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Effect of cadmium on the rhizospheric microorganisms of the sunflower

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Abstract We studied the effect of cadmium (Cd) at a dose of 100 mg/kg of soil on changes in the rhizospheric microbiota in four varieties (P62LE122, P63LE113, P64HE144 and P64LE136) of sunflower (*Helianthus annuus*). Undemanding aerobic heterotrophic bacterial species, microscopic fungi, *Azotobacter* sp., and *Rhizobium* sp. were evaluated. The variety P64LE136 had increased root biomass as well as total amount of rhizosphere microflora, significantly especially *Rhizobium* sp., under Cd conditions compared to the control. We attribute this phenomenon to better nitrogen availability for the roots, due to the symbiotic relationship with bacteria. In contrast, in variety P63LE113 we found that all four groups of examined microbiotas were inhibited by Cd without a significant relationship to root biomass. The highest total number of examined microorganisms (2.8. 10^7 CFU) arose in the rhizosphere of variety P62LE122, and the lowest (1.2. 10^7 CFU) in the rhizosphere of variety P64HE144.

Key words Cadmium, sunflower, rhizospheric microbiota

1. INTRODUCTION

Environmental degradation by heavy metals (HM) has always been a serious problem, and it continues to cause global concern. Cadmium (Cd) is considered one of the most potentially toxic trace elements in the environment (Rizwan et al., 2018; Dutta et al., 2020) due to its toxicity, persistence, bioaccumulation, and transmission through the food chain (Cui et al., 2017; Niu et al., 2021). The application of phosphorous fertilizers, various wastes in the form of composts, and sludge from wastewater treatment plants (McLaughlin, 2021) in particular contribute to increasing Cd content in soils.

Plants have variable sensitivity to Cd, with tolerance often determined by their ability to absorb and accumulate the element in tissues, its dose, exposure time, soil composition, as well as soil pH. Soil microorganisms also play an important role in the process of tolerance and accumulation of Cd. The rhizosphere is a microhabitat that contains roots and the immediate 1-2 mm of soil around them, where an intensive chemical dialogue between plants and microorganisms takes place. The two coexist and develop synergistic relationships that can support plant functions and productivity, as well as their ability to respond to stressful conditions, including HM contamination. Root exudates, which promote the growth of fungi and bacteria in the rhizosphere, play an important role in the formation of rhizosphere microbiota, and their density is much higher than the population in the surrounding bulk soil (Shilev, 2001; Barra Caracciolo and Terenzi, 2021).

Current ecological problems and increasing emphasis on human health are creating pressure towards searching for nature-friendly uses or decontamination of infested soils. Bioremediation uses the ability of a wide range of biological substrates (both metabolically active and inactive) to reduce or remove toxic substances of various origins, including HM. Microorganisms have strong potential for use in bioremediation processes - their structure is very complex, with numerous ways for metal cations and microorganisms to interact. These interactions depend on the type of microorganism and its metabolic activity, as well as on the form of the element (Šimonovičová et al., 2013). Of bacteria, species of the genera *Pseudomonas, Flavobacterium, Achromobacter, Micrococcus, Bacillus, Acinetobacter, Nocardia* and others are the most widely used in biological remediation. The second group of microorganisms is the form of microscopic filamentous fungi, which is very often used in bioremediation. For example, the species *Aspergillus niger* significantly reduces the content of Cd^{2+}, Zn^{2+}, and accumulates and volatilizes As. *Aspergillus nidulans* accumulates Ni^2 and its mutant strains are considered Cd^{2+} tolerant. *Aspergillus oryzae, Cladosporium cladosporioides, Stachybotrys chartarum, Scopulariopsis brevicaulis, Verticillium marquandii, Neosartorya fischeri, Trichoderma viride, Cunninghamella blakesleeana* and many species of the genus *Penicillium* also have a wide range of uses (Urík et al., 2007; Urík

et al. 2009,). Better knowledge of interactions between plants and microorganisms is important for the development of correct agronomic management and natural-based solutions, such as phytotechnology for remediation. This is needed as an ecological approach, which takes into account the biotic and abiotic interactions between plants and site-specific microorganisms (Barra Caracciolo and Terenzi, 2021).

Sunflower (*Helianthus annuus* L.) is considered a suitable candidate for the bioaccumulation and phytoremediation of HM, since it has stronger potential for HM uptake and tolerance than e.g. corn, ricinus, alfalfa and mustard (Niu, 2007; Vinothkumar and Senthil Valavan, 2018). Relatively few studies are devoted to evaluating the composition of the rhizospheric microbiota of sunflowers exposed to cadmium ions.

The main aim of this study was to evaluate the effect of Cd on the abundance of selected groups of rhizospheric microorganisms in four varieties of annual sunflower.

2. MATERIAL AND METHODS

The pot experiment took place in Phytotron II, and we used four varieties of sunflower: P62LE122, P63LE113, P64HE144 and P64LE136. We weighed 1 kg of Klasmann KTS 2 Fine growing substrate type into each of the pots (pH 5.5 to 6.5, EC in 400S 400, N mg/l 140, P in mg/l 160, K in mg/l 180, Mo in mg/kg dry matter 3.0 to 35, trace elements (Mg, Mo, Cu, B, Fe, Mn, Zn in low concentrations) and we sowed 12 seeds of selected sunflower varieties in 23 cm-wide pots. In the initial watering of 1000 ml of pure water we added control variant (K), and in pots with cadmium (Cd) we applied an equal volume of aqueous CdCl2 in a dose of 100 mg Cd per kg of soil dry matter. The plants were grown in a light mode for 12 hours light and 12 hours dark at a temperature 23°C/20°C. After 30 days, we terminated the pot experiment and continued by treating the plant material and rhizosphere soil. All of the experiments were performed in three repetitions.

2.1 Fresh weight of sunflower roots

We separated the shoots from the roots of the plants. We picked the roots together with the rhizospheric soil, and thoroughly cleaned and washed them with water after removing the rhizosphere soil. After sucking water from the root surfaces, we weighed them on analytical scales.

2.2 Isolation of rhizospheric microorganisms

To isolate and quantify bacteria from the soil rhizosphere of sunflower, we used the following nutrient agars: 1. Nutrient agar medium (NA), which is rich in organic nutrients and is a suitable nutrient medium for undemanding aerobic heterotrophic bacterial species. 2. CDA medium generally intended for cultivating microscopic filamentous fungi and yeasts. 3. BA medium for the isolation of free nitrogenic bacteria of the genus *Azotobacter*. 4. RA medium for the isolation of symbiotic nitrogenic bacteria of the genus *Rhizobium*. The agar media were prepared according to the manufacturer's instructions, and poured into sterile petri dishes.

Preparation of soil solution and its dilution
We separated the sunflower shoots from the roots. Subsequently, we used a shovel to select 3 roots and from them we shook the rhizosphere soil onto convex clock glass. We mixed the soil and weighed 0.5 g of each soil sample on an analytical balance, and poured into a labelled tube. Subsequently, 5 ml of physiological saline containing 0.1% gelatine (thus obtaining a dilution of 10^{-1}) was added to each tube (Štyriak et al., 2002). Soil samples prepared in this way were vortexed vigorously for 1 minute, and placed on a shaker (200 RPM) for two hours. Then the samples were centrifuged and dilutions were prepared from the supernatant using the decimal dilution method up to a dilution of 10^{-5}.

Inoculation and cultivation of soil microorganisms
We inoculated 0.25 ml of supernatant on all agar plates and left to cultivate. We spread the microbial suspension on the agar plate surface using sterile bent sticks in the shape of a stick. At NA we inoculated soil solution from dilutions 10^{-3} and 10^{-4}, at CDA we used only dilutions 10^{-4} and 10^{-5}, at BA we inoculated dilutions10^{-3} and 10^{-4}, and at RA we used dilutions10^{-3} and 10^{-4}. The cultivation of soil microorganisms took place under aerobic conditions at temperature optimums for individual groups of microorganisms (NA - 30 ° C/24 hours, BA - 30 ° C/48 hours, CDA - 28 ° C/6 days, RA - 28 ° C/24 hours).

Counting colony forming units of soil microorganisms and processing the results. After the cultivation, we counted the individual colony forming units (CFU) with a manual counter, converted to one dilution, and finally to the number in 1 g of rhizospheric soil.

We used MS EXCEL to evaluate the results, and for statistical analysis we chose a two- sample Student's T-test.

3. RESULTS AND DISCUSSION

We did not observe any visual symptoms of toxicity on the roots of the monitored sunflower varieties. Cadmium didn´t have a negative effect on the root biomass, and in the case of variety P64LE136 there was a 26% increase in biomass (Fig. 1).

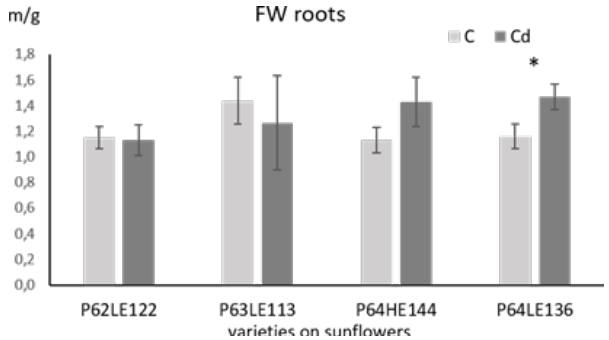

Fig. 1. The changes in the fresh weight of sunflower roots by the action of cadmium

Explanations: C - variant control, Cd - variant with cadmium, error bars show the standard deviation of the mean from three replicates, significant difference at $p<0.05$ *.

The recorded number of microorganisms in the rhizosphere depended on the variety, experimental variant, and type (Figures 2 and 3).

We observed a reduced number of microscopic filamentous fungi and yeasts under the influence of Cd in the rhizosphere of variety 122 (by 28%) and variety P63LE113 (by 61%) (Fig. 2). In the case of these varieties, we also recorded a decrease in the number of aerobic heterotrophic bacteria (by 33% and 57%). The number of

bacteria of genus *Azotobacter* sp. also decreased in the rhizosphere of variety P63LE113 (by 8%) (Fig. 2). The number of bacteria of genus *Rhizobium* sp. decreased in the rhizosphere of variety P63LE113 (by 79%), while it contrast it increased in the rhizosphere of variety P64LE136 (by 372%) (Fig. 2).

The increased number of microorganisms due to Cd in the rhizosphere of variety P64LE136 may be due to this variety binding Cd from the soil earlier and more than other varieties during growth. Niu et al. (2021) reported that the number of different groups of microorganisms increases after phytoremediation, hence after a decrease in Cd in the soil. It can also be a tolerant variety that can produce various phytocompounds that support the growth of microorganisms. In contrast, the inhibition of microorganisms in the rhizosphere of variety P63LE113 may be due to a different composition of root exudates and the variety's response to Cd ions. Similarly, Shilev et al. (2001) isolated NA bacteria from the rhizosphere of sunflower and monitored their tolerance to many heavy metals (concentration of 10-30 g/l of soil) including Cd. They found that bacterial tribes indicated significant variability in HM in general, and the presence of bacteria caused an increase in metal uptake in both the sunflower's root and aboveground parts.

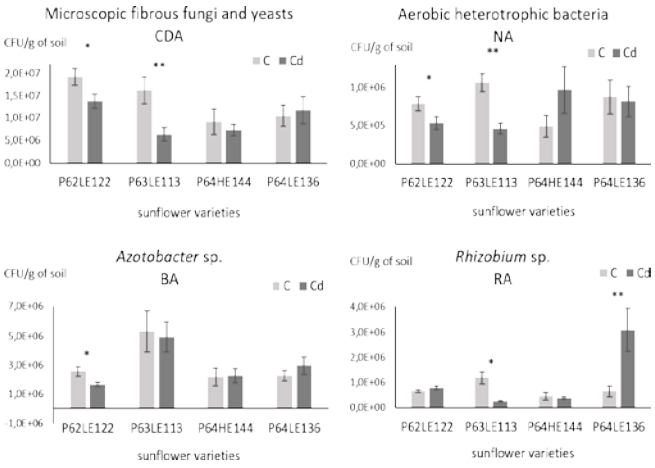

Fig. 2. Influence of cadmium on the number of monitored groups of rhizospheric microorganisms in four varieties of sunflower - comparison of the number of microorganisms within varieties

Explanations: C - variant control, Cd - variant with cadmium; error bars represent the standard deviation of the mean from three repetitions; significant differences at p<0.05 *, p<0.01 **.

Cardoso et al. (2020) examined the effect of Cd on *Rhizobium*, and found that at low concentrations of phytocompounds Cd stress on bacteria is reduced, while at higher concentrations it is the opposite. Thus, the presence and amount of bioactive compounds produced by plants in the soil can affect the tolerance of microorganisms to toxic substances, and may alter their environmental impact (Cardoso et al., 2020). Prasad et al. (2020) report a reduced number of *Azotobacter* sp. and heterotrophic bacteria (p <0.05) caused by Cd, the most harmful being the concentration (5 and 10 mg/kg of soil, respectively), i.e. 20 and 10 times lower than we used. Similar results have been reported by other authors (Khan et al., 2010, Ahmad et al. 2005). Toxicity tests in the study by Diacon et al. (2020) demonstrated the negative effect of Cd on the growth of *Azotobacter* sp. and yeast *Pichia* sp., manifested by a decrease in biomass by more than 50% at heavy metal concentrations of 150-300 mg/l. They found that these microbes could tolerate heavy metal stress only at low Cd concentrations. In our experiment, the values of *Azotobacter* sp., heterotrophic bacteria, and microscopic fungi also declined in most cases.

The largest number of microscopic fungi (CDA, 10-20 times more than other groups of microorganisms) was recorded in the control, as well as in Cd-treated samples. That was followed by bacteria Azotobacter sp. (BA), and finally Rhizobium sp. (RA) and aerobic heterotrophic bacteria (NA) occurred (Figs. 2, 3). The highest total number of microorganisms was in the rhizosphere of variety P62LE122, and the least in the rhizosphere of variety P64HE144 (Fig. 4).

The increased number of symbiotic nitrogenic bacteria Rhizobium sp. in the Cd rhizosphere of the treated P64LE136 plants may be related to the higher root biomass of this variety. Increased root biomass may be due to increased nitrogen availability due to the symbiotic relationship. The total number of evaluated microorganisms depended on the variety (Fig. 4).

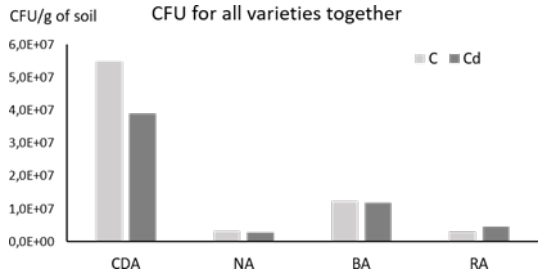

Fig. 3 Influence of cadmium on individual groups of sunflower rhizosphere microbiota

Explanations: CDA - microscopic fungi, NA - aerobic heterotrophic bacteria, BA - *Azotobacter* sp., RA - *Rhizobium* sp.; C - variant control, Cd - variant cadmium.

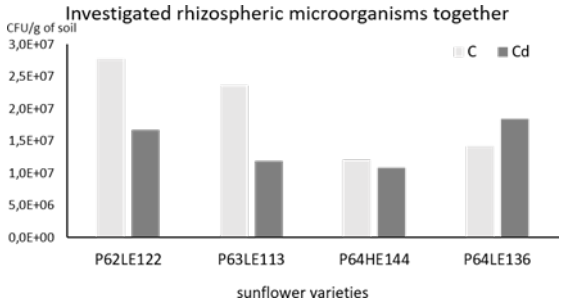

Fig. 4 Influence of cadmium on the total rhizospheric microbiota

Explanations: C - variant control, Cd - variant cadmium.

The results of Sun et al. (2022) showed that Cd- contaminated soil affected microbial diversity and soil composition, and bacterial diversity was affected more than fungal diversity in Cd-contaminated soil. The reduced number of soil microbial community and its composition due to Cd was highlighted by Sun et al. (2022). In our experiment, both the diversity of fungi and bacteria were affected. The differences showed most in the numbers of rhizospheric microorganisms for individual sunflower varieties.

4. CONCLUSIONS

Our results provide new insights into the effects of Cd contamination on microbial communities in sunflower rhizosphere. In conclusion, only variety P64LE136 (of the four studied varieties) had increased root biomass and total amount of rhizosphere microflora under cadmium conditions compared to the control variant. The number of symbiotic nitrogenic bacteria *Rhizobium* sp. was the highest by the same variety. We explain this phenomenon by the better nitrogen availability for the roots due to the symbiotic relationship. In contrast, in variety P63LE113 we found that all four examined groups of microbiotas were inhibited by cadmium without a significant relationship to root biomass. The highest total number of microorganisms occurred in the rhizosphere of variety P62LE122, and the least in the rhizosphere of variety P64HE144. In this study, we pointed out the different sensitivity of the rhizospheric microbiome of individual sunflower varieties to Cd ions. These differences are probably determined by the ability of individual varieties to absorb and accumulate Cd, and their ability to tolerate the metal. The content of root exudates in individual varieties probably also plays an important role. A deeper understanding of the mechanisms of Cd impact on the rhizospheric microbiome can contribute to the efficient use of sunflower in the remediation process of Cd-contaminated soils.

In further research of this problem, it would be more appropriate to use the extraction of microbial DNA from the sunflower rhizosphere to identify microbial diversity, and subject it to the 16S rRNA amplicon sequencing technique. This would provide more informative value about the complex diversity of the rhizospheric microflora.

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