

Number of Microorganisms in the Soil Cultivated with Legume-Grass Mixtures Supplied with Phytohormones and Nitrogen

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Abstract

The study concerned the number of microorganisms colonizing the humus layer and under plough soil from the cultivation of perennial ryegrass mixtures with red clover, which were fed by biostimulant based on the phytohormones and different doses of nitrogen. Nitrogen was applied at four levels-control (no nitrogen), 50, 100 and 150 kg N · ha⁻¹, biostimulant in two combinations - with and without preparation. Soil material to assess the size of individual groups of microorganisms was collected from each experimental plot in autumn 2010 from the humus (0-20 cm) and under plough layers (20-40 cm). The analysis of soil samples for a total number of bacteria, actinomycetes and fungi was conducted at the Department of Agricultural Microbiology IUNG-PIB in Pulawy. Results of studies have shown that more colonies of bacteria, actinomycetes and fungi were colonized the humus horizon. Biostimulant application in this cultivation led to a decrease of the total number of colonies of the all soil microorganisms groups but improved the ratio of bacteria to fungi. Increasing doses of mineral nitrogen by the limiting of the bacteria number and intensive development of soil fungi, helped to narrow the B/F ratio.

Keywords: bacteria, fungi, nitrogen, phytohormones, soil

Introduction

The conventional system of crop production was conducted in order to maximize yield through the intensive fertilization and plant protection treatment that has a major influence on soil characteristics. These practices caused the deterioration of biological and physico-chemical properties of the soil. (Martyniuk *et al.*, 2001). Soil microorganisms affect not only the health of crops, but also the availability of nutrients for them, especially those that are released in the soil as a results of biological degradation and transformation of both fresh and stable soil organic matter (Nannipieri *et al.*, 2000; Polyanskaya and Zvyaginstsev, 2005; Sveshnikova *et al.*, 2001; Zvyaginstsev, 2011). The intensity of microbial transformation of the organic nitrogen and phosphorus compounds is very important in diversified fertilization conditions. Therefore, the soil fertility in macro-and micronutrients, and therefore its productivity are associated with the stable influx into the soil organic matter from plant residues, by the use of proper crop rotation with

legumes or mixtures of grasses (Jodelka *et al.*, 2011; Sveshnikova *et al.*, 2001). Apart from microbial transformation of organic and inorganic nitrogen compounds, an important source of this element in soil may also be the microorganisms, that assimilate atmospheric nitrogen for example: free-living nitrogen fixing bacteria of the *Azotobacter* genus, especially nodules bacteria - *Rhizobium* and *Bradyrhizobium* - binds N₂ in symbiosis with the roots of legumes (Ivanova *et al.*, 2013; Martyniuk *et al.*, 2001; Zvyaginstsev, 2011). In phosphorus transformation in the soil, both bacteria and fungi play an important role. Among the organisms mentioned above, a specific group stated mycorrhizal fungi. They create symbiotic systems with the roots of crop plants enabling them to take not only the available forms of phosphorus, but also other components such as micronutrient, increasing the absorptive surface of roots and producing appropriate enzymes (phosphatases) (Alabouvette, 2000). Mycorrhizal fungi also play an important role in creating a lumpy soil structure (Crecchio *et al.*, 2007; Polyanskaya *et al.*, 2012).

In addition, soil microorganisms contribute to the distribution and detoxification of various soil pollutants (xenobiotics) (Burd *et al.*, 1998; Gajda *et al.*, 2010). They also put restraint to the development of pests and plant pathogens. This microbial activity is mostly connected with the occurrence of competition in the soil for food, among its inhabitants but also with the phenomena of antagonism and hyper parasitism. A few examples for these relationships are the inhibition by some soil bacteria, the development of phytopathogenic fungi, which produce different types of antibiotic substances or enzymes, decomposed by the fungal hyphen. Conversely, some soil fungi have the capability of riding on the nematodes and insects (Alabouvette, 2000; Compant *et al.*, 2005; Jodełka *et al.*, 2011).

In recent years, there has been observed the increase of interest in searching for new preparations, which application can improve the productivity and health of crops, reducing the use of mineral fertilizers and pesticides. Examples for this could be the substances derived from marine algae, which are considered as the most important group of living organisms that can be used on a large-scale in crop production. Literature data (Lipa and Pruszyński, 2010; Zodape, 2001) based on studies about algae extracts, indicate their positive impact on crops. The plants treated with extracts, are characterized by an increased yield, resulting mainly from increased plant unprofitable to adverse environmental factors (drought, frost), greater resistance to pests and pathogens and intensive nutrient uptake from the soil. Among the clusters of algae biostimulation effects on plants were observed. Green algae species are the most frequently mentioned: *Cladophora dalmatica*, *Enteromorpha intestinalis*, *Ulva lactuca*. Also red algae: *Corralina mediterranea*, *Jania rubens* and *Pterocladia pinnata* and brown algae: *Ascophyllum nodosum*, *Ecklonia maxima*, *Sargassum* spp. (Bai *et al.*, 2007) have been mentioned. Biostimulation effects of the algae extracts are correlated with a large presence of plant hormones, mainly cytokines. Individual algae species differ in cytokinins content and in its proportions in relation to the other plant hormones. Therefore, the effect of algae extracts is not uniform (Reitz and Trumble, 1996; Verkleij, 1992). In addition, algae are considered as a rich source of micro- and macronutrients, which are necessary for plant growth. The action of algae extracts is strongly dependent on the concentration, application methods even as the species and varieties of the crop (Sultana *et al.*, 2005).

The aim of this study was to determine the number of bacteria, actinomycetes and fungi located in the humus and under plough layer from the cultivation area of perennial ryegrass in combination with red clover supplied with phytohormones and different nitrogen dose.

Materials and methods

Soil material subjected to microbiological analysis came from a field experiment from the cultivation of perennial ryegrass 75%, 'Diamond' in a mixture with red clover 25% cv. 'Parade'. It was established in the third decade of April 2009, by a randomized block design with three replications, in the experimental field of the Siedlce, University of Natural Sciences and Humanities. Experimental plot area was 6 m². In the year of sowing only infestation cuts was carried out. The full period of this experiment was in the years 2010-2011. The soil on which the experiment was realized was classified in the medium soils category as anthropogenic soil. Before the experiment was established, the soil was characterized by a neutral reaction (pH in KCl = 7.2), high abundance in humus (3.78%), available phosphorus (P₂O₅ - 600 mg·kg⁻¹) and magnesium (Mg-74 mg·kg⁻¹), average content of total nitrogen (N-1.6 g·kg⁻¹) and available potassium (K₂O - 16 mg·kg⁻¹).

The experimental factors were as follow: nitrogen dose: A1-control (no nitrogen), A2-50, A3-100; A4-150 kg·ha⁻¹ (nitrogen fertilizer applied in three doses in the form of 34% ammonium nitrate), biostimulant: B1 - control object (without biostimulant), B2 - biostimulant.

Growth regulator was used at a dose of 2 dm³·ha⁻¹, diluted in 350 dm³ of water. This growth regulator contain natural plant hormones such as auxin (11 mg·dm⁻³) and cytokinins (0.03 mg·dm⁻³) which was prepared from brown algae *Ecklonia maxima* - 32.2%.

The preparation was used for all the regrowth in the form of spray.

Along the experimental years, on all plots potassium (60% potassium salt) was used, which like nitrogen fertilization, were used on the regrowth in quantities of 160 kg K₂O·ha⁻¹ per year. Phosphorus (46% superphosphate) at 40 kg P₂O₅·ha⁻¹ per year was applied once in the spring.

The soil samples were collected from each plot in October 2011 from two levels: C1 - humus layer (0-20 cm), C2 - under plough layer (20-40 cm). The analysis tests on the overall number of bacteria, actinomycetes and fungi was conducted at the Department of Agricultural Microbiology IUNG-PIB in Pulawy.

The following methods and individual microbiological units were used:

- The total number of bacteria and actinomycetes (10⁷cfu·g⁻¹ DM of soil) was determined by Wallace and Lockhead (1950).

- The total number of fungi (10⁷cfu·g⁻¹ DM of soil) was determined by Martin (1950) method.

The obtained results were evaluated statistically using the analysis of variance. The mean differentiation was verified by Tukey's test, at significance level p≤0.05.

The climate conditions of the research area are typical for the IX - eastern agro-climatic district of Poland. The annual average temperature varies within 6.7-6.9 °C. In summer, the average daily temperature reaches 15 °C. Annual precipitation levels reach 550 - 650 mm, while they are not frequent, but heavy. The vegetation period begins on the first decade of April and ends in the third decade of October therefore, it lasts from 200 to 220 days. Meteorological data from the years of research were obtained from the Hydrological and Meteorological Station in Siedlce. However, in order to determine the temporal and spatial variability of meteorological elements and their effects on vegetation, it was calculated the hydrometrical ratio of Sielianinow. Data presented in Tab. 1 indicate that the most favorable distribution and amount of rainfall, with optimum air temperatures within the growing period of plants was the year 2010. In that year, there were no months with strong drought in contrast to the year 2011.

Results and discussion

In some studies (Doran and Parkin, 1994; Marinari *et al.*, 2006; Myśków and Zięba, 1997) was underlined the fact that some of the parameters, on comprising the microbiological properties of soils,

can be modified by both the direct impact of some agricultural treatment on microbial activity as well as indirectly by changing the physical and chemical properties of the soil.

The assessment of the microorganisms number (Tabs. 2, 4) indicates that, the application of biopreparation in cultivation of perennial ryegrass with red clover, regardless of the nitrogen dose and sampling levels of soil resulted in more than 48% reduction in the number of soil bacteria, 16% of actinomycetes and over 26% of fungi. Noteworthy is the fact that the use of biostimulant without nitrogen fertilization (Tab. 2) has resulted in a significant increase in the number of bacterial colonies from 41.71 to 118.1 $10^7 \text{cfu} \cdot \text{g}^{-1} \text{D.M. soil}$.

Wyszkowska *et al.* (2000) showed that different groups of organisms react differently on the application of plant hormone compounds. Only the application of gibberellic acid caused an increase in the number of bacterial colonies compared to control. Indolilobutyric acid, α -naphthaleneacetic, triacontanol and benzyladenine significantly limited the development of these organisms. Actinomycetes preferably reacted to indolilobutyric acid, and fungi on benzyladenine treatment. It should have to be emphasized that hormones, used in the cited studies at least degree, limiting the number of fungi, which was not confirmed in our study.

Jodełka *et al.* (2011), Wyszkowska *et al.* (2000) in

Tab. 1. Value of hydrometrical index of Sielianinow (K) in individual months of vegetation

Year	Month						
	IV	V	VI	VII	VIII	IX	X
2010	0.40	2.21	1.19	1.18	1.79	2.81	0.53
2011	1.10	0.89	0.72	2.19	0.84	0.78	0.94
0,5 - severe drought; 0,51 – 0,69 - drought; 0,70 – 0,99 – poor drought; over 1 – no drought							

Tab. 2. The total number of bacteria ($10^7 \text{cfu} \cdot \text{g}^{-1} \text{D.M. soil}$), depending on the nitrogen dose, biostimulant and soil level

Factors		Level (C)		\bar{X}
Nitrogen dose (A)	Biostimulant (B)	C1	C2	
A1	B1	45.29	38.13	41.71
	B2	129.7	106.5	118.1
	A1	87.40	72.35	80.05
A2	B1	258.0	172.4	215.2
	B2	40.53	32.90	36.72
	A2	149.2	102.6	125.8
A3	B1	213.4	163.8	188.6
	B2	53.97	41.09	47.5
	A3	133.6	102.4	118.0
A4	B1	72.89	56.66	64.77
	B2	67.79	55.32	61.56
	A4	70.35	55.99	63.16
B	B1	147.5	107.7	127.6
	B2	73.00	58.98	65.98
	\bar{X}	110.27	83.37	-
LSD _{0.05} : A – 29,52; B – 53,30; C – 21,70; AxB – 46,52; Ax C – r.n.; BxC – 32,50; Ax BxC – 31,09				

Nitrogen: A1-control (no nitrogen), A2 - 50, A3 - 100, A4 -150 $\text{kg} \cdot \text{ha}^{-1}$, biostimulant: B1 - control object (without biostimulant), B2 - biostimulant, the level of sampling: C1 - a layer of humus (0-20 cm), C2 - under plough layer (20-40 cm)

their studies highlight the impact of mineral fertilizers, especially nitrogen on soil microbial life, which is reflected in its numbers. The highest number of bacteria (approximately $122 \cdot 10^7 \text{ cfu} \cdot \text{g}^{-1}$ D.M. soil) occurred in the samples from plots fertilized with 50 and 100 $\text{kg N} \cdot \text{ha}^{-1}$. Increasing doses of nitrogen to 150 $\text{kg N} \cdot \text{ha}^{-1}$ reduced the growth of these organisms, and caused intensive growth of fungi colonies. This is confirmed in other publications (Rekosz-Burlaga and Russel, 1999) and must be explained by over fertilization soil with the mineral nitrogen, which leads to an excessive concentration of nitrosamines in soil solution, which are toxic for bacteria.

The most strongly developed actinomycetes have developed in soils, which were supplied with 150, and 50 $\text{kg N} \cdot \text{ha}^{-1}$ and their number varied from 40.95 to $45.97 \cdot 10^7 \text{ cfu} \cdot \text{g}^{-1}$ D.M. soil (Tab. 3) and exceeded 20%. This number was higher than the total number of colonies observed in the soil material came from the control objects and fertilization with 100 $\text{kg N} \cdot \text{ha}^{-1}$.

Microbiological quantitative analysis for the soil level samples indicates that significantly more colonies of bacteria, actinomycetes and fungi were colonized the humus horizon. However nitrogen and biostimulant were applied, there were average 24% more bacteria, 26% actinomycetes, and 23% fungi

Tab. 3. The total number of actinomycetes ($10^7 \text{ cfu} \cdot \text{g}^{-1}$ D.M. soil), depending on the nitrogen dose, biostimulant and soil level

Factors		Level (C)		\bar{X}
Nitrogen dose (A)	Biostimulant (B)	C1	C2	
A1	B1	34.64	25.49	30.07
	B2	40.47	29.18	34.83
A1	B1	37.56	23.17	32.44
	B2	66.68	42.63	54.65
A2	B1	42.31	32.22	37.27
	B2	54.50	37.43	45.97
A3	B1	32.75	24.78	28.77
	B2	43.33	34.14	38.74
A3	B1	38.04	29.47	33.75
	B2	58.63	47.83	53.24
A4	B1	33.73	23.60	28.68
	B2	46.19	35.72	40.95
B	B1	48.17	35.18	41.69
	B2	39.96	29.79	34.88
\bar{X}		44.06	32.48	-

LSD_{0.05} : A – 4,44; B – 3,98; C – 7,04; Ax B – 10,54; Ax C – 9,70; Bx C – 8,97; Ax Bx C – 3,33

Nitrogen: A1-control (no nitrogen), A2 - 50, A3 - A4 100 - 150 $\text{kg} \cdot \text{ha}^{-1}$, biostimulant: B1 - control object (without biostimulant), B2 - biostimulant, the level of downloads: C1 - a layer of compost (0-20 cm), C2 - under plough layer (20-40 cm)

Tab. 4. The total number of fungi ($10^7 \text{ cfu} \cdot \text{g}^{-1}$ D.M. soil), depending on the nitrogen dose, biostimulant and soil level

Factors		Level (C)		\bar{X}
Nitrogen dose (A)	Biostimulant (B)	C1	C2	
A1	B1	23.45	13.41	18.43
	B2	9.510	5.960	7.740
A1	B1	16.49	9.69	13.08
	B2	41.21	30.40	35.81
A2	B1	25.95	21.48	23.71
	B2	33.58	25.94	29.76
A3	B1	33.80	26.08	29.94
	B2	28.54	24.10	26.33
A3	B1	31.18	25.09	28.13
	B2	50.18	41.39	45.79
A4	B1	42.45	32.19	37.32
	B2	46.31	36.79	41.56
B	B1	37.17	27.83	32.50
	B2	26.62	20.93	23.77
\bar{X}		31.89	24.38	-

LSD_{0.05} : A – 4,44; B – 3,98; C – 7,04; Ax B – 10,54; Ax C – 9,70; Bx C – 8,97; Ax Bx C – 3,33

Nitrogen: A1-control (no nitrogen), A2 - 50, A3 - A4 100 - 150 $\text{kg} \cdot \text{ha}^{-1}$, biostimulant: B1 - control object (without biostimulant), B2 - biostimulant, the level of downloads: C1 - a layer of compost (0-20 cm), C2 - under plough layer (20-40 cm)

Tab. 5. The ratio of bacteria to fungi, depending on the nitrogen dose applied, biostimulant and soil level

Factors		Level (C)		\bar{X}
Nitrogen dose (A)	Biostimulant (B)	C1	C2	
A1	B1	1.93	2.84	2.39
	B2	13.6	17.8	15.7
	A1	7.78	10.36	9.07
A2	B1	6.26	3.04	4.65
	B2	1.56	1.53	1.55
	A2	3.91	3.80	3.86
A3	B1	6.31	6.28	6.30
	B2	1.89	1.70	1.80
	A3	4.10	3.99	4.05
A4	B1	1.45	1.72	1.59
	B2	1.60	1.74	1.67
	A4	1.53	1.73	1.63
B	B1	3.99	3.47	3.73
	B2	4.67	5.71	5.19
	\bar{X}	4.33	4.59	-
NIR _{0,05} dla: A – 3,33; B – 1,44; C – r.n.; Ax B – 5,02; Ax C – r.n.; Bx C – r.n.; Ax Bx C – 4,21				

Nitrogen: A1-control (no nitrogen), A2 - 50, A3 - A4 100 - 150 kg·ha⁻¹, biostimulant: B1 - control object (without biostimulant), B2 - biostimulant, the level of downloads: C1 - a layer of compost (0-20 cm), C2 - under plough layer (20-40 cm)

(Tab. 4) in this layer. According to a research, concerning the evaluation of biological properties of soil from cultivation of amaranth (Skwaryło-Bednarz, 2008) the difference between the quantity of these microorganisms can reach 50%. It is worth noting that, according to cited research, the number of microorganisms in different layers also depend on crop variety and the season of the year. In autumn samples, aside from the other experimental factors, the number of microflora was much higher in the humus layer and in the subsoil layer than in the spring samples. This increase it is attributable to the fact that more precipitation occurred in this period.

Similar results were obtained by analyzing the biological properties of the soil in the cultivation of winter wheat (Frąc *et al.*, 2011). Independently of the production system (conventional or organic), the number of bacteria and fungi were higher in 0-10 cm of soil layer. The increase was caused by the utilization of the organic matter introduced into soil by microorganisms with after-harvest residue due to the rotation system applied and due to the secretion of root crops. In addition, the reduction of microbial number in the lower layer of the soil profile was the consequence of the access limitation by microorganisms to use carbon substrates.

According to production, an important characteristic of microorganisms is to create a symbiotic association with plants (Martyniuk, 2009; Martyniuk, 2011; Sveshnikova *et al.*, 2001). The most studied example of symbiosis is the coexistence of papillary bacteria (rhizobia) with legume plants. The exchange of the nutrients between these partners takes place in root nodules in which rhizobia transmits the nitrogen taken from the atmosphere to plants. In return, they collect carbohydrates as a source of energy necessary to carry out the process of bacteria to reduce atmospheric nitrogen. This process is beneficial from both ecological and agricultural

point of view, because it contributes to the reduction of nitrogen fertilization. Another type of symbiosis is mycorrhiza symbiosis, which exists between fungi species from the soil and plant roots. In this case, the mycorrhiza fungus facilitates for partner the collection of water and mineral salts and also protects plant roots from pathogenic fungi (Martyniuk, 2011). It is well known the fact that soil microorganisms through transformation processes of organic matter, are largely responsible for the availability of nutrients for plants. Therefore, the large number, activity and diversity of microorganisms are influential factors for a good quality and productivity of the soil (Marinari *et al.*, 2006; Masto *et al.*, 2006; Polyanskaya and Zvyaginstsev, 2005). A good indicator for estimation of the soil quality fertility is the ratio of the number of bacteria to fungi - B/F (Buryukhaev *et al.*, 1985; Gajda *et al.*, 2010; Myśków and Zięba, 1997). Higher values of this parameter inform about the relatively weak development of fungi, but less proved about their strong participation.

Due to the phytopathogenic and toxic characteristics of fungi, their increased development is negative in terms of fertility and productivity of the soil (Bis, 2006; Buryukhaev *et al.*, 1985; Gajda *et al.*, 2010; Wielgosz and Szember, 2006). It is worth noting, that the most profitable value of B/F (Tab. 5), aside from the level of sample collection, reach an average of 18,4. This average resulted from the object without nitrogen -A1, powered only by phytohormones. Higher doses of nitrogen decreased the index value limiting the effect of biostimulant. It should be noted, that the lowest B/F at 1.63 was obtained for samples taken from objects fertilized with nitrogen in an amount of 150 kg per ha.

Interaction between biostimulant and soil sample collection depth did not affect significantly the variation in question characteristics. In addition, statistical analysis showed that aside from other

experimental factors, soil sample levels did not significantly affect the evolution of the ratio of bacteria to fungi.

Conclusions

Biostimulant application based on the phytohormones in the cultivation of perennial ryegrass in a mixture with red clover, caused a decrease in the total number of colonies of all groups of soil microorganisms, but increased the ratio of the number of bacteria to fungi.

Increasing the dose of mineral nitrogen to 150 kg·ha⁻¹ contributed to more intensive growth of soil fungi and reduced the number of bacterial colonies, which indeed resulted in the deterioration of soil biological quality expressed as the number ratio of bacteria to fungi.

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