EXUDATES FROM *MISCANTHUS* X *GIGANTEUS* CHANGE THE RESPONSE OF A ROOT-ASSOCIATED *PSEUDOMONAS PUTIDA* STRAIN TOWARDS HEAVY METALS

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Abstract

Plants shape their rhizosphere microbiome by excreting root exudates, which for some metallophytic plants function also as a defense mechanism to resist/tolerate contamination. The composition of root exudates is modulated by several environmental factors, and it remains unclear how that affects beneficial rhizosphere microorganisms under heavy metal (HM) contamination. Therefore, we evaluated the transcriptional response of *Pseudomonas putida* E36, a beneficial *Miscanthus* x *giganteus* isolate, to Cd, Pb and Zn in an *in vitro* study implementing root exudates from *M.* x *giganteus* grown under HM and control conditions.

Our results indicated higher exudation rate for plants challenged with HM. Further, out of 29 organic acids identified and quantified in the root exudates, 8 of them were significantly influenced by HM. For example, salicylic acid and terephthalic acid concentrations were increased (11.1 and 2.9 $\log_2$ fold change, respectively) in the root exudates of HM-treated plants. The transcriptional responses of *P. putida* E36 were significantly affected by the treatments. As expected, HM addition to the growth medium significantly increased the expression of several efflux pumps and stress response-related functional units. The additional supplementation of the growth medium with root exudates from HM-challenged plants resulted in a downregulation of 29 % of the functional units upregulated in *P. putida* E36 as a result of HM addition to the growth medium. In addition, the transcription of several functional units linked to carbohydrate and lipid metabolism were upregulated in the presence of root exudates. Surprisingly, root exudates + HM downregulated the expression of *P. putida* E36 functional units related to plant colonization (e.g., chemotaxis, motility, biofilm formation) compared to the control treatment without HM.

Our findings suggest that root exudates may alleviate *P. putida* E36 HM-induced stress mainly by provision of nutrients. That might offer an insight for the future *in vivo* studies contributing to improvements in phytoremediation of HM contaminated soil.

Keywords (maximum 6): *Miscanthus*; phytoremediation; *Pseudomonas putida*; root exudates; plant growth promotion

1. INTRODUCTION

Elevated heavy metal (HM) concentrations in soils are of global concern (Li et al., 2019). They negatively impact plants, microorganisms, and pose threats to human health, especially when transferred across the food chain (Ahmadpour et al., 2012; Lian et al., 2019). As a consequence, HM pollution is increasing land use conflicts, as the area of land suitable for food and feed production is strongly reduced (Fargione et al., 2008). Taking recent models for a growing world population into account, this development of reduced availability of land for covering our demands for high quality crops is a major concern (Godfray et al., 2010).

Recent strategies of managing HM contaminated sites suggest that such sites could be at least used for fibre production, which could indeed reduce land-use conflicts and trigger the reclamation process via phytoremediation, a cost-efficient way for removing pollutants from soils (Schröder et al., 2018) and can be considered environmentally friendly since greenhouse gas emissions from such sites are low (Fargione et al., 2008). The perennial grass *Miscanthus* x *giganteus* is a bioenergy plant combining high biomass production, good metal tolerance, and phytoextraction and phytostabilization properties. Therefore, it has been proposed as a suitable candidate for phytoremediation of HM polluted areas (Pavel et al., 2014; Novo et al., 2018).

Metallophytic plants, including *M.* x *giganteus*, have developed different strategies to resist/tolerate increased HM concentrations, either with internal tolerance mechanisms (hyperaccumulators) or exclusion mechanisms, the latter of which prevent metals from entering root cells by secreting metabolites (i.e., amino acids, organic acids, sugars, phenolics, polysaccharides, proteins) into the rhizosphere (Bais et al., 2006; Zhu et al., 2011). One of the most studied mechanisms is exudation of low-molecular-weight organic acids. Many of these molecules can function as chelators forming stable complexes with metals such as Cd, Pb, Al,

Ga, Cu and Mn, to prevent their rapid uptake and by that alleviate metal toxicity (Chen et al., 2017). In addition, root exudates can induce plant adaptation and survival under metal stress by the stimulation of microbial activity (Ma et al., 2016). Root exudates also attract microorganisms and shape microbial composition of the rhizosphere that extends the capacity of plants to adapt to their environment (Bulgarelli et al., 2013). Growth of bacteria in the rhizosphere depends on their substrate preferences and chemical composition of root exudates, specifically organic acids (Zhalnina et al., 2018).

A possible strategy to enhance yields and phytoremediation efficiency of plants growing in HM contaminated soils is the use of plant growth promoting (PGP) microbial strains, which improve plant growth and health in contaminated soils (Chen et al., 2014; Babu et al., 2015; Schmidt et al., 2018). PGP bacteria improve plant growth and development by provision of nutrients (e.g., phosphorus solubilization and fixation of atmospheric nitrogen), production of growth hormones (e.g., auxins, gibberellins and cytokinins), siderophores, organic acids and amino-cyclopropane carboxylic acid (ACC) deaminase, and defence against pathogens (chitinase, glucanase) (Asad et al., 2019). PGP bacteria can also alter metal availability (biosurfactants, organic acids, phytohormones, chelating agents, altering the soil pH and driving redox reactions) and assist plants by reducing HM toxicity through reactive oxygen species (ROS) neutralizing systems, such as peroxidases and catalases (Asad et al., 2019). Nevertheless, inoculated strains need to compete with soil microbes for the colonization of the root-soil interface. Thus, the composition of root exudates is an important factor that determines the success of colonization of microbial inocula. However, both, the composition and quantity of root exudates vary depending on plant genotype, growth stage, environmental conditions like pH, temperature, CO_2, light, moisture and nutrients (Badri and Vivanco, 2009), and was also shown to change under HM pollution (Luo et al., 2014). Therefore, there is a lack

104 of knowledge in defining the best inoculation strategies of microbiota into HM contaminated
105 soils.

106 Here we carried out an experiment where we investigated transcriptional responses of
107 *Pseudomonas putida* E36, a strain which has been isolated from the roots of *M.* x *giganteus*
108 plants grown in HM contaminated soils, in the presence of *M.* x *giganteus* root exudates under
109 Cd, Pb and Zn stress. Root exudates from control plants and plants exposed to heavy metals
110 were used and their composition was analysed. Since plants use root exudation as a defence
111 mechanism against metal pollution, we anticipated that *P. putida* E36 would tolerate HM stress
112 better in the presence of root exudates. Pathways, differentially expressed in *P. putida* E36
113 challenged with HM, were expected to be downregulated in the presence of root exudates.

114

115 **2. MATERIAL AND METHODS**

116 **2.1. Isolation of *P. putida* E36 and genome sequencing**

117 The bacterial strain *Pseudomonas putida* E36 (DSM 114018) was isolated from sterilized *M.*
118 x *giganteus* roots grown on a Cd, Pb and Zn-contaminated field in Bytom, Upper Silesia,
119 Poland (Institute for Ecology of Industrial Areas'experimental field) in August 2014. The
120 medium used for isolation was Tryptic Soy Agar (TSA) supplemented with 0.5 mM $CdCl_2 \cdot$
121 H_2O. Plates were incubated at 28 °C for 2-3 days. The strain tested positively for indole-3-
122 acetic acid synthesis (modified from (Gordon and Weber, 1951)), siderophore production (Ali
123 et al., 2014), HCN production (Bakker and Schippers, 1987), motility (minorly modified from
124 (Atkinson et al., 2006), and cellulolytic enzymes production (Kasana et al., 2008), and
125 negatively to phosphate solubilization ability (Nautiyal, 1999).

126 To assess the genome of the strain, genomic DNA isolation, library preparation and sequencing
127 using Illumina MiSeq platform were performed as described by Nesme et al. (2017). After

quality filtering and PhiX decontamination clean reads were assembled using SPAdes (Bankevich et al., 2012) and gene calling was performed with Prodigal (Hyatt et al., 2010). The genomic sequence data of *P. putida* E36 have been deposited with links to BioProject accession number PRJNA766417 in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/).

2.2. Preparation of root exudates

Rhizomes of *Miscanthus* x *giganteus* J. M. Greef & Deuter ex Hodk. & Renvoize (approximately 10 cm long; Energene Ltd., Poland) were grown in a greenhouse using 3.8 kg quartz sand at 23/18 °C day/night temperature, relative humidity 65 % and a photoperiod of 12 h. One rhizome per pot was planted. The pots used (15 x 15 x 20 cm) were beforehand disinfected with ethanol and sand was autoclaved and rinsed with sterile water to remove the dust. Pots with two weeks old seedlings were treated with $Cd(CH_3CO_2)_2 \cdot 2H_2O$ (2.1 mg Cd kg^{-1}), $Pb(CH_3CO_2)_2 \cdot 3H_2O$ (54.7 mg Pb kg^{-1}) and $Zn(NO_3)_2 \cdot 6H_2O$ (217.5 mg Zn kg^{-1}). Treatments with CH_3COOH (43.5 mg kg^{-1}) and NH_4NO_3 (265.8 mg kg^{-1}) were used as controls. Each treatment was applied in five replicates. Plants were watered weekly from below with 250 mL of autoclaved tap water and received a dose of sterile 1x strength Hoagland's No. 2 basal salt mixture (Sigma-Aldrich, Deisenhofen, Germany). Sampling was carried out 5 weeks after treatment application.

Root exudates were obtained from 5-week-old *M.* x *giganteus* plants using the dipping method described by Marx et al. (2007). Prior to the collection of root exudates, roots were rinsed with sterile water to remove sand. Intact roots were then immersed in 400 mL of autoclaved deionized water for 2 h. Extracts were filter sterilized using a 0.22 µm pore-size filter (Stericup, Merck-Millipore, Darmstadt, Germany) and frozen at -20 °C until lyophilization. Further, 20

152 mL of each root exudate extract, spiked with ^{13}C-benzoic acid, was lyophilized for the chemical
153 composition analysis, and a 60 mL mixture of 5 replicates per each treatment of root exudate
154 extracts was lyophilized (Martin Christ Alpha 1-4 LD plus, Osterode am Harz, Germany) for
155 *P. putida* E36 challenge experiment.

156 After collection of root exudates plant roots, rhizomes and above-ground parts (shoots) were
157 separated and dried at 80 °C for at least 48 h for dry weight determination. Concentrations of
158 Cd, Pb and Zn were determined (Perez Przyk and Held, 2010) in root exudates and pulverized
159 dry root and shoot biomass (Fig. S2) as follows: homogenized samples were subjected to
160 pressure digestion with nitric acid in a high-pressure digestor (Seif, Unterschleißheim,
161 Germany) and subsequently total Cd, Pb and Zn concentrations were measured by inductively
162 coupled plasma – atomic emission spectrometry (ICP-AES, Spectro Arcos, Spectro Analytical
163 Instruments, Kleve, Germany).

164

165 **2.3. Chemical composition of root exudates**

166 Total C and total N was determined in root exudates (ca. 10 mL) on DIMATOC 2000
167 (DIMATEC, Langenhagen, Germany) without additional sample preparation steps.

168 For the analysis of organic and amino acids, 20 mL sample of each root exudate extract was
169 spiked with the internal standard (IS, ^{13}C-benzoic acid at a final concentration of 0.8 mg/L) and
170 immediately frozen until lyophilization (Martin Christ Alpha 1-4LD plus). The final extract
171 was resuspended in 400 µl of Milli-Q water with 0.2% of formic acid and further used for
172 organic acids (OA, 350 µL) and amino acids (AA, 50 µL) quantification. Before analysis,
173 organic acid samples were passed through a 0.20 µM nylon filter with 13 mm diameter (Millex,
174 Millipore, Ireland). Amino acids samples were first subjected to protein precipitation by adding
175 45.8 g/L 5-sulfosalicylic acid (99.9%, Sigma-Aldrich, Taufkirchen, Germany; 1:1, v:v)

followed by a vortex and centrifugation (10 min at 18620 x g) step. Finally, 200 µL of a second internal standard (DL-norvaline at a final concentration of 1 mg/L) was added and the protein-free supernatant was filtered as indicated above (Henderson and Brooks, 2010).

Amino acids were quantified on an Agilent 1100 system (Agilent, Waldbronn, Germany) equipped with a binary pump, a four-channel degasser, a pre-treatment autosampler, a column oven and a fluorescence detector. A column Agilent Xorbax Eclipse plus C18 (250 x 4.6 mm, 5 µM) fitted with the corresponding guard column at a constant flow of 1.5 mL/min and a temperature of 40 °C was used. The fluorescence excitation and emission wavelengths were 340 nm and 450 nm, respectively. The gain was set to 16. Mobile phase A was prepared by dissolving 2.8 g disodiumhydrogenphosphate, 7.6 g disodiumtetraborate decahydrate and 64 mg sodium azide in approximately 1.9 L of water, adjusting the pH to 8.2 with 25 % hydrochloric acid and then filling to a final volume of 2 L. Mobile phase B consisted of acetonitrile:methanol:water 45:45:10 (*v:v:v*). The mobile phase gradient was as follows: 0-0.84 min, 2 % B. 33.4 min, 57 % B. 33.5 min, 100 % B. 43.5 min 100 % B. 45.55 min, 2 % B. The supernatant was transferred to an HPLC vial and derivatized with OPA solution, according to well-established methods (Henderson and Brooks, 2010).

Organic acids were determined using an Ultimate 3000 LC system (ThermoFisher, Dreieich, Germany) coupled to an ultra-high-resolution maXis 4 g plus QTOF mass spectrometer (Bruker, Bremen, Germany) equipped with an electrospray source (LC-UHR-Q-TOF-MS). The TOF–MS was operated in negative polarity with active focus under the following conditions: Capillary voltage, 4000 V; nitrogen dry gas temperature, 225 °C; dry gas flow, 10 L/min; nebulizer pressure, 2 bar. Low tune mass parameters were used. Each run was recalibrated using the high-performance calibration algorithm by infusing a 10 mM sodium formate calibrant solution into the TOF at the beginning of each run via a 6-port valve, as suggested by the manufacturer (Daltonics, n.d.). The LC conditions were as follows: the

analytical column was a Nucleodur C18-Gravity-SB 150 x 4 mm; particle size 3 µm (Macherey-Nagel, Feucht, Germany) fitted with the corresponding guard column. The flow rate was 0.35 mL/min and the oven temperature was 40 °C. Mobile phase A contained water with 0.2% formic acid (*v:v*) and mobile phase B was 100 % methanol. The elution gradient was as follows: 0–4 min, 98% A; 15 min, 100 % B; 21 min, 100 % B; 21.1 min, 98 % A. The total run time was 27 minutes. The injection volume was 20 µL.

The performance of the methods was checked using method blanks (solvent controls), quality controls (two different concentrations from the calibration curve levels), fortified samples, and daily calibration curves (Appendix B). The limits of detection (LODs) and quantification (LOQs) were defined as LOD = 3.3((α)/S) and LOQ = 10(α/(S)); α denoting the standard deviation of the response, and S the average slope of the calibration curves (Appendix B). The amino acids LODs ranged between 0.4 and 14.1 µmol L^{-1} and LOQs between 1.0 and 16.0 µmol L^{-1} (Table S1). The organic acids LODs varied from 0.03 to 3.01 mg L^{-1} and LOQs ranged from 0.08 to 9.12 mg L^{-1} (Table S2); precision and accuracy were evaluated following the criteria established by IUPAC Technical Report (Thompson et al., 2002). Every amino acid analytical series was controlled by using two commercially available quality control products (ClinChek® Level I and II, #10282, Recipe, Munich, Germany).

2.4. *P. putida* E36 challenge experiment with HM and root exudates

P. putida E36 was routinely cultivated on Luria-Bertani (LB) agar plates (Gerhardt, 1994; Russell and Sambrook, 2001) supplemented with 0.5 mM Cd as $Cd(CH_3CO_2)_2 \cdot 2H_2O$ and 2 mM Zn as $Zn(NO_3)_2 \cdot 6H_2O$ at 28 °C and was stored in 50 % glycerol at -80 °C. For all experiments, the bacterium was grown in MES buffered minimal medium (MBMM) with low metal-chelation characteristics, which makes it more suitable for determination of metal

toxicity (Rathnayake et al., 2013). The medium contained (in g L^{-1}): 2-(N-morpholino) ethanesulfonic acid, MES (1.95), Na_2HPO_4 (0.01), NH_4Cl (0.05), $CaSO_4$ (0.14), $MgSO_4 \cdot 7H_2O$ (0.24), KCl (0.02), $FeSO_4 \cdot 7H_2O$ (0.004), glucose (0.2 %) and SL7 trace element solution (1) consisting of (in mg L^{-1}): $ZnCl_2$ (70), $MnCl_2 \cdot 4H_2O$ (100), H_3BO_3 (60), $CoCl_2 \cdot 6H_2O$ (200), $CuCl_2 \cdot 2H_2O$ (20), $NiCl_2 \cdot 6H_2O$ (20), $NaMoO_4 \cdot 2H_2O$ (40) and 1 mL L^{-1} of 25 % HCl. The pH was adjusted to 6.4 and the medium was sterilized by autoclaving.

Growth curves of *P. putida* E36 were assessed in 50 mL tubes using 30 mL of MBMM medium, overnight, to reach a starting optical density (OD_{600}) 0.03. Bacterial cultures were grown at 28 °C with shaking at 130 rpm. The optical density (OD_{600}) was measured at wavelength of 600 nm hourly for 174 h on a Spectra Max 190 plate reader (Molecular Devices, California, USA) using the PathCheck option.

Cd, Pb and Zn minimal inhibitory concentrations (MIC) were determined according to Andrews et al. (2001) and Wiegand et al. (2008) in MBMM medium containing a gradient of $CdCl_2$ (0.08, 0.02, 0.03, 0.06, 0.1, 0.3, 0.5, 1.0, 2.0, 4.0 mM), $PbCl_2$ (0.03, 0.06, 0.1, 0.2, 0.5, 0.9, 1.9, 3.8, 7.5, 15.0 mM) or $ZnCl_2$ (0.08, 0.02, 0.03, 0.06, 0.13, 0.3, 0.5, 1.0, 2.0, 4.0 mM), respectively. *P. putida* E36 was grown in liquid pre-cultures to reach approximately 1-2 10^8 CFU mL^{-1}. 96-well microtiter plates were inoculated in triplicates and incubated for 72 h in plate incubator at 28 °C and 100 rpm. Sterility control and growth control were included on each plate. Bacterial growth was assessed spectrophotometrically at 600 nm. For the HM challenge experiment 0.5 MIC metal concentrations were used.

For the HM challenge experiment, *P. putida* E36 pre-cultures from the mid-exponential phase were used to inoculate 50 mL tubes containing MBMM to reach a final OD_{600} of 0.4, which is equivalent to approximately 4-8 10^8 CFU mL^{-1}. The MBMM was supplemented according to the treatments as follows: i) Cd, Pb, Zn (**H**), ii) Cd, Pb, Zn and root exudates extracted from

control plants (**EC+H**), and iii) Cd, Pb, Zn and root exudates extracted from HM-treated plants (**EH+H**). HM were added to reach final concentrations of 0.5 mM $CdCl_2$, 0.12 mM $PbCl_2$, and 2 mM $ZnCl_2$ (**H, EC+H and EH+H**). In control treatments without HM (**C**, **EC** and **EH**), DEPC-treated Milli-Q water was added instead of HM solutions. Each treatment was performed in triplicates. Lyophilized root exudates (60 mL, a mixture of 5 replicates per each treatment) were reconstituted in MBMM medium to reach a 2-fold carbon concentration relative to the initial extract.

To measure the transcriptomic responses of *P. putida* E36, bacterial cultures were incubated for 1 h after inoculation at 28 °C with shaking at 130 rpm. Before cell harvest the OD_{600} of the cultures was measured using a Spectra Max 190 plate reader. Each sample was divided into 3 technical replicates, cells were centrifuged (3255 xg, 10 min) and the supernatant was removed. Pellets were snap frozen in liquid nitrogen and stored at -80 °C until RNA extraction.

2.5. RNA extraction and analysis of bacterial transcriptomes

RNA was extracted from each technical replicate using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and the DNA was digested using DNase I (Qiagen, Hilden, Germany) according to manufacturer's instructions. Subsequently, the three technical replicates were pooled and purified following the RNA Cleanup protocol (RNeasy Mini Kit) according to manufacturer's instructions. Extractions with no sample template served as a blank extraction control. A successful removal of DNA from extracted RNA was confirmed with a PCR targeting bacterial 16S rRNA gene using primers FP16S (5' -GGTAGTCYAYGCMSTAAACG- 3') and RP16S (5' -GACARCCATGCASCACCTG- 3') (Bach et al., 2002). Amplifications were carried out in a PeqStar 96x universal thermal cycler (Peqlab, Fareham, UK) using 1x NebNext High Fidelity PCR Master Mix (New England Biolabs, Hitchin, UK), 0.3 % BSA, and 5 pmol of

each primer to a final volume of 25 µL. The cycle conditions were as follows: initial denaturation at 98 °C (1 min), 25 cycles of denaturation (98 °C, 10 s), annealing (55 °C, 30 s) and elongation (72 °C, 30 s), and the final elongation at 72 °C (5 min). Further, 1 µg of total RNA was used for rRNA depletion and cDNA library preparation using the ScriptSeq Complete Kit, Bacteria (Epicentre, Madison, Wisconsin, USA), according to manufacturer's protocol. The cDNA libraries were purified using Agencourt AMPure XP Kit (Beckman Coulter, Brea, California, USA) and sequenced on MiSeq platform (Illumina, San Diego, California, USA). The quantity and quality of RNA and cDNA were measured using Qubit 4 Fluorometer (Invitrogen, Waltham, Massachusetts, California, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany), respectively.

To assemble the transcriptome data, the raw reads were processed using Trimmomatic version 0.36 (Bolger et al., 2014). First, the adapter sequences were removed; retained reads were filtered using the maximum information quality trimming parameter (target length of at least 25 bp and strictness parameter 0.4, MAXINFO:50:0.4). Contaminant reads from PhiX, which is routinely added to sequencing runs as a quality control, were removed by mapping against the PhiX genome using bowtie2 (Langmead and Salzberg, 2012). The raw sequence data of 18 libraries was deposited into the NCBI Sequence Read Archive (SRA) with links to BioProject accession number PRJNA766417 in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/).

Since the draft genome of *P. putida* E36 strain was not closed, we performed *de novo* transcriptome assembly for each condition to obtain a reference genome for mapping (Grabherr et al., 2011). The methods of mapping using the draft genome and the *de novo* approach were compared, and the *de novo* transcriptome assembled contigs showed better results regarding the number of recovered unique genes and gene families than the draft genome method (more about the comparison of the two methods in the Supplementary information and Fig. S7). The

reads which passed the quality control from the same treatment were pooled together (triplicates) and assembled using megahit version 1.1.3-0 (Li et al., 2015). So generated transcriptome assembly was used for mapping of contigs from the respective treatments.

For the functional annotation of the transcriptome data, the assembled clean reads, namely contigs, were provided ORF prediction and protein sequences using prodigal version 2.6.3 (Hyatt et al., 2010). Such predicted protein sequences of each treatment were used as input into the KEGG internal annotation tool KOFAMKOALA (Aramaki et al., 2020) for KEGG Orthology (KO) annotations. Those genes that had an adaptive score higher than the predefined threshold or an E-value ≤ 0.01 were assigned a KO number. Furthermore, all good quality clean sequencing reads (in total comprising 18 libraries) belonging to the same treatment were mapped against the respective transcriptome assembly using bbmap (Bushnell, 2014). The gene-count data thus generated was normalised using transcripts per million (TPM) transformation and was used to obtain the functional profile and used for further analysis. A sample from H treatment (H5) was by number of reads an outlier and therefore excluded from further analysis.

The KEGG orthologs from each treatment were organised into the KEGG modules, further referred as functional units. The fraction of functional units present in a particular condition (KEGG module completeness, cmp) was obtained using R package 'metQy' (Martinez-Vernon et al., 2018). This allowed us to characterize the functional capabilities of the bacterium based on the complete or incomplete presence of the functional units.

The differentially expressed functional units were obtained using DESeq2 (Love et al., 2014). The functional units with a $\log_2$ fold change ($\log_2$FC) > 2, p value < 0.001 (adjusted for multiple comparisons) and functional unit's completeness (in at least one condition under comparison) ≥ 0.5 (meaning at least 50 % of the functional unit's constituting genes were expressed) were

322 considered significantly differentially expressed in comparison to the respective control. Only
323 those functional units were considered as relevant.

324 The relative abundance of KEGG Module profiles of the samples from treatments under study
325 were used to perform LefSE analysis (Segata et al., 2011). Those functional units with a
326 Kruskal-Wallis p value < 0.05 and LDA score higher than 3162x fold change ($\log_{10}FC > 3.5$)
327 were considered to be potential functional biomarkers characterizing the differences between
328 the treatments (Fig. 3B).

329 To identify genes related to stress response in the transcriptome dataset, the antibacterial
330 biocide and metal resistance genes database BacMet version 2 (Pal et al., 2014) was compared
331 against KEGG database using KofamScan (Aramaki et al., 2020). Those stress response genes
332 that had an adaptive score higher than the predefined threshold or an E-value ≤ 0.01 were
333 assigned to the specific KO numbers. The genes with the same KO numbers were organised
334 and their gene counts were summed to obtain the corresponding stress response KO gene
335 profiles.

336

337 **2.6 Statistical analysis**

338 All statistical analysis were performed using R software version 3.5.2 (R Core Team, 2018)
339 and RStudio interface version 1.1.463 (RStudio Team, 2018), including packages “reshape2”
340 (Wickham, 2007), “ggpubr” (Kassambara, 2018), “dplyr” (Wickham et al., 2017), “stringr”
341 (Wickham, 2018), “tidyr” (Wickham and Henry, 2018), “ggrepel” (Slowikowski, 2018),
342 “svglite” (Wickham et al., 2020), and “NMF” (Gaujoux and Seoighe, 2010). For data
343 manipulation and graphical visualization, a package “ggplot2” (Wickham, 2009) was used.

344 From amino acid and organic acid average concentrations the blank measurement was
345 subtracted, the values were divided by dry root biomass and expressed per 1 h of exudation.

Overall effect of HM treatment on the amino acid and organic acid composition of root exudates, as well as for normalised transcripts per million (TPM), was evaluated using two-way PERMANOVA analysis (function adonis from R package "vegan" (Oksanen, 2018)). For the Principal Component Analysis (PCA), and Principal Coordinates Analysis (PCoA) based on Bray-Curtis distances "ape" (Paradis et al., 2004) and "ellipse" (Sharma et al., 2020) R packages were used.

Statistical significance of treatment on the *M.* x *giganteus* biomass and composition of root exudates, including total C and N, amino acids and organic acids concentrations, was analysed by Kruskal-Wallis rank sum non-parametric test (kruskal.test).

Heatmap of transport and antimicrobial resistance modules related to stress response with row scaling (z-score of relative abundance) and hierarchical clustering was created using "pheatmap" (Kolde, 2019) and "dendextend" (Galili, 2015) R packages.

3. RESULTS

3.1 Heavy metal treatment influenced the composition of *M.* x *giganteus* root exudates

HM treatment significantly affected the growth of *M.* x *giganteus*, which showed 69 % and 36 % lower shoot ($p < 0.05$) and root biomass ($p > 0.05$) compared to control plants, respectively in the used semi-axenic system (Fig. S1). When the composition of root exudates extracted from both control and HM-treated plants was analysed, total nitrogen was significantly higher in exudates from plants grown under HM stress compared to the exudates from control plants (Fig. 1). Although not significant, the same trend was observed for the total carbon contents. Those results indicate higher exudation rates per dry mass of plants under HM treatment.

368 Overall, 15 amino acids and 29 organic acids were identified and quantified in the root exudates
369 (Table S1, Table S2). The HM treatment caused a significant change ($p = 0.034$) in the
370 composition of eight organic acids (Fig. 2A), including: acetylsalicylic acid, adipic acid, citric
371 acid, glyoxylic acid, salicylic acid, shikimic acid, succinic acid, and terephthalic acid (Fig. 2B,
372 Fig. S3). Only concentrations of salicylic and terephthalic acid were significantly increased
373 (11.1 and 2.9 $\log_2$FC, respectively) in the root exudates from plants grown in HM contaminated
374 sand. The other six organic acids, which were identified as responders to the HM addition were
375 negatively affected by the HM. In contrast, amino acid concentrations in root exudates did not
376 differ significantly between plants grown on sand with or without the presence of HM (Fig.
377 2C).

378

379 **3.2 Gene expression pattern of *P. putida* E36 challenged with HM and root exudates**

380 In order to characterize the influence of exudates on *P. putida* E36, we compared the
381 transcriptional response of *P. putida* E36 in presence of HM and root exudates after one hour
382 of incubation. In total 14,846,983 raw sequences were obtained. After quality filtering with
383 Trimmomatic 9,415,560 read pairs were retained, and finally after PhiX removal 9,398,809
384 clean read pairs remained.

385 Transcriptional pattern illustrated on the principal coordinates analysis plot (PCoA, Figure 3A)
386 revealed significant differences between the treatments ($p \leq 0.001$). Samples treated with HM
387 solution clustered together (H, EC+H, EH+H) and clearly separated from the non-treated
388 samples at the first PCoA axis (C, EC, EH). Interestingly, the expression pattern of *P. putida*
389 E36 incubated with root exudates from HM stressed plants (EH) and control plants (EC) clearly
390 differed, the latter one being more similar to the control without any amendment (C). The
391 expressed genes were organized into functional units based on KEGG modules.

392

393 3.2.1 Stress response of *P. putida* E36 challenged with HM

394 A total of 116 functional units were differentially expressed in H in comparison to C (Fig. 3B,
395 Fig. 4, Fig. 5 and Appendix C). As expected, the majority of the functional units differentially
396 expressed in H were assigned to the category environmental information processing,
397 representing 49 % of the total. From all differentially expressed functional units, 64 were
398 downregulated in H. The majority of the downregulated functional units was related to the
399 categories "carbohydrates and lipid metabolism" (e.g., glycolysis and glyconeogenesis) and
400 "nucleic acid and amino acid metabolism" (e.g., valine and isoleucine biosynthesis),
401 representing 19 % and 23 %, respectively. In contrast, only three functional units upregulated
402 in H were assigned to carbohydrate metabolism. Moreover, many functional units upregulated
403 in H were related to stress response (e.g., envelope stress response 2-component regulatory
404 system (2-CRS) and osmotic stress response 2-CRS) and efflux pumps (e.g., multidrug R efflux
405 pump MexEF-OprNm, multidrug R efflux pump AbcA and fluoroquinolone R efflux pump
406 LfrA).

407

408 3.2.2 Root exudates and stress response of *P. putida* E36 to HM

409 We further addressed the role of root exudates in the response of *P. putida* E36 to HM stress.
410 We detected 67 differently expressed functional units in EH+H in comparison to H (Fig. S4).
411 The category "environmental information processing" represented 64% of the differently
412 expressed functional units (Appendix C and Fig. 3B). Among 29 % of the functional units that
413 were upregulated in response to HM (H) but downregulated in the presence of exudates
414 (EH+H), there were only a few directly related to stress response and efflux pumps. For
415 example, osmotic stress response 2-CRS, anoxic redox control 2-CRS, and nickel tolerance

(Fig. 5 and Appendix C). Interestingly, many functional units related to antibiotic resistance were upregulated in the presence of root exudates. Also, more functional units related to the category "carbohydrate and lipid metabolism" were upregulated in the presence of root exudates (EH+H). In general, metal transporters and antimicrobial resistance modules involved in multidrug efflux were less expressed in EH+H than in H, whereas the majority of modules related to metal transport and efflux were more expressed in EH+H compared to H (Fig. 6 and Appendix C). This confirmed that *P. putida* E36 responded differently to HM stress in presence or absence of root exudates.

3.2.3 Changes on *P. putida* E36 gene expression pattern caused by root exudates

In order to understand how HM stress influences the signalling between plant-associated bacteria and their host, response of *P. putida* E36 to exudates from non-stressed plants (EC) was compared to controls without exudate amendment (C). In EC treatment, 63 functional units were upregulated compared to C (Fig. S6, coloured green, Appendix C, and Fig. 7). Mainly functional units from the category "nucleotide and amino acid metabolism" were upregulated in EC (40 %), among those shikimate, tryptophan, cysteine and threonine biosynthesis pathways were completely expressed. Furthermore, 24 % of the upregulated functional units belonged to the category "environmental information processing". Many of these functional units were highly expressed and with a high degree of completeness, such as multiple sugar transport system, multidrug resistance EfrAB transporter, multidrug resistance efflux pump QacA, gamma-aminobutyric acid (GABA) biosynthesis and alginate production 2-CRS. On the contrary, 46 functional units were downregulated in EC in comparison to C. From those, 65 % belonged to the category "environmental information processing", and among others

were 5 multidrug resistance efflux pumps (e.g., VexEF-TolC and MacAB-TolC), multiple sugar transport systems, and 15 2-CRS (e.g., cell-to-cell signalling).

3.2.4 HM treatment affected the interaction between *P. putida* E36 and *M.* x *giganteus* via root exudates

In total, 57 functional units were downregulated in EH+H treatment compared to EC (Fig. 7 and Appendix C), mostly belonging to the categories "nucleotide and amino acid metabolism" and "carbohydrate and lipid metabolism". Mainly pathways for the biosynthesis of amino acids were downregulated in EH+H. Moreover, pathways usually related to biofilm formation were downregulated in EH+H, e.g., alginate production 2-CRS, and fimbriae and flagellar synthesis 2-CRS. In contrast, 53 % of the functional units upregulated in EH+H were assigned to the category "environmental information processing". Interestingly, some functional units, such as catechol ortho-cleavage, insecticidal toxin regulation 2-CRS and those associated to bacitracin resistance, seem to be characteristic for EH+H, as they were upregulated in EH+H in comparison to both, EC and H treatments (Fig. 3B, Fig. 7 and Fig. S6).

4. DISCUSSION

4.1. Plants challenged with HM showed an increased root exudation

Increases in root exudation are part of the mechanisms of plant response to HM, which involve the exudation of organic acids and phytochelatins and allows further detoxification processes (Benzarti et al., 2008). In our study, the treatment of *M.* x *giganteus* induced changes in the composition of root exudates, mainly organic acids. From those, particularly terephthalic acid and salicylic acid were detected in higher concentrations in the exudates of HM-challenged

plants. Terephthalic acid was also detected in root exudates of the hyperaccumulator *Sedum alfredii* when treated with high concentrations of cadmium, and might be therefore involved in tolerance mechanisms (Luo et al., 2014). Salicylic acid was shown to play a role in the response of plants to HM stress (Liu et al., 2016), as well as in the recruiting of soil microbes (Lebeis et al., 2015). Therefore, it is possible that salicylic acid exudation helps *M.* x *giganteus* plants to cope with HM stress by stimulating the synthesis of antioxidant compounds and enzymes (A. Sharma et al., 2020), but also by recruiting beneficial microorganisms. In fact, when exposed to root exudates from HM-challenged plants (EH+H), *P. putida* E36 upregulated the pathway catechol ortho-cleavage, which is involved in the degradation of salicylic acid (Hamzah and Al-Baharna, 1994; Li et al., 2018).

4.2 Root exudates improved the response of *P. putida* E36 to HM mainly due to increase in nutrient availability

We hypothesized that root exudates would decrease the toxicity of HM for *P. putida* E36, as it is known that compounds present in exudates are chelating agents, e.g., citric acids, which can actually reduce HM toxicity (Panchal et al., 2021). In our study, many functional units of *P. putida* E36, which were upregulated in response to the HM treatment in the minimal medium, were downregulated in the presence of root exudates; however, mostly those not directly associated with HM stress. Thus, the improved response of *P. putida* E36 to the HM stress was mainly related to increased availability of nutrient sources due to the exudation, also evident by the high number of functional units related to carbohydrate metabolism in EH+H compared to H treatment. The increased cell density one hour after root exudate amendment points to the positive effects on bacterial growth (data not shown). Though the reduction of toxicity due to the chelation of heavy metals by organic acids cannot be excluded, although the upregulation

of many efflux pump pathways as well as secretion stress response 2-CRS in EH+H indicates that bacterial cells were still under stress. Moreover, we detected higher expression levels of glutathione S transferase in all H and EH+H samples (Fig. 6, Fig. S5). This enzyme was shown to play a central role in the Cd detoxification, since higher glutathione concentrations allowed higher efficiency of cytosolic Cd complexation, reducing Cd deleterious effects, as it was shown for two *Rhizobium* strains (Cardoso et al., 2018).

4.3 HM stress modifies the interactions between *P. putida* E36 and *M.* x *giganteus*

As expected, root exudate amendment led to an increase in the expression of the pathways related to carbohydrate and nitrogen metabolism. Interestingly, exudates of control plants (EC) triggered the expression of many functional units related to chemotaxis (e.g., flagella and fimbriae) and biofilm formation (alginate), which were downregulated in *P. putida* E36 when challenged with HM (EH+H). When tested *in vitro*, organic acids, such as malic, fumaric, citric, succinic and oxaloacetic acids attracted the PGP bacterium *Bacillus velezensis*, leading to an increase in synthesis of the exopolysaccharide alginate and biofilm formation (M. Sharma et al., 2020). Therefore, HM might reduce the ability of *P. putida* E36 to recognize and colonize *M.* x *giganteus* roots, as the above-mentioned traits are essential for PGP bacteria to colonize plant roots.

Remarkably, when challenged with EH+H, *P. putida* E36 upregulated the expression of functional units related to the insecticidal toxin regulation and those associated to bacitracin resistance in comparison to EC. It is known that bacteria upregulate the synthesis of antibiotics and antibiotic resistance genes in presence of root exudates, as they prepare themselves to compete with other microbes for the colonization of the host. Biocides, antibiotics and HM

resistance genes are found to co-occur in environments where microbes are subjected to selective pressure, e.g., metal and radionuclides contaminated soils (Thomas et al., 2020).

5. CONCLUSIONS

Our results point to a positive feedback loop between the PGP bacterium *P. putida* E36 and its host plant *M.* x *giganteus* under heavy metal stress, which is mediated by root exudates. We observed that under heavy metal stress *M.* x *giganteus* changes its root exudation pattern and might release specific compounds, e.g., salicylic acid, that can attract *P. putida* E36. In turn, root exudates improve the response of *P. putida* E36 to HM stress, possibly due to the improved nutrient availability. Considering the strong influence of site-specific conditions, including soil type, nutrient status or climate for the quality and quantity of root-exuded substances, future studies should be carried out under *in vivo* conditions to evaluate how would inoculation of *M.* x *giganteus* with *P. putida* E36 affect the uptake of HM from contaminated soils, and possibly lead to enhanced soil remediation and biomass production.

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