 11 using 50 mM buffer (sodium acetate, pH 3.0-6.9, Tris-HCl, pH 7.0-8.9; and glycine buffer, pH 9.0-11.0).

Sodium dodecyle sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in 12.5% polyacrylamide gels according to the method of Laemmli (1970). The separated proteins were stained with Coomassie Brilliant Blue R-250 (Fluka, Switzerland) and their molecular weights were determined by comparison with low range molecular weight markers (Bio-Rad).

Isolation of total DNA from Trichoderma harzianum isolates

DNA was extracted from 50 mg of mycelium using Qiagen Kit for DNA extraction. The extracted DNA was dissolved in 100 μl of elution buffer. The concentration and purity of the obtained DNA was determined by using "Gen qunta" System-Pharmacia Bio-Tech. The purity of the DNA was between 90-97%. Concentration was adjusted at 50 ng/µl using TE buffer pH 8.0.

Isolation of β 1-3 glucanase gene

PCR amplification was carried out using *glu* forward and reverse specific primers; A synthetic oligonucleotide primer based on the sequence of an internal peptide was designed to clone the cDNA corresponding to BGN13.1. The amplification reaction includes approximately 50 ng of fungal genomic DNA as template, one unit of *Taq* DNA polymerase, 0.2 mM each dNTPase, 1× PCR buffer, 3 mM

$Mgcl_2$ and 10 *p*mol of each primer in total volume 25 µl, using *T-gradient* thermal cycler (Biometra). PCR was carried out for 35 cycles (94°C, 1 min; 56°C, 1 min; 72°C, 3.5 min) followed by a 10 min extension at 72°C. The amplified product were separated on 1.5% agarose gel at 100 constant volt to check for product size and purity, using 1× TBE buffer followed by staining in ethidium bromide solution (1 µg/ml) and determine with UV transilluminator.

Gene clean purification system (QiAquick Gel Extraction Kit, Qiagen) was used to purify DNA fragment from the agarose gel according to manufacture procedure.

Cloning of β 1-3 glucanase gene

The purified DNA was ligated into pGEM-T-Easy vector (system 1) (Promega, Madison, WI, USA). The ligation mixture transformed into competent JM109 *E. coli* cells (Promega USA) with subsequent ampicillin selection. QiA prep R Miniprep Kit (Qiagene) was used to isolate pure super-coiled plasmid DNA with high yields (15 µl) according to instruction manual. Validation of cloning took place by PCR to select the transformed colonies with recombinant pGEM -T-Easy vector by using specific *glu* forward and reverse primers as well as the universal primers (M13 forward and reverse). The amplified product was separated on 1.5% agarose gel at 75 constant volt to check for product size and purity, using 1×TBE buffer followed by staining in ethidium bromide solution (1 µg/ml) and was determined by UV transilluminator.

Nucleotide sequencing of β 1-3 glucanase

Partial nucleotide sequence of *β* 1-3 glucanase gene was sequenced by ABI PRISM TM 310 genetic analyzer by using dye-primer and dye terminator method at Gene Link DNA Sequencing service, New York, USA and Gene Analysing Unite. The resulting sequences gene was then compared to published sequences in the Gene Bank.

RESULTS AND DISCUSSION

The main objective of the present study was to identify the most promising *Trichoderma* isolate for the management of root rot. Therefore, dual culture technique was carried out for studying the interaction between *Trichoderma* species viz. *T. harzianum*, *T. viride* and *T. longibrachiatum* with *Fusarium oxysporium*. Different species of *Trichoderma* with respect to suppression of mycelial growth of the test pathogen was recorded. It is evident from the data that *T. harzianum* suppressed the radial growth of *Fusarium* significantly (79.30%) followed by *T. viride* (68.5%) and *T. longibrachiatum* (44.3%). As shown in Fig. (1)

The promising antagonistic activity of the isolates *T. harzianum*, *T. viride* and *T. longibrachiatum* on dual culture may be

due to mycoparasitism. The ability of *Trichoderma* to inhibit the growth of plant pathogens like *Fusarium sp*. has been reported by Srivastava *et al.* (2014). The antagonistic action of *Trichoderma* species against phytopathogenic fungi might due to secretion of extracellular hydrolytic enzymes (Schirmbock, 1994). The present finding was also supported by several workers (Obaiue and Oti, 2007). Singh *et al*. (2013) also revealed that 30 isolates of *Trichoderma* collected from various districts of U.P. were found highly antagonist against three of the tested pathogens (*Fusarioum oxysporium f. sp. udum, Fusarioum oxysporium f. sp. cicero, Fusarioum oxysporium f. sp. lenties*).

β-1,3-glucanase has been purified from many plant species, usually by multistep procedures employing ion exchange columns followed by gel filtration. There is increasing evidence that they play important roles in mycoparasitism (Kauffmann *et al.*, 1987; Kurosaki *et al.*, 1991). In the present study, it was found that when the dialyzed protein solution, obtained by ammonium sulphate precipitation of the culture supernatant was loaded on DEAE-Sephacel column, the three major peaks with 11.5 purification fold and 23.7% recovery were detected (Table 1 and Fig. 2A).

As the eluted peaks of high Glucanase activity were subsequently submitted to further purification in Sephadex G-100, single activity peak was observed with final enzyme activity of 21 U mL^{-1}, specific activity of 4 U mg^{-1} protein, purification fold 20 and recovery 11.9% of the crude preparation (Fig. 2B). The apparent molecular mass of the purified Glucanase was estimated to be 30 kDa by SDS-PAGE (Fig. 3). The molecular masses of β-1,3-glucanases produced by *Trichoderma* appear to vary considerably, not only between organisms, but also within the same species. Molecular masses of exo-β 1,3-glucanases from *T. harzianum* T-Y were in a high range of 75 kDa (Ramot *et al*., 2000), while in the range of 29, 31 and 40 kDa β 1,3-glucanase also have been isolated and characterized (Noronha and Ulhoa, 2000). The optimal pH for the enzyme activity was found to be 7.5 (Fig. 4) meanwhile Pitson *et al*. (1993) found the most optimal activities of fungal β 1,3-glucanases are usually in the range of 4.0 and 7.0. The optimum temperature for the enzyme activity was found to be 55°C (Figure 5), and it is in agreement with exo- β-1,3-glucanases from *T. harzianum* TC (Noronha and Ulhoa, 2000).

Detection of β 1-3 glucanase gene from T. harzianum

In this study, we isolated β -glucanase gene (*bgn13.1*) from *T. harzinum* which had previously exhibited the highest antagonistic effect. To isolate the *bgn13.1* gene, the oligonucleotide primers were designed based on the related DNA sequences available in the GenBank database. PCR amplification that was performed on the genomic DNA generating a specific band of approximately 600 bp. (Fig. 6). The primer pair specif-

ic to *β* 1,3 glucanase gene was used in order to amplify a PCR product of about 600 bp from the sample which is agreement with Shinya *et al*. (2006). It has been suggested that β-1,3-glucanases contribute to the mycoparasitic activities of several fungal species by facilitating penetration through host cell wall structures (Lorito *et al.* 1994).

The strategy used for cloning of *β* 1-3 glucanase into *E. coli* was based on a direct gene cloning of the generated DNA fragments after gene clean using QiAquick Gel Extraction Kit (Qiagene) eluted into 50 µl of autoclaved deionized water and quantified by spectrophotometer and gel electrophoresis. The DNA concentration was checked by measuring the OD at 260/280 nm. The eluted DNA was ligated into prokaryotic expression pGEM-T-Easy-vector. The ligation reaction was transformed into competent *E. coli* JM109. The obtained ampicillin resistant white colonies containing recombinant plasmid were further selected for isolation of DNA plasmids containing the *β* 1,3 glucanae gene by plasmid minipreparation using Qiaprep® miniprep kit (Qiagene).

One ampicillin resistant colony, named *T1glu,* containing recombinant plasmids was validated by PCR to confirm the presence of *β* 1,3 glucanase gene in the right orientation. Plasmid was amplified using *glu* forward and reverse primers as well as forward and reverse of universal primers M13. The results showed amplified product of 1500 bp by M13 primers, where amplified product was 600 bp by the specific primers as shown in Fig. (7).

T1Glu clone was sequenced using forward primer used in PCR technique. Partial nucleotide sequence of *β*,1-3 glucanase from *T. harzianum* was performed on an applied Biosystems 310 genetic analyser (Applied Biosystems) using Big Dye terminator cycle sequencing ready reaction mix according to manufacturer's instruction. The obtained sequence was aligned to published *β* 1,3 glucanase in Gene bank using DNAMAN V 5.2.9 package. Madison. Wisconsin, USA. Comparison of partial nucleotide sequence of *T1Glu* showed 97% sequence homology with other published sequence of *T. harzianum* mRNA for endo1,4 β-glucanase under the accession number X84085.1 and 96% sequence homology with the other sequences *Trichoderma viride* clone T1#36endo1,3(4) β-glucanase mRNA, complete cds (Accession no. Kj603460) and *Trichoderma viride* β-,3glucanase (*glu*) gene, complete cds (Accession no. EF176582), as shown in Fig. (8).

The most pathogenesis related proteins have damaging action on the structure of parasite PR1 and PR5 interact with the plasma membrane. Whereas *β*-1,3 glucanase (PR2) and attack *β* 1,3 glucans which is component of the cell walls in most higher fungi such as reported by Odjakova and Hadjiivanova (2001). Most of these proteins have been shown to exhibit antifungal activity *in vitro* (Ponstein *et al.*, 1994).

With regard to the importance of yield losses due to the contribution of fungal diseases, some research has been conducted to develop transgenic crop plants that have increased expression levels of pathogen related (PR) protein genes in hopes of producing fungal disease resistant varieties (Lawrence *et al*., 2000; Vleeshouwers *et al.*, 2000; Gau *et al.*, 2004). Our study showed that *T. harzianum* isolate is a high glucanase enzyme activity source, from which *Trichoderma* glucanase gene, *bgn13.1*, was isolated and amplified. There are several reports indicating transgenic plants expressing *bgn13.1* gene have shown enhanced fungal disease resistance in different species including apple (Bolar *et al.*, 2000 & 2001), creeping bent grass (Wang *et al*., 2003), and rice (Liu *et al*., 2004). This fragment was sub cloned in order to transfer it into plant vector. Various strategies have been suggested to utilize genes encoding cell wall-degrading enzymes of mycoparasitic fungi. First, superior fungal strains that exhibit greater biocontrol activities have been produced by genetic transformation, (Migheli *et al.*, 1998; Limon *et al.*, 1999). Second, transgenic plants that exhibit increased resistance to fungal pathogens have been obtained by introducing genes of fungal origin (Lorito *et al.*, 1998; Bolar *et al.*, 2000). Finally, expression of these genes in heterologous hosts may yield enzyme production at a commercially reasonable scale (Margolles *et al*., 1996). This work is still in progress to reach the ultimate goal of this study. In order to produce transgenic plants with these defense genes.

SUMMARY

Dual culture technique was used to evaluate the effect of three species of *Trichoderma* that showed a potential control of *Fusarium oxysporium*. *Trichoderma harzianum* showed maximum growth inhibition (79.3%) followed by *Trichoderma viridi* (68.5%) and *Trichoderma longibrachiatum* (44.3%). β-1,3-glucanases was purified from *Trichoderma harzianum* to homogeneity by ion exchange chromatography on DEAE-Sephacel and gel filtration on Sephadex G100. A typical procedure provided 20-fold purification with 11.9% yield. The apparent molecular mass was 30 kD and it was active on a broad pH range, however the maximal activity was detected at pH 7.5. The optimum temperature of the β-1,3-glucanase was 55°C. Polymerase chain reaction (PCR) was used to amplify a fragment about 600 bp from β-1,3 gluanase gene using specific *glu* forward and reverse primers. The eluted DNA was ligated into pGEM-T-Easy vector and transformed into competent *E. coli* JM109. White transformed colony, named ***T1glu,*** containing recombinant plasmid was validated by PCR using both *glu* forward and reverse and M13 forward and reverse primers to confirm the presence of β 1,3 glucanase gene insert in right orientation whereas, the fragment amplified with *glu* forward and *glu* reverse primers was 600 bp. Partial sequence of the amplified DNA fragment showed 97% sequence homology with the other published sequences.

REFERENCES

Abo-Elyousr, K. A. M., I. Sobhy, I. Abdel-Hafez and I. Abdel-Rahim (2014). Isolation of *Trichoderma* and evaluation of their antagonistic potential against Alternaria porri. J. Phytpathol., 162: 567-574.

Benítez, T., A. M. Rincón, M. C. Limón and A. C. Codón (2004). Biocontrol mechanisms of *Trichoderma* strains. Int Microbiol., 7: 249-260.

Bolar, J. P., J. L. Norelli, K. W. Wong, C. K. Hayes, G. E. Harman and H. S. Aldwinckle (2000). Expression of endo-chitinase from Trichoderma harzianum in transgenic apple increases to apple scab and reduces vigor. Phytopathology, 90: 72-77.

Bolar, J. P., J. L. Norelli, G. E. Harman, S. K. Brown and H. S. Aldwinckle (2001). Synergistic activity of endo-chitinase and exo-chitinase from Trichoderma atrovireade (*T. harzianum*) against the pathogenic fungus (*Venturia inaequalis*) in transgenic apple plants. Transgenic Reserch, 1067: 1-11.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem., 72: 248-254.

Burner, R. L. (1964). Determination of reducing sugar value 3,5-dinitrosalicylic acid method. Methods Carbohydr. Chem., 4: 67-71.

El-Katatny, M. H., W. Somitsch, K. H. Robra, M. S. El-Katatny and G. M. Gübitz (2000). Production of Chitinase and β-1,3 glucanase by *Trichoderma harzianum* for Control of the Phytopathogenic Fungus *Sclerotium rolfsii*. Food Technol. Biotechnol., 38: 173-180.

Gau, A. E., M. Koutb, M. Piotrowski and K. Kloppstech (2004). Accumulation of pathogenesisrelated proteins in apoplast of a susceptible cultivar of apple (*Malus domestica* cv. Elstar) after infection by *Venturia inaequalis* and constitutive expression of PR genes in the resistant cultivar Remo. Eur. J. Plant Pathol., 110: 703-711.

Kauffmann, S., M. Legrand, P. Geoffroy and B. Fritig (1987). Biological function of pathogenesis-related protein: Four PR proteins of tobacco-leaves have β-1,3-glucanase activity. EMBO Journal, 6: 3209-3212.

Kurosaki, U., Y. Tokitoh and A. Nishi (1991). Purification and characterization of wall-bound β-1,3-glucanase in cultured carrot cells. Plant Science, 77: 21-28.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly

of the head of bacteriophage T4. Nature, 227: 680-685.

Lawrence, C. B., N. P. Singh, J. Qiu, R. G. Gardner and S. Tuzun (2000). Constitutive hydrolytic enzymes are associated with polygenic resistance of tomato to *Alternaria solani* and may function as an elicitor release mechanism. Physiol. Mol. Plant Pathol., 57: 211-220.

Limón, M. C., J. A. Pintor-Toro and T. Benítez (1999). Increased antifungal activity of *Trichoderma harzianum* transformants that overexpress a 33 kDa chitinase. Phytopathology, 89: 254-261.

Liu, M., Z. X. Sun, J. Zhu, T. Xu, G. E. Harman and M. Lorito (2004). Enhancing rice resistance to fungal pathogens by transformation with cell wall degrading enzyme genes from Trichoderma atroviride. Journal of Zhejiang University Science, 5: 133-136.

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

Lorito, M., S. L. Woo, I. Garcia, G. Colucci, G. E. Harman, J. A. Pintor-Toro, E. Filippone, S. Muccifora, C. B. Lawrence, A. Zoina, S. Tuzun and F. Scala (1998). Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. Proc. Natl. Acad. Sci. USA, 95: 7860-7865.

Margolles-Clark, E., C. K. Hayes, G. E. Harman and M. E. Penttilä (1996). Improved production of *Trichoderma harzianum* endochitinase by expression in *Trichoderma reesei*. Appl. Environ. Microbiol., 62: 2145-2151.

Migheli, Q., L. González-Candelas, L. Dealessi, A. Camponogara and D. Ramón-Vidal (1998). Transformants of *Trichoderma longibrachiatum* overexpressing the β-1,4-glucanase gene *egl1* show enhanced biocontrol of *Pythium ultimum* on cucumber. Phytopathology, 88: 673-677.

Mondéjar, R. L., M. Ros and J. A. Pascual (2011). Mycoparasitism-related genes expression of *Trichoderma harzianum* isolates to evaluate their efficacy as biological control agent. Biol. Control., 56: 59-66.

Morton, D. T. and N. H. Stroube (1955). Antagonidtic and stimulatory effects of microorganism upon Sclerotium rolls. Phytopathology, 45: 419-420.

Mukherjee, M., P. K. Mukherjee, B. A. Horwitz, C. Zachow, G. berg and S. Zeilinger (2012). Trichoderma-

Plant-Pathogen Interactions: Advances in Genetics of Biological Control. Indian J. Microbiol., 52: 522-529.

Noronha, E. F. and C. J. Ulhoa (2000). Characterization of a 29-kDa β-1,3-glucanase from *Trichoderma harzianum*. FEMS Microbiol. Lett., 183: 119-123.

Obaiua, A. O. and E. Oti (2007). Antagonistic properties of *Trichoderma viridi* on post-harvest cassava root rot pathogens. African Journal of Biotechnology, 6: 2447-2450.

Odjakova, M. and C. Hadjiivanova (2001). The complexly of pathogen defense in plants. Journal of Plant Physiology. 27: 101-109.

Pandey, S., M. Shahid, M. Srivastava, A. Sharma, A. Singh and V. Kumar (2014). Isolation purification and characterization of glucanase enzyme isolated from antagonistic fungus *Trichoderma* species. Int. J. Sci. Eng. Res., 5: 646-649.

Pitson, S. M., R. J. Seviour and B. M. McDougall (1993). Noncellulolytic fungal β-glucanases: their physiology and regulation. Enz. Microbiol. Technol., 15: 178-192.

Ponstein, A. S., S. A. Bres-Vloeman, M. B. Sela-Buurloge, P. J. M. Van-den Elzen, L. S. Melchers and B. J. C. comelissen (1994). A novel pathogen and wound-inducible tobacco (*Nicotiana tabacum*) protein with antifungal activity. Plant Physiology, 104: 109-118.

Ramot, O., R. Cohen-Kupiec and I. Chet (2000). Regulation of β-1,3-glucanase by carbon starvation in the mycoparasite *Trichoderma harzianum*. Mycol. Res., 104: 415-420.

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

Singh, A., S. Mohd, M. Srivastava, V. Kumar and A. Bansal (2013). Antagonistic activity of *Trichoderma viridi* isolate against different pathogens of *Fusarium oxysporium* isolate from legume crop of UP. Progressive Research 8: 47-50.

Shinya, T., R. Menard, I. Kozone, H. Matsuoka, N. Shibuya, S. Kauffmann, K. Matsuoka and M. Saito (2006). Novel β-1,3, 1,6 oligoglucan elicitor from *Alternaria alternata* 102 for defense response in tobacco. FEBS Journal, 273: 2421-2431.

Srivastava, M., A. Singh and D. K.

Srivastava (2014). Morphological and molecular characterization of *Trichoderma* isolates: An antagonist against soil borne pathogens. International Journal of Science and Research (IJSR) Vol. 3, Issue 7, July 2014.

Vleeshouwers, V. G. A. A., W. Van Dooijeweert, F. Govers, S. Kamoun and L. T. Colon (2000). Does basal PR gene expression in Solanum species contribute to non-specific resistance to Phytophthora infestans?. Physiol. Mol. Plant Pathol., 57: 35-42.

Wang, Y., A. P. Kausch, J. M. Chandlee, H. Luo, B. A. Ruemmele, M. Browing, N. Jackson and M. R. Goldsmith (2003). Co-transfer and expression of chitinase, glucanase, and bar genes in creeping bentgrass for conferring fungal disease resistance. Plant Science, 165: 497-506.

Table (1): Purification steps of β 1,3-glucanase produced by *Trichoderma harzianum* isolate.

Purification step	Total protein (mg)	Total activity (Unite)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Culture filtrate	845.0	177	0.20	100.0	1.0
Ammonium sulphate precipitation	485.0	123	0.25	69.5	1.2
DEAE-Sephacel column	18.3	42	2.30	23.7	11.5
Sephadex G-100 column	5.2	21	4.00	11.9	20.0

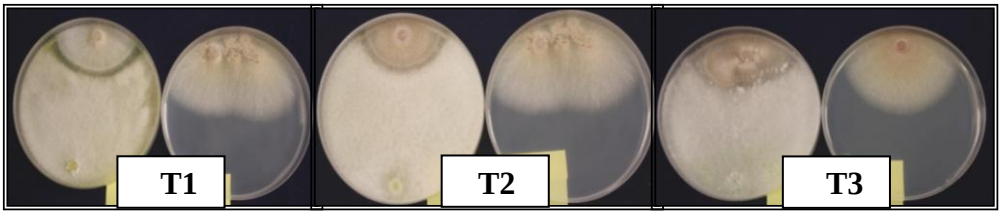


Fig. (1): Antifungal activity of culture filtrate of four isolates of *Trichoderma viride* (T1) *Trichoderma harzianum* (T2) and *Trichoderma llongibrachiatum* (T3) against *Fusarium oxysporium.*

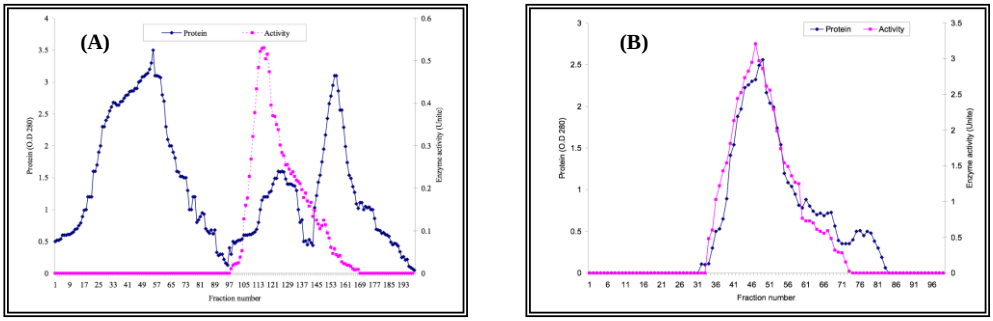
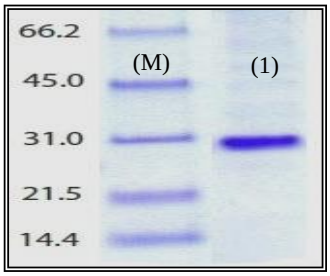


Fig. (2): Typical elution profile for the behavior of β-1,3-glucanases (A) DEAE-Sephacel, (B) Sephadex G-100.

Fig. (3): SDS-PAGE for purified β 1,3-glucanases; lane (M) Protein marker (kDa), and lane (1) purified enzyme.



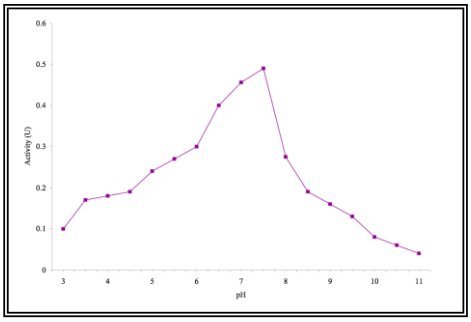


Fig. (4): Effect of pH on the activity of purified β -1,3-glucanases.

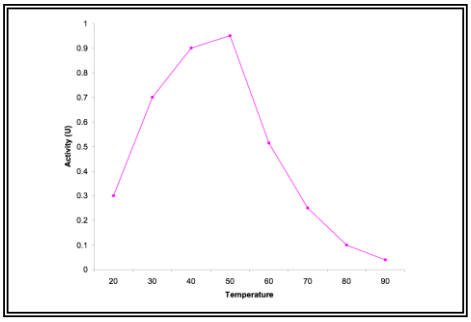


Fig. (5): Effect of different temperatures on the activity of purified β -1,3-glucanases.

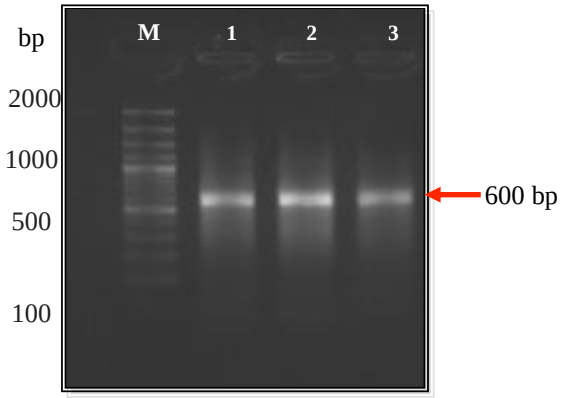


Fig. (6): PCR pattern using glu forward and glu reverse primers. *Trichoderma harzianum*, (lane 1), *Trichoderma viride*, (lane 2), *Trichoderma longibrachiatum*, (lane 3). M: 100 bp DNA ladder marker.

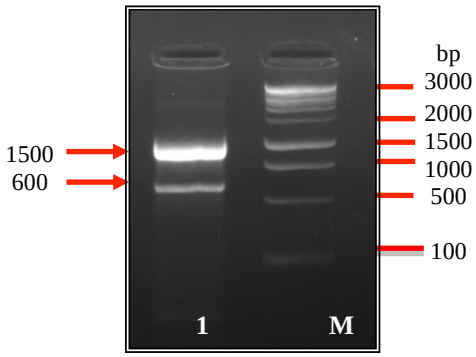


Fig. (7): Validation of cloning by PCR. Recombinant plasmid was screened by plasmid mini-preps and PCR amplification using the specific primers (GluF & GluR) and universal primers (M13 F & M13 R). M:100 pb DNA Ladder marker.

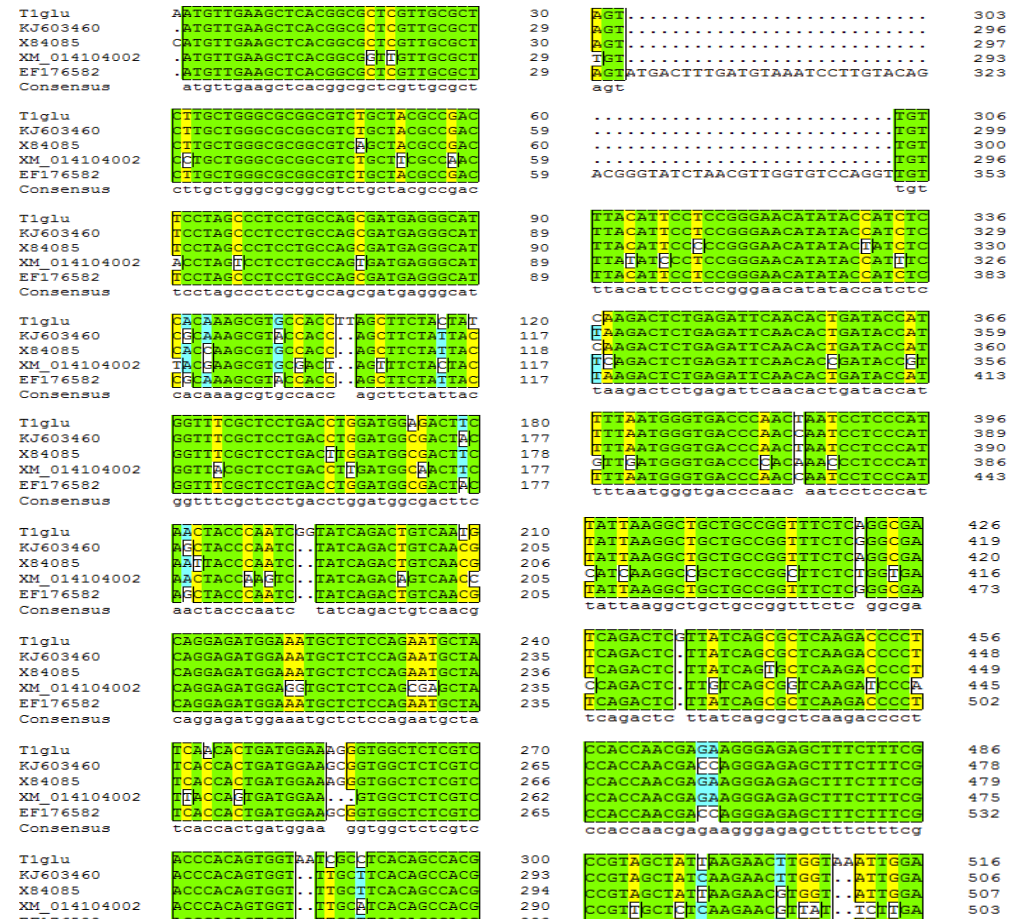


Fig. (8):

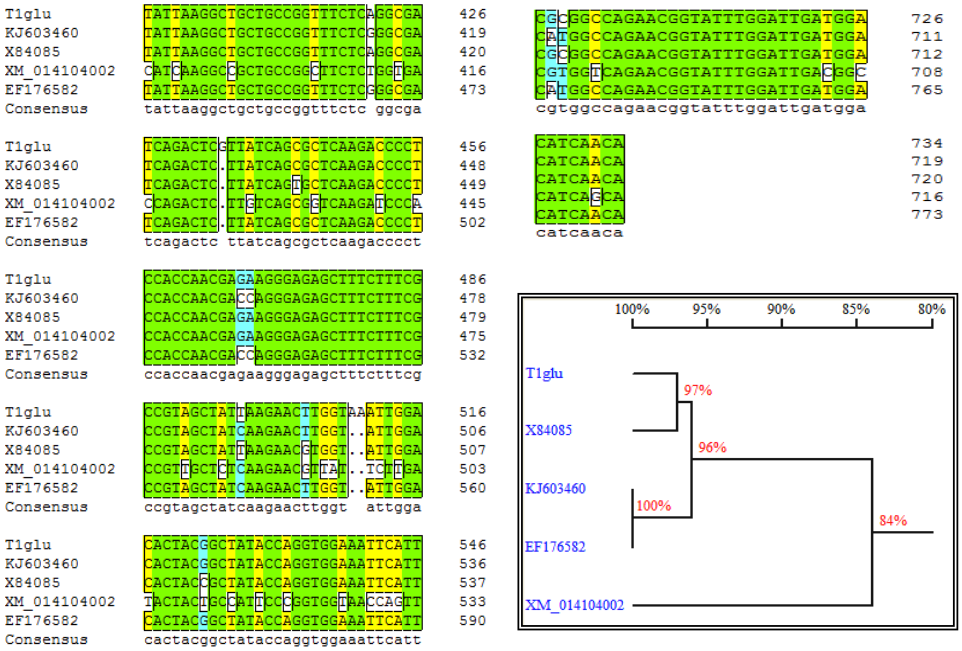


Fig. (8): Clustral multiple sequence alignment of partial nucleotide encoding β-,1-3 glucanase of *Trichoderma harzianum* in comparison with the published B-1,3 glucanase