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Using gamma-ray to increased exoglucanase activity in *Trichoderma* and improvement of *Sclerotinia* rot of canola biocontrol

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ABSTRACT: *Trichoderma* due to several mechanisms of cell wall degrading enzymes such as chitinase and glucanase is known as a biocontrol agent. The purpose of this study was to investigate the production of exogluconase enzymes in wild type and 20 motant isolates (gamma ray) of *Trichoderma harzianum* to raise to be antagonistic against the pathogen *Sclerotinia sclerotiorum*. At first, the dual culture performed between the wild type and mutant strains of the pathogen. Then Exogluconase activity enzymes is done using substrates such as cellulose and Avicel crystal, and the protein concentration determined by Bradford. Results showed that the induction 9 irradiated mutant isolates, leading to increased activity of the Exogluconase enzyme. The ability of these isolates in dual culture with pathogens and production of Exogluconase enzymes is a significant relationship. Comparing protein profiles from SDS-PAGE analysis of proteins of the extracellular fluid TFM upper fermentation medium of *T. harzianum* and its mutant enzyme was observed Cel 7A (CBH I) with molecular weight 67KDa and Cel 6A (CBH II) with KDa 58.8 molecular weight.

Keywords: Gamma Radiation, exoglucanase, biological control, *Trichoderma harzianum*, *Sclerotinia sclerotiorum*.

INTRODUCTION

Trichoderma have been recognized as the potential biocontrol agents (BCA) with the ability to produce antimicrobials and cell wall-degrading enzymes in plant pathogenic fungi. Glucanase enzymes are appeared as the most recognized cell wall-degrading enzymes which play a potential role in Biocontrol mechanisms of *Trichoderma* strains. *Sclerotinia* stem rot due to Soil Fungi of *Sclerotinia sclerotiorum* (Lib.) de Bary has been recognized as the plant pathogen agent with wide host geographic range and one of the most important oilseed rape diseases (Bolland & Hall, 1994). This disease due to the symptoms which appear with them is called with different names such as white blight, white rot, stem blight, canker of oilseed rape (Gaetn & Madina 2005). Infected stems may be cracked, under which a large number of regular, spherical and white to black *Sclerotinia sclerotiorum* will raise inside tissue-specific stem cells (Kolte, 1985). *Trichoderma* fungal species produce exoglucanase (EC 3.2.1.58) (Cellobiohydrolase) including Cel 6A, CBH II, Cel 7A, CBH I that specifically attack to glucan bonds (Sutherland, 1999).

Exoglucanase or Cellobiohydrolase (EC 3.2.1.91) which serve in reducing or non-reducing ends of the cellulose chain release oligosaccharides, Cellobiose and Glucose as the important products. According to previous studies, it has been indicated that β-1,3 glucanases have role of complementary feeding in Saprophytes and Mycoparasitism mechanism (Chet, 1997). In addition, β-1,3 glucanases have appeared as plant defense responses against pathogen attack (Simmons, 1994).

MATERIALS AND METHODS

The fungus *Trichoderma harzianum* and 20 mutant strains induced by gamma irradiation have been used as antagonist inoculum (Naseripour *et al.* 2014) and the fungus *S. sclerotiorum* separated from *Brassica napus* L. were used as pathogen (Golestan, Iran). The antagonistic activity of *T. harzianum* against *S. sclerotiorum* was determined by dual culture technique. Mycelia discs (5 mm diameter) were cut out from actively growing pure cultures of both strains (mutants or parent culture of *T. harzianum* and *S. sclerotiorum*) on PDA at 28°C for 3 days and placed at the opposite sides, 25 mm apart, of 100 mm petri plates containing PDA. After 3 days percent inhibition of mycelia growth of the pathogen was calculated. The valuation of inhibition by mutants or parent culture of *T. harzianum* estimated by calculating the percentage inhibition of mycelia growth by the following formula:

$$I\% = (1-C_n/C_o) \times 100.$$

C_n is the average diameter of colonies of pathogen in the presence of the antagonist and Co is the average diameter of colonies of control. Activity of exoglucanase enzymes is measured by microcrystallization of cellulose (Teeri, 1997).

Exoglucanase activity was determined by measuring the amount of glucose released from substrates by the dinitrosalicylic acid (DNS) method with glucose as the standard (Gamma and Mota, 1980). The protein content in the TFM (*Trichoderma* fermentation medium) supernatant (Wen *et al.*, 2005) were estimated after 48 hour by the dye binding method of Bradford (1976).The amount of protein was calculated using bovine serum albumin (BSA) as a standard . Protein samples (40 ml) from TFM supernatants were precipitated with equal volume of acetone and precipitated proteins were resuspended in double distilled water in final volume of 1 ml, frozen and kept at -70°C until they were used. The molecular weight of the cellulase was determined by sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE) with a 4% (stacking) and 12.5% (separating) polyacrylamide gel based on Laemmli (1970). The experimental data were subjected to analysis of variance (ANOVA) followed by a Duncan's test. Significance was defined at $P < 0.05$. The SPSS (developer, 13) program was used for all statistical analysis.

RESULTS AND DISCUSSION

Comparison of mean of inhibition of wild-type and mutant strains in fungus of *T. harzianum* against Fungal pathogen *S. sclerotiorum* indicates that all the mutant strains have a significant difference at 0.05 level. Inhibition of growth of pathogen varied from 51.88% to 72.17%. All the mutant strains showed higher antagonistic activity than *Trichoderma harzianum*. The highest amount of inhibition activity was observed in strains Th M8, Th M17, Th M15, Th M10, Th M9, Th M2, Th M11, Th M6 with over 67% inhibition of fungal growth. Comparison of mean of activity of exoglucanase enzyme (U/ml) and Estimation of protein and extracellular cellulase activity *Trichoderma harzianum* and its mutant strains was assayed in *Trichoderma* fermentation medium after centrifugation at 4500 × g for 7 min at 4°C (Fig. 1).

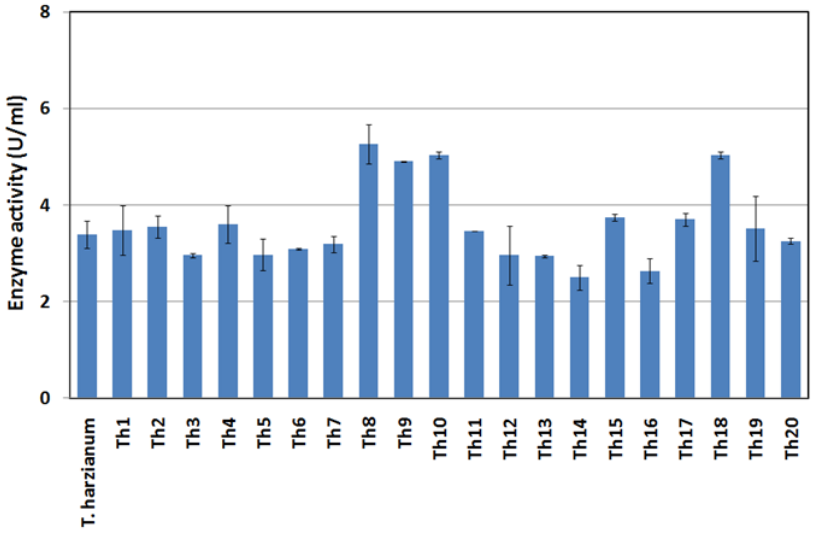

Fig. 1. Comparison of mean of enzymatic activity (U / ml) in mutant strains in *Trichoderma harzianum* fungus via Microcrystalline Cellulose substrate to measure activity of extracellular enzyme produced in the upper liquid fermentation medium TFM containing Colloidal microcrystalline cellulose.

The highest amount of activity of exoglucanase enzyme in mutant strains Th M8, Th M10, Th M18 and Th M9 were observed equal to 5.27, 5.04, 5.04 and 4.91. Amount of changes in exoglucanase enzyme varied from 2.50 to 5.27. The lowest amount of enzyme activity was observed in mutant strain Th M14.Yet, since strain Th M16 shows higher enzyme activity at lower amount of protein, it will indicate special enzyme activity among mutants. The protein profiles obtained from SDS-PAGE jels representing extracellular proteins in supernatant of TFM medium of from the studied fungi and mutant strains (Fig. 2). The *Trichoderma* species produces at least two exoglucanases (cellobiohydrolases, CBHs, EC 3.2.1.91) Cel6A (CBHII) and Cel7A (CBHI) for cellulose degradation (Grishutin *et al.*, 2004). The highest expression of the enzyme Cel 7A (CBH I) with a molecular weight KDa 64 was observed in mutant strains Th M2, Th M4, Th M5 and Th M1 (Fig. 2. a). Amount of protein concentration Cel 7A (CBH I) for the mutant strain Th M3 has been close to expression of this protein in fungus of *T. harzianum*. On the other hand, expression of protein Cel 6A (CBH II) in mutant strain Th M3 has been greater than expression of enzyme Cel 7A (CBH I) in this strain as well as other mutant strains (Th M1, Th M2, Th M4 and Th M5). Protein profile indicates glucanase enzymes in mutant strains Th M6 to Th M11. All the samples under study in this protein profile enjoy different enzyme bands at the range of molecular weight 11-245 KDa.

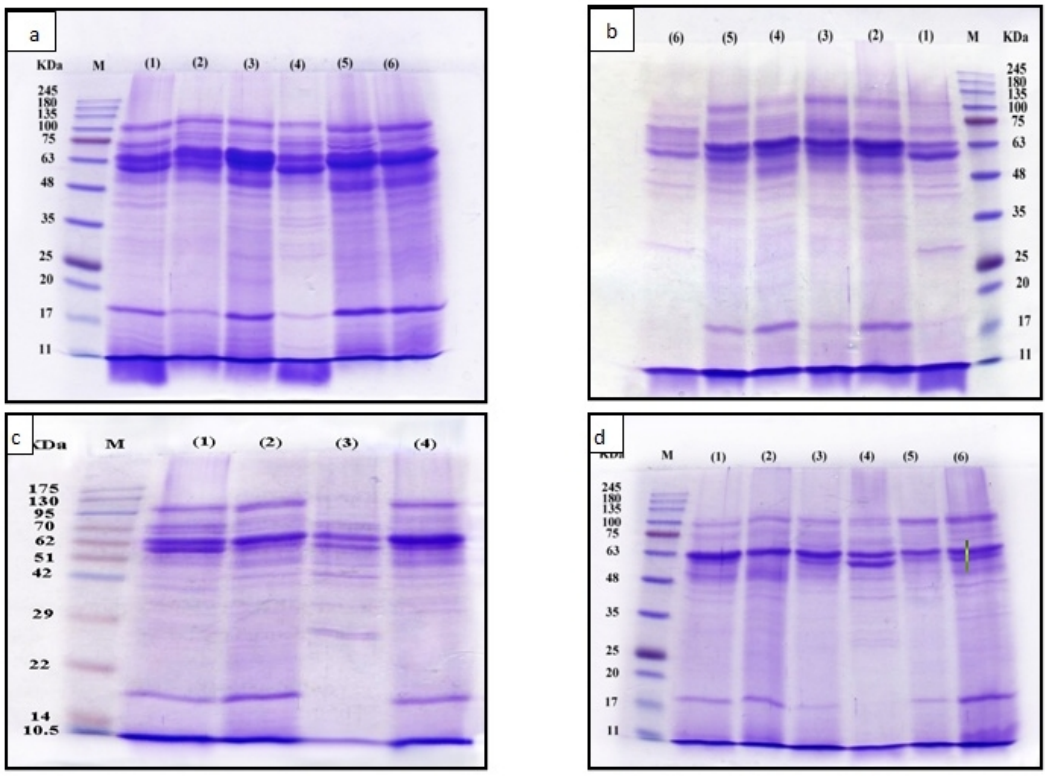

Fig. 2. Comparison of protein profile (Electrophoresis pattern) obtained from SDS-PAGE test representing extracellular proteins in fluid TFM upper fermentation medium of *T. harzianum*: A- M: protein marker, (1) *T. harzianum* (wild type), (2): Th M1, (3): Th M2, (4): Th M3, (5): Th M4, (6): Th M5, b (1) :Th M6, (2): Th M7, (3): Th M8, (4): Th M9, (5): Th M10, (6): Th M11 and M: protein marker, C- (1): Th M12, (2): Th M13, (3): Th M14, (4): Th M15, (5): Th M16, (6): Th M17 and M: protein marker, d-1): *T. harzianum*, (2): Th M18, (3): Th M19, (4): Th M20 and M: protein marker.

The major protein band among these strains was observed at molecular weights of 63 kDa protein that the highest protein concentration was observed in this profile in mutant strain Th M7. This molecular weight associates to enzyme Cel 7A (CBH I). Further, Cel 6A (CBH II) was observed at the range of molecular weight 57 KDa in this protein profile that the highest concentration of this protein band was observed in mutant strain Th M6. Expression of enzyme Cel 7A (CBH I) has had the lowest protein band concentration in mutant strain Th M11. All the mutant strains in this protein profile have had enzymes Cel 7A (CBH I) & Cel 6A (CBH II) with molecular weight of 63 and 57 KDa. The highest protein expression of enzyme Cel 7A (CBH I) was observed in mutant strains Th M7, Th M9, Th M10, Th M8 and Th M6 and also in mutant strain Th M11. Mutant strain of Th M11 showed more specific protein band Cel 6A (CBH II) than other protein bands especially protein band for enzyme Cel 7A (CBH I); nevertheless, this protein Cel 6A (CBH II)) shows less concentration than other mutant strains Th M6, Th M10 and Th M7. Comparison of enzyme protein profile (Fig. 2.c) represents mutant strains Th M12 to Th M17 that there are different protein bands at the range of molecular weight of 11-245 KDa in all the samples under study. All the mutant strains had a sharp protein band at molecular weight of 63 KDa pertaining to enzyme Cel 7A (CBH I). Protein profile represents cellulase enzymes in *T. harzianum* and mutant strains Th M18 to Th M20 (Fig. 2.d). All the mutant strains under study in this protein profile had different protein bands at molecular weight of 10.5-175 KDa. Protein profile of mutant strains Th M18, Th M19, Th M20 similar to protein profile of *T. harzianum* had a sharp protein band with molecular weight of 64.5 KDa pertaining to enzyme Cel 7A (CBH I). The highest concentration of this enzyme in this protein profile was observed in mutant strain Th M20 and the lowest concentration of this enzyme was observed in mutant strain Th M19. Enzyme Cel 6A (CBH II) was observed in mutant strain Th M19 as well as in mutant strains Th M18 and Th M20. Nevertheless, this enzyme has been low in *T. harzianum* strain. Enzyme Cel6A (CBH II) was observed in protein profile of *T. harzianum* at molecular weight 58.2 KDa. Cel6A is a GH family 6 CBH. The enzyme has an estimated molecular weight of 47 kDa, 53 kDa on a SDS-PAGE, and it has a pI of 5.9 (Bhikhabhai *et al.*, 1984).

Cel6A is a processive enzyme that hydrolyzes the glycosidic bonds in cellulose using the inverting mechanism and it has been shown that the enzyme preferably hydrolyzes the cellulose chain from the non-reducing end (Boisset *et al.*, 2000). Protein profile of fungus of *T. harzianum* contains three major protein bands with molecular weights of 16, 34.42 and 58.20. Previous reports have stated that the *T. harzianum* produces different β-1, 3-glucanase enzymes with molecular weights 17, 31, 36, 67, 74, 75, 78 and 110 KDa (El-Katatny *et al.*, 2004). In general, the results from this research indicate that induced mutation through gamma ray affects strengthening *Trichoderma* glucanase enzymes. Different factors can associate to changes in enzyme activity of a mutant.

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