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EFFECT OF UREA PHOSPHATE ON THE *Bacillus* sp. POPULATION IN SOIL AND ANTIFUNGAL ACTIVITY OF SELECTED STRAINS ON *Fusarium* sp.

WPŁYW FOSFORANU MOCZNIKA NA LICZEBNOŚĆ BAKTERII Z RODZAJU *Bacillus* W GLEBIE I AKTYWNOŚĆ PRZECIWGRZYBOWĄ WYBRANYCH SZCZEPÓW NA *Fusarium* sp.

Abstract: The main objective of the examination was to assess the influence of urea phosphate (UP) on the number of *Bacillus* sp. and antifungal activity both *Bacillus subtilis* and *Bacillus amyloliquefaciens* on *Fusarium* sp. Fungal growth was evaluate on the basis of fungal spore germination and rate index of mycelium growth. Studies revealed positive influence of objects with urea phosphate on the number the *Bacillus* sp. The number of those microorganism was higher than in the non-manured and manured with FYM soil assayed throughout the experiment period. The spore germination of all *Fusarium* species were strongly inhibited but strain *Fusarium tricinctum* showed the highest sensitivity to metabolites of *Bacillus subtilis* I7. *Bacillus amyloliqefaciens* III14 demonstrate the highest activity against mycelium growth of all tested moulds.

Keywords: urea phosphate, *Bacillus* sp., antifungal activity, *Fusarium* sp.

Soil's microorganisms are very important factors which decide about biological processes taking place in the soil. The basic factor modifying soil's quantitative microorganisms composition and its activity is the applied fertilizer. The *urea phosphate* (UP) has a practical application in the food industry, fertiliser industry and environment protection as well. The prior investigations proved that this compound used as the farmyard disinfectant, did not reduce the activity of ammonificators, denitrificators and nitrificators in the soil [1].

Bacillus sp. usually inhabit the soil and are important factor of the natural environment. Strains of many *Bacillus* and relatives possess nitrogenase and are able to

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fix atmospheric nitrogen, to increase nutrient availability in the rhizosphere for example phosphorus (*B. megaterium*), to produce of plant growth regulators such as auxin, cytokinins and/or giberelins. Moreover, they may exhibit biological control activity through direct antagonism of the soil-borne fungal and bacterial plant pathogens by metabolize many different bioactive compounds and antibiotics (phospholipides or lipoprotein). From the ecological point of view, natural fungicides are a potential alternative for the use of chemical pesticides, which have a harmful influence on the environment [2–5]. The aim of this study was to estimate the influence of urea phosphate on *Bacillus* sp. population in the soil and the antifungal properties of the *B. subtilis* and *B. amyloliquefaciens* against 4 species of *Fusarium*.

Materials and methods

The studied material consisted of samples brown soil, pH_{KCl} 5.5, 27, 50 mg · kg^{-1} soil N-NO_3, 23.80 mg · kg^{-1} soil N-NH_4, sampled from the layer of soil from 0 to 25 cm in the autumn period. The pot experiment design included four treatments: 1 – control soil, without fertilization, 2 – soil + *farmyard manure* (FYM), 3 – soil + urea phosphate (UP), 4 – soil + urea phosphate + FYM. Soil was watered to 60 % of total water capacity and incubated at a temperature of 20 ^{o}C, with moisture content being kept constant over the entire experimental period. Analyses were carried out in three replications, in: day 7 – I term, day 30 – II term and day 90 of the experiment – III term and involved counts of the number of *Bacillus* sp. on DDG medium that consist of (g · dm^{-3}) yeast extract – 3, glucose –10, K_2HPO_4 – 0.5, agar Difco – 20, of soil extract 300 cm^3, distilled water 700 cm^3. The number of *Bacillus* sp. was assayed with the incubation-plate method.

Bacterial strains were selected among a collection of 45 *Bacillus* sp. isolates from samples of soil fertilized with urea phosphate whereas fungal strains from soil fertilized with farmyard manure. Bacterial phenotypic characterization by physiological and biochemical tests were performed according to the Bergey's Manual of Determinative Bacteriology [6] and API 50CHB system (bioMerieux, France). The *Bacillus* were identified as strains of *Bacillus subtilis* – No. B I7, B II11 and *Bacillus amyloliquefaciens* – B III14. They were inoculated into a flask containing 50 cm^3 nutrient broth and incubated at 30 ^{o}C for 24 h. Subsequently the 15 cm^3 of nutrient broth was inoculated with a concentrated suspension of *Bacillus* sp. (2.0 OD at λ = 550 nm) and incubated at 30 ^{o}C for various times to give 6, 12, 24 h cultures (working culture). *Fusarium* species were identified according Domsh *et al* [7], Kwasna *et al* [8] and identified as *Fusarium solani* (F5), *Fusarium tricinctum* (F8), *Fusarium oxysporum* (F45), *Fusarium sporotrichoides* (F87). They were cultivated on Czapek-Dox medium (plates and slants) at 25 ± 2 ^{o}C for 5–7 days.

The antagonistic activity of the *Bacillus* strains was evaluate on the basis of fungal spore germination and rate index of fungal growth.

Determination of influence of *Bacillus* strains on fungal spore germination. Analysis of the effect of these bacteria on fungal spore germination was performed with the modifying slide germination method. Fungal cultures were cultivated on Czapek-

-Dox slants at 25 ± 2 °C for 7 days and the well-developed fungal culture was rinsed with 10 cm^3 of sterile water containing 0.05 % Tween 80. Mycelium was filtered from sterile gauze and the suspension was adjusted to 10^6 conidia/cm^3 in a hemocytometer. Subsequently 50 mm^3 of working culture of *B. subtilis and B. amyloliqefaciens* and 25 mm^3 of fungal spores were transferred on 1 cm^2 clean glass slides. Conidia germination in broth was used as a control. The slides were incubated in humid chambers in triplicate at 25 ± 2 °C for 24 h. After this period the number of germinated and non-germinated conidia were counted under a microscope using x 200 magnification. Conidia germination was presented as a *rate index of conidia germination* (Ig %) and was obtained using the formula [9]:

$$Ig = \frac{\sum (n \cdot a)}{N \cdot 4} \cdot 100\%$$

where: n – the number of germinate spore at a given stage;
a – bonitation scale of the stage: 0 – no germination, 1 – germination hypha length shorter than conidium, 2 – germination hypha length similar to conidium, 3 – germination hypha length was bigger than two lengths of conidium, 4 – germination hypha length was bigger than three or more lengths of conidium and branched;
N – the total number of the macroscopic analysed spores;
4 – the high stage of the scale.

Determination of influence of *Bacillus* strains on mycelium growth. Fungal mycelial-disks (diameter of 10 mm) obtained from growing cultures of test fungal isolates were placed in the centre of Czapek-dox and PDA medium containing 0.5 cm^3 working cultures of *Bacillus* (in four replications). A control was made only with fungal mycelial-disks on both medium. After incubation at 27 °C for 14 days, plates were observed for antifungal activity (at 2 days intervals) and estimated as the rate index of fungal growth (Ifg) using the formula [10]:

$$Ifg = \frac{A}{D} + \frac{b_1}{d_1} + \ldots + \frac{b_x}{d_x}$$

where: A – the mean from colony measurement,
D – the experiment duration,
$b_1, \ldots, b_x$ – the increase a colony diameter from lasted measurement,
$d_1, \ldots, d_x$ – the number days from lasted measurement.

Statistical calculation were performed by the variance method with the use of Duncan's test.

Results and discussion

The influence of urea phosphate on *Bacillus* growth and antifungal activity of selected *Bacillus* strains was studied. The strong disinfecting proprieties of the urea

phosphate were affirmed in earlier investigations. However inhibitory it actions on some microorganisms physiological groups was not noticed.

Changes in the numbers of *Bacillus* sp. in soil are presented in Fig. 1. Among all the treatments, in the presence of urea phosphate (in S + UP as well in S + UP + FYM objects) significant higher count of *Bacillus* sp. was observed when compared with objects containing no urea phosphate or farmyard manure. The highest increase of the number those bacteria after 7 days (206×10^3 cfu/g) and 90 days (140×10^3 cfu/g) was recorded in fertilized soil (S + UP + FYM). Similarly, the greatest growth of *Bacillus* sp. was observed in non-fertilized soil (S + UP) 125×10^3 cfu/g after 7 days and 112×10^3 cfu/g after 90 days. Simultaneously, the FYM treatment showed significantly lower value than UP treatments, but significantly higher than in the non-manured soil [1].

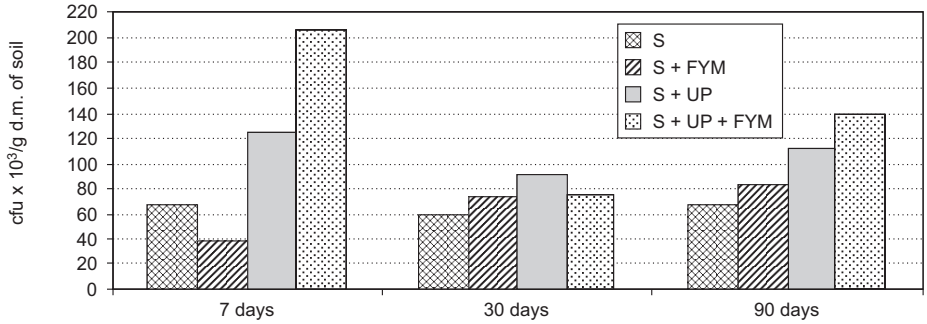

Fig. 1. Number of *Bacilus* sp. in 1 g d.m. of soil depending on fertilization

The antifungal *Bacillus* isolated from fertilized soil was identified as *B. subtilis* (strains BI7 and BII11) and *B. amyloliquefaciens* (BIII14). Members of the *Bacillus* genus and relatives are generally found in soil and most of these bacteria *eg B. subtilis*, *B. cereus*, *B. mycoides*, *B. amyloliquefaciens* are known to suppress several fungal pathogens growth such as *Rhizoctonia* sp., *Sclerotina* sp., *Phytium* sp. [11]. A mechanism by which *Bacillus* sp. suppresses disease is explained in different ways by various authors. Many species of them are capable to produce biologically active substances (*eg* extracellular chitinase) able to disintegrate fungal cell walls [12] as well as growth on the mycelium as a sole carbon source [13]. Some of them (iturin, surfactin) were found to act upon the sterol present in the cytoplasmic membrane of the fungi [14, 15]. *Bacillus subtilis* is one of the major producers of metabolites with antifungal properties in the genus. They possess the activity against some fungi from different taxonomic groups (*eg Penicillium chrysogenum, Aspergillus wentii*, *Trichotecium roseum*, *Botritis cinerea*, *Rhizoctonia solani*) and are even capable of partially controlling a natural infection of plant [4, 16–18]. Some investigators have suggested that biological control or use of microbial fungicides may be an alternative strategy to chemical fungicides [4, 5].

In the present work, antifungal effect of *B. subtilis* and *B. amyloliquefaciens* was determined as the fungal spore germination and the rate index of fungal growth.

The degree of fungal spore germination was different, depending on the strains of *Bacillus* sp. and also age of the culture applied. The results are shown in Table 1.

Table 1

The index rate germination of *Fusarium* (Ig [%] / inhibition of germination [%])

Bacillus strains		*Fusarium solani*	*Fusarium tricinctum*	*Fusarium oxysporum*	*Fusarium sporotrichoides*	Mean
Bacillus subtilis I 7	control	96.69	97.73	97.23	100.00	97.91 c
	6 h	94.11/2.67	78.50/19.68	94.49/2.82	80.00/20.00	86.77 b
	12 h	88.63/8.33	41.66/57.37	93.80/3.53	86.35/13.65	77.61 a
	24h	76.92/20.45	92.22/5.64	84.42/13.17	87.50/12.5	85.26 b
	mean	89.08 b	77.52 a	93.17 c	88.46 b	97.91
Bacillus subtilis II 11	control	96.69	97.73	97.23	100.00	97.91 d
	6 h	93.55/3.25	66.66/31.79	85.7111.85	84.61/15.39	82.63 a
	12 h	95.00/1.75	94,61/3.19	100.00/+2.85	93.38/6.62	95.74 c
	24 h	92.38/4.45	90,527.37	92.70/4.65	95.04/4.96	92.66 b
	mean	94.45 b	87.38 a	93.91 b	93.25 b	92.24
Bacillus amyloliquefaciens III 14	control	96.69	97.73	97.23	100.00	97.91 d
	6 h	95.00/1.75	90.00/7.91	92.46/4.91	100.00/0.0	94.36 c
	12 h	78.9918.30	86.54/11.45	97.17/0.06	100.00/0.0	90.67 b
	24 h	61.11/36.80	97.65/0.08	95.45/1.83	100.00/0.0	88.55 a
	mean	82.94 a	92.98 b	95.57 c	100.00 d	92.87

Small letters – significant difference at the level $p < 0.05$.

In this experiment 6 h, 12 h and 24 h cultures of *Bacillus* sp. were used as an inhibition factor. The strongest inhibition of fungal spore germination was observed when *B. subtilis* I7 was used for 12 h, *B. subtilis* II 11 for 6 h, *B.amyloliquefaciens* for 24 h cultures against all tested fungal strains. *B. subtilis* (strains I7 and II 11) had the highest inhibitory effect for spore germination of *Fusarium tricinctum* (57.37 and 31.79 %, respectively), *B. amyloliquefaciens* for *Fusarium solani* (36.80 %) whereas the spore germination of *Fusarium oxysporum* and *Fusarium sporotrichoides* were weakly inhibited by all of tested *Bacillus* strains. The *B. subtilis* I7 was more efficient than the others *Bacillus* strains.

These observations agree withe the data of some authors, who reported that antifungal metabolites production is strongly conditioned by factors such as strain of the producing microorganisms, age of the culture (growth phase), the chemical composition of the mycelium and the incubation condition [19]. Toure *et al* [18] observed that surfactins were mainly produced during exponential growth with the higher value for cell productivity observed after 12 h. By contrast, iturins and fengycins were mostly synthesized once the culture entered the stationary phase to reach optimal production rate after 72 h. Whereas Feio *et al* [20] showed that synthesis of mycobacilin and

bacilysin occurs after the exponential phase of development in *B. subtilis*. Chitarra *et al* [21] detected an antifungal compound produced by a strain of *B. subtilis* that permeabilises fungal spores and blocks germination of *Penicillium roqueforti*. Besides, different sensitivities of the fungi to the various *Bacillus* genus may indicate the production of different metabolites or antifungal products at different concentrations, or both [19].

The antifungal activity of *Bacillus* sp. grown on 2 different media was evaluated towards *Fusarium* sp. as the rate index of fungal growth. The antifungal activity of *Bacillus* strains was dependent on the age of the culture applied and the growth media used. Table 2 shows the results obtained with PDA medium.

Table 2

Effects of the *Bacillus subtilis* and *Bacillus amyloliquefaciens* on *Fusarium* growth on PDA medium (diameter of the mycelium in mm/mycelium growth inhibition in %)

Bacillus strains		*Fusarium solani*	*Fusarium tricinctum*	*Fusarium oxysporum*	*Fusarium sporotrichoides*	Mean
Bacillus subtilis I 7	control	33.03	25.71	34.41	34.01	31.79 c
	6 h	32.00/3.12	27.68/+7.66	32.08/6.77	31.32/7.91	30.81 b
	12 h	32.33/2.12	31.94/+24.23	33.69/2.09	31.06/8.67	32.25 d
	24 h	27.16/17.77	28.69/+11.59	35.16/+2.18	29.54/13.14	30.14 a
	mean	31.14 b	28.55 a	33.83 c	31.48 b	31.25
Bacillus subtilis II 11	control	33.03	25.71	34.41	34.01	31.79 b
	6 h	25.24/23.58	24.14/6.11	25.68/25.37	18.02/47.02	23.27 a
	12 h	25.09/24.04	24.26/5.64	23.68/31.18	19.47/42.75	23.12 a
	24 h	26.29/20.41	26.26/+2.14	24.01/30.22	17.01/49.98	23.38 a
	mean	27.40 c	25.09 b	26.94 c	22.13 a	25.39
Bacillus amyloliquefaciens III 14	control	33.03	25.71	34.41	34.01	31.79 b
	6 h	21.58/34.66	29.06/+13.03	20.06/41.70	14.59/57.10	21.31 a
	12 h	22.34/32.36	25.51/0.78	20.30/41.00	16.06/52.78	20.30 a
	24 h	22.21/32.76	24.43/4.98	20.37/41.80	14.32/57.89	20.35 a
	mean	24.03 b	26.18 b	23.78 b	19.76 a	23.43

Small letters – significant difference at the level $p < 0.05$.

Among the *Fusarium* species, *F. solani, F. oxysporum, F. sporotrichoides* were the most sensitive to metabolites produced by all tested *Bacillus* strains. In case of *F. tricinctum* all the *Bacillus* strains had a very little inhibitory properties and even a small stimulatory properties in case of *B. subtilis* I7 (7.66–24.23 %). It is appear that inhibitory activities of *B. amyloliquefaciens* was significantly higher than all the other *Bacillus* sp., especially towards *F. sporotrichoides* (52.78–57.89 %). A similar influence of *Bacillus* sp. was observed on Czapek-Dox medium. The results are reported in Table 3.

Table 3

Effects of the *Bacillus subtilis* and *Bacillus amyloliquefaciens* on *Fusarium* growth on Czapek-Dox medium (diameter of the mycelium in mm / mycelium growth inhibition in %).

Bacillus strains		*Fusarium solani*	*Fusarium tricinctum*	*Fusarium oxysporum*	*Fusarium sporotrichoi-des*	Mean
Bacillus subtilis I 7	control	36.62	35.56	36.76	38.31	36.81 d
	6 h	27.98 /23.59	36.47/+2.56	31.94/13.11	30.31/20.88	31.82 b
	12 h	29.41/19.69	36.95/+3.91	27.72/24.59	30.54/20.28	31.15 a
	24h	29.60/19.17	36.78/+3.43	36.76/0.0	29.40/23.26	33.12 c
	mean	30.90 a	36.44 d	33.26 c	32.30 b	33.22
Bacillus subtilis II 11	control	36.62	35.56	36.76	38.31	36.81 c
	6 h	11.33/69.06	34.07/4.19	26.94/26.71	28.03/26.83	25.09 a
	12 h	11.79/67.80	34.43/3.18	26.09/29.03	27.28/28.79	24.90 a
	24 h	25.48/30.42	34.82/2.08	30.06/18.23	26.00/32.13	28.87 b
	mean	21.08 a	34.73 c	29.96 b	29.90 b	28.91
Bacillus amyloliquefaciens III 14	control	36.62	35.56	36.76	38.31	36.81 c
	6 h	17.54/52.10	35.65/+0.25	29.16/20.67	27.46/28.32	27.45 b
	12 h	11.55/68.46	17.99/49.41	28.71/21.90	24.58/35.84	20.70 a
	24 h	25.30/30.91	32.13/9.65	29.60/19.48	22.53/41.19	27.39 b
	mean	22.75 a	30.33 c	31.05 c	28.22 b	28.09

Small letters – significant difference at the level $p < 0.05$.

It was found, that the inhibitory activities of *B. amyloliquefaciens* III 14 and *B. subtilis* II 11 was significantly higher than the *B. subtilis* I7. Growth of *Fusarium solani* and *Fusarium sporotrichoides* was strongly inhibited, whereas growth of *Fusarium tricinctum* was poorly inhibited by all *Bacillus* strain. Among the *Fusarium* species, *F. solani*, *F. oxysporum*, *F. sporotrichoides* were the most sensitive to metabolites produced by *B. amyloliquefaciens* III 14 (6 h and 24 h old culture) whereas *F. tricinctum* was a very little inhibited or a very little stimulated. In contrast to PDA medium, 24 h culture of *Bacillus* sp. exerted the higher inhibiting effect on growth all *Fusarium* species on Czapek-Dox medium.

Conclusion

1. The applied of urea phosphate as the disinfectant of farmyard manure would have the beneficial effect on the increase of the population of *Bacillus* sp. in soil.

2. The inhibitory properties of *Bacillus* species depend on the kind of the ones: age of the culture applied and /or strains of *Bacillus.*

3. *Bacillus subtilis* (strains I 7 and II 11) had the highest inhibitory effect for spore germination of *Fusarium tricinctum* whereas *Bacillus amyloliquefaciens* for *Fusarium solani.*

4. Among the *Bacillus* strains*, B. amyloliqefaciens* had the highest inhibitory properties for mycelium growth of *Fusarium* strains.

5. *Fusarium solani* and *Fusarium sporotrichoides* shows higher sensibility of mycelium to all tested *Bacillus* sp., whereas *Fusarium tricinctum* was a very little inhibited or even stimulated.

6. The present investigations indicated, that urea phosphate is indirectly influenced by plant pathogen increase the number of *Bacillus sp.* with antifungal properies.

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WPŁYW FOSFORANU MOCZNIKA NA LICZEBNOŚĆ BAKTERII Z RODZAJU *Bacillus* W GLEBIE I AKTYWNOŚĆ PRZECIWGRZYBOWĄ WYBRANYCH SZCZEPÓW NA *Fusarium* sp.

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Abstrakt: Celem badań była ocena wpływu fosforanu mocznika (FM) na liczebność bakterii z rodzaju *Bacillus* i ocena aktywności fungistatycznej *Bacillus subtilis*, jak również *Bacillus amyloliquefaciens* na *Fusarium* sp. Przeprowadzone badania wykazały korzystne oddziaływanie obiektów z udziałem fosforanu mocznika na liczebność bakterii z rodzaju *Bacillus* sp. W ciągu całego okresu badań liczebność ich była większa niż w glebie nienawożonej i nawożonej obornikiem. Zdolność kiełkowania zarodników grzybów z rodzaju *Fusarium* była hamowana przez *Bacillus* sp., lecz największą wrażliwość na *Bacillus subtilis* I 7 wykazał *Fusarium tricinctum*. Natomiast największą aktywność w stosunku do grzybni wszystkich testowanych grzybów wykazał *Bacillus amyloliquefaciens* III 14.

Słowa kluczowe: fosforan mocznika, *Bacillus* sp., aktywność przeciwgrzybowa, *Fusarium* sp.