EFFECT OF BIOEFFECTORS ON TOMATO AND PHOSPHORUS UTILIZATION OF SOIL

PH.D. THESIS

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1. BACKGROUND AND OBJECTIVES

One of the biggest challenges of the 21st century is to develop sustainable and environmentally friendly cultivation. A common feature of current agricultural technologies is of using large quantities of fertilizers, which more particularly on a long-term level might result high environmental risk. The use of microbial soil inoculum can be an alternative method of sustainable agricultural cultivation. Several microbial biofertilizers are commercially also available as PSP, “plant-strengthening-product”. Those biofertilizers are containing biologically effective (bioeffector) microorganisms that might improve the soil fertility and also can enhance plant growth and development. The effectiveness of bioeffectors is being in less direct relation with the inorganic or organic nutrient-addition, but they dissolve nutrients originally present in the soil and they establish a beneficial plant-microbe relationship.

In Hungary, there is a wide range of PSP biofertilizer products containing bioeffectors, and this large supply makes it difficult to select the most suitable inoculums for the cultivated plant. External environmental factors can significantly affect the number and composition of these soil-borne organisms. The effectiveness of the microorganism-plant interactions highly depends on the genotype of the plants, the composition of the soil microbial community, and also many abiotic environmental factors, such as temperature, light intensity, physical, chemical soil properties. In this research we would like to investigate, how those biotic and abiotic environmental factors are affecting on the PSP applications.

Our experiments are focusing mainly for the soil fertility and understanding of biological processes in healthy plant production. Experiments are designed from pot to the field conditions, in an “upscaling” way. Our research was carried out within the framework of the BIOFECTOR project (The Use of Bioeffectors in European Crop Production) supported by the European Union's 7th Framework Program (FP7/2007-2013).

The effects of PSP biofertilizer commercial inoculants were studied with a tomato test plant under laboratory and field conditions. Using a slightly humus-rich sandy soil of Soroksár, we were investigating the correlations between the different bioeffector products and the phosphorus (P) uptake, as well as the quantity and quality of tomatoes.
The following objectives are assigned to the PhD study:

1) To investigate how the phosphorus (P) content and availability in the soil might be improved by using of the different inoculums.
2) To investigate how the P-uptake of the plant and the yield of tomatoes might be modified.
3) To study on the positive interactions among the applied bioeffectors and the yield of tomato, furthermore its fruit-quality, i.e. the tasty, measured by instrumentally.
4) We assume, that biofertilizer inoculations increase the number and/or activity of the soil microorganisms, and this can have a direct or indirect beneficial effect on plant-growth and development.
5) We hypothesized that the results of the pot experiments could be confirmed by the field experiments, despite of the various biotic and abiotic environmental (stress) factors.
6) Our aim is also to develop applicable advisory recommendations about the potential use of bioeffector products, containing P-mobilizing microorganisms in the practice.
2. MATERIALS AND METHODS

2.1. Experimental details

Our experiments with tomato (*Solanum lycopersicum* L. var. ‘Mobil’) were carried out between 2014–2016. In the experiments we were using biologically effective (bioeffector) bacteria for soil inoculation: **RV** (RhizoVital 42 Fl; ABiTEP GmbH; *Bacillus amyloliquefaciens*) is a one-component inoculum from Germany, and **BR** (Biorex, Chem-Trade Kft.; **BR-1**: *Bacillus subtilis, B. thuringiensis, B. megaterium*; **BR-2**: *Azotobacter chroococcum; Azospirillum lipoferum; Pseudomonas putida*) is a two-component inoculum from Hungary. We investigated the single and combined use of those inoculums between *in vitro* and *in situ* circumstances.

The pot experiments were carried out in the light room of Department of Soil Science and Water Management, University of SZIE (Szent Istvan, today it is MATE) under controlled conditions. It was performed with 24 °C day (14 hours, 14000 LUX) and 18 °C temperature night (10 hours). The water content of soil was 40% of total water-holding capacity (WHC). Tomato growth was 2 weeks of the seedling and 16 weeks of the full-plant vegetation time. Two types of **phosphorus (P) fertilizers** were added to the soils: 1) water-soluble, easily absorbed triple superphosphate (TSP), and 2) hardly available natural rock phosphate (RP). Inoculums (RV and BR) were given to the plants two times in concentrations recommended to the manufacturers' instructions: first time at sowing and second time when seedlings were at four weeks of age. Treatments were set up in quadruplicate (n=4).

The field experiments were carried out at Experimental Farm of SZIE University (Soroksár, Hungary), which area has been a certified eco-area for more than 20 years. The seedlings were grown in an unheated greenhouse for 9 weeks and then planted into field in the 10th week. The vegetation period was 17 weeks. Based on the starting soil tests, the phosphorus content at the experimental site was relatively high, so only 29 g/plant **Viano** (15.5% N) and 53 g/plant **Patentkali** (30% K₂O+18% S+10% MgO) were added to the plants. We combinations of the inoculums were also tested. The RV and Biorex-2 were mixed in the suggested 1:2 ratio. The first inoculation was performed when seedlings were four-week-ago and the second inoculation was carried out when the seedlings were planted into the field.
2.2. Methods of investigation

Soil samples were collected for nutrient- and microbiological investigations and also at the end of the experiment, plant samples were taken, as well. The assessment of phosphorus (P) content of soils was performed by the standard method (MSZ 20135: 1999), of using ammonium lactate (AL) extractant. During the experiments, phenological studies were performed at the 10th, 14th and 18th weeks. The following parameters were measured: shoots length, number of flowers, number of fruits, the ratio of diseased and healthy tomato; at the end of the experiments, we collected plant samples and measured their dry and wet weights. Leaf and shoot samples were collected to determine their phosphorus (P) content (MSZ-08-1783-28: 1985). Healthy crop samples were prepared and their sugar content (Brix), pH, sugar (glucose, fructose) and acid content (citric acid, malic acid) of the fruits were determined instrumentally (MSZ EN 12143: 1998). The most probable number (MPN) method (MSZ 21470-77: 1988) was used to estimate the abundance of different microorganisms (countable number of germs) in soil, while FDA (fluorescein diacetate) and phosphatase enzyme assays were performed to investigate the enzymes produced by the microorganisms.

2.3. Statistical analysis

For the evaluation of the results, one-way ANOVA test was used with IBM SPSS Statistics 22 and Ms Office Excel program. Normality assumption was proven by Kolmogorov-Smirnov or Shapiro-Wilk test (p>0.05), and the homogeneity of variances was checked by Levene’s test. Estimation was investigated by Tukey HSD post hoc test if the scattering homogeneity was satisfied or Games-Howell post hoc tests if the homogeneity was not accepted. Significant differences were set at a 95% confidence level (p<0.05). Possible correlations between the variables were examined by Pearson’s correlation analysis.
3. RESULTS AND DISCUSSION

3.1. Effect of microbial inoculation on soil properties

3.1.1. Inoculation and the uptake of phosphorus

In the experiments, we found that the phosphorus (P) content was significantly higher in the soils which contain inoculums compared to the control soils (p <0.01). We also detected more phosphorus in the soil samples that contained only inoculant and no added phosphorus fertilizer. The German biofertilizer product (RV) proved to be more effective than the Hungarian one (BR) (p <0.05), but we measured higher P-content with both inoculations compared to the control soil. Among the field experiments, however, we did not find any correlations between the different inoculations and the P-content of the tomato plants in neither years.

3.1.2. Soil biological properties

The microbiological activity of the soils was examined by several methods. Measuring fluorescein diacetate (FDA) activity in the soil, the enzyme activity was higher in the inoculated soils compared to the control and soils which contain only P fertilizer (RP, TSP), but the difference was statistically significant only in the soil with German bioeffector (RV) (p = 0.012) in the pot experiment. During the measurement of phosphatase activity for detection of phosphate solubilizing microorganisms, control soils containing phosphorus but no inoculum (TSP, RP) showed no difference compared to the control, while increased enzyme activity was measured in soils containing added biofertilizer inoculums. Based on the results, the microorganisms were not active without added phosphorus source, but they increased their activities with the addition of fertilizers. This was especially true in soils added rock phosphate (RP). The effect of RV inoculation with the addition of RP was outstanding (p = 0.052), while the combination of RV+BR2 also showed increased phosphatase activity with RP (p = 0.076). Due to the large variances, however, these values were not significantly improved (p <0.05). The results are also consistent with the literature, if available phosphorus is present in the soil, P-mobilizing bacteria are less active (Allison et al., 2007b). Among the field conditions, the soil enzyme tests, were not performing on the same results with the pot experiment. Neither FDA activity, nor phosphatase activity could be resulted any significant effect between the treatments (p> 0.05). At FDA enzyme activity, values of the control soil were almost the same with the German (RV) and Hungarian (BR) bioeffectors, as well as with combination
(RV+BR2). The results of phosphatase activity were not showing differences from the control, in any of the years. Soil was well supplied with the phosphorus, so P-mobilizing bacteria were not activated in large amounts. It was also potentially possible, that the native microorganisms in the soils could replace the introduced biofertilizer microbes, so that the inoculums were not able to exert the same beneficial effect, then it was found at the controlled conditions. This is especially true in the field, which has been a certified organic area for more than 20 years.

3.2. Effect of microbial inoculation on the tomato plant

3.2.1. Effect of single inoculation

Although there was no difference in the morphology of the plants in the pot experiments, the examination of the plant element content showed that under the controlled conditions the plants could absorb more phosphorus with the “help” of used bioeffectors. The shoots of plants treated with RV bioeffector contain more phosphorus (P) than control ones. Compared to all controls (untreated control, rock phosphate control, and triple-superphosphate control), higher amounts of P were measured in shoots added RV, although this was not significant (p_{K_{RV}}=0,058; p_{RP_{RP+RV}}=0,052; p_{TSP_{TSP+RV}}=0,063). In case if P fertilizers were added to the soils, still no any change in the P content of the plants was found. The possible reason was that the amount of available P was enough for the plant-growth in the soil, so no additional fertilizers was needed. Although statistically there were no significant differences, the inoculated plants could contain higher P, than the control plants. There were no visible morphological differences between the treatments regarding the shape of tomato plants. It was especially found under the field conditions (p> 0.05). P-content was found in almost of the same quantities in plants (roots and shoots), and even less P at the combination of German and Hungarian soil-inoculums (RV+BR2).

3.2.2. Effect of combined inoculation

In pot experiments, the inoculum combination showed significantly higher phosphatase activity with the presence of rock phosphate (RP+RV+BR2) than single inoculum (RV; p = 0.035) and than control soils containing P fertilizer (TSP; p= 0.012) (RP; p=0.021). In contrast, under field conditions, this positive effect was no longer detected: the FDA and phosphatase activity of the combination was not outstanding compared to the single inoculum. Although the soil samples treated with RV+BR2 had the highest phosphorus content in the field, no significant difference was found compared to the other treatments (p> 0.05). In morphological investigation of tomatoes, we also found that plants added combined inoculum showed no significant differences compared to single
bioeffectors. Although there was a minimal difference in the dry root and shoot weight with combinated bioeffectors, but the difference was not statistically detectable due to the large variance (p> 0.05).

3.3. Effect of microbial inoculation on tomato quality and taste

3.3.1. The size and tasting-value of tomatoes

In addition to the vegetative parts of the tomato, we also examined the quantitative and qualitative parameters of the fruits. In the pot experiments, the content properties of the fruits were outstanding. The average number of fruits per plant was higher in all treatments compared to the control, except Biorex (BR) treatment. Due to the large variance, however, statistically only the effect of German bioeffector added superphosphate (TSP+RV) treatment has significant difference (p=0.44). Results of the average fruit weight (due to the large variance) and the difference between the treatments could not be statistically justified. All added P fertilizers and/or inoculants, however, increased the average fruit weight compared to the control. Among them, both superphosphate (TSP) and RhizoVital (RV) treated plants and their combination (TSP + RV) were also outstanding. At the end of the field experiment, average total berry numbers and total berry weights were measured. As in the other field treatments, there was no significant difference between the treatments, and neither bioeffector increased either the number of berries (p = 0.75) or their weight (p = 0.119) compared to the control. Plants treated with the Hungarian bioeffector (BR), have better yields minimally than plants treated with the German bioeffector (RV). However, the combination of inoculums (RV+BR2) also did not produce the expected synergistic results.

Phosphorus not only can affect the morphological properties of the plants but also, the effect can be realized in tomato fruit taste values (Di Cesare et al, 2010; Wass-Matics, 2018). Many components contribute to the pleasant taste of tomatoes, such as sugars (fructose and glucose) and acids (malic acid and citric acid). These parameters can be determined to measuring the pH value and Brix of the fruits, and examining the concentrations and ratios of sugars and acids with HPLC. In the pot experiments, the fruits treated with RhizoVital (RV) bioeffector were the least pH of the fruits. Compared to the control, the fruits with added P-forms proved to be more acidic, but their pairs treated with bioeffector already increased the pH of the berries (pH> 5). For the manufacturing industry it is appropriate that pH has to be below 4.35, so from this point of view, inoculums did not improve the pH-value of tomato. Water-soluble dry matter content of tomatoes has to be between 4-7 ° Bx (Brandt, 2007). In the homogenized fruits which are collected at the end of the experiment, compared to the control, less sucrose was measured in the fruit-pulps treated with Biorex (BR) and Biorex with rock
phosphate (RP+BR). For all other treatments, the fruits contained more sugar than the control. Among these, the yield with RhizoVital (RV) had an outstanding sugar content (p = 0.014). High performance liquid chromatography (HPLC) was used to measure glucose and fructose levels. Based on the results, although RV showed no significant difference in glucose content compared to the control, it was outstanding compared to the Biorex treatments (p = 0.046). As a result of BR and RP+BR treatments, the sugar content was lower in the fruits, as in the Brix measurement. The same result was observed for the citric acid content as for the sugar content: BR and RP+BR treatments had less citric acid in the fruits, while we measured the highest malic acid level in RP+BR. Less citric acid was measured in TSP+BR treatment also. The acid content of the other treatments was almost the same as in the control plants, although less citric acid and malic acid were measured in the RhizoVital (RV) treatments.

The pH, Brix, and HPLC were also used for the assessments at the end of field experiments. The pH of the RV inoculum-treated fruits were almost identical to control plants, while the pH of tomato fruits, treated the single (BR) and combined (RV+BR2) Hungarian bioeffector proved to be more acidic (pH 4.5-4.52).

Regardless, the pH of each treatment is between the optimal 4-5, and the optimal pH of 4.35 for the manufacturing industry was best, resulted with the treatments containing Biorex (BR; RV+BR2). The water-soluble dry matter content of tomato fruits was significantly lower than in the pot experiment plants. This weak Brix value is perhaps due to the inadequate precipitation distribution, because in the vegetation period it was bad in 2014. Based on the HPLC results, higher fructose and glucose contents were measured in the RV and BR treatments, but because of to the large standard deviation, there was no significant differences in the fruits between the treatments on field. The results of the RV+BR2 combination were almost identical to the values of the control plants. When measuring the acid content, we did not find a significant difference between the treatments in the case of malic acid either, but citric acid, which strongly correlated with the sugar content, was higher in the fruits of the treated plants. Plants inoculated with the German bioeffector also had outstanding values when examining of the fruit quality. There were statistically significant differences between treated and untreated plants not only in the pot experiments but also in field-grown tomatoes.

3.3.2. Market characteristics of tomatoes

During the phenological phase of ripening, large and sudden amounts of precipitation fell in July and August, 2014. The average temperature was also lower then. The effect of these two factors can cause phytophthora infection (Phytophthora infestans). The area treated with Biorex (BR) had the
highest average yield, but RhizoVital (RV) treated crops had the best healthy:sick fruits ratio. This ratio is probably due to the biopesticide effect of *Bacillus amyloliquefaciens*, but due to the unfavorable environmental conditions it could not be explained very well. However, statistically none of the treatments showed significant differences from the control. The year 2015 was very dry, but the precipitation distribution was better. The phytophthora disease could not multiply as much as in the previous year, and the tomato fruits did not crack either. The average yield was lower in 2015, both in number and weight, but the proportion of healthy berries was higher. As a result of water-scarce, non-ideal conditions, the bioeffector was also able to prevail more effectively. Although it was almost the same amount of total fruit counts compared to the control, the RV-treated fruits had larger size and more characteristic taste. Statistically, when comparing the two treatments (Control and RV), the fruits weight was significantly higher in both healthy and diseased production treated with the bioeffector than the control.

### 3.4. Comparison of results in pot and field experiments

The effectiveness of inoculation is confirmed with the fact that after controlled greenhouse experiments, similarly favorable results are obtained under field conditions. This process is called “upscaling”. Our results did not support the potential positive effects among the field conditions. The treatments showing a significant difference in the pot experiments, in the field on the other hand they were identical to the results of the control treatments. In the soil treated with the German bioeffector (RV) under controlled conditions contained significantly more phosphorus than the control soils (p=0.034). The P-mobilizing bacterium *Bacillus amyloliquefaciens* was able to dissolve more phosphorus (P) from the soil in optimal conditions. On the other hand, under field conditions, we measured nearly identical results in the control and in the inoculated soils. We got the same results in FDA activity, as well. While in the pot experiments RV-treated soil had higher enzyme activity compared to the untreated soils (p=0.012), there was no detectable difference between them in field (p_{RV}=0.708; p> 0.05). As in the P-content in plant shoots and roots shows that under controlled conditions, where the amount of soluble phosphorus in the soil is higher and/or the activity of P-mobilizing microorganisms is higher, the plant will be able to take more of the phosphorus (p_{shoot}=0.026). In contrast under field conditions, as in the soil, there is no detectable difference in P-content in the plants, neither in the shoots nor in the roots (p_{shoot}> 0.05).

As a main result of the experiments, however, there were significant differences in the quality of the fruits. Soil inoculation, also improved the total water-soluble dry matter content (p_{pot}=0.014; p_{field}=0.019) and citric acid content of the fruits.
The abiotic and biotic stressors of bioeffectors can be significant reasons for the ineffectiveness of field inoculation. Perhaps the most important of these is that the microorganisms took in with the inoculum are unable to multiply and colonize the roots of the plants on field because the native, indigenous organisms might displace them. Native (abundant) microorganisms, which have already adapted to the certain environmental conditions of the area, are better able to perform their activity than the introduced, inoculated bacteria. This is especially true on the area, that we studied, which has been a certified eco-area for more than 20 years. The native, indigenous organisms can occur in higher numbers than in the traditional cultivated soils. The conclusion, that the native microorganisms in the field-soils might be displaced by the introduced bioeffectors is consistent with the international results of the project partners. It has been found that the efficiency of bioeffectors among the field conditions generally is lower and less reproducible. Various stressors (drought, extreme temperature conditions, nutrient supply constraints) strongly influence the expression of bioeffectors in the soil-plant systems.
4. CONCLUSIONS AND SUGGESTIONS

Based on the results in pot experiments under controlled conditions, the bioeffectors used proved to be potential phosphor solubilizing and plant growth promoting microorganisms. In contrast, field experiments showed that inoculums did not improve the growth of tomatoes in the organic farming system and did not increase the phosphorus content of either the soil or the plant.

Soil type is important for the success of effective inoculation. The soil of Soroksár we studied has poor in humus, and it has a sandy texture. Low number and low quality of aggregates can greatly reduce the colonization and survival ability of microorganisms used inoculums. The nutrient content of soils is also one of the critical issues in soil fertilization. In nutrient-rich, well-supplied soils, the growth-promoting effect of microorganisms may be lower than in soils which are poor in nutrients. In soils which are rich in available phosphorus (P) forms that can be taken up by plants, the activity of bacteria is negligible, their effect is not perceptible at all in the growth of plants (Deubel et al., 2007). Based on our soil test results, the examined Soroksár soil proved to be well supplied with phosphorus, so it is presumably also influenced the relative ineffectiveness of phosphorus solubilizing bacterial treatments on field. We also proved with the phosphatase enzyme-activity measuring that the activity of microorganisms were weaker due to the presence of available P under field conditions.

According to several reports, soil inoculation also fails because native microorganisms displace bioeffectors from their habitat, and they are unable to compete with native organisms. Indigenous, local organisms were better adapted to the certain local conditions (van Veen et al, 1997; Yarzábal, 2010). We have to pay attention to the richer soil life of organic soils when apply inoculations. The study area can show a certified organic farming for 20 years, so the number and competitiveness of the microorganisms in the soil is also better among those soil-conditions.

On the other hand, the inside quality content of the tomato fruits showed a positive effect of the used bioeffectors under the field conditions. Both the sugar content measured in Brix and the citric acid content showed significantly higher values as a result of the bioeffector treatments compared to the control plants in both the pot and field experiments. In this way we can achieve a better and more harmonious fruit, which is a very important aspect in vegetable and of organic plants.

Our results demonstrated the need to examine bieffectors efficacy in the overall system. Despite the uncertainties, the number of commercially available microbial inoculums has increased. One reason for that it is not necessary demonstrate sufficient efficiency in different soils, under different environmental conditions for legal approval. Distributors want to produce “universal” inoculums that
primarily consider ideal plant-microbial relationships, but pay little attention to the influencing effects of the biotic and abiotic environmental (stress) factors.

Based on our results, I have the following suggestions:

When examining soil inoculums, we have to monitor not only the quantity of the crops, but also their quality changes, as they can also be affected by the applied treatments. Inoculants can also affect soil-plant biomass properties, which can indirectly exert their beneficial properties.

Field experiments are essential for more accurate elaboration of the inoculation practical application. According to the bioeffective approach, not only the potential properties of the microorganisms can be considered, but we have to ensure also the conditions that allow the possible manifestation of the activity and functionality of inoculated strains. The present dissertation highlighted the attention to some of the influencing factors.

Field experiments should be performed for several consecutive years; learning about the annual effects, which is an important step to understanding the properties of bioeffectors and learn the effects as much as it is possible.
5. NEW SCIENTIFIC RESULTS

1) When evaluating the effect of bioeffectors, it is not enough to evaluate the yield-effectiveness only. Our studies confirmed that the quality characteristics, the market values or the quality value and taste (acid-sugar ratio) of the tomato fruits could be also changing favorably depending on the type of inoculum strains. In case of soil inoculations the possibility of improving the effects on crop protection and quality must be considered, as well.

2) Based on our results of Rhizovital and Biorex bioeffectors, the complex effects of the combined formulations were not clearly confirmed compare with the single-strain inoculations. This indicates that during the selection of inoculated microorganisms the consideration of their potentially synergistic, mutually reinforcing properties could be suggested.

3) The effect of the pot experiments was not proven in the field experiments in sandy soil, at the ecologically certified area (Soroksár) of using the commercial products with *Bacillus* strains. The effect of inoculums and the result of the “upscaling” are also strongly influenced by the biotic and abiotic environmental conditions and the native, indigenous microorganisms in the soil. Those parameters have to be taken more carefully.

4) The beneficial effect of the Hungarian inoculum (Biorex) could result the same potential effect with the German product (RhizoVital), which draws attention to the possibilities of inoculum development in our country, as well. Possibility of the locally adapted strains and industrial products can be highlighted from the study.

5) When planning the application of phosphorus-containing nutrients, it is suggested to consider the the phosphorus supply of the soil-plant systems and the presence, the amount and competitiveness of soil microorganisms involved in P-mobilization.
6. PUBLICATIONS RELATED TO THE DISSERTATION

Lectured and referred scientific full-length papers in relation with the dissertation:

   IF: 0,61

   IF: 0,68


Other:

   (Egyéb konferenciakötet)

Publications not in main relation with the dissertation: