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Effect of Nitrogen Mineral Fertilisers on Crop Inoculation Efficiency With use of Associative and Symbiotic Nitrogen Fixers

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Abstract: High efficiency of pea inoculation with symbiotic bacteria complex *Rhizobium leguminosarum* and diazotrophs *Klebsiella planticola* and *Pseudomonas sp.* was first determined. Similar inoculation with bacteria complex was found to be effective twice the high mineral nitrogen rate (80 kg/ha), thereby contributing to a better utilisation of soil and atmospheric nitrogen by pea and, subsequently, to a higher yield and higher N quantity. The complex of bacteria *Rhizobium leguminosarum* + *Klebsiella planticola* decreased nitrates amounts in the soil, whereas that of *Rhizobium leguminosarum* + *Pseudomonas sp.* caused their increase. Complex inoculation *Rhizobium leguminosarum* + *Klebsiella planticola* and *Rhizobium leguminosarum* + *Pseudomonas sp.* didn't visibly change absorption $(^{15}NH_4)_2SO_4$ by plants, indicating neither the accumulation of ^{15}N stable isotope in the plants nor the presence of N which had been fixed by bacteria in the structure of peayield.

Keywords: plant, fertilisers, inoculation, microorganisms, nitrogen fixation

Introduction

Agricultural trend towards working out and application of the ecologically pure and energetically sparing methods for higher crop productivity has brought about partial substitution of the mineral, in the first place nitrogen, fertilisers with microbe agents composed of the microorganism cultures able to improve nitrogen and phosphorus plant nutrition at the cost of hormones production and thus raise nutritional matter intake from soil. Contrary to chemism, "microbiologisation" of agriculture, bears no negative effects and meets fully the demands of agricultural production -"ecologisation". This stems from the fact that microorganisms, used in agriculture, are isolated from the nature representing their product so that, contrary to biological law of buffering, they cannot be accumulated more nor disturb the ecological equilibrium.

An array of microbe agents, whose production is based upon symbiotic and associative nitrogen fixers, has come into use in recent time with the aim of "ecologisation" of agriculture and obtainment of the ecologically pure products. Still, despite widely used nitrogen fixing microorganisms towards reducing mineral fertiliser amounts and lower deficit of forage proteins, many of the natural and antropogenous factors contributing to proteins effective use have little been studied so far.

Therefore, we tended to examine what effects soil - ecological conditions and nitrogen mineral fertilisers might have on the agricultural crops inoculation by means of associative and symbiotic nitrogen fixers.

Materials and Method

Soil diazotrophic bacteria of *Pseudomonas* and *Klebsiella* and symbiotic ones of *Rhizobium* strain have served as the main object of studies which were performed in three vegetation experiments.

The first experiment used diazotrophic bacteria *Pseudomonas sp.* (strain A1), obtained at the Institute of Microorganisms Biochemistry and Physiology of the Russian Academy of Sciences (Puscino).

The second and third one used bacteria *Rhizobium leguminosarum* (strain Pis 4), diazotrophs of *Pseudomonas sp.* (strain $PsIA_{12}$) and *Klebsiella planticola* (strain TSHA - 91). Bacterial strains *Rhizobium leguminosarum* Pis 4 and *Pseudomonas sp.* $PsIA_{12}$ were obtained at the Institute of Ecogeophysiology of Primary Production in Mjuunherberg, Germany (Institute fur Okophysiologie der Primarproducktion, Miincherberg). Bacterial strain *Klebsiella planticola* TSHA - 91 was obtained at the Department of Microbiology MSHA (Moscow Agricultural Academy). The effect of these microorganisms was studied on two crops: table beetroot "Bordo" and seeding pea "Progres No.7".

The first vegetation experiment was set up at the Institute of Pedology and Photosynthesis of The Russian Academy of Sciences in Puscin (Moscow area) with table beetroot grown on various soils, as follows: I - turf podzolic moderate clayey; II Brown forest moderate clayey without sand; III Brown forest with addition of sand in the ratio 1:1; IV Flooded moderate clayey with addition of 4kg/dish manure. 36 kg air dry soil was placed in botomless dishes containing four plants each.

Concurrently with treatments without inoculation, those with addition of nitrogen fixing pseudomonas (*Pseudomonas sp.*) were also used, showing a high nitrogenous activity. The bacteria Pseudomonas were introduced in the soil in the form of a suspension during seed sowing with 107 cells counted per plant. Cell counting in the suspension was performed in the chamber *Gorjaeva.* In view of optimality of the soil environmental factors, the flooded ones (treatments IV and V) proved to be best. They are featured with abundance of organic matter

(1.87% and 2.45% C) and high availability of nutritional elements - mobile phosphorus (60.0 and 76.2 mg/100 g soil) and potassium (28.4 and 55.6 mg/100g soil). The manure introduced had a positive role (300 t/ha) in improving indicators mentioned. The content of Ca and Mg was excellent in flooded soils.

Turf-podzolic soil was much poorer than forest, and particularly, flooded soil in the light of the most important soil-ecological indicator - organic matter content, showing its minimal content (0.58%C), but being twice as much of the readily available phosphorus and potassium in relation to brown forest soil. The addition of sand to this soil have considerably changed soil -ecological factors, in the first place, lower humus content (nearly twice) and that of exchangeable potassium and calcium. Excepting brown forest, soil acidity was nearly equal in the remaining soils having neutral response of the medium (pH=6.93 - 7.00). The soil was irrigated with distilled water up to 60% MVK.

After completion of the vegetation period, betroot shoot and root yield and nitrogen uptake in the leaves and root, were determined. The content of bacteria, actinomycetes and fungi was determined both at at the start and at the end of vegetation.

Vegetation trials with seeding pea (2 and 3) were set up at the Humbolt University in Berlin (Germany) according to the following scheme:

2. trial: I Control, II. 20 kgN/ha, III *Rhizobium*, IV. 20 kg/ha N/ha + *Rhizobium leguminosarum* (strain Pis 4), V. 40 kg N/ha + *Rhizobium leguminosarum* (strain Pis 4), VI. 80 kg N/ha + *Rhizobium leguminosarum* (strain Pis 4).

3. trial: I Control, II 20kg N/ha, III *Rhizobium* IV.20 kg N/ha + *Rhizobium leguminosarum* (strain Pis 4), V. 20kgN/ha + *Rhizobium leguminosarum* (strain Pis4), VI + *Klebsiella planticola* (strain TSHA-91), VI 20 kgN/ha + *Rhizobium leguminosarum* (strain Pis 4) + *Pseudomonas sp.* (strain PsIA12).

Trials proceeded with six replications on turf-podzolic light clayey soil. 1.5kg air dry soil was placed into Micherlih's dishes each. The dishes were sown with eight germinated seeds each. Seed germinating lasted five days at 22°C. After the occurrence of germs, five plants were placed in each dish. Ammonium sulfate $(^{15}NH_4)_2SO_4$ with 90.2% ^{15}N was used as nitrogen fertiliser which was introduced in the form of water solution equally spread in the soil.

Microorganisms were introduced in the form of peat agents, with 10^9 cells per 1g peat agent, calculated with 1g peat agent per 50 seed pea grains.

After crops removal, N exudation in roots, leaves and legumes and their yield were determined. The content of total, nitrate and ammonium nitrogen in soil as well as that of ^{15}N in the plants, were also determined.The content of total nitrogen in plant matter (first trial) was determined using phenol method (Kudejarov, 1972). Plant leaves and roots (100g) were burnt in diluted sulfur acid (1 part H_2SO_4) : 2 parts H_2O) with catalyser (containing sulfur potassium), sulfur copper and metal selenium and zinc). In so degraded sample, N content was determined photometrically by means of phenolate-hypochloride reaction.

In the second trial, the content of total N in soil and plants was determined using Kjedahl method (Arimuskin, 1970) and that of mobile N forms (nitrate and ammonium form) in soil, using Cotte-Kahane method (modification, 1985). In principle, this method is based upon release of ammonium ions from soil - absorption complex by means of alumo - potassium alaun. 30 g air dry soil was first soaked with 100 ml alumo - potassium alaun ($KAl(SO_4)_2 \cdot 12H_2O$), then stirred and filtered. The content of ammonium ions (NH_4^+) in filtrate, was determined by means of titration with 0.01 n sulfur acid upon distillation on Kjeldahl apparatus with addition of NaOH and must indicator. The content of nitrate ions (NO_3^-) in the filtrate was also determined by means of titration with 0.01 n H_2SO_4 upon distillation on Kjeldah apparatus with the addition of iron-sulfate and silver-sulfate dissolver.

Stable isotope ^{15}N percent share was determined on the analyser NOI-6PC in liquid sample of ammonium chloride (NH_4Cl). Quantitative analysis of 15N nitrogen isotope share in per cent, was made spectrometrically upon ammonium oxidation up to molecule N. Assimilation of $^{15}N_2$ was established on the basis of he per cent increase of ^{15}N compared to its natural value. This value, which is calculated as the difference between ^{15}N per cent share in the sample and the size of its natural content, is designated as the maximum - atom % ^{15}N or enrichment (Barnard, 1957).

The number of bacteria, actynomicetes and fungi was determined as usual ("Methods of soil microbiology...", 1991). The microorganisms using organic sources of nitrogen, were determined on the mesopeptonic agar (MPA, pH=7.0 - 7.2). The total microorganism and *Pseudomonas* number was also determined on MPA. Microorganisms using mineral N sources were determined on starch - ammonium agar (KAA, pH=7.2 -7.4). Bacteria and actinomycetes were determined on KAA.

Microscopic fungi were determined on must agar (SA, pH=4.5-5.0). Analyses were made with three replicates.

Results were processed by means of dispersion method analysis (Dospehov, 1968).

Results and Discussion

Effect of soil-ecological factors on table beetroot inoculation efficiency with *Pseudomonas* strain bacteria. Analyses have shown an essential effect of soil conditions on beetroot inoculation efficiency with *Pseudomonas sp.* (tab.1.). Thus, beetroot root had the best growth and the highest yield with Pseudomonas inoculation, on the soil of poorer fertility - brown forest with addition of sand. It could be interesting to mention that the effect of inoculation on the forest brown soil without addition of sand was almost none. It is possible that in the treatment with sand, mechanical composition of soil has had a certain impact (the number of soil particles sized 1-1.25mm), thereby providing for loose and highly water-permeable soil of up to 66.59% and good aeration and suitable conditions of *Pseudomonas sp.* bacteria growth, accordingly.

Similar results were obtained on inundated soil, though of better fertility yet suitable for *pseudomonas* life. Inoculation had no effect on the soil of fairly high fertility - inundated soil (+manure), which is in agreement with already known literature data favouring high efficiency bacteria fertilisers on the soils of poor fertility.

Low efficiency of pseudomonas bacteria on turf-podzolic soil appeared to be an obvious effect of the specific soil conditions, particularly of the higher Al, Fe content and the like.

Tab.1. Effect of soil-ecological factors on table beetroot inoculation efficiency with *Pseudomonas* strain bacteria (*Pseudomonas sp.*)

Variants			Turf-podzolic soil	Brown forest soil	Brown forest soil + sand	Flooded soil	Flooded soil + manure	Lsd 0.05
Surface and underground (fruit) mass weight, g/dish	Leaves (dry mass)	Without inoculation	63.2	48.2	43.9	43.2	57.7	4
		With inoculation	75.7	52.4	68.3	65.8	67.3	4
		Inoculation efficiency, %	120	109	156	152	117	
	Fruit (dry mass)	Without inoculation	184.4	95.6	114.3	118.1	160.5	16
		With inoculation	167.6	110.0	148.7	157.5	173.3	9
		Inoculation efficiency, %	91	115	130	133	108	
	Fruit and leaves (dry mass)	Without inoculation	247.6	143.8	152.2	161.3	218.2	12
		With inoculation	243.3	162.4	217.0	223.3	240.6	16
		Inoculation efficiency, %	98	113	137	138	110	
	Fruit (raw mass)	Without inoculation	1077	621	635	642	991	12
		With inoculation	10.5	697	922	743	725	18
		Inoculation efficiency, %	103	114	145	116	73	

<u>Effect of soil-ecological factors on nitrogen uptake on table beetroot with *Pseudomonas* inoculation</u>. Since *Pseudomonas* strain, used for beetroot inoculation, indicated the ability of nitrogen fixation, it seemed to be of some interest to study the effect of these microorganisms on N uptake by the plants grown on the soils of differing fertility (tab.2).

From tab.2., one can see that even in the case of effective inoculation, table beetroot was bacterised on the brown forest soil (+sand), with N uptake being the highest on the flooded soil. Beetroot inoculation was also found to favour higher N uptake in other soil-ecological conditions which was proved by a strong pseudomonas nitrogen fixation ability as well as by their actively stimulating root system growth which, in turn, exerted a stronger utilization of the soil nitrogen.

Tab. 2. Effect of soil-ecological factors on nitrogen uptake on table beetroot with *Pseudomonas* inoculation (*Pseudomonas sp.* A_1)

Variants			Turf-podzolic soil	Brown forest soil	Brown forest soil + sand	Flooded soil	Flooded soil + manure	Lsd 0.05
Nitrogen uptake by plants, mg/dish	Leaves	Without inoculation	1213	947	826	977	1285	21
		With inoculation	1816	1442	1693	1622	1747	27
		Inoculation efficiency, %	150	152	205	166	136	
	Fruit	Without inoculation	2740	1498	1782	1581	3099	15
		With inoculation	3190	2203	2926	2849	2944	18
		Inoculation efficiency, %	116	147	164	180	95	
	Total	Without inoculation	3953	2445	2608	2558	4384	28
		With inoculation	5005	3645	4619	4471	4691	34
		Inoculation efficiency, %	127	149	177	175	107	

Effect of soil-ecological factors on soil microbe cenosis compositon with beetroot inoculated with *Pseudomonas* strain bacteria. Soil inoculated with *Pseudomonas* bacteria just before beetroot seeding affected microbe cenosis composition of all the soil types studied.

The microorganisms, using organic nitrogen forms, were reported to be higher in number after the harvest in all inoculated than in non-inoculated treatments. Thus, their number increased in brown forest soil, brown forest soil with the addition of sand, and in inundated soil with manure added. After the harvest, these microorganisms continued to increase in brown forest soil, brown forset soil with the addition of sand and in floded soil with addition of amnure. Notably, at the end of vegeattion *Pseudomonas* bacteria were higher in number in the inoculated treatments, as evidenced by their higher number upon harvest in brown forest soil, brown forest soil with sand and, particularly in flooded one with manure.

Pseudomonas inoculation - exerted much stronger effect on the number of the microorganisms using mineral nitrogen forms. Thus, by the end of vegetation, these microorganisms in the inoculated treatments on brown forest soil were five, on that with sand three, and on inundated 2.1 times higher than those in the same but non-inoculated treatments. Certain increase in actinomycetes and micromycetes was also observed in most of inoculated soils.

Therefore, *Pseudomonas* strain bacteria had a positive effect on the microbe cenosis of a series of bacteria, which is judged by an increase in microorganisms mainly using mineral nitrogen forms. This is apparently related to the activity of inoculant bacteria which contribute to the release of mobile nitrogen forms, and that is manifested by a stronger nitrogen uptake by table beetroot, in the first place, on the brown forest, brown forest with addition of sand and inundated soil.

The fact that *Pseudomonas* bacteria number has been higher compared to non-inoculated treatments which revealed the efficiency of infection performed, seems to be none the less significant.

Therefore, current studies have shown that high efficiency of plant inoculation with *Pseudomonas* strain bacteria resulted from a series of soil-ecological factors (mechanical composition of soil, air regime, moisture and organic matter amount), which raised both growth and yield of beetroot as well as the traits of soil microbe cenosis and transforming processes of N compounds, favouring its stronger uptake by plants.

Effect of Nitrogen Mineral Fertilisers on Pea Inoculation Efficiency with Symbiotic Associative Microorganisms. Considering differing meaningfulness of the question related to the kind of effect and amounts of nitrogen mineral fertilisers exerting legume microbe inoculation efficiency, three vegetation trials were set up (2 and 3) with pea plants grown using different N amounts. Initial N rate (20 kg/ha) and those twice or even four times the initial one (40 and 80kg/ha), with simultaneously inoculated plants with symbiotic bacteria *Rhizobium leguminosarum* strain Pis4 and with associative bacteria *Klebsiella planticola* strain TSHA - 91 and *Pseudomonas sp.* strain $PsIA_{12}$, particularly those of the complex, have been introduced.

Effect of N mineral fertilisers on pea inoculation efficiency with *Rhizobium leguminosarum* (trial 2) has shown that not only high, but also the initial (20kg) mineral fertiliser rates had a strong inhibition effect. Truefully, higher N amounts contributed to higher total pea biomass, which was only due to mineral fertilisers impact, whereas *Rhizobium* functioning was simultaneously being depressed. Attention should be paid to a significant detail which occurred over the trial - pea root system growth inhibition due to higher mineral N amounts (tab.3).

Once more, a question is raised about how necessary further research towards working new microbiological routes out for legume yield increase is (instead of raising mineral N rates (tab.3), not only using highly effective Rhizobium strains, but also other nitrogen fixing microorganisms able to biosynthesize growth matter and antibiotics. In this regard, the trial with pea grown on one N rate of 20 kg (trial 3), has been set up, but with application of the complex plant inoculation with *Rhizobium leguminosarum* sp. strain $AsIA_{12}$ which are characterized with both nitrogenous activity and antibiotics and growth matter producing ability (Emtsev et al., 1994).

From Figs.1-4., one can clearly see that complex *Rhizobium+Klebsiella* and *Rhizobium+Pseudomonas* pea inoculation favour high legume yield rather than that with Rhizobium only. Pea phytomass and, to some extent, root mass have also been enhanced. All this once more confirms that complex inoculation enables better plant supply with nitrogen and exerts growth factors due to ability of the bacteria used of performing phytohormones biosynthesis. It seems to be of some interest to mention that effect of symbiotic and associative bacteria complex used underneath legumes essentially, almost twice, that of rather high mineral N rate (80kg/ha), which is of prime ecological importance when discussing agricultural production and environmental concerns, allowing for the use of bacterial cultures which enables production with minimal nitrate pollution thus contributing environmental protection.

Tab. 3. Effect of Different Nitrogen Mineral Fertilisers Rates on Pea Inoculation Efficiency with Symbiotic Associative Microorganisms. (*Rhizobium leguminosarum*, strain Pis4)

Variants		Control	20 kg/ha nitrogen	R. *leguminosarum*	20 kg/ha N + *R. leguminosarum*	40 kg/ha N + *R. leguminosarum*	80 kg/ha N + *R. leguminosarum*	Lsd 0.05
Dry mass, g/dish	Leaves	1.07	1.44	1.43	1.35	1.64	1.67	0.12
	Control %	100	135	134	126	153	156	
	Seed	1.13	1.49	1.56	1.31	138	1.32	0.20
	Control %	100	132	138	116	122	117	
	Legume	0.59	0.73	0.52	0.69	0.64	0.73	0.21
	Control %	100	124	88	117	108	124	
	Surface mass	2.79	3.66	3.51	3.35	3.66	3.72	0.30
	Control %	100	131	126	120	131	133	
	Root	0.61	0.88	0.89	0.85	0.83	0.90	0.11
	Control %	100	144	146	139	136	148	
	Total phytomass	3.40	4.54	4.40	4.20	4.49	4.62	0.38
	Control %	100	134	129	124	132	136	

Fig. 1. Effect of complex pea inoculation (mass of leaves g/dish)

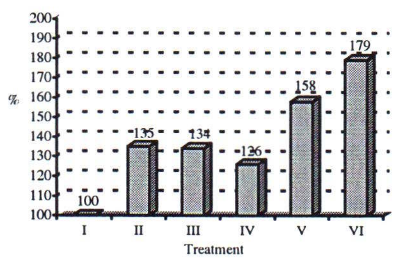
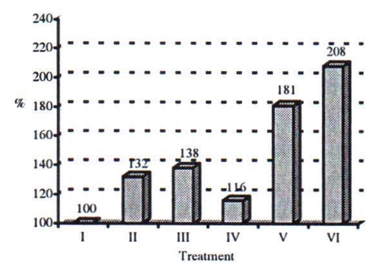


Fig. 2. Effect of complex pea inoculation (seed mass g/dish)



I-Control; II-20 kg/ha N; III-*Rhizobium*; IV-20 kg/ha N + *R.leguminosarum* (strain Pis 4)
V-20 kg/ha N + *R.leguminosarum* (strain Pis 4) + *K. planticola* (strain TSHA-91)
VI-20 kg/ha N + *R.leguminosarum* (strain Pis 4) + Pseudomonas sp. (strain $PsIA_{12}$)

Fig. 3. Effect of complex pea inoculation (root mass g/dish)

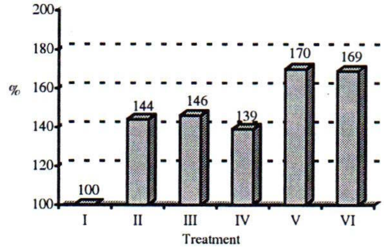
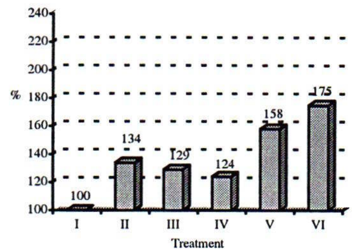


Fig. 4. Effect of complex pea inoculation (total mass g/dish)



I-Control; II-20 kg/ha N; III-*Rhizobium*; IV-20 kg/ha N + *R.leguminosarum* (strain Pis 4)
V-20 kg/ha N + *R.leguminosarum* (strain Pis 4) + *K. planticola* (strain TSHA-91)
VI-20 kg/ha N + *R.leguminosarum* (strain Pis 4) + Pseudomonas sp. (strain $PsIA_{12}$)

From given data, it is obvious that increased N rates essentially decreased pea inoculation efficiency with *Rhizobium*, which proves inapropriateness of using chemical nitrogen for growing of legumes inoculated with *Rhizobium*. On the other hand, the efficiency of pea inoculation with knotty bacteria Rhizobium coupled with associative diazitrophs *Klebsiella* and *Pseudomonas* is twice that of the high mineral N rate (80 kg/ha), as a result of the complex effect exerted by the microorganisms entered (N availability, growth matter and phytosanoitary protection).

<u>Effect of different N mineral fertilisers on N uptake by pea plants inoculated with symbiotic bacterium *Rhizobium* and associative diazotrophs *Klebsiella* and *Pseudomonas*.</u> Studies of effects differing mineral N fertilisers had on N uptake by pea plants inoculated by *Rhizobium* have shown that N uptake increase is exerted by symbiotic bacteria *Rhizobium*. Thus, for example, in the control treatment with N uptake by legume pea plants of 40.3mg/dish, plant inoculation by *Rhizobium* would raise N uptake up to 65.8mg/dish. Simultaneously, 20 kg N/ha entered somewhat reduced, while greater rates (40 and 80kg/ha) increased, but only slightly, N uptake by pea inoculated with *Rhizobium*. In other words, if inoculation with *Rhizobium* favours N uptake of 56.8 mg /dish, then entering of 20, 40 and 80kg N/ha will help N uptake of 53.2, 57.5 and 58.9mg/dish, respectively (tab.4).

Lower legume nitraginisation efficiency has been observed when using mineral N, accordingly. Higher N rates favoured both, higher N uptake by the above-ground (mainly by leaves and ...) and root part of pea.

Thus, a question of how significant complex pea inoculation to symbiotic, associative bacteria is in N uptake by plants in the role of the mineral N.

Experimental results are given in tab.5. As can be seen from tab.5., higher N uptake by pea proceeded not only due to *Rhizobium leguminosarum*, but also due to common impact of *Rhizobium* and *Klebsiella* and *Rhizobium* and *Pseudomonas* (at rate of 20kg N/ha). Addition of diazotrophs Klebsiella and Pseudomonas Rhizobium has enabled entire N uptake by pea plants to increase from 119.2 mg/dish in the treatment with Rhizobium only to 159 and 178.5 mg/dish in the treatments with *Klebsiella* and *Pseudomonas*.

Tab. 4. Effect of different N mineral fertilisers on N uptake by pea plants inoculated with symbiotic bacterium *Rhizobium leguminosarum*,strain Pis4

Variants		Control	20 kg/ha nitrogen	R. *leguminosarum*	20 kg/ha N + *R. leguminosarum*	40 kg/ha N + *R. leguminosarum*	80 kg/ha N + *R. leguminosarum*	Lsd 0.05
Nitrogen uptake by plants, mg/dish	Leaves	20.54	26.44	27.69	31.52	37.51	41.42	2.52
	Control %	100	129	135	153	183	202	
	Seed	40.29	54.99	56.80	53.24	57.49	58.98	7.71
	Control %	100	136	141	132	143	146	
	Legumes	11.56	14.88	10.62	18.05	19.25	27.07	5.19
	Control %	100	129	92	156	167	234	
	Surface mass	72.39	96.31	95.11	102.81	114.25	127.47	6.07
	Control %	100	133	131	142	158	176	
	Root	10.44	12.76	14.67	16.42	14.30	22.38	2.02
	Control %	100	122	141	157	137	214	
	Total phytomass	82.82	109.07	109.78	119.23	128.56	149.86	10.63
	Control %	100	132	133	144	155	181	

Tab. 5. Effect of different N mineral fertilisers on N uptake by pea plants inoculated with symbiotic bacterium *Rhizobium leguminosarum* strain Pis 4 and associative diazotrophs *Klebsiella planticola*, strain TSHA-91 and *Pseudomonas* sp. strain $PsIA_{12}$

Variants		Control	20 kg/ha nitrogen	R. *leguminosarum*	20 kg/ha + *R. leguminosarum*	40 kg/ha N + *R. leguminosarum + K. planticola*	80 kg/ha + *R. leguminosarum + Pseudomonas sp.*	Lsd 0.05
Nitrogen uptake by plants, mg/dish	Leaves	20.54	26.44	27.69	31.52	39.90	45.21	2.52
	Control %	100	129	135	153	194	220	
	Seed	40.29	54.99	56.80	53.24	84.05	97.04	7.71
	Control %	100	136	141	132	209	241	
	Legumes	11.56	14.88	10.62	18.05	14.11	15.69	5.19
	Control %	100	129	92	156	122	136	
	Surface mass	72.39	96.31	95.11	102.81	138.06	157.94	6.07
	Control %	100	133	131	142	191	218	
	Root	10.44	12.76	14.67	16.42	21.01	20.60	2.02
	Control %	100	122	141	157	201	197	
	Total phytomass	82.82	109.07	109.78	119.23	159.07	178.54	10.36
	Control %	100	132	133	144	192	216	

Therefore, the effect of bacteria Klebsiella and *Pseudomonas* used together with *Rhizobium* at rate of 20kg N/ha was even higher than that of 80kg N/ha and inoculation with *Rhizobium* used.

Higher mineral N rates essentially affect pea yield and N uptake from the soil. Complex pea inoculation with symbiotic *Rhizobium* bacteria and associative diazotrophs *Klebsiella* and *Pseudomonas* have thus contributed to better utilisation of the soil and atmospheric N by pea plants and to its higher uptake with yield.

Effect of differing mineral N fertilisers on the total N content and on its mobile forms (NH_4^+ and NO_3) in the soil under pea inoculated with *Rhizobium* and associative diazotrophs *Klebsiella* and *Pseudomonas*. The results of these studies have shown that, while using mineral nitrogen in the soil of the vegetation trial, its content increases to a certain extent, which is however particularly pronounced in the microbe inoculation of pea plants. Thus, if using of 80 kg N/ha (+ *Rhizobium*) increases N content in the soil up to 116%, then the inoculation of pea with *Rhizobium* + *Klebsiella* and *Rhizobium* + *Pseudomonas* (at the rate of 20kgN/ha) will increase its content up to 118 and 121%, respectively. It should be noted that, regardless of appreciable N uptake through yield markedly increasing applying bacteria *Rhizobium* + *Klebsiella* and *Rhizobium* + *Pseudomonas*, no decline in N content, but quite contrary, its increase takes place.

The total N content in the soil of the treatments *Rhizobium* + *Klebsiella* and *Rhizobium* + *Pseudomonas* has presumably increased at the cost of accumulation of the biological nitrogen fixed by the bacteria complex.

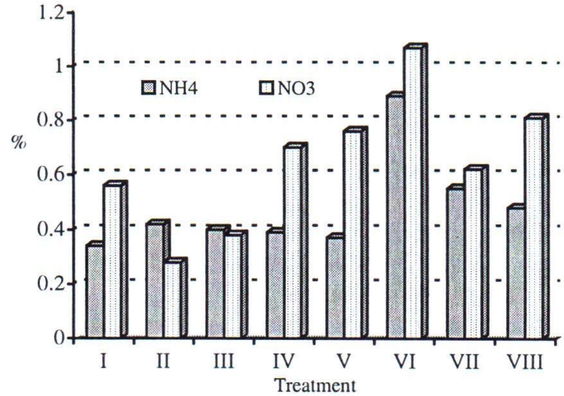
In addition, the use of bacteria complex on the basis of the minimal initial nitrogen rate (20 kg/ha) has evidently shown that it can be more effective than that of 80kg/ha N mineral fertilisers + *Rhizobium*.

Among mobile nitrogen forms existing in soil, special role may be attributed to the nitrate ones affecting agricultural production quality and environmental circumstances, as well. In this regard, the effect of mineral nitrogen fertilisers on its mobile forms content in the soil under he pea, inoculated with Rhizobium and *Rhizobium*+associative diazotrophs, could be also interesting (Fig.5.).

In our studies, the total mobile N content (NH_4^+ and NO_3^-) in the soil largely increased upon introducing of the mineral nitrogen fertilisers, whilst microbe inoculation of the plants caused even higher accumulation of these compounds. The use of the microorganism complex *Rhizobium* + *Klebsiella* and *Rhizobium* + *Pseudomonas* (based upon 20 kgN/ha) has increased the sum amount of mobile nitrogen forms in soil by 1.3 and 1.4 times, respectively, compared to the control. Compared with control, the decrease in the sum mobile nitrogen forms amount has been noticed in the treatments with introduced 20 kg N/ha and *Rhizobium*, which is likely due to more intense denitrifying process.

As regards changes in the content of particular mobile nitrogen compounds forms, nitrate nitrogen in the soil has been found to increase with increase in nitrogen fertilisers. Thus, at the rate of 80 kg N/ha + *Rhizobium*, nitrate nitrogen content in the soil has increased twice. Microbe inoculation of the pea plants at differing mineral N rates has shown differing effects. *Rhizobium* used without N fertilisers, and with 20 kgN/ha, produced considerably low content of the nitrate nitrogen in the soil compared with the control treatment.

Fig. 5. Effect of different mineral N fertilisers on the total N content and on its mobile forms (NH_4^+ and NO_3) in the soil under pea inoculated with *Rhizobium leguminosarum* strain Pis4 and associative diazotrophs *Klebsiella planticola* strain TSHA-91 and *Pseudomonas sp* strain $PsIA_{12}$.



I-Control; II-20 kg/ha N; III-*Rhizobium*; IV-20 kg/ha N + *R.leguminosarum*
V-40 kg/ha N + *R.leguminosarum;* VI-80 kg/ha N + *R.leguminosarum*
VII-20 kg/ha N + *R.leguminosarum* + *K. planticola*
VIII-20 kg/ha N + *R.leguminosarum* + *Pseudomonas sp.*

Nitrates were differently accumulated under the influence of the complex *Rhizobium* + *Klebsiella* and *Rhizobium* + *Pseudomonas.* Thus, the rate of 80kgN/ha + *Rhizobium* doubled nitrate nitrogen content in the soil. Differing nitrogen rates caused differing effects with pea microbe inoculation. *Rhizobium* used without N fertilisers, and with only 20 kgN/ha produced low nitrate nitrogen content in the soil compared with control.

The complex *Rhizobium* + *Klebsiella* and *Rhizobium* + *Pseudomonas* on the nitrates accumulation was aslo differing. The bacteria *Rhizobium* + *Klebsiella* increased mobile N content in the soil to some extent at the cost of ammonium form, simultaneously decreasing nitrates content (1.17mg/100g soil-total mobile N content, 0.62 mg/100g nitrate N and 0.55 mg/100g ammonium N, respectively).

Rhizobium + *Pseudomonas* increased mobile N in the soil to some extent at the cost of nitrate forms (1.29mg/100g soil - the total content of mobile N forms, 0.81mg/100g nitrate N and 0.48mg/100g ammonium N, respectively).

In general, bacteria complex *Rhizobium* + *Klebsiella* and *Rhizobium* + *Pseudomonas* allowed ammonium nitrogen content in soil to increase by1.6 and 1.4 times compared to the control.

Pea plants bacterised with symbiotic *Rhizobium* and associative *Klebsiella* and *Pseudomonas* complex on the basis of the minimal "starter" N rate (20 kg/ha) contributed to some increase in N content in the soil on the one hand, and to lesser nitrate accumulation, one the other. That complex inoculation of the legume plants with symbiotic and associative bacteria on the basis of rate (starter) 20 kgN/ha proved to be purposeful is beyond doubt, while more rates

(40 and 80 kgN/ha) simultaneously used levelled off the significance of inoculation itself.

The Role of Nitrogen Mineral Fertilisers and of Biological Nitrogen in its Accumulation in Pea Plants in Soil introducing *Rhizobium*, *Rhizobium* + *Klebsiella* and *Rhizobium* + *Pseudomonas* using Stable Isotope ^{15}N. Previous studies have shown that, when increased mineral nitrogen has a comletely negative effect on the pea bacterisation efficiency, while its "starter" rates provide for the optimal conditions for plant inoculation with symbiotic and associative diazotrophs, which elevates pea productivity and N accumulation in the plants and in soil. Since the experimental results confirmed an essential rise in yield and nitrogen uptake in pea, the vegetation experiment with use of stable isotope ^{15}N has been set up for determining the nature of nitrogen which itself induced such an increase.

As it is known, the method of designated atoms (particularly stable isotope ^{15}N) is widely used for studying of symbiotic and associative nitrogen fixation (Kalininskaja, 1971; Kalininskaja, Miller, 1989). Modern spectrophotometry techique makes it possible to determine ^{15}N content in the objects in question with high precision and to notice the differences in the amount of ^{15}N existing in the sample, of the level 0.001 - 0.002 maximal atomic % isotope ^{15}N.

Since ^{15}N exists in the mineral nitrogen fertiliser $[(^{15}NH_4)SO_4)]$, per cent of stable isotope is presumed to exist in the yiueld of the chemical nitrogen. Analogously, per cent of ^{15}N existing in soil imparts nitrogen of the mineral fertiliser. In other words, the amount of fertilisers introduced should stand in direct proportion with ^{15}N stable isotope content in the plants and in the soil.

The results of studying ^{15}N in different plant organs are outlined in tab.6.

From tab.6., one can see that introducing of *Rhizobium* + *Klebsiella* and *Rhizobium* + *Pseudomonas* (rate of 20 kgN/ha) doesn't visibly affect change in absorption of ^{15}N by plants. The fact that no accumulation of ^{15}N took place in the plants suggests its presence in the yield structure of nitrogen pea having been fixed by the microorganisms, at the cost of which as well as of the microbe stimulators, pea yield rose.

If we go back to tables 3 and 5 and figs. 1-4 in which yield and N uptake by pea are presented and compare these data with those in tab.6., we may conclude that increase in N uptake in the treatments with entering of the bacteria *Rhizobium* + *Klebsiella* and *Rhizobium* + *Pseudomonas* (at the rate of 20 kgN/ha) and subsequently higher pea yield, basically, occurred at the cost of nitrogen delivered by nitrogen fixing organisms imparting pea yield more in these than in the remaining treatments. With entering of the higher N fertiliser rates, biologocal N share decreased for which substituted mineral N.

Considerable increase in stable isotope ^{15}N in all the pea plant parts, particularly in the fruits and waists (commercial yield part) - in the treatments with 40 kgN/ha + *Rhizobium* and 80 kgN/ha + *Rhizobium* indicated higher share of the mineral nitrogen in the case of using higher mineral fertilisers rates. Thus, a possible danger of polluting the commercially valuable plant yield with nitrates while using the high mineral fertilisers rates.

Tab. 6. Stable isotope ^{15}N content in pea plants, grown with different nitrogen rates and inoculated with *Rhizobium leguminosarum* strain Pis4, *Rhizobium leguminosarum* strain Pis4 + *Klebsiela planticola* strain TSHA-91 i *Rhizobium leguminosarum* strain Pis4 + *Pseudomonas* sp. strain $PsIA_{12}$

Variants	Stable isotope ^{15}N content, total nitrogen %			
	Leaf	Fruit	Legume	Root
Control	0.439	0.472	0.466	0.491
20 kg/ha nitrogen	6.480	8.391	8.717	6.477
Rhizobium leguminosarum	0.429	0.460	0.708	0.610
20 kg/ha +*R. leguminosarum*	5.868	8.563	8.419	6.864
40 kg/ha +*R. leguminosarum*	12.018	16.563	16.511	12.338
80 kg/ha +*R. leguminosarum*	23.375	29.842	29.371	23.465
40 kg/ha + *R. leguminosarum* + *K. planticola*	6.279	8.348	8.031	6.529
80 kg/ha +*R. leguminosarum* + *Pseudomonas sp.*	6.405	7.847	8.096	6.610
Lsd 0.05	0.410	0.088	0.078	0.079

Study results of stable isotope ^{15}N in the soil are given in tab.7.

As can be seen, with increase in N fertiliser rate up to 40 and 80kg/ha (+ inoculation *Rhizobium*), per cent share of ^{15}N in the soil practically increases proportionally. At the same time, a certain drop of stable isotope ^{15}N (%total N) in the soil into which *Rhizobium* + *Klebsiella* + *Pseudomonas* have been entered (at the rate of 20 kgN/ha) is noticed.

Tab. 7. Stable isotope ^{15}N content in the soil under pea, grown with different nitrogen rates and inoculated with *Rhizobium leguminosarum* strain Pis4, *Rhizobium leguminosarum* strain Pis4 + *Klebsiela planticola* strain TSHA-91 i *Rhizobium leguminosarum* strain Pis4 + *Pseudomonas* sp. strain $PsIA_{12}$

Variants	^{15}N content in soil, % of		
	total	NH_4^+	NO_3^-
Control	0.666	1.896	1.435
20 kg/ha nitrogen	1.162	2.505	3.954
Rhizobium leguminosarum	0.506	1.598	1.172
20 kg/ha +*R. leguminosarum*	1.203	2.467	6.701
40 kg/ha +*R. leguminosarum*	1.779	3.638	10.683
80 kg/ha +*R. leguminosarum*	3.163	11.469	23.291
40 kg/ha + *R. leguminosarum* + *K. planticola*	1.147	2.068	4.742
80 kg/ha +*R. leguminosarum* + *Pseudomonas sp.*	1.111	1.424	4.898
Lsd 0.05	0.128	0.756	0.278

Certain effect of the bacteria complex *Rhizobium* + *Pseudomonas* (at the rate 20 kgN/ha) was exhibited on the nitrate nitrogen form in the soil. The content ^{15}N in per cent of its nitrate form somewhat declined in the soil with use of the bacteria *Rhizobium* + *Klebsiella* and *Rhizobium* + *Pseudomonas* (at the rate of 20 kgN/ha) compared to *Rhizobium* culture at the rate 20 kgN/ha with 6.7% up to 4.7 and 4.9%, respectively. At the same nitrogen rate, the complex *Rhizobium* + *Pseudomonas* caused lowerance in ammonium nitrogen content in the soil from 2.5% to1.4%.

Lowerance in nitrogen mobile forms in the soil - ammonium and nitrate while using bacteria complex *Rhizobium* + *Klebsiella* and *Rhizobium* + *Pseudomonas* alleviated danger of polluting water basins and groundwaters with nitrates, which is one more confirmation of the practical significance of agricultural plants inoculation with pure, and particularly mixed microorganism cultures able to fix nitrogen and biosynthesize growth matter and antibiotics.

Conclusions

The effect of soil-ecological circumstances and nitrogen mineral fertilisers on the agricultural plants inoculation efficiency with associative and symbiotic bacteria was studied. Based upon study results, the following may be concluded:

- inoculation efficiency of table beetroot with *Pseudomonas* strain bacteria is dependent on soil conditions. Beetroot inoculation was the most efficient on the brown forest soil with sand and on inundated soil (root yield increased by 177 and 175%, respectively), N uptake enhanced by this crop). The sand entered into the brown forest soil obviously improved aeration, and a high content of coloid particles in the unindated soil contributed to the inoculants aeration and to their more efficient impact on the plants.

- table beetroot inoculation with *Pseudomonas* on the brown forest (+sand) and on inundated soil has also shown certain effect on the microbe cenosis state. This is particularly evidenced by an increase in the soil microorganisms using mineral nitrogen forms that appeared with pseudomonates activity, thereby inciting nitrogen mineral forms release and enhanced nitrogen uptake by plants.

- when increased, mineral nitrogen fertiliser rates (40-80 kg/ha) essentially (1.6-1.7 times) reduced productivity of pea inoculation with symbiotic bacteria of the *Rhizobium* strain. Therefore, the use of higher rates of mineral N under legumes (particularly pea) inoculated with Rhizobium has proven ineffective.

- pea inoculation with symbiotic bacteria complex *Rhizobium leguminosarum* and associative diazotrophs *Klebsiella planticola* and *Pseudomonas* sp. was manifested as highly efficient. Thus, the complex inoculation of soil with these bacteria was efficient twice that with high mineral N fertiliser rate (80kg/ha) as a result of synergistic effect of the microorganisms entered - plant supplies with N, growth matter and phytosanitary protection.

- higher N mineral fertiliser rates have shown no essential effect on pea yield and, therefore, on N uptake from the soil, with inoculation efficiency with Rhizobium being somewhat reduced. On the other hand, complex inoculation of pea with symbiotic bacteria *Rhizobium leguminosarum* and associative diazotrophs *Klebsiella planticola* and *Pseudomonas sp.* favoured better utilising of the soil and atmospheric N by pea plants, thereby increasing their yield and, therefore, N uptake, coupled with yield.

- pea plants bacterization with the *Rhizobium leguminosarum* symbiotic bacteria complex and associative diazotrophs *Klebsiella planticola* and *Pseudomonas sp.* on the basis of starter N rate (20 kg/ha) somewhat incrased entire and, to some extent, ammonium nitrogen content in the soil. The lower nitrates in soil due to complex *Rhizobium leguminosarum* + *Klebsiella planticola*, the higher their quantity in the soil due to *Rhizobium leguminosarum* + *Pseudomonas sp.* bacteria complex.

- the application of stable isotopes (^{15}N) has enabled complex inoculation *Rhizobium leguminosarum* + *Klebsiella planticola* and *Rhizobium leguminosarum* + *Pseudomonas sp.* to display visible effect on the exchange of absorption $(^{15}NH_4)_2SO^4$ by plants, being the proof of absent accumulation of stable isotope ^{15}N in the plants and its presence in the yield of pea nitrogen fixed by the bacteria.

- in brief, it is in these studies that complex legume cultures inoculation with symbiotic bacteria *Rhizobium leguminosarum* and associative diazotrophs *Klebsiella planticola* and *Pseudomonas sp.* has been thoroughly approached for the first time, representing a new and a highly perspective trend in agricultural biotechnology which is most likely to impart practical significance to bio-correction of the polluted soil environment and production of the ecologically pure products.

- synergistic use of the symbiotic bacteria *Rhizobium leguminosarum* and associative nitrogen fixers *Klebsiella planticola* and *Pseudomonas sp.* has therefore enabled mineral nitrogen fertilisers to be largely reduced or completely skipped.

References

Arinuškina, E.V. (1970): Rukovodstvo po himičeskomu analizu počv. Moskva: Izd. MGU, 487s.

Bernard, D. (1957): Sovremennaja mass - spektrometrija. Moskva: Izd. inostr. lit.,415s.

Dospehov, B. A. (1968): Metodika polevogo opita. Moskva: Kolos, 336s.

Emcev, V. T., Selickaja, O. V., Kubareva, O. G., Petrov- Spiridonov, A.A. (1994): Associativnij simbioz i jevo rolj v produktivnosti seljsko-hozjajsvennih rastenij. Timirjazev i biologičeskaja nauka, SPb - M, s. 106 - 119.

Kalininskaja, T. A. (1971): Ispoljzovanije ^{15}N dlja opredelenija azot fiksirajušćej aktivnosti počvi. Mikrobiologičeskie i biohimičeskie metodi issledovanija počv. Kijev: Urožaj, s. 176 - 180.

Kalininskaja, T. A., Miller Ju. M. (1989): Ispoljzovanie izotopa ^{15}N pri izučemii nesimbiotičeskoj azotfiksacii. V. kn.: " Biologičeskij azot v seljskom hozjajstve SSSR" - Moskva: Nauka, s. 156 - 165.

Kudejarov, V. N. (1972): Kolorimetričeskoe opredelenie ammonijnovo azota v počvah i rastenijah fenolovim metodom. Agrohimija No. 6, s. 146 -151.

UTICAJ AZOTNIH MINERALNIH ĐUBRIVA NA EFEKTIVNOST INOKULACIJE POLJOPRIVREDNIH BILJAKA ASOCIJATIVNIM I SIMBIOZNIM AZOTOFIKSATORIMA

-originalni naučni rad-

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Rezime

Najpre je utvrđena visoka efektivnost inokulacije graška kompleksom simbioznih bakterija. *Rhizobium leguminosarum* i asocijativnih diazotrofa *Klekbsiella planticola* i *Pseudomonas sp.* Slična inokulacija kompleksom bakterija dva puta je efektivnija od prilično visoke doze mineralnog azota (80kg/ha), doprinosi boljem iskorišćavanju zemljišnog azota, kao i atmosferskog azota od strane graška, što je uslovilo povećanje njegovog prinosa i, usled toga, povećanje iznosa azota sa prinosom.Dokazano je da kompleks bakterija *Rhizobium leguminosarum* i *Klebsiella planticola* izaziva sniženje nitrata u zemljištu, dok, istovremeno, kompleks bakterija *Rhizobium leguminosarum* +*Pseudomonas sp.* ,nasuprot, izaziva povećanje nitrata u zemljištu.Utvrđeno je da kompleksna inokulacija *Rhizobium leguminosarum* + *Klebsiella planticola* i *Rhizobium leguminosarum* + *Pseudomonas sp.* ne ispoljava primetan uticaj na izmenu apsorpcije $(^{15}NH_4)_2SO_4$ od strane biljaka, što ukazuje na izostanak nakupljanja stabilnog izotopa ^{15}N u biljkama i prisustva azota, kojeg su fiksirale bakterije, u strukturi prinosa graška.