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Full Length Research Paper

Microbial activity of soil during the inoculation of soya bean with symbiotic and free-living nitrogen-fixing bacteria

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Soya bean has a great importance in the organic production due to its ability to coexist with nitrogen-fixing bacteria of the genus *Bradyrhizobium*. The soya bean seed inoculated with the symbiotic nitrogen-fixing bacteria *Bradyrhizobium japonicum* and with a mixture of nitrogen-fixing bacteria was observed in this study. The following highly efficient strains of free nitrogen-fixing bacteria were in the liquid inoculum: *Azotobacter chroococcum, Azospirillum lipoferum, Beijerinckia Derx* and *Klebsiella planticola.* Beside bacterization, different rates of pure nitrogen per hectare were applied (0, 40, 60, 80 kg N/ha). The effect of nitrogen-fixing bacteria at different levels of mineral nitrogen on the abundance of those groups of soil microorganisms and on the amounts of (NO_3+NH_4)- N/ha at the end of the growing season was determined. Three-year bacterization resulted in the significant increase of the observed parameters. The highest values when inoculation was done with mixed nitrogen-fixing bacteria were obtained with the application of 40 kg N/ha. The application of both types of nitrogen-fixing bacteria in the soya bean production is a very beneficial activity from ecological and economical aspects. Therefore, this measure should be accepted, especially in relation to soils contaminated with excess use of mineral nitrogen fertilisers.

Key words: Fertilising, nitrate and ammonium nitrogen, nitrogen-fixing bacteria, soya bean.

INTRODUCTION

In the future, agriculture will have a conclusive role in many key situations that would be faced by humans. The time is coming in which agriculture will again be a centre of the development, as agricultural production will have to provide sufficient food for ever growing human population, with respect and protection of natural resources. After several-decade anxiety over petrol, the decades of food domination in global stock exchanges are coming. This statement is confirmed by the historical price record of soya bean. The world experts claim that this situation will not be significantly altered in following years. Little was known about soya bean at the beginning of the 20th century, but towards the end of the century it became a leading field crop in the world. When the first soya bean harvest was performed in Serbia 30 years ago, it was said that this industrial plant would be a crop of the future. Now, it can be freely said that that future has already become the present time. The greatest soya bean areas in Serbia were in 2000 (141,559 ha), ranking 19th in the world for the last five years, and ranking seventh in the world based on the obtained yields when Serbia is compared with countries that have equal or greater soya bean areas. In 2009, soya bean areas amounted to 147,613 ha, greater by 2% than in the previous year, while the production was higher by 12.3% than in 2008 (Source: Bureau of Statistics of the Republic of Serbia). It can be stated that soya bean has spread

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over areas in Serbia and that only a slight increase of this areas can be expected in future (Hrustic et al., 2002).

Soya bean is not only a raw material for ever growing number of food products, but it also very important for the organic agriculture development. It is considered that soya bean is a very good preceding crop, especially for winter wheat genotypes, and it is a good crop for mixed cropping (Yusuf et al., 2009; Lazic and Babovic, 2008). Sowing of soya bean as a preceding, mixed or stubble crop is, according to the FAO and WHO definition, a method to manage the production that promotes the recovery of the ecosystem, including biodiversity, biological cycles and methods that, to the greatest extent, exclude or correct the input application (Kadar, 2007; Pepo, 2007). Soya bean is an important plant in organic food production because it requires less amounts of mineral nitrogen, and its root is suitable environment for activity of bacteria that fix atmospheric nitrogen. Cessation of intake of mineral fertilizers will not affect a rapid reduction of nitrogen in the soil; also, the proceeding of soybean seed is not followed with the use of pesticides. In addition, microbial biomass C in the rhizosphere of soybean according to the study of Yusuf et al. (2009) from 30 to 200% was higher than in the rhizosphere maize (*Zea mays L.*), which is very important in the rotation. The three-year research of influence of nitrogen fertilizers on soybean yield by Djukic et al. (2009) found that application of small doses of nitrogen fertilizer led to increase of yields, while higher amounts of nitrogen of 50 kg / ha led to reduced yields.

The practical application of the information on a biological cycle of nitrogen, as well as on microorganisms that actively participate in this process has been increasing in the organic plant production. Only several genera of prokaryotic organisms among microorganisms that are active biofertilisers are particularly important. The greatest share of biological nitrogen in agricultural lands originates from the activity of symbiotic nitrogen-fixing bacteria and free-living nitrogen-fixing bacteria coexisting with plants. In this process, microorganisms fix inert atmospheric nitrogen and convert it into forms availabe for plant nutrition, having positive effect on yield (Çakmakçı, 1987). According to Wani et al. (1994), approximately 175 million tons of nitrogen is bound by biological nitrogen fixation. Fixation soil systems make approximately 79% of the mentioned amount. Nitrogen fixation is an integral part of metabolism of bacteria belonging to the genera of *Bradyrhizobium* and *Rhizobium*. The first stable product of such nitrogen fixation is ammonia that is immediately transported to the plant cytoplasm and directly built in organic compounds. In such a way, nitrogen requirements by soya bean plants are satisfied in the amount of 75%, which is very important from the aspect of lowering mineral nitrogen fertiliser incorporation and possible soil degradation. Also, the economic effect is enormous, as the input of mineral nitrogen fertilisers is reduced (Cvijanovic et al., 2005, 2007a). As these micro-organisms developing and dwelling in the soil are mixed with plant roots, their activity affects the plant development and yields by products of their metabolic activity (hormones, vitamins, auxins and gibberllin). Moreover, their cell structure affects the maintenance of the soil structure, while their biomass affects the maintenance and the increase of the soil organic matter (Lupway et al., 2011).

The incorporation of associative nitrogen-fixing bacteria into the soil induces the development and the enzymatic activity of the existing soil autochthonous beneficial microbial population, which further affect changes of basic productive capacities of the soil (Sharma et al., 2007; Singh et al., 2007). As shown by several serological tests, there are relatedness between symbiotic and some free-living, associative nitrogen-fixing bacteria. The number of studies aimed at the results of effects of soya seed bacterization with different strains of nitrogen-fixing bacteria on elements of soil biogeny have been incresed in the recent times. Moreover, inoculation of seeds has to be a required agrotechnical method in the production of soybean, as it affects the increase in yield and grain quality. By using the inoculation, grain protein content of soybean is significantly larger than the variations without inoculation (Djukic, 2010). Results obtained on the effects of fertilizing soya bean with different types of nitrogen-fixing bacteria and different rates of mineral nitrogen stimulated the summary of the effect of their application on groups of microorganisms within a cycle of nitrogen that were at the same time good parameters of soil biogeny.

MATERIALS AND METHODS

This study was carried out in the experimental field of the Maize Research Institute in Zemun Polje (Serbia), on slightly calcareous chernozem. All cropping practices were of high quality and were applied on the optimum dates. The four-replicate trial was set up according to the randomised complete block design with inoculation of seeds of the soya bean cultivar ZP 015 and with the application of different rates of mineral nitrogen.

Seed inoculation was performed before sowing in the shady place in the following way: variant I - without seed inoculation, variant II - inoculation with symbiotic bacteria *Bradyrhizobium japonicum* and variant III - inoculation with the mixture of *B. japonicum* and selected high efficient strains of the following associative species: *Azotobacter chroococcum* strain 84, *Azospirillum lipoferum* strain 4, *Beijerinckia Derx* strain 4 and *Klebsiella planticola* strain 2. All selected strains belonged to the collection of the Department of Microbiology, Faculty of Agriculture, University of Novi Sad. Cell titre of used associative nitrogen-fixing bacteria was 20×10^8/ml.

The mineral nitrogen fertiliser was applied in the trial as follows: basic fertilising was done in autumn with complex NPK fertilisers at the ratio of 8:24:16, while 46% N was applied in spring. The amounts and types of incorporated nitrogen fertilisers expressed the values of the pure nutrient: N_0 - without fertilising, N_{40} - 40 kg N/ ha, 60 kg P_2O_5 and 40 kg K_2O; N_{60} - 60 kg N/ha, 90 kg P_2O_5 and 60 kg K_2O 60; N_{80} - 80 kg N /ha, 120 kg P_2O_5 and 80 kg K_2O. Sowing was done manually and sowing density amounted to 550,000 plants/ha.

Standard microbiological methods (Pochon and Tardeaux 1962)

Table 1. Average of total microorganisms number (×10^7/g soil) and index level (I.L.).

Fertilizer (kgN/ha)	Inoculation of variant	I Pheno-phase		II Pheno-phase		III Pheno-phase	
		Average (2006 - 2008)	I.L.	Average (2006 - 2008)	I.L.	Average (2006 - 2008)	I.L.
0	II	8.9	119.9	71.9	174.3	218.4	376.4
	III	15.8	212.6	100.4	243.4	103.9	179.2
	I	7.4	100	41.2	100	58.03	100
40	II	11.0	132.2	90.0	207.8	141.9	224.0
	III	134.3	161.2	110.9	255.9	260.4	411.1
	I	83.3	100	43.3	100	63.3	100
60	II	80.8	133.7	80.8	205.1	119.9	196.1
	III	102.5	169.5	98.6	250.1	218.5	357.4
	I	60.5	100	39.4	100	61.16	100
120	II	92.2	136.5	126.4	256.8	177.5	304.7
	III	80.2	118.5	101.6	206.3	245.3	420.0
	I	67.5	100	49.2	100	58.40	100
Average	II	93.1	130.3	92.1	212.6	160.9	267.2
	III	118.7	166.2	102.8	237.5	225.6	443.3
	I	71.4	100	43.3	100	60.2	100

I.L., Index level.

Table 2. Factors and interactions.

Factor and interaction	2006			2007			2008		
	F-test	LSD 5%	LSD 1%	F-test	LSD 5%	LSD 1%	F-test	LSD 5%	LSD 1%
A inoculated	2017.137**	6.981	9.220	260.22**	17.220	22.740	222.390**	8.341	11.020
B fertilization	26.051**	8.061	10.650	1.922	-	-	14.062**	9.632	12.720
A × B	12.566**	13.96	18.440	7.812**	34.440	45.480	9.700**	16.68	22.030
C pheno-phase	2367.238**	6.981	9.220	968.28**	17.220	22.740	181.030**	8.341	11.020
A × C	112.420**	12.09	15.970	48.501**	29.820	39.390	19.846**	14.45	19.080
B × C	18.764**	13.96	18.440	1.250	-	-	19.134**	16.60	22.030

LSD, Least significant difference.

were used for the determination of the abundance of certain groups of microorganisms. Easy available forms of nitrogen in the soil were determined by the Kjeldahl method.

RESULTS

Soil as a polydisperse system has also its living phase; hence the total number of microorganisms is a good indicator for the soil status evaluation – that is a good bioindicator of all changes that can occur under effects of different factors. On the average for the investigated period, the lowest abundance of total microbial number was determined in the variant without inoculation, while the highest abundance in the variant III with inoculation in all was observed in developmental pheno-phases (Table 1). All observed factors and their interactions affected the increase of the total number of microorganisms (Table 2), which certainly affects the increase of their biomass that is a prerequisite for the increase of productive capacities of the soil.

Oligonitrophylic bacteria are very important group of free-living nitrogen-fixing bacteria that reduce atmospheric nitrogen and convert it into organic forms. The analyses of the obtained results show that this group is the most abundant in the pheno-phase of the pod formation when nitrogen requirements by plants are great (Table 3). Also, the variant III with inoculation significantly affected the increase of their abundance (Table 4), especially at the

Table 3. Average number of oligonitrophylic bacteria (x10^6 / g soil) and index level (I.L.).

Fertilizer (kg N/ha)	Inoculation of variant	I Pheno-phase		II Pheno-phase		III Pheno-phase	
		Average (2006-2008)	I.L.	Average (2006-2008)	I.L.	Average (2006-2008)	I.L.
0	II	22.4	148.8	49.5	174.3	22.8	273.7
	III	43.0	285.3	55.6	196.1	27.2	156.2
	I	15.1	100	28.4	100	8.3	100
40	II	27.4	158.6	48.6	149.5	17.4	168.0
	III	29.5	170.7	65.5	201.3	34.1	329.3
	I	17.3	100	32.5	100	10.4	100
60	II	38.9	172.8	53.8	143.3	16.7	136.5
	III	30.6	136.1	48.3	128.8	15.9	130.1
	I	22.5	100	37.5	100	12.2	100
120	II	34.6	147.0	41.4	130.4	16.4	115.5
	III	23.1	98.36	53.6	168.6	29.2	204.9
	I	23.5	100	31.8	100	14.2	100
Average	II	30.8	157.9	48.3	148.5	18.3	162.4
	III	31.6	161.8	95.7	171.3	30.8	272.8
	I	19.51	100	32.55	100	11.3	100

Table 4. Factors and interactions.

Factor and interaction	2006			2007			2008		
	F-test	LSD 5%	LSD 1%	F-test	LSD 5%	LSD 1%	F-test	LSD 5%	LSD 1%
A inoculated	1741.625**	5.364	7.085	226.571**	14.670	19.370	63.469**	17.830	23.540
B fertilization	5.526**	6.194	8.181	12.262**	13.960	18.440	6.728**	20.580	27.190
A × B	12.805**	10.730	14.170	13.190**	29.340	38.750	9.634**	35.650	47.090
C pheno-phase	191.870**	5.364	7.085	1927.29**	14.670	19.370	426.496**	17.830	23.540
A × C	34.241**	9.291	12.270	48.736**	25.410	33.560	16.254**	30.880	40.780
B × C	20.836**	10.730	14.170	1.875	-	-	10.773**	35.650	47.090

LSD, Least significant difference.

end of growing season. In addition, ammonifiers have an important role in the cycle of nitrogen. They represent a great group of microorganisms that convert proteins and other nitrogen compounds, whereby ammonia is released. Hence this process is important from the aspects of the plant assimilates formation and from the aspect of the maintenance of productive capacities of the soil, as extracted ammonia is mainly built in the microbiological protein. Obtained results (Tables 5 and 6) show that the abundance of this group in the examined variants illustrates that the processes of nitrogen fixation were intensive till the end of the growing season. The number of these bacteria at the end of growing season was higher by 26.23; 92.26% in the variant II and variant III with inoculation, respectively.

The abundance of *Azotobacter* is a very important indicator of all changes in the soil. *Azotobacter* is the most distributed genus of the associative nitrogen-fixing bacteria dwelling in the rhizosphere zone. They are very sensitive and respond by their number and enzymatic activity to any changes in the habitat, hence they can be used, besides the total number of microorganisms, as a good indicator of the soil status. Seasonal dynamics of changes in their number in the pheno-phases II and III were not great (Tables 7 and 8). A higher number of *Azotobacter* was established in the variant III with inoculation. Moreover, the highest abundance of this group was estimated when the soil was fertilised with 40 kg N/ha, while the lowest number of *Azotobacter* was noticed at variants without nitrogen fertilizing nor seed

Table 5. The average of total number of ammonifiers (10^7/g soil) and index level (I.L.).

Fertilizer (kg N/ha)	Inoculation of variant	I Pheno-phase		II Pheno-phase		III Pheno-phase	
		Average (2006-2008)	I.L.	Average (2006-2008)	I.L.	Average (2006-2008)	I.L.
	II	29.11	153.7	65.5	201.2	47.8	155.4
0	III	40.9	216.5	73.4	225.5	109.2	354.5
	I	18.9	100	32.5	100	30.8	100
	II	36.4	158.1	70.3	174.6	52.6	612.9
40	III	43.1	187.1	109.1	270.9	31.9	371.6
	I	23.0	100	40.2	100	8.6	100
	II	36.4	153.6	95.2	168.4	38.3	571.1
60	III	41.1	173.6	62.0	109.7	35.7	532.6
	I	23.7	100	56.5	100	6.7	100
	II	34.7	140.4	61.7	131.1	31.4	245.4
120	III	38.6	156.2	87.5	185.8	21.9	171.7
	I	24.7	100	47.1	100	12.8	100
	II	31.1	120.1	73.2	165.9	55.4	126.2
Average	III	40.4	157.8	83.0	188.2	84.5	192.3
	I	25.9	100	44.1	100	43.9	100

Table 6. Factors and interactions.

Factor and interaction	2006			2007			2008		
	F-test	LSD 5%	LSD 1%	F-test	LSD 5%	LSD 1%	F-test	LSD 5%	LSD 1%
A inoculated	812.971**	9.938	13.130	285.059**	14.470	19.110	181.151**	8793	11.610
B fertilization	15.228**	11.480	15.160	16.021**	16.710	22.070	5.813**	10.150	13.140
A × B	10.397**	19.880	26.250	14.362**	28.940	38.220	1.657	-	-
C pheno-phase	297.896**	9.938	13.130	1671.097**	14.470	19.110	875.080**	8.793	11.610
A × C	49.578**	17.210	22.173	40.128**	25.060	33.100	69.769**	15.230	20.110
B × C	10.726**	19.880	26.250	6.459**	28.940	38.720	4.236**	17.590	23.230

LSD, Least significant difference.

inoculation.

According to the correlation analysis (Figure 1), it can be concluded that inoculation during the investigation period significantly affected the increase of amounts of easy available nitrogen forms in the soil. The highest amount of (NO_3+NH_4)-N /ha was determined when 40 kg N ha^{-1} was applied and in the variant III with inoculation. Variants II and III with inoculation significantly affected the content of kg (NO_3+NH_4)-N/ha in relation to the variant I with inoculation.

DISCUSSION

Nowadays, it is known that the association between the bacteria *B. japonicum* and soya bean is the most ideal type of the symbiosis in which each member benefits and therefore this association is called mutualism. Previous investigations confirmed the positive effect of bacteria inoculation on plant yield (Raicevic et al., 2002; Zaidi and Khan, 2006), specially pulse (Uslu and Esendal, 1998). Bacteria *B. japonicum* form nodules on the soya bean roots and by their production of growth substances, especially indole (indole 3-pyruvic acid, tryptophan), phenol (coumarin and gallic acid) and gibberellin, they affect plants over metabolic processes by which the adsorptive root area is enhanced and consequently the amount of absorbed nutrients is greater. It is known that there is a genetic variability of compatibility of the host plant and the microsymbiont, and the amount of nitrogen that is

Table 7. The average of Azotobacter number (10^3 / g soil) and index level (I.L.).

Fertilizer (kg N/ha)	Inoculation of variant	I Pheno-phase		II Pheno-phase		III Pheno-phase	
		Average (2006-2008)	I.L.	Average (2006-2008)	I.L.	Average (2006-2008)	I.L.
	II	2.8	187	1.9	211	1.7	170
0	III	3.6	240	2.0	222	2.4	240
	I	1.5	100	0.9	100	1.0	100
	II	2.5	147	1.9	135	2.1	161
40	III	3.2	188	2.1	150	2.2	169
	I	1.7	100	1.4	100	1.3	100
	II	2.6	118	1.7	142	1.9	158
60	III	3.1	141	1.7	142	2.0	167
	I	2.2	100	1.2	100	1.2	100
	II	2.3	128	1.3	144	1.7	142
120	III	2.5	139	1.2	133	1.7	142
	I	1.8	100	0.9	100	1.2	100
	II	2.5	139	1.7	154	1.8	164
Average	III	3.1	172	1.7	154	2.1	191
	I	1.8	100	1.1	100	1.1	100

I.L., Index level.

Table 8. Factors and interactions.

Factor and interaction	2006			2007			2008		
	F-test	LSD 5%	LSD 1%	F-test	LSD 5%	LSD 1%	F-test	LSD 5%	LSD 1%
A inoculated	376.706**	3.972	5.247	1019.389**	5.653	7.467	125.741**	11.800	15.590
B Fertilization	130.901**	4.587	6.058	15.175**	6.528	8.622	9.569**	13.630	18.000
A × B	22.482**	7.945	10.490	4.311**	11.310	14.930	14.517**	23.610	31.180
C pheno-phase	27.903**	3.972	5.247	217.963**	5.653	7.467	965.958**	11.800	15.590
A × C	21.090**	6.881	9.088	73.654	9.792	12.930	34.271**	20.450	27.010
B × C	20.616**	7.945	10.490	7.165**	11.310	14.930	14.015**	23.610	31.180

fixed in that association. Associative nitrogen-fixing bacteria populate the root rhizosphere and enter the relation with the plant; this symbiosis is called synoikia.

Nitrogen-fixing bacteria incorporated into the soil in any way (seed bacterization, incorporation, spraying the soil area and plant seedlings) are advantageous for the association formation in relation to other soil microflora. Once incorporated into the soil their multiplication and the relationship with the present microbes are different. They stimulate the plant development by their inhibitory effects on competitive, harmful and parasitic microflorae present in the spermatoshpere and the rhizosphere, and enter into competitive relationships for space and food with microorganisms within a microbial association, which causes changes in the microbial association (Cvijanovic et al., 2007b; Milosevic, 2008). Entering into the association with the root and using root exudates for their metabolic processes these bacteria increase their biomass and activity by which they increase soil biogeny (Jarak et al., 2005). Hence, cycling of necessary elements is more intensive and thereby plants are better supplied with necessary nutrients. Moreover, nitrogen-fixing bacteria incorporated under conditions of different presence of mineral nitrogen differently affect elements of soil biogeny and productive capacities of the soil and thus the yield quality and quantity (Nemeth, 2006). Since nitrogen fixation is an energy process, factors controlling the level of photosynthesis and the photosynthetic

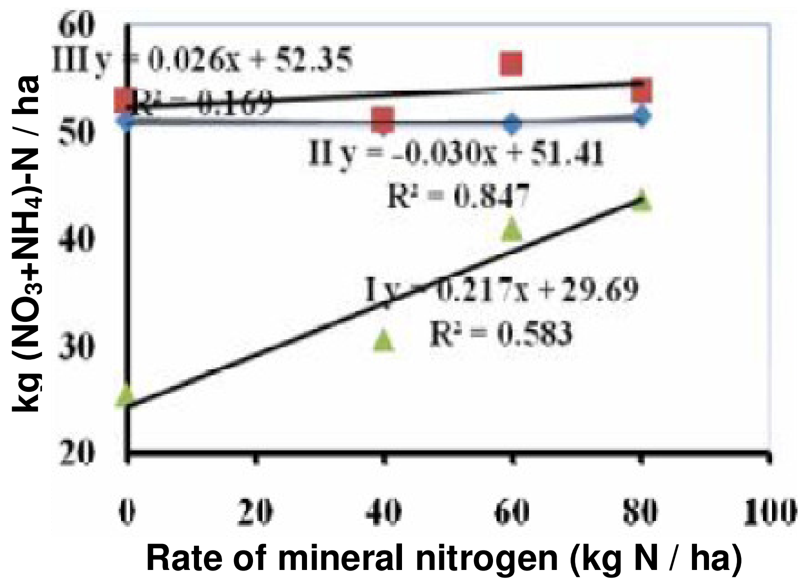

Figure 1. Effects of inoculation on the content of (NO_3+NH_4)-N in the soil in dependence on the type of nitrogen-fixing bacteria and rates of mineral nitrogen. R^2-I = 0.583** coefficient of correlation in the variant without inoculation; R^2-II = 0.847* coefficient of correlation in the variant with symbiotic nitrogen- fixing bacteria; R^2-III = 0.169** coefficient of correlation in the variant with a mixture of symbiotic and associative nitrogen- fixing bacteria.

distribution in the plant also affect nitrogen fixation. Excessive doses of mineral nitrogen are not favourable for soybeans because of nitrogen fixation absence, leading to reduced yield (Djukic, 2009).

Beside biotic factors affecting the number of certain systemic and physiological groups of microorganisms under field conditions, mineral nutrition of plants can also be significant (Lee, 1994). Soya bean is a protein plant,and for producing a great amount of proteins (35 to 50%) great amounts of nitrogen are necessary and it is realistic to expect a strong response of soya bean plants to nitrogen fertilising. According to obtained results, it is determined that the abundance of observed groups of microorganisms is greater when lower rates on mineral nitrogen are applied. This conclusion is in accordance with the results obtained by Dozet (2006). Furthermore, the analysis of these results shows that there is a significant difference in the amount of mineral nitrogen in the soil at the end of the growing season depending on the type of inoculation. In addition, from the obtained results it can be concluded that the activity of nitrogen-fixing bacteria can be enhanced with lower rates of mineral fertilisers. The possibility of soya bean seed bacterization with symbiotic and associative nitrogen-fixing bacteria is a very beneficial activity from ecological and economical aspects and therefore this measure should be accepted, especially in relation to the organic food production (Cvijanović et al., 2010). Results also show that significant amounts of nitrogen remain in the soil after soya bean harvest. This nitrogen is in an organic form and will be available after cell lysis, which is very important in the process of humification.

In conclusion, the application of soya bean seed bacterization is very useful from ecological and economical aspects and therefore this measure should be accepted, especially in relation to soils contaminated with excess use of mineral nitrogen fertilisers. Moreover, further studies should be aimed at finding compatible types of associative nitrogen-fixing bacteria that could be applied in soya bean inoculation with symbiotic nitrogen-fixing bacteria.

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