Utilization of the biological nitrogen fixation for soil evaluation

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ABSTRACT

Non-symbiotic nitrogen fixation (potential nitrogenase activity – PNA) of soil samples originating from different plots of long-term field experiments (selected variants: Nil, NPK [mineral fertilisation: 64.6–100 kg N/ha/year], FYM [farmyard manure], and FYM + NPK from three blocks III, IV and B with different crop rotation) was determined in laboratory experiments. The symbiotic nitrogen fixation (total nitrogenase activity – TNA) of the same soil samples was evaluated in hydroponic experiments with pea (2001, 2002) and lucerne (2001) in which the soil samples were used as a natural inoculum. The high values of PNA were found in the variants fertilised with FYM in all three blocks and all experiments. Simultaneously, the variants fertilised with mineral NPK reached low values of PNA. The farmyard manuring enhanced the number of free-living bacteria *Azotobacter* spp. that were identified in all soil samples. In the hydroponic experiments with pea, the highest nonsignificant values of TNA were found in variants B 284 (FYM + NPK) and III 254 (FYM + NPK) in 2001, and B 214 (FYM) and III 214 (FYM) in 2002. Plants inoculated with soil from these variants formed also high amounts of nodules (significant differences in block IV in 2001) and plant biomass. In the experiments with lucerne, the nonsignificantly highest TNA values were found in variant III 154 (NPK). Variants from block III (214, 254) and IV (114 and 154) showed the nonsignificantly lowest TNA values. The rhizobia that effectuate symbiosis with pea were more active in the soil samples in 2001 than those forming nodules on lucerne.

Keywords: long-term field experiments; non-symbiotic nitrogen fixation; symbiotic nitrogen fixation; pea; lucerne

Biological nitrogen fixation is an important part of the microbial processes. This process is catalyzed by nitrogenase, an enzyme possessed by all the nitrogen fixers, both free-living and symbiotic. Perhaps 80% of the biologically fixed N_2 comes from symbioses involving legume plants and rhizobia (Graham and Vance 2000). Biological nitrogen-fixation potential in soils is influenced (positively or negatively) by management, cultivation, fertilisation, and other agronomic interventions. The determination of the biological nitrogen-fixation activity can be used to characterize the changes in the soil properties and quality as a consequence of the farming system and/or soil contamination. Biological nitrogen fixation can be determined by several methods. The common method – acetylene reduction assay (ARA) – uses the reduction of acetylene to ethylene. Ethylene originating from the enzymatic reaction is measured by gas chromatography. The method allows to compare various N_2 fixing systems (Hardy et al. 1968, Šimek 1993, etc). These systems can cover the symbiotic apparatuses of legume plants and trees or soil samples (intact soil cores or sprinkled soil samples). The aim of this study was to determine the symbiotic and non-symbiotic nitrogen fixation in selected soil samples coming from log-term field experiments and to evaluate the efficiency of this process.

MATERIAL AND METHODS

Soil samples. Soil samples were taken from selected plots of long-term experiments in Prague. Soil type is Orthic Luvisol, clay-loam, developed on dilluvial sediments mixed with loess; soil reaction is neutral (pH_{KCl} is 6.8–7.1) in the whole profile. Four variants (Nil – no fertilisation, NPK – mineral fertilisation, FYM – farmyard manure and FYM + NPK) from three blocks (III, IV and B) were selected for this study. Crop rotation and organic and mineral N fertilisation is shown in Table 1. Crop rotation in blocks III and IV differed just in one-year shift of the cultivated crops. Soil samples were taken from 0–200 mm layer in all plots three times (autumn 2001, spring 2002 and autumn 2002). Cultivated crops – block III: 2001 – winter wheat, 2002 – sugar beet, block IV: 2001 – sugar beet, 2002 – spring barley, and block B: 2001 – sugar beet, 2002 – spring wheat.

Non-symbiotic nitrogen fixation (potential nitrogenase activity). Potential nitrogenase activity (PNA) was determined according to Šimek (1993). Fresh samples were sieved through a 2mm sieve and weighed into 100ml incubation flasks (equivalent to 15 g of dry soil) in three replications. 2 ml of 7.5% glucose solution was added as the energy source for microorganisms. Flasks were tightly closed with rubber stoppers and 10% of the volume in flasks was supplemented with the same volume of acetylene. The flasks were incubated at room temperature for 48 hours and were shaken two times during this period of time. After the incubation, gas samples were taken into the 2ml syringes and the amount of ethylene was analyzed on gas chromatograph Hewlett-Packard using the FI detector. As a standard, the calibration mixture of gases Supelco (Germany) was used for the calculation. The flasks were opened and filled with water to determine the

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Table 1. Selected blocks and variants

	Crop rotation	Variants	Average N doses (kg N/ha/year)
Block III	since 1955 (9 years)	114 Nil	0
	lucerne, lucerne, winter wheat,	154 NPK	64.6
	sugar beet, spring barley, potatoes,	214 FYM	38.6
	winter wheat, sugar beet, spring barley	254 FYM + NPK	103.2
Block IV	since 1955 (9 years)	114 Nil	0
	lucerne, lucerne, winter wheat,	154 NPK	64.6
	sugar beet, spring barley, potatoes,	214 FYM	38.6
	winter wheat, sugar beet, spring barley	254 FYM + NPK	103.2
Block B	since 1965	114 Nil	0
	alternatively sugar beet and spring wheat	184 NPK	100
		214 FYM	57
		284 FYM + NPK	157

Nil = no fertilisation, NPK = mineral fertilization, FYM = farmyard manure

volume of the incubation atmosphere. PNA of the samples was expressed in nmol/g soil/48 h. The data obtained were processed by analysis of variance followed by the Tukey test that evaluates the significance of differences between the variants.

Symbiotic nitrogen fixation (total nitrogenase activity of native rhizobia in symbiosis with pea and lucerne). Hydroponic experiments with perlite were used for the determination. The set of two pots sterilized in autoclave was used in the experiments. The upper pots (volume 500 cm^3) were filled with perlite (grains 1–3 mm) while the bottom pots contained the nutrient solution without nitrogen (Šimon 1991). Both pots were connected with glass fibres that conducted the flow of nutrients to the plant roots. The nutrient solution was regularly replaced. Portions (2 g) of the selected soil samples were added to each set as an inoculum 20 mm under the surface of perlite before the planting of pea and lucerne seedlings. Such experiments permit to determine the presence and activity of rhizobia present in the soils, and to eliminate the effect of other soil characteristics. Each set had three replications. The plants were cultivated in a greenhouse. Sodium-vapour lamps provided additional light and the temperature was partly regulated (20–24°C day/12–16°C night). A 16-hour photoperiod was used. The total nitrogenase activity (TNA) (µmol/plant/hour) was measured according to Hardy et al. (1973) at the onset of anthesis using the system described above for non-symbiotic N_2 fixation. The roots were separated from the shoots, the number of nodules and their dry weight were determined in pea and dry matters of pea and lucerne roots and shoots were estimated by weighing.

RESULTS AND DISCUSSION

Non-symbiotic nitrogen fixation expressed as PNA in soil samples is shown in Table 2. The individual values of PNA measured in autumn 2001 did not differ significantly. The highest value of PNA in block III was found in the variant fertilized with farmyard manure (38.33 nmol/g soil/48 h). Similarly, in block IV the variant FYM (IV 214) had a highest value of PNA – 33.39 nmol/g soil/48 h that was two or three times higher than those in the other variants. In block B, the highest PNA value was found in the control variant without fertilisation (B 114) (36.66 nmol/g soil/48 h). All other variants of organic or mineral fertilisation showed a half or two-thirds lower values. Interesting results were received in spring 2002. The absolutely highest value of PNA was found in variant B 214 (FYM) (410.60 nmol/g soil/48 h) that differed significantly compared to all other variants. Nevertheless, also other two variants fertilised with FYM in block III (III 214) and IV (IV 214) reached the highest values of PNA inside these blocks. On the contrary, low values were found in the variants fertilised with NPK (block B, IV) and Nil variant in block III. The results of measurements in autumn 2002 confirmed the results from the spring of that year for block III where the FYM variant III 214 reached the highest value of PNA (331.90 nmol/g soil/48 h). In block B, the highest value was found for variant B 284 (FYM + NPK) (419.06 nmol/g soil/48 h) that was the absolutely highest value of all measurements. In block IV, the measured values were low especially in variant IV 154 fertilised by NPK.

The results of measurement confirm that there exists a non-symbiotic nitrogen-fixation potential in the soils used for the experiments. The PNA can be mainly attributed to the free-living nitrogen fixing bacteria (*Azotobacter* spp.) that were identified in soil samples from the same plots by Nováková (2002, personal communication). The number of colony-forming units per gram of soil (CFU/g) ranged between 100 and 1000. Another nitrogen fixers occurring in some soils belong to *Azospirillum* spp. associated with *Graminae* spp. and *Acetobacter* spp., and *Herbaspirillum* spp. associated with sugar cane; they were isolated from the rhizosphere of these plants by Rennie et al. (1982), Li and MacRae (1992) and Döbereiner et

Table 2. Non-symbiotic nitrogen fixation (PNA) in soil samples (mean values)

Variant	PNA (nmol/g soil/48 h)		
	autumn 2001	spring 2002	autumn 2002
B 114 0	36.66NS	141.65a	358.87c
B 184 NPK	15.90	83.14a	63.42ab
B 214 FYM	17.38	410.60b	146.46abc
B 284 FYM + NPK	24.11	162.10a	419.06c
III 114 0	34.28	2.76a	33.71a
III 154 NPK	34.80	19.13a	224.97abc
III 214 FYM	38.33	97,00a	331.90bc
III 254 FYM + NPK	37.16	19.15a	63.62ab
IV 114 0	10.97	14.16a	21.60a
IV 154 NPK	17.47	4.84a	2.50a
IV 214 FYM	33.39	22.44a	16.51a
IV 254 FYM + NPK	18.17	12.30a	14.47a

NS = nonsignificant differences
Means within the column followed by the same letter do not differ significantly as determined by Tukey multiple range test ($p < 0.05$)

al. (1995). Organic fertilisation with farmyard manure as a source of nutrients and organic carbon seems to enhance the incidence and activity of the free-living nitrogen-fixing microorganisms. Hardarson et al. (1987) reported that high N_2 fixation by free-living microorganisms is strongly dependent upon the presence of substantial amounts of easily oxidizable organic matter in soil. On the contrary, the fertilisation with NPK without farmyard manuring leads to a decrease in the soil microbial activity including the free-living nitrogen-fixing microorganisms. The determination of the presence or absence of these microorganisms and their activities can thus indicate the soil quality and its immediate condition. Such a determination can also be indicative about the soil health because the free-living nitrogen fixing microorganisms are very sensitive to the soil contamination (Skujins et al. 1986).

Nitrogenase activity using two host plants is shown in Tables 3 and 4. In the first experiment with pea (2001), the nonsignificantly highest values of TNA were found in variants B 284 (FYM + NPK) and III 254 (FYM + NPK). The plants inoculated with soil from these variants formed also the highest amounts of nodules and plant biomass (Table 3). On the other hand, the mineral-fertilised variant in block IV revealed the lowest values of all the characteristics measured. The results, however, did not differ significantly except the nodule number per plant where variant III 114 had a significantly higher

Table 3. Nodule and growth characteristics of pea – 2001, 2002 (mean values)

Variant	TNA (μmol/plant/h)		Nodule number/plant		Nodule dry weight (mg/plant)		Shoot dry weight (g/plant)		Root dry weight (g/plant)	
	2001	2002	2001	2002	2001	2002	2001	2002	2001	2002
B 114	6.70 ± 0.91	0.81 ± 0.39	62 ± 8	32 ± 10	69.33 ± 52.55	19.00 ± 12.69	1.01	0.76	0.17	0.13
B 184	7.90 ± 3.27	0.69 ± 0.17	54 ± 28	57 ± 25	68.33 ± 55.94	23.33 ± 9.02	1.23	0.78	0.16	0.12
B 214	7.64 ± 2.50	1.60 ± 0.28	69 ± 19	67 ± 24	63.33 ± 23.44	30.33 ± 4.01	1.33	1.09	0.22	0.21
B 284	10.73 ± 1.37	0.69 ± 0.08	87 ± 24	82 ± 4	61.00 ± 7.21	31.67 ± 10.50	1.38	1.19	0.20	0.17
III 114	6.49 ± 3.55	1.33 ± 0.87	10 ± 5	82 ± 33	71.33 ± 56.88	27.33 ± 17.61	1.14	1.13	0.18	0.15
III 154	6.23 ± 1.95	0.42 ± 0.36	86 ± 3	74 ± 11	49.33 ± 9.02	23.00 ± 3.00	1.25	0.82	0.22	0.12
III 214	8.08 ± 3.36	2.03 ± 1.51	77 ± 5	92 ± 33	81.33 ± 42.15	34.67 ± 24.69	1.21	1.22	0.18	0.20
III 254	8.57 ± 1.84	0.78 ± 0.62	98 ± 11	43 ± 25	68.33 ± 25.38	21.00 ± 10.54	1.40	0.72	0.20	0.12
IV 114	8.06 ± 2.13	1.17 ± 0.94	75 ± 24	87 ± 35	48.67 ± 12.22	25.33 ± 21.83	1.20	1.04	0.17	0.14
IV 154	3.27 ± 3.12	1.47 ± 0.87	42 ± 25	60 ± 18	28.33 ± 25.78	21.67 ± 11.93	0.68	0.81	0.11	0.10
IV 214	6.99 ± 2.69	0.62 ± 0.76	86 ± 22	28 ± 10	76.67 ± 28.93	8.33 ± 6.66	1.01	0.55	0.16	0.06
IV 254	4.87 ± 1.17	0.73 ± 0.44	45 ± 17	86 ± 42	40.67 ± 15.37	19.00 ± 6.56	0.69	0.84	0.12	0.11

Table 4. Growth characteristics of lucerne (2001, mean values)

Variant	TNA (μmol/plant/h)	Shoot dry weight (g/plant)	Root dry weight (g/plant)
B 114 0	1.64 ± 1.38	0.30 ± 0.13	0.28 ± 0.15
B 184 NPK	0.72 ± 0.55	0.10 ± 0.04	0.09 ± 0.02
B 214 FYM	1.49 ± 1.01	0.33 ± 0.22	0.21 ± 0.14
B 284 FYM + NPK	0.65 ± 0.57	0.07 ± 0.06	0.18 ± 0.15
III 114 0	0.90 ± 0.37	0.09 ± 0.03	0.19 ± 0.11
III 154 NPK	2.30 ± 1.21	0.30 ± 0.18	0.40 ± 0.26
III 214 FYM	0.21 ± 0.12	0.04 ± 0.02	0.06 ± 0.03
III 254 FYM + NPK	0.03 ± 0.01	0.01 ± 0.01	0.08 ± 0.04
IV 114 0	0.15 ± 0.08	0.20 ± 0.10	0.20 ± 0.08
IV 154 NPK	0.04 ± 0.03	0.06 ± 0.05	0.10 ± 0.05
IV 214 FYM	1.50 ± 1.38	0.36 ± 0.12	0.38 ± 0.18
IV 254 FYM + NPK	1.63 ± 1.40	0.24 ± 0.09	0.28 ± 0.08

number than variant IV 154 (data not shown). In the second experiment with pea (2002), the highest nitrogenase activity was found in variants fertilised with FYM in blocks B and III (Table 3). In block IV, the highest value was found in variant IV 154. The differences between the variants were not significant. The plants inoculated with soil samples from plots fertilised with FYM formed high amounts of nodules and plant biomass except block IV where the unfertilised variant formed the most nodules and plant biomass.

In symbiosis with lucerne, there were higher differences in TNA between blocks III, IV, B. However, the differences within the individual variants were also high (Table 4). Therefore, no significant differences were found between the blocks and variants. The highest TNA activity was found in variant III 154 (NPK). The lowest values were found for the variants from block III (214, 254) and IV (114 and 154). In all soil samples, approximately 10^4–10^5 CFU of native rhizobia per 1 g were determined by microbiological analysis according to Vincent (1970). Bacteria of *Rhizobium leguminosarum* biovar *viciae* that effectuate symbiosis with pea roots were more active in the first experiment (2001) than *Rhizobium meliloti* that forms nodules on lucerne roots. It is surprising especially in block III and IV where lucerne was included in the crop rotation, and where the rhizobia nodulating lucerne should have been promoted by the regular planting of this legume. It seems that-although rhizobia were present in those variants-they have a low efficiency and posses a poor ability for symbiosis with lucerne. It is known that a high nitrogen content in soil depresses the N_2 fixation (Pate and Atkins 1983). Actually, in symbioses with pea the variants fertilised with mineral NPK in blocks III and IV (2001) as well as in B and III (2002) revealed a decreased TNA. If nitrogen originated from FYM or FYM + NPK, it did not depress the TNA of pea (Table 3). This effect could be combined with the well-known positive effect of organic fertilisation on the contents of organic matter and microbial biomass, and on the enzymatic activities in soils (Kubát et al. 1999).

The intention to use PNA/TNA as a parameter of the biological nitrogen fixation by native diazotrophs in soil samples is useful but due to the very high variability of the results obtained it seems that the application of these methods for the evaluation of the soil biological activity is questionable. The use of a higher number of soil samples taken from the experimental variants and a better standardisation of the growth conditions of the host plants in hydroponic experiments could overcame the disadvantages of these tests.

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ABSTRAKT

Využití biologické fixace dusíku pro hodnocení půd

V inkubačním pokusu byla u vybraných půdních vzorků z dlouhodobého polního pokusu (varianty: bez hnojení, NPK, hnůj, hnůj + NPK, bloky III, IV a B s různým osevním postupem) měřena potenciální nitrogenázová aktivita (PNA) jako ukazatel nesymbiotické fixace dusíku. Následně byl založen hydroponický pokus s hrachem (2001, 2002) a vojtěškou (2001) s cílem stanovit celkovou nitrogenázovou aktivitu (TNA) jako ukazatel symbiotické fixace dusíku. Stejné vzorky půd jako v prvním případě sloužily jako přirozené inokulum. Každá varianta měla tři opakování. Vysoké hodnoty PNA byly zjištěny u variant hnojených hnojem ve všech třech termínech odběru u všech bloků. Naopak nízké hodnoty PNA byly zaznamenány u variant hnojených minerálními hnojivy. V půdních vzorcích všech variant byly identifikovány bakterie rodu *Azotobacter*. V hydroponickém pokusu s hrachem byly nejvyšší hodnoty TNA zjištěny u variant B 284 (hnůj + NPK) a III 254 (hnůj + NPK) v roce 2001 a B 214 (hnůj) a III 214 (hnůj) v roce 2002. Rozdíly oproti ostatním variantám nebyly signifikantní. Rostliny inokulované půdou z těchto variant tvořily též nejvíce hlízek (signifikantní rozdíly byly zjištěny u variant z bloku IV v roce 2001) a měly nejvyšší hmotnost biomasy rostlin. V hydroponickém pokusu s vojtěškou byly zjištěny nejvyšší nesignifikantní hodnoty TNA u varianty III 154 (NPK). Některé varianty, především varianty bloku III (214, 254) a IV (114 a 154), vykazovaly nejnižší hodnoty TNA, rostliny špatně rostly a málo fixovaly dusík. Rhizobia nodulující hrách byla v půdních vzorcích v roce 2001 aktivnější než rhizobia nodulující vojtěšku.

Klíčová slova: dlouhodobé polní pokusy; nesymbiotická fixace dusíku; symbiotická fixace dusíku; hrách; vojtěška

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