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EGYETEM**

GÖDÖLLŐ

**SZENT ISTVÁN UNIVERSITY
DOCTORAL SCHOOL OF ENVIRONMENTAL SCIENCES**

Effects of biodiesel by-products on the soil-plant system

THESES OF THE DOCTORAL (PhD) DISSERTATION

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Introduction and objectives

Increasing energy consumption results in increased environmental exposure. One of the most significant effects is climate change due to the large amounts of CO₂ and other greenhouse gases emitted into the atmosphere. Nearly one sixth of the increase in the emission is due to transportation. The use of biomass stocks as a conditionally renewable energy source could be an alternative in the reduction of gaseous emissions into the atmosphere. The European Union's regulatory system requires the use of an increasing proportion of biofuels, which is the goal to be achieved by the Member States by 2020. While Germany is at the forefront of producing biodiesel in Europe, Hungary is far behind on the list of the Member States, despite the fact that Hungary has very large useable areas for growing rapeseed and sunflowers crops in terms of agricultural and climatic conditions. However, it would also be necessary to increase the growth of domestic biodiesel plants to achieve the EU-required share ratio. During the production of biodiesel a large amount of contaminated glycerol by-product arises, which can only be sold to the cosmetic and chemical industries after purification. Utilizing the residual by-product is the most significant cost-cutting factor in the production chain.

Since I started my research, more and more publications have been concerned with effect of the by-products on the nutrient management of the soil, with the aim of examining the carbon backflow into the soil. The possible toxic effect of impurities and glycerol, which is the largest part of the biodiesel by-product, have been discussed in several publications in the literature. On the other hand, only a few international papers have made a detailed examination of the most significant impurities present (glycerol and methanol) in the contaminated glycerol by-product. The Hungarian literature also lacks information about this topic.

The aim of my doctoral dissertation was to examine the contaminated glycerol by-product and the impurities produced during the process of biodiesel production. In my research, I studied the effects of the above-mentioned materials on the nutrient management of the soil, on the germination of plants and on the nutrient supply of the soil. For each experiment, sandy soil was used as a planting medium because the most acute effects were expected with this type of treatments.

I was looking for answers for the following question:

- How does glycerol treatments affect the change of mineral nitrogen forms in the soil?
- How can glycerol affect the germination of plants (ryegrass and rape) in the soil?

- What can be the impact of the contaminants in the biodiesel by-product?
Can the effects be toxic?
- What are the effects of glycerol in soil on plant growth by application of ryegrass as an indicator plant?
- Can the glycerol cause stress effect on plant development?

Materials and methods

During my research, I examined the effects of contaminated glycerol by-product and other impurities on sandy soil. The soil incubation experiment was followed by germination experiments with the ryegrass (*Lolium perenne* L.) and rape (*Brassica napus*). Further experiments were carried out with ryegrass (*Lolium perenne* L.) as an indicator plant to observe the effects of the biodiesel by-product on plant growth.

The following substances were used as treatments in the experiments:

- 99.5% laboratory pure **glycerol**,
- 96%-os laboratory pure **methanol**,
- contaminated glycerol by-product, with 86% of glycerol, 10% of methanol, 2% of KOH, protein, lipid, phosphate compounds and oily soap 2%.

The following soil was used during the experiments:

- Lime sandy textured soil from Fót. The main parameters of the soil: $K_A=27$, $CaCO_3\% = 8\%$, $pH(H_2O) = 8,2$, $H\% = 1,4 \%$, $AL-P_2O_5 = 95$ ppm, $AL-K_2O = 120$ ppm.

Sandy soil was used as a planting medium because the most acute effects were expected with this type of treatments. Due to its low humus content, this type of soil responds the most sensibly to the effects caused by the treatments. The soil samples were dried out and sifted through a 2 mm sieve.

Effect of soil incubation on the change of mineral nitrogen content of the soil

In order to study the effect on the mineral nitrogen content of the soil, a four-week long soil incubation experiment was carried out by adjusting soil moisture content to soil moisture at room temperature (hereinafter maturing). In addition to control and nitrogen treatments, the maturation experiment used different glycerol and by-product doses, which were performed in four-replicates on sandy soil. Potassium sulphate, potassium phosphate and ammonium nitrate were measured on an analytical scale.

200 g of air dried soil was placed in each plastic vessel. The materials used for the treatments were dissolved in a solution and mixed in the soil samples.

The following treatments were used during the experiment:

- Treatment 1: 100 ppm P_2O_5 + 100 ppm K_2O (in the form of KH_2PO_4 and K_2SO_4) – **Control (PK)**
- Treatment 2: PK + 100 ppm N (in the form of NH_4NO_3) – **Nitrogen (NPK)**

- Treatment 3: NPK + 1% glycerol as carbon source – **1% C glycerol**
- Treatment 4: NPK + 0,5% by-product as carbon source – **0,5% C by-product**
- Treatment 5: NPK + 1% by-product as carbon source – **1% C by-product**

50-50 g of soil samples were taken from the vessels and extracted with 1% KCl solution. Parnass-Wagner water vapor distillation equipment was used to determine the contents of the extracts $\text{NH}_4\text{-N}$ and mineral nitrogen ($\text{NH}_4\text{-N} + \text{NO}_3\text{-N}$) (Bacsó et al. 1972). Nitrate content was determined from the difference between the two results.

Investigation of the effect on the germination of ryegrass

In the first step of germination experiments, ryegrass (*Lorium perenne L.*) was used as an indicator plant and sandy soil was used as a planting medium. Carbon sources at different ratios of treatments were administered in the form of glycerol, methanol, glycerol-methanol blends and by-product.

Two separate cases were investigated. In the first step, the indicator plant germination responses were observed with varying concentrations of glycerol, methanol, glycerol-methanol and by-product on their fifth day stage. In the next step, the previous experiment was repeated with the addition that the seeds were planted immediately in one half of the replicates, while in the other half was planted in two-week long incubated soil.

After that, an examination was made on how many seeds germinated from the seeds affected by treatments. The first experiment was carried out in four replicates, the second one in two-two replicates. The solutions used for the treatments were transferred to the soil samples by mixing.

Petri dishes were used in the germination experiment. In each dish, 90 g of sandy soil and 100 seeds of ryegrass were placed in each dish. Pretreatment was not used in order to stop the resting state. The ryegrass seeds were scattered evenly so that neither the seeds nor the growing seedlings could contact each other.

The moisture content of the soil was adjusted to 60% of the moisture content of the „Arany-féle kötöttség” using distilled water to ensure a loose and uniform wet planting medium for ryegrass seeds. Then it was covered with a cover layer that was previously removed from the soil samples. During the experiments, the irrigation of the dishes were compensated by weighing. The following treatments were used during the experiment:

- Treatment 1: 100 ppm P_2O_5 + 100 ppm K_2O (in the form of KH_2PO_4 and K_2SO_4) – **Control (PK)**
- Treatment 2: PK + 100 ppm N (in the form of NH_4NO_3) – **Nitrogen (NPK)**

- Treatment 3: NPK + 0,5% glycerol as carbon source – **0,5% C glycerol**
- Treatment 4: NPK + 0,5% methanol as carbon source – **0,5% C methanol**
- Treatment 5: NPK + 0,5% glycerol-methanol blend as carbon source, 50% of glycerol and 50% of methanol – **0,5% C 50% glycerol + 50% methanol**
- Treatment 6: NPK + 0,5% glycerol-methanol blend as carbon source, 90% of glycerol and 10% of methanol – **0,5% C 90% glycerol + 10% methanol**
- Treatment 7: NPK + 0,5% by-product as carbon source – **0,5% C by-product**
- Treatment 8: NPK + 1% glycerol as carbon source – **1% C glycerol**
- Treatment 9: NPK + methanol as carbon source – **1% C methanol**
- Treatment 10: NPK + 1% glycerol-methanol blend as carbon source, 50% of glycerol and 50% of methanol – **1% C 50% glycerol + 50% methanol**
- Treatment 11: NPK + 1% glycerol-methanol blend as carbon source, 90% of glycerol and 10% of methanol – **1% C 90% glycerol + 10% methanol**
- Treatment 12: NPK + 1% by-product as carbon source – **1% C by-product**
- Treatment 13: NPK + 0,25% by-product as carbon source – **0,25% C by-product**

Investigation of the effect on the germination of rape

The germination experiment with rape (*Brassica napus*) was carried out using the same methodology as described above. Details of the treatments were been presented in the previous chapter. The interaction between treatments and incubation was investigated in two-two replicates, immediate sowing, and two-week long incubation, and then analyzed on the ninth day state.

Calibration model of the growth experiment

At the Department of Soil Science and Agrochemistry, a hardware and software system was developed on pot experiment and image processing, which allows automated observation of plant growth at any time and without damaging the plants.

Thus the designed equipment can provide information about the dynamics, the rate of growth and the factors that ensure or inhibit development, over a longer period of time.

Under optimized laboratory conditions, colored images were taken from different angles of the growing plants. A software was developed to calculate the green pixels on the pictures and the dry mass of the plants was also measured. The target software improved the statistical accuracy of the experiment by using statistical methods of the different angles and treatment replicates. The schematic illustration of the equipment used for optical observation is shown below (Figure 1).

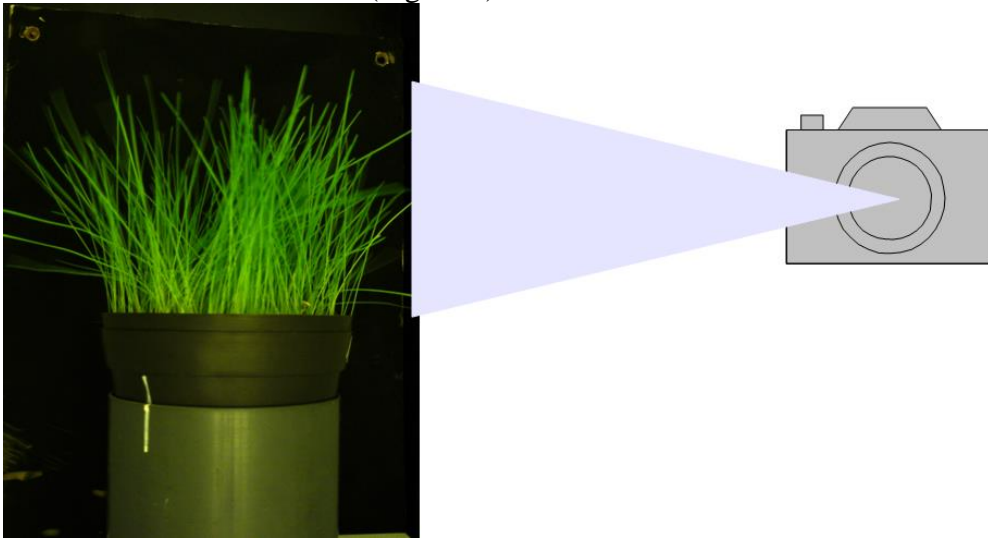


Figure 1. Design of the optical observation

For the calibration of the method, a plant experiment consisting of four replicates was set up. Ryegrass indicator plants were cultured four times in 10 500 cm³ pots containing 900 g of sandy soil. In the upper layer of the soil, 1-1 grams of ryegrass seeds were sowed. Every second day, the shoots were cut off, dried out and measured on a scale.

As a result, dried shoot mass and optical data were simultaneously obtained at 10 times (4, 6, 8, 10, 12, 14, 16, 18, 20, 22 days). A linear model was fitted on the data.

The figure below shows that there is a close linear relationship ($R^2 = 0.8841$) between the number of green pixels obtained from the image processing analysis and the dried out weight of the plants.

The fitted model is in line with the literature (Tolner et al. 2010a, Kovács et al. 2011c, Vasseur et al. 2017). The regression coefficient is practical to determine plant production day-by-day without damaging the plants (Figure 2).

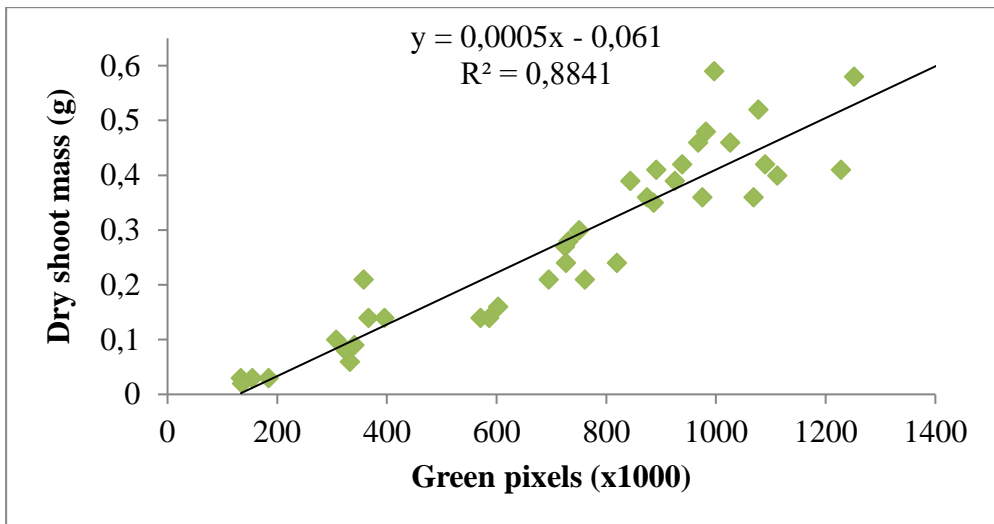


Figure 2. Calibration model of the plant growth

Introduction of the growth experiment with ryegrass

500 cm³ plastic pots were used and 900 g of sandy soil as planting medium. All treatments were performed in four replicates.

The following treatments were used during the growth experiment:

- Treatment 1: 100 ppm P₂O₅ + 100 ppm K₂O (in the form of KH₂PO₄ and K₂SO₄) – **Control (PK)**
- Treatment 2: PK + 100 ppm N (in the form of NH₄NO₃) – **Nitrogen (NPK)**
- Treatment 3: NPK + glycerol as carbon source – **0,5% C glycerol**
- Treatment 4: NPK + methanol as carbon source – **0,5% C methanol**
- Treatment 5: NPK + 0,5% glycerol-methanol blend as carbon source, 85% of glycerol and 15% of methanol – **0,5% C 50% glycerol + 50% methanol**
- Treatment 6: NPK + 0,5% glycerol-methanol blend as carbon source, 85% of glycerol and 15% of methanol – **0,5% C 85% glycerol + 15% methanol**
- Treatment 7: NPK + 0,5% by-product as carbon source – **0,5% C by-product**

After the application of the treatments into the soil, the top layer of soil was sowed with 1-1 grams of ryegrass seeds. The seeds were evenly scattered.

The moisture content of the soil was adjusted to 60% of the moisture content of the „Arany-féle kötöttség”. The moisture content was maintained daily with irrigation. The plants were cultured in a closed space under uniform artificial illumination.

The development of ryegrass was observed by optical method. Under the same conditions, pictures were taken from the plants with a high resolution

camera on a daily or three day basis, depending on their rate of growth. In all cases, rotations were used to take photos of the plants from eight different angles (0°, 45°, 90°, 135°, 180°, 225°, 270°, 315°).

Investigation of the effect on the growth of ryegrass through optical observation

Additional growth experiment was set up in order to examine whether the stress effects caused by treatments can be statistically proven by the use of green pixels obtained through image processing. In this case, 900 g of sandy-soiled soil was used as a planting medium in 500 cm³ plastic culture vessels. All treatments were performed in 4 replicates.

The following treatments were used during the growth experiment:

- Treatment 1: PK + 100 ppm N (in the form of NH₄NO₃) – **Nitrogen (NPK)**
- Treatment 2: NPK + 0,5% glycerol as carbon source – **0,5% C glycerol**
- Treatment 3: NPK + 0,5% methanol as carbon source – **0,5% C methanol**
- Treatment 4: NPK + 0,5% glycerol-methanol blend as carbon source, 85% of glycerol and 15% of methanol – **0,5% C 50% glycerol + 50% methanol**
- Treatment 5: NPK + 0,5% glycerol-methanol blend as carbon source, 90% of glycerol and 10% of methanol – **0,5% C 90% glycerol + 10% methanol**
- Treatment 6: NPK + 0,5% by-product as carbon source – **0,5% C by-product**

After the application of the treatments into the soil, the top layer of soil was sowed with 1-1 grams of ryegrass seeds. The seeds were evenly scattered.

The moisture content of the soil was adjusted to 60% of the moisture content of the „Arany-féle kötöttség”. The moisture content was maintained daily with irrigation. The plants were cultured in a closed space under uniform artificial illumination.

The development of ryegrass was observed by optical method. Under the same conditions, pictures were taken from the plants with a high resolution camera on a daily or three day basis, depending on their rate of growth. In all cases, rotations were used to take photos of the plants from eight different angles (0°, 45°, 90°, 135°, 180°, 225°, 270°, 315°).

Results and discussion

Effect of incubation on the change of mineral nitrogen content of the soil

In case of the untreated soil samples (1. PK), the mineralization of the organic nitrogen forms is a consequence of the favorable moisture content over time (Figure 3).

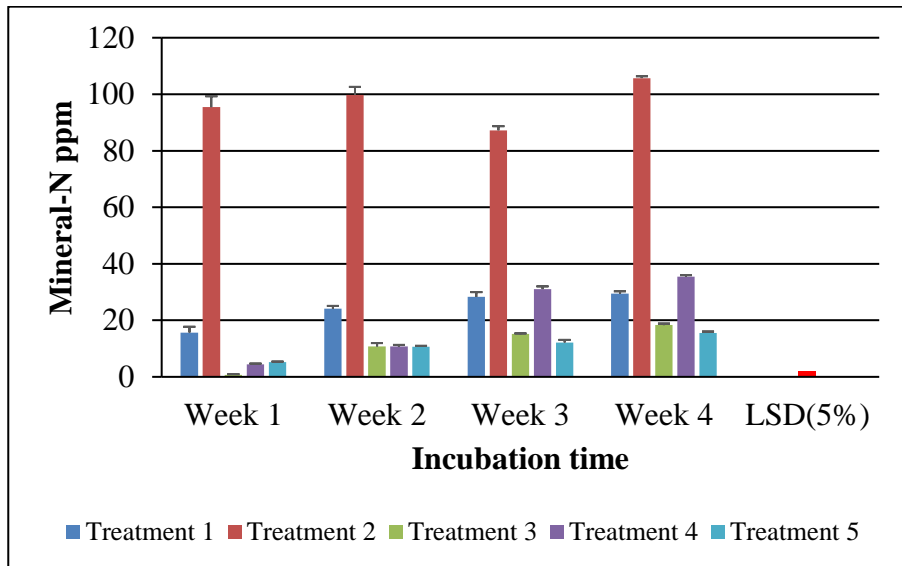


Figure 3. Total mineral-N content in the function of the incubation periods and the treatments

* 1. PK, 2. NPK, 3. 1% C glycerol, 4. 0,5% C by-product, 5. 1% C by-product, LSD (5%) - Least Significant Difference on 5% level

In the case of Treatment 2 (NPK), the additional nitrogen content of the treatment was increased the mineral nitrogen content of the soil samples. Its concentration in the soil corresponds to the amount of the ammonium nitrate treatment (100 ppm). This concentration fluctuates during the four weeks, but the change in time did not show a definite tendency (Figure 3).

It can be seen that if the nitrogen-free carbon source was added along with nitrogen fertilizer, the nitrogen content of the fertilizer and the soil's mineral-N content were also immobilized by the effects of glycerol-containing treatments. For treatments with 1% carbon source, the effect of pure glycerol was faster (3. 1% C glycerol) than the by-product (5. 1% C by-product). This treatment reduced the mineral-N content of the soil samples almost to 0 after one week.

Over time, some of this amount was gradually released at about the same rate as the nitrogen was mineralized in the case of the untreated soil samples. Both the immobilization and the mobilization were slower in the case

the by-product treatments (4. and 5.). In the case of 0.5% C by-product, intensified N mobilization was observed from the third week (Figure 3).

Results of the nitrate-N (Figure 4) can be interpreted similarly as seen in the case of the total mineral-N results.

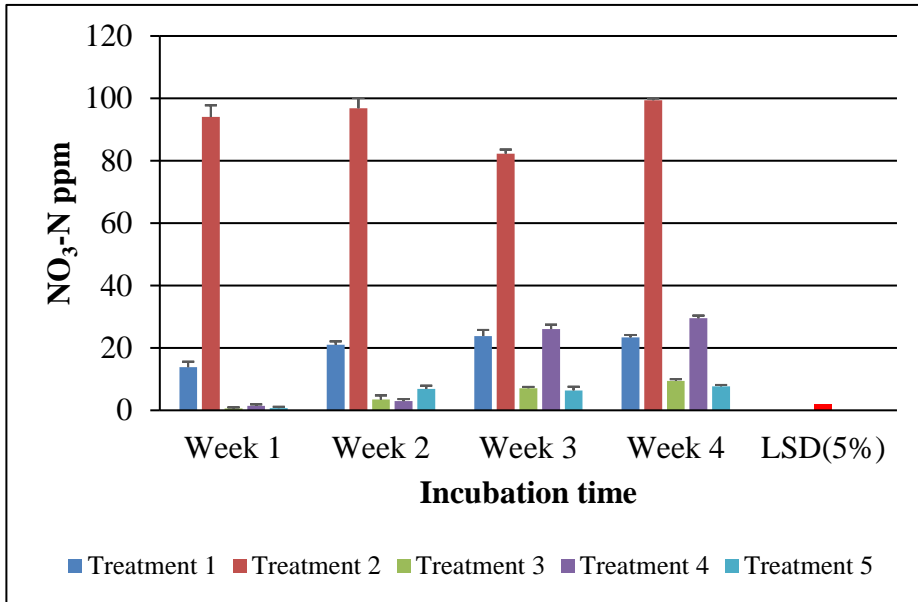


Figure 4. Nitrate-N content in the function of the incubation periods and the treatments
 * 1. PK, 2. NPK, 3. 1% C glycerol, 4. 0,5% C by-product, 5. 1% C by-product, LSD (5%) - Least Significant Difference on 5% level

It can be seen clearly from Figure 4 in Figure 3 that in the case of Treatment 2 (NPK), a significant amount of the total nitrogen content was presented in the form of nitrate-N from the first week. In the first week all the three treatments as carbon sources (3., 4. and 5.) were immobilized the total nitrate-N content of the soil. The formation of nitrate-N remained slow until the end of the observation. The difference between the total mineral-N and nitrate-N remained in the form of the increasing proportion of ammonium-N (Figure 4).

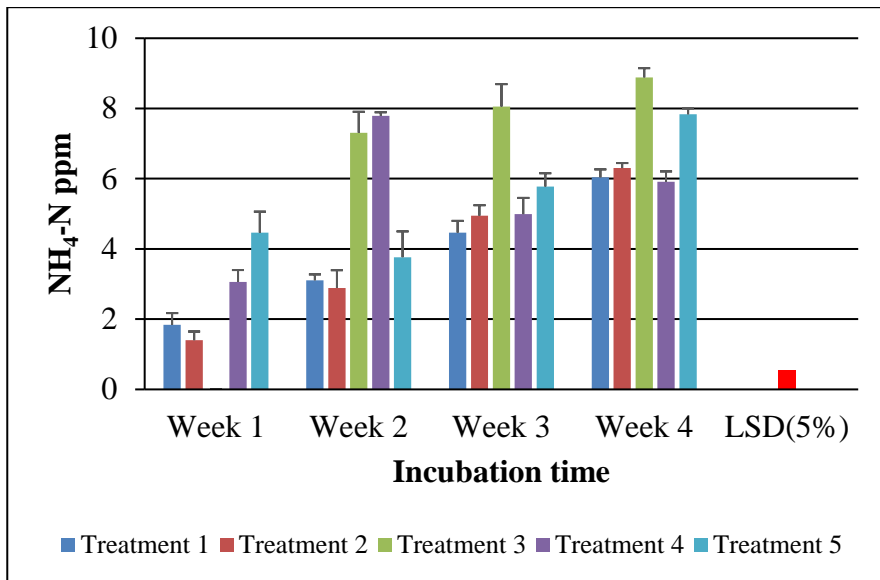


Figure 5. Ammonia-N content in the function of the incubation periods and the treatments
 * 1. PK, 2. NPK, 3. 1% C glycerol, 4. 0,5% C by-product, 5. 1% C by-product, LSD (5%) - Least Significant Difference on 5% level

After two weeks, the effect of the 0.5% C by-product treatment (4.) on the ammonium-N value was similar than the treatments without carbon source (1. and 2.) in the third and fourth week. Due to the 1% C glycerol (3) treatment, nitrogen in ammonium-N form was immobilized during the first week. The mobilization of the ammonium-N form was greater than the other treatments from the second week. This suggests that this treatment inhibited the nitrification. In case of 1% C-by-product (5.) treatment, this effect was observed from the 3rd and especially in the 4th week (Figure 5).

In summary, it can be concluded that the contaminated glycerol by-product and the glycerol were immobilized the nitrogen content of the soil and the added fertilizer within one week. The subsequent mobilization is slow and its speed depends on the concentration of the glycerol in the treatments. Inhibition effect on mobilization was greater for the nitrification. The other impurities of the glycerol-containing by-product did not exert any adverse effects.

Based on the soil incubation experiment, it was found that in the case of glycerol-containing treatments, the same immobilisation effect was observed as the pentozane effect known in the literature (Füleky 1999).

Effects of glycerol treatments on the germination of ryegrass and rape

Increasing the amount of organic matter added in the form of glycerol proportionally ($R^2 = 0.9847$) reduced the germination ability of ryegrass in the case of immediate sowing. Glycerol as 1% carbon source (Treatment 8), caused complete inhibition in germination (Figure 6).

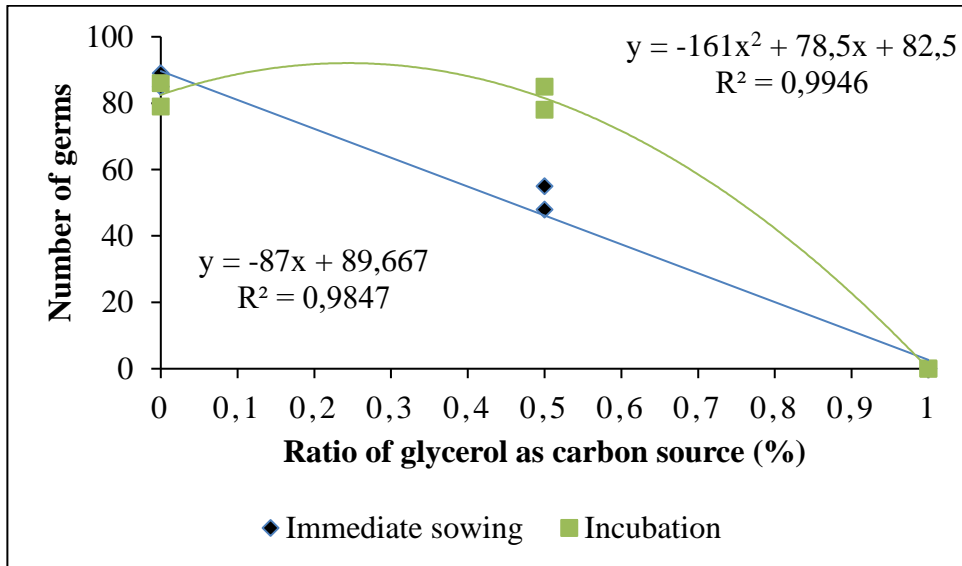


Figure 6. The effect of glycerol concentration on the germination of ryegrass in the case of immediate sowing and incubation

As a result of incubation, glycerol, even at 0.5% C source, did not result a significant decrease compared to 1. (PK). The inhibitory effect of glycerol decreased after two-week long incubation (Figure 6).

It is also observed that 8. (1% C glycerol) treatment caused complete inhibition in germination after incubation (Figure 6).

Increasing the amount of organic matter added in the form of glycerol proportionally ($R^2 = 0.9637$) reduced the germination ability of rape in the case of immediate sowing. Glycerol as 1% carbon source (Treatment 8), caused complete inhibition in germination (Figure 7).

As a result of incubation, glycerol, even as a 0.5% C source, did not result a significant decrease compared to 1. (PK). However, at 1% C source, the number of germs significantly decreased (Figure 7).

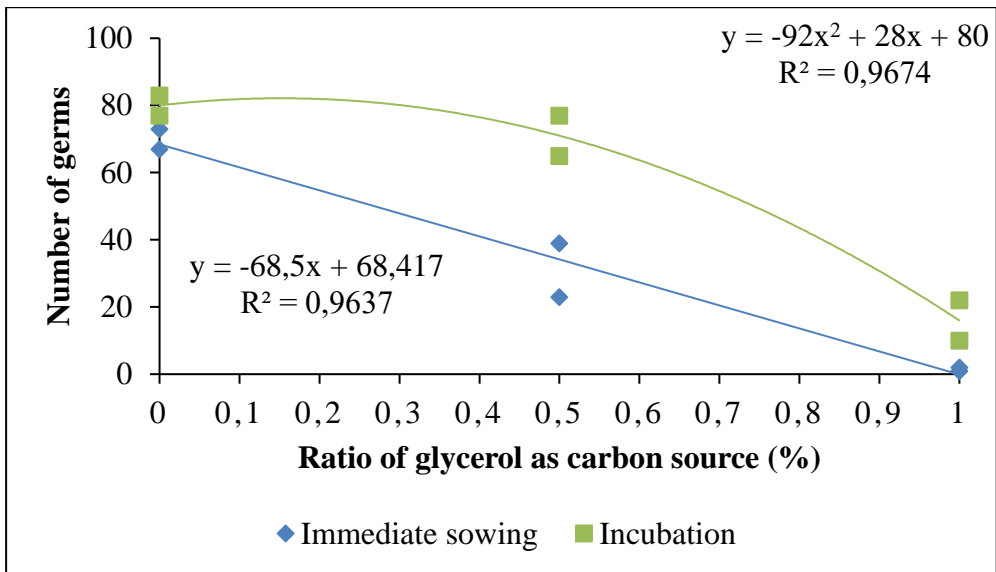


Figure 7. The effect of glycerol concentration on the germination of rape in the case of immediate sowing and incubation

Effects of glycerol-methanol treatments on the germination of ryegrass and rape

The results of the treatments 1. (PK), 5. (0.5% C 50% glycerol + 50% methanol), 6. (0.5% C 90% glycerol + 10% methanol), 10. (1% C 50% glycerol + 50% methanol), 11 (1% C 90% glycerol + 10% methanol) can be seen on Figure 8.

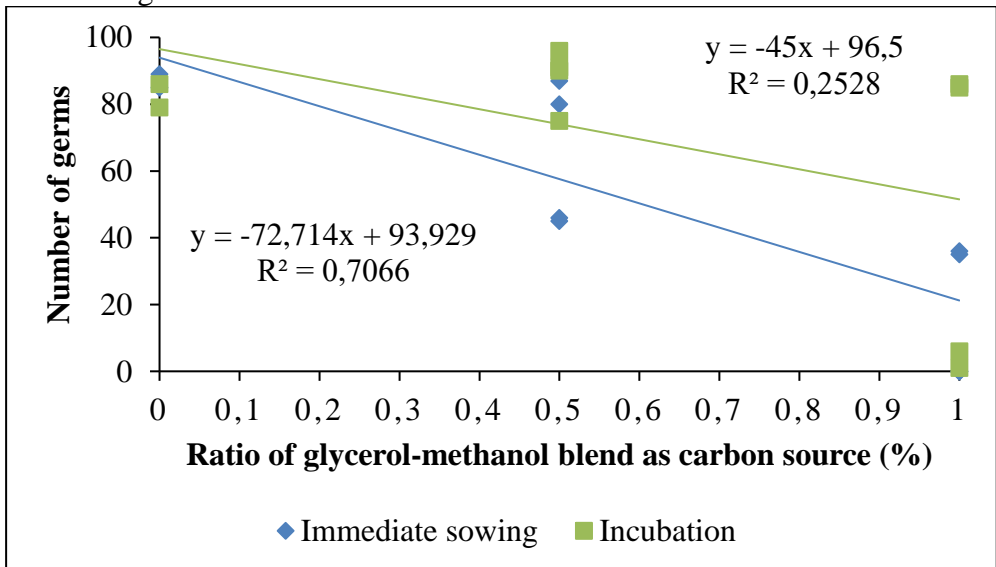


Figure 8. The effect of glycerol-methanol blend concentration on the germination of ryegrass in the case of immediate sowing and incubation

The negative linear relationship between the glycerol phase of treatments and the number of germs was proven by the regression coefficient

($R^2 = 0.7066$) in the case of immediate sowing. As the concentration of the glycerol phase increased in the blend, a greater inhibitory effect was observed (Figure 8).

After the incubation, the adverse effect of glycerol-methanol treatments on germination was not significant ($R^2 = 0.2528$). The inhibitory effect of glycerol decreased after incubation. After 2 weeks of incubation of the soil, there was no inhibitory effect on germination in the case of 0.5% C glycerol-methanol (treatment 10), while 1% carbon-containing glycerol-methanol (treatment 11) resulted inhibition in germination (Figure 8).

The results of the treatments 1. (PK), 5. (0.5% C 50% glycerol + 50% methanol), 6. (0.5% C 90% glycerol + 10% methanol), 10. (1% C 50% glycerol + 50% methanol), 11 (1% C 90% glycerol + 10% methanol) can be seen on Figure 9.

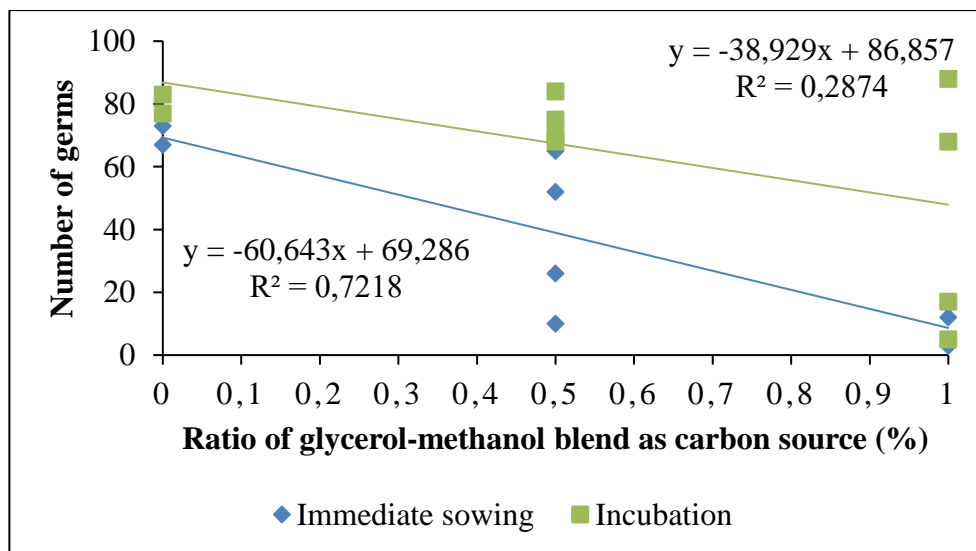


Figure 9. The effect of glycerol-methanol blend concentration on the germination of rape in the case of immediate sowing and incubation

The negative linear relationship between the glycerol phase of treatments and the number of germs was proven by the regression coefficient ($R^2 = 0.7218$) in the case of immediate sowing. As the concentration of the glycerol phase increased in the blend, a greater inhibitory effect was observed (Figure 9).

After the incubation, the adverse effect of glycerol-methanol treatments on germination was not significant ($R^2 = 0.2874$). The inhibitory effect of glycerol decreased after incubation. After 2 weeks of incubation of the soil, there was no inhibitory effect on germination in the case of 0.5% C glycerol-methanol (treatment 10), while 1% carbon-containing glycerol-methanol (treatment 11) resulted inhibition in germination (Figure 9).

Effects of methanol treatments on the germination of ryegrass and rape

Using ANOVA, in the case of ryegrass, immediate sowing did not 4. (0.5% C methanol), but (1% C methanol) treatment 9 significantly reduced the average number of germs compared to treatment 1 (PK). After incubation, non of the methanol treatments deviated significantly from the control (PK) (Figure 10, LSD (5%) = 34.4).

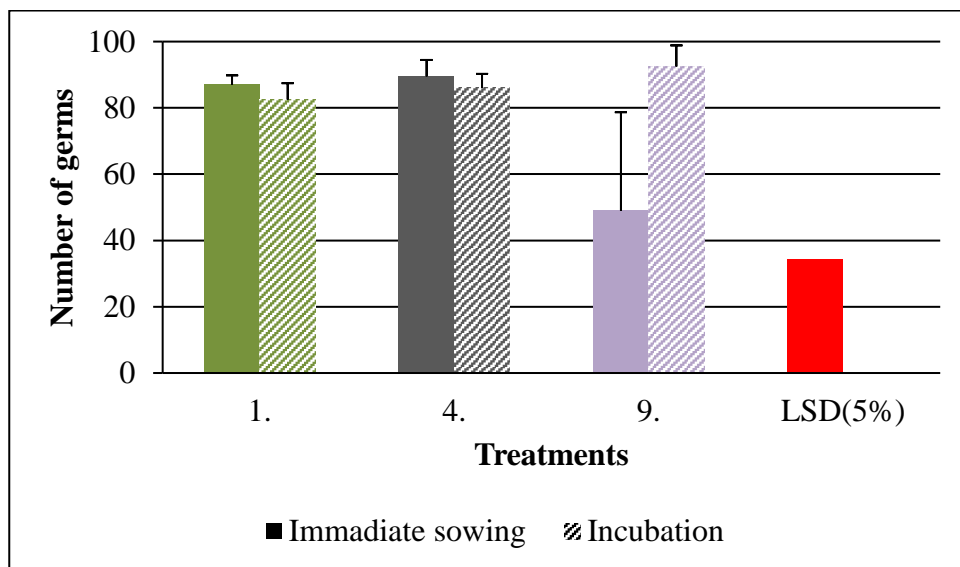


Figure 10. The effect of methanol on the germination of ryegrass in the case of immediate sowing and incubation

* 1. control (PK), 4. 0,5% C methanol, 9. 1% C methanol, LSD(5%) - Least Significant Difference on 5% level

In the case of rape, non of the methanol treatments reduced the average number of germs compared to the control treatment (PK). By variation analysis, in the case of angioedema, immediate sowing was not 4. (0.5% C methanol), but 9. (1% C methanol) treatment had significantly reduced the average number of bacteria compared to 1. (PK). After incubation, non of the methanol treatments deviated significantly from treatment 1 (PK) (Figure 11, LSD (5%) = 12.19).

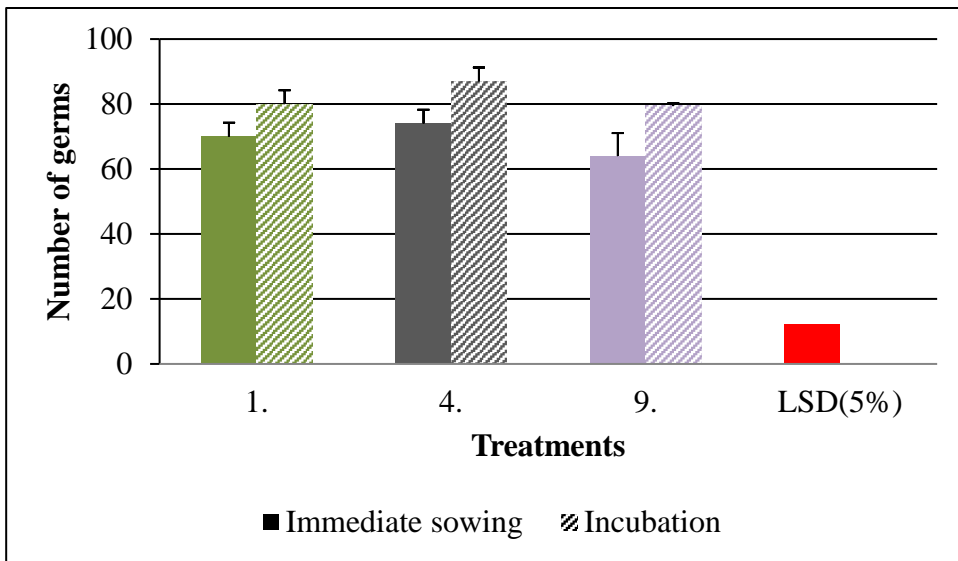


Figure 11. The effect of methanol on the germination of rape in the case of immediate sowing and incubation

* 1. control (PK), 3. 0,5% C glycerol, 9. 1% C methanol, LSD(5%) - Least Significant Difference on 5% level

In summary, the results of the incubation experiments with ryegrass and rape, it can be concluded that the inhibitory effects of glycerol-containing treatments on germination were only observed at a higher, 1% of the concentration carbon source. This suggests that the two-week long soil incubation period was not sufficient for the soil microorganisms to degrade the glycerol. Based on the results of the methanol treatments, it can be concluded that incubation did not significantly change the rate of germination. This is in line with the literature (Kovács et al. 2011a). The adverse effect of by-product and methanol decreased after incubation.

Impact on the growth of ryegrass

Logistic functions were fitted according to the calibration model presented in the materials and methods chapter. These sigmoid curves were well illustrated the effects of the treatments on the growth rate of ryegrass (Tolner et al. 2010a, Kovács et al. 2011a, Kovács et al. 2011b, Kovács et al. 2011c, Figure 11).

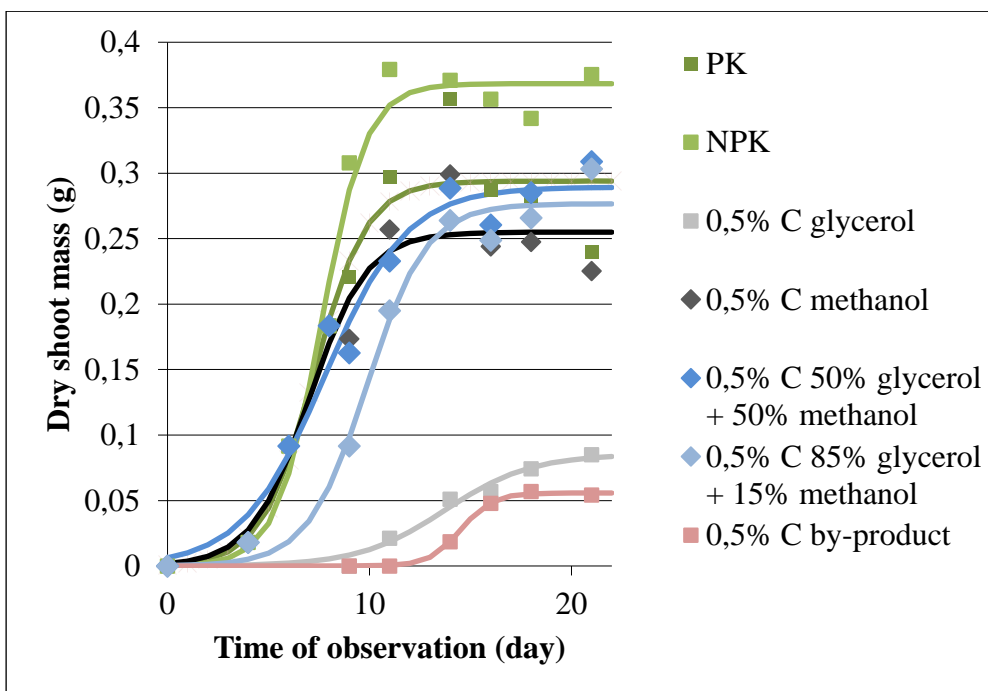


Figure 12. The effects of treatments on the dry shoot mass of ryegrass
 The equation of the fitted logistic function is the following:

$$y = \frac{A}{(1 + e^{k*(t-t_0)})}, \text{ where}$$

- A: the maximum of the air dried mass [g],
- k: the kinetic parameter characterizing the slope of growth [1/days],
- t: the elapsed time [days],
- t₀: time of the maximum growth rate (inflection point of the curve) [days].

It is clearly visible in the above diagram that pure glycerol and by-product slowed down the development of the plant and significantly reduced the plant production (Figure 12).

The following conclusions can be made according to the parameters of the fitted curves (Table 1):

Table 1. Parameters of the model fitted on the treatments

Parameters	Treatments						
	PK	NPK	0,5% C glycerol	0,5% C methanol	0,5% C 50% glycerol + 50% methanol	0,5% C 85% glycerol + 15% methanol	0,5% C by-product
A	0,29	0,37	0,09	0,25	0,29	0,28	0,06
k	-0,77	-0,90	-0,47	-0,70	-0,49	-0,68	-1,29
t ₀	7,25	7,60	13,75	7,01	7,77	9,88	14,54

- The parameter A (maximum air dried mass) showed that the effect of the added nitrogen (NPK treatment) resulted the highest plant production. Methanol (4.) and glycerol-methanol (5. and 6.) treatments added as 0.5% carbon source resulted almost the same plant production as the control treatment (PK). The pure glycerol (3.) and the by-product (7.) treatments added as 0.5% carbon source reduced significantly the plant production (Table 1, Figure 12).
- It can be seen on the parameter k (growth of the slope), the higher amount of the added glycerol, the slower the growth rate became (Table 1, Figure 12).
- Examining the parameter t_0 (time of the maximum growth rate – curve's inflection point), the control (PK) and the nitrogen (NPK) treatments were appeared at about the same time. Methanol (4.) and glycerol-methanol (5.) treatments added as 0.5% carbon source did not significantly differ from control (PK) or nitrogen (NPK) treatments. It is also noted that the glycerol ratio of pure glycerol (3.), glycerol-methanol (6.) and by-product (7.) delayed significantly the time of the maximum growth rate (Table 1, Figure 12).

In summary, it was found that the application of glycerol, glycerol-methanol and contaminated glycerol by-product at 0.5% concentrations as a carbon sources delayed the germination, slowed down the growth rate of the plants and resulted in decreased plant production. These effects were most pronounced when glycerol and contaminated glycerol by-product were applied. Methanol and glycerol-methanol treatments decreased only the plant production.

Impact on the growth of ryegrass through optical observation

With the complex analysis of the large amount of optical data obtained from the image analysis process, valuable information was gained from the development of the plants. The plants reacted differently to the different stress effects. This was manifested in the germination at different times and in deviations of the developmental rate.

Plant specimens were cultivated under the same conditions showed optically detectable differences. Rotation of the pots were made from 8 different angels. By analyzing the differences in the different shoots, quantifying the unevenness of the plant production can be quantified.

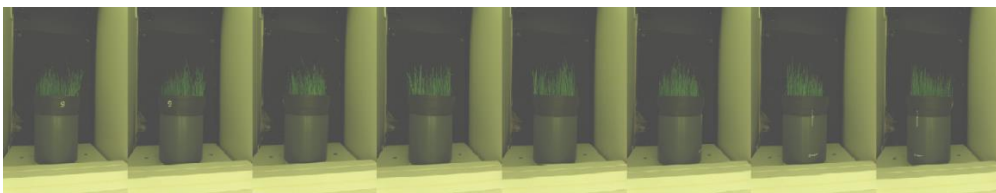


Figure 13. Pictures from a plant breded in a pot from eight different angles

It can also be seen on Figure 13 that the plants showed different images from 8 different angles. Table 2 shows the average number of green pixels (1/1000 part of green pixels) taken from eight different angles depending on the treatments and the time of the observation in four replicates. The coloring of the table indicates the average deviation (CV%) of data from eight different angles. The white background is below 10, light gray is between 10 and 20, and dark gray corresponds to CV% above 20.

Table 2. Growth of plants between the first and the 23rd day of the observation (number of green pixels/1000)

Treatments	Day 1	Day 2	Day 5	Day 8	Day 11	Day 14	Day 17	Day 20	Day 23
Nitrogen (NPK)	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 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 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% glycerol + 50% methanol	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 90% glycerol + 10% methanol	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

A statistical method was needed to prove that these deviations are significantly differs from each others. The examination was performed individually on the data of the 9 days (1, 2, 5, 8, 11, 14, 17, 20, 23) with Cochran Probe at the 5% level of significance. The calculation is presented on the data of the first day of the observation (Table 3). Since the standard deviations depend on the size of the basic data, the comparison was done by standardized data. For the calculation of standardized data (Table 3), the pixel numbers of the different angles were divided by the average number of pixels of the 8 images.

Table 3. Standardized data. Normalized average pixel numbers from the pictures of eight different angles of a plant bred in a pot on the first day of the observation

Treatments	0°	45°	90°	135°	180°	225°	270°	315°	σ	σ^2
Nitrogen (NPK)	1,00	0,99	0,95	0,99	1,04	1,01	0,99	1,03	0,029	0,00086
	1,03	1,01	0,98	0,98	0,95	0,95	1,04	1,05	0,039	0,00155
	0,99	0,99	0,95	0,98	1,02	1,04	1,01	1,01	0,028	0,00079
	1,02	1,00	1,01	0,96	0,97	0,99	1,02	1,02	0,022	0,00049
0,5% C glycerol	1,19	1,12	1,09	1,00	0,82	0,73	0,96	1,07	0,157	0,02473
	1,13	1,05	0,95	0,92	0,88	0,81	1,10	1,17	0,130	0,01696
	1,22	1,22	1,01	0,94	0,76	0,75	0,92	1,18	0,193	0,03708
	1,02	1,19	1,12	1,14	1,06	0,94	0,77	0,77	0,162	0,02610
0,5% C methanol	0,97	0,99	1,07	1,10	1,03	0,97	0,94	0,93	0,060	0,00360
	1,10	0,99	0,92	0,90	0,88	0,97	1,06	1,18	0,104	0,01087
	0,94	0,95	0,96	0,96	1,03	1,09	1,06	1,01	0,056	0,00315
	0,93	0,94	0,94	0,96	1,01	1,08	1,07	1,07	0,066	0,00441
0,5% C 50% glycerol + 50% methanol	1,00	1,03	1,03	0,97	0,99	1,02	0,98	0,98	0,024	0,00059
	0,97	0,95	0,98	1,03	1,07	1,05	0,98	0,97	0,044	0,00194
	1,02	1,01	0,97	0,97	0,99	1,03	1,02	0,98	0,025	0,00061
	0,95	1,05	1,06	0,99	1,00	0,97	0,98	0,99	0,038	0,00141
0,5% C 90% glycerol + 10% methanol	1,03	1,05	1,16	1,09	1,00	0,95	0,91	0,81	0,111	0,01239
	1,13	1,07	1,00	0,96	0,90	0,81	1,00	1,12	0,111	0,01228
	0,99	1,01	1,00	1,05	1,06	1,04	0,88	0,97	0,058	0,00339
	1,02	1,04	0,90	0,79	0,95	1,08	1,13	1,08	0,112	0,01252
0,5% C by-product	1,00	1,02	1,00	1,00	0,97	0,94	1,03	1,04	0,032	0,00105
	0,95	0,99	1,00	1,02	0,96	1,03	1,03	1,01	0,030	0,00088
	0,99	0,93	0,98	0,99	1,01	1,03	1,04	1,03	0,036	0,00132
	0,92	0,97	1,03	1,01	1,00	1,01	1,04	1,02	0,039	0,00153

The values of the standard deviation and the variance of the green pixel numbers measured on the 8 different (0° - 315°) angles of the pots were determined (columns σ and σ^2 in Table 3).

In the first step, examination was made whether there was an offset value between the obtained 24 variances, if it was determined as offset, then this process was continued with the rest of the 23 variances. The procedure was continued until offset variance was found.

For the calculation, the spreadsheets (σ^2) in Table 3 were copied and sorted into Table 4.

Table 4. Examination of the normalized dispersions of green pixel numbers measured on the potting vessels with Cochran's test on the first day the observation (Sváb 1981)

Ordinal number	Original number	Ordered σ^2	Cochran C	Critical C	K (number of samples)
1.	4.	0,00049			
2.	13.	0,00059	0,5425	0,8332	2
3.	15.	0,00061	0,3606	0,6530	3

4.	3.	0,00079	0,3176	0,5365	4
5.	1.	0,00086	0,2576	0,4564	5
6.	22.	0,00088	0,2092	0,3980	6
7.	21.	0,00105	0,1985	0,3535	7
8.	23.	0,00132	0,2001	0,3185	8
9.	16.	0,00141	0,1764	0,2901	9
10.	24.	0,00153	0,1607	0,2666	10
11.	2.	0,00155	0,1396	0,2440	11
12.	14.	0,00194	0,1494	0,2299	12
13.	11.	0,00315	0,1949	0,2148	13
14.	19.	0,00339	0,1735	0,2030	14
15.	9.	0,00360	0,1556	0,1911	15
16.	12.	0,00441	0,1600	0,1833	16
17.	10.	0,01087	0,2828	0,1750	17
18.	18.	0,01228	0,2422	0,1676	18
19.	17.	0,01239	0,1963	0,1608	19
20.	20.	0,01252	0,1656	0,1501	20
21.	6.	0,01696	0,1832	0,1490	21
22.	5.	0,02473	0,2108	0,1438	22
23.	8.	0,02610	0,1820	0,1390	23
24.	7.	0,03708	0,2054	0,1286	24

The data in the last line (24th) of the table was calculated by dividing the largest variance value (0.03708) in this row by the sum of all (24) variance squares (ordered table σ^2 in Table 4). The Cochran C value (0,2054) thus obtained was compared to the Cochran test critical values (k = 24, FG = 7, p = 5%) (0,1286).

Since the calculated Cochran C value exceeded the critical value, it can be justified with a probability error of up to 5% that the observed variance value (0.03708) is offset. Therefore, this numeric value and the Cochran C (0,2054) were marked in bold.

Disregarding the row 24 of the above table, the procedure was repeated with the data of the first 23 lines (**0.02610**, **0.1820**, 0.1390, k = 23, FG = 7, p = 5%). Since the variance in this line is offset to its lower values, this is why this value was also marked in bold. Similarly to the before mentioned calculation to the second line, it was found that the variance of **0.01087** in the 17th row and the variance values above this were all concerned as offset values. These values were marked in bold. The corresponding treatments to these offset data was marked in bold in Table 2.

The most significant stress effect was observed in the case of glycerol-containing treatments. The offset standard deviation values corresponding to treatments were marked in bold in Table 2.

The data was evaluated by two-way ANOVA (Table 5 and Table 6).

Table 5. Development of plants in the average of the four replicates between day one and day 23 (number of green pixels/1000)

Time of observation (day)	Nitrogen (NPK)	0,5% C glycerol	0,5% C methanol	0,5% C 50% glycerol + 50% methanol	0,5% C 90% glycerol 10% methanol	0,5% C by-product	B mean
Day 1	759	23	208	255	34	419	283
Day 2	917	39	328	391	80	532	381
Day 5	958	106	500	601	235	667	511
Day 8	1041	240	631	750	386	777	638
Day 11	924	317	702	779	467	791	663
Day 14	1049	438	890	920	549	826	779
Day 17	1138	559	1104	1137	700	982	937
Day 20	1237	657	1063	1200	762	957	979
Day 23	1093	751	1102	1017	839	876	946
A mean	1013	348	725	783	450	758	678

Table 6. Table of two-way ANOVA for treatments. The development of plants in the average of four replicates between day one and day 23 (number of green pixels/1000)

Source	SS	DF	MS	F	F-0.1%	F-1%	F-5%	F-10%	LSD(5%)	LSD(10%)
Total	25913112877	215			***	**	*	+		
Replication	239630859	3	79876953	9,86	5,69	3,91	2,66	2,12		
Treatment	24385634101	53	460106304	56,81	1,92	1,64	1,42	1,31	125,68	97,76
A factor	10547930391	5	2109586078	260,45	4,34	3,13	2,27	1,88	41,89	32,59
B factor	12327854088	8	1540981761	190,25	3,48	2,62	2,00	1,71	51,31	39,91
A x B	1509849622	40	37746241	4,66	2,04	1,72	1,47	1,35		
Error	1287847918	159	8099672					CV%=	13,2	

It can be seen that the effect of treatment combinations ($F = 56.81$ ***), the effects of treatments ($F = 260.45$ ***) and time ($F = 190.25$ ***) were highly significant (Table 6).

Compared to control, each treatment caused stress. This was less in the case of methanol (0.5% C methanol), glycerol-methanol blend (0.5% C 50% glycerol + 50% methanol) and contaminated glycerol by-product (0.5% C by-product).

No significant differences were observed between t effect of methanol and by-product treatments. Mostly, pure glycerol affected adversely the plants ($LSD(5\%) = 125.68$).

The development of plants in time can be seen on the figure below (Figure 14). It can be seen that the initial significant differences caused by the

treatments were gradually compensated over time. This was proved by the highly significant ($F = 4.66$ ***) interaction value in the variance table (Table 6).

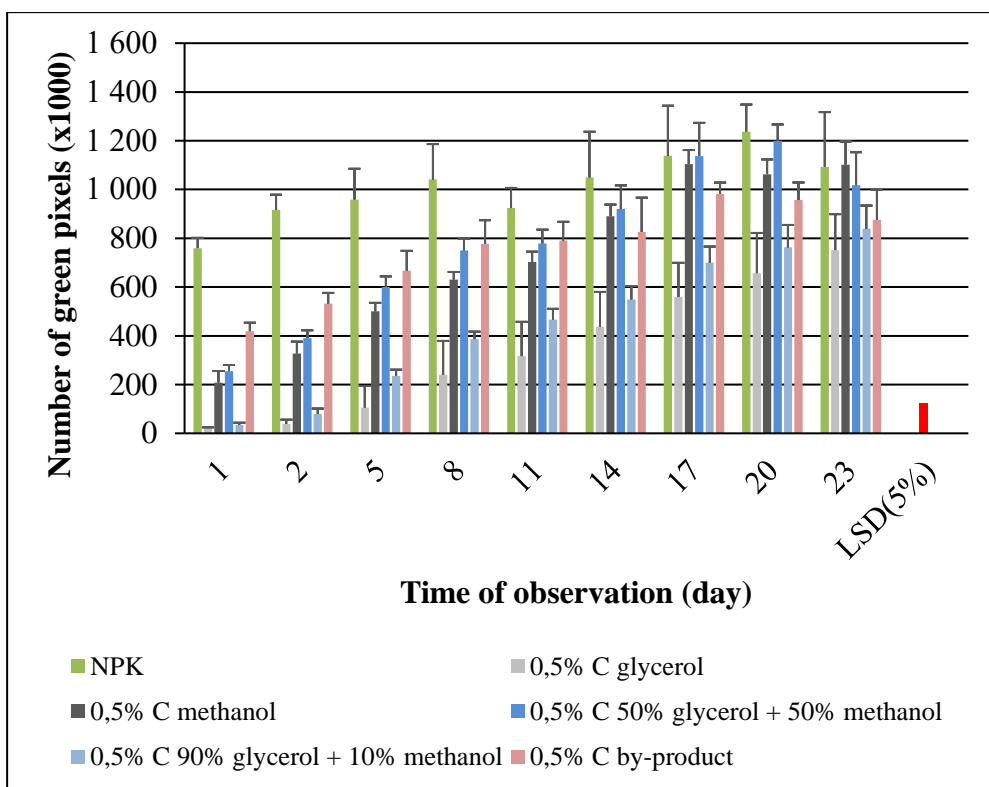


Figure 14. Effects of treatments by time on plant development (number of green pixels/1000)

In summary, the data were analyzed using a complex statistical method and it was shown that glycerol, glycerol-methanol, methanol and contaminated glycerol by-product all had a stress effect on plant growth, which was manifest in uneven growth and development of the plants. The standard deviations of the green pixel numbers obtained from image processing were analyzed using the Cochran test. It was statistically proven that the treatments 0.5% glycerol as a carbon source caused stress, which delayed germination and slowed down the development of the plants. These differences gradually decreased over time.

New scientific results

- Glycerol contaminated biodiesel by-product immobilized the nitrogen content of the soil and the added fertilizers within a week. The subsequent mobilization is slow and its speed depends on the rate of glycerol treatments. Inhibition effect on mobilization for nitrification is greater. The other contaminants of the glycerol contaminated by-product was not caused any adverse effects.
- Based on the experiments with germination of ryegrass and rape, it can be concluded that glycerol delayed germination. This effect was decreased after two weeks of soil incubation.
- Considering the impact of methanol contamination in the by-product, it can be concluded that it did not cause inhibition in germination even of immediate sowing.
- The effects of glycerol and glycerol contaminated by-product treatments caused delay in the germination and slowed down the growth of the plants.
- The statistical analysis of the image analysis process was proved the stress effect of the glycerol treatments, which appeared in the delayed germination, in the uneven plant development and the decrease in plant production.

Conclusions and suggestions

Since the beginning of my research, the exploitation of glycerol by-product, which arises from the production of biodiesel has been studied by several publications. The Hungarian literature lacks information about this topic. The effect of the glycerol and methanol phases present in the by-product has already been studied. However, a detailed examination of the effects of the glycerol contaminated by-product on the change of the mineral nitrogen content of the soil, the germination of plants and the development of plant have never been studied before. This information could have benefits in the utilization of the biodiesel by-product.

Glycerol treatments were resulted a change in the amount of the total nitrogen content of the soil by time, because the glycerol phase in the by-product had influenced the microbial activity. However, by the addition of the proper concentration of glycerol, the immobilisation and subsequent mobilization of nitrogen in the fertilizer becomes controllable.

Based on this fact, a similar technology can be developed for the use of slow-acting nitric fertilizers. Continuous release of controlled concentrations of nitrate content can reduce nitrate decomposition and the unfavorable effects of nitrogen over-fertilization (eg. grain bending).

In the case of immediate sowing, the glycerol treatments added as a 1% C source is presumably inhibited the primary environmental condition of germination due to its high water absorption capacity. Thus, glycerol may had prevented the first step of germination, the water absorption, resulting reduction germination or it had maintained the initial resting phase.

The number of germ numbers obtained from the soil incubation experiment showed that the 2-week-long incubation period was not sufficient for the microorganims to degrade glycerol in 1% carbon source.

Biodiesel by-product: Mildew experienced during germination experiments was due to the fact that other substances in the by-product (such as trace of elements, vitamins and lipids) were added to the glycerol contaminated by-product phase, which further increased the effect of glycerol as a source of carbon. The by-product-treated samples could be colonized. This effect was favoured the mould of the soil. The odor associated with mould was also characteristic of the by-product treated samples, which is evidently the proof of the microbial activity.

Methanol: Based on the germination experiments, it can be concluded that the methanol did not affect the potential of germination of the plants. After two weeks of soil incubation, the effect on germination proved to be more favorable.

Glycerol-methanol: Compared to the effects of methanol and glycerol-methanol blend treatments, it can be concluded that decrease in germination the case of glycerol-methanol treatments was imputed by the glycerol phase.

Based on the experience of the growth experiment, it can be concluded that the glycerol phase of the treatments resulted a negative effect on plant production, the growth rate and the time of the maximum of the plant growth rate. This effect can be explained by that the microorganisms had less time to degrade the glycerol, therefore this amount of nitrogen was not accessible for the plants.

The results from the soil incubation experiment (Tolner et al. 2010b) was proved the immobilizing effect of by-product and glycerol. As a result, the amount of nitrogen capacity in the soil was reduced, which could slow down the development of plants.

The information obtained from the image analysis process indicates that the increase in stress, caused delay in sprouting, slowed down the growth and caused uneven plant development. The effect was observed mainly at the early stage of the development of plants, which could caused by moulding, which was experienced during the germination experiments. However, these differences were gradually decreased over time.

Based on the experiments carried out from my doctoral work and from the literature the following suggestions can be concluded for further researches:

- to carry out similar experiments on different soil types, including other plants,
- to have a better understand of the adverse effect of glycerol, it would be advisable to establish field experiments with at least 3 week-long incubation period,
- to offset the adverse effect on the nitrogen management of the soil, the contaminated glycerol by-product should be applied more than two weeks before sowing. Such a technological recommendation exists also in current fertilization practice (use of urea fertilizer).

Related publications

1. Peer-reviewed research articles

1.1. With impact factor (according to WEB OF SCIENCE), in English

1.1.1. Hungarian publisher

Kovács A., Tóth, M., Somogyi, F., Tolner, L., Czinkota, I., Béres, A., Wilk, T., Aleksza, L.: Comprehensive investigation of biodiesel by-product on germination and plant growth Experiments, APPLIED ECOLOGY AND ENVIRONMENTAL RESEARCH 13(4): 1171-1181. (2015) **(18 points)**

(IF = 0,5)

1.1.2. International publisher

1.2. In English, without IF

1.2.1. Hungarian publisher

1.2.2. International publisher

1.3. In Hungarian, without IF

2. Professional full text article,

2.1. Professional full text article

4. Conference proceedings with ISBN, ISSN or other certification

4.1. Full text article in Foreign language, peer-reviewed

Tolner L., Bondor B., Czinkota I., Vadkerti Zs., Kovács A.: Investigation of soil fertility affected by biodiesel by-product in microcosm experiment. XII. Alps-Adria Scientific Workshop Opatija, Croatia, Növénytermelés **62** Suppl. 433-436. (2013) **(5 points)**

Kovács A., Czinkota I., Vágó I., Kovács A.: Regulation of soil nitrogen supply by using glycerol as a biodiesel by-product, 2011 Növénytermelés Volume 60. Supplement p. 247-250. (2011) **(5 points)**

Tolner L., Rétháti G., Kovács A.: Examination of an alternative way to prevent nitrate leaching in soil by using glycerol as a biodiesel by-product. XI. Alps-Adria Scientific Workshop Smolenice, Slovakia, Növénytermelés 61 Suppl. 267-270. (2012) **(5 points)**

4.2. Full text article in Hungarian, peer-reviewed

Tolner L., Vágó I., Kovács A., Tolner I., Füleky Gy.: Energiaerdővel a környezetkímélő tápanyaggazdálkodásért. (Energy forests for the environmentally compatible nutrient management.) Zöldenergia, földhő és

napenergia hasznosítása a hőtermelésben Konferencia, Gyöngyös 2010.05.20., Konferenciakiadvány 106-109. ISBN: 978-963-9941-12-0 (2010) **(3 points)**

Tolner L., Zódi M., Kovács A., Kertész B.: Biodízelgyártás by-producteként keletkező glycerol hatása a talaj ásványi nitrogén tartalmára. (The effect of glycerine, a by-product of biodiesel production, on the mineral nitrogen content of soil.) Zöldenergia, földhő és napenergia hasznosítása a hőtermelésben Konferencia, Gyöngyös, 2010.05.20. Konferenciakiadvány 110-114. ISBN: 978-963-9941-12-0 (2010) **(3 points)**

Tolner L., Kovács A., Kovács A., Vágó I., Czinkota I.: Ellentmondások a biodízelgyártás by-product mezőgazdasági hasznosíthatóságában. (Contradictions in agricultural Utilization of biodiesel byproduct.) Zöldenergia termelés és hasznosítás rendszere c. Tudományos Konferencia, Gyöngyös, 2011.09.27. Konferenciakiadvány (ISBN: 978-963-9941-24-3) 154-159. (2011) **(3 points)**

Kovács A., Czinkota I., Kovács A., Tolner L.: Biodízel by-product alkalmazása a talajvédelemben. Erdei Ferenc VI. Tudományos Konferencia, 2011. augusztus 25., Kecskemét, Konferencia kiadvány III. kötet (ISBN 978-615-5192-01-2) 375-380. (2011) **(3 points)**

5. Conference proceeding without certification

5.1. Full text article in Foreign language

5.2. Full text article in Hungarian

5.3. One page summary in Foreign language or Hungarian

Tolner L., Kovács A.: Nitrát kimosódás vizsgálata talajoszlop kísérletben. Talajtan a mezőgazdaság, a vidékfejlesztés és a környezetgazdálkodás szolgálatában. Talajtani vándorgyűlés, Miskolc, 2012. Talajvédelem (Különszám) 513-521. (2013) **(2 points)**

Tolner L., Czinkota I., Vadkerti Zs., Kovács A., Rétháti G.: Glycerol hatása az angolperje csírázására és kezdeti növekedésére. Magyar Epidemiológiai Társaság VII. és a Közép-európai Kemoprevenációs Társaság I. közös Nemzetközi Kongresszusa 2013.04.05-06., Pécs. Magyar Epidemiológia (Hungarian Epidemiology) 10 Suppl. S.46. (2013) **(2 points)**

Kovács A., Czinkota I., Nagy A., Issa I., Tolner L. : Use of biodiesel byproduct in agriculture. 6th ISMOM, International Symposium of Interactions of Soil Minerals with Organic Components and Microorganisms, 26th June-1st July 2011, Montpellier, France (2011) **(2 points)**

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