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BIOMASS PRODUCTION OF GIGANTIC GRASSES *ARUNDO DONAX* AND *MISCANTHUS* × *GIGANTEUS* IN THE DEPENDENCE ON PLANT MULTIPLICATION METHOD

MARCELA GUBIŠOVÁ*, JOZEF GUBIŠ, ALŽBETA ŽOFAJOVÁ

National Agricultural and Food Centre – Research Institute of Plant Production, Piešťany, Slovak Republic

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The effect of plant propagation method on growth parameters and the yield of above-ground biomass in two species of gigantic grasses were measured during three growing seasons. Plants were multiplied in explant culture and through traditional methods – by rhizome segments (*Miscanthus* × *giganteus*) or by stem cuttings (*Arundo donax*). In the case of *M.* × *giganteus*, *in vitro*-multiplied plants produced more shoots with significantly lower diameter, but the differences in the number of shoots, plant height and the yield of dry biomass were not statistically significant. Different results were observed for *A. donax*, where *in vitro*-multiplied plants showed significantly weaker results in all parameters, with the exception of the number of shoots in the first measured season. In both the species, there was observed the strong effect of the year. While in *M.* × *giganteus* the yield of dry biomass gradually decreased during the measured years, it increased in the case of giant reed.

Key words: energy plants, vegetative propagation, *in vitro* multiplication, rhizomes, stem cuttings

Increasing consumption of fossil fuel and the request for biofuels and bioenergy production has led to an increase in the importance of species with high biomass production. In the past decade, growers in Slovakia have shown interest in cultivation of biomass plants due to profitability from the crop. *Miscanthus* × *giganteus* and *Arundo donax* belong to the introduced species of family *Poaceae*, which cannot be overcome in biomass production by native species and are therefore excellent candidates for marginal land utilization. These two plant species comply with most of the requirements for energy plants, such as perennial character, huge amount of yearly harvested above-ground biomass, low need for pesticides and fertilisers, sequestration of nutrients to the underground parts before harvesting, adaptability to different conditions and tolerance to drought and frost. However, a significant disadvantage of giant reed is the high moisture content during harvesting (about 50%) and the high ash content (3.5–5.5%) (Ceotto & Candilo 2010).

Species from the genus *Miscanthus* belong to perennial rhizomatous grasses. Within the genus, only one clone, *Miscanthus* × *giganteus* Greef et Deuter, is considered to be the most valuable for biomass production, and is grown commercially (Xue *et al*. 2015). *M.* × *giganteus* is the natural triploid infertile hybrid of diploid *M. sinensis* and tetraploid *M. sacchariflorus* (Greef & Deuter 1993; Hodkinson *et al*. 2002), which was sampled in 1935 and introduced to Europe from Japan by Danish botanist A. Olson (Greef *et al*. 1997). *Miscanthus* belongs to C4 plants with high photosynthetic and water use efficiency (Atkinson 2009). It is considered an attractive and

Mgr. Marcela Gubišová, PhD. (*Corresponding author), Ing. Jozef Gubiš, PhD., Ing. Alžbeta Žofajová, PhD., National Agricultural and Food Centre – Research Institute of Plant Production, Bratislavská cesta 122, 921 68 Piešťany, Slovak Republic. E-mail: gubisova@vurv.sk

environmentally friendly bioenergy plant with high production of ligno-cellulose biomass, which usually reaches annual yields ranging from 15 to 40 tons of dry matter per hectare (Clifton-Brown *et al*. 2001b).

A. donax L. is also a perennial rhizomatous grass originated from Asia. Plants are very adaptable to different conditions; they grow in tropical to warm-temperate regions, also on contaminated or salinized soils (Ceotto & Candilo 2010). Plants similar to bamboo can grow up to 10 m in height, and due to its vigorous growth, the species is considered invasive (Pilu *et al*. 2013). Despite the C3 character of photosynthesis, the biomass yield can reach up to 78 tons per hectare annually, and due to hollow canes, it is easily processed into chips (Bass *et al*. 2014). Despite its invasiveness, in July 2013, the U.S. Environmental Protection Agency stated that giant reed yields three times as much ethanol per acre as maize, and qualified this plant as a cellulosic renewable fuel [http://www.newenergyfarms.com/crops/arundo-donax/]. Giant reed is known as a multipurpose plant that is used in paper and pulp industry, as a building material, for making musical instruments, fishing rods, walking sticks and stakes for plants (Pilu *et al*. 2012). It is also used in Ayurvedic medicine, as an ornamental plant and for phytoremediation of contaminated areas (Alshaal *et al*. 2014).

Both species do not produce viable seeds and can be multiplied by the vegetative methods only. On the other hand, the absence of seed production minimizes the risk of potential invasiveness. Even though these grasses belong to rhizomatous plants, the rhizome (Figure 1a) propagation method in giant reed is expensive and impractical (Ceotto & Candilo 2010). Therefore, considering traditional methods, propagation by shoot cuttings offers greater potential. Although, for *M.* × *giganteus,* traditional propagation by rhizome segments (Figure 1b) is still predominant (Boersma & Heaton 2014a), high demand for propagules needs simpler and more effective

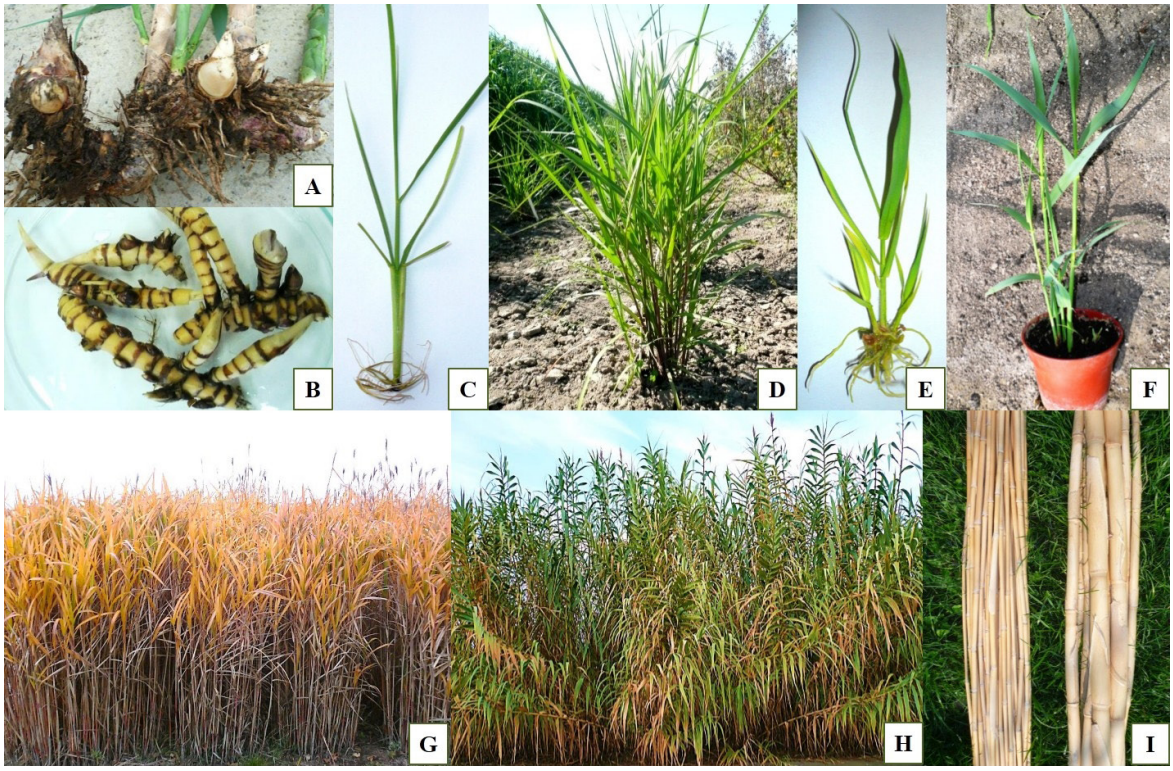

Figure 1. Propagation and biomass production of *Arundo donax* and *Miscanthus* × *giganteus*: A) rhizomes of *A. donax*, B) rhizomes of *M.* × *giganteus*, C) *in vitro*-multiplied plantlet and D) acclimatized plant of *M.* × *giganteus* after the transplantation into the field, E) *in vitro*-multiplied plantlet and F) acclimatized plant of *A. donax*, G) plants of *M.* × *giganteus* and H) *A. donax* at the end of October, I) dried biomass of *M.* × *giganteus* (left) and A. *donax*

propagation systems (Atkinson 2009). Mann *et al*. (2013) compared ramet growth from whole shoots or shoot fragments of giant reed and miscanthus grass in California and found higher biomass production by whole shoots in both species. While axillary buds of miscanthus grass regenerated immediately after shoot cutting in spring and summer only, giant reed shoots regenerated throughout the year. Regeneration of miscanthus grass was lower (25–32%) compared to giant reed (49–74%), which propagates more readily from stem segments.

In vitro techniques (tissue cultures) offer an alternative tool for plant multiplication and have been commercially used for many plant species. It enables rapid multiplication of plants by direct shoot multiplication omitting the callus phase; indirectly through induction of callus followed by whole plant regeneration; or by a combination of both procedures. Moreover, tissue cultures also enable the production of pathogen-free plants and storage of plant material for a long time in controlled aseptic conditions. The important advantage of this method is the possibility of multiplying plants throughout the year and using *in vitro* breeding methods. *In vitro* techniques for the multiplication of *A. donax* (Cavallaro *et al*. 2011; Herrera-Alamillo & Robert 2012; Antal *et al.* 2014; Gubišová *et al.* 2016) and *M.* × *giganteus* (Holme & Petersen 1996; Lewandowski 1997; Glowacka *et al*. 2010; Gubišová *et al*. 2013) have already been described. In praxis, these plants are often called as "meristem plants". While the comparison of such plants with those propagated by traditional methods has been done for *Miscanthus* (Lewandowski 1998; Clifton-Brown *et al*. 2007), until now there was no data available for *A. donax*.

The aim of our experiments was to compare growth parameters and biomass production of plants multiplied by *in vitro* techniques and classical vegetative methods in two species of gigantic grasses – *A. donax* and *M.* × *giganteus*.

MATERIAL AND METHODS

Plant material

Two species of gigantic grasses were used in our experiments: *M.* × *giganteus* Greef et Deu (miscanthus grass) and *A. donax* L. (giant reed). Mother plantation for experiments was established from rhizomes in both cases. Rhizomes of miscanthus grass were kindly provided by Mr. L. Sovák (SWHG Ltd., Valašské Meziříčí, Czech Republic).

Characterization of the experimental site

Field experiments were established in the locality of Piešťany (west part of Slovakia) at an altitude of 163 m and a continental character of climate with long-time average annual precipitation of 608 mm and temperature of 9.2°C. Soil type was Luvi-Haplic Chernozem; the locality belongs to a maize production type. The actual data of average monthly temperature and total monthly rainfall in the growing seasons 2013–2015 is given in Figure 2.

Plant multiplication methods and field establishment

Plants of *M.* × *giganteus* were multiplied *in vitro* via callus culture induced from immature inflorescences. Immature inflorescences were taken from 1-year-old mother plants. After regeneration from calli, the shoots were multiplied by *in vitro* tillering, elongated and rooted in the culture, and then transplanted to the soil (Figure 1c, d). The method is discussed in detail by Gubišová *et al*. (2013). Plantlets were acclimatized to *ex vitro* conditions and 10 weeks later, in June 2011, they were transplanted into the field. Control plants of miscanthus grass were established from rhizomes planted in the month of May of the same year.

Plants of *A. donax* were multiplied by *in vitro* tillering. The culture was established from stem segments with axillary bud of 1-year-old mother plants. Shoots regenerated from buds were multiplied and rooted *in vitro,* and then transplanted into the soil and acclimatized to *ex vitro* conditions (Figure 1e, f). The method is discussed in detail by Gubišová *et al.* (2016). Plants were transplanted into the field in June 2012. Control plants of giant reed were prepared from stem cuttings taken from mother plants in October 2011. Stem segments were cultivated in the sand, and regenerated and rooted shoots were transplanted into the garden substrate. During the winter 2011/2012, plants were cultivated under greenhouse conditions and transplanted into the field in the month of May 2012.

In the field, plantlets or rhizomes were planted at a spacing of 1×1 m. Plants were cultivated without

irrigation and fertilisers. Plants growing along the edge of the plots were excluded from evaluation.

Data measurement and evaluation

Biomass production (Figure 1g, h, i) and growth parameters of plants cultivated in growing seasons of years 2013, 2014 and 2015 were evaluated during harvest in the month of March 2014, 2015 and 2016, respectively. The plants were evaluated for: the number of shoots per plant; thickness of the shoots (diameter [mm], measured at the base of the shoot); plant height [cm]; biomass moisture [%], measured by ((fresh weight − dry weight)/fresh weight) × 100]; and yield of dry biomass per plant (100% dry matter mass; content of dry matter mass was measured from three samples of each variant). Shoot diameter was the average shoot diameter (calculated from five randomly measured shoots per plant) in the case of miscanthus grass, or diameter of the thickest shoot in the case of giant reed. Twenty plants were evaluated for each parameter in both species. Experimental data were analysed by the analysis of variance (ANOVA) and means were then separated by *LSD* test (the least significant difference) at $\alpha = 0.05$ using the statistical software STATGRAFICS Centurion XVI.II (Statpoint Technologies, Inc., Virginia, USA).

RESULTS AND DISCUSSION

Growth parameters and the yield of dry biomass of *M.* × *giganteus* were measured in the third (2013), fourth (2014) and fifth (2015) year of cultivation when these parameters are supposed to be already stabilized (Clifton-Brown *et al*. 2001a; Christian *et al*. 2008), although Polish experiments showed yield stabilization only after the third growing season (Jezowski *et al*. 2011). No statistically significant differences were observed between the two propagation methods (e.g. rhizomes versus *in vitro*-multiplied plantlets) for the number of shoots, plant height and the yield of above-ground biomass harvested in early spring. Statistically significant differences were observed only for shoot diameter, which was higher for rhizome-derived plants (Figure 3, Table 1). Lewandowski (1998) also mentioned that *in vitro*-multiplied plants showed smaller shoot diameter. Similar to our results, she also observed a higher number of shoots for *in vitro*-multiplied plants and no differences in shoot height. In our experiments, the yield of above-ground biomass was slightly higher for *in vitro*-multiplied plants in 2013 and 2014 (Figure 3). Lewandowski (1998) observed higher biomass production for *in vitro*-multiplied plants at one locality in Germany, but no differences at the other one. Clifton-Brown *et al*. (2007) found no differences in the biomass yield between rhizome-developed and *in vitro*-multiplied plants during a 16-year experiment in Southern Ireland.

Differences among years were statistically significant, except for the number of shoots. Higher rainfall in the year 2014 (124.6 mm for June and July together when the growth of plants is the most abundant, and 526.2 mm for the whole growing period) positively affected shoot height despite the low-

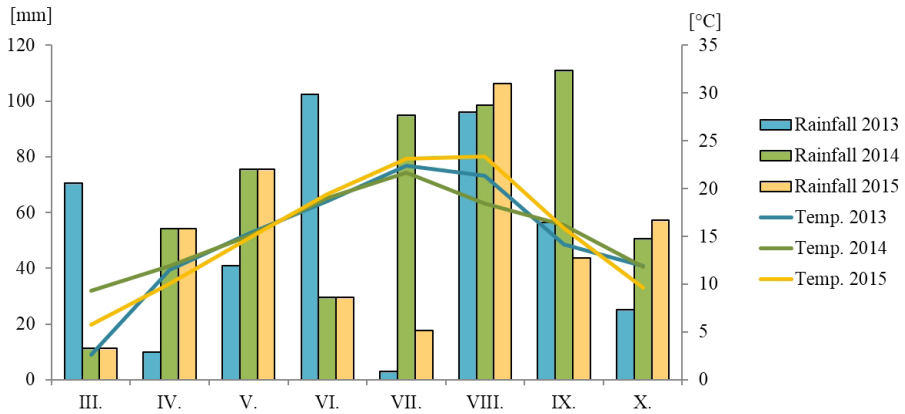

Figure 2. The average monthly temperature and total monthly rainfall during the growing seasons in the years 2013–2015

est temperatures during this year, mainly in July and August, while in the dry growing season of the year 2015 (47.1 mm for June and July together, 272.6 mm for the whole growing period) (Figure 2), significantly shorter and thicker shoots were observed. Moisture content in harvested biomass was about 16% in March 2016 and 2014, and 28% in March 2015. Such differences may have been caused by higher rainfall in the season of 2014 or different weather conditions during the winter before harvest. Despite different weather conditions in the monitored growing seasons, the yield of above-ground biomass gradually decreased from the year 2013 to 2015 (Figure 3, Table 1).

Lower winter freeze survival was mentioned for *in vitro*-multiplied plants (Lewandowski 1998). Plants are considered to be most susceptible to winter

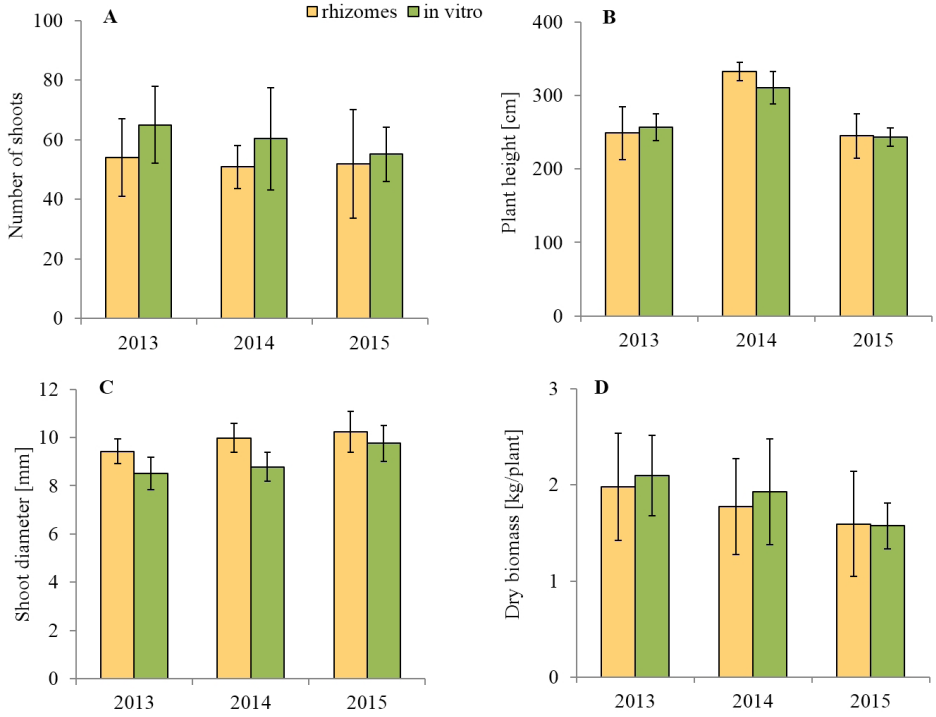

Figure 3. *Miscanthus* × *giganteus*. Growth parameters and biomass production during the third, fourth and fifth year of cultivation: A) the number of shoots per plant; B) plant height; C) diameter of shoots; D) production of dry biomass, calculated as kg/plant; average value ± standard deviation

T a b l e 1

Miscanthus giganteus – statistical evaluation of measured parameters

Parameter	Propagation method	Year	
	P-value	*P*-value	Statistical differences by *LSD* (2013/2014/2015)
No. of shoots	0.0518	0.4807	a/a/a
Plant height	0.4176	**0.0000**	a/b/a
Shoot diameter	**0.0000**	**0.0003**	a/a/b
Dry biomass	0.5180	**0.0198**	b/ab/a

P-value by analysis of variance (bold font indicates statistically significant difference at α = 0.05); *LSD* (the least significant difference) test was used as a multiple range test for evaluated years (different letters indicate statistically significant difference at α = 0.05)

frost during the first winter after field establishment (Clifton-Brown & Lewandowski 2000; Jezowski *et al*. 2011). In our experiment, *in vitro*-multiplied plants of *M.* × *giganteus* were transplanted into the field in the year 2011. The winter of 2011/2012 was characterised by very low temperature here. During the first two weeks of February 2012, the minimal temperature was measured from –10.3°C to –16.0°C and the soil remained frozen throughout the day (the minimal soil temperature in these days was from –1.9 to –4.6°C), which has not been a common occurrence in this locality during the past few years. Despite this, we did not observe any plant losses during this winter (2011/2012), as well as no establishment losses in the first year. There were only some losses (4.9%) during the phase of acclimatization to *ex vitro* conditions. Plant survival may have also been affected by the fact that plants transplanted to the field were strong and vital, as they were appropriately pre-cultivated in pots with 0.25 dm^3 of the garden substrate for a period of ten weeks. Based on worldwide experiences, Clifton-Brown and Lewandowski (2000) suggested that larger plants can survive better than smaller ones. Boersma and Heaton (2014b) compared the survival of rhizome and stem-propagated plants of miscanthus grass in the field and did not find statistically significant differences between these two groups. They observed much higher mortality in the phase of the field establishment compared to winter losses (23.7% vs 1.2%). The interesting fact is that they also observed lower number of shoots per plant and higher basal circumference for rhizome-propagated plants. Authors explained that the higher number of shoots in stem-propagated plants may be due to the inherent characteristics of aerial organs, including native hormone levels. These observations were the same for the comparison of rhizome versus *in vitro* propagated plants in our experiment and the experiments of Lewandowski (1998).

A completely different situation was observed for plants of *A. donax*. Under *in vitro* conditions, plants were regenerated directly from axillary buds and then multiplied. Control plants were also regenerated from axillary buds but were then stored under greenhouse conditions for the whole winter. It caused that plants from stem segments had been stronger with probably more enlarged underground

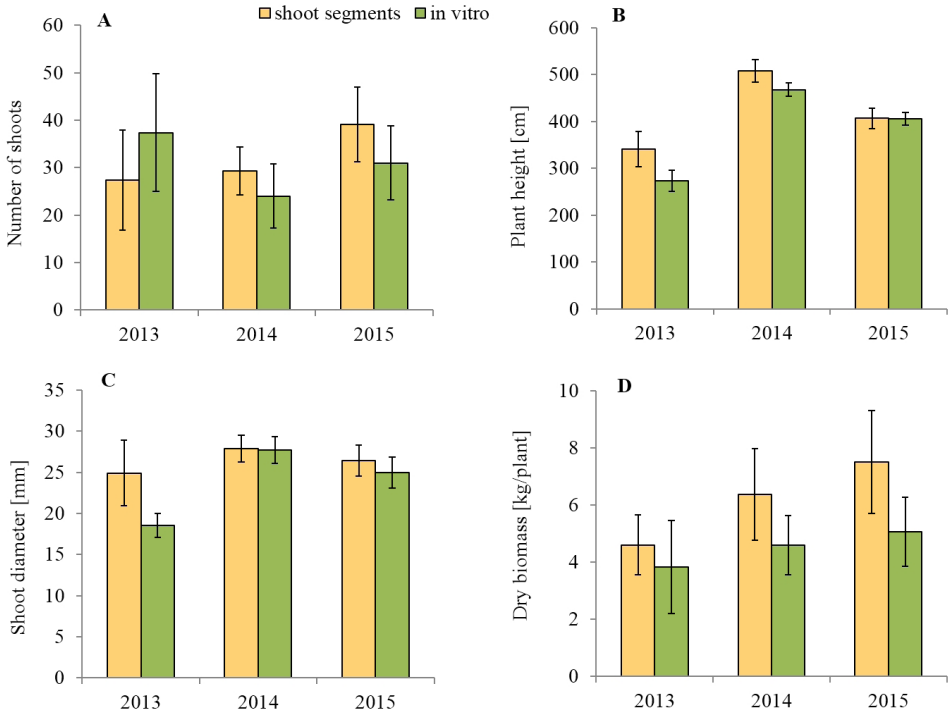

Figure 4. *Arundo donax*. Growth parameters and biomass production during the second, third and fourth year of cultivation: A) the number of shoots per plant; B) plant height; C) diameter of the thickest shoot; D) the production of dry biomass, calculated as kg/plant; average value ± standard deviation

part compared to plants multiplied *in vitro*. It could redound to the advantage of these plants in the field conditions and may have been the important one, but not the only, reason for higher biomass production. It is important to mention that there were no losses (as well for *M.* × *giganteus*) during field establishment or in winter in both groups of plantlets, and losses during acclimatization to *ex vitro* conditions were only 4.4%.

In the first year of evaluation of giant reed, the plants were only in the second year of cultivation, contrary to miscanthus grass. Different results were observed in the number of shoots compared to next years (Figure 4). Statistical evaluation of the complete results is shown in Table 2, and with the exception of the number of shoots, significant differences between propagation methods were observed for other measured parameters. When the first year, 2013, was excluded from the evaluation, significant differences between propagation methods were measured also for the number of shoots ($P = 0.0065$). In the first year, the number of shoots was higher for *in vitro*-multiplied plants, but from the second year onwards, it turned reverse (Figure 4). The higher number of shoots in the first year may have been caused by the effect of plant growth regulators used for the induction of *in vitro* tillering, which persist in plant tissues and confer a residual hormonal response (Boersma & Heaton 2014a), or it can be explained by the greater development of meristems forming buds at the base of the *in vitro*-multiplied plantlets.

If only the second and third years of measurement were compared, the difference in shoot diameter between stem-propagated and *in vitro*-propagated plants were not statistically significant ($P = 0.1569$). Nevertheless, there was visually detectable difference between the circumferences of the sheaf of cut shoots on the basal side. Parameter stem diameter may have been slightly distorted here because the diameter of the thickest shoot only was measured in the case of giant reed plants. It was measured that way because the diameter of the shoot of giant reed is, in contrast to miscanthus grass, variable (varies from 0.8 to 30 mm) and it would be too laborious to determine the exact average value. Therefore, the circumference of the shoot sheaf was measured in the third year, and it was 60.17 ± 6.36 cm for stem-propagated plants and 50.67 ± 9.27 cm for *in vitro*-multiplied plants. It is clear from these measurements that the stem-propagated plants had a higher proportion of thicker shoots, although the diameter of the thickest shoot was not different.

Generally, the stem-propagated plants of giant reed showed better results in all measured parameters than the *in vitro*-multiplied plants (Figure 4). Differences among years were statistically significant for all parameters, also in the case when only the years 2014 and 2015 were compared. Shoot height and diameter were highest in the year 2014 (Figure 4). Contrary to *M.* × *giganteus*, in the case of *A. donax*, the yield of above-ground biomass gradually increased, and the highest yield was measured in the year 2015 despite deficient rainfall. Probably, the age of plants or higher temperatures in that year may have been the stronger factor affecting the growth of above-ground biomass. It is noteworthy also that plants growing on the edge of the plot gave much more biomass than other plants, and due to this

T a b l e 2

Arundo donax – statistical evaluation of measured parameters

Parameter	Propagation method	Year	
	P-value	*P*-value	Statistical differences by *LSD* (2013/2014/2015)
No. of shoots	0.6607	**0.0120**	ab/a/b
Plant height	**0.0000**	**0.0000**	a/c/b
Shoot diameter	**0.0002**	**0.0003**	a/c/b
Dry biomass	**0.0007**	**0.0053**	a/ab/b

P-value by analysis of variance (bold font indicates statistically significant difference at $\alpha = 0.05$); *LSD* (the least significant difference) test was used as a multiple range test for evaluated years (different letters indicate statistically significant difference at $\alpha = 0.05$)

fact, these plants were excluded from evaluation. The comparison was accomplished in the spring of 2015 and biomass yields were 4.59 kg/plant versus 8.53 kg/plant (plants on the edge of the plot). The situation was very similar in the case of *M.* × *giganteus* also.

An interesting fact is that, in our field conditions, the differences in biomass yield of giant reed and miscanthus grass were much higher in comparison with the long-term experiment of Angelini *et al*. (2009) in Italy, where the average yield of *A. donax* was 37.7 tons and *M.* × *giganteus* was 28.7 tons of dry matter per hectare.

Moisture content in harvested biomass was about 43% in March 2014 and 2015 but only 31% in March 2016. In contrast to *M.* × *giganteus*, where the atypically high moisture content in the harvested biomass was measured after the "wet" growing season of 2014, in *A. donax*, the typical moisture content was measured in this year, but unusually low moisture content was measured after the "dry" season of 2015.

CONCLUSIONS

We can conclude that the propagation method may affect morphological and yield parameters of miscanthus and giant reed plants. One of the alternative methods of vegetative propagation is *in vitro* multiplication via tissue cultures. Apart from the cost of plant propagation, *in vitro* multiplication of vegetatively propagated gigantic grasses is considered an interesting tool for rapid plant multiplication, particularly when a new clone or cultivar has to be propagated in a very short time. Even though the growth of plants in the field conditions may be affected by the method of propagation, strong influence of plant size and vitality independent of propagation method and growth conditions, including date of planting, soil quality and weather conditions, should be considered. In our experiment, the effect of year connected with different weather conditions was stronger than the effect of the method of plant propagation in the case of miscanthus grass. In the case of giant reed, significant effect of both factors was observed. In our study, we confirmed the results of previous studies on *M.* × *giganteus* and obtained new information concerning the differences in growth parameters of *A. donax* plants multiplied by conventional vegetative propagation and *in vitro* method.

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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?

KATARÍNA ONDREIČKOVÁ[1*], ALŽBETA ŽOFAJOVÁ[1], MICHAELA PILIAROVÁ[2], JOZEF GUBIŠ[1], MARTINA HUDCOVICOVÁ[1]

[1]National Agricultural and Food Centre – Research Institute of Plant Production, Piešťany, Slovak Republic
[2]University of SS. Cyril and Methodius in Trnava, Slovak Republic

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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

Mgr. Katarína Ondreičková, PhD. (*Corresponding author), Ing. Alžbeta Žofajová, PhD., Ing. Jozef Gubiš, PhD., Mgr. Martina Hudcovicová, PhD., National Agricultural and Food Centre – Research Institute of Plant Production, Bratislavská cesta 122, 921 68 Piešťany, Slovak Republic. E-mail: ondreickova@vurv.sk
Mgr. Michaela Piliarová, University of SS. Cyril and Methodius in Trnava, Nám. J. Herdu 2, 917 01 Trnava, Slovak Republic

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-

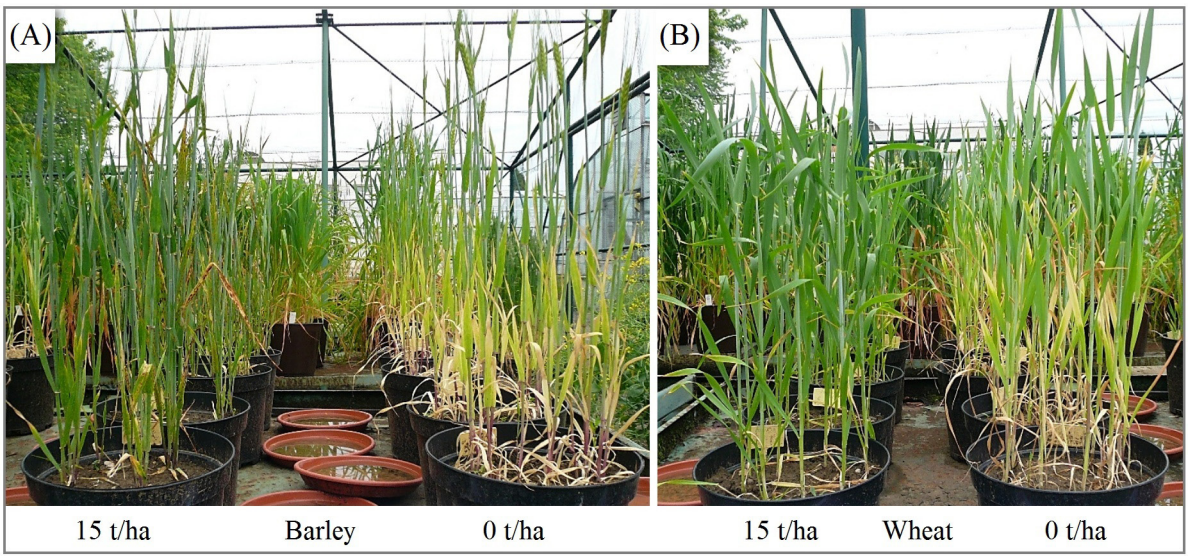

Figure 1. The pot experiment with addition of 15 t/ha of soil additive derived from sewage sludge and agricultural byproducts represented by wastes from grain mill industry and crushed corn cobs. (A) spring barley, cultivar Levan; (B) spring wheat, line PS-6.

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-

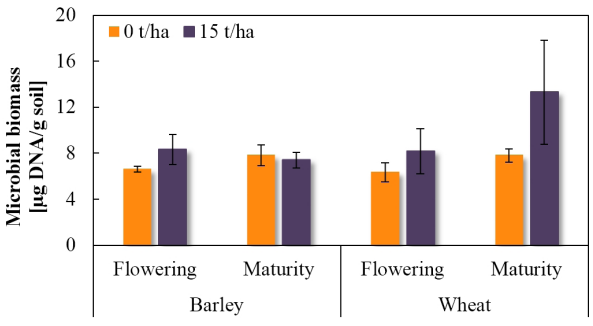

Figure 2. Total microbial biomass expressed as a metagenomic DNA extracted from the rhizosphere of barley and wheat from soil without and with addition of soil additive at a concentration of 15 t/ha. Bar represents standard deviation (n = 3).

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

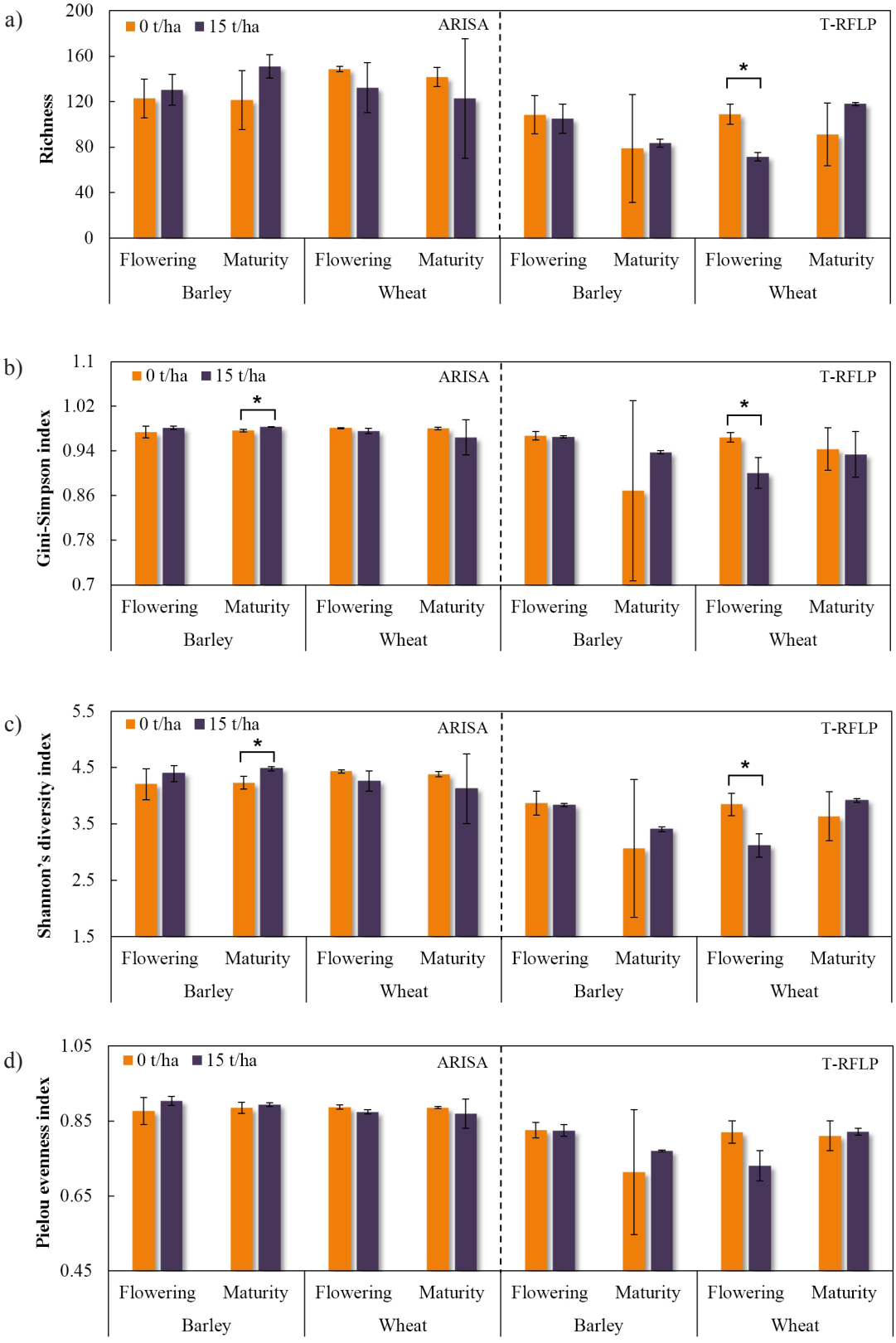

Figure 3. Diversity indices and evenness detected in barley and wheat rhizosphere from soil without and with addition of soil additive at a concentration of 15 t/ha. Bar represents standard deviation (n = 3). *denotes statistically significant difference (*LSD*, α = 0.05).

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

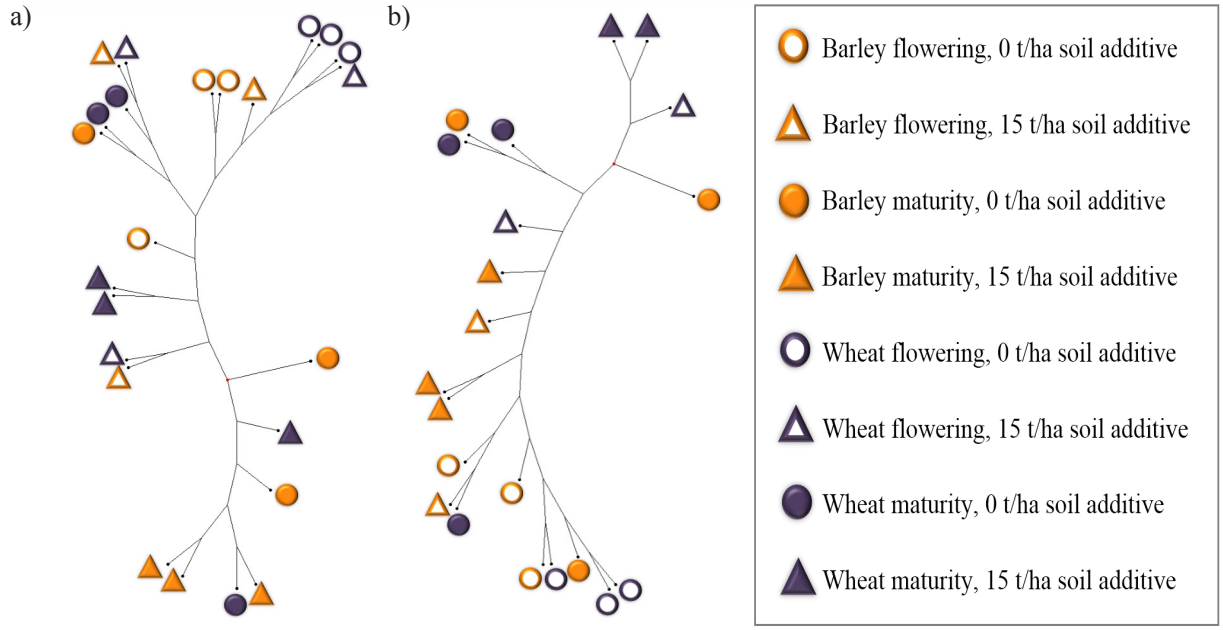

Figure 4. Cluster analysis constructed from a) ARISA binary data and b) T-RFLP binary data of bacterial communities from barley and wheat rhizosphere from soil without and with addition of soil additive at a concentration of 15 t/ha.

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

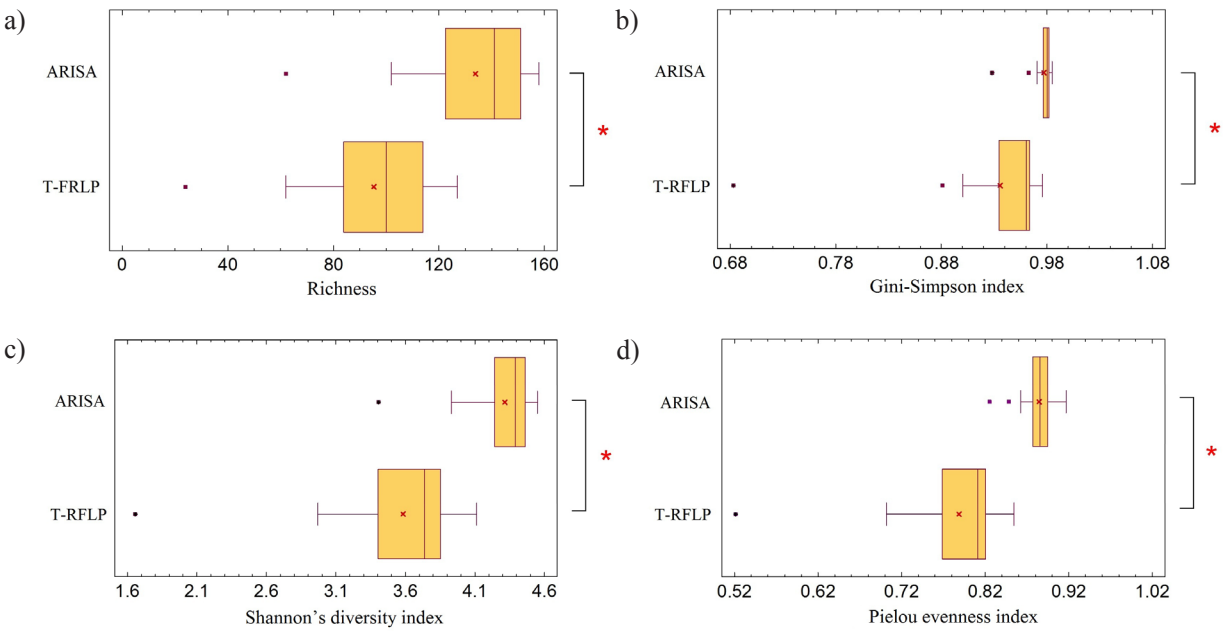

Figure 5. The comparison of ARISA and T-RFLP methods using Box and Whisker plots that were created using data from diversity indices and evenness. *denotes statistically significant difference (*LSD*, α = 0.05).

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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EFFECT OF DIFFERENT PLANT ARRANGEMENTS ON MAIZE MORPHOLOGY AND FORAGE QUALITY

JANA JIRMANOVÁ, PAVEL FUKSA*, JOSEF HAKL, VÁCLAV BRANT, JAROMÍR ŠANTRŮČEK

Czech University of Life Sciences Prague, Czech Republic

JIRMANOVÁ, J. – FUKSA, P. – HAKL, J. – BRANT, V. – ŠANTRŮČEK, J.: Effect of different plant arrangements on maize morphology and forage quality. Agriculture (Poľnohospodárstvo), vol. *62*, 2016, no. 2, pp. 62–71.

A study was carried out in Central Bohemia to understand the effect of row spacing and stand density on plant morphology, productivity and quality of silage maize in two row spacing treatments (0.70 m and 0.35 m) at two stand densities (92,000 plants/ha and 110,000 plants/ha). The results of the study showed that row spacing and stand density had no effect on plant height or weight; however, significantly higher ear ratio and dry matter content was found in narrow rows at 110,000 plants/ha. It was observed that plant morphology was affected more by the interaction between row spacing and stand density than by a single effect of tested factors. Significantly higher dry matter yield was recorded at higher stand density, but there was no row spacing × stand density interaction. Row spacing had no impact on the whole plant neutral detergent fiber (NDF) content, crude protein of stover and starch content of ear, while narrow rows resulted in almost significantly higher stover NDF content. Our results suggest that narrow rows could be advantageous for maize morphology and quality in cases where higher stand density is applied.

Key words: row spacing, stand density, plant height, plant parts, NDF, crude protein, starch content

Maize plant arrangement is one of the most important management tools to improve solar radiation interception and can be done through changes in plant density, row spacing and distribution of plants in the row with the aim of optimizing its use and maximizing the yield (Modolo *et al.* 2010). Generally, plant arrangement is a function of used stand density per area unit and plant spacing in this area.

The effect of plant density on the yield is usually significant and generally predictable (Stone *et al.* 2000). Silage or grain yield increases gradually with increasing plant densities up to plateau, and then the yield decreases. Gözübenli *et al.* (2004) found the highest yield at a density of 90,000 plants/ha, and Çarpici *et al.* (2010) found the same effect at density of 180,000 plants/ha. Regarding plant morphology, minimal or no effect of stand density was recorded on plant height, leaf number and the number of ears per plant (Turgut *et al.* 2005).

However, increasing the plant density reduced the nutritive quality of forage maize. As plant density increases, crude protein (CP) and dry matter digestibility (DMD) decrease, but acid detergent fiber (ADF) and neutral detergent fiber (NDF) increase (Widdicombe & Thelen 2002). The reduction in forage quality with increasing plant density is attributed to the decline in leaf to stalk ratio, as well as reduced ear to whole plant ratio (Baghdadi *et al.*

Ing. Jana Jirmanová, Ing. Pavel Fuksa, PhD. (*Corresponding author), doc. Ing. Josef Hakl, PhD., prof. Ing. Jaromír Šantrůček, CSc., Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resource, Department of Forage Crops and Grassland Management, Kamýcká 129, 165 21, Prague 6-Suchdol, Czech Republic. E-mail: fuksa@af.czu.cz
doc. Ing. Václav Brant, PhD., Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resource, Department of Agroecology and Biometeorology, Kamýcká 129, 165 21, Prague 6-Suchdol, Czech Republic

2012). However, Çarpici *et al.* (2010) recorded no effects of stand density on leaf percentage, CP and NDF.

The reported influence of row spacing on maize yield and quality has been inconsistent. Alternative spatial arrangement should theoretically decrease plant-to-plant competition, alleviate crop crowding stress and improve yields (Robles *et al.* 2012). Reduction in row spacing provides a more uniform distribution among plants, which can increase yield (Strieder *et al.* 2008). The benefits of narrow row spacing can depend on the kind of crop management system (Strieder *et al.* 2008), the hybrid used (Baron *et al.* 2006) and the environmental conditions (Balkcom *et al.* 2011).

Regarding forage quality, the narrow row production system neither had any impact on the nutritive value of maize, such as DMD, ADF, NDF and CP (Widdicombe & Thelen 2002), nor on the concentration of starch, simple sugars and digestibility of NDF (Beres *et al.* 2008). However, Baron *et al.* (2006) and Skonieski *et al.* (2014) recorded higher content of CP for conventional rows compared with the narrow rows.

The variable results of the effect of row spacing suggested that this effect is strongly related to the environment, as well as other crop management tools including plant density. Experiments with alternative spatial arrangement have been conducted predominantly in the USA (Widdicombe & Thelen 2002; Balkcom *et al.* 2011), Argentina (Barbieri *et al.* 2012), Brazil (Strieder *et al.* 2008; Modolo *et al.* 2010), Turkey (Turgut *et al.* 2005; Çarpici *et al.* 2010; Gözübenli 2010), as well as in Pakistan (Maqbool *et al.* 2006), Iran (Ramezani *et al.* 2011) and New Zealand (Stone *et al.* 2000). For Central Europe region, there is a lack of results for alternative spatial arrangement, especially for maize forage quality.

The aim of this study was to investigate the effect of row spacing on plant morphology, productivity and nutritive value of silage maize in the Czech Republic. In view of the parallel effect of plant density on evaluated traits, two levels of stand density were used in this study.

MATERIAL AND METHODS

The field experiments were conducted at two locations of Central Bohemia, Czech Republic, in the growing season of the year 2013.

Experiment A: Plot experiment was established on the experimental field of the Faculty of Agrobiology, Food and Natural Resources at the Czech University of Life Sciences Prague (50°7′39″ N, 14°22′19″ E, 286 m a.s.l.). The long-term annual air temperature at the experimental field is 9.1°C and the total sum of precipitation is 495 mm. Daily sums

Table 1

Monthly sums of precipitation and mean air temperature for the period from 1.4.2013 to 30.9.2013 (Experiment A – Prague, Experiment B – Budihostice)

	Experiment A		Experiment B	
	Air temperature	Precipitation	Air temperature	Precipitation
	[°C]	[mm]	[°C]	[mm]
April	9.6	25.3	9.3	24.3
May	12.7	106.5	12.8	104.7
June	16.8	173.4	16.6	127.6
July	20.6	54.3	20.0	44.7
August	18.5	89.5	18.4	89.7
September	13.1	37.5	13.3	58.5
Vegetation period	15.3	486.5	15.1	449.5

of precipitation and mean air temperature for the vegetation period are shown in Figure 1. Monthly sums of precipitation and mean air temperature are presented in Table 1. The soil was Haplic Chernozem. Soil types were determined according to the World Reference Base (IUSS Working Group WRB 2014).

The tested stand densities of maize were 92,000 and 110,000 plants/ha at row spacing treatments 0.70 and 0.35 m, respectively. The experiment was designed at Latin square with four replications of the main plot. Plot size was 2.8 × 5 m with four and eight rows for conventional and narrow rows, respectively. Maize (hybrid Kuxxar, FAO 300) was hand-sown to a depth of 4 cm on 29 April 2013.

At the experimental site, the silage maize had been continuously cultivated since 2004 under conventional tillage practices. Fertilisers were applied prior to seeding at rates of 120 kg N/ha (ammonium sulphate), 45 kg P/ha (superphosphate) and 120 kg K/ha (potassium chloride). Weed control was ensured using post-emergent herbicide Laudis (100 g tembotrione/ha and 50 g isoxadifen-ethyl/ha). Two (0.70 m row spacing) or four (0.35 m row spacing) rows at the centre of each plot were manually harvested at optimal silage maturity on 11 September 2013.

Experiment B: An additional experiment verifying the influence of row spacing in field conditions was established in the experimental area situated near village Budihostice (50°19′7″ N, 14°15′42″ E, 233 m a.s.l.). In this area, the long-term annual air temperature is 9.6°C and the total sum of precipitation is 582 mm. Daily sums of precipitation and mean air temperature are presented in Figure 1, and monthly averages are summarized in Table 1. According to the World Reference Base (IUSS

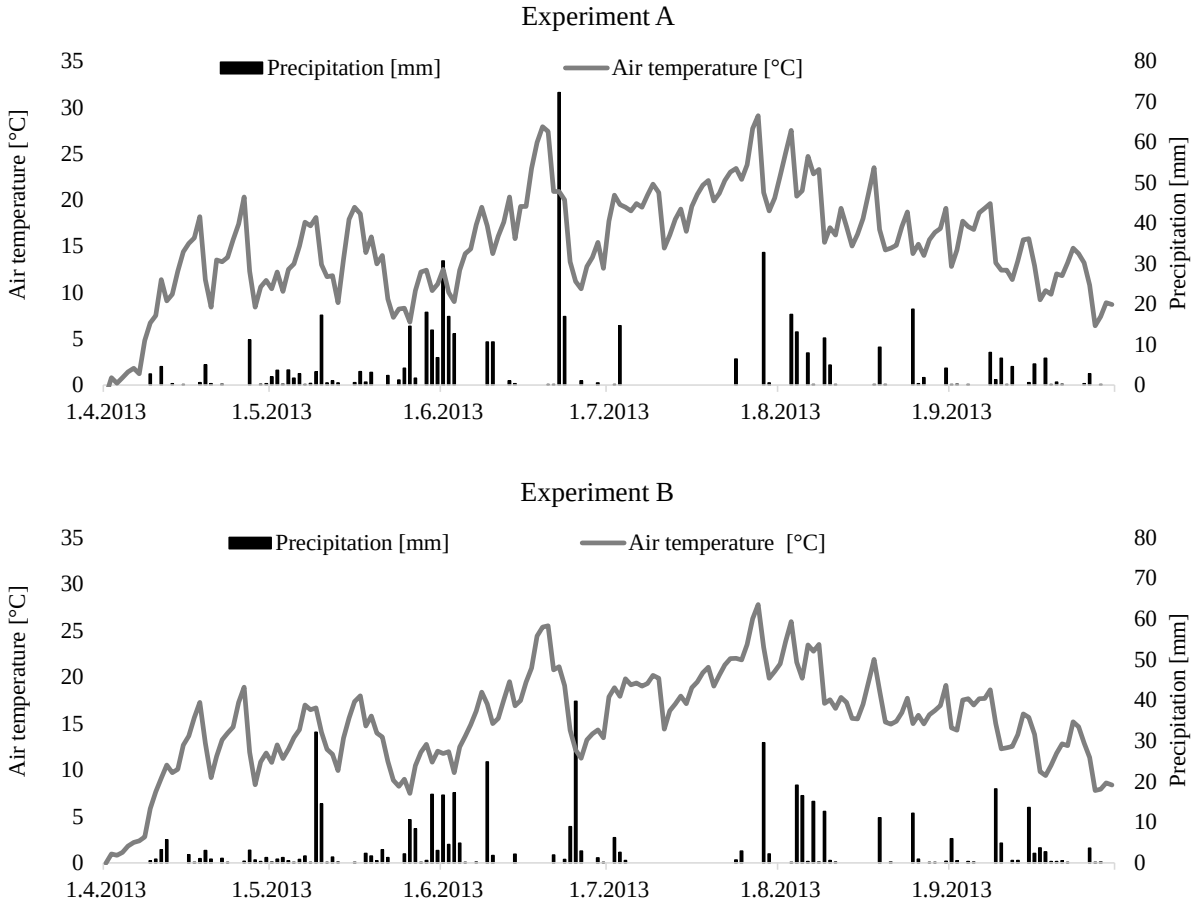


Figure 2. Daily sums of precipitation and mean air temperature for the period from 1.4.2013 to 30.9.2013 (Experiment A – Prague, Experiment B – Budihostice)

Working Group WRB 2014), the soil was Haplic Chernozem.

Row spacing treatments were 0.75 and 0.45 m, with the average number of plants per hectare being 89,000 plants/ha and 88,000 plants/ha, respectively. Hybrid PR38N86 (FAO 290) was sown on 19 April 2013 by six-row seeder Kverneland Accord Optima HD. Experimental plot size was 1 ha for each treatment and sampling was realised within each plot. The maize followed the winter wheat (*Triticum aestivum* L.). Tillage practices, fertilisation and weed control were identical with Experiment A. The

T a b l e 2

Effect of row spacing (RS) and stand density (SD) on plant height, plant weight, proportion of plant parts and dry matter yield
(Experiment A – Prague, Experiment B – Budihostice)

Site	Row spacing [m]	Stand density [plants/ha]	Plant height [m]	Plant weight [g]	Leaf	Stalk	Ear	Yield [t/ha]
					[g/kg]			
Experiment A	0.35	92,000	2.37	270	124	286	590^{ab}	20.0
		110,000	2.37	248	133	250	616^{a}	22.7
	0.70	92,000	2.39	245	121	285	594^{ab}	19.2
		110,000	2.43	296	130	294	576^{b}	21.5
	P	RS	0.709	0.526	0.633	0.090	0.089	0.281
		SD	0.817	0.446	0.236	0.270	0.660	0.013
		RS × SD	0.819	0.066	0.994	0.081	0.041	0.805
Experiment B	0.45	88,000	3.14	289	113	287	599	24.0
	0.75	89,000	3.11	281	117	279	603	25.3
	P	–	0.724	0.602	0.315	0.381	0.714	0.223

P = probability; different letters indicate statistical differences for Tukey *HSD* (α = 0.05)

T a b l e 3

Effect of row spacing (RS) and stand density (SD) on the dry matter content of whole plant (DMC_{WP}), neutral detergent fiber of whole plant (NDF_{WP}), neutral detergent fiber of stover (NDF_S), crude protein of stover (CP_S) and starch of ear
(Experiment A – Prague, Experiment B – Budihostice)

Site	Row spacing [m]	Stand density [plants/ha]	DMC_{WP}	NDF_{WP}	NDF_S	CP_S	$Starch_{EAR}$
			[g/kg]				
Experiment A	0.35	92,000	318^{a}	410	604	51	467
		110,000	347^{b}	405	616	45	463
	0.70	92,000	327^{ab}	416	595	45	470
		110,000	333^{ab}	411	571	46	472
	P	RS	0.663	0.484	0.099	0.663	0.817
		SD	0.015	0.563	0.700	0.706	0.974
		RS × SD	0.085	0.923	0.256	0.530	0.913
Experiment B	0.45	88,000	327	436	663	69	551
	0.75	89,000	316	444	687	63	552
	P	–	0.264	0.375	0.136	0.406	0.947

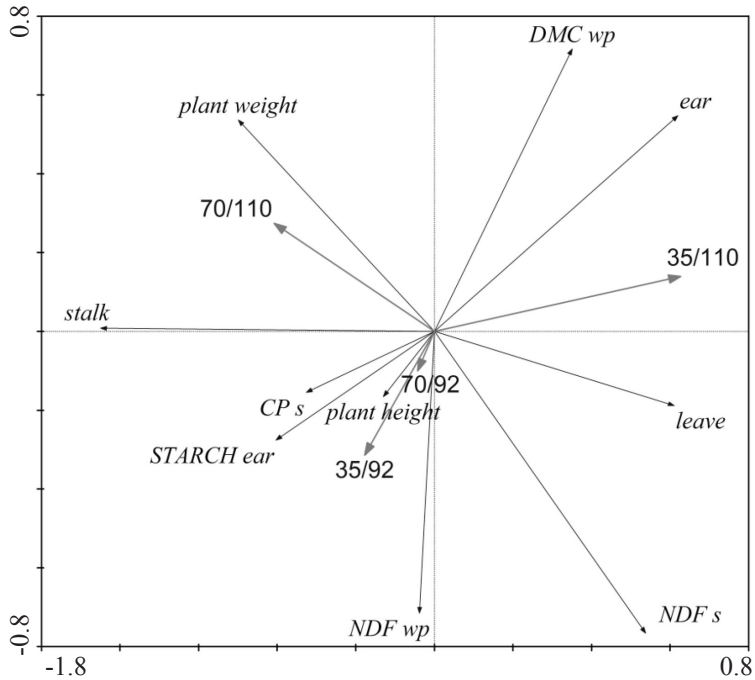
P = probability; different letters indicate statistical differences for Tukey *HSD* (α = 0.05)

maize was harvested at optimal silage maturity on 10 September 2013. The yield was assessed using the average plant weight (evaluated twenty plants per treatment) and the number of plants per hectare.

Harvest measurements and plant morphology: The measurement of plant height and sampling of above-ground biomass were realized during harvest time. In Experiment A, plant height was measured at 20 plants in the centre of two rows of each plot from the soil surface to the tip of tassel (m), and four plants were randomly selected (from each plot) to determine dry matter content (DMC) and forage quality characteristics. In Experiment B, 4 × 10 plants were measured for height and 4 × 4 plants from each tested spatial arrangements were selected for the following analyses. The sampled fresh plants were divided into ear, leaf and stalk, and dried at 60°C in a forced-air dryer for calculation of their weight percentage ratio.

Forage quality: Dried samples of plant parts from each plot were ground in a mill on sieve with 1 mm mesh size. Mixed samples of leaves and stalks (i.e. the stover) were analysed for crude protein (CP, N x 6.25) by Dumas combustion method (Dumatherm N analyser). Amylase-treated neutral detergent fibre (2002.04) in stover and starch in ear by the amylase method (920.40) were assessed according to Association of Official Analytical Chemists (2005). In the ear, the NDF content 283 g/kg was considered to be constant value. The NDF of whole plant was calculated from ear ratio and NDF content in the stover.

Statistical analysis: The data of maize yield, plant morphology and forage quality were statistically evaluated by using two- and one-way analysis of variance in Experiments A and B, respectively. All analyses were performed using Statistica 12 (2013) followed by Tukey post-hoc test ($\alpha = 0.05$). Ordination biplot of principal component analysis (PCA) was created in CanoDraw (Microcomputer Power, Ithaca, NY) for graphical visualization of the relationship between maize morphology and quality (dependent variables), and row spacing and stand density (combination of groups used as supplementary variables). All ordination analyses were



DMC_{WP} – dry matter content of whole plant; NDF_{WP} – neutral detergent fiber of whole plant; NDF_S – neutral detergent fiber of stover; CP_S – crude protein of stover; treatments are described as row spacing (70 and 35 cm)/ stand density (92,000 and 110,000 plants/ha)

Figure 2. Ordination biplot of PCA shows the relationship between maize plant morphology and forage quality (dependent variables) regarding different row spacing and stand density (supplementary variables), Experiment A – Prague

performed in CANOCO for Windows 4.5 program (ter Braak & Šmilauer 2002).

RESULTS

Maize plant morphology and dry matter yield in relation to row spacing and stand density are summarized in Table 2. In the field Experiment A, the impact of row spacing was almost significant for ear and stalk proportion. Stand density showed significant effect only on the yield. However, the interaction row spacing × stand density was significant or almost significant for plant weight, stalk and ear proportion. Within 0.35 m rows, increasing the stand density reduced plant weight and stalk proportion, whereas ear proportion was significantly higher than those in 0.70 m rows under higher stand density. In the field Experiment B with one level of stand density, any significant difference was found for all evaluated traits in line with lower density in Experiment A.

Differences in DM content and forage quality over different row spacing and stand density are shown in Table 3. In Experiment A, the effect of row spacing was almost significant just for stover NDF content, where higher values were observed within 0.35 m rows regardless of stand density. Higher stand density tends to increase of DM content but this effect was visible only within 0.35 m rows where the highest value was observed. In Experiment B, any differences between row widths were not visible at all evaluated traits.

The effect of row spacing and stand density on relationship among maize plant morphology and forage quality in Experiment A is illustrated in the ordination biplot of PCA (Figure 2). The most important first (horizontal) canonical axis represents the positive correlation between the stalk opposite to ear and leaves proportion; the second (vertical) axis represents the negative relation between DM content and plant NDF. Both axes were strongly affected by the negative relationships between plant weight and stover NDF, and between ear ratio and ear starch concentration. Regarding the external supplementary factors, both treatments with lower stand density were located in the centre of the figure regardless of row spacing. In contrast to this, higher stand density separated treatments according to row spacing: 0.35 m to right and 0.70 m to left side (see Figure 2). Narrow rows under higher density were associated with higher ear and leave ratio, as well as DM content and stover NDF. Plant weight and stalk proportion were related to 0.70 m row spacing.

DISCUSSION

The presented values of plant morphology and forage quality traits were within the usual ranges published about maize plot experiments (Cox & Cherney 2001; Millner & Villaver 2005). Our results showed that the impact of different row maize spacing on morphology and forage quality was not meaningful, similar to the results presented by Widdicombe and Thelen (2002) or Stone *et al.* (2000). Also, the differences in stand density did not show any significant changes within the given ranges, except for DM content. It must be taken into account that evaluated year 2013 was humid; 98% and 77% of long-term annual sum of precipitation was achieved over vegetation period in localities of Experiment A and B, respectively. In 2013, the yield was considerably influenced by the climatic condition of the growing season. In this year, in the Czech Republic, the average yields of silage maize were lower by 19.6% and 19.1% in comparison to years 2012 and 2014 according to Czech Statistical Office (2015), respectively. Significant single effect of the year on the yield of grain and silage maize was described by many authors (Çarpici *et al.* 2010; Novacek *et al.* 2013), but significant year × row spacing or year × stand density interactions were not determined (Barbieri *et al.* 2012; Novacek *et al.* 2013).

In spite of a humid experimental year, almost significant interactions between row spacing and stand density were observed. It appeared that the effect of plant spacing on evaluated traits was enhanced due to an increase in the plant population density in the present experiment.

Plant morphology and dry matter yield

Regarding the plant morphology under higher population density in our experiment, wide rows increased the plant weight and stalk proportion, while the ear ratio was significantly reduced. In spite of

these changes in proportion, the plant height as well as leaves ratio were relatively stable across all treatments. Reduced ear ratio under increased plant yield was in accordance with the results reported by Çarpici *et al.* (2010). According to Baghdadi *et al.* (2012), the reduction in forage quality with an increasing stand density (from 90,000 to 130,000 plants/ha) was attributed to the decline in leaf to stalk ratio, as well as reduced ear to whole plant ratio. On the contrary, Iptas and Acar (2006) did not observe any significant changes in ear proportion between different row spaces; however, changes in stand density was not independent of row spacing in their experiment. Millner and Villaver (2005) or Ramezani *et al.* (2011) did not find any significant effect of stand density on plant part proportion and plant weight.

Plant height is an important component that helps in the determination of growth attained during the growing period (Zamir *et al.* 2011). In our experiment, this trait was not affected by stand density or row spacing arrangement. This is in agreement with Çarpici *et al.* (2010) or Ramezani *et al.* (2011) for different stand densities and with Turgut *et al.* (2005) for various row spacings. However, some effects on plant height were previously described by Gözübenli (2010) who reported significantly higher plant height in narrow rows compared with conventional rows (2.07 vs. 2.00 m) on average of two years, while Ramezani *et al*. (2011) observed the opposite effect (2.10 vs. 2.23 m).

Our result suggests an increase in ear ratio under higher density level in narrow rows, which corresponds with Modolo *et al.* (2010), who reported some tendency of narrow rows to produce higher grain yield. It is possible to assume that ear ratio reduction together with increase in the stalk proportion could be observed, where row spacing, stand density or their interaction increase the plant weight.

Significantly higher dry matter yield at higher plant stand density corresponded with the results of many authors. Across two years, Çarpici *et al*. (2010) published a significantly higher dry matter yield (21.26 t/ha) at 180,000 plants/ha in comparison to 60,000 and 100,000 plants/ha (18.72 and 19.45 t/ha, respectively). Baron *et al*. (2006) found greater impact of population density on yield increase (+6.4%) than row spacing and hybrid choice during two-year experiments. In Experiment A, the yield (4.2% at 92,000 plant/ha and 5.6% at 110,000 plant/ha) increased when the row spacing was narrowed from 0.70 to 0.35 m. Comparable increase of yield (5.4%) for narrow rows was presented by Widdicombe and Thelen (2002). Similar to Baron *et al.* (2006), no row spacing × stand density interaction was found in our experiment as well. In Experiment B, higher yield in narrow rows was not observed, which corresponded with Skoniesky *et al.* (2014). These results highlighted that hybrids evaluated in two experimental localities did not show the same effect in similar row spacing, as was published by Turgut *et al.* (2005).

Forage quality

Maize DM content parameter is closely connected with harvest timing (Lynch *et al.* 2012). Non-significant differences in maize DM content were found under various row spacings (Iptas & Acar 2006) or stand densities (Millner & Villaver 2005). Similarly, Beres *et al.* (2008) did not observe any effect of stand density, row spacing or their interaction on maize DM content under applied irrigation. In the present experiment, lower stand density significantly reduced DM content but this effect was visible only for narrow rows. Within the same density, row effect was not significant. It appeared that simple row spacing effect on DM content was small but could be enhanced by interaction with plant population density. However, this is also modified by environmental conditions where these changes seem to be eliminated under irrigation (Beres *et al.* 2008). In present experiment, significantly reduced DM content was observed at narrow rows treatment under drier end of vegetation period.

Regarding plant NDF concentration, the differences between row spacing were not significant at both sites. It is in line with Widdicombe and Thelen (2002) that the narrow row production system did not impact maize NDF content. However, some impact of row spacing in Experiment A was visible in stover NDF content, where narrow rows resulted in almost significantly higher stover NDF content. Higher difference was found at 110,000 plants/ha than at 92,000 plants/ha. Similarly, at lower stand density in Experiment B, minimal difference in stover NDF content was observed. Our

result suggests that the effect of row spacing on NDF was higher in stover than in the whole plant. Similar tendency for whole plant NDF content was reported by Beres *et al.* (2008). Under various stand densities, Çarpici *et al.* (2010) and Marsalis *et al.* (2010) had not found any significant effect on plant NDF, which is in accordance with our results. However, Widdicombe and Thelen (2002) recorded an increase in NDF (from 441 to 456 g/kg) as the stand density increased from 64,200 to 88,900 plants/ha. Cox and Cherney (2001) found significantly higher NDF concentrations (473 g/kg) at 116,000 plants/ha compared with 80,000 plants/ha (460 g/kg).

Starch content in the ear was not affected by plant spacing arrangement in both the experiment sites. Contrary to this, Beres *et al.* (2008) reported an almost significant reduction in starch content in whole plant maize biomass under narrow rows treatment. This discrepancy could probably be explained by the changes in plant part proportions. It seems that starch content in maize biomass can be influenced by row spacing but our result shows that it is more closely connected with ear proportion than starch content in the ear.

Similar to starch, CP content in the stover was also not affected by either row spacing or stand density. It is in line with the research by Çarpici *et al.* (2010) and Marsalis *et al.* (2010) in which they reported no significant effect of stand density on CP content. Conversely, Widdicombe & Thelen (2002) recorded a decrease in CP (from 76 g/kg to 72 g/kg) as the stand density increased from 64,200 plants/ha to 88,900 plants/ha. Also Cox and Cherney (2001) found significantly lower CP concentrations at 116,000 plants/ha (52 g/kg) compared with 80,000 plants/ha (55 g/kg). Regarding row spacing, Skonieski *et al.* (2014) recorded a significantly higher content of CP (70 and 68 g/kg) for conventional rows (0.60 and 0.80 m) compared with narrow rows (54 g/kg at 0.40 m). Also, Baron *et al.* (2006) found marginally higher protein concentration (74 g/kg) in comparison with narrow rows (72 g/kg). It is, therefore, possible to conclude that the reported effects of stand density or row spacing for CP content could be significant; however, the differences observed by the above-cited studies were generally marginal for absolute values. The expression of these effects in CP content has also been related to site conditions (Cusicanqui & Lauer 1999), intensity of nitrogen fertilisation (Cox & Cherney 2001) and applied levels of stand density (Çarpici *et al.* 2010).

CONCLUSIONS

In the humid year 2013, a significant increase in maize yield was observed under higher stand density. The influence of row spacing effect on maize yield, morphology and quality was not meaningful at both the sites. In experiment A, plant morphology (ear ratio) and quality (stover NDF) were more affected by the interaction between row spacing and stand density than by a row spacing effect. In line with Experiment B, it seems that observed differences in these traits were smaller under low stand density. Using of narrow rows showed benefit in higher yield; however, achieved results reveal that forage quality should be also considered. It appeared that narrow rows could increase ear ratio under higher stand density but tendency for higher stover NDF content was also observed.

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EFFECT OF CADMIUM ON GROWTH, PHOTOSYNTHETIC PIGMENTS, IRON AND CADMIUM ACCUMULATION OF FABA BEAN (*VICIA FABA* CV. AŠTAR)

BEÁTA PIRŠELOVÁ[1*], ROMAN KUNA[1], PETER LUKÁČ[2], MICHAELA HAVRLENTOVÁ[3]

[1]Constantine the Philosopher University in Nitra, Slovak Republic
[2]West Slovakia water company, a.s. Nitra, Slovak Republic
[3]National Agricultural and Food Centre – Research Institute of Plant Production, Piešťany, Slovak Republic

PIRŠELOVÁ, B. – KUNA, R. – LUKÁČ, P. – HAVRLENTOVÁ, M.: Effect of cadmium on growth, photosynthetis pigments, iron and cadmium accumulation of faba bean (*Vicia faba* cv. Aštar). Agriculture (Poľnohospodárstvo), vol. *62*, 2016, no. 2, p. 72–79.

The influence of different concentrations of cadmium (Cd) ions (50 and 100 mg/kg soil) on growth, photosynthetic pigment content, Cd, and iron accumulation in faba bean (*Vicia faba* L. cv. Aštar) was studied under laboratory conditions. No significant changes were observed in the growth parameters of shoots (length, fresh, and dry weight). Both tested Cd doses resulted in decrease in root fresh weight by 31.7% and 28.68% and in dry weight by 32.2% and 33.33%, respectively. Increased accumulation of Cd was observed in roots (125- and 173- fold higher than in control) and shoots (125- and 150- fold higher than in control) as a result of applied doses of Cd. Increased accumulation of iron was detected in roots (1.45- and 1.69-fold higher than in control). Decrease in the content of chlorophyll *a* (by 25.52 and 24.83%, respectively) and chlorophyll *b* (by 6.90%) after application of Cd 100 as well as decrease in carotenoids (by 40.39 and 38.36%, respectively) was detected. Weak translocation of Cd from roots to shoots pointed to low phytoremediation potential of the tested bean variety in contaminated soil. However, the high tolerance of this cultivar, its relative fast growth, as well as priority of Cd accumulation in roots presume this plant species for phytostabilisation and revegetation of the Cd-contaminated soils.

Key words: faba bean, cadmium, tolerance, photosynthesis, oxidative stress, remediatory potential

Contamination of soils with Cadmium (Cd) is a major threat to ecosystems. Cd is rapidly taken up by plant roots and can be loaded into the xylem for its transport to leaves. Many species accumulate toxic metals mainly in the roots (Benavides *et al*. 2005); according to Wu (1990), about 70–85% of the absorbed Cd remains in the roots in various plants. The differences in Cd accumulation capacity and localisation appear to be the major factors in determining plant tolerance to Cd exposure (Obata & Umebayashi 1993). The toxic effect of Cd is related to its ability to generate reactive oxygen species (ROS) resulting in unbalanced cellular redox homeostasis (Schützendübel *et al*. 2001). The ROS generation is indirect because Cd does not participate in Fenton-type reactions; therefore, it is a non-redox metal (Romero-Puertas *et al*. 2004). In plants, exposure to Cd causes inhibition of growth, activation or inhibition of enzymes, reduction of transpiration rate and water content (Benavides *et al.* 2005). Stomatal closure due to entry of Cd into the guard cells in competition to Ca^{+2} (Perfus-Barbeoch

RNDr. Beáta Piršelová, PhD. (*Corresponding author), doc. RNDr. Roman Kuna, PhD., Department of Botany and Genetics, Faculty of Natural Sciences, Constantine the Philosopher University in Nitra, Nábrežie mládeže 91, 949 74 Nitra, Slovak Republic. E-mail: bpirselova@ukf.sk; rkuna@ukf.sk
RNDr. Peter Lukáč, West Slovakia water company, a.s., Nábrežie za hydrocentrálou 4, 949 01, Nitra, Slovak Republic. E-mail: rndr.peter.lukac@gmail.com
RNDr. Michaela Havrlentová, PhD., National Agricultural and Food Centre – Research Institute of Plant Production, Bratislavská cesta 122, 921 68 Piešťany, Slovak Republic. E-mail: havrlentova@vurv.sk

et al. 2002) and reduction in stomata count per unit area are also characteristic symptoms of Cd stress resulting in lesser conductance to CO_2 (Pietrini *et al*. 2010), which consequently lead to the overall inhibition of photosynthesis. In addition, Cd may disturb plant mineral metabolism. For example, Cd almost completely inhibits iron (Fe) translocation from roots to shoots, leading to increased root Fe concentrations in plants (Muradoglu *et al.* 2015).

Many studies have attempted to clarify the mechanism of Cd toxicity in plants (Békésiová *et al.* 2008; Tamás *et al.* 2012; Balestri *et al.* 2014); however, relationships between growth inhibition and physiological processes under Cd condition are still discussed. Mainly because of the fact that its toxic effects are expressed in relation to plant species or varieties. The toxicity of Cd is also greatly influenced by the concentration of Cd^{2+} ions, their form and availability in the soil, duration of their application, as well as by other different factors of the environment (pH of the soil, soil humidity, and others). There are also no univocal reports on the relationships between Cd stress and some physiological processes (e.g., water relations) since Cd can interfere in several ways on the parameters that affect these physiological processes in leaves (Barceló & Poschenrieder 1990). Knowledge of mechanisms of plants' tolerance to heavy metals ions provides an opportunity of breeding varieties suitable for phytoremediation. Besides, metal hyper-accumulating plants, non-accumulating Cd, and high biomass crops are also considered for phytoextraction purposes, but it has been suggested that the success of this approach might be limited by Cd-induced phytotoxicity problems (McGrath *et al.* 2001). Although plants belonging to family *Fabaceae* are sensitive to high concentrations of heavy metals (Kuboi *et al.* 1987), several studies indicated that plant such as *Lupinus albus or Vicia faba* are used in re-vegetation and phytostabilization of cadmium contaminated soils (Vazquez *et al*. 2006; Pichtel & Bradway 2008).

In the presented article, the influence of different concentrations of Cd ions (50 and 100 mg/kg soil) on growth, photosynthetic pigment content, Cd and Fe accumulation in faba bean (cv. Aštar) is presented. In addition, the potential of broad bean for the phytoremediation of Cd in a contaminated soil was presented.

MATERIAL AND METHODS

Plant material and growth conditions

Seeds of beans (*Vicia faba* cv. Aštar) were surface-sterilized with 5% sodium hypochloride for 15 min and planted in pots containing mix of soil (BORA, pH 6–7, 1.0% N; 0.3% P_2O_5; 0.4% K_2O) and perlite (4:1). The plants were cultivated in a growth chamber at 20°C, 12 h light/12 h dark period (illumination of 400 lux), and relative humidity 60–70%. Pots were watered daily to 60% water-holding capacity of the soil. When the first assimilating leaves were developed, plants were supplied with distilled water (control) or two doses of Cd: 50 (Cd 50) and 100 (Cd 100) mg/kg of soil, respectively. Cd was added as $Cd(NO_3)_2.4H_2O$.

The test concentrations of cadmium were used due to predicted toxicity of this element to bean plants (Piršelová *et al*. 2015).

Growth parameters

On day 10 after application of metal solutions (BBCH 31-2 visibly extended internodes), roots were separated from the above-ground part of the plants, washed with tap water, and growth parameters (length and fresh weights) were determined. After washing, the plant samples were oven-dried at 70°C for 24 h to constant dry weight, and this parameter was also determined. Three replicates were used per treatment and eight plants from each pot were analysed (altogether 24 plants).

Photosynthetic pigments determination

For photosynthetic pigments (chlorophyll *a* and *b*, carotenoids) analysis, fully developed trifoliate leaves were extracted with 80% acetone. Pigments contents were determined spectrophotometrically (UV-VIS spectrophotometer, Shimadzu) at the following wavelengths: 663, 646 and 470 nm and calculated according to Lichtenthaler and Wellburn (1983). The experiment was performed in four replicates.

Determination of tolerance index

Tolerance index (TI) was calculated as a ratio of the mean dry weight of plants grown in the presence of Cd and the mean dry weight of control plants expressed as percentage.

In vivo detection of H_2O_2 in leaves

Diaminobenzidine (DAB) was used for the detection of H_2O_2 staining in leaf tissues (Thordal-Christensen *et al.* 1997). On day 10 after application of metal solutions, fully developed leaves (the first bifoliate – developmental stage 1 and second trifoliate – developmental stage 2) excised from Cd-treated plants (50 and 100 mg Cd^{2+}/kg soil) or from untreated plants were placed in Petri dishes containing DAB solution (1 mg/ml). Plates were left in a climate chamber at 24°C in darkness, and DAB staining was assessed visually 12 h later. Leaves were bleached by immersing in boiling ethanol to visualize the brown spots characteristic of the reaction of DAB with H_2O_2.

Measurements of metal content in leaves and roots

Dried plant material (0.5 g roots and shoots) was digested in the mixture of 5 ml water, 5 ml of concentrated HNO_3 p.a. (Merck, Darmstadt, Germany), and 1.5 ml of H_2O_2 p.a. (Slavus, Bratislava) by using the microwave oven Mars Xpress (CEM Corporation, Matthews, USA). Decomposition temperature was 140°C, ramp time 15 min, and hold time 13 min. After digestion, the solution was diluted to 25 ml with deionised water and filtered through an acid-resistant cellulose filter (Whatman No. 42). Blank samples were prepared in a similar way. The elements (Cd and Fe) were determined by electrothermal atomic absorption spectroscopy (AAS Perkin Elmer 1100B, Norwalk, Connecticut, USA).

The biological accumulation coefficient for cadmium - BAC, biological transfer coefficient - BTC and biological concentration factor - BCF were determined (Tukura *et al.* 2012).

BAC = (metal content in the above-ground part of plant/metal content in soil) × 100

BTC = (metal content in the above-ground part of plant/metal content in root) × 100

BCF = (metal content in root/metal content in soil) × 100

Statistical analysis

Data were analysed by one-way ANOVA or Kruskal-Wallis tests using XLSTAT software. The significance of differences between the concentrations of heavy metals in plant tissues was shown by using the Student's t-test, $P < 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Plant growth

Growing in a contaminated soil, the bean plants did not show any apparent visual symptoms of intoxication by the metal. Similar conclusion was also reached by Dobroviczká *et al*. (2013) at cultivation of soybean (*Glycine max* cv. Bólyi 44, cv. Cordoba) in soil contaminated with Cd in concentration of 50 mg/kg soil and by Pinto *et al.* (2004), who exposed sorghum (*Sorghum* sp.) to various doses of Cd.

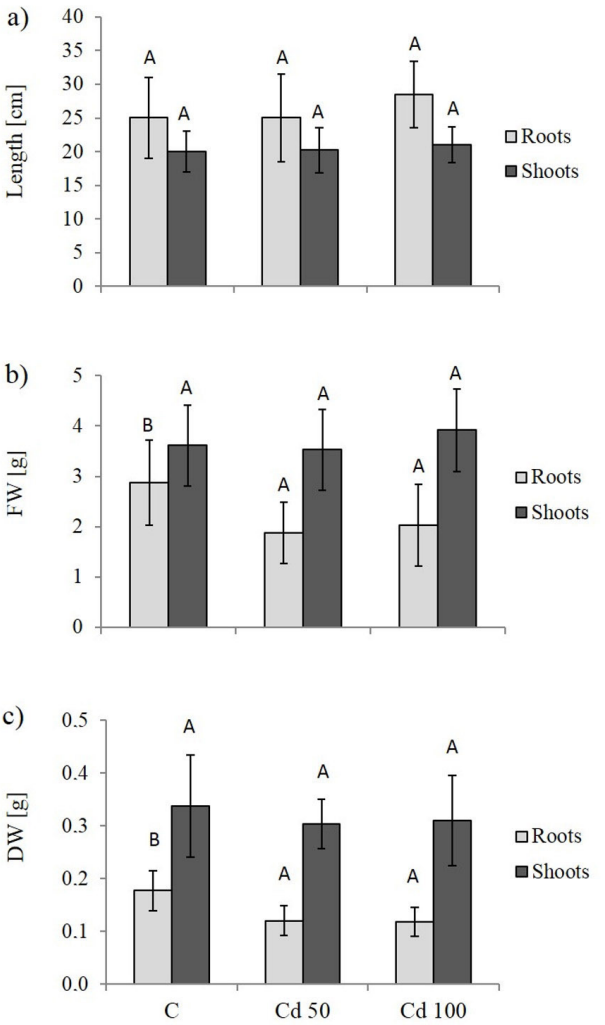

Figure 1. Effect of cadmium on length – a, fresh weight (FW) – b, and dry weight (DW) – c of roots and shoots of bean plants. Data are presented as means ± SD, n = 24. Different letters indicate significant differences at $p < 0.05$.

Plant length, fresh and dry weight of shoots were not significantly affected by Cd (Figure 1); however, each of the tested doses of Cd resulted in decrease of root fresh weight by 31.70 and 28.68% and dry weight by 32.2% (TI = 67.80) and 33.33% (TI = 66.67), respectively (Figure 1). Decrease in root biomass after exposure to Cd was also observed by others (Kochlar *et al*. 2004; Rodriguez-Serrano *et al.* 2009). By contrast, low doses of Cd often cause increase in the amount of fresh biomass of shoots (Pinto *et al.* 2004; Shah *et al*. 2008). In our experiment, due to doses Cd 50 and Cd 100, the length of shoots was also increased by 1.35% and 5.08% (Figure 1a), and fresh biomass of shoots was increased by 0.82 and 4.41%, respectively (Figure 1b). Detected TI calculated on the dry mass of roots and shoots (66.67–91.99) suggests high tolerance of the given variety to Cd. Plants with TI higher than 60 are considered as tolerant (Lux *et al.* 2004).

Accumulation of Cd and Fe in plant tissue

With increased concentration of the applied metal, also the increased accumulation of Cd in roots (125 and 173-more compared to the control) and in shoots (125 and 150-more compared to the control) of faba bean was observed (Table 1). Our results indicate that the majority of Cd was accumulated in the roots, which suggests a strong Cd retention during its long distance transport from roots to shoots, which might be a plant mechanism to tolerate the metal stress (Zornoza *et al.* 2002). Increased Fe accumulation was detected only in roots (1.5 and 1.69-more compared to control). In shoots, just the same content of Fe was detected in control as well as in stressed samples (Table 1). Our results correspond to the results of Luo *et al.* (2012), who observed increased accumulation of given metal and Fe mainly in roots influenced by Cd concentration. The intake of Fe from the soil by roots in non-graminaceous monocots and dicots is primarily regulated by the Fe transporter IRT1 (Curie & Briat 2003). Several studies also provide strong evidence that the Fe transporter IRT1 is also primarily responsible for Cd^{2+} influx into root cells (Vert *et al.* 2002).

Although no leaves chlorosis and no changed Fe content in shoots were observed in our experiments, strong differences in the Fe content in roots and shoots indicate inhibition of Fe translocation from roots to shoots. Although the mechanism underlying Cd-induced Fe deficiency in plants has not been identified, there are several possible explanations. The root Fe-deficiency-inducible enzyme Fe(III)-chelate reductase is inhibited by Cd (Parmar

T a b l e 1

Cadmium (Cd) and iron (Fe) content in roots and shoots [μg/g dry weight]

Variant of experiment	Root		Shoot	
	Cd	Fe	Cd	Fe
Control	0.50 ± 0.01	1,035 ± 103.00	0.11 ± 0.03	117 ± 1.53
Cd 50	62.26 ± 9.60^{+}	1,503 ± 175.00^{+}	13.73 ± 3.27^{+}	108 ± 0.71
Cd 100	86.40 ± 0.99^{+}	1,754 ± 104.00^{+}	16.53 ± 4.37^{+}	119 ± 11.72

Data are presented as means ± SD; n = 3; $^{+}$indicate the level of significance at $p < 0.05$

T a b l e 2

Effect of soil pollution with cadmium on the biological accumulation coefficient (BAC), biological transfer coefficient (BTC), and biological concentration factor (BCF)

Variant of experiment	BAC	BTC	BCF
Cd 50	0.275	0.221	1.245
Cd 100	0.165	0.191	0.864

et al. 2013), suggesting that Cd may directly impair Fe acquisition. Also, Cd usually accumulating in roots, almost completely inhibits Fe translocation from roots to shoots, leading to increased root Fe concentrations in strawberry (Muradoglu *et al.* 2015) and mung bean (Liu *et al.* 2000).

As a result of the Cd accumulation in roots, the BAC and BTC values were very low and less than 1 (Table 2). Despite the relative high value of BCF at lower concentration of Cd (BCF > 1) was determined, bean are not suitable for phytoremediation of soils contaminated with Cd because of low BAC and BTC values. Plants exhibiting BTC (particularly BCF) value less than one are unsuitable for phytoextraction (Fitz & Wenzel 2002). However, higher BCF values (Table 2) presume this plant species for phytostabilisation and revegetation of the Cd-contaminated soils. By the influence of higher doses of Cd, low decrease in values of BAC, BCF, and BTC were observed (Table 2), probably as an effect of Cd toxicity. Cd-dependent increase of BTC at lower concentration and decrease at higher concentration of Cd were also observed by de Maria *et al.* (2013) in sunflower.

Pigment content and H_2O_2 accumulation in leaves

Upon the exposure to both doses of Cd, decreases in content of chlorophyll *a* (by 25.52% and 24.83%, respectively), chlorophyll *b* (by 6.90% upon application of Cd 100 only) as well as carotenoids (by 40.39% and 38.36%, respectively) were detected (Figure 2). These decreases were statistically significant. Reduction of the pigment contents in our study is comparable with the results of Kumar *et al*. (2000), who observed reduction of chlorophyll *a* by 38.37%, chlorophyll *b* by 26.27% and carotenoids by 31.27% in broad bean leaves treated with Cd (120 mg/kg soil).

The results of the effect of Cd on the ratio of chlorophyll *a* and *b* diverge. The results of many authors suggest that Cd ions cause degradation of chlorophyll *a* more rapidly than chlorophyll *b*, resulting in decreased Chl *a*/*b* ratio (Myśliwa-Kurdziel & Strzałka 2002; Kummerová *et al.* 2010). On the contrary, increased Chl *a*/*b* ratio was observed by some authors (Azevedo *et al*. 2005). From the data available in the literature, it is difficult to conclude to what extent the changes in the Chl *a/b* ratio

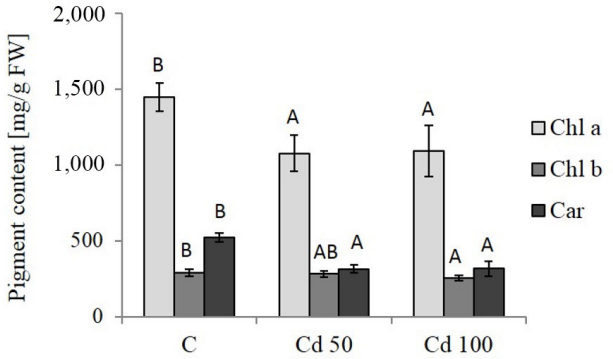

Figure 2. Chlorophyll *a* (Chl a), chlorophyll *b* (Chl b), and carotenoids (Car) contents in leaves affected by Cd (50 or 100mg/kg soil). Data are presented as means ± SD, n = 4. FW – fresh weight. Different letters indicate significant differences at $p < 0.05$.

caused by metal stress are the result of the inhibition of the enzymatic activity converting Chl *a* to Chl *b* and to what extent they derive from different rate of degradation of both chlorophyll species (Myśliwa-Kurdziel & Strzałka 2002). Carotenoid content in plants exposed to Cd also does not exhibit a set pattern, and may either increase or decrease. The increase was observed in *Cucumis sativus* (Burzynski & Zurek 2007) and *Nicotiana tabacum* (Procházková *et al.* 2014). Oppositely, decrease was also observed, for example, in *Pisum sativum* (Hattab *et al.* 2009).

Inhibitory effect of Cd on photosynthetic apparatus has previously been reported by many other authors (Kummerová *et al.* 2010; Wang *et al*. 2013), although the opposite reaction has also been observed (Bindhu & Bera 2001). Reduction of chlorophyll content could result in enzymatic degradation of these pigments or inhibition of their biosynthesis, which could be connected with Cd-induced deficiency of Fe and zinc, decrease of magnesium content or Cd bond to essential thiol groups in various enzymes (Parmar *et al.* 2013). Cd does not participate in Fenton-type reactions; therefore, it can only indirectly lead to oxidative stress (Romero-Puertas *et al.* 2004). Thus, it is much more likely that Cd-related oxidative stress is a consequence of inhibition of photosynthesis, especially in leaves. This fact is supported by the results of histochemical staining of bean leaves with DAB for detection of H_2O_2 (Figure 3).

Despite the fact that on leaves no symptoms of toxicity have been observed, Cd induced a significant accumulation of H_2O_2 especially in older bean leaves treated with higher dose of Cd (Figure 3). While the content of Cd was not examined in different developmental stages of leaves, higher accumulation of H_2O_2 was observed in older leaves, which may indicate increased accumulation of Cd in older leaves compared with younger. The high Cd concentration, found mainly in roots and old leaves, suggests that plants tend to avoid toxicity in the physiologically most active portions of the plants by reducing Cd translocation to the epigeous portion, and by promoting the re-translocation of toxic metals from shoots to roots (de Maria *et al.* 2013).

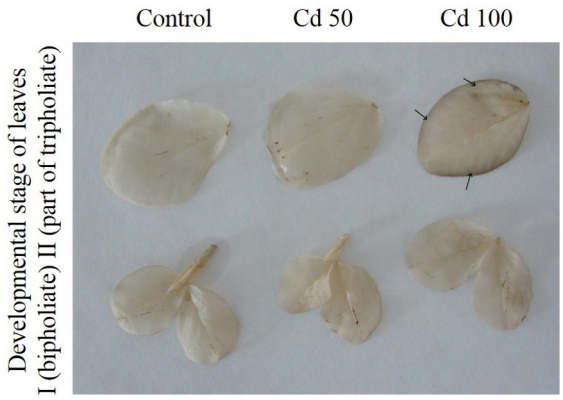

Figure 3. Histochemical detection of H_2O_2 in faba bean leaves. Arrows indicate brown deposits of H_2O_2.

CONCLUSIONS

The tested concentrations of cadmium (Cd) resulted in no visible symptoms of toxicity on faba bean cv. Aštar. Our results clearly demonstrated that photosynthetic apparatus of faba bean responded sensitively to the tested doses of Cd despite the high tolerance of the tested cultivar (TI > 60); however, disruption of photosynthetic apparatus is probably not the direct effect of Fe deficiency in shoots, but by Cd-induced changes in content of active iron (Fe) in cells (Luo *et al.* 2012) by emergent oxidative stress or other mechanisms. Low values of BAC and BTC show low phytoremediation potential of the given plant species in contaminated soils; however, the high tolerance of this cultivar, its relative fast growth, high biomass as well as priority of Cd accumulation in roots presume this plant species for phytostabilisation and revegetation of the Cd-contaminated soils. More in-depth biochemical and molecular biological analyses can contribute to revealing some further potential mechanisms of resistance of this faba bean variety to Cd.

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