THE EFFECT OF BIODIESEL BY-PRODUCTS ON GERMINATION AND PLANT GROWTH

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Abstract. The aim of this paper is to investigate the effects of agricultural utilization of biodiesel by-products. The hydrophilous by-products of transesterification, such as proteins, carbohydrates, minerals, vitamins, potassium hydroxide used as catalyser and methanol are concentrated in the phase of glycerol during biodiesel production. Agriculture can utilize these components but some effects of glycerol in soil do not serve the needs of plants. Several studies have investigated the different effects of glycerol in soil. The specific focus of our research is to analyse the relationship between seed germination and glycerol content of soil. During this research the inhibitor effect of glycerol, biodiesel by-product, and methanol on seed germination of ryegrass (*Lolium perenne* L.) and oilseed rape (*Brassica napus*) were investigated. In the case of some treatments, a different percentage of glycerol and methanol was applied, in order to establish the methanol content of soil that can balance the inhibitor effect of glycerol. Based on the obtained information, pot experiment was established with ryegrass to examine the effects on plant growth. Our research has not only studied the impact of biomass production, but variations in the rate of plant growth as an effect of different treatments were observed as well.

Keywords: *biodiesel by-product, glycerol, germination, microbe*

Introduction

The industry of biofuels is one part of reneweable energy sources which is gaining ground in leaps and bounds. Between 2000 and 2007, global biofuel production tripled from 4.8 billion gallons to 16 billion gallons. Biodiesel production was about 3 billion gallons in 2007 and more than 50% of this quantity was produced by EU (Coyle, 2007). By prognosis, this process will increase, which raises the issue of by-product utilization. The newest, third generation possibility is to produce biofuels from algae. Algae were cultivated as biofuel feedstock mainly due to their high productivity of oil and less land requirement. Microalgae do not compete for land and can grow anywhere, even in brackish saline water (Sanjay et al., 2013; Piyushi et al., 2014).

During biodiesel production a huge amount of useable by-products are produced with high glycerol content. Outputs of biodiesel production depend largely on oil type and quality. Approximately 100 litres of vegetable oil, 25 litres of methanol, and 0.8 kg KOH catalyser are used to produce 75 litres of biodiesel and 25 litres of crude glycerol (Wilkie, 2008). Generally the glycerol is used by the cosmetic and chemical industry but biodiesel by-product is contaminated by vegetal parts. But this „vegetal contamination" makes it perfect for utilizing this by-product as a fertilizer on agricultural fields.

The glycerol is an easily available and adequate carbon source for microorganisms (Lee et al., 2001; Tickell, 2003). It can intensify microbial activity which can help to increase the availability of vegetal nutrient. The large quantities of microbes increase organic nutrient content, the organic part of soil, which can increase humus content and results good conditions in soil. One part of nutrients can concentrate in the phase of glycerol, so it can balance the vegetal absorption. To sum up, glycerol has direct and indirect effects on productivity and nutrient content of soil.

Review of literature

During biodiesel production vegetable oils and animal fats are transesterificated by methanol or ethanol in the presence of a KOH or NaOH catalyser. At the end of this process, the results are alkyl ester and glycerol (Furfari, 2008). Solubility behaviour of the two residues can help to separate the hydrophilous glycerol from hydrophobic ester (Holser, 2008). If methanol is used during transesterification, the result is fatty acid methyl ester (FAME), whereas if ethanol is used, the result is fatty acid ethyl ester (FAEE). In the European Union biodiesel is rapeseed-oil methyl ester (Kovács, 2000).

Glycerol of biodiesel production is well utilized by microorganisms (Papanikolaou et al., 2008; Temudo et al., 2008). Studies have investigated whether the utilization of glycerol by microorganisms is a great possibility to convert that into value-added products (Barbirato et al., 1998; Johnson and Taconi, 2007; Yazdani and Gonzalez, 2007). The measurment of biologically active functions of organic matter, such as microbial biomass C, N, P and potential C, N and P mineralization were better able to reflect changes in soil quality and productivity (Patel et al., 2010). Biebl et al. (1998) have analysed fermentation of glycerol by *Klebsiella pneumoniae* (Biebl et al., 1998; Zeng et al., 1993). Biebl also investigated fermentation of glycerol in the case of *Clostridium pasteurianum* batch (Biebl, 2001).

Soil microorganisms adsorb nitrogen instead of plants and retard their development. Provisionally, microbes immobilize nitrogen of chemical fertilizers and the mineral nitrogen part of soil, but later mobilize them (Tolner et al., 2010b). This process prevents the wash-out of nitrogen and subsequently the destruction of microbes nitrogen will be available for plants again. The glycerol increases the storage capacity of soil and helps adsorption of nutrients by solvent action. Dispersive capacity and coagulability of glycerol can cause friable texture of soil (Czinkota, 2007).

The nutrition providing ability of the soil can be tested with soil tests and plant experiments. The growth rate of plants reacts sensitively to the current nutrition supply. The development of plants, thus the dry matter accumulation, is not linear in time, in the vegetation period it is connected to certain stages of development (Lásztity et al., 1984; Waldren and Flowerday, 1979; Prew et al., 1985), showing changes which are genetically determined yet influenced by external ecological factors, it is the result of the interaction of all these (Lásztity, 2006). The growth rate depends significantly on the available nutrient (Jocic 1981; Lásztity and Kádár, 1978). The development of the plants can be followed by visual bonitation and computer processing. Narumalani et al. (2009) and Auda et al. (2008) tried to gather information on the spread of the invasive plant species. Sanyal and Patel (2008) estimated the health and nutrition ability of rice plant by the shape and size of the plant. Timmermnas and Hulzebosch (1996) used

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image analysis for isolating the growth stages and plant parts. Behrens and Diepenbrock (2006) scanned the development of the swede rape with the help of image analysis. It was established in a former experiment that linear relationship stands between the number of green pixels and plant green mass.

Materials and Methods

Two plant species were investigated in complex experimental series to gain information about the reaction of plants for the glycerol content of soil which is the most determinant component of biodiesel by-product.

In our paper, on the one hand **germination tests** of ryegrass (*Lolium perenne* L.) and oilseed rape (*Brassica napus*) will be presented, which were carried out in order to study the germination retarding effect of glycerol. After the results, on the other hand, **plant growth test** was carried out which was performed only in the case of ryegrass (*Lolium perenne* L.) in order to investigate the growth inhibitor effect of glycerol in the later stage of plant life.

Arenosol from Fót was applied for the treatments. The main parameters of this soil were: plasticity index of soil according to Arany K_A = 28.33, lime content ($CaCO_3$%) = 8%, pH(H_2O) = 8.2, humus content (H%) = 1.4%., AL-P_2O_5 = 95 ppm, AL-K_2O = 120 ppm. The water content of the soil was 60% of field capacity. We used analytical quality materials for treatments in both experiments (*Table 1*).

Table 1. *Different treatments applied for experiments*

Treatments in germination experiments	Treatments in growth experiments
PK as control	
NPK as control	Control
0.5% C glycerol	0.5% C glycerol
0.5% C methanol	0.5% C methanol
0.5% C 50% glycerol + 50% methanol	0.5% C 50% glycerol + 50% methanol
0.5% C 90% glycerol + 10% methanol	0.5% C 90% glycerol + 10% methanol
0.5% C by-product	0.5% C by-product
1% C glycerol	
1% C methanol	
1% C 50% glycerol + 50% methanol	
1% C 90% glycerol + 10% methanol	
1% C by-product	
0.25% C by-product	

Germination experiment

90 grams of soil and 100 pieces of seed were placed in every single Petri dish. We did not use pretreatment to break the resting period of plants. After this Petri dishes were set into a refrigerator to reach the required moisture content for seed germination.

In the first stage, the effects of glycerol, glycerol-methanol combination, methanol and biodiesel by-product were investigated. In the second stage, the modification effect of a two-week incubation period was analysed. After the preparation of treatments,

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seeds of ryegrass and oilseed rape were not placed into soil, only after the two-week incubation.

We wanted to ascertain that the two-week incubation is enough for microorganisms to metabolize glycerol content of soil.

The first experiment was accomplished in four replications. The second experiment, in the case of the two-week incubation, was carried out in two replications, and the immediate seeding was also carried out in two replications. Plantlets were counted in Petri dishes on the fifth day. We tookthist as a basis of absolute germination.

Plant growth experiment

The second column of *Table 1* shows the treatments that we started to apply in pot experiments on 2^{nd} of September 2010. Every pot contained 900g soil which were treated before the sowing of a 1 g seed of *Lolium perenne* L. (ryegrass). The water content of soil was 60% of field capacity which was kept continuously at this value by sprinkling. The culture pots were set in a closed place and were kept under artificial daylight. Treatments were carried out in four replications.

The images were taken at certain times depending on the plant growing status. There were 24 pots and 6 treatments. All experiments were accomplished in four replications. Eight pictures were taken of each pot from different angles (at 45^o degrees). On the plant images the image processing program counts the green pixels representing the colour of leaves and thus, we could convert the plant growing status to numerical data. After the pixel numbers were gained, a calibration was made which was able to transform the numbers to plant leaf mass measured in grams. In all treatments the soil samples were mixed with solutions. The calculated amount of materials for the treatments were put in the soil, dissolved in this amount of water. The observation started on the 5^{th} day and ended depending on the plant growing status (Tolner et al., 2010a).

Evaluations

The outlier mean square deviations were examined by Cochran test (Sváb, 1981). The effect of treatments was evaluated by analysis of variance. We used the ANOVA program on Visual Basic algorithm in Microsoft Office Excel which was based on Sváb (1981). This algorythm has been successfully applied in several studies (Kátai et al., 2013; Kovács et al., 2013; Szabó et al., 2013).

Results

Results of germination experiment

The effect of treatment combinations were evaluated by ANOVA with 0.1% probability of error. Reduction in the number of germs were significant in almost every treatment in the case of both species, where glycerol or biodiesel by-product were incorporated into the soil as compared to NPK control.

The inhibitor effect of glycerol was total in 1% C glycerol and the number of germs was zero. There was no significant difference between the results of 0.5% C glycerol and 0.5% C by-product, both of them had inhibitor effect. Moreover there was no significant difference between the results of 1% C glycerol and 1% C by-product

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(*Figure 1*). Thus, these results verified that the inhibitor effect on germination was caused by the glycerol content of by-product.

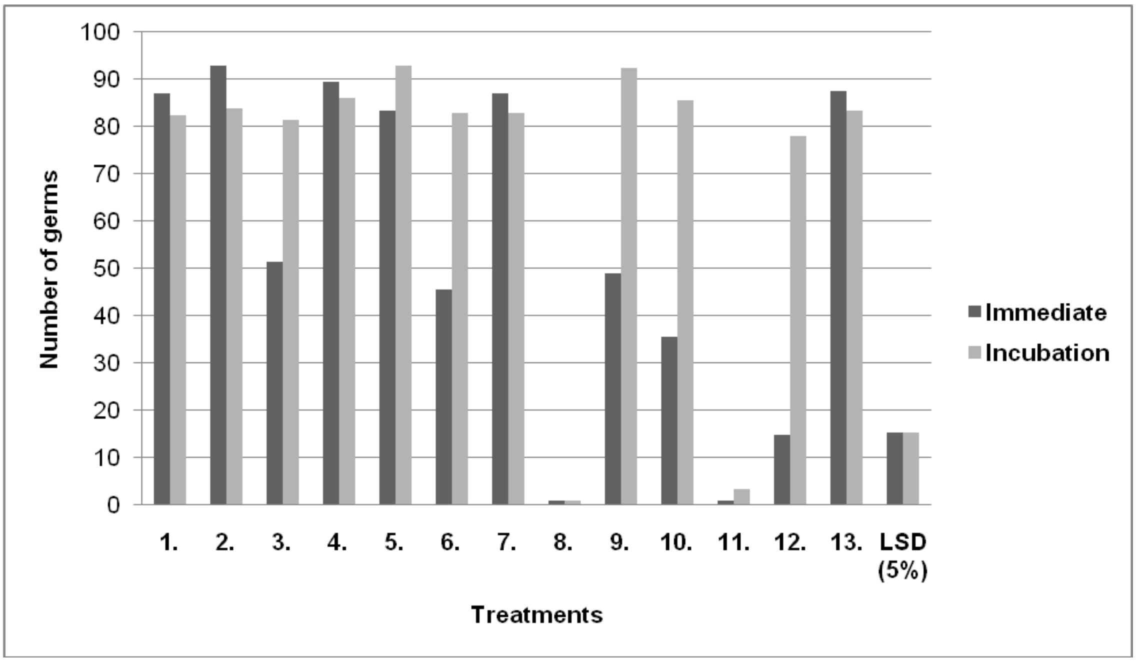

Figure 1. *Number of germs in case of ryegrass*

Blight was experienced in treatments where by-product or glycerol were appiled. Generally Petri dishes of the by-product included more colonies than Petri dishes of glycerol and glycerol-methanol combinations. The reason for this phenomenon was the nutrient content of by-product. During biodiesel production, by-products of transesterification (proteins, carbohydrates, minerals, vitamins, potassium hydroxide used as catalyser, in the case of transesterification used methanol) were concentrated in the phase of glycerol which could increase the effect of glycerol as a carbon source. The smell of blight was typical which was also sign of microbial intensification.

The treatments having methanol content did not inhibit the germination (*Table 2*). Since the methanol does not have a significant inhibition effect on germination, glycerol treatments became comparable to each other.

Table 2. *Inhibitor effect of the pure methanol treatmennts compared to control*

Treatments	Immediate	Incubation	**Mean**	LSD(5%)
2. Nitrogen (NPK)	77	88	**82**	11
4. 0,5% C Methanol	74	87	**81**	
9. 1% C Methanol	64	80	**72**	
Mean	**72**	**85**		
LSD(5%)	9			

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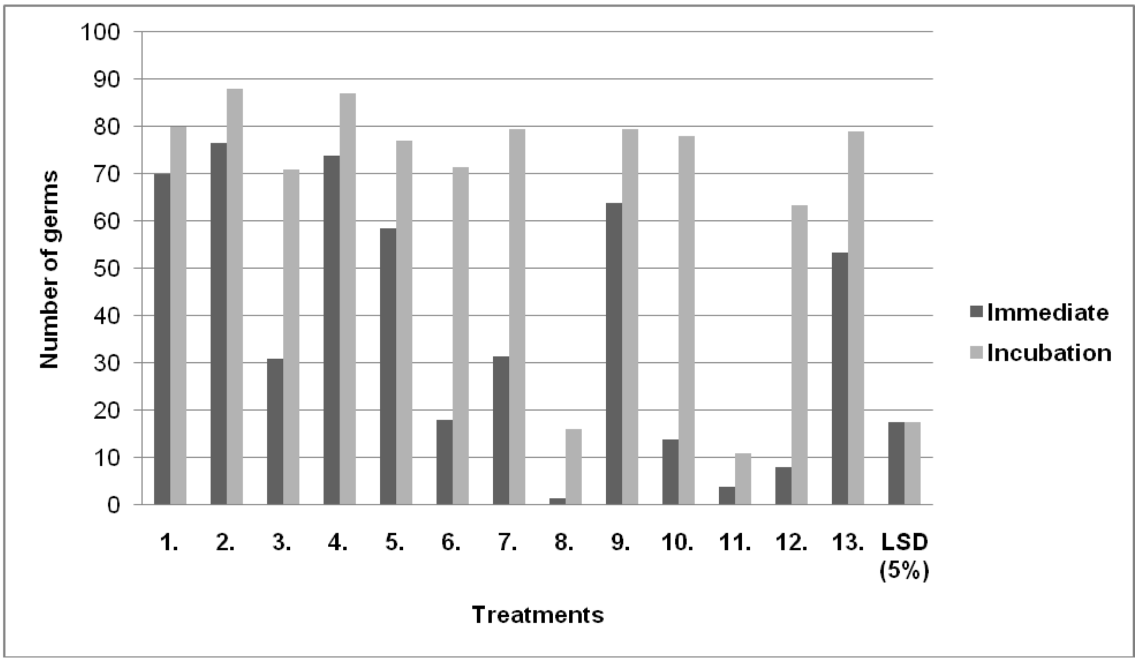

Figure 2. *Number of germs in case* of oilseed rape

The experimental dates of 13 levels of treatments in two incubation periods were evaluated using two-way ANOVA. There were significant differences between the germination of seeds in the functions of treatments and incubation periods as well (p<0,1%).

The two factors had a strong interaction (F-rate=5,69 p<0,1%). The case of immediate sowing treatments resulted in significant differences, but after the incubation period the dependency by treatments became less harmful.

There was a strong interaction between the number of germinating seeds and the C% ratio in the glycerol phase. It can be seen in *Figure 1* and *2* that without incubation the germination was more sensitive to C% amount in glycerol, than after the two-week incubation.

However, this negative effect of glycerol could be moderated by two-week incubation. Comparing the reactions of biodiesel by-product of the two plants, it was obvious that *Lolium perenne* L. (ryegrass) has a higher tolerance for negative conditions than *Brassica napus* (oilseed rape).

Results of plant growth experiment

After analysiing the pictures a lot of information was gained about plant growth. The complex analysis of the huge database gave appraisable information. During the experiments, the reactions of plants were intensive to different stresses. That was experienced in differences between length of germination times and velocity of growth. Also there were differences between plant individuals within the same culture plates. For quantity determination of differences, photos were taken from eight different angles.

Table 3. shows thousandths of average number of pixels of eight photos in four replications, with different treatments, and at different times of the experiment. Variations of gray backgrounds demonstrate the coefficient of variation (CV) of data

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originated from the eight different angles. White shows CV lower than 10%, light gray shows CV between 10% and 20%, dark gray shows CV greater than 20%.

Table 3. *Development of plants between 11th September to 3rd October (number of pixels/1000)*

Sowing 2.09	11.09	12.09	15.09	18.09	21.09	24.09	27.09	30.09	03.10
Control	768	956	988	1048	1017	1088	1146	1210	898
	733	880	911	968	828	857	**881**	1098	973
	721	850	817	905	891	958	1141	1279	1092
	815	981	1117	1241	960	1292	1384	1361	1408
0,5% C 100% glycerol	**22**	36	**108**	261	339	469	539	705	815
	26	**64**	**230**	428	507	626	763	869	929
	22	**25**	**44**	**142**	215	336	465	539	647
	22	**31**	**40**	129	205	320	471	514	614
0,5% C 100% methanol	254	374	533	662	726	913	1025	985	1185
	146	**263**	457	593	645	823	1113	1094	1107
	200	320	486	619	694	891	1117	1124	967
	233	354	524	649	743	933	1162	1049	1147
0,5% C 50% methanol 50% glycerol	284	424	624	776	815	937	1208	1291	1043
	227	353	542	685	694	786	963	1200	1032
	243	376	597	745	795	938	1104	1178	834
	267	409	639	794	810	1018	1275	1132	1160
0,5% C 10% methanol 90% glycerol	**24**	**62**	**216**	388	498	596	757	840	949
	47	**111**	**258**	413	495	564	700	751	862
	34	**69**	**208**	342	404	469	608	636	718
	32	**77**	**257**	**401**	470	567	736	821	826
0,5% C by-product	417	530	678	788	833	775	1037	1053	1045
	406	503	605	725	744	764	973	879	**773**
	385	500	606	688	710	730	925	955	883
	467	595	778	908	876	1035	992	940	801

At every measuring moment, the values of mean-square deviation of standardized data of photos from 6 treatments, from 4 replications were studied using the Cochran test at value P = 5%. In calculation of standardized data, the number of pixels of every single photo was divided by the average number of pixels of eight photos. So the standard deviation of different development stages of plants could be comparable. After that the first 24 mean-square values were investigated to determine outlier data. That process was continued until outlier data was found.

The heterogeneity of plants stand of culture plates were characterized by relative deviation. So the number of pixels were normalized by the mean of pixels of eight photos. The standard deviation and mean-square deviation of normalized values of pixels also were determined.

Data shows that an increase in stress caused not only retardation of growth of germinated plants but also augmentation of irregularity in shooting and in growth. That effect is mainly perceptible in the first section of the growing phase. Between elder plants differences would be equalized progressively. In the case of elder plants the different treatments (Control, By-product) resulted outlier deviation values sporadically which was caused by technical measuring problems. The longer spears were bent over so the optical observing based investigation would become improper.

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In the following analysis the standard deviation of pixels of 8 photos was disregarded, the different growth status of plants were characterized only by means of thousandth pixel number of 8 photos.

The homogeneity of standard deviations of different treatments at different times was investigated using the Cochran test but outlier values were not received. After that the whole experiment was evaluated by two-way analysis of variance.

The effect of treatments and changes in time were strongly significant. In accordance with control every treatment caused depression. This was lower in the case of 100% methanol, 50% methanol-50% glycerol and 100% by-product. Significant difference was not experienced between the harmful effects of 100% methanol and by-product. The most retarding effect was observed in the case of treatment 100% glycerol. The changes in time are demonstrated in *Figure 3.*

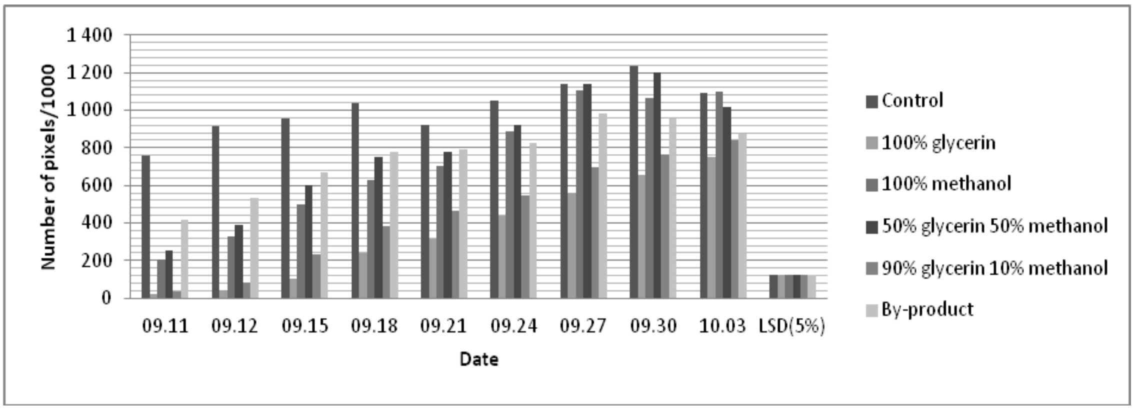

Figure 3. Result of the plant growth experiment with ryegrass

Conclusions

Theresults of our research are important to define the best time for fertilization of by-product.

Glycerol and biodiesel by-product had a significant negative effect on the germination of ryegrass in an arenosol, 1% concentration of glycerol inhibited the germination.

The same inhibitor effect on germination of glycerol and by-product verified the glycerol content of by-product.

The microbial stimulant effect of glycerol bred blight (*Aspergillus* spp.) colonising Petri dishes where glycerol and by-product were applied.

The inhibitor effect of glycerol was decreased in treatments of 50% glycerol - 50% methanol combination. This combination resulted good conditions for plants. In treatments of 1% C 50% glycerol - 50% methanol the improving effect of two-week incubation was significant.

During the research we could not verify clearly the inhibitor effect of methanol in treatments.

Generally we can state that biodiesel by-product was more useful for microorganisms of soil than for plants.

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