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MONITORING OF RHIZOSPHERE BACTERIAL COMMUNITIES IN SOIL WITH SEWAGE SLUDGE ADDITION USING TWO MOLECULAR FINGERPRINTING METHODS: DO THESE METHODS GIVE SIMILAR RESULTS?

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In this study, bacterial genetic diversity from the rhizosphere of barley and wheat were studied. The plants were sown in pots with aliquot amount of 15 t/ha concentration of soil additive derived from sewage sludge and agricultural by-products represented by wastes from grain mill industry and crushed corn cobs. The plants sown in pots without the addition of soil additive represented control samples. The rhizosphere samples were collected on two dates (plant flowering and maturity) and the composition of bacterial communities were detected using two molecular fingerprinting methods – automated ribosomal intergenic spacer analysis (ARISA) and terminal restriction fragment length polymorphism (T-RFLP). Microbial biomass expressed as the amount of metagenomics DNA was higher in soils with addition of soil additive, except during maturity stage in barley rhizosphere. Nevertheless, statistically significant differences between control and sludge samples were not detected in any case. Similarly, no changes were detected in the composition of bacterial community between control and sludge samples in barley and wheat rhizosphere by using cluster analysis. Only minor temporal changes in the composition of bacterial community between flowering and maturity periods were observed. These changes were related to the samples collected in the plant maturity stage. In this stage, plants were completely mature and their impact on the rhizosphere bacterial communities in the form of root exudates was limited. Statistically significant differences between ARISA and T-RFLP methods were detected in all measured values of diversity indices. Despite these differences, both methods gave results leading to similar conclusions.

Key words: ARISA, bacterial community, genetic diversity, rhizosphere, sewage sludge, T-RFLP

Sewage sludge is the final product of wastewater treatment process and its production in the Slovak Republic has increased from 54,000 tons in 1998 to 58,706 tons in 2012 (Ministry of Environment of the Slovak Republic). This sludge is mechanically dewatered and anaerobically stabilized, allowing its use as a raw material in the production of compost, or direct application to agricultural soil. Sludge from municipal wastewater treatment in the Slovak Republic is classified according to the Act no. 223/2001 Z. z. as waste. An advantage of sewage sludge application to agricultural land is its use as a valuable source of plant micro- and macronutrients, and organic matter (Moffett *et al*. 2003). The high content of organic matter and the favourable ratio of C:N (18:1) lends relevance to the use of sewage sludge as a fertiliser substrate. On the other hand, sewage sludge may be a source of chemical (heavy metals) and biological contamination (thermo-tolerant coliform bacteria, faecal streptococci, and

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others). Simultaneously, concentrations of heavy metals may limit its acceptability for application to agricultural land. For these reasons, in Europe, its direct application to agricultural soil is governed by Council Directive 86/278/EEC of 12 June 1986 on the protection of the environment, particular of the soil when sewage sludge is used in agriculture, and in the Slovak Republic by Act no. 188/2003 Z. z. In both these acts, inter alia, a table about the limits of concentration of hazardous substances (heavy metals) in sewage sludge is mentioned. Also, the acts set rules on how farmers can use sewage sludge as a fertiliser to prevent it from harming the environment and human health by compromising the quality of the soil or surface and ground water (http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A31986L0278). Only treated sludge may be applied to agricultural soil, in which the concentration of hazardous substances does not exceed the limits in any of the monitored indicators, simultaneously complies with microbiological criteria and has a minimum of 18% dry matter content. Maybe due to these strict criteria, application of sludge directly to agricultural land in 2012 represented only 1.9% of the total amount of sludge produced in the Slovak Republic.

Sewage sludge is a rich source of organic matter, nutrients and trace elements, and can significantly improve the physico-chemical and biological soil properties. The basic condition for sewage sludge application in agriculture is that its use should not cause soil and groundwater contamination. Nowadays, direct application of sewage sludge to soil is generally considered one of the best ways of returning organic matter and nutrients to the soil. Direct use of sewage sludge is linked to hygienic harmlessness in terms of content of hazardous elements and pathogenic microorganisms. Application of treated sludge to the soil as a fertiliser benefits plants, but the effect of sludge addition on rhizosphere microorganisms is less known. Generally, microorganisms in the rhizosphere play important roles in the growth and ecological fitness of their plant host, and the huge amount of organic carbon secreted by plant roots forms, sustains and drives this rhizosphere web (Buée *et al*. 2009). Different factors such as soil type, soil pH, plant species (cultivars), plant developmental stage, or agricultural management practices have been described to have a direct effect on the composition of bacterial community in the rhizosphere of agricultural plants (Berg & Smalla 2009; Berg *et al*. 2014).

For this reason, the aim of this study was to monitor and evaluate changes in the bacterial genetic diversity of the rhizosphere of barley and wheat as a result of the impact of soil additive derived from sewage sludge and agricultural byproducts represented by wastes from grain mill industry and crushed corn cobs. Whereas via cultivation only slightly to 1% of soil microorganisms can be detected, to accomplish our goal, two culture-independent methods were chosen – automated ribosomal intergenic spacer analysis (ARISA) and terminal restriction fragment length polymorphism (T-RFLP). Subsequently, an additional aim was the comparison of these two molecular fingerprinting methods in order to determine which is more suitable for the detection of bacterial genetic diversity.

MATERIAL AND METHODS

Characteristics of soil additive and experimental design

The sewage sludge used in all the experiments was collected from the wastewater treatment plant Pannon-Víz Zrt. (Győr, Hungary) and was denoted as concentrated, anaerobically digested, dewatered and dried. This sewage sludge was one part of the soil additive and agricultural by-products, represented by wastes from grain mill industry and crushed corn cobs, another part (Top Feed & Cargo Hungary Holding Zrt., Hungary). The final soil additive was prepared in the ratio of 1:1.5 (sewage sludge : agricultural by-products) using the low capacity granulator equipment designed by Energy Agency Public Nonprofit Ltd. (Budapest, Hungary). The low capacity granulator provided the mixing of both primary composites and thermal treatment to ~ 75°C for inhibition of present microorganisms. The elemental composition of the soil additive was: As – 6.5 ppm; Ca – 3.21%; Cd – < 2 ppm; Cr – 67.5 ppm; Cu – 583 ppm; Fe – 3.13%; Mg – 0.21%; Mn – 0.03%; Ni – 44 ppm; Pb – 26 ppm; Sb – < 2 ppm; Se – < 1 ppm; Zn –

1,510 ppm (Šuňovská *et al*. 2013). For better characterisation of used sewage sludge and soil additive, see article by Šuňovská *et al*. (2013).

This research was conducted at the Research Institute of Plant Production (RIPP), Piešťany. The pot experiment (5 kg of arable land/pot) was established by randomised complete block design in three replications using two agricultural plants: spring barley, cultivar Levan and spring wheat, line PS-6. Control samples represented rhizosphere from pots without the addition of soil additive. Sludge samples represented rhizosphere from pots with the addition of 15 t/ha of soil additive. Both plants were planted in the pots with arable land from the field of RIPP Piešťany (for characterisation of used land see article by Ondreičková *et al*. 2014), and the seeding rate was 10 seeds per pot (Figure 1).

Rhizosphere sampling and DNA isolation

The samples were collected from the rhizosphere of barley and wheat in two stages – flowering – 10.5.2 stage by Feekes (June 2014) and plant maturity – stage 11.4 by Feekes (July 2014) (Large 1954). Each sample was taken individually from separate pots – 3 pots/3 individual controls, 3 pots/3 individual sludge samples. These three replicates of the samples were collected as follows: plants were taken out from soil, the soil residues were gently removed from roots and the rhizosphere soil was scraped from roots with sterile scalpel, subsequently cooled and stored before analysis at 4°C.

Metagenomic DNA was extracted from the 300 mg of fresh rhizosphere samples using the PowerSoil™ DNA Isolation kit (MoBio Laboratories, Inc., Carlsbad, USA) according to the manufacturer's protocol, but the extracted DNA was dissolved in 50 µl of nuclease-free water. The quantity and purity of DNA was detected by NanoDrop-1000 Spectrophotometer (Thermo Scientific, USA), and samples were diluted to the same final concentration (20 ng/µl). DNA was stored at –20°C before use. DNA was isolated immediately after sampling but the subsequent ARISA and T-RFLP analyses were conducted with all samples at once.

Automated ribosomal intergenic spacer analysis

The ITSF/ITSReub (Cardinale *et al*. 2004) primer set with 6-FAM fluorescent dye on the 5′ end of the reverse primer was used for amplification of the 16S-23S rRNA intergenic transcribed spacer region from the bacterial rRNA operon. DNA amplification was carried out in 50 µl reaction mixture containing 1 × PCR buffer (Invitrogen, Thermo Fisher Scientific Inc., Waltham, USA), 1.5 mmol Mg^{2+}, 0.25 µmol of both primers, 200 µmol of each dNTP (Invitrogen, Thermo Fisher Scientific Inc., Waltham, USA), 1 U Taq DNA polymerase (Invitrogen, Thermo Fish-

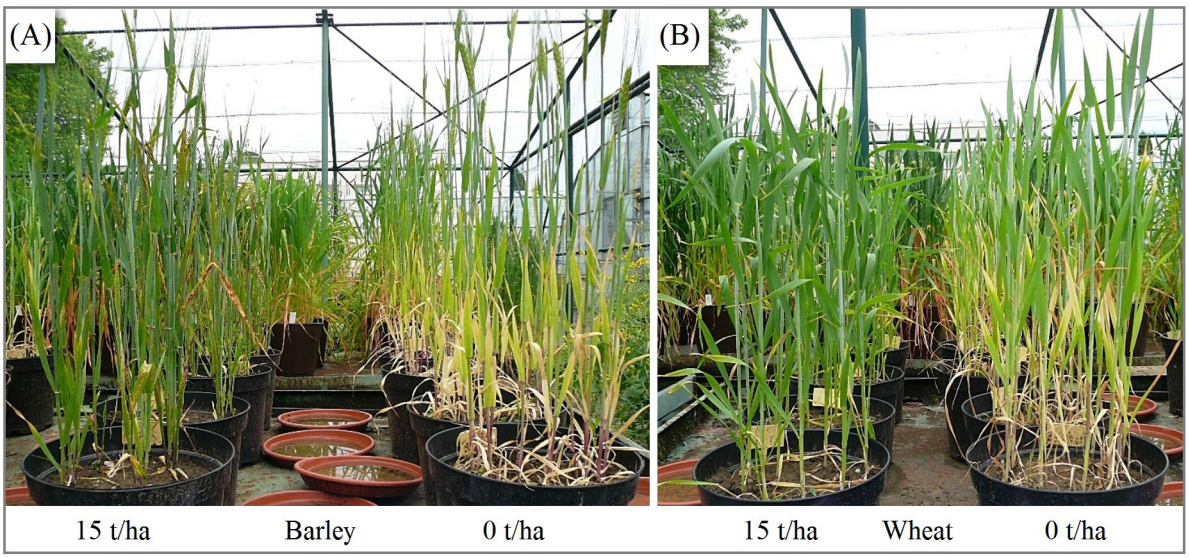

Figure 1. The pot experiment with addition of 15 t/ha of soil additive derived from sewage sludge and agricultural byproducts represented by wastes from grain mill industry and crushed corn cobs. (A) spring barley, cultivar Levan; (B) spring wheat, line PS-6.

er Scientific Inc., Waltham, USA), and 1 µL (20 ng) of DNA extracted from the rhizosphere. The PCR was performed in a GeneAmp PCR System 9700 (Applied Biosystems, Thermo Fisher Scientific, Inc., USA) using the following conditions: initial heat denaturation at 94°C for 3 min, followed by 35 cycles each consisting of a denaturation step at 94°C for 45 s, annealing at 60°C for 1 min, extension at 72°C for 2 min and a final extension step at 72°C for 7 min. PCR amplification was confirmed by horizontal electrophoresis on a 1% (w/v) agarose gel in 1 × TBE buffer (1.1% (w/v) Tris-HCl; 0.1% (w/v) $Na_2EDTA\ 2H_2O$; 0.55% (w/v) boric acid), pre-stained with 0.10 µl/ml of ethidium bromide and visualised using ultraviolet illumination. PCR products were purified by the PCR Purification & Agarose Gel Extraction Combo kit (Ecoli s.r.o., Slovakia) and dissolved with 10 µl of sterile water. One microlitre of purified product was added to 9 µl formamide containing LIZ1200 size standard (Applied Biosystems, Thermo Fisher Scientific, Inc., USA), denatured at 95°C for 3 min and separated by capillary electrophoresis using ABI 3100 Prism Avant (Applied Biosystems, Thermo Fisher Scientific, Inc., USA). The electropherograms were analysed by Peak Scanner 2 (Applied Biosystems, USA). Only fragments within the range 200–1002 bp were used for evaluation with minimum peak height threshold of 50 fluorescence units.

Terminal restriction fragment length polymorphism

This analysis was realised according to Ondreickova and Kraic (2015), but purified PCR products were digested with *Msp*I restriction enzyme (Promega Corp., Madison, USA) and terminal-restriction fragments (T-RFs) between 62 bp and 662 bp were used for evaluation. Only peaks above the threshold of 50 fluorescence units were considered.

Statistical analyses

Statistical significant differences among samples were tested by using the Fisher's least significant difference (*LSD*) procedure at the 95.0% confidence level. *LSD* was performed using the software Statgraphics X64 (Statpoint Technologies, Inc., Warrenton, USA). Diversity indices were calculated from standardized profiles of individual soil samples by using the number and height of peaks in each profile as representations of the number and relative abundance of phylotypes. The Gini-Simpson index (Jost 2006) was calculated as follows: $1 - \lambda = \Sigma(p_i^2)$, where λ is Simpson diversity index and p is the proportion of an individual peak height relative to the sum of all peak heights. The Shannon's diversity index (Shannon & Waever 1948) was calculated as follows: $H' = -\Sigma(p_i)\ (\ln p_i)$ and this index is commonly used to characterize species diversity in a community. Pielou evenness index (Pielou 1966) was derived from Shannon's diversity index and was calculated as follows: $J' = H'/H'_{max}$, where $H'_{max} = \ln(S)$ where S represents the total number of species. Diversity indices were calculated using Excel 2013. Cluster analysis was conducted using the binary system – operational taxonomic unit (OTU) and terminal restriction fragment (T-RF) peaks were classified as present (1) or absent (0) in each sample. The unweighed pair group method of cluster analysis using arithmetic means was used for grouping of genotypes. Dendrograms were constructed based on Jaccard´s similarity coefficient using DARwin 5.0.158 statistical software (http://darwin.cirad.fr/darwin; Perrier & Jacquemoud-Collet 2006).

RESULTS AND DISCUSSION

Total microbial biomass

Metagenomic DNA extracted from the rhizosphere samples was used as a measure of microbial biomass (Figure 2). Microbial biomass, except during maturity stage in barley rhizosphere, was higher in soils with soil additive. Interestingly, the highest and also the lowest microbial biomass were detected during maturity stage in wheat and bar-

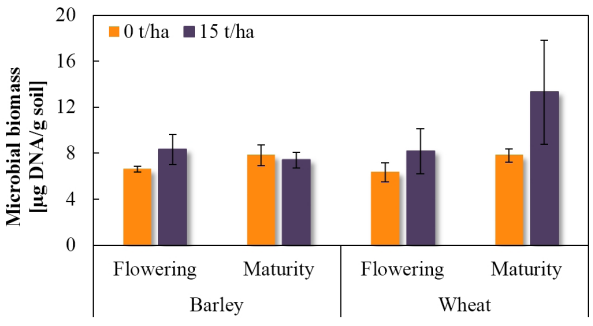

Figure 2. Total microbial biomass expressed as a metagenomic DNA extracted from the rhizosphere of barley and wheat from soil without and with addition of soil additive at a concentration of 15 t/ha. Bar represents standard deviation (n = 3).

ley rhizosphere, respectively. At the same time, the highest difference in measured microbial biomass between control and sludge samples was detected in wheat during the maturity stage; nevertheless, statistically significant differences between control and sludge samples were not detected in any case (*LSD*, $\alpha = 0.05$).

Bacterial genetic diversity

Biological diversity can be quantified in many different ways. One possibility is to measure the richness, in our case bacterial richness, which corresponds to the number of different species represented in each rhizosphere samples. From Figure 3a, it can be observed that richness value (No. of OTUs/T-RFs) varies between control and sludge samples, but a statistically significant difference between them was detected only in one case, during the flowering stage in wheat rhizosphere using T-RFLP analysis (Figure 3a). On the other hand, diversity indices provided more information about community composition than simply species richness. The Gini-Simpson index equals the probability that the two entities taken at random from the dataset of interest represent different types (Jost 2006). The differences in measured values of this index using ARISA between control and sludge samples were slight. But it is surprising that statistical difference was detected at very similar values of control and sludge samples, i.e. barley rhizosphere in maturity stage (Figure 3b). T-RFLP analysis yielded higher differences between control and sludge samples, and statistically significant difference was detected also only in one case, in wheat rhizosphere during flowering stage (Figure 3b). Shannon's diversity index, like the above-mentioned index, accounts for both abundance and evenness of the species present (Shannon & Waever 1948). Also, the Shannon index (H') increases as both the richness and the evenness of the community increase, and high values of H' would be representative of more diverse communities (Magurran 2004). Higher index values were obtained with the ARISA analysis than using T-RFLP, as well as these values were more balanced between the control and sludge samples. In both methods, one statistical difference was detected between the said samples (Figure 3c). Poulsen *et al*. (2013) by using pyrosequencing detected higher Shannon index, which is logical because of the huge number of used sequences. The Shannon diversity index, with 20,000 sequences, showed that the untreated (control) soil had the index value of 7.09 and the sludge soil had the value of 7.16. They also observed that the analysis using all detected sequences, showed a positive correlation between the number of sequences and H'. Evenness index compares the similarity of the population size of each species present (Mulder *et al*. 2004). Unlike previous diversity indices, no statistical differences between control and sludge samples were detected by using Pielou evenness index (Figure 3d). Overall, Figure 3 shows that the heights of each column (no values) are similar across all diversity indices.

Impact of sewage sludge on the composition of rhizosphere bacterial communities

To study the impact of soil additive derived from sewage sludge and agricultural byproducts represented by wastes from grain mill industry and crushed corn cobs on the composition of bacterial community in the barley and wheat rhizosphere, the samples were statistically processed using cluster analysis. In T-RFLP analysis, three samples for unforeseen problems in capillary electrophoresis did not give any product, i.e. one sample from barley, one sample from wheat rhizosphere in flowering stage with 15 t/ha of soil additive and one sample from wheat rhizosphere in maturity stage with 15 t/ha of soil additive. For this reason, these three samples were not included in subsequent statistical evaluation.

Cluster analysis was constructed using binary data and fluorescence intensity was not taken into account. Dendrograms constructed from ARISA and T-RFLP data showed essentially similar results (Figure 4). The impact of the growth stage of barley and wheat on the composition of bacterial community is noticeable in both dendrograms. Bacterial communities in control samples from rhizosphere of both plants in flowering were very similar. These controls are located at the top (ARISA, Figure 4a) or the bottom (T-RFLP, Figure 4b) of the dendrograms. Samples collected from rhizosphere of mature plants were more dispersed within the whole dendrograms. It was probably due to the fact that, during the maturity stage, the plants were dry and

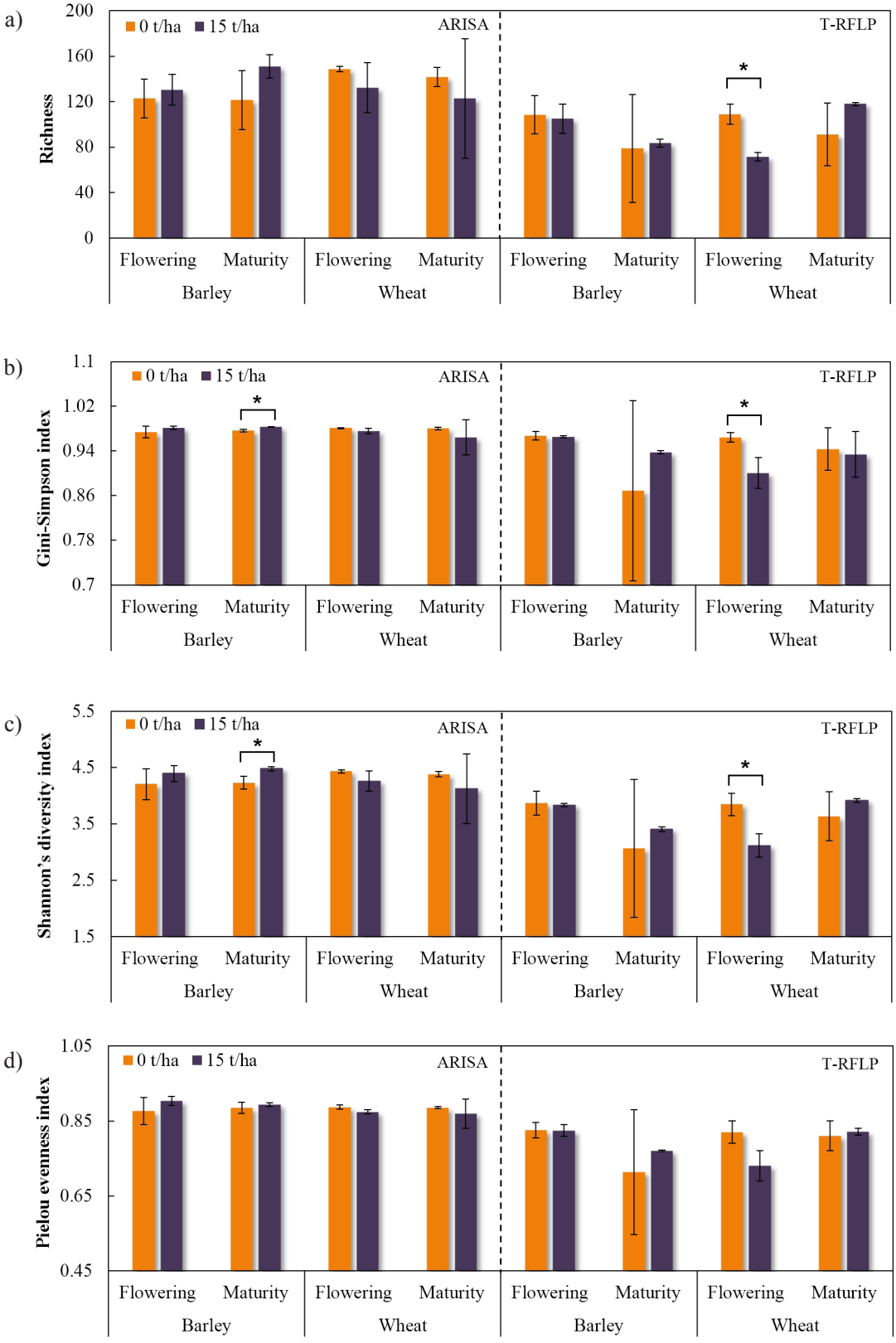

Figure 3. Diversity indices and evenness detected in barley and wheat rhizosphere from soil without and with addition of soil additive at a concentration of 15 t/ha. Bar represents standard deviation (n = 3). *denotes statistically significant difference (*LSD*, α = 0.05).

Abbreviations: ARISA – automated ribosomal intergenic spacer analysis; *LSD* – least significant difference; T-RFLP – terminal restriction fragment length polymorphism

their roots showed no or very low metabolic activity. Therefore, this metabolic inactivity could result in overgrowth of various types of bacteria, independent of root exudates secreted by barley or wheat. It is known that microbial population and mainly their activity in soil is significantly influenced by plant roots (Bais *et al*. 2006). Furthermore, the plant growth stage may be an important factor that shapes the composition of bacterial community in the rhizosphere (Herschkovitz *et al*. 2005; Lerner *et al*. 2006) because production and dispersion of root exudates are also affected by plant development (Hamlen *et al*. 1972). These exudates create a selective microbial stimulation (Miller *et al*. 1989), which varies in function of time due to the plant age (Cavaglieri *et al*. 2009). In the dendrograms, the partial separation of soil samples with the addition of soil additive from the control samples is also visible. Nevertheless, this separation is not very significant and the impact of sewage sludge as a soil additive on the bacterial composition in the barley and wheat rhizosphere cannot be clearly confirmed.

The impact of sewage sludge on the composition of bacterial community was not significant in this study. This is most likely due to the fact that, in our case, it was a pot experiment, using the same soil type. It is known that land use and soil type are the main drivers that may cause the changes in microbial community composition (Acosta-Martínez *et al*. 2008; Lauber *et al*. 2008; Drenovsky *et al*. 2010), and likewise, the particle size fractions are more important than the type of fertiliser applied (Sessitsch *et al*. 2001). That statement was supported by the results of MacDonald *et al*. (2011). They studied the impact of metal-rich sludge additions at seven experimental sites (five were under arable and two under grassland management) and detected the strong effect of site on microbial community structure. Also, the metal effects were weak compared to the effect of different site. Another approach has been used by Poulsen *et al*. (2013). They studied the impact of different urban waste and reference fertilisers on prokaryotic diversity at one field site and found only small changes in the community composition due to different fertiliser treatments. Similarly, Nakatani *et al*. (2011) published that two sequential annual applications of tannery sludge to agricultural soils did not have negative impacts on the microbial properties evaluated but denaturing gradient gel electrophoresis showed different profiles at different sampling times. This was probably due to a rearrangement of bacterial communities in different treatments as a result of the exhaustion of easily degradable substrates towards the end of each

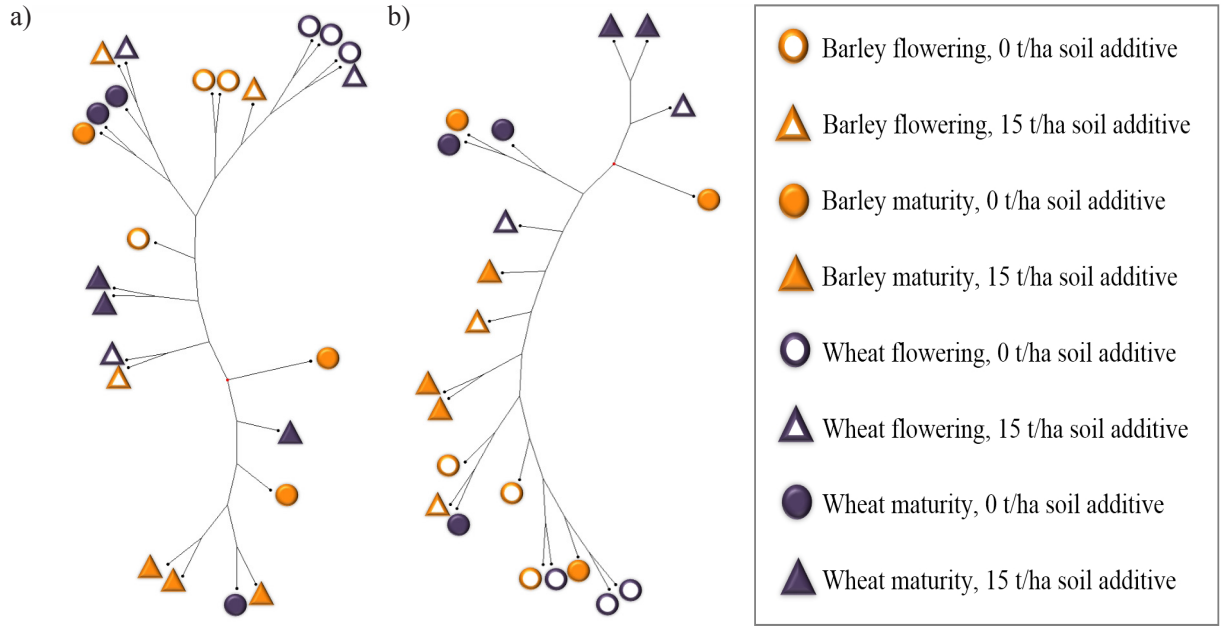

Figure 4. Cluster analysis constructed from a) ARISA binary data and b) T-RFLP binary data of bacterial communities from barley and wheat rhizosphere from soil without and with addition of soil additive at a concentration of 15 t/ha.

cycle of tannery sludge application. There are many studies with different results about the impact of sludge on the soil microbial composition but some of the differences between the studies may be due to the use of different methods and also primers, which have different biases (Poulsen *et al*. 2013). Mattana *et al*. (2014) in their study of three sewage sludge fractions (fresh, composted and thermally dried) and its impact on soil microbial community recommended that composting rather thermal drying can represent a more appropriate post-digestion process to make sewage sludge suitable for use as soil conditioner in agriculture.

Mutual comparison of ARISA and T-RFLP

ARISA and T-RFLP belong to the molecular fingerprinting methods and, in principle, are very similar. The main difference is in the DNA region, which is used for PCR amplification – functional gene in T-RFLP or highly variable intergenic spacer in ARISA. This determines the subsequent steps in these methods. Results obtained from both methods about diversity indices were statistically significant (Figure 5). In this statistical evaluation, plant species, plant growth stages and addition of soil additive were not taken into account. As a result of using the hypervariable intergenic spacer, it is understandable that the number of OTUs were statistically higher in ARISA than in T-RFLP. However, the range between the smallest and the largest number of OTUs was approximately the same in both methods (Figure 5a). For other diversity indices, the range between the lowest and the highest value was lower in ARISA than in T-RFLP, which indicates that the ARISA method yielded less variable values or, in other words, more consistent results. This is surprising in view of the fact that in T-RFLP, the conserved gene region is used, where we assumed more consistent results. The differences in the values of diversity indices between the two methods were statistically significant (*LSD*, α = 0.05).

CONCLUSIONS

Our pot experiment with the addition of soil additive derived from sewage sludge and agricultural byproducts, represented by wastes from grain mill industry and crushed corn cobs, to arable land at the rate 15 t/ha did not reveal differences between

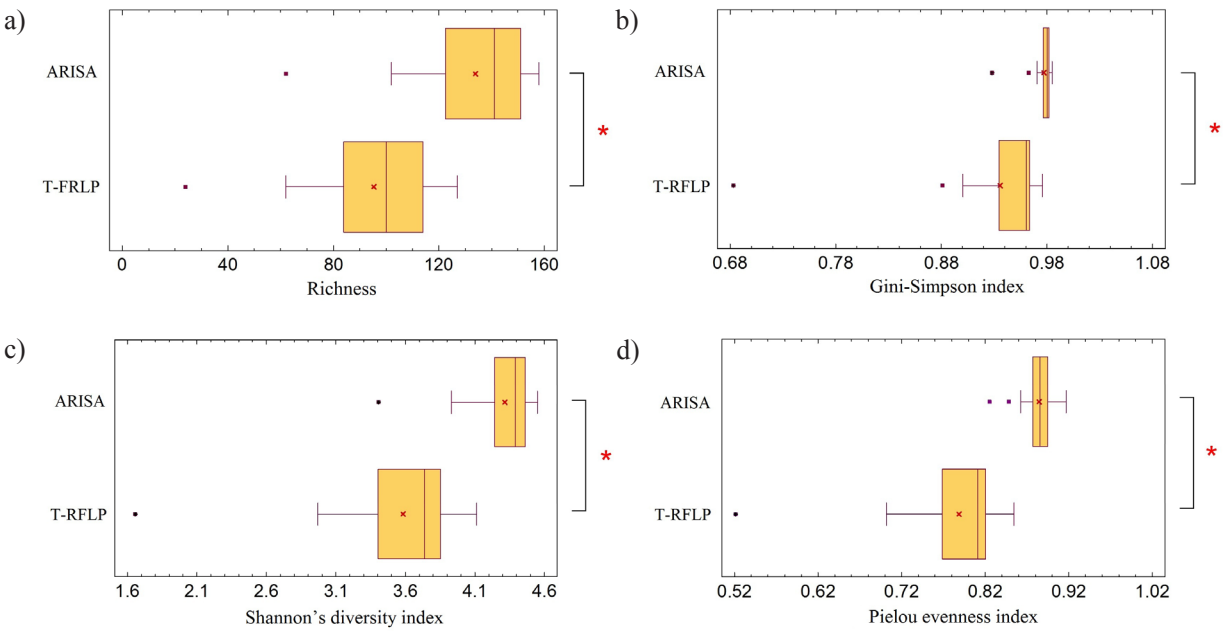

Figure 5. The comparison of ARISA and T-RFLP methods using Box and Whisker plots that were created using data from diversity indices and evenness. *denotes statistically significant difference (*LSD*, α = 0.05).

Abbreviations: ARISA – automated ribosomal intergenic spacer analysis; *LSD* – least significant difference; T-RFLP – terminal restriction fragment length polymorphism

control and samples with sludge in the composition of bacterial community in barley and wheat rhizosphere. Only minor temporal changes in the composition of bacterial community between flowering and maturity periods were observed. These changes were related to the samples collected during the plant maturity stage. Whereas the plants were mature and probably showed low metabolic activity, their impact in the form of root exudates on the composition of bacterial communities was reduced. This, in turn, caused the mutual diversity in these samples, which was confirmed by cluster analysis. Significant differences between measured values of diversity indices were also detected by using ARISA and T-RFLP methods. Despite these differences, both methods gave results leading to similar conclusions.

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