New generation of phosphate fertilizer from bones, produced by bacteria

1 Introduction

Utilization of phosphate solubilizing bacteria is a very attractive and promising method for mitigation of the phosphate problem. Two ways of utilization of microbial solubilization are found in literature: 1 – dissolution of mineral phosphorus from freshwater sediment in aquatic ecosystem, 2 – production of phosphate fertilizers in agriculture [1,2]. In the first case, the freshwater sediment contains between 8 and 82% of phosphorus of the total aquatic phosphorus, however only part of it is available compared with in the soil [3]; the content of phosphorus in the soil is 0.02 to 0.5%, but only 1% is available to plants. In both cases phosphorus is bound by cations (Ca, Al, Fe, Mn) and unavailable for plants [1,4].

Each year, large amounts of phosphate fertilizers are applied to the fields to provide the suitable amounts of this macronutrient to ensure the proper development and growth of crop plants [5,6]. The primary method of phosphate fertilizers production is digestion of high-grade phosphate rock by sulfuric acid in a wet process producing phosphoric acid and superphosphates [5]. However, the reserves of high-quality phosphorite could be depleted in the next 3–4 decades and after that only a low-grade deposits, contaminated by cadmium or uranium and costly to extract and purify, will remain [3,7]. Additionally, production of conventional phosphate fertilizers leads to environmental problems related to creation of a huge amount of waste phosphogypsum contaminated by radionuclides (pCi > 25) [6,8]. It is possible to use the waste resources of phosphorous, such as bone (about 19% P$_2$O$_5$), in the production of fertilizers [9]. Digestion of low quality phosphate raw materials can be carried out under conditions which are more environmently friendly, for example by using microbial solubilization [3]. The relationship between soil, microorganisms, and plants is very important for distribution of nutrient to plants. For example, the phosphate solubilizing bacteria, through acidification of the environment via production of low molecular weight organic or mineral acids, release soluble phosphates from otherwise unavailable forms [10].
In this work, utilitarian properties of a biofertilizer obtained from poultry bones via solubilization process performed by *Bacillus megaterium*, are presented.

## 2 Material and methods

### 2.1 Biofertilizer production

The biofertilizer, obtained from poultry bones, was produced in a microbial solubilization process, in sterile reactors of the capacity from 250 ml to 10 liter for 8 days in 34°C. After the solubilization of bones the neutralized culture medium was used in *in vivo* tests. Phosphorus concentration was found to be 5.56 mg L\(^{-1}\) in the final formulation.

### 2.2 Hydroponic tests

Twelve glass tubes were prepared and each tube was filled with 0.3 g of wadding and spring wheat. All tubes were stratificated at 0°C for three days (Fig. 1a).

### 2.3 Germination test

50 cress seeds were planted on each of the 12 dishes containing 15 g of universal garden soil. *Lepidium sativum* was selected as the plant of choice. All dishes were stratificated at 0°C for three days (Fig. 1b). The seed trials...
were prepared according to International Safe Transit Association (ISTA) Standards.

Three groups in four replicates, comparable to the hydroponic tests (Fig. 2), were prepared. The doses of P$_2$O$_5$ in the tests were 16.7 mg/plate.

After 7th day of the field trials, the plants were collected, measured and counted. The green color of leaves was determined. The whole mass of plants was dried to constant weight, mineralized, and underwent multielemental analyses.

### 2.4 Content of elements in the plants

The samples were mineralized with spectrally pure 69% HNO$_3$ (Suprapur, Merck) in a closed system by using microwave oven Milestone Stard D. After mineralization, the solutions were diluted to 25 g.

The concentrations of elements were determined by ICP–OES Varian-Vista MPX (Australia) with ultrasonic nebulizer CETAC U5000AT+ . The results are the arithmetic mean of the three measurements. The analyses were prepared in a laboratory accredited by Polish Centre of Accreditation (PCA) according to PN-EN ISO/IEC 17025:2005.

### 2.5 Statistical methods

The results of the experiments were analyzed statistically using the program Statistica ver. 10. The normal distribution of the variable was tested using the Shapiro-Wilk test. The Brown Forsythe test was used to verify the homogeneity of the variance (for a normal distribution).

For groups with a homogeneous variance, the test F of significance differences was performed. For a distribution other than normal, the Kruskal-Wallis test was used. Statistically significant differences were for p < 0.05.

### 3 Results and discussion

#### 3.1 Hydroponic test

In the first stage of the hydroponic test, a dose of the biofertilizer was chosen. From five different doses of the fertilizer, 0.75 mg P$_2$O$_5$ per tube was found to be the best for wheat (Table 1). In this sample a significant increase in the green parts and roots as well as the greatest mass of the plants was observed compared to the control.

During the main part of the experiment the following parameters of the cress growth were determined: length of the leaf, length of the root, intensity of green color, and the dry mass of plants (Table 2). The increase in the dry mass of plants obtained from the group where the biofertilizer was applied compared with the dry mass of plants obtained for both control groups (control 1 and control 2) was found to be 44% (p < 0.05) and 22.6% respectively (Fig. 2).

Statistically significant differences between the length of the green parts (increase of 53%) and the roots (increase of 66%) were found when the group with the biofertilizer was compared to the control group 1 (with water only). There were no statistically significant differences between the groups were the biofertilizer or the conventional fertilizer were used.

Additionally, statistically significant differences in the intensity of the green color were found between the experimental and the control groups. The highest intensity was observed for the group with the biofertilizer applied — the increase of 33% compared to the control 1 group.

#### 3.2 Germination test

A statistically significant difference was found between the dry mass of plants from the group where the biofertilizer was used as a source of phosphorus compared to the group
Figure 2: The growth parameters of plants in hydroponic test; a – length of the leaf, b – length of the root, c – intensity of green color, d – the dry mass of plants

Table 2: Statistically significant differences between wheat growth parameters in hydroponic tests; LL - length of the leaf, cm, LR - length of the root, cm, G - intensity of green color, SPAD, MP – the dry mass of plants, g

| Growth parameter | Units | Groups                          | Control                  | Experimental |
|------------------|-------|---------------------------------|--------------------------|--------------|
|                  |       | With water only                 | With classical NPK fertilizer |               |
|                  |       | average ±SD                     | average ±SD              | average ±SD  |
| LL               | cm    | 14.0 A,B ± 0.7                  | 20.6 A                   | 21.4 A       |
| LR               | cm    | 3.45 A,A ± 0.55                 | 5.35 A                   | 5.72 A       |
| G                | SPAD  | 20.5 A,B ± 0.7                  | 26.8 A                   | 27.3 A       |
| MP               | g     | 0.0180 A,B ± 0.0008             | 0.0212 A,B,c             | 0.0260 A,B,c |

A-C p<0.050. A – p<0.100

Table 3: Differences between cress growth parameters in germination tests; LP - length of plant, cm, G - intensity of green color, SPAD, MP – the mass of plants, g, NG - the number of germinated seeds

| Growth parameter | Units | Groups                          | Control                  | Experimental |
|------------------|-------|---------------------------------|--------------------------|--------------|
|                  |       | With water only                 | With classical NPK fertilizer |               |
|                  |       | Average/median ±SD              | Average/median ±SD       | Average/median ±SD |
| NG               |       | 43.2 ± 6.0                      | 45.0 ± 2.94              | 47.2 ± 2.36  |
| MP               | g     | 0.0666 A ± 0.0042               | 0.0796 ± 0.0087          | 0.0819 A ± 0.0004 |
| G                | SPAD  | 25.9 A ± 5.1                    | 32.5 ± 4.71              | 35.5 A       |
| LP*              | cm    | 9.47 A ± 2.66                   | 9.14 ± 1.02              | 7.90 A       |

A- p<0.050, *other than normal distribution
with no fertilization (control 1 group). The dry mass of the plants in the group with the biofertilizer was about 23% (p < 0.05) greater than in the control 1 group (Table 3).

Additionally, statistically significant increase in the intensity of the green color of plants from the group where the biofertilizer was used (33%, p < 0.05) compared to the control 1 group was recorded (Fig. 3).

Since the higher values of utilitarian parameters (the mass of plants and intensity of green color) were observed for the groups where the phosphorus fertilizers (both conventional and experimental) were used, the effect of the biofertilizer on the growth and development of plants was confirmed.

### 3.3 Multielemental composition of plants

After the experiment, the collected plants were mineralized and the multielemental composition of the plants was determined (Table 4). The highest content of phosphorus was found for the groups where the phosphate fertilizer was used compared to the control 1 group; 8.3% and 8.4%

| Content of elements, mg/kg | With water only | Control With classical NPK fertilizer | Experimental With biofertilizer |
|---------------------------|-----------------|---------------------------------------|-------------------------------|
|                           | Average/Median  | ±SD                                   | Average/Median  | ±SD                                    | Average/Median  | ±SD                                    |
| Ag*                       | 0.610           | 0.398                                 | 0.100            | 0.430                                   | 1.10            | 0.38                                   |
| Al                        | 273             | 295                                   | 93.6             | 274                                      | 0.00            | 0.00                                   |
| As                        | 24.8            | 18.1                                  | 19.7             | 0.00                                     | 0.00            | 0.00                                   |
| B*                        | 60.6            | 38.5                                  | 5.8              | 39.7                                     | 3.9             |
| Ba                        | 11.6            | 7.28                                  | 1.67             | 9.17                                     | 0.25            |
| Be                        | 0.0100          | 0.0120                                | 0.00             | 0.00                                     | 0.00            | 0.00                                   |
| Ca                        | 15971^A,A^      | 1376                                  | 11802^A          | 1799                                     | 9504^A          | 392                                     |
| Cd                        | 0.930           | 0.650                                 | 0.230            | 1.65                                     | 0.10            |
| Co                        | 3.11            | 1.39                                  | 1.25             | 1.18                                     | 0.04            |
| Cu*                       | 10.0            | 8.10                                  | 6.89             | 7.88                                     | 1.02            |
| Fe                        | 527             | 684^A                                 | 111              | 450^A                                    | 6.05            |
| K                         | 48427           | 53609                                 | 12223            | 55866                                    | 1629            |
| Mg                        | 5771            | 5836                                  | 862              | 6016                                     | 149             |
| Mn                        | 69.8^A          | 135^A,A^                             | 25               | 70.0^A                                   | 3.9             |
| Mo*                       | 1.49            | 7.97                                  | 4.35             | 0.00                                     | 7.60            |
| Na                        | 958^A           | 3320^A,A                             | 213              | 1203^A                                   | 73              |
| Ni                        | 4.05            | 0.00                                  | 0.00             | 5.66                                     | 9.80            |
| P                         | 16542           | 17926                                 | 804              | 17917                                    | 631             |
| Pb                        | 14.6            | 27.0                                  | 17.4             | 19.0                                     | 8.35            |
| S                         | 22006           | 19005^A                               | 1271             | 26028^A                                  | 1298            |
| Sb*                       | 9.75            | 10.5                                  | 9.1              | 0.00                                     | 3.04            |
| Se                        | 29.9            | 27.2                                  | 21.8             | 43.9                                     | 2.00            |
| Si                        | 638             | 465^A                                 | 162              | 721^A                                    | 15              |
| Ti                        | 28.0            | 31.8                                  | 7.0              | 38.5                                     | 2.4             |
| Tl                        | 25.2            | 0.00                                  | 3.19             | 0.00                                     | 3.42            |
| V                         | 5.91            | 5.01                                  | 4.08             | 1.56                                     |
| Zn                        | 145             | 105                                   | 15               | 103                                      | 8               |

A, B- p < 0.050, *other than normal distribution
for the groups where the biofertilizer and the NPK fertilizer were used, respectively (Fig. 4). No statistical significant difference was found between the content of phosphorus in the experimental group and the control 2 group.

Transfer factor of phosphorus is defined as a percent of phosphorus mass in plant biomass to a phosphorus dose from fertilizer [11].

\[ TF_P = \frac{m_{\text{plants}}}{m_{\text{fertilizer}}} \times 100\% \]

The \( TF_P \) for both groups where the phosphate fertilizers were used (conventional fertilizer and biofertilizer) were similar and there were respectively 19.6\% and 20.1\%.

Statistically significant differences were observed in the content of elements such as: Ca, Na, S, Fe and Si. Silicon is an essential element for plants, responsible for protection against various adverse environmental parameters (pathogens) [12]. Silicon has also synergistic relationship with titanium, which has a positive effect on the growth of plants. This was confirmed by the correlation coefficient \( r = 0.56 \) (Table 4).

Sulfur is also very important for proper development of plants. It is a part of some proteins and amino acids [13]. Since bones are a good source of sulfur the highest content of sulfur was observed for the group where the biofertilizer obtained from bones was applied.

The matrix of correlation coefficients of elements was elaborated (Table 5). Some elements may improve the uptake of other elements by plants (synergism) or inhibit their uptake (antagonism). The highest synergistic and statistically significant relationship was found between Na and Fe \( (r = 0.72, p < 0.05) \) and between Mn and Na \( (r = 0.89, p < 0.05) \). Synergism was also shown between P/Mg \( (r = 0.64) \) and P/K \( (r = 0.58) \). The same relationship was also found by Karaköy and co-workers [14]. The positive impact of magnesium, phosphorus, and potassium is essential for the proper development of plants [15].

Statistically significant high antagonism was observed in P/Cu \( (r = -0.84, p < 0.05) \). Application of phosphate fertilizer, which leads to a high content of phosphate in soil, can cause the decrease of copper uptake by plants [15].

Antagonism was also found among S/Mn \( (r = -0.68, p < 0.05) \), Si/Na \( (r = -0.78, p < 0.05) \), and P/Zn \( (r = -0.72, p < 0.05) \). Other negative correlations such as: P/Co \( (r = -0.60) \), S/K \( (r = -0.67) \), S/Na \( (r = -0.65) \), Se/S \( (r = -0.49) \) were also found. The relationship between macro- and micronutrient and toxic elements was shown as: Mg/Al \( (r = 0.78, p < 0.05) \), S/Cd \( (r = 0.86, p < 0.05) \), Fe/Al \( (r = 0.61) \), Si/Cd \( (r = 0.58) \), Se/As \( (r = -0.81, p < 0.05) \), S/As \( (r = -0.58) \), Se/Pb \( (r = -0.49) \).
Table 5: Correlation matrix between elements in plant (N=12). Italic – statistically significant differences

|     | Ag  | Al  | As  | B   | Ba  | Be  | Ca  | Cd  | Co  | Cu  | Fe  | K   | Mg  | Mn  | Mo  | Na  | Ni  | P   | Pb  | S   | Sb  | Se  | Si  | Ti  | Zn  |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Ag  | 1.00|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Al  | -0.22|1.00|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| As  | -0.66|0.30| 1.00|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| B   | 0.22| 0.17| 0.34| 1.00|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Ba  | 0.47| 0.26| -0.03|0.83| 1.00|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Be  | -0.09|0.23| 0.38| 0.26| -0.081| 1.00|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Ca  | -0.14|0.18| 0.64| 0.92| 0.63| 0.24| 1.00|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Cd  | 0.59| 0.01| -0.53|0.12| 0.33| -0.65| -0.281| 1.00|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Co  | -0.22|0.21| 0.73| 0.57| 0.25| 0.82| 0.62| -0.56| 1.00|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Cu  | -0.11|0.25| 0.38| 0.25| -0.081| 0.24| -0.67| 0.81| 1.00|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Fe  | -0.42|0.61| 0.19| 0.10| -0.07|0.29| 0.20| -0.37|0.09| -0.29| 1.00|     |     |     |     |     |     |     |     |     |     |     |     |     |
| K   | -0.13|0.26| -0.21|0.33| -0.04|0.46| -0.26| 0.21| -0.47|0.42| -0.26| 1.00|     |     |     |     |     |     |     |     |     |     |     |     |
| Mg  | -0.15|0.78| 0.36| 0.08| 0.30| -0.33|0.13| 0.24| 0.16| -0.33|0.15| 0.20| 1.00|     |     |     |     |     |     |     |     |     |     |
| Mn  | -0.74|0.48| 0.37| -0.27|0.44| -0.28|0.02| -0.48|0.12| -0.27|0.80| 0.15| 0.30| 1.00|     |     |     |     |     |     |     |     |     |
| Mo  | -0.30|0.26| 0.07| -0.36|0.56| 0.04| -0.24|0.49|0.15| 0.06| 0.17| 0.37| -0.16|0.52| 1.00|     |     |     |     |     |     |     |     |
| Na  | -0.61|0.12| 0.05| -0.46|0.64| -0.27|0.26| -0.48|0.37| -0.26|0.72| 0.17| -0.12|0.89| 0.63| 1.00|     |     |     |     |     |
| Ni  | 0.68| -0.09|0.28| 0.26| 0.42| -0.21| 0.06| 0.41| -0.13|0.25| 0.17| 0.17| -0.34|0.23| -0.39| 1.00|     |     |     |     |     |
| P   | -0.02|0.42| -0.18|0.32| 0.00| -0.85|0.28| 0.44| -0.60|0.84| 0.29| 0.58| 0.64| 0.48| 0.20| 0.34| 0.27| 1.00|     |     |     |
| Pb  | -0.65|0.31| 0.23| -0.47|0.60| 0.45| -0.28|0.61| 0.19| 0.48| 0.16| 0.34| -0.22|0.28| 0.47| 0.37| -0.50|0.27| 1.00|     |     |     |
| S   | 0.74| -0.24|0.58| -0.04|0.42| -0.38|0.28| 0.86| -0.40|0.38| -0.67| 0.39| 0.15| -0.68|0.29| -0.65|0.65| 0.29| -0.41| 1.00|     |     |
| Sb  | -0.75|0.21| 0.71| 0.06| -0.30|0.60| 0.34| -0.76|0.59| 0.62| -0.06|0.10| -0.12|0.27| 0.36| 0.20| -0.44|0.43| 0.79| -0.59| 1.00|     |     |
| Se  | 0.69| 0.16| -0.81|0.08| 0.39| -0.24|0.28| 0.41| -0.37|0.25| 0.11| -0.18|0.14| -0.33|0.34| -0.34|0.28| 0.06| -0.69| 0.40| -0.78| 1.00|     |
| Si  | 0.47| 0.39| -0.03|0.25| 0.60| -0.04|0.06| 0.58| 0.24| -0.06|0.45| 0.00| 0.65| -0.51|0.55| -0.78|0.43| 0.18| -0.41| 0.66| -0.37|0.25| 1.00|
| Ti  | 0.84| 0.46| -0.92|0.71| 0.63| 0.07| -0.22|0.19| -0.14|0.09| 0.63| 0.16| -0.54|0.88|0.11| 0.79| 0.40| -0.11| 0.06|0.18| -0.43|0.95| 0.15| 1.00|
| Zn  | -0.04|0.11| 0.45| 0.56| 0.26| 0.91| 0.52| -0.63|0.89| 0.92| -0.23|0.32| -0.13|0.27| -0.02|0.36| -0.06|0.72| 0.30| -0.30|0.58| -0.20|0.10| 0.31| 1.00|

4 Conclusion

Our studies proved that bones are a good renewable source of phosphates for production of phosphate biofertilizers. Phosphorus delivered to plants in the form of a biofertilizer was characterized by the same bioavailability to plants when compared with the phosphates delivered in the form of a conventional mineral fertilizer. Plants from the group where the biofertilizer was applied were characterized by a higher yield. No phytotoxicity effect was shown in any group. The performed experiments confirmed that the biofertilizer produced by phosphate solubilizing bacteria can be used as an alternative to a conventional phosphate-fertilizer.

Acknowledgements: This project has been financed in the framework of PBS 2/A1/11/2013 grant entitled: „Phosphorus renewable raw materials — a resource base for new generation of fertilizers.” provided by the National Center for Research and Development.

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