Current Trends in Natural Sciences
Vol. 12, Issue 24, pp. 56-64, 2023
https://doi.org/10.47068/ctns.2023.v12i24.006
Current Trends in Natural Sciences (on-line)
ISSN: 2284-953X
ISSN-L: 2284-9521
Current Trends in Natural Sciences (CD-Rom)
ISSN: 2284-9521
ISSN-L: 2284-9521

ENDO-ß-1,4-GLUCANASE ENZYME ACTIVITIES OF SOME *Trichoderma* spp. AGAINST *Rhizoctonia solani* Kühn.

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Current Trends in Natural Sciences

Abstract
Some enzymes are secreted by biocontrol agents, which are increasingly preferred in agricultural control, and suppress the pathogen of diseases and decrease its growth and effects. Rhizoctonia solani Kühn., one of these pathogens, causes many losses in plants by causing many diseases such as root cancer, root rot and collapsing. One of the microorganisms that damage the cell walls of such phytopathogens and is therefore preferred as a biocontrol agent is Trichoderma spp. In this study, the reaction of Trichoderma spp. against R. solani and changes in the synthesis of endo-ß-1,4-glucanase enzyme were determined. Eleven different Trichoderma spp. and R. solani Kühn. isolates were used as materials. The enzyme activity results indicated that the cellulase enzyme activity of the R. solani samples increased significantly compared to the control group. TVD3 showed the highest enzyme activity while TH4 was the lowest. The most significant difference from the control group was in TVD3 isolate. These results suggested that Trichoderma spp. showed a biochemical response for defending the plant against R. solani. The results also support the effectiveness of Trichoderma spp. as a biological control agent and potential for the development of new eco-friendly commercial products.

Keywords: Cellulase, Rhizoctonia solani Kühn., Trichoderma spp.

1. INTRODUCTION

Pesticides applied intensively to increase vegetative production leads to deterioration of the physical structure of the soil. Thus, biological control has become preferred in order to prevent these problems. Some microorganisms living in the rhizosphere split up two main groups as plant-growth promoting microorganisms (PGPM) and biocontrol agents (BCA). PGPMs directly promotes plant growth if BCAs have indirect effects on plant growth through controlling phytopathogens. Major microorganisms known as biocontrol agents are *Azospirillum* spp., *Rhizobium* spp., *Trichoderma* spp., *Pseudomonas* spp. and *Glomus* spp. *Trichoderma* spp. are the most studied biocontrol microorganisms (Kucuk and Guler, 2009) and show antagonistic effect by the way of synthesis toxic antibiotics or some lytic enzymes. They sold as registered commercial products by name “F-Stop”, “BinabT”, “PlantShield” and “T-22 Planter Box” in different countries due to their influences on plant pathogens. These products are especially used against soil borne fungi based damping off and fusarium diseases on fruits, ornamental plants, grass, and vegetables (Kavanagh, 2017).
BCAs also produce some metabolites like hormones and play a significant role by dissolving the nutrients in the soil. So, they increase productivity and boost the plant immune system (Kleifeld and

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Chet, 1992; Altomare et al., 1999; Yedidia et al., 2003; Vinale et al., 2006). Otherwise, they show antifungal effects to fungal diseases and promote the host response. Fungal pathogens are responsible for a great majority of disease factors in plants. One of them, *Rhizoctonia* spp., is found in the soil as a saprophyte or on plant residues. They cause root and stem rot diseases in many plants (Kavanagh, 2017). Many microorganisms secrete some enzymes to block the growth of phytopathogens and promote plant resistance. It has been reported that *Trichoderma* spp. secrete lytic enzymes such as cellulases, glucanases, proteases, chitinases and degrade the cell wall structure of pathogen fungi (Chet et al., 1990).

Main purposes of this study are 1) Determination of endo-ß-1,4-glucanase enzyme activity produced by *Trichoderma* spp. in the presence and absence of *R. solani*; 2) Comparison of the results and determine whether the presence of *R. solani* causes changes in endo-ß-1,4-glucanase enzyme activity; 3) Verification the molecular weight of produced fungal cellulase by SDS-PAGE method.

Increased cellulase enzyme synthesis of *Trichoderma* spp. in the presence of *R. solani* is the sign of a biochemical response. It means *Trichoderma* spp. degrade the cell wall of *R. solani* by lytic enzymes. Accordingly increased enzyme activity provides information that *Trichoderma* spp. may be effective in biological control against *R. solani*. As demonstrated by other studies on this subject, *Trichoderma* spp. contributes to biological control by both attacking *R. solani*'s cell wall and increasing the resistance of plants. In this study, mentioned characteristics are based on experimental results and biological control, which is an environmentally friendly form of struggle, is emphasized. It has been underlined that *Trichoderma* spp. or commercial products to be produced from these species will provide many agricultural benefits without harming the environment. Also, the fungal cellulase produced may be utilized in many industrial areas. It is hoped that this study may shed light on the importance of using *Trichoderma* spp. in making moves that are environmentally friendly and economical.

2. MATERIALS AND METHODS

In this study, *R. solani* Kühn. and a total of 11 *Trichoderma* isolates were used; 2 different *T. viride* (TVD1 and TVD3), 3 different *T. harzianum* (TH2, TH3, and TH4), 3 different *T. virens* (TVN1, TVN2, and TVN3) and 3 different *T. asperellum* (TA1, TA2, and TA3). *Trichoderma* isolates were obtained from Van Yuzuncu Yil University, Department of Plant Protection (Figure 1).

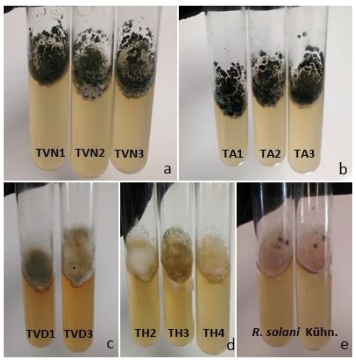

Figure 1. ***10 days old T. viride (a), T. harzianum (b), T. virens (c), T. asperellum (d) and R. solani (e) isolates in PDA slants***

Protein markers supplied from MyBioSource (10 – 250 kDa) and Sigma-Aldrich (29 – 200 kDa) were used in SDS-PAGE analyzes to to confirm the produced fungal cellulase.

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Enzyme Assays

The pH value of the Endo-ß-1,4-glucanase production media prepared according to the Mandel and Weber (1969) method was adjusted to 5.0. Medium components were Urea (0.3 g/L); $(NH_4)_2SO_4$ (1.4 g/L); KH_2PO_4 (2 g/L), $MgSO_4.7H_2O$ (0.3 g/L), $CaCl_2$ (0.3 g/L), $MnSO_4.H_2O$ (1.56 mg/L), $ZnSO_4.7H_2O$ (1.4 mg/L), $FeSO_4.7H_2O$ (5 mg/L), and $CoCl_2$ (2 mg/L). 1 % (w/v) Carboxymethyl cellulose (CMC) was added to the medium as a carbon source and the medium was inoculated with isolates. Incubation was carried out by shaking during 5 days at 30°C temperature, 150 rpm.

Potato Dextrose Agar (PDA) petri dishes were inoculated with 1 cm^2 diameter mycelial discs which were taken from *Trichoderma* spp. and *R. solani*. Then were incubated for 10 days at 30°C temperature and the antagonism were observed morphologically.

A standard curve (A540) was generated by preparing different concentrations of glucose to calculate Endo-ß-1,4-glucanase activity. Activity assays were carried out by following Ghose (1987) method. The following equation is used to calculate the enzyme activity (Ghose, 1987; Guruk and Karaaslan, 2020).

Enzyme activity = (Absorbance x Regression equation) / Incubation time

Enzyme activity values were calculated from absorbance measurements. The difference between the activity amounts in the presence and absence of *R. solani* was determined by t test using SPSS 10 statistic package software.

Protein determination

A bovine serum albumin (BSA) standard curve prepared with different concentrations of BSA was generated to calculate protein amounts. Protein determination was carried out by following the Bradford (1976) method. The absorbance values and the regression equation were used for calculating.

SDS polyacrylamide gel electrophoresis

The purity of the enzyme was checked by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Firstly, the samples were carried out using 40 V for 20 minutes, then 80 V for 2 hours. After electrophoresis, the gel was shaken gently in a gel-fixing solution for 20 minutes. Then, it was removed from this solution and taken into the dying one, where it was gently shaken again for 2 hours. After this stage, it was taken into the washing solution and shaken gently until the bands became visible. At the last stage, the gel was displayed.

Kinetic parameters

Different conditions were tested to determine the effects of pH value, temperature, substrate specificity and substrate concentration on endo-ß-1,4-glucanase enzyme activity. Microorganism were inoculated into different culture medias with pH values of 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0, respectively to observe the effect of pH value. The effect of temperature was determined by heating an enzyme production media with pH 5,0 to 20°C, 30°C, 40°C and 50°C respectively and the enzyme activity were measured in these temperatures. Substrate specificity was determined by using 1% (w/v) concentrations of CMC, wheat bran, cotton, and shavings as substrate. The effect of substrate concentration was detected using 0,5%, 1%, 1,5% and 2% (w/v) concentrations of CMC.

3. RESULTS AND DISCUSSIONS

The absorbance values obtained by following the 3,5-Dinitrosalicylic acid (DNS) method were used in enzyme activity calculation (Table 1). Enzyme activities of all *Trichoderma* isolates used in the presence and absence of *R. solani* were statistically significant.

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Table 1. ***Cellulase activities of Trichoderma spp. in the presence and absence of R. solani***

Microorganism	Endo-ß-1,4-glucanase activity (U/ml)	Endo-ß-1,4-glucanase activity in the presence of *R. solani* (U/ml)
TVN1	0,103	1,376
TVN2	0,649	1,165
TVN3	0,436	0,649
TH2	0,010	1,177
TH3	0,695	1,068
TH4	0,032	0,117
TA1	0,353	1,068
TA2	0,295	0,498
TA3	0,047	0,124
TVD1	0,064	0,443
TVD3	0,428	2,45

The highest value of the quantitative protein amounts calculated from the absorbance values was recorded from TA2 isolate and TA1, TA3 and TH3 isolates followed this value (Table 2).

Table 2. ***Protein amounts of the samples where R. solani and Trichoderma spp. were coexisted (mg/ml)***

Microorganism	Protein (mg/ml)
TVN1	0,033
TVN2	0,038
TVN3	0,081
TH2	0,075
TH3	0,335
TH4	0,052
TA1	0,336
TA2	0,337
TA3	0,335
TVD1	0,045
TVD3	0,017

TH2, TH3, TVN1, TVN2 and TVN3 samples had a distinct band at 29 kDa. The bands of TH2 and TH3 samples were comparatively unclear at 25 kDa. TVD1 and TA2 samples had distinct bands at 26 kDa while TA1, TA3, and TVD3 had at 25 kDa (Figure 2 and Figure 3).

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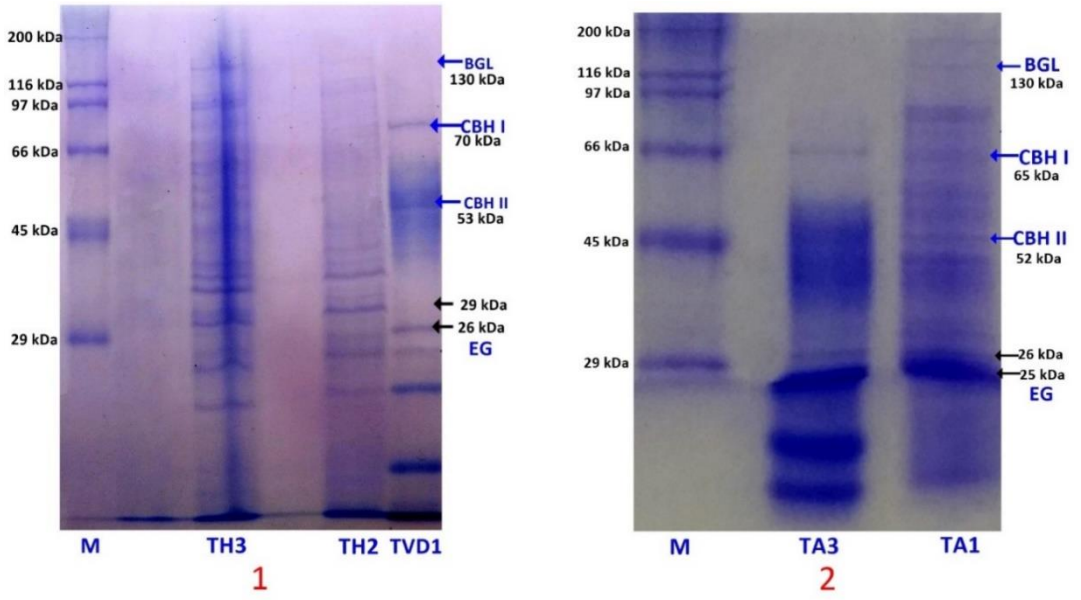

Figure 2. ***SDS-PAGE images of the endo-ß-1,4-glucanase (EG) enzyme produced from TH3, TVD1 (Gel No.1), TA3 and TA1 (Gel No.2) isolates***

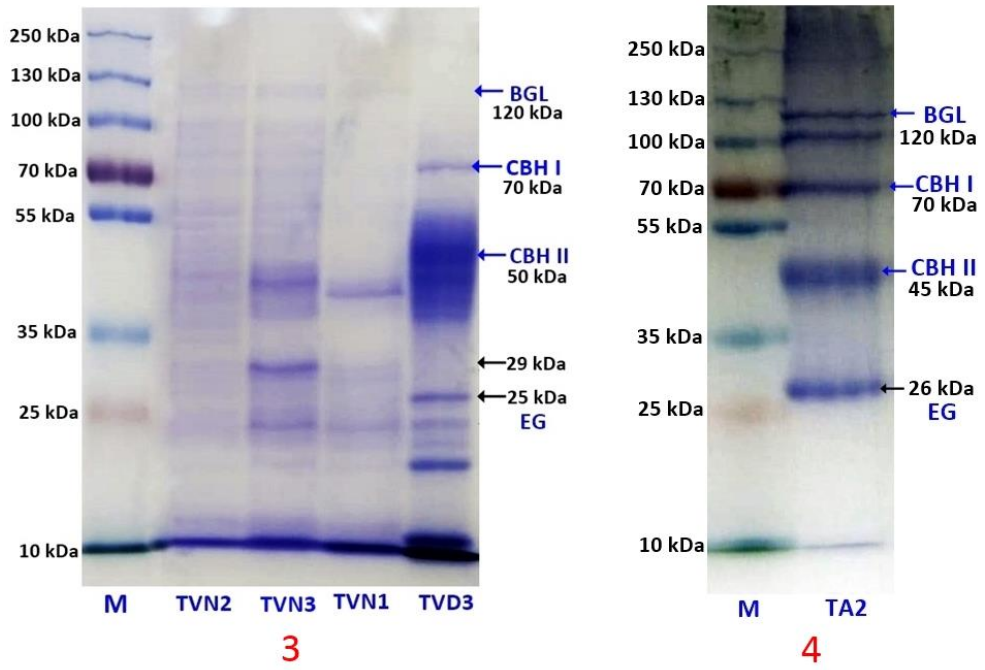

Figure 3. SDS-PAGE images of the endo-ß-1,4-glucanase (EG) enzyme produced from TVN2, TVN3, TVN1, TVD3 (Gel No.3) and TA2 (Gel No.4) isolates

The experiment results indicated that endo-ß1,4-glucanase activity increased when pH value approaches 5.0, and it reached the maximum value when pH was 5.0. Enzyme activity decreased as the pH was above 5.0 and completely stopped when it was 8.0 (Figure 4).

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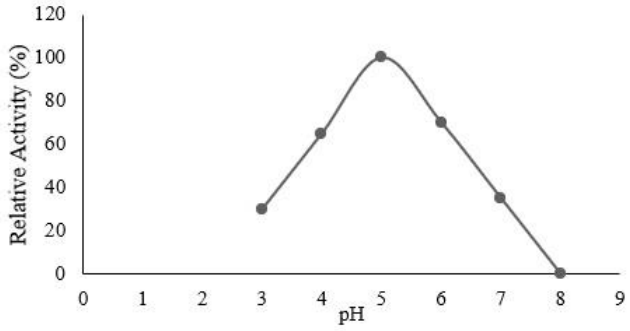

Figure 4. ***Effects of different pH values on endo-ß-1,4-glucanase enzyme activity***

The enzyme activity increased as the temperature of enzyme production media approached to 30°C, and reached the maximum value at 30°C. When the temperature reached 50°C, the enzyme activity was lost (Figure 5).

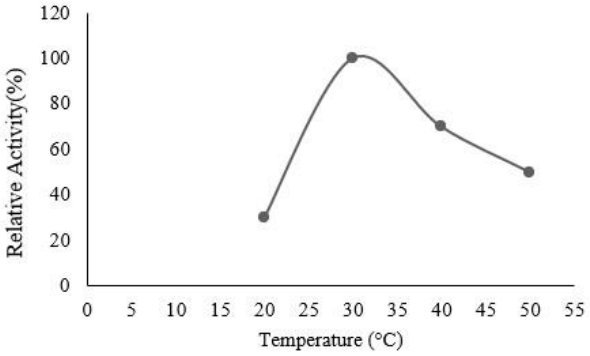

Figure 5. ***Effects of different temperatures on endo-ß-1,4-glucanase enzyme activity***

Data obtained by testing different substrates indicated that endo-ß-1,-glucanase enzyme production was quite higher in the presence of CMC. Wheat bran, cotton, and shavings did not give positive results due to top re-treatment application were needed (Figure 6).

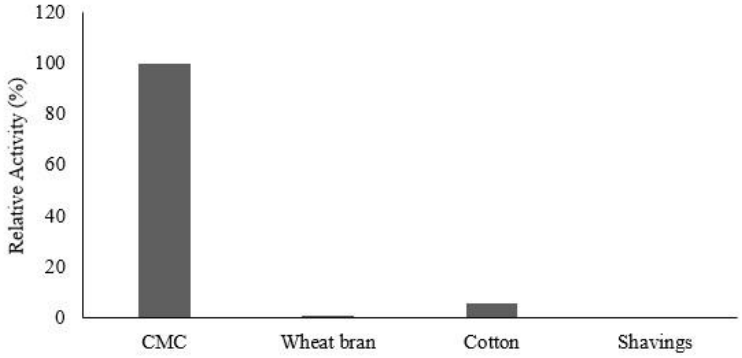

Figure 6. ***Effects of different substrates on endo-ß-1,4-glucanase enzyme activity***

As the CMC concentration used in the study increased, an increase in enzyme activity was observed; but when the substrate concentration rises above 2%, the activity is fixed (Figure 7).

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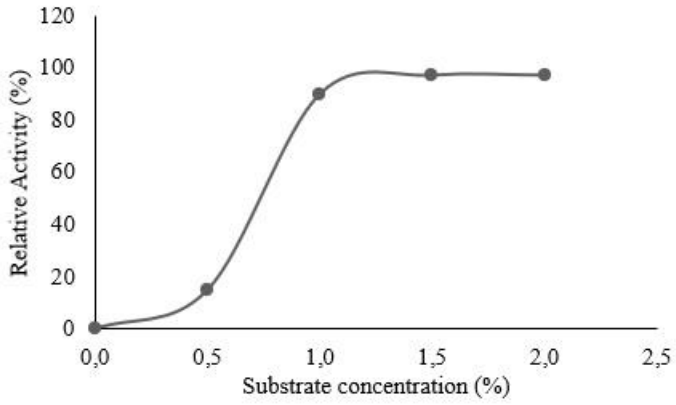

Figure 7. ***Effects of different substrate concentrations on endo-ß-1,4-glucanase enzyme activity***

4. CONCLUSIONS

Rhizoctonia solani Kühn. was known as the reason for different plant diseases like damping off, root rot, rot and root crown cancer, and causes serious damages in agricultural production. Pesticides that used to struggle against such pathogens like *R. solani*, have many side effects on both the environment and human health. Alternative solutions have become more popular to minimize the dangerous effects of chemicals. The usage of antagonistic microorganisms is one of the eco-friendly methods known as a biological struggle.

Supressor and regressive effects of *Trichoderma* spp. on *R. solani* have been the subject of many studies. Antagonistic behavior of *Trichoderma* spp. is possible by force of some enzymes. One of these lytic enzymes, endo-ß-1,4-glucanase, could be produced naturally by *Trichoderma* spp. under favorable conditions. However, an increase of endo-ß-1,4-glucanase production in the presence of *R. solani* has been determined in previous studies.

There is some information in the literature supporting our findings that endo-ß-1,4-glucanase (EG) enzyme gives bands at 29 – 25 kDa. One of these reported that *T. harzianum* gave band at 29 kDa, and the other one indicated *T. harzianum* showed bands at 66 – 25 kDa (Noronha and Ulhoa, 2000; Saravanakumar et al., 2016). These results also support our findings that the endo-ß-1,4-glucanase enzyme has been producted by *Trichoderma* spp. in the study.

The cellulase system consists of three enzyme groups as endoglucanases, exoglucanases, and ß-glucosidases (Lynd et al., 2013). In this study, it was possible to see other enzyme groups of cellulase systems since the endo-ß-1,4-glucanase enzyme was not obtained in pure form. A study about the cellulase complex of *T. reesei* observed bands at 130 kDa BGL; 70 kDa CBH I; 53 kDa CBH II and 25 kDa EG (Ng et al., 2014). As shown in Figure 1 and Figure 2, ß-glucosidase (BGL) showed bands at 130 and 120 kDa in different samples. Cellobiohydrolases I and II (CBH I and CBH II) showed bands at 35 – 70 kDa and 45 – 53 kDa. Endoglucanase (EG) has given 29, 26 and 25 kDa bands.

When the effects of different parameters were examined; it was detected the fungal cellulase produced *in vitro* shows high specificity against CMC and the optimum pH value of enzyme production media was 5.0. Some researchers detected the optimum pH for ß-1,4-glucanase enzyme production by *T. viride* was 4.5 (Shoemaker and Brown, 1978). Another group of researchers observed that *T. reesei* unfolds that optimum pH is 4.6 and the optimum pH is 5.0 for *T. ouroviride* (Farid and el-Shahed, 1993; Sahin et al., 2013).

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In this study, the optimum temperature for endo-ß-1,4-glucanase enzyme production media was found at 30°C. A study about this subject also detected the optimum temperature was 30°C in a similar study (Sahin et al., 2013). In other studies about ß-1,3-glucanase production by *Trichoderma* spp.; it was found as 30°C, 28°C and 25°C (Witkowska and Maj, 2002; Parmar et al., 2015; Gupta et al., 2016).
It was observed that a 2% concentration of CMC provides maximum amounts of enzyme activity. Similarly, a study using *Trichoderma spp*. and remarked that a 2% concentration of CMC was optimum for cellulase enzyme activity (Guruk and Karaaslan, 2020). Another study about *T. harzianum* presented the 2.5% concentration of CMC makes the enzyme activity maximum (Kim et al., 2003).
In this study, microorganisms in biotechnological methods produced overcosting cellulase enzyme cheaper by and the effect of *Trichoderma* spp. against *R. solani* in the biological struggle was presented. It was confirmed that endo-ß-1,4-glucanase enzyme production has increased in the presence of *R. solani*. In addition, it was observed in dual culture assays that the presence of *Trichoderma* spp. in the media suppresses *R. solani* growth and limits the spreading area (Figure 8).

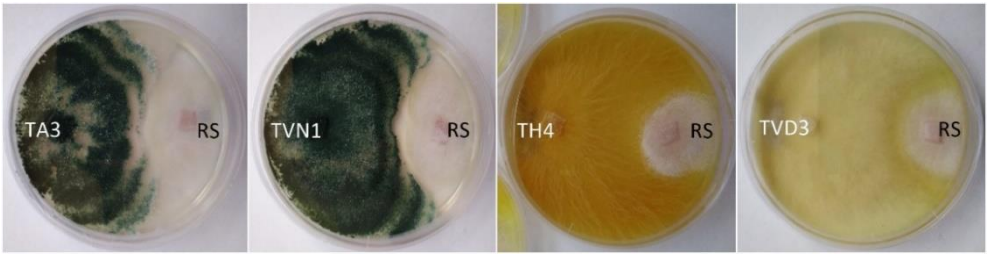

Figure 8. ***Antagonistic effects of TA3, TVN1, TH4, and TVD3 isolates against R. solani Kühn. (RS) (10 days old dual cultures)***

The bands obtained by means of SDS-PAGE were evaluated; the results were compared with similar studies in the literature and bands from the requested enzyme were observed. Also, existences of exoglucanase and ß-glucosidase enzymes were detected through bands on the gel.
Trichoderma spp. showed an increment in the synthesis of cellulase enzyme to damage the cell wall of phytopathogens such as *R. solani*. These properties bring *Trichoderma* spp. to an important place in biological control. It is hoped that this study will encourage usage of *Trichoderma* spp. in biological struggle and this kind of struggle will provide economic and environmental advantages.

5. ACKNOWLEDGEMENTS

This paper was generated from the master thesis of Ceren Elibol Ileri. This study was supported by the Scientific Research Projects Fund of Eskisehir Osmangazi University by the project number 2018-2219.

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