BIOCHEMICAL AND MICROBIOLOGICAL STUDY CONCERNING IDENTIFICATION ROLE OF HYDROLASE ACTIVITIES FROM *TRICHODERMA HARZIANUM* AND *TRICHODERMA KONINGII* IN PATHOGENIC FUNGUS *F. OXYSPORUM* INHIBITION

STUDIU BIOCHIMIC ŞI MICROBIOLOGIC PRIVIND IDENTIFICAREA ROLULUI ACTIVITĂŢILOR DE HIDROLAZĂ DIN *TRICHODERMA HARZIANUM* ŞI *TRICHODERMA KONINGII* ÎN INHIBAREA CIUPERCII PATOGENE *F. OXYSPORUM*

RADHI M. N.[1], PETRISŞOR Cristina[2], ROŞCA I.[1], FARHOOD Hattf Bazool[3], CRĂCIUN N.[3], STOIAN Gh.[3]
e-mail: mradhi84@yahoo.com

Abstract. *The genus Trichoderma are a very large group of microorganisms that play a significant role in plant protection. Several Trichoderma spp. like Trichoderma harzianum and Trichoderma koningii strongly affected plants by stimulating plant growth, and protecting plants from fungal and bacterial pathogens such as Fusarium oxysporum. They are used as a biological plant protection as bio fungicides. Members of the Trichoderma spp. are also utilized in different branches of industry - principally in the areas of enzymes, antibiotics, and other metabolites. In this study we focus in the effect of T. harzianum strain ICCF 417 and T. koningii strain ICCF 418 on F. oxysporum (ZUM 2407) by microbiologic and enzymatic tests. Where the results of fungal growth speed of the malt medium showed that the fungus T. koningii was the fastest in growing, followed by T. harzianum and F. oxysporum after 72 hours of culture. While the degree of antagonism was 1 according to Bell scale in petri dish on the PDA medium the ability of fungi T. harzianum and T. koningii to overcome on fungus F. oxysporum. The results of the study showed the susceptibility of bio-fungi on production of an enzyme FPase was 32.3 % in T. harzianum comparative to T. koningii after 14 days of fermentation, the amylase was 84.5% in T. harzianum comparative to T. koningii while the CMCase was 36.6 % in T. harzianum comparative to T. koningii. Our results showed that hydrolase activities studied in this experiment play an important role in pathogenic fungus F. oxysporum inhibition and the degree of effect is different.*

Key words: *Trichoderma harzianum, Trichoderma koningii, F. oxysporum* hydrolases

Rezumat. *Genul Trichoderma este un grup foarte mare de microorganisme care joacă un rol semnificativ în protecţia plantelor. Mai multe specii ale genului Trichoderma cum ar fi Trichoderma harzianum şi Trichoderma koningii, afectează puternic plantele prin stimularea creşterii plantelor şi protejarea acestora de agenţii*

[1]University of Agricultural Sciences and Veterinary Medicine Bucharest, Romania
2Development and research for Plant Protection, Bucharest, Romania
3Faculty of Biology. The University of Bucharest, Romania

patogeni fungici şi bacterieni cum ar fi Fusarium oxysporum. Ele sunt utilizate ca protecție biologică a plantelor, ca fungicide bio. Specii ale genului Trichoderma sunt, de asemenea, utilizate în diferite ramuri ale industriei, în principal domeniul enzimelor, antibioticelor şi altor metaboliți. În acest studiu ne concentrăm asupra efectului tulpinii T. harzianum ICCF 417 şi tulpinii T. koningii ICCF 418 asupra F. oxysporum (ZUM 2407) prin teste microbiologice şi enzimatice. În cazul în care rezultatele vitezei de creştere a fungilor din mediul de malț au arătat că fungul T. koningii a fost cel mai rapid în creştere, urmat de T. harzianum şi F. oxysporum după 72 de ore de cultură. În timp ce gradul de antagonism a fost 1 în funcție de scala Bell în vasul Petri pe mediul PDA capacitatea fungiilor T. harzianum şi T. koningii de a depăşi pe ciuperca F. oxysporum. Rezultatele studiului au arătat că susceptibilitatea bio-fungilor la producerea unei enzime FP-a fost de 32,3% în T. harzianum comparativ cu T. koningii după 14 zile de fermentație, amilaza a fost de 84,5% în T. harzianum comparativ cu T. koningii în timp ce CMCaza a fost de 36,6% în T. harzianum comparativ cu T. koningii. Rezultatele noastre au arătat că activitățile de hidrolază studiate în acest experiment joacă un rol important în inhibarea fungicidelor patogene F. oxysporum, iar gradul de efect este diferit.

Cuvinte cheie: *Trichoderma harzianum, Trichoderma koningii, F. oxysporum,* enzime hidrolitice

INTRODUCTION

The genus *Fusarium* spp. is known for a long time as important plant pathogens, *Fusarium* spp. is a one fungal species belonging to class Deuteromycota as it includes *Fusarium* spp more than 90 species, and these species have adapted to live within range of environmental and broad stretches across the world, in addition the types that include in it described as the important causes of pathogen of the plant (Booth, 1971). The wilt disease caused by *F. oxysporum* is an important tomato crop diseases and the disease was knew for the first time in the world in 1895 in the English Channel Islands (Walker, 1971). Recently scientists interested in biological control, including the fungus *Trichoderma*, this genus *Trichoderma* comprises a great number of fungal strains that act as biological control agents, the antagonistic properties of which are based on the activation of multiple mechanisms. A lot of methods of action have been proposed to clarify the biocontrol of plant pathogens by *Trichoderma*; these methods consist of the production of antibiotics and cell wall de grading enzymes, competition for key nutrients, parasitism, stimulation of plant defense mechanisms and combination of these possibilities. The double role of antagonistic action against plant pathogens and plant growth promoter make *Trichoderma* strains attractive alternatives to severe fumigants and fungicides. The aim of the study inhibition factors from in live and dead cells of *T. harzianum*, strain ICCF 417 and *T. koningii* strain ICCF 418 against pathogenic fungus *F. oxysporum* strain ZUM 2407.

MATERIAL AND METHOD

Liquid medium of malt extract was prepared by adding 20 grams of ready medium for a liter of distilled water, then it was sterilized and left to cool down, after

that antibiotic chloramphenicol was added to it (1 mg/100 mL). The density of 0.05 for 120 mL was prepared for each fungi using dilution law and mixing well and distributed by 10 ml of 12 tube and then was measured by the change in density within 12 hours. Total FPase activity in the culture filtrate was determined according to the standard method of Hankin and Anagnostakis (1975). CMCase activity was measured using a reaction mixture containing 1 ml of 1% carboxymethyl cellulase (CMC) in 0.5 M citrate acetate buffer (pH 5.0) and aliquots of suitably diluted filtrate. Cellobiose activity was measured using a reaction mixture containing 1 mL of 0.02 cellobiose in 0.5 M citrate acetate buffer (pH 5.0) and aliquots of suitably diluted filtrate. Reducing sugar produced was determined by DNS method (Murao *et. al.*, 1988). Amylase activity was determined by measurement of maltose released from starch according to the method of Miller (1959). The enzymatically liberated reducing sugar was calculated from a standard curve using maltose. The protein concentration (mg/mL) was determined by the method of Lowery method (Lowery *et. al.*, 1951), using bovine serum albumin (BSA) as a standard. The enzymes activity and protein were measured after 1, 2, 3 and 4 weeks of culture. In order to determine the optimum pH value for the enzyme obtained after fermentation, the activity of the enzyme was assayed between the pH values of 3.0-9.0. The ability of fungi *T. koningii* and *T. harzianum* to produce enzymes in synthetic medium, fungi's were grown in 250 mL Erlenmeyer flask that contained 100 ml of synthetic medium SM then we add the enzymes inducer for each enzyme, the cultures were incubated and the ability of production was determined after 1, 2, 3 and 4 weeks, also enzymes were used diluted to 100%, 50% and 10%. The fungi weight was determined by weighting an empty tube, then put (1 mL) of the fungi from each period of culture into the weighted tube after that drying and centrifuging and taking the precipitate which dried at 50^0 C for 3 days the weighted again. Preparation of cell lysate was done by taking (10 mL) of each culture and centrifuged, the precipitate re-suspended in 5 mL of phosphate buffer (pH 7.4) with vortex, then adding some of sand to the tube, put it in IKA® ULTRA TURRAX device at 6000 rpm for 30 s then put it at ice for 1 min, replay for three times and centrifuged at 10000 rpm for 10 min at 4^0 C. Filtered by Millipore 0.22µm, aliquots of the supernatant were used for next assays. All cultures were put at 100^0 C for 15 min, filtered by micro filter, aliquots of the supernatant were used for next assays. Characterization of enzymes that produced from *T. koningii* and *T. harzianum* that effect on *F. oxysporum* was done in PDA medium using a petri dish divided into 6 sections, at the first one we put 10 µL synthetic medium SM, the second 10 µL of dead cell of enzymes culture, the third one 10 µL of living cell of enzymes culture, the forth section 10 µL of dead cell from fungi culture, the fifth with 10 µL of dead lysate, the last one with 10 µL living lysate. And for control we put a petri dish of *T. koningii*, *T. harzianum* and *F. oxysporum* alone and a petri dishes divided into two part one with *Trichoderma* and the other with *F. oxysporum.* All experiments were in triplicate.

RESULTS AND DISCUSSIONS

The results regarding fungi growth in liquid malt medium extract after 12 hours from culture, showed that *F. oxysporum* reached the highest growth (0.42) of the fungus followed by fungus us *T. harzianum* and *T. koningii* (0.33) and (0.31) respectively, after 72 hours, *F. oxysporum* reached the highest growth (1.3) of the fungus followed by fungus us *T. harzianum* and *T. koningii* (0.95) and (0.87) respectively. The fungi weight/1 mL was 0.0097 for *T. harzianum* 0.009

for *F. oxysporum* and 0.0016 for *T. koningii*. Our results of the optimum pH for the enzymes showed that the optimum pH for all enzyme, for all fungi, was at pH=6 (fig. 1), Lee *et al.*, (2002) found that the best pH for FPase and CMCase was at the range of 4-7, also Refaz *et al.*, (2013) mention that the best production of FPase and CMCase enzymes was at pH= 5.6, Yassien *et al.*, (2014) proved that the best productivity of FPase enzyme was at pH= 5.5-7.5.

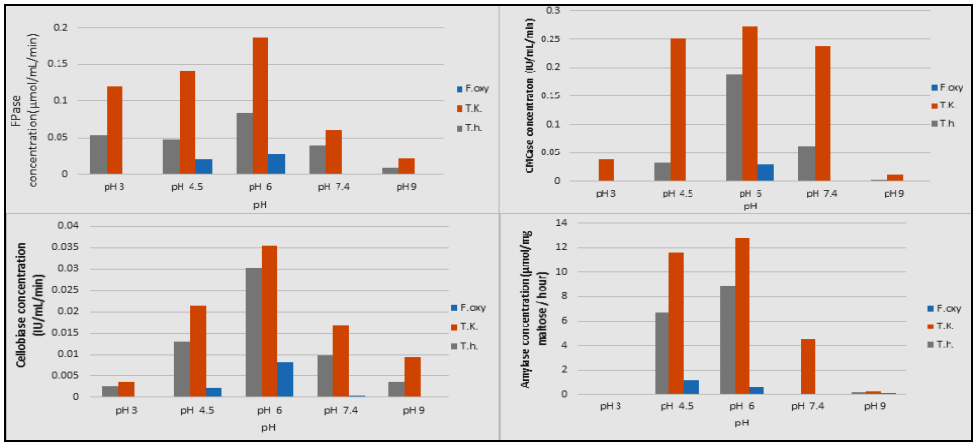

Fig. 1 The optimum pH for FPase, CMCase, Cellobiose and amylase enzymes in *T. harzianum*, *T. koningii* and *F. oxysporum*

The results of FPase enzyme showed that the two fungi can produce the FPase but its level on *T. koningii* was higher than *T. harzianum*, and for the two fungi the period of 14 days was the highest production, also the dilution ratio 100% was the highest, as shown in figure 2. The levels of FPase enzyme was 0.24 and 0.07 µmol/mL in *T. koningii* and *T. harzianum* respectively, several studies have relied on the values of this enzyme in production, (Ojumu *et al.*, 2003).

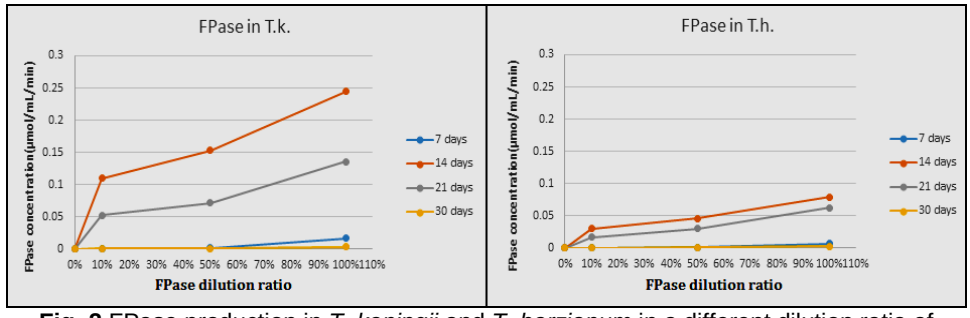

Fig. 2 FPase production in *T. koningii* and *T. harzianum* in a different dilution ratio of enzyme and for a period 1, 2, 3 and 4 weeks

The production of CMCase enzyme in synthetic medium in *T. koningii* was higher than *T. harzianum* and the second week was the highest period of production, as shown in figure 3.

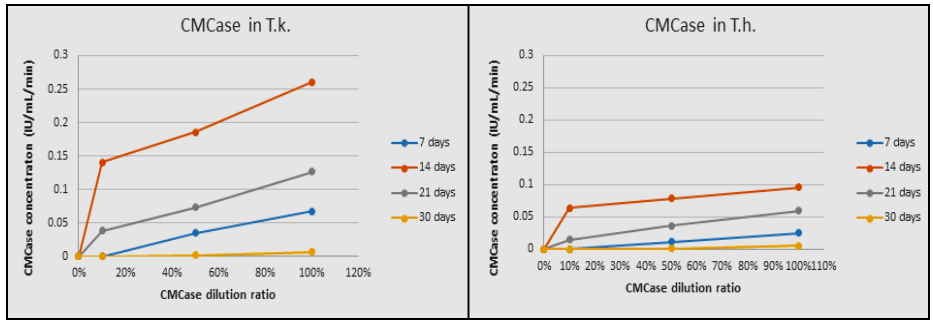

Fig. 3 CMCase production in *T. koningii* and *T. harzianum* in a different dilution ratio of enzyme and for a period 1, 2, 3 and 4 weeks

In figure 4 are presented the results of cellobiose production in *T. koningii* and *T. harzianum* respectively, cellobiose levels on *T. koningii* was higher than *T. harzianum*, and for the two fungi the period of 14 days was with the highest production, also the dilution ratio 100% was the highest increasing.

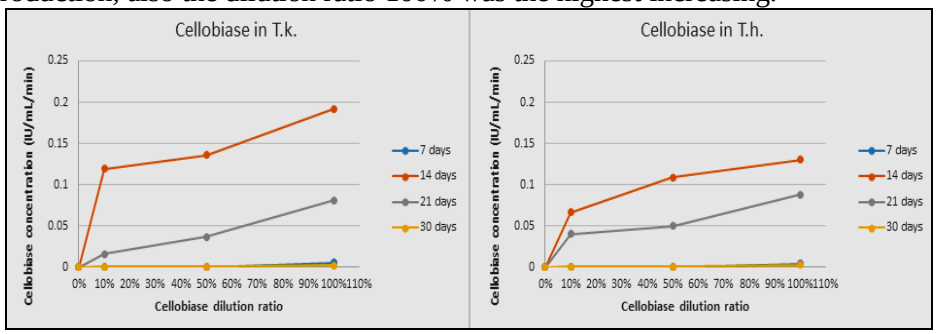

Fig. 4 Cellobiose production in *T. koningii* and *T. harzianum* in a different dilution ratio of enzyme and for a period 1, 2, 3 and 4 weeks

The production of amylase enzyme in synthetic medium in *T. koningii* was higher than *T. harzianum* and the second week was the highest period of production, as shown in figure 5. Generally, the protein levels were in out cell higher than the lysate for all enzymes.

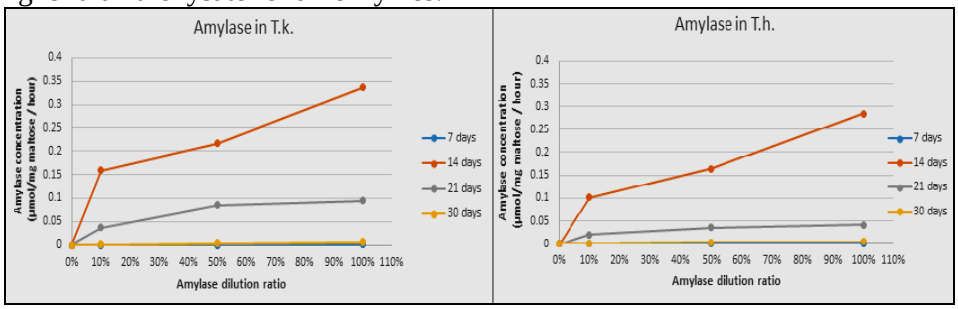

Fig. 5 Amylase production in *T. koningii* and *T. harzianum* in a different dilution ratio of enzyme and for a period 1, 2, 3 and 4 weeks

The protein levels in FPase enzyme were in *T. koningii* higher than the *T. harzianum*, and the second week had the highest levels of protein in the two fungi, as shown in figure 6.

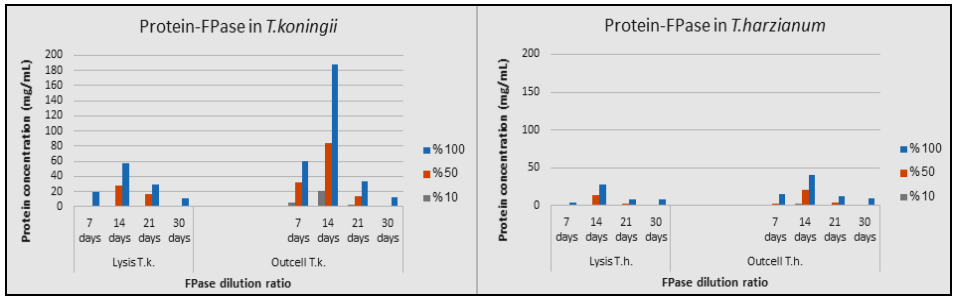

Fig. 6 Protein levels in *T. koningii* and *T. harzianum* in a different dilution ratio of enzyme and for a period 1, 2, 3 and 4 weeks

The highest protein level in the CMCase enzyme medium was *T. koningii* in comparison with its level in *T. harzianum*, but also the period of 14 days was the highest level of protein production in comparison with the rest periods, as shown in figure 7.

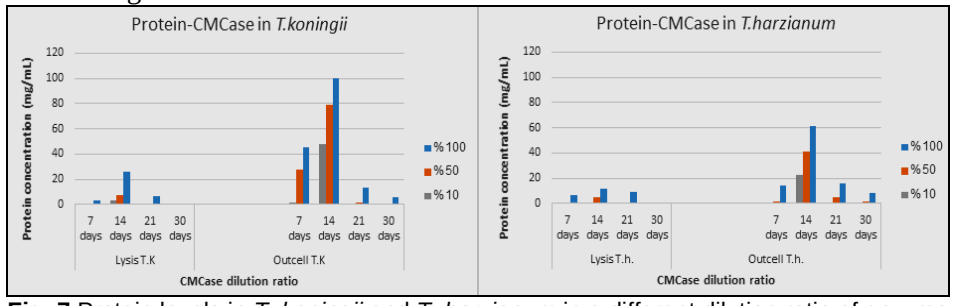

Fig. 7 Protein levels in *T. koningii* and *T. harzianum* in a different dilution ratio of enzyme and for a period 1, 2, 3 and 4 weeks

The result of the level of protein from the cellobiose enzyme are the same as the results of protein levels in FPase enzyme which was the highest level of protein in the second week and in the fungus *T. koningii*. The cellobiose activity was 0.19 IU/mL in *T. koningii* and 0.13 IU/mL in *T. harzianum*, *Trichoderma* spp. are known to produce FPase, the cellobiose in one of the FPase enzymes complex, this enzyme works on the hydrolysis of cellobiose to glucose (De Marco *et. al.*, 2003). The amylase enzyme production was 0.33 IU/mg in *T. koningii* and 0.28 IU/mg in *T. harzianum*, *T. harzianum* produces substantial amounts of lytic enzymes, including amylases, the protein levels in enzymes' medium were proportional proportionality with the levels of enzymes, also the levels in out cell were higher than the cell lysate and the second week had the highest level of protein for all out cell and the lysate.

In figure 8 are presented the levels of protein in amylase enzyme synthetic medium in *T. koningii* and *T. harzianum* respectively, protein levels on *T. koningii* was higher than *T. harzianum*, and for the two fungi the period of 14 days was the highest production. The levels of protein in *T. koningii* were 187.54, 100.24, 28.59 and 86.95 mg/mL for FPase, CMCase, cellobiose and amylase respectively in out of cell, while in cell lysate were in FPase 57.40 mg/mL, CMCase 25.88 mg/mL, cellobiose 16.24 and amylase 34.82 mg/mL. In *T. harzianum* the protein levels were 39.98, 61.30, 21.98 and 72.96 mg/mL for FPase, CMCase m cellobiose and amylase respectively in out of cell, while in cell lysate were in FPase 28.53 mg/mL, CMCase 11.75 mg/mL, cellobiose 10.33 and amylase 25 mg/mL.

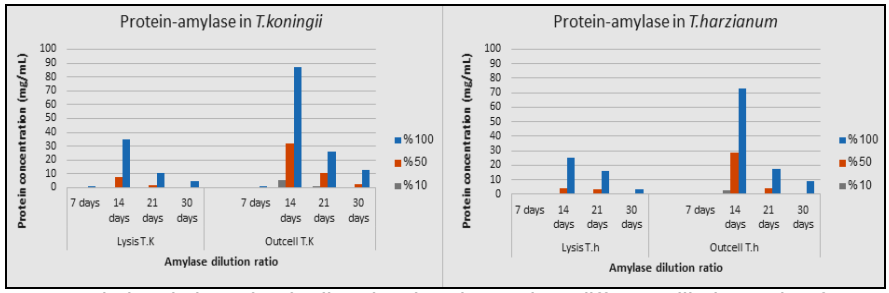

Fig. 8 Protein levels in *T. koningii* and *T. harzianum* in a different dilution ratio of enzyme and for a period 1, 2, 3 and 4 weeks

The results of the effect of *T. harzianum* and *T. koningii* on *F. oxysporum* showed that these fungi have a high ability against the pathogenic fungi *F. oxysporum,* the ratio reached to 1, according to the scale of (Bell *et al.*, 1982). While the cellobiose and the amylase enzyme were not effective on *F. oxysporum* for all period of incubation, also the first, the third and the forth weeks of FPase and CMCase enzymes were not effective. Also table 1 shows the weight results of fungi in the synthetic medium of the enzymes.

Table 1

The weight of fungi in the synthetic medium of the enzymes

	T. koningii			
	Week 1	Week 2	Week 3	Week 4
FPase	0.0032	0.0151	0.01185	0.00135
CMCase	0.0048	0.1138	0.10865	0.00394
Cellobiose	0.00125	0.00355	0.00255	0.000992
Amylase	0.00115	0.01055	0.00755	0.001058
	T. harzianum			
	Week 1	Week 2	Week 3	Week 4
FPase	0.001775	0.00925	0.06245	0.001276
CMCase	0.0026	0.0221	0.012	0.0015
Cellobiose	0.001	0.00315	0.00105	0.000875
Amylase	0.0021	0.00535	0.0039	0.001008

CONCLUSIONS

1. There is an inhibitory effect of *T. harzianum*, strain ICCF 417 and *T. koningii* strain ICCF 418 against pathogen *F. oxysporum* strain ZUM 2407 by production of protein and extracellular enzymes which may effect on the action *F. oxysporum*.

2. The CMCase has the highest weight in comparison with the others enzymes, while the cellobiose and the amylase enzyme were not effective on *F. oxysporum* for all period of incubation.

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