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Effect of wheat straw and cellulose degrading fungi of genus *Trichoderma* on soil respiration and cellulase, betaglucosidase and soil carbon content

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Abstract. *Due to the intensive soil exploitation and increased mineral fertilization, the degradation of plant residues in the soil is becoming more difficult and slower over the years. This disturbs the structure of the soil and the nutritional balance and leads to a reduction in soil fertility. To solve the problem, microorganisms capable of degrading plant residues in the soil can be used. The purpose of this study was to investigate the effect of fungi of genus Trichoderma on the biodegradation of wheat straw in the soil by observation of the change in cellulase enzyme activity in the soil and the increase in soil biological activity. The highest basal soil respiration was noted at T2TUR (65.76 μgCO_2) and T6 (53.69 μgCO_2). During the entire straw degradation period, the highest endoglucanase activity was observed at T4 (285.0 μgGlu) and T6 (275.56 μgGlu), whereas the highest β-glucosidase was noted at T6 (5220.3 μgPNP/g/h) and T1UKR (5020.0 μgPNP/g/h). The presence of cellulose-degrading fungi positively affected the increase in the total amount of microbial biomass at the end of the study period, whereas the amount of Corg was increased in all straw amended variants. At the beginning of the process, CMCase correlated with the microbial carbon (r=0.896 for Cmic) and β-glucosidase activity was closely connected with both soil organic carbon and microbial carbon (r=0.819 for Corg and r=0.866 for Cmic). At the end of the investigated period a stronger correlation with Corg was observed.*

Keywords: *Trichoderma*, soil respiration, endoglucanase, β-glucosidase, straw degradation

Abbreviations: β-Glu - β-glucosidase; CMC - carboxymethyl cellulose; CMCase - carboxymethyl cellulase; CN ratio - carbon to nitrogen ratio; MUB - modified universal buffer; ONA - organic nitrogen amendment; PNG - p-Nitrophenyl-β-D-glucoside; PNP - p-Nitrophenol; Corg - soil organic carbon; Cmic - soil microbial carbon.

Introduction

The degradation of plant residues is a major process in the biogeochemical cycle that provides nutrient resources to the soil and microbial communities. Plant residues have an important role to save the soil moisture. In the last decades they have been considered to be the basis of the preservation of organic matter and the recovery of nutrients in the soil. Microorganisms play a key role in the distribution of nutrients in the soil and the regulation of primary products.

Wheat straw is one of the most abundant agricultural residues and has high content of lignin and cellulose, which impedes degradation in soil (Wang and Bakken, 1997). Each 1000kg^{-1} of wheat exports about 7.8kg of phosphorus (P_2O_5), 5.6kg of potassium (K_2O) and 2.0kg of magnesium (MgO) from the soil (HGCA, 2009). The incorporation of straw will bring back these elements into the soil and will help to preserve the soil structure and productivity. In this way, the waste biomass from agricultural production becomes a useful product that maintains the physical, chemical and biological conditions in the soil and in general improves the ecological balance in it (Krishna et al., 2004). The degradation of crop residue depends on three main factors: soil microorganisms, physical conditions (temperature, moisture, soil structure and presence of O_2) and plant residue properties (baseline C:N ratio). Plant residues with a C:N > 40 ratio are mineralized much more slowly than those with less than 40. The species from family *Poaceae* such as wheat, oats and barley have a high C:N ratio and they are hardly degradable. For wheat straw C:N ratio reaches 97 (Soon and Arshad, 2002).

The wheat straw that remains in the soil hinders the sowing the next crop. To solve this problem, farmers often make the choice to burn the stubbles. Regarding Bulgarian agriculture this is an old practice for stubble cleaning and ensuring trouble-free sowing. Stubble burning causes environmental pollution. A possible solution of this problem is to inoculate microorganisms into the soil as an additive that will accelerate straw degradation. Microorganisms capable of digesting lignocellulose have two types of extracellular enzyme systems: 1) hydrolytic enzyme system - produces hydrolases responsible for the degradation of cellulose and hemicellulose; 2) oxidative lignolithic system - responsible for the depolymerization of lignin (Perez et al., 2002).

To this date, very few studies have been carried out on the use of cellulose degrading microorganisms in the soil. According to van Veen et al. (1997), soil systems act as a buffer against imported microorganisms and they must be extremely adaptable to survive and colonize the soil. In China, the use of microbial inoculants to increase straw degradability was introduced several years ago (Li et al., 2011, 2012; Ogunniyi et al., 2013). There is still insufficient scientific research in this area - the activity of the introduced microorganisms and changes in the soil conditions. Optimizing the potential of these products and examining their catabolic activity is the key to their successful implementation.

The amount of organic carbon in the soil influences soil quality and has direct relevance to its chemical, physical and biological properties. Indicators of soil biological properties are microbial carbon and soil respiration, microbial populations and soil enzymes (Tabatabai, 1994; Kandeler et al., 2006; Marriot and Wander, 2006; Suman et al., 2006; Joachim et al., 2008).

Crop residues such as wheat straw are a suitable habitat for many soil and plant borne pathogenic fungi. The selection of microorganisms to accelerate the degradation of straw should be carried out not only on the basis of their ability to produce celluloses and hemicelluloses, but also on their ability to inhibit the growth of

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plant pathogenic fungi. One of the most investigated microorganisms for the production of extracellular enzymes and the most widely used for the biological control of plant pathogenic fungi are the species of the genus *Trichoderma*. In the present study, five isolates of *Trichoderma* spp. selected in our previous study were tested for their ability to accelerate the decomposition of wheat straw directly into the soil. The enhancement of straw degradation in the soil was evaluated by measuring the soil cellulase activity, β-glucosidase activity, soil organic carbon and the microbial organic carbon.

Materials and methods

Straw and soil preparation

The soil used in this study was collected from 0-20cm depth at field located at the experimental farm of Agriculture University – Plovdiv, Bulgaria. The soil had the follow characteristics: water capacity 56.8%, total N 2.25 g/kg, organic carbon 14.2 g/kg, pH 6.32. The soil samples were crushed and sieved to remove the organic material. The wheat straw (cv. Enola) was collected from crop harvested during the 2015-2016 growing season, air-dried and then crushed into 2-9 mm-long pieces. The straw was mixed with the soil in ratio 10 g/kg of soil (2.5 t da^{-1}, respectively). The wheat straw had the following characteristics: total N - 5.02 g/kg, total C - 412.8 g/kg, and the C/N ratio was 82.2.

Microorganisms

Five fungal isolates, belonging to genus *Trichoderma* were used in this study. They were chosen from the group of 37 isolates, scanned for their ability of wheat straw degradation and biocontrol of soil borne plant pathogenic fungi (Draganova et al., 2017).

Experimental design and sampling

The experiment was performed in growth chamber at 27-28°C for 123 days and consisted of seven treatments: K1 - soil; K2 - wheat straw and soil; T6, T4, T2TUR, T1UKR and T1I - wheat straw and soil inoculated with 1 ml spore suspension ($1*10^6$ spores/ml) of different fungus isolates. A total of 100g of soil mixed with 1g of wheat straw was put into plastic pots. The C:N ratio of the straw was adjusted to 28 with amino acid fertilizer containing 44% of dry matter, 35% of which amino acids and 3.8% total nitrogen. The soil moisture was maintained about 80% of the field capacity with distilled water. The sampling was performed at 24, 48, 72, 96, 144, 192, 240, 288, 336, 504, 624 and 2952 h.

β-glucosidase activity

The reaction mixture consisting of 1g soil, 4 ml MUB (pH 6.0) and 1ml 25 mM PNG (p-Nitrophenyl-β-D-glucoside), was incubated at 37°C for 1h. After incubation 1ml of 0.5 M $CaCl_2$ and 4ml of 0.1 M Tris buffer, pH 12 were added. The absorbance values were read at 400nm on Spectroquant Prove 300. The unit of β-glucosidase activity is expressed as the amount of released μg of PNP (p-Nitrophenol) per 1h by 1ml of enzyme (Tabatabai, 1994).

Endoglucanase (CMCase) activity

The soil cellulase activity was determined by measuring the release of reducing sugars from the substrate carboxymethyl cellulose (CMC) by Somogyi-Nelson method. The reaction mixture consisted of 5g of air-dried soil with 0.5ml of toluene and 20ml of 1% CMC at 0.05M acetate buffer, pH 5.5. The mixture was incubated at 30°C for 24h, and centrifuged at 15000 rpm for 1h. One ml of soil extract was mixed with 5ml of distilled water and a 2-ml mixture of Somogyi I and II (4:1). Six ml of water served as a blank. The final solution was heated in a boiling water bath for 20min. The test tube was cooled in cold water and mixed with 2ml of the Nelson reagent. The absorbance was measured on Spectroquant Prove 300 at 710nm. The cellulase activity was defined as microgram glucose equivalent per gram of soil per 24h (Schinner and von Mersi, 1990).

Soil organic carbon (C_{org})

One gram of soil was put into a 500ml conical flask treated with 10ml of N $K_2Cr_2O_7$ and 20ml of concentrated H_2SO_4. The flask was immediately swirled vigorously for 1min, allowed to stand for 30min, and treated with 200ml of water and concentrated H_3PO_4 (10ml). The residual dichromate was determined by titration with 0.5M Fe^{2+}(Walkley and Black, 1934)

Soil microbial carbon (C_{mic})

Carbon in the soil microbial biomass was determined by fumigation extraction procedure. Twenty-five grams of soil were fumigated with chloroform and incubated for 24h in the dark at 25°C. After that the chloroform was allowed to evaporate for 30min and the soil was extracted with 100ml of 0.5 M K_2SO_4 solution. The soil in the control variant was not fumigated. After mixing on shaker for 30min, the extracts were filtered. Eight ml of soil extract were mixed with 2ml of 0.4N $K_2Cr_2O_7$ and 15ml mix from H_2SO_4 and H_3PO_4 (ratio 2:1). Cooled samples were added to 2 5ml water. After that they were titrated with a solution of 0.01 N $Fe(NH_4)_2(SO_4)_2$ $6H_2O$ in 0.4 M H_2SO_4 with 4-5 drops of o-phenanthroline as indicator (Vance et al., 1987).

Basal microbial respiration

Basal microbial respiration was measured as carbon dioxide (CO_2) released from the soil (Alef, 1995). The CO_2-C output was determined using an alkali absorption technique. The evolved CO_2 from 20g of soil (oven-dry basis) was trapped for 6 hours in 20ml of 0.05 M KOH, placed in a beaker, put into stoppered glass jars. The released CO_2 was determined by titration of the excess KOH with 0.05 M HCl to the phenolphthalein endpoint, after precipitating the carbonate with $BaCl_2$. The results are presented as μg CO_2, released from 1g of soil per hour.

Statistical analysis

All statistical calculations were performed by SPSS statistical software (IBM SPSS Statistics 19.0). The relationships between the data were analyzed by Pearson correlation test at $p<0.01$ and $p<0.05$.

Results and discussion

Straw returning to the soil after harvest is a common method to improve the soil properties but straw residues have high lignocellulose content and high carbon to nitrogen (C:N) ratios and decompose slowly in the fields creating difficulties for farming operations. To estimate the potential as an inoculant to accelerate straw biodegradation after returning straw to the field, five cellulose degrading fungal isolates, belonging to genus *Trichoderma* were inoculated into wheat straw-amended soil in the laboratory.

Basal microbial respiration

Basal microbial respiration is an indicator of the overall biological activity of the soil. During the study period, biological activity remained unchanged and did not exceed 8 μgCO_2/g soil/h in the control variant K1. The change in soil biological activity in all straw containing variants followed a general trend, increasing rapidly to 72h, followed by a decrease until 200h and showed a slight change at the end of the investigated period (2952h). The control variant K2 showed increase in biological activity to 24.1 μg CO_2/g soil/h at 72h and decreased at 144h. After that time, until the end of the observation (2952 h), the activity remained relatively constant. During the evaluation period, soil respiration with added straw was higher than the one without addition of straw (Figure 1).

The same tendency was observed in all variants amended with microorganisms. The reached values were significantly higher compared to K1 and K2. At 24h, samples with microorganisms showed about 6 times higher activity than K2 and about 15 times than K1. Increasing release of CO_2 as a result of microbial activity was observed until 72h. The highest amount of CO_2 was measured at T2TUR (65.76 μgCO_2), followed by T6 (53.69 μgCO_2), T1UKR (50.73 μgCO_2), T1I (49.42 μgCO_2), and T4 (45.20 μgCO_2). In the present study, the increase of soil respiration in variants with microorganisms could be associated with their ability to degrade wheat straw. Similarly, Chen et al. (2007) and Ogunniyi et al. (2013) stated increase of soil respiration evaluated straw degradation by microorganisms.

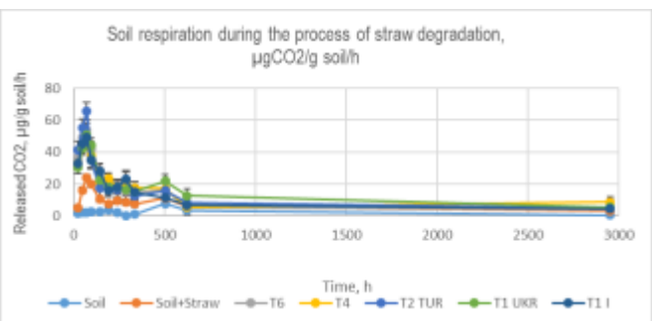

Figure 1. Basal microbial respiration of soil

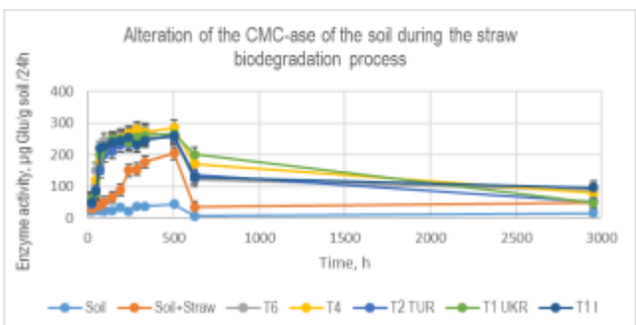

Figure 2. Soil carboxymethyl cellulose activity

The other variants had a maximum enzyme activity at 504h. The highest activity was measured in the variant T4 - 285.0 μgGlu, followed by T1UKR - 265.32 μgGlu, T1I - 260.75 μgGlu, T2TUR - 256.22 μgGlu, K2 - 204.55 μgGlu, and K1 - 49.95 μgGlu.

β-glucosidase activity

A gradual increase in enzyme activity in all amended with microorganisms variants was observed (Figure 3). The highest enzyme activity was observed in variant T6 - 5220.3 μgPNP/g soil/h at 288h, followed by a decline in enzyme activity to 3107.6 μgPNP/g/h at 2952h. The activity of T6 remained the highest compared with the other variants at the end of the observation period. High enzyme activity was also measured in variant T1UKR - 5020 μgPNP/g/h at 288h. This variant ranked second at the end of the process, with activity of 2950.5 μgPNP/g/h. In contrast to T6 and T1UKR, the maximum of the enzyme activity of T2TUR, T4 and T1I was measured at 624h (4848.1, 4746.3, and 4423.6 μgPNP/g/h, respectively).

Maximum enzyme activity of K2 was measured at 240h – 3540.7 μgPNP/g/h which (with slight fluctuations) remained constant to 624h (3498.9 μgPNP/g/h), and decreased to 2045.1 μg PNP/g/h at the end of the observation. During the study period β-glucosidase activity of K1 was approximately 2000 μgPNP/g/h.

During the entire straw degradation period, the variants with added microorganisms had higher total cellulolytic activity compared to the control variants (soil with and without straw). The highest endoglucanase activity was observed at T4 and T6, whereas

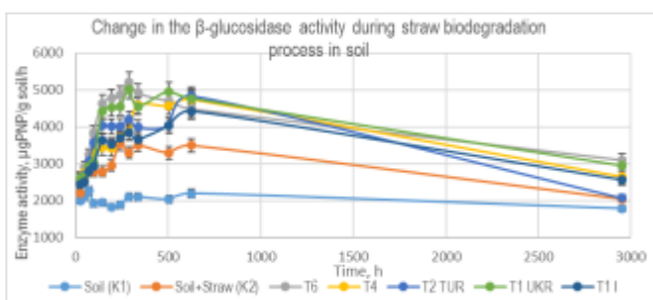

Figure 3. Alteration of the β-glucosidase of the soil during the straw biodegradation process

the highest β-glucosidase was noted at T6 and T1UKR. Endoglucanase is a part of the enzyme complex responsible for degradation of cellulose and attacks the polymer chain in the interior, detaching different fragments of it. The β-glucosidase is a component of the cellulolytic enzyme complex, responsible for the degradation of cellobiose to glucose. After activation of the endoglucanase (carboxymethylcellulose), accumulation of short strands of glucose units resulting in inclusion of β-glucosidase occurs.

Ogunniyi et al. (2013) studied the influence of different microbial systems on the degradation of wheat straw. The authors reported that the soil incorporated with straw showed from 71.6 to 88.5% increased cellulase activity than the soil without the straw. Li et al. (2012) stated that the crop straw dramatically enhances the soil

organic matter and increases the enzyme activity, particularly those of cellulases. Additionally, both research groups reported increase of enzyme activities involved in crop residues degradation. Piotrowska and Koper (2010) observed higher β-glucosidase activity in the soil enriched with farmyard manure.

Determination of total and microbial C in soil

The determination of C_{mic} and C_{org} was performed twice during the study period, in the baseline period (72h) where the biological activity of the soil was the highest and at the end of the study period (2952h) where the processes of active biodegradation were completed and the soil condition could be accepted as their consequence (Table 1). In all straw amended variants with microorganisms, the amount of microbial carbon (C_{mic}) was increased about 6 times compared to the control K1 at 72h. At the same time the increase for K2 was 2.9 times (from 0.31 to 0.91g/kg soil) compared with K1. C_{mic} in the variants with microorganisms exceeds twice the value of C_{mic} at K2. Since the samples contained equal amount of nutrients, the increase of C_{mic} early in the process was due to the added fungi, capable of releasing accessible to the soil microorganism substances from the straw. Soil microbial biomass of the controls K1 and K2 was equal at 2952h. In variants with microorganisms the value of C_{mic} was increased between 1.3 (T4) and 2.1 times (T2 TUR), compared with the controls K1 and K2.

Table 1. Total and microbial C in soil during straw degradation process

Variant	C_{mic}, g/kg soil		C_{org}, g/kg soil		Increase in C_{org} over control (K1), %	
	72h	2952 h	72h	2952 h	72h	2952 h
K1 (Soil)	0.31^{a}	0.38^{a}	14.22^{a}	14.01^{a}	-	-
K2 (Soil+Straw)	0.91^{b}	0.37^{a}	15.22^{ab}	20.64^{b}	7.0	47.3
T6	1.99^{c}	0.60^{c}	20.72^{c}	20.70^{b}	45.7	47.8
T4	2.08^{c}	0.49^{b}	18.00^{c}	20.57^{b}	26.6	46.8
T2 TUR	2.01^{c}	0.78^{c}	18.50^{c}	20.57^{b}	30.1	46.8
T1 UKR	1.84^{c}	0.77^{c}	19.05^{c}	19.81^{b}	34.0	41.4
T1 I	1.83^{c}	0.57^{bc}	14.50^{a}	20.04^{b}	2.0	43.0

*Figures with the same letter in the same column are not significantly different at p≤0.05

The soil organic carbon (C_{org}), measured at 72 h, increased for all straw amended variants compared to K1. A statistically significant increase in the range of 26.6% (T4) to 45.7% (T6) was observed for variants with added microorganisms except for T1I. At the end of the observation period, the amount of C_{org} for all straw amended variants increased as compared to the beginning of the observation. There were no statistically significant differences in the content of C_{org} in all samples with added straw.

Carbon comes into the soil mainly from plant residues. It is related to the processes of humus formation and its quantity is determinative for the physical, chemical and biological properties of the soil and for the soil fertility. The addition of easily hydrolysable C sources to the soil leads to quick microbial biomass increase because the soil is most often C-limited environment (Ocio et al., 1991). In this context, wheat straw amendment and inoculation with microorganisms capable to degrade it, increased soil organic carbon (C_{org}) and microbial carbon (C_{mic}). This is in agreement with the results, obtained by Cayuela et al. (2009), Li et al. (2012) and Ogunniyi et al. (2013).

At the beginning of the process (72h), most of the examined indicators were related to microbial activity (Table 2). High dependence was observed between soil respiration, the amount of microbial biomass on the one hand and the tested enzyme activities on the other. CMCase correlates with the amount of microbial carbon at the beginning of the process (r=0.896 for C_{mic}) more than with C_{org} (r=0.475 for C_{org}). At the end of the process, it is more related to the amount of soil carbon (r=0.721 for C_{org}) than to the available microbial biomass (r=0.214 for C_{mic}).

Table 2. Pearson correlation coefficient of the soil respiration, β-glucosidase, CMCase, soil organic carbon (Corg), and microbial biomass (Cmic) at 72 and 2952h

72h	SR	β-Glu	CMCase	C_{org}	C_{mic}	2952h
SR	1	0.605	0.643	0.712	0.385	SR
β-Glu	0.864*	1	0.679	0.551	0.466	β-Glu
CMCase	0.822*	0.604	1	0.721	0.214	CMCase
C_{org}	0.693	0.819*	0.475	1	0.431	C_{org}
C_{mic}	0.950**	0.866*	0.896**	0.708	1	C_{mic}

Correlation is significant: (*) at the 0.05 level, (**) at the 0.01 level

β-glucosidase activity at the beginning of process of straw decomposition was closely connected with both soil organic carbon and microbial carbon (r=0.819 for C_{org} and r=0.866 for C_{mic}). Piotrowska and Koper (2010) also reported positive correlation between β-glucosidase activity and soil organic carbon and total nitrogen content (r=0.611-0.770 for C_{org}, and r=0.844-0.912 for N_{org}).

Conclusion

The results of our study showed that incorporation of wheat straw and addition of fungi of genus *Trichoderma* into soil can increase soil enzymes activity indicating their ability to improve straw degradation. An increase was found in microbial activity and soil organic carbon accumulation that are important for nutrient cycling and soil fertility. The most intense increase in the basal soil respiration was observed up to 72h, cellulase activity was maintained at maximum values up to 504h and β-glucosidase up to 624h. These indicators are positively influenced by the addition of straw and cellulose-degrading fungi. The highest basal soil respiration was noted at T2TUR and T6. During the entire straw degradation period, the highest endoglucanase activity was observed at T4 and T6, whereas the highest β-glucosidase was noted at T6 and T1UKR. The addition of straw resulted in an increase in the amount of microbial biomass. This increase was even more pronounced after the addition of fungal isolates. At the end of the process the amount of microbial biomass increased only in the variants with added fungi. Conversely, at the end of the study period the amount of Corg was increased in all straw amended variants, without difference between K2 and all tested fungal isolates. At the beginning of the process, most of the examined indicators were related to microbial activity. High dependence was observed between soil respiration, the amount of microbial biomass, on the one hand, and the tested enzyme activities, on the other. At the end of the process, where nutrients are depleted, the processes began to depend more heavily on the amount of soil carbon than the available microbial biomass. Loss of soil organic matter content and soil fertility due to intensive cropping system can be reverted by spraying stubble with fungi of genus *Trichoderma* and incorporation of wheat straw into the soil. This residue management technology may help to preserve the soil structure and productivity and it can be an option of eco-friendly technology for effective utilization of plant residue.

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Genetics and Breeding

Nutrition and Physiology

Production Systems

CONTENTS 2 / 2

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The manuscript should be structured as follows: Title, Names of authors and affiliation address, Abstract, List of keywords, Introduction, Material and methods,Results, Discussion, Conclusion, Acknowledgements (if any), References, Tables, Figures.

The title needs to be as concise and informative about the nature of research. It should be written with small letter /bold, 14/ without any abbreviations.

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Thesis:

Hristova D, 2013. Investigation on genetic diversity in local sheep breeds using DNA markers. Thesis for PhD, Trakia University, Stara Zagora, Bulgaria, (Bg).

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