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INFLUENCE OF FOUR *BACILLUS STRAINS*, ISOLATED FROM CALCAREOUS SOILS, ON PHOSPHATE SOLUBILIZATION

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Abstract: The phosphate solubilization by four strains belonging to the species *B. subtilis* and *B. amiloliquefaciens* were studied. The strains were isolated from rhizosphere of Cichorium intybus (Common chicory), inhabiting calcareous soils. The phosphate solubilizing activity was determined by using Pikovskaya's medium. The concentration of dissolved phosphate increased by 28 to 56 mg/l for different strains. Also, all four strains produce Indole-3-acetic acid. The effect of strains on growth of *Lepidium sativum* L. (Cress) in vivo was evaluated in pot vegetative experiments. Two type of substrates were used: perlite and perlite mixed with 0,1% $Fe_3(PO_4)_2$. In the first case the plants are fed with a nutrient solution which contains all required nutrients. The nutrient solution for plants, growing in perlite mixed with $Fe_3(PO_4)_2$ didn't contain any source of phosphorous. The fresh and dry shoot weight of treated plants in both experiments were above those of controls by 8 to 50%.

INTRODUCTION

The rhizosphere is a habitat for microorganisms from different taxonomic groups – *Acetobacter, Agrobacterium, Alcaligenes, Azoarcus, Azomonas, Azospirillum, Azotobacter, Bacillus, Burkholderia, Clostridium, Comamonas, Derxia, Herbaspirillum, Enterobacter, Erwinia, Klebsiella, Microbacterium, Micrococcus, Paenibacillus, Pseudomonas, Rhizobium, Variovorax, Xanthobacter* etc. (Joseph et al., 2007, Krishnaveni, 2010, Han et al., 2011). Rhizosphere microorganisms stimulate growth via direct and indirect mechanisms such as nitrogen fixation of atmospheric nitrogen, modification of soil organic carbon, transformation of poorly soluble phosphorous compounds to easy assimilated by

bacterial phosphatases, increase of nitrate assimilation, production of siderophores chelating the iron into plant bioavailable form, synthesis of physiologically active substances, variations in root cell membrane permeability, protection against stressful environmental factors, phytopathogens, etc. (Glick, 2012).

The role of rhizosphere microflora is very important for the mineral nutrition of plants in calcareous soils, characterized with low content of useful biogenic elements. In Bulgaria such soils are found in Thracian Plain (Dimitrov et al, 2009). Calcrete is present in soil mainly as calcite and dolomite, as sands, nodules and layers of soil and it leads to the formation of alkaline conditions. Alkaline values of pH in soil obstruct the assimilation of elements such as – P, N and K, as well as some important microelements - Fe, Cu, Zn and B (Rahma et al., 2011).

The plants take up phosphorus from the soil in the form of soluble orthophosphate ions; $H_2PO_4^{1-}$, HPO_4^{2-} and PO_4^{3-} (Yadavand and Verma, 2012). In arid soils most of these phosphate compounds are presented in inorganic form as calcium, iron and aluminium phosphate. The original natural source of phosphorous is the mineral apatite, a calcium phosphate that is nearly insoluble. Apatite is present in small quantities because this mineral easily transforms to the more insoluble forms. Iron and aluminium phosphate are involved probably as hydroxyl phosphates such as strengite - iron phosphate and variscite - aluminium phosphate. Both minerals are too insoluble to contribute much to plant nutrition.

The organic phosphorus is about 50% of the total P in soils. Most of the organic P compounds are esters of orthophosphoric acid and have been identified primarily as inositol phosphates, phospholipids and nucleic acids.

The principal mechanism for mineral phosphate solubilization is the excretion of organic acids. There are various heterotrophic microorganisms, which produce organic acids (citric acid, lactic acid, gluconic acid, 2-ketogluconic acid, oxalic acid, tartaric acid and acetic acid etc. (Ivanova et al., 2006). These organic acids dissolve phosphoric minerals by lowering the pH of soil, or chelate cationic partners such as Ca, Al and Fe of the phosphate ions and directly release phosphorous into the soil (Awasthi et al., 2011).

Acid and alkaline phosphatases use organic phosphate as a substrate to transform it into an inorganic form. Acid phosphatases play a major role in the mineralization of organic phosphorus in soil. Many P solubilizing bacteria produce these enzymes (Tarafdar et al., 2003; Aseri et al., 2009). Also, some fungi produce phytase, an enzyme which releases soluble inorganic phosphate from organic P compound (inositol hexaphosphate) (Yadav and Tarafdar 2011). Population of phosphate solubilizing bacteria (PSB) in soil depends upon its chemical and physical properties and also on organic matter and phosphorus content of soil.

Various authors established the high potential of representatives of *Pseudomonas, Bacillus* etc. for improvement of plant mineral nutrition in calcareous soils via phytochromes, enzymes and organic acids production.

Oliveira et al., (2009) reported that among the bacterial isolates, *Bacillus* sp. and *Burkholderia* sp. were the most efficient P-solubilizing strains from P-Ca source culture solution.

The role of phosphate solubilizing microorganisms in the native P solubilization and increasing crop yield under some arid ecosystem has been studied (Yadav and Tarafdar, 2010; Yadav and Tarafdar, 2011). It was found that the effectiveness of inoculation with phosphate solubilizing microorganisms vary with the soil physico-chemical properties and the test crop.

Talboys et al. (2014) studied the influence of secretion of IAA on the root P uptake. In their study, seed of *Triticum aestivum*, treated with *B. amyloliquefaciens FZB42*, an auxin-producing rhizobacterium, increased root growth at low environmental P concentrations, but significantly repressed root P uptake.

The objectives of this study were to investigate P solubilizing potential at 30°C of four strains of *Bacillus* genera, isolated from rhizospheres of two plants *Cichorium intybus* (Common chicory), to study their potential for IAA production and observed their impact in vivo of vegetation of *Lepidium sativum* L. (Cress) at temperature 20 – 22°C.

MATERIALS AND METHODS

1. Bacillus strains

The strains R1, R2, R3 and R4 were isolated from rhizospheres of two plants *Cichorium intybus* (Common chicory), inhabited calcareous soils. 16S rDNA gene nucleotide sequences were used for identification of four strains (Bratkova et al., 2012a). The strains R1 and R3 were found to belong to the species *Bacillus subtilis* and the strains R2 and R4 to the species *Bacillus amyloliquefaciens*.

2. Phosphate solubilizing activity

To detect the phosphate solubilizing activity, strains were streaked onto Pikovskaya's agar medium, which contains (per litre): 10g dextrose, 0.5g yeast extract, 5g $Ca_3(PO_4)_2$, 0.5g $(NH_4)_2SO_4$, 0.2g KCl, 0.1g $MgSO_4.7H_2O$, 0.0001g $MnSO_4.H_2O$, 0.0001g $FeSO_4.7H_2O$ and 15g agar.

Determination of phosphate solubilizing activity by the strains was carried out following standard method (Das et al., 2003). Cells were grown in 200 ml liquid Pikovskaya's medium at 30°C in a rotary shaker (200 rpm) up to 12 days and in 1, 2, 5, 7, 9 and 12 days an aliquot of 10 ml was collected and cells were removed by centrifugation at 9,000 rpm for 20 min. The phosphate concentration was determined by the molybdenum-blue ascorbic acid method. pH variation in Pikovskaya's medium during the growth of each strain was observed.

Production of Indole-3-acetic acid (IAA) was also determined at 12 days following the standard method (Sasirekha et al., 2012). One ml of the supernatant was mixed with equal volume of Salkowski reagent (0.5 M $FeCl_3$ in 35 % perchloric acid). The contents were mixed by shaking and allowed to stand at

room temperature for 30 min till the development of pink colour. Quantitative estimation of IAA was made by spectroscopic absorbance measurements at a wavelength of 535 nm. Uninoculated broth served as control.

3. Experiments with *Lepidium sativum* L. (Cress)

The volumes of each pot for different variants were 700 ml each and the depths - 5 cm. Two types of substrates were used in pot vegetative experiments: perlite and perlite mixed with 0,1% $Fe_3(PO_4)_2$. One hundred seeds of *Lepidium sativum L.* (Cress) were planted in each pot. The nutrient solution for plants, growing in perlite has the following content: 0,12g KNO_3, 0,038g KH_2PO_4, 0,010g $NH_4H_2PO_4$, 0.025g $MgSO_4.7H_2O$, 0,275g, $Ca(NO_3)_2$, 0,015 g urea and 2 ml solution of microelements ($FeSO_4.7H_2O$ 500 mg/l, $NaMoO_4.2H_2O$ - 30 mg/l, $MnCl_2.4H_2O$ -50 mg/l, $CuSO_4.5H_2O$ - 5 mg/l, $ZnSO_4.7H_2O$ - 50 mg/l, Na_3BO_3 - 5 mg/l, Na-EDTA - 1.0 g/l). The nutrient solution for plants, growing in perlite mixed with $Fe_3(PO_4)_2$ contains (per litre): 0,14g KNO_3, 0.025g $MgSO_4.7H_2O$, 0,275 g $Ca(NO_3)_2$, 0,025 g urea and 2 ml solution of microelements.

The bacterial cultures were obtained into the medium, which contains (per litre): 25,0g sucrose, 3,0g $(NH_4)_2SO_4$, 1,0g KH_2PO_4, 0,5g $MgSO_4.7H_2O$, 1g yeast extract, 1,0g peptone. For this experiment, pure cultures were grown in nutrient broth at 30°C in a rotary shaker (200 rpm) and diluted to a final concentration of 10^8 cfu/ml in sterile distilled water.

The seeds were treated with nutrient solution after planting with the addition of 2 ml/l bacterial culture at start of the experiment. In the first control no bacteria were added, neither microbial media. In the second control 2 ml of sterile microbial medium was added during the first day of the experiment in order to evaluate the effect of the therein present nutrients.

The fresh weight of shoots was determined at 15 days. The plant materials were kept in a hot air oven at 80°C for 24 hours and then their dry weight was also determined.

RESULTS AND DISCUSSION

The previous data about biochemical characteristics of R1 and R3 strains, belonging to the species *Bacillus subtilis* and the R2 and R4 strains from the species *Bacillus amyloliquefaciens* showed that all four strains are positive for starch hydrolysis and casein decomposition as well as they produce acid from glucose, arabinose, and mannitol. It was established in previous experiments with *Medicago sativa* (alfalfa) on calcareous soil that these isolates improve the mineral nutrition of plants, which leads to an increase in their shoot biomass (Bratkova et al., 2012b). The important effect of applied microorganisms was a decrease of concentration of active $CaCO_3$. The active $CaCO_3$ reduced P availability in alkaline soil by the reaction of P with calcium. These calcium phosphate minerals are specific with their lowest solubilities at about pH 8.

1. Phosphate solubilizing activity

Initially the phosphate solubilizing activity of four *Bacillus* strains was detected by halozone formation after 3 days cultivation at 30°C onto Pikovskaya's agar medium.

The P solubilization activity of four *Bacillus* strains was estimated by use of liquid Pikovskaya's medium at 30°C in a rotary shaker (200 rpm) up to 12 days.

The concentration of dissolved phosphorus was increased over time, as the highest phosphate solubilizing potential has strain R2, belonged to the species *Bacillus amyloliquefaciens*. The concentration of dissolved phosphate was raised to 48 mg/l for 5 days, then retains in the range from 50 to 56 mg/l. For the other tested strains concentration of solubilized phosphate was between 28 to 40 mg/l at the end of the experiment. These data correspond with the changes of pH of the media (Figure 2). Due to the microbial produced organic acids the pH decreased in all variants, as the lowest values were measured at the cultivation of strain R2.

The phosphate solubilization of species *Bacillus cereus* and *Bacillus subtilis* were studied by Maheswar and Sathiyavani (2012). The authors have studied the effect of various parameters such as pH (7-9), temperature (30°C - 45°C) and nutrient supplementation on phosphate solubilizing activity. Studies on different carbon sources like glucose, sucrose, lactose, mannitol and sodium acetate on this process revealed that incorporation of glucose followed by lactose increased solubilization of phosphate and enhanced acid production efficiently. Similar experiments, but with the *Pseudomonas fluorescens* strain GRS1, PRS9 and their cold tolerant mutants were performed by Das et al., (2003). Maximum P solubilization among all tested strains was established on the 4th day, 19.40% at 10°C and 23.99% at 25°C.

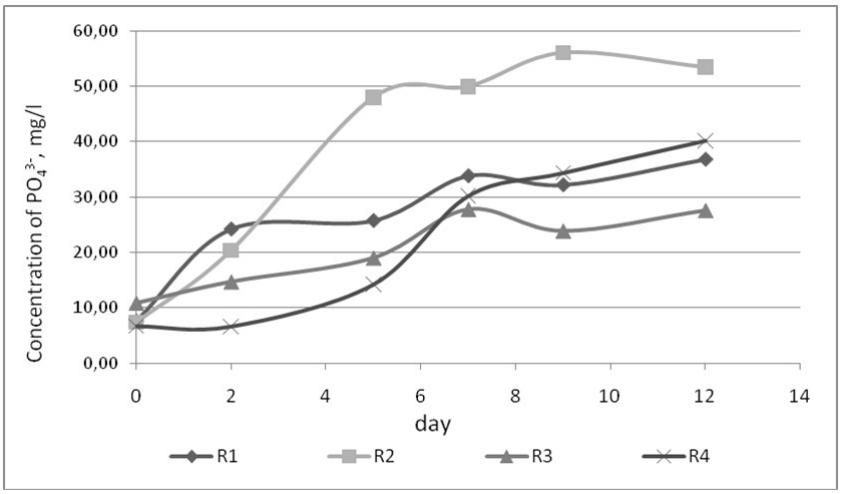

Figure 1. Comparative P solubilization potential of *Bacillus* strains.

Chatli et al. (2008) have studied phosphate solubilizing microorganisms (bacteria and fungi) associated with *Salix alba* Linn. The authors have found that *Bacillus* strains mobilized P in the range from 57 mg/l to 100 mg/l, using Pikovskaya's agar medium. The data from figure 1 showed, that four Bacillus strains, isolated from calcareous soils have lower phosphate solubilizing activity.

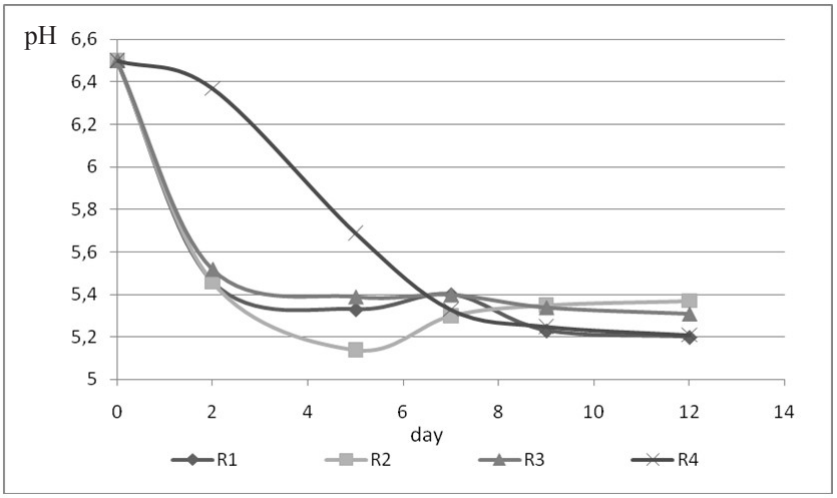

Figure 2. Dynamic of pH of medium during phosphate solubilization.

Joseph et al. (2007) reported that strains related to genera *Bacillus*, isolated from rhizosphere of *Cicer arietinum* L (chickpea) produce indole-acetic acid. Figure 3 shows the values for IAA produced at 12th day onto liquid Pikovskaya's medium at 30°C. The isolates R1 and R3 belonging to the species *Bacillus subtilis* produced higher IAA, respectively 0,039 and 0,044 mg/l, then strains of *Bacillus amyloliquefaciens* R2 and R4, which produced 0,023 and 0,021 mg/l IAA. Further research is needed in increasing the production of IAA from the studied *Bacillus* strains. First, it is optimization of medium components and the presence of the precursor, L-tryptophan for improved (IAA) production. Sasirekha et al., (2012) reported that yeast extract, tryptophan and EDTA were identified as significant components influencing IAA production by *Pseudomonas aeruginosa*, using the Plackett-Burman method.

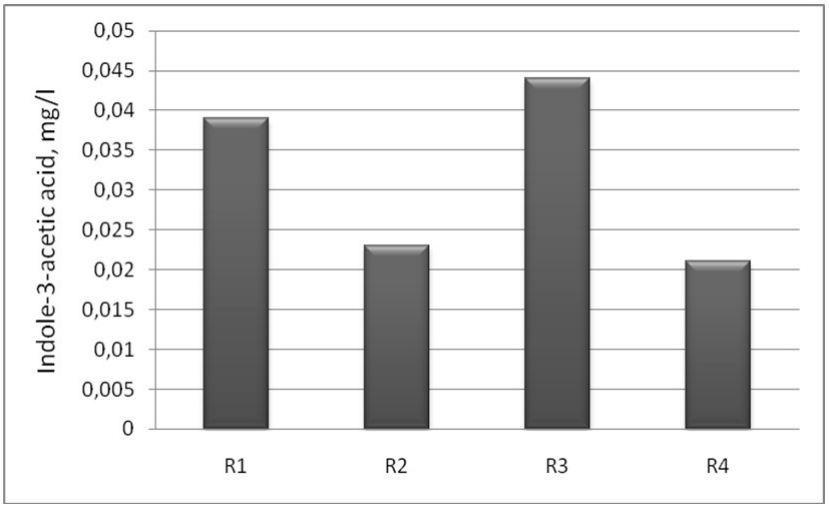

Figure 3. IAA produced at 12th day by *Bacillus* strains.

2. Experiments with *Lepidium sativum* L. (Cress)

The first vegetative pot experiment with *Lepidium sativum* L. (Cress) was conducted using perlite as substrate and nutrient solution containing all the required nutrients for plants.

The data presented in Figure 4 show that the sterile microbial medium added increases the fresh and dry shoot weight of plants respectively by 3,7 and 5,6%. The strains of *Bacillus subtilis* - R1 and R2 proved less effective on plant growth than the strains of *Bacillus amyloliquefaciens* – R2 and R4. The last two strains increase fresh shoot weight by 14.5 and 21.7%, and dry shoot weight by 31 and 50%.

Idris et al., 2004 provide some lines of evidence that *Bacillus* species enhance plant growth via synthesis of plant growth hormones, which are synthesized under specific growth conditions. In another study (Idris et al., 2002) the authors reported that *B. amyloliquefaciens* is able to produce and secrete significant amounts of IAA, whereas production of gibberellin was not detected, as the main route of IAA biosynthesis in this bacterium is dependent on tryptophan.

In its thorough study Chen et al., (2007) demonstrated that *B. amyloliquefaciens* FZB42 genome reveals an unexpected potential to produce secondary metabolites, including the polyketides bacillaene and difficidin. Probably in present study, although the strains R1 and R3 produce higher amounts of IAA than R2 to R4, production of other unexplored microbial metabolites play important role in plant nutrition in studied conditions, which is reason for the higher yield of plants, inoculated with *B. amyloliquefaciens*.

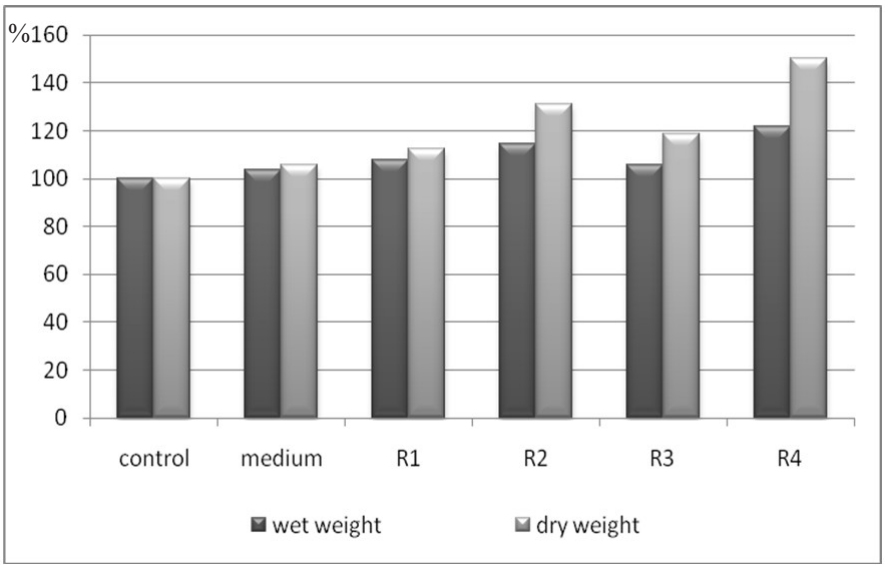

Figure 4. Fresh and dry shoot weight, % of control of *Lepidium sativum L.* (Cress), planted on perlite.

Data about shoot biomass of cress, growth on perlite mixed with $Fe_3(PO_4)_2$ are presented in figure 5. An effect on plant nutrition by adding of 2 ml sterile microbial medium at the start of the experiment was not found. Inoculation of cress with Bacillus strains however has increase of fresh shoot weight by 13,5 to 21,6% as result. Also, in conditions of unavailable source of phosphorus to plants is established increase of their dry shoot biomass by 8 - 19,3%. There were no significant differences in the effects of the four tested strains. Although the data in the studies on phosphate solubilizing activity (Figure 1) showed that strain R2 have the highest potential, the similar results received about the influences of all Bacillus strains are related to complex factors as dynamic of the number of bacteria in time, their production of different organic acids and as well as the specific environmental conditions, which were not studied. The results, however, clearly show the high potential of the strains isolated from rhizosphere of plants, inhabiting calcareous soils to be involved in phosphate solubilization and improvement of plant mineral nutrition.

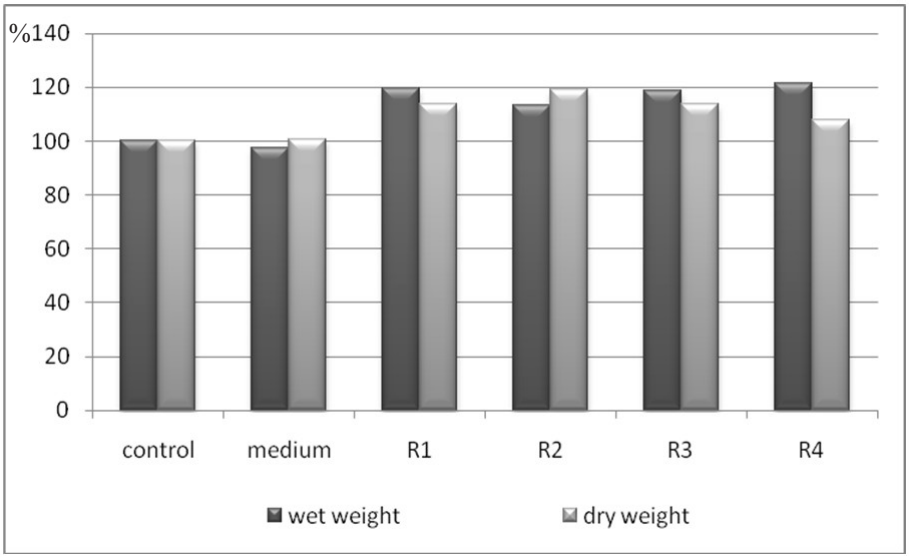

Figure 5. Fresh and dry shoot weight, % of control of *Lepidium sativum L.* (Cress), planted on perlite mixed with 0,1% $Fe_3(PO_4)_2$.

CONCLUSIONS

Phosphate solubilizing *Bacillus* strains, isolated from rhizosphere of plants, inhabiting calcareous soils will attract more attention in the field of bio-fertilization with their beneficial properties. They are very effective for increasing plant available P in substrate containing insoluble forms of this biogenic element, as well as for improving the growth and yield of crop plants. Also, these bacteria synthesize and release greater amounts of IAA as a secondary metabolite because of the substrates exuded from the roots. The isolates have a potential to be used as plant bio-fertilizer as well as the opportunities for their usage for the improvement of soil fertility.

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