UNIVERSITY OF SILESIA
IN KATOWICE

Katowice, 06.07.2023

dr hab. Tomasz Płociniczak
Associate Professor
Institute of Biology, Biotechnology and Environmental Protection
Faculty of Natural Sciences
University of Silesia in Katowice
Jagiellońska 26/28 Street
40-032 Katowice, Poland
Phone +48667125783
E-mail tomasz.plociniczak@us.edu.pl

Review of the Ph.D. dissertation written by M.Sc. Piotr Koczorski entitled Root microbiomes as controls of P use efficiency in woody crops

According to European Union Directive 2009/28/E.C., 20% of all energy in Europe should be produced from renewable sources. The subsequent E.U. Directive 2018/2001 states that the share of energy from renewable sources in the Union's gross final energy consumption in 2030 is at least 32 %. These sources include short rotation coppices (SRC), the subject of research performed as part of this doctoral dissertation. *Salix* fields were located in two different geographical regions in Europe: Uppsala (Sweden) and Rostock (Germany). In general, *Sailix* species are efficient in P mobilization, suggesting that microorganisms associated with willows promote P availability in soil and maintain it at a stable level.

The first study evaluated the nutrient availability and P mobilization under two willow species, *Salix dasyclados* var. 'Loden' (L) and *S. schwerinii* × *viminalis* cross var. 'Tora' (T) grown as pure and mixed cultures (L.T.). These plants were cultivated at non-fertilized former arable sites in Germany and Sweden, and these two field sites differed in climatic and soil conditions. The hypothesis that mixtures of *Salix* species can induce a higher nutrient mobilization and soil nutrient availability than pure stands **by their higher microbial diversity** and activity in the rhizosphere was verified. For this purpose, the impact of the growth of *Salix* species/varieties in pure and mixed cultures on the nutrient availability in the soil at two test sites with different soil and climate conditions and the effects of mixed *vs.* monocultures on soil enzymatic P mobilization by phosphatases were evaluated.

str. 1

The laboratory assays made as part of publication no. 1 (**P1**) included biochemical characteristics of soils such as:

- the activity of acid and alkaline phosphatases;
- the total carbon (T.C.) and total nitrogen (T.N.) concentrations;
- pH and soil organic carbon (SOC);
- and plant available fractions of P, Mg, and K.

The methodological description, however, shows that the diversity of soil microbial communities was not determined ***at this stage of the research,*** *so the hypothesis could not be fully verified in the present form.*

The average parameters of precipitation and temperature in both places of willow cultivation in 2014-2016 were comparable, but no information was provided on the air temperature and soil moisture on the days of soil sampling. Similarly, information about such parameters in the years 2017-2018 was not provided, and these are essential data because samples were also taken in 2018 (after the first cutting cycle). Therefore, I am asking whether the doctoral student recorded such data, and I request to provide it during the doctoral thesis defense if available.

The results are presented transparently, facilitating their analysis; however, there are some inaccuracies, e.g., *The activity of acid phosphatases significantly increased in the early plantation stages of willow species than in the late plantation stages in Rostock.* It suggests the existence of an even earlier measuring point, but the methodological description clearly states that there were two measuring points.

All the obtained results were statistically analyzed and presented in a descriptive and graphical form. The main finding was that the biomass production at the test site Rostock was higher than at the test site Uppsala; however, no significant differences were observed between species and pure *vs.* mixed culture plots. Additionally, biomass production was not correlated with the plant availability of one of the tested nutrients (Mg, K, P); however, a correlation between the phosphatase activities and biomass production was indicated. Moreover, willows planted as monocultures caused higher soil nutrient reduction than mixed cultures.

Please specify the exact participation of the Ph.D. student in publication no. 1 because only the writing of the original draft was listed for P.K. in the Author contributions part.

In the second study (**P2**), the impact of environmental conditions, plant genotype, and type of fungal association with plants on the abundance and diversity of phosphate solubilizing fungi (PSF) was tested. In general, authors hypothesized that in the rhizosphere soil, the number of PSF will be higher than in the endosphere of *Salix* plants, and plant genotype and the experimental site will influence the fungal diversity. To verify such hypotheses, the following methods were applied:

- soil physicochemical analysis (the total organic carbon, TOC, total nitrogen, T.N., pH, and available phosphorus, P_{av});
- isolation of cultivable fraction of fungi (PDA medium) and PSF (NBRIP, PVK and DCP media) from rhizosphere soils and roots of different cultivars of willow;
- molecular identification of 22 strains of PSF;
- and metagenomic analysis of fungi from rhizospheric soil and endosphere of roots of willows.

All the assays were conducted correctly, and the necessary controls were applied. All the obtained results were subjected to statistical analysis. Importantly, the isolation of phosphate-dissolving fungi was carried out using three different media: NBRIP and PVK (containing tricalcium phosphate) and DCP containing dicalcium phosphate. This approach increases the chances of more efficient isolation of fungal strains of a cultivable fraction.

Soils taken from tested experimental sites differed significantly. The abundance of fungi was higher in the rhizosphere soil than in the endosphere and higher in roots at mixed sites (L.T.) than under monocultures (L or T). Generally, the highest fungal density was recorded in L.T. samples from Sweden and T samples from Germany. Among the most frequently isolated fungal strains at both test sites was the *Penicillium* (Germany 30%, Sweden 70%), the only fungal genus occurring at both locations. Additionally, at the German site, similarly to *Penicillium*, *Clonostachys* also reached a 30% share of the cultivable fraction of fungi. The site-specific genera were also indicated; *Clonostachys* (isolated from the rhizosphere and endosphere) was specific to the German site, whereas *Juxtiphoma*, *Talaromyces*, and *Beauveria* were specific to the Swedish site.

Molecular analysis revealed that the alpha diversity of the fungal community was not influenced by genotype but by site or the level of fungi association with the plant (rhizosphere *vs.* endosphere). Diversity (H'), species richness (observed OTU number), and evenness (E) were higher

Str. 3

in the rhizosphere than in the endosphere. The beta diversity revealed that the experimental site drives the fungal community structure. A surprising result is that the *Penicillium* sequences were not detected in the metagenomic analysis, even though this genus dominated the cultivable fraction of PSF. The authors stated that this may be due to the presence of other abundant fungal genera that may mask its presence, or this is due to universal primer use. Thus, I would like to ask, was this phenomenon described in the literature earlier, and what actions can be taken to detect these sequences? I suspect that the method of DNA isolation is crucial in this case. Still, my question is: whether other actions were taken (at the level of bioinformatics analyses, used databases) to demonstrate the presence of this genus?

The obtained results enabled the complete verification of the research hypothesis. They supported the expectation that the rhizosphere is a more suitable niche for isolation P solubilizing fungi than the root endosphere of willow.

In conclusion, the authors stated that the level of fungal community association with the plant (rhizosphere *vs.* root endophytes) is the most important factor shaping its diversity. The site, season, and planting design have a lower impact. The fungal diversity at the same level of plant association was mainly driven by soil properties such as T.N., TOC, and pH.

Similar research hypotheses were also verified in the manuscript no. 3 (**P3**). However, in this article, bacteria capable of increasing the availability of phosphorus in the soil were investigated. The cultivable fraction of bacteria was isolated on an R2A medium, and the microbiological media mentioned above (**P2**) were used to isolate the phosphate-solubilizing bacteria (PSB). The remaining research steps are consistent with those described for work no. 2.

In general, the tests showed a significant difference in endophytic bacterial abundance between the tested variants of the experiment (mono and mixed cultures). During the investigation, 88 (41 endophytic and 47 rhizospheric) strains with the ability to solubilize P were isolated. Fifty-one strains that could solubilize P at the German and 37 at the Swedish sites were obtained. These bacteria were dominated by *Proteobacteria* (74 strains, 85%). The representatives of *Actinobacteria* (6 strains, 6%) and *Firmicutes* (8 strains, 9%) were also isolated. The dominant taxa among *Proteobacteria* included species of *Pseudomonas* with 12 strains (13%), *Erwinia* with 17 (19%), and *Rahnella* with 10 (11%). Similarly to fungi, the bacterial alpha diversity was mainly determined by the level of association with the plant (endosphere *vs.* rhizosphere), whereas in the case of endophytes, it was additionally determined by the experimental sites. The bacterial population in the rhizosphere soil was more diverse, and a significant difference between the rhizospheric and endophytic bacterial diversity was found in the Shannon's H' and the number of OTUs.

The undoubted advantage of the research conducted by the doctoral student is the simultaneous use of classical microbiological methods and advanced molecular analyses. This approach allows us to obtain more complete results, such as the isolation and identification of fungi of the genus *Penicillium* by cultivation methods. At the same time, molecular analyzes did not show the presence of sequences belonging to this species.

The results described in manuscripts 1 - 3 provide a detailed insight into the structure of rhizospheric and endophytic bacteria and fungi in monocultures and mixed willow cultivation. In addition, the influence of many factors on the bioavailability of minerals and enzyme activity has been investigated. The crucial effect of the research conducted so far is also the establishment of the collection of microorganisms (bacteria and fungi) capable of increasing the bioavailability of phosphorus in the soil. The two selected strains of bacteria *Paenibacillus* sp. and *Pantoea agglomerans* were then used to perform a pot experiment with bioaugmentation of soil planted with willow growing in three tricalcium phosphate (TCP) concentrations. The results of such an experiment, mainly the plant growth parameters and changes in gene expression, were described in manuscript no. 4 (**P4**).

Strains used for pot experiments were chosen based on their ability to solubilize TCP in liquid media. Monocultures of Loden and Tora variants treated with tested strains and non-treated controls were watered with fertilizer solutions containing P at two concentrations. The obtained plant material was subjected to length and weight measurements, total P concentration estimation, and RNA isolation for transcriptome analysis. Since the supplementary materials to article no. 3 (Koczorski et al., 2022, not 2021, as stated by the author in the Materials and Methods section, page 76, P-solubilizing bacterial strain collection) refers to two strains of *Pantoea agglomerans* marked with the symbol B23 and B50 (GT1_3_EP5 and GT1_3_ED1 strain description in GenBank, respectively), it is impossible to indicate which of them was selected for plant inoculation. Using the analysis of the sequence of the 16S rRNA gene fragment, it was impossible to determine the species affiliation of the second isolate; therefore, it was designated as *Paenibacillus* sp. A more detailed analysis, such as MLSA (multilocus sequence analysis), is strongly recommended in this case. Unambiguous species affiliation of inoculants is essential but often limited to the 16S rRNA gene sequence analysis. Such precise identification of known or new species enables, for instance, a comparison of the obtained results with the works of other authors.

Similarly, no information was provided about inoculation (method of inoculum preparation, number of CFU of particular strain per one gram of sand, survival, and activity of inoculants in soil). These are crucial factors in the context of effective bioaugmentation, and I am asking to provide these data during the thesis defense if such results are available. *Since the tested strains originated from internal tissues of roots of the Tora variety growing in Germany, it would be very interesting to confirm if they could cooperate with the Loden willow. Similarly, sterile sand is not a typical niche for introduced strains. Although such an environment reduces competition with indigenous microorganisms, it may inhibit the survival and multiplication of inoculants after their introduction. On the other hand, due to the strongly reduced competition, it can be assumed that the total activity of microorganisms in the soil and the number of colonies isolated on the plates (after morphological, biochemical, or genetic verification) will come mainly from the introduced strains. Such results would support the hypothesis of a positive role of inoculants in willow growth and their impact on P bioavailability in soil.*

Back to the obtained results, all the experimental factors (P concentration, willow species, and bacterial treatment) showed statistically significant effects on most plant growth parameters. High P concentration in the soil caused different effects against roots (higher biomass) and leaves (lower biomass), regardless of the bacterial treatment. In high P concentration, bacterial inoculation did not significantly affect the P content in the leaves. In contrast, in the soil, an increase in phosphorus content was observed, adequate to the applied concentration. What is important, the stimulating role of inoculants in P supplying to plants was limited, and plants treated with *P. agglomerans* showed an even decrease in P content in L.P. and H.P. experimental groups compared to N.P. Thus the author stated that it was difficult to determine whether different P levels, bacterial inoculation, or a combination of both caused the changes. However, I would like to ask for an attempt to explain the reason for obtaining such results. Valuable and interesting are the results concerning changes in the plant's transcriptome after applying the tested factors. Transcriptomic analysis showed that the inoculation of Tora willow with P-solubilizing bacteria significantly affects the leaf's transcriptome and causes the upregulation of most genes, especially those related to photosynthesis, which are highly influenced by phosphorus. In the case of Loden willow, a general reduction in gene expression was observed for genes involved in ion transport, transcriptional regulation, and chromosomes.

According to fold-change = 1, I would like to ask about the reason for such a value. In most literature, values of 1.5 - 2 are recommended. Were analyses with a higher value of fold-change also performed? Regarding Figures 4-6 and 7-9, please clearly indicate the analyses presented in them (G.O. enrichment vs. KEGG enrichment). Were all differentially expressed genes (DEGs) included in the analysis shown in Fig. 4 -6? If yes, I recommend separate analysis for up- and down-regulated genes. Please use "top 30 terms" instead of "top 30 genes" in the figure description.

My last question is connected with final conclusion no. 3. Was the first part of this conclusion proposed based on the results of publication no. 4?

The **strengths** of the doctoral dissertation submitted for review include the research task's importance and international cooperation in the project. The consistency and novelty of the research, the high quality of the obtained results, and their detailed statistical analysis are also considerable advantages. Similarly, the high application potential of the obtained results is also essential. **It is worth noting that three scientific articles with a total Impact Factor of 15.1 have been published so far**. The Ph.D. student was listed as the first author in these articles, similar to the fourth not published manuscript. The **weaker** points are mainly connected with manuscript 4 (**P4**), which was not reviewed yet. The most critical issues are a too-general description of the methodology for preparing plant inoculation with the tested strains, the superficial description of the methods of transcriptomic data analysis, and editorial shortcomings in the last manuscript. Since two strains were used for inoculation, I believe their identification should be unambiguous, and more microbiological analyses should be done to determine the inoculation consequences in the soil environment. In the delivered version of the thesis, I also did not find supplementary materials for manuscript no. 4, which the doctoral student repeatedly refers to.

The Ph.D. thesis of M.Sc. Piotr Koczorski undoubtedly brings significant input into the knowledge on the diversity, abundance, and impact of P-solubilizing microorganisms associated with willows on their biomass production. Notably significant and innovative are the results of the effect of bioaugmentation on the plant transcriptome. Besides scientific values, such knowledge is crucial for effectively managing short rotation coppices. Overall, I consider the research and Ph.D. dissertation as valuable study, which present novelty and a high scientific

level. The Ph.D. candidate has proven his scientific skills by designing and conducting advanced scientific research and analyzing the results.

As a result of my review, I declare that the Ph.D. thesis written by M.Sc. Piotr Koczorski **meets the criteria** pursuant to art. 187 of Act of 20 July 2018, The Law on Higher Education and Science (Journal of Laws of 2018, item 1668, as amended) **and request that the Research Discipline Council of Biological Sciences of the Nicolaus Copernicus University in Toruń accepts M.Sc. Piot Koczorski for further stages of doctoral proceedings.**

Tomasz Płociniczak

I am convinced that most of my comments will be addressed during the doctoral thesis defense. Since the advantages of the thesis submitted for review, which I mentioned, are crucial and significantly outweigh the potential weaknesses, I, hereby, request that the thesis is accepted with distinction.

Tomasz Płociniczak
