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Inoculated Seed Endophytes Modify the Poplar Responses to Trace Elements in Polluted Soil

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Abstract: Seed endophyte inoculation can enhance the plant tolerance to pollutants, which allows plant cultivation on trace element (TE) polluted soils. *Methylobacterium sp*. CP3 and *Kineococcus endophyticus* CP19 were tested *in vitro* for their tolerance to Zn and Cd and their plant growth promotion traits. The in vivo effects of bioaugmentation with individual strains or both strains were tested using two poplar cultivars, *Populus deltoides x* (*P. trichocarpa x P. maximowiczii*) 'Dender' and 'Marke', grown in TE polluted soil for six weeks. *Methylobacterium sp.* was found to grow on media enriched with 0.4 and 0.8 mM Cd, and both endophytes tolerated 0.6 and 1 mM Zn, due to the presence of genes involved in Zn and Cd tolerance and transport. *Methylobacterium sp.* showed an extracellular ion sequestration mechanism. Production of indole-3-acetic acid by *Methylobacterium sp.* and *K. endophyticus*, as well as phosphorus solubilization by *Methylobacterium sp.* were observed. Bioaugmentation with both endophytes increased the shoot length of *Populus* 'Marke' and enhanced the Mg uptake in both cultivars. Inoculation with *Methylobacterium sp.* reduced the bioaccumulation of Zn in 'Marke', conferring it an excluder strategy. *Methylobacterium sp.* and *K. endophyticus* seemed to improve the plant nutritional status, which can alleviate abiotic stress.

Keywords: cadmium; *Kineococcus endophyticus* CP19; *Methylobacterium sp*. CP3; plant growth promotion; populus; zinc

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1. Introduction

Soil pollution by trace elements (TE) is a worldwide problem due to the negative consequences to soil quality, food safety, and human health. The main sources of TE pollution, include mining, industrial waste, phosphate fertilizers, pesticides as well as atmospheric deposition by industrial activities [1]. Globally, around 10 million sites are considered polluted, of which more than 50% by TE [2]. In the EU, about 6.2% of agricultural land is potentially polluted by TE [3], including an area of 280 km^2 in the northeast of Belgium [4]. In this agricultural region, the activities of several pyrometallurgical smelters resulted in soil pollution by Zn, Cd and Pb [4,5]. The cultivation of TE-tolerant non-food crops in this area would allow to lower the negative impacts of the polluted soil on the environment as well as to support the local economy [6–9]. Suitable candidates belong to the *Salicaceae* family, which includes some woody species (i.e., poplars and willows), with

high biomass production and the capability for TE phytoextraction [9–13]. In particular, *Populus* 'Dender' and 'Marke, which select cultivars of controlled crossings (*P. deltoides x* (*P. trichocarpa x P. maximowiczii*)), show fast growth and they are suitable for short rotation biomass production [14]. The cultivation of TE tolerant and fast-growing trees can allow the valorization of polluted agricultural land, through the production of biomass as for energy or other purpose as well as a gradual soil depollution by extracting TE [15,16].

Trace element-tolerant microorganisms can improve plant establishment and growth under conditions of TE phytotoxicity, and thus improve the overall phytostabilization performance. Amongst these plant-associated microorganisms, endophytes interact very intimately with their host. In this mutual symbiosis, plants provide nutrients to the microorganisms, and the latter can enhance plant growth and health [17]. Specifically, endophytic bacteria can improve plant growth through the production of plant growth regulators, nitrogen fixation and by increasing the availability of some elements such as phosphorous and iron. In addition, some endophytes may possess ion sequestration capacities, reducing their phytotoxicity [18,19]. Sequestration mechanisms include the bioaccumulation of ions inside the cell, as well as the physical adsorption to the cell wall, binding ions to anionic functional groups and to polysaccharides on cell surfaces [20]. Seed endophytes can provide benefits to their host plants through the growth promotion and increasing their tolerance to abiotic stresses, as TE presence in soil. The core seed microbiome in wheat includes endophytes able to produce auxins and siderophores, as well as to solubilize phosphate [21]. Similar traits have been observed in cucurbit seed endophytes, resulting in increased nutrient uptake and plant growth [22]. *Nicotiana tabacum* possesses seed endophytes with the capability to enhance biomass production, as well as to increase Cd concentrations in inoculated tobacco plants under Cd stress [17]. Moreover, the transmission of such endophytes through several seed generations supports the adaptation process of plants to polluted conditions [23–26]. The exploitation of seed endophytes that occur in metallophytes can be a strategy to improve the plant tolerance to TE polluted soils [27]. *Crotalaria pumila* is an herbaceous plant colonizing metalliferous soils in Mexico, with a phytoextraction potential for Zn. The bacterial seed endophytic communities of *C. pumila* have been described, including *Kineococcus endophyticus* CP19 and *Methylobacterium sp*. CP3 strains, over three seed generations. *Methylobacterium* is the most abundant genus of the *C. pumila* core microbiome, with the potential to improve the nutrient uptake and TE tolerance of the host plant. Moreover, as observed in *Arabidopsis thaliana*, plant colonization by *Methylobacterium* involves a migration towards aerial tissues through xylem vessels [28–30].

To the best of our knowledge, little information is available regarding the role and fate of inoculated endophytic bacteria in trees, like poplar, growing on TE polluted soil. For the purpose of improving our knowledge, *Methylobacterium sp.* CP3 and *Kineococcus endophyticus* CP19 strains were further characterized with regard to their TE tolerance and capability to support plant growth. Subsequently, an inoculation experiment was performed to assess the effects of inoculation of these selected endophytes on poplar TE-tolerance and uptake using *Populus* 'Dender' and 'Marke' cultivars, growing in soil originating from a TE polluted area (Lommel, Belgium). Additionally, we also investigated the fate of the inoculated strains, separately and in association, in plant organs, in order to elucidate whether the observed effects are due to an indirect or direct effects of the bacteria colonizing the plant.

2. Materials and Methods

2.1. Bacterial Strains Characterization

2.1.1. *In Vitro* Plant Growth Promotion Traits and TE Tolerance

Methylobacterium sp. CP3 and *Kineococcus endophyticus* CP19 were characterized in vitro for their potential plant growth promotion traits, production of indole-3-acetic acid

(IAA) and phytate mineralization, as well as their TE tolerance and production of siderophores and organic acids. Before testing, bacterial strains were grown overnight in 869 medium [31], and the different assays were performed after washing the bacterial cells with 10 mM MgSO4.

IAA production was assessed according to Patten and Glick method [32]. 25 µL of bacterial suspension was incubated for 4 days at 30 °C in 1 mL 1/10 strength 869 medium supplemented with 0.5 g l^{-1} tryptophan. 1 ml of Salkowski reagent (0.5 M $FeCl_3$ and 35% $HClO_4$) was added to 0.5 ml of supernatant and colorimetrically evaluated. Phytate mineralisation by the bacteria was estimated by measuring the halo-zones produced around colonies, growing on National Botanical Research Institute's phosphate solid medium at 28 °C for 12 days [33]. Siderophore production was verified according to the colorimetrical method proposed by Schwyn and Neilands [34]. An amount of 20 µL of bacterial suspension was incubated in 800 µL selective 284 medium [35], containing carbon sources and supplemented with 0, 0.25 or 3 µM Fe(III)citrate (deficient, optimal and oversupplying Fe conditions, respectively) at 30 °C. After 5 days, 100 µL of the blue chrome-azurol S (CAS) reagent was added and change of color was evaluated. Organic acid production was evaluated using the method proposed by Cunningham and Kuiack [36]. An amount of 800 µL sucrose tryptone medium was added to 20 µL of bacterial suspension. After a 30 °C incubation, for 5 days, 100 µL of 0.1% (v/v) Alizarine red S was added and the production was qualitatively evaluated. The TEs with values above the threshold for Flanders legislation were selected for bacterial tolerance. The maximum inhibitory concentrations for selected bacteria were also assessed: A total of 20 µL of bacterial suspension was plated on selective 284 medium supplemented with a carbon source [35], and 0.0, 0.4 and 0.8 mM $CdSO_4$ or 0, 0.6 and 1.0 mM $ZnSO_4$. After 14 days incubation at 30 °C, bacterial growth was assessed.

2.1.2. Scanning Electron Microscopy (SEM) and EDX analysis

Based on TE tolerance results, *Methylobacterium sp.* CP3 and *Kineococcus endophyticus* CP19 were chosen for scanning electron microscopy (SEM) and EDX analysis. *Methylobacterium sp.* CP3 was grown in liquid 284 medium supplemented with 1 mM $ZnSO_4$ and 0.8 mM Cd $CdSO_4$, *Kineococcus endophyticus* CP19 in 1 mM $ZnSO_4$ supplemented medium. Cell fixation was performed according to the method of Holmes et al. [37]. Briefly, bacteria pellets were washed 3 times with 0.01 M phosphate-buffered saline buffer (PBS, pH 7.0) to remove unbound ions, sugars and proteins. Pellets were resuspended in 2% glutaraldehyde for 1h at room temperature. Subsequently, samples were centrifuged for 3 min at 3000 *x g* and pellets were washed 3 times with Millipore water. A total of 1 µl of each sample was placed on a sample holder, covered with carbon conductive tape. Then, samples were coated with a gold layer and analyzed using a Scanning Electron Microscope (FEI Quanta 200F FEG-SEM with ThermoFisher Pathfinder Alpine EDS system with UltraDry Premium (60 mm^2 active area) EDS detector). Images were taken using accelerating voltages of 7.5, 12.5 kV or 15 kV.

2.1.3. Genome Sequencing and Assembly

Total DNA extraction and sequencing of *Methylobacterium sp.* CP3 and *Kineococcus endophyticus* CP19 were carried out in a previously study [26]. The RAST annotation system was used to perform the open reading frame prediction and gene annotation [38]. The prokaryotic genome automatic annotation pipeline of the National Center for Biotechnology Information [39], and the platform MicroScope were performed using the tool Magnifying Genomes, MaGe (http://www.genoscope.cns.fr, access date: 3/02/2021) [40]. Clusters of Orthologous Genes [41] and metabolic pathway reconstruction was carried out using the databases of Kyoto Encyclopedia of Genes and Genomes (KEGG) [42] and MetaCyc [43].

2.2. Inoculation Experiment

2.2.1. Soil and Plant Collection

Polluted soil was sampled in Lommel (Belgium, 51°12'41'' N; 5°14'32'' E), in an abandoned agricultural area at 500 m north-east of a Zn smelter. The soil has a sandy texture with a pH of 4.6–5 [9,44]. Cuttings of two poplar cultivars ('Dender' and 'Marke') were provided by Sylva nurseries (Lievegem, Belgium).

2.2.2. Plant Inoculation

The inoculation experiment was carried out in March and April 2019. Cuttings of the *Populus* cultivars 'Dender' 'Marke' were placed in water to allow root development, under greenhouse conditions (25 °C day/ 19 °C night; 60% humidity; 500 mL per day nebulised watering; 400 µmoL cm^{-2} s^{-1} PAR). After the cuttings developed roots, bacterial inoculations were performed. *Methylobacterium sp*. CP3 and *Kineococcus endophyticus* CP19 were incubated at 30 °C in 869 liquid medium for 2 days, until mid-exponential phase and an optical density of 1 U at 600 nm (≈10^8 CFU/mL) was achieved (visible spectrophotometer Novaspec Plus, Holland). The bacterial cultures were centrifuged (15 min at 4000 rpm) and bacterial pellets were washed 3 times with 10 mM $MgSO_4$ solution. Afterwards, the pellets were resuspended in 100 ml of tap water and added to the cuttings in water. Cuttings of each cultivar were placed in a total of four 10 l containers (one container per inoculation condition). Conditions were the following: Non-inoculated (Control), *Methylobacterium sp.* CP3 inoculation (M), *Kineococcus endophyticus* CP19 inoculation (K), and *Methylobacterium sp.* CP3 + *Kineococcus endophyticus* CP19 inoculation (M+K) (Figure 1). Seven days after the inoculation, 17 cuttings of each cultivar were transferred to plastic pots, filled with 3.5 kg of polluted soil and kept in greenhouse conditions. Cuttings were grown on polluted soil for 6 weeks to avoid possible plant stress to improve growing conditions.

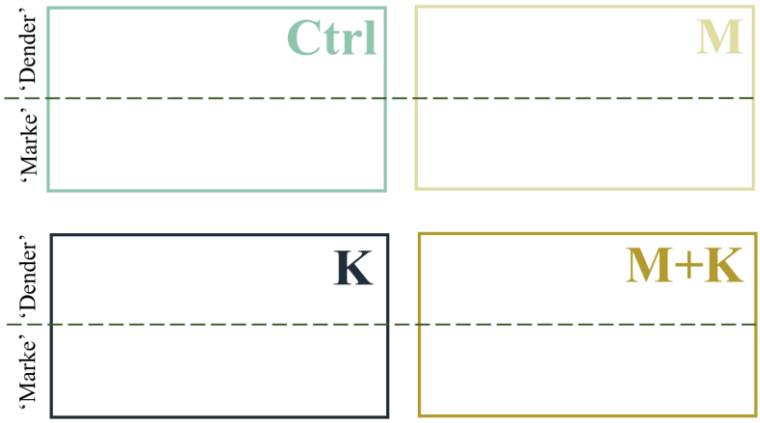

Figure 1. Scheme of inoculation setup using four 10 l containers, filled with tap water, in which the poplar cuttings were inoculated. Ctrl (Control): Non-inoculated cuttings; M: *Methylbacterium sp.* CP3 inoculation; *K: Kineococcus endophyticus* CP19 inoculation; M+K: *Methylbacterium sp.* CP3 and *Kineococcus endophyticus* CP19 inoculation.

2.3. Specific Automated Ribosomal Intergenic Spacer Analyses (ARISA)

After 6 weeks of growth, total DNA was extracted from the roots and leaf samples of 3 inoculated cuttings per condition, and from *Methylobacterium sp.* CP3 and *Kineococcus endophyticus* CP19 pure colonies, using for all the samples the E.Z.N.A. Bacterial DNA kit (Omega Bio-Tek, VWR, Leuven, Belgium). Plant samples were surface sterilized before DNA extraction with 5% NaOCl for 3 min, ethanol 70% for 3 min and rinsed three times with sterile water. Surface sterilization was checked by plating 1 mL of the rinsing water on Petri dishes filled with 869 medium. In circumstances where no colonies were observed after an incubation time of two weeks, sterilization was considered to be adequate. The

16S-23S internal transcribed spacer (ITS) regions were amplified by PCR with ITSF (5-GTCGTAACAAGGTAGCCGTA-3) and ITSreub (5-GCCAAGGCATCCACC-3) primers [45], using the FastStart High Fidelity PCR system, dNTPack (Sigma-Aldrich, St. Louis, USA). Afterwards, 1μL of DNA samples were loaded on a DNA-chip (DNA 1000 kit, Agilent Technologies, Diegem, Belgium) and analyzed on a 2100 Bioanalyzer (Agilent Technologies, Diegem, Belgium). Expert Software (Agilent Technologies) was used to digitalize the ARISA fingerprints, resulting in electropherograms in ASCII formats. ASCII formats were processed using the StatFingerprints package [46].

2.4. Plant Health and Growth

Poplar growth was examined once per week, in terms of shoot height, leaf number and area of the first three fully expanded leaves. The chlorophyll content was also estimated using a CCM-200 plus Chlorophyll Content Meter (OPTI-SCIENCES, Inc., Hudson, USA). In addition, the efficiency of photosystem II (PSII) was evaluated by the detection of chlorophyll *a* fluorescence, using the Plant stress kit (ADC Bioscientific Ltd, Hoddesdon, UK). More specifically, the maximum quantum efficiency of PSII was measured as F_v/F_m, where F_v is the variable fluorescence, calculated as the difference between maximum fluorescence (F_m) and the minimum fluorescence yield (F_0). Fm was measured after application of a saturating light pulse [6000 μmoL (photon) m^{-2} s^{-1}]. After 6 weeks of growth, shoots and roots were harvested for determining fresh (FW) and dry weight (DW). Shoots and roots were dried in an oven at 60°C until unchanged weight and DW biomass were measured.

2.5. Trace Element and Nutrient Concentrations in Soil

Trace element and nutrient concentrations were evaluated in the soil used prior to the greenhouse experiment. Oven-dried soil samples were analyzed for total Zn and Cd concentrations and nutrients (Ca, Mg, Mn, K, Cu), using the USEPA 3051 HNO3-microwave assisted digestion method [47]. Amounts of 37% HNO_3 and 37% HCl solution (1:3 v:v) were added to 0.5 g of soil, and soil samples were digested in a microwave over (Milestone 1200 MEGA, Milestone Systems, Belgium) at 160 °C (25 min ramp time, 10 min ventilation). The obtained extracts were diluted to a final volume of 50 mL with Millipore water and subsequently analyzed with inductively coupled plasma optical emission spectrometry (ICP-OES, Agilent Technologies, 700 series, Belgium). Plant available metal fractions were estimated by determining the exchangeable metal concentrations, while 20 ml 0.01 M $CaCl_2$ was added to 2 mg of dry soil sample, and after a 4h incubation at 25 °C, soil samples were centrifuged (15 000 g; 15 min) and extracts were filtered. Cd and Zn concentrations in the extracts were measured using ICP-OES. All samples were analyzed in triplicate. For the quality control, blank and standard references were included in the analysis (sewage sludge amended soil, Standard Reference Material 143R, Commission of the European Communities).

2.6. Element Concentrations in Plant Tissues

To determine total Cd, Zn, micro- (Cu, Mn) and macronutrient (Ca, Mg, K) concentrations in plant biomass, 0.2 g of dried plant roots and leaves were powdered and digested in glass tubes in a heating block, using the USEPA 3050B Acid Digestion of Sediments, Sludges, and Soils [48]. Three digestion cycles were carried out for each sample. The first one in 1 ml HNO_3 (70%) followed by one cycle in 1 ml HCl (37%) at 120 °C for 4 h. After the digestion, samples were dissolved in HCl (37%) and diluted to a final volume of 5 ml (2% HCl) with Millipore water. The extracts were analyzed using the same ICP-OES, as mentioned before. All samples were analyzed in triplicate. Blanks (only HNO_3) and standard references (NIST Spinach 1570a) were included.

2.7. Bioaccumulation and Translocation Factors

To evaluate the phytoextraction capabilities of inoculated and non-inoculated *Populus* 'Dender' and 'Marke' cultivars, the bioaccumulation (BAF) and translocation (Tf) factors were calculated after 6 weeks in polluted soil. The BAF was calculated as the ratio of total Cd and Zn concentrations in each plant organ to total the Cd and Zn concentrations detected in soil, while Tf as ratio of total Cd and Zn concentrations in leaves to root ones [49].

2.8. Statistical Analysis

After confirmation of normality (Shapiro-Wilk normality test) and homoscedasticity (Bartlett test) of the data, one-way ANOVA, followed by Tukey's Multiple Comparison Test ($p \leq 0.05$), was applied to plant growth and photosynthetic parameters, as well as to TE concentration data in plant tissues and relative indices (bioaccumulation and translocation factors). Statistical analyses were performed using R 3.6.0. Principal Component Analysis (PCA) was computed with the main results of the experiment, in order to investigate the correlations between variables (shoot height, biomass, element concentrations, Tf and BAF) within *Methylobacterium sp.* CP3 and *Kineococcus endophyticus* CP19 inoculations. PCA that explained more than a single parameter alone (eigenvalues >1) were considered, using Orange: Data Mining Toolbox in Python.

3. Results

3.1. Characterization of the Bacterial Strains

The in vitro TE tolerance and plant growth promotion traits of *Methylobacterium sp*. CP3 and Kineococcus endophyticus CP19 are presented in Table 1. *Methylobacterium sp*. CP3 could grow in media supplemented with 0.6 and 1 mM Zn, as well with 0.4 and 0.8 mM Cd, while K. endophyticus CP19 only developed in Zn enriched medium (0.6 and 1 mM Zn). Both bacterial strains produced IAA and only *Methylobacterium sp*. CP3 showed the capacity to solubilize phosphate.

Table 1. Trace element tolerance and in vitro plant growth promotion (PGP) traits of *Kineococcus endophyticus* CP3 and *Methylobacterium sp.* CP19. + = positive response; − = negative response.

	Metal tolerance				**PGP traits**			
Bacteria strains	**Cd (0.4 mM)**	**Cd (0.8 mM)**	**Zn (0.6 mM)**	**Zn (1 mM)**	**IAA**	**Sid**	**Org acid**	**P-mi**
K. endophyticus CP19	-	-	+	+	+	-	-	-
Methylobacterium sp. CP3	+	+	+	+	+	-	-	+

IAA = auxine production; **Sid** = siderophore production; **Org acid** = organic acid production; **P-mi** = phytate mineralization.

A functional characterization of *Methylobacterium sp*. CP3 and Kineococcus endophyticus CP19 and their interaction with Cd and Zn was performed (Table 2). The draft genomes of *Methylobacterium sp*. CP3 and Kineococcus endophyticus CP19 disclosed the presence of genes involved in Zn and Cd transport and tolerance, especially genes of the operons czc and znu involved in the tolerance to Cd and Zn. In addition, gene annotation revealed the presence in Kineococcus endophyticus CP19 of the iaaH gene which is encoding for indole-3-acetamide hydrolase in the pathway of IAA production. The draft genome of *Methylobacterium sp*. CP3 included the phoA gene, which is encoding for alkaline phosphatase that is involved in the mineralization of phosphate. Moreover, the absence of genes for the production of some organic acids was confirmed by the genome annotation.

Table 2. Main genes and relative encoded protein involved in *Kineococcus endophyticus* CP19 and *Methylobacterium sp.* CP3 responses to Cd and Zn. ABC = ATP-binding cassette.

Trace element	Gene	Encoded protein/enzyme	Bacteria
Cd, Zn	*czcD*	CzcD, cation efflux system protein	*Methylobacterium* *K. endophyticus*
Cd, Zn	*czcA*	CzcA, proton antiport (CzcCBA chemiosmotic transporter)	*Methylobacterium*
Cd, Zn	*czcB*	CzcB, proton antiport (CzcCBA chemiosmotic transporter)	*Methylobacterium* *K. endophyticus*
Cd, Zn	*cadA3*	Cd^{2+}- P-type exporting ATPase	*Methylobacterium* *K. endophyticus*
Zn, Cd	*zntA*	Zn^{2+}-P-type exporting ATPase	*Methylobacterium* *K. endophyticus*
Zn	*Zur*	Zur, Zinc uptake regulation protein	*Methylobacterium* *K. endophyticus*
Zn	*znuA*	ZnuA, Z^{2+} ABC transporter, periplasmic-binding protein	*Methylobacterium* *K. endophyticus*
Zn	*znuB*	ZnuB, Zn^{2+} ABC transporter, inner membrane permease subunit	*Methylobacterium* *K. endophyticus*
Zn	*znuC*	ZnuC, Zn^{2+} ABC transporter, ATP-binding subunit	*Methylobacterium* *K. endophyticus*

SEM-EDX analysis enabled the physical interaction between bacterial strains and trace elements to be observed. Cadmium (Figure S1) and Zn (Figure S2) are clearly present on/in the bacterial cell. After 2 days of growth in liquid cultures, enriched with 0.8 mM Cd (Figure S1) and 1 mM Zn (Figure S2), it was possible to distinguish trace element rich zones as being the brighter regions on the cell wall of *Methylobacterium sp*. CP3.

3.2. Inoculation Experiment

3.2.1. Trace Element Concentrations in Soil

The total TE concentrations of soil were: 223.4 ± 9.74 mg Zn kg^{-1} and 3.6 ± 0.18 mg Cd kg^{-1}. $CaCl_2$-extractable Zn and Cd concentrations were 5.72 ± 0.032 and 0.13 ± 0.008 mg kg^{-1}, respectively. The total Zn and Cd concentrations exceeded the background values (59 and 0.69 mg kg^{-1}, respectively) for sandy arable soil in Flanders legislation [50]. The nutrient concentrations in soil were: 1611 ± 160.5 mg kg^{-1} Ca; 409.7 ± 46.9 mg kg^{-1} K; 695 ± 83.1 mg kg^{-1} Mg; 66 ± 6.0 mg kg^{-1} Cu.

3.2.2. .Plant Growth and Biomass Production

In order to evaluate the possible effects of bacterial inoculation on the performance of Populus 'Dender' and 'Marke' cuttings, growth and photosystem II (PSII) efficiency were examined during the experiment. Growth was assessed by measuring shoot height, number of leaves and leaf area. Populus 'Marke' cuttings showed a significant increase in shoot length after inoculation with both *Methylobacterium sp*. CP3 and Kineococcus endophyticus CP19, compared to the control (Figure 2).

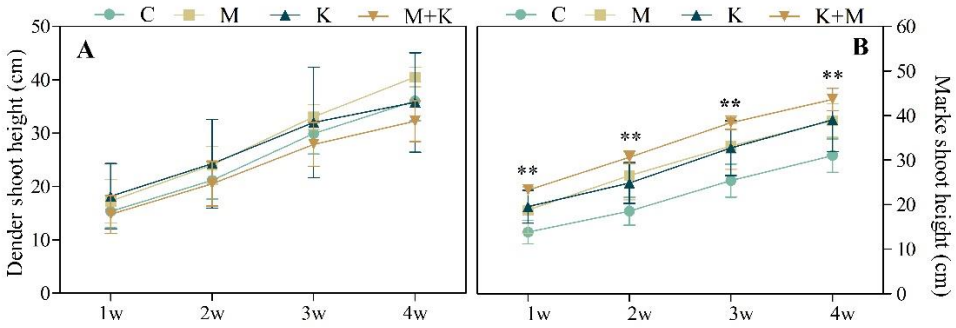

Figure 2. Shoot height in *Populus* 'Dender' (**A**) and Populus 'Marke' (**B**) cultivars detected during the 4-weeks inoculation experiment. Ctrl (control): non-inoculated cuttings; M: *Methylobacterium sp.* CP3 inoculation; K: *Kineococcus endophyticus* CP19 inoculation; M+K: *Methylobacterium sp.* CP3 and *Kineococcus endophyticus* CP19 inoculation. Bars represent ±SD.

No significant differences were detected between inoculated and non-inoculated control cuttings, in terms of number of leaves and leaf area (Figure S3). Chlorophyll a fluorescence confirmed the good health status of the poplar cuttings. Both cultivars showed a good PSII performance: F_v/F_m, with values between 0.75 and 0.85. In addition, no significant differences between the chlorophyll contents were detected between the different treatments (Figure 3).

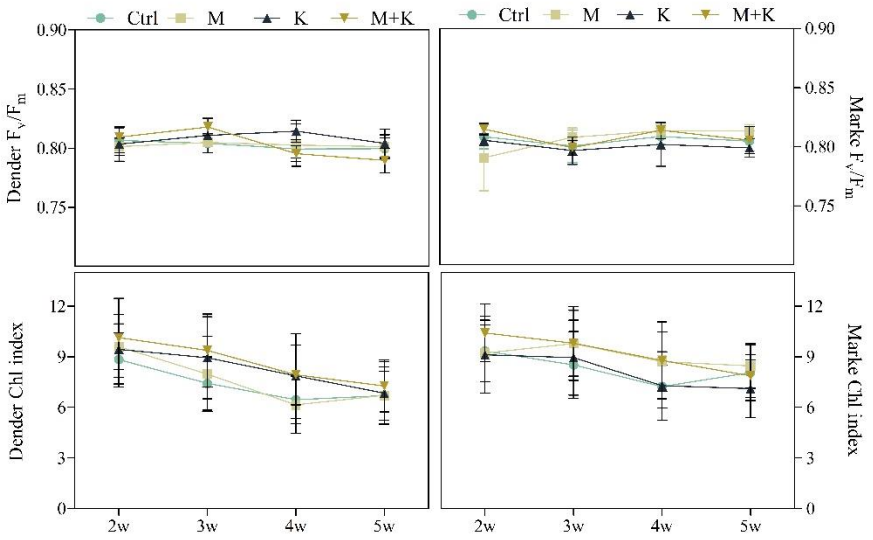

Figure 3. F_v/F_m and chlorophyll content (Chl index) of leaves of *Populus* 'Dender and Populus 'Marke' determined during the 4-week experiment. Ctrl (control): Non-inoculated cuttings; M: *Methylobacterium sp*. CP3 inoculation; K: *Kineococcus endophyticus* CP19 inoculation; M+K: *Methylobacterium sp.* CP3 and *Kineococcus endophyticus* CP19 inoculation. Bars represent ±SD.

In Figure 4, shoot and root dry weights of Populus 'Dender' and 'Marke' are presented. Shoot and roots were collected after 6 weeks of growth in trace element polluted soil. Bacterial inoculation did not have any significant effect on the dry weights of both poplar cultivars.

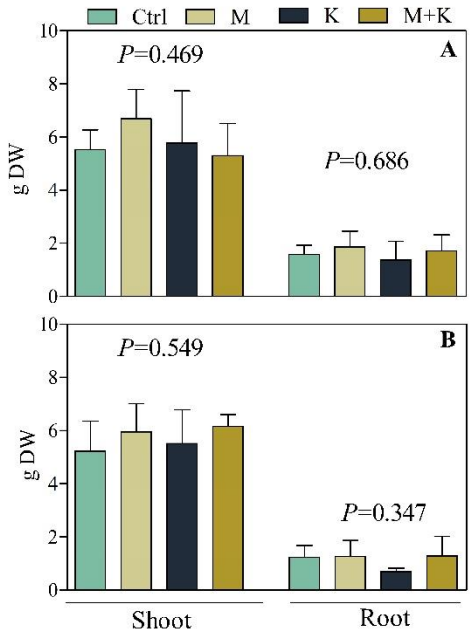

Figure 4. Dry weights (g) of shoots and roots of *Populus* 'Dender (**A**) and *Populus* 'Marke' (**B**) after 6 weeks of growth in trace elements polluted soil. Ctrl (control): non-inoculated cuttings; M: *Methylobacterium sp*. CP3 inoculation; K: *Kineococcus endophyticus* CP19 inoculation; M+K: *Methylobacterium sp.* CP3 and *Kineococcus endophyticus* CP19 inoculation. Statistical significance was tested with one-way ANOVA followed by Tukey's Multiple Comparison Test ($p \leq 0.05$).

3.2.3. Element Concentrations in Plant Leaves and Roots

After 6 weeks of growth in the polluted soil, the total Cd and Zn concentrations were determined in leaves and roots of both poplar cultivars. One-way ANOVA indicated no significant differences for Cd and Zn concentrations between inoculated plants and non-inoculated ones, for both poplar cultivars (Figure 5), as well as for Cd and Zn translocation factors (Figure S4). Bioaccumulation factors (BAF, Figure 6) showed that the average values are higher than 1 for Cd and Zn in leaves and roots of cuttings of both cultivars. The concentration of Zn was significantly lower (−40%) in roots of cuttings of cultivar 'Marke' inoculated with *Methylobacterium sp*. CP3 (1.3 ± 0.06), compared to the non-inoculated ones (2.2 ± 0.76).

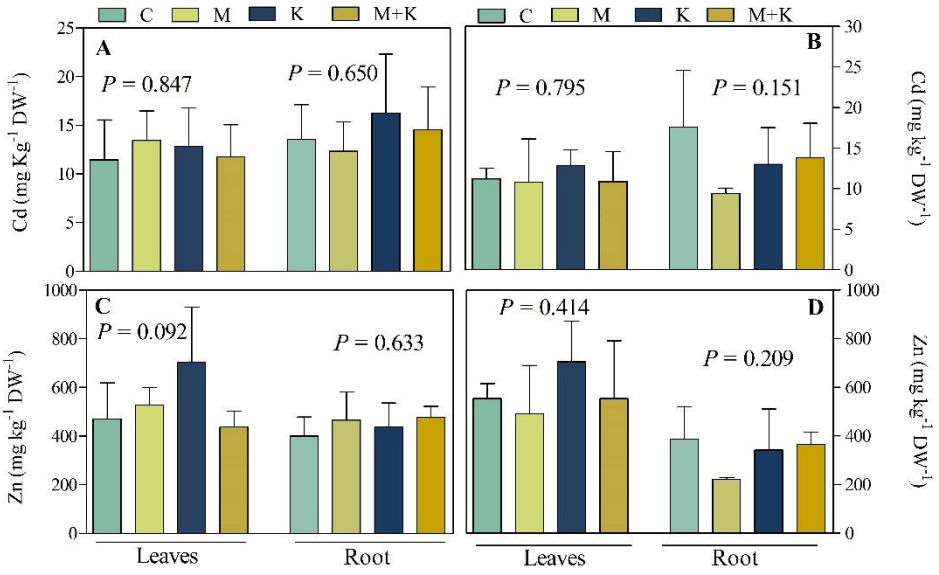

Figure 5. Cd and Zn concentrations (mg kg^{-1} dry weight) in leaves and roots of cuttings of Populus cultivars 'Dender' (A, C) and 'Marke' (B, D) after 6 weeks of growth in trace elements polluted soil. Ctrl (control): Non-inoculated cuttings; M: Methylobacterium sp. CP3 inoculation; K: Kineococcus endophyticus CP19 inoculation; M+K: Methylobacterium sp. CP3 and Kineococcus endophyticus CP19 inoculation. Statistical significance was determined with one-way ANOVA followed by Tukey's Multiple Comparison Test ($p < 0.05$);

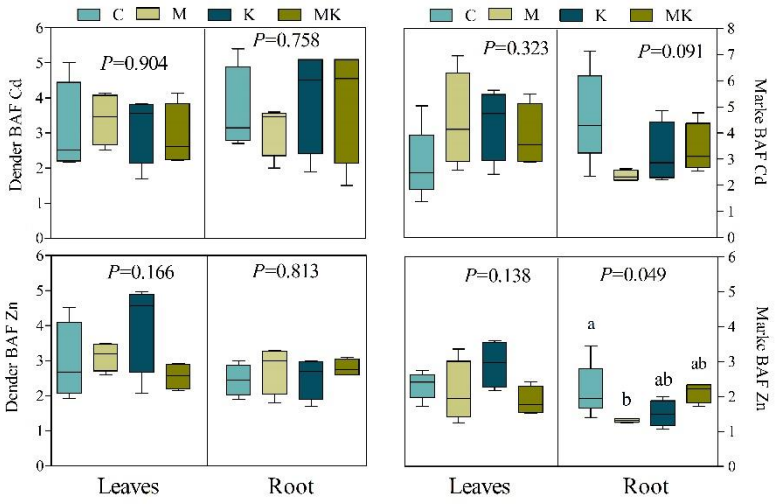

Figure 6. Bioaccumulation factors (BAF) for Cd and Zn calculated after 6 weeks of growth in trace elements polluted soil in leaves and roots of cuttings of the Populus cultivars 'Dender' and 'Marke'. Ctrl (control): non-inoculated cuttings; M: Methylobacterium sp. CP3 inoculation; K: Kineococcus endophyticus CP19 inoculation; M+K: Methylobacterium sp. CP3 and Kineococcus endophyticus CP19 inoculation. Statistical significance was determined with one-way ANOVA followed by Tukey's Multiple Comparison Test ($p \leq 0.05$).

3.2.4. PCA Results

PCA was performed to elucidate the poplar responses, in terms of growth and trace element accumulations, to inoculations with *Methylobacterium sp*. CP3 and K: *Kineococcus endophyticus* CP19 (Figure 7). The highest eigenvalues were achieved for two principal components which explained a 93% in total of the variability. The first principal component was determined by root biomass, Tf for Cd and Zn, BAF for Cd and Zn in root and BAF for Cd in shoot, while the second one was represented by shoot biomass, BAF Cd in root, BAF Zn in shoot and shoot height. PCA revealed that the differentiation between inoculated and non-inoculated plants was mainly determined by these factors.

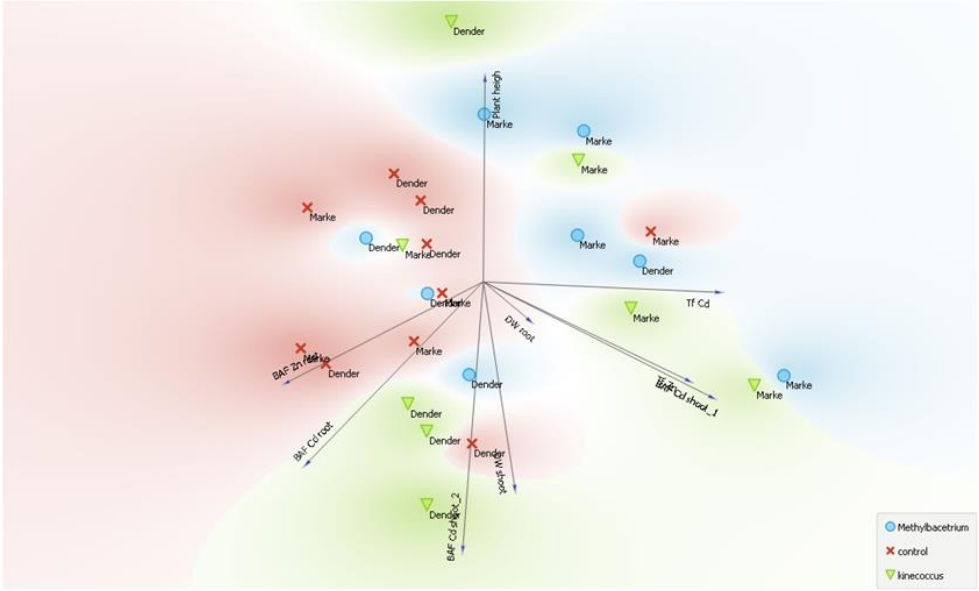

Figure 7. Principal component analysis computed with the main results of the experiment. Plant height refers to shoot length. Data obtained from different types of inoculation were reported in

different colors and symbols. Control as red crosses (non-inoculated cuttings); Methylobacterium sp. CP3 inoculation as blue points; Kineococcus endophyticus CP19 inoculation as green triangle.

3.2.5. Plant Nutritional Status

Tables 3 and 4 show the micro-and macronutrient concentrations in the roots and leaves of cuttings after 6 weeks of growth in polluted soil. The Mg concentration was significantly higher (+16%) in leaves of cuttings of the Populus 'Dender' cultivar inoculated with *Methylobacterium sp*. CP3 and *Kineococcus endophyticus* CP19, compared to non-inoculated cuttings (Table 3). In leaves of cuttings of the 'Marke' cultivar the Mg concentrations were higher (+10%) when inoculated with with *Methylobacterium sp*. CP3 and *Kineococcus endophyticus* CP19, compared to non-inoculated cuttings (Table 4).

Table 3. Micro- (Cu, Mn) and macronutrient (Ca, Mg, K) concentrations (mg kg^{-1} DW) in leaves (L) and roots (R) of cuttings of the Populus cultivar 'Dender' after 6 weeks of growth in trace elements polluted soil. Ctrl (control): Non-inoculated cuttings; M: Methylobacterium sp. CP3 inoculation; K: Kineococcus endophyticus CP19 inoculation; M+K: Methylobacterium sp. CP3 and Kineococcus endophyticus CP19 inoculation. Statistical significance was determined with one-way ANOVA followed by Tukey's Multiple Comparison Test ($p \leq 0.05$).

	organ	Ctrl	M	K	M+K	*ANOVA*
Cu	*L*	6 ± 1.35	8 ± 1.5	9 ± 2.8	6.3 ± 0.8	0.3019
	R	33 ± 9.9	40 ± 8.5	34 ± 7.5	41 ± 1.0	0.051
Mn	*L*	14.1 ± 3.5	16 ± 2.6	26 ± 16.2	16.5 ± 1.4	0.311
	R	49 ± 22.3	81 ± 11.9	71 ± 4.9	73 ± 4. 8	0.214
Ca	*L*	8771 ± 1574.1	10167 ± 1521.7	11443 ± 2510.3	10712 ± 728.6	0.160
	R	5007 ± 667.8	5015 ± 823.0	5130 ± 597.7	5062 ± 684.1	0.993
Mg	*L*	1906 ± 105.3b	2118 ± 189.4b	2091 ± 190.0b	2286 ± 66.6a	**0.015**
	R	1532 ± 133.6	1400 ± 129.9	1451 ± 181.1	1402 ± 49.8	0.135
K	*L*	3374 ± 306.2	3792 ± 562.3	3765 ± 828.9	4229 ± 456.8	0.058
	R	2960 ± 524.4	2636 ± 462.9	3699 ± 701.5	2553 ± 431.1	0.581

Table 4. Micro- (Cu, Mn) and macronutrient (Ca, Mg, K) concentrations (mg kg^{-1} DW) in leaves (L) and roots (R) of cuttings of the Populus cultivar 'Marke' after 6 weeks of growth on trace elements polluted soil. Ctrl (control): non-inoculated cuttings; M: Methylobacterium sp. CP3 inoculation; K: Kineococcus endophyticus CP19 inoculation; M+K: Methylobacterium sp. CP3 and Kineococcus endophyticus CP19 inoculation. Statistical significance was determined with one-way ANOVA followed by Tukey's Multiple Comparison Test ($p \leq 0.05$).

		Ctrl	M	K	M+K	*ANOVA*
Cu	*L*	6 ± 1.1	6 ± 0.8	6 ± 0.9	6 ± 1.2	0.971
	R	28 ± 11.3	23 ± 1.5	22 ± 6.1	34 ± 5.2	0.1212
Mn	*L*	14.2 ± 3.1	16 ± 4.1	18 ± 4.8	16 ± 3.6	0.652
	R	35.3 ± 23.5ab	21 ± 2.5ab	22.5 ± 7.6b	41.4 ± 14.6a	**0.029**
Ca	*L*	10767 ± 948.1	11099 ± 1756.6	12904 ± 1732.7	11661 ± 956.3	0.233
	R	4284 ± 765.5ab	3880 ± 231.4ab	3630 ± 410.9b	4842 ± 283.4a	**0.022**
Mg	*L*	2030 ± 129.6a	2203 ± 221.3ab	2196 ± 67.6ab	2267 ± 57.9b	**0.029**
	R	1442 ± 2018.4	1269 ± 71.9	1282 ± 119.4	1502 ± 176.7	0.135
K	*L*	3799 ± 429.0	3818 ± 661.8	4134 ± 366.3	4442 ± 497.3	0.241
	R	2941 ± 514.0	2435 ± 461.5	2354 ± 56.2	2339 ± 320.2	0.107

3.2.5. Specific Automated Ribosomal Intergenic Spacer Analyses (ARISA)

In order to evaluate the plant colonization by the two bacterial strains *Methylobacterium sp*. CP3 and *Kineococcus endophyticus* CP19, an ARISA analysis was performed (Figure S5). Heatmaps and the relative Pearson correlation analysis revealed the possible presence

of *Kineococcus endophyticus* CP19 in some root samples of Populus 'Marke' (Figure S5A) and leaves of Populus 'Dender' cuttings (Figure S5B) inoculated with one or both strains. *Methylbacterium sp*. CP3 was not detected in none of the roots or leaves of both cultivars.

4. Discussion

The study of the seed microbiome of metallophytes revealed interesting endophytes, demonstrating high TE tolerance and plant growth promotion properties [28]. A bioaugmentation approach using endophytes previously isolated from metallophytes, can improve the tolerance of plants to trace elements [27]. Therefore, two seed endophytic bacteria isolated from *C. pumila* colonizing a mining area in Mexico, were characterized and inoculated on cuttings of two poplar cultivars growing in TE polluted soil.

In vitro tests indicated that the two seed endophytes possess potential plant growth promoters (Table 1). Both *Methylobacterium sp.* CP3 and *Kineococcus endophyticus* CP19 can synthetize the plant hormone indole-3-acetic acid (IAA). Some of the effects of IAA production are positively related to root elongation and the numbers of root branches and hairs, which can lead to a higher water and nutrient uptake potential [32]. The production of IAA by bacteria is mostly promoted by the release of certain molecules (amino acids, sugar and organic acids) by the host plants [51]. In addition, *Methylobacterium sp.* CP3 showed the ability to mineralize phytate in vitro, most likely through the production of organic and inorganic compounds as well as phosphatase enzymes [52,53], suggesting some capability to convert insoluble phosphorous into an available form for plants [54]. Therefore, *Methylobacterium sp*. CP3 can be considered as a phosphobacteria, like other bacterial strains belonging to the genera *Pseudomonas*, *Serratia*, *Pantoea*, *Bacillus*, *Agrobacterium* and *Micrococcus* [55,56]. These in vitro plant growth promoting traits have been verified in an in vivo experiment in which cuttings of *Populus* 'Marke' were inoculated with *Methylobacterium sp.* CP3 and *Kineococcus endophyticus* CP19. In vitro, *Methylobacterium sp*. CP3 could grow on 0.4 and 0.8 mM Cd, as well as 0.6 and 1 mM Zn supplemented media (Table 1), confirming the endophyte tolerance for these TEs [29]. *Kineococcus endophyticus* CP19 showed only tolerance for Zn (0.6 and 1 mM). Earlier, copper tolerance has been reported in the genus *Kineococcus* [57]. This study increased the knowledge bank and applicability for such endophyte.

Generally, different mechanisms can be involved in bacteria TE defense, including the reduction of the TE uptake through enzymatic transformation [58]. Redox reactions may decrease the mobility and/or toxicity of TE (e.g., Cr) and methylation enables the transformation of some TE into a gaseous state, due to the volatility of the methylated TE [59]. Other TE tolerance mechanisms in bacteria include the sequestration of TE as physical adsorption of Zn and Cd through binding sites [60], for instance carboxyl groups in gram-negative (e.g., *Methylobacterium sp.* CP3) or involving extracellular polymer substances (EPS). Such high molecular weight polyanionic polymers which can contribute to metal immobilization, outside the bacterial cell [61–63]. The EPS properties to bind metals, i.e. Cu and Pb, have been studied in other bacterial species of the genus *Methylobacterium* [64]. In our study, the capability of *Methylobacterium sp.* CP3 to interact with trace elements was highlighted via SEM-EDX imaging (Figure S1 and S2) and the gene annotation (Table 2). Bacteria can actively transport ions outside the membrane through ion antiporter systems and the subsequent increase in pH leads to the precipitation of TE as insoluble forms, e.g., carbonates [65,66]. In our study, the presence of Cd and Zn in/on the bacterial surfaces can be related to the presence of *czc* ion efflux system, protecting the bacterial cell in a TE polluted environment. In fact, the *czc* is an ion antiporter efflux system and the *czc*-mediated efflux of cations, followed by the precipitation of TE carbonates in bacteria walls, has been observed in bacterial cultures, growing in high TE concentrations [67,68]. The dissimilar responses to high TE concentrations amongst studied endophytes could be related to the different TE resistance systems (Table 2). The ability of *Methylobacterium sp.* CP3 to tolerate high concentration of Zn and Cd could reflect the presence of encoded *CzcA*, *CzcB* and *CzcD* proteins, while *Kineccoccus endophyticus* CP19 encoded *CzcB* and *CzcD*. The *CzcA*

protein is mainly involved in Cd and Zn resistance and coupled with *CzcB* and *CzcD* contributes to the efflux of TE outside the bacterial cell [69]. The Zn tolerance detected at high Zn concentrations in both bacterial strains could be related to the encoded *ZnuABC* systems (Table 2), mainly involved in Zn homeostasis [70]. However, deeper investigation into this area is required to fully unravel the specific mechanisms adopted by the studied bacteria, especially influenced by growing media. In fact, the composition of the medium can affect the detoxification strategies adopted by bacteria to cope with non-physiological concentrations of TE, as investigated for Cd by Holmes [37].

In an in vivo experiment, the inoculation with *Methylobacterium sp*. CP3 led to a decrease of the bioaccumulation factor of Zn in roots of cuttings of the *Populus* 'Marke' cultivar (Figure 6). Previous studies also reported a lower bioaccumulation of Zn in tomato plants inoculated with other species of the genus *Methylobacterium* [71], and in *Spartia maritima* inoculated with other TE tolerant and PGP endophytes [72]. Due to such a protective role through the reduction in Zn uptake, *Methylobacterium sp.* CP3 may confer to the *Populus* cultivar 'Marke' an excluder strategy, thus also conferring the ability to grow in soils with Zn concentrations that are phytotoxic for non-inoculated plants [73]. Trace element pollution in soils can lead to nutrient deficiency in plants, as TE ions and essential nutrients are taken up by the same plant transporter proteins [74]. We observed higher Mg concentrations in the leaves in both poplar cultivars, inoculated with both *Methylobacterium sp*. CP3 and *Kineococcus endophyticus* CP19 (Tables 3 and 4). Magnesium is the central element in chlorophyll molecules and an important co-factor in several enzymes [75]. Moreover, the role of Mg in alleviating TE toxicity has been demonstrated. This element is involved in plant protection mechanisms that include synthesis of organic acids and sequestration of TE ions. In addition, maintaining a good Mg nutritional status in plants is effective to limit photooxidative damage due to ROS production under abiotic stress [76,77]. These positive effects of *Methylobacterium sp*. CP3 and *Kineccoccus endophyticus* CP19 on the Mg concentration in leaves might increase the tolerance of poplar to TE exposure in the long-term.

The ARISA confirmed the presence of the inoculated *Kineococcus endophyticus* CP19 in certain of the plant tissues, especially in *Populus* 'Marke' (Figure S5). The increased plant growth and nutrient uptake in *Populus* 'Marke' could be related to a direct effect of *Kineococcus endophyticus* CP19 in such cultivar. In *Populus* 'Dender', the effects of inoculation on trace element bioaccumulation and nutrient uptake (Figure 7; Table 3 and 4) are observed, although the presence of the inoculated endophytes was not confirmed. More extensive and robust investigations of inoculation success, using specific primers, is required. This will also hypothesize the eventual indirect effects of inoculation.

5. Conclusions

Methylobacterium sp. CP3 and *Kineococcus endophyticus* CP19 are seed endophytes, whose tolerance and close physical interaction with Zn and Cd have been confirmed in this study. In addition, the ability to produce IAA by both strains and the phytate mineralization by *Methylbacterium sp.* CP3 were demonstrated in vitro. The inoculation of both bacteria positively affected the growth of the *Populus* cultivar 'Marke'. This study also puts forward *Methylobacterium sp.* CP3 as a possible candidate for the increase in Zn tolerance of the host plant. Apparently, Zn uptake is lowered, while the Mg concentration in poplar leaves is enhanced by inoculation of both *Methylobacterium sp.* CP3 and *Kineococcus endophyticus* CP19, already after six weeks of plant growth. The improvements in TE tolerance of poplar in the long-term, as well as the inoculation success need to be more deeply investigated. In addition, the link between the bacteria TE tolerance and the presence of certain genes needs to be clarified through transcriptomic analyses.

Supplementary Materials: The following are available online at www.mdpi.com/article/10.3390/agronomy11101987/s1, Figure S1: SEM image of *Methylobacterium sp*. CP3 culture and

relative EDX spectra in a 0.8 mM Cd containing medium, Figure S2: SEM image of *Methylobacterium sp.* CP3 culture and relative EDX spectra in 1mM Zn containing medium, Figure S3: Number of leaves and leaf area in *Populus* 'Dender and *Populus* 'Marke' cul-tivars detected during the 4-weeks inoculation experiment, Figure S4: Translocation factors for Cd and Zn calculated for cuttings of the *Populus* cultivars 'Dender' and 'Marke' after 6 weeks of growth in trace elements polluted soil, Figure S5: Heatmaps derived by ARISA analysis for Pop-ulus 'Marke' roots and 'Dender leaves.

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