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Dynamic interactions of *Trichoderma harzianum* TS 143 from an old mining site in Turkey for potent metal(oid)s phytoextraction and bioenergy crop farming

Necla Pehlivan [a,*], Kenan Gedik [b], Rengin Eltem [c], Ertugrul Terzi [d]

[a] *Recep Tayyip Erdogan University, Biology Department, Rize, Turkey*
[b] *Vocational School of Technical Sciences, Recep Tayyip Erdogan University, Rize, Turkey*
[c] *Ege University, Department of Bioengineering, Izmir, Turkey*
[d] *Kastamonu University, Faculty of Fisheries, Kastamonu, Turkey*

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ABSTRACT

Despite high pollution risk, the termination of mining practices is not in question in the current era in line with the growing needs of beings. Instead, the rehabilitation by phytoremediation restores the economic and aesthetic values of the damaged locale. Here, potentially toxic elements (PTEs) tolerant 29 *Trichoderma* isolates from mining sites located foothills of Turkey`s NE Black Sea coast were isolated. The highest tolerant strain (As 1400 mg L^{-1}, Cd 1200 mg L^{-1}, Cu 2000 mg L^{-1}, Pb 2100 mg L^{-1}, Zn 3000 mg L^{-1}) was characterized with translation elongation factor1 alpha (*tef-1α*) barcode and deposited in the GenBank. The PTEs removal strength of novel *Trichoderma harzianum* TS143 was highest for Pb (58%) and the lowest for As (8.5%) in the order of Pb > Cd > Cu > Zn > As. While bioleaching capacity was highest in Cd with 30%, the lowest was for As (8%). TS143 was found remarkably effective on all the physicochemical parameters in the shoot and root tissues of maize. The increase in the carbohydrate content (33.50%) proves the potential usage of the contaminated maize plants in bioenergy production. Core sustainable agents with their mesh type robust hyphal structure enfolding PTEs such as TS143 contribute to the phytoremediation technology along with potential plant biomass management for the biodiesel industry.

1. Introduction

Turkey is rich in metallic mineral deposits as gold, copper, mercury, zinc, silver, and so on. These deposits include two crucial active and passive sulfide ores. The first one is an abandoned open-pit mining area, the Kutlular mine (Trabzon), one of the vast volcanogenic sulfide deposits in Turkey, which is located Black Sea coastline of the north Anatolian zone (Abdioglu and Arslan, 2009). The Kutlular, is currently passive for almost three decades, was operated between the years of 1986-1992 with an approximate 0.04% Pb, 1.5% Zn, and 2.4% Cu grade. The other one is a joint venture, Çayeli shallow underground copper-zinc mine established in 100 km west of the Turkish border with Georgia. Canadian and two other Turkish partners are still in charge of operations since 1994 with an initial of 70,641 tons of yellow (Cu-rich) and black ore (Zn-rich) operation capacity at 600 m depth (Yumlu, 2001).

The massive contribution of mines, one of our most critical natural resources to the modern world economy and wealth, cannot be neglected, however, particularly the abandoned open-pit mines turn into ecological burdens after long operation periods. These mines not only are the risk factors for their locations but also for the surrounding niche that is contaminated with the elevated concentrations of trace potentially toxic elements (PTEs). Especially in abandoned mining areas, toxicity can further be moved to the agricultural areas with the help of surface flows, rainfall washings, winds, etc. (Párraga-Aguado et al., 2013). Even though particularly Cu and Zn are essential elements owing to their vital roles for protein function for normal plant growth (White and Brown, 2010), their toxicity along with other metal(oids) leads to significant yield losses in agricultural production by interfering with plant photosynthesis or respiration (Babu et al., 2014). Beside yield losses, the mines whose operation has been terminated, disrupt the biodiversity and, in some cases, the aesthetic appearance of forests,

* Corresponding author.
E-mail addresses: neclapehlivan@gmail.com (N. Pehlivan), kenan.gedik@erdogan.edu.tr (K. Gedik), rengin.eltem@ege.edu.tr (R. Eltem), eterzi@kastamonu.edu.tr (E. Terzi).

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finally cause an economic loss because these areas are prone to lose their suitability for farming (Mendez and Maier, 2008; Sousa et al., 2012). In order to eliminate such disadvantages, improvement for the sustainable post-mining land use should be considered by putting these areas back into a suitable condition for farming.

Amongst rehabilitation methods, phytoremediation is frequently preferred in recent years, owing to its low-cost and agronomically applicable characteristics (Wu et al., 2006). For instance, several perennial woody plants such as poplar and willow, along with some herbaceous ones, are used in the phytoremediation of minefields (Yu et al., 2019). However, if these plants are used for the rehabilitation, the agricultural land, and plants grown in this land, both will not be able to be used in the food economy due to the harmful potentially toxic elements (PTEs) accumulations. Yet, maize which is one of the main agricultural products in the Black Sea rural area is a much better candidate among these herbaceous plants with its high PTEs accumulation capacities, modest growth needs, and most importantly high carbohydrate content indicating its biofuel production potential. If the plants such as maize are used in the phytoremediation, even though they obviously are not candidates for food available for human or animal consumption due to the high PTEs, resulting maize biomass after the remediation process could be a crucial resource for a sub-industry as bioethanol/biogas production. Conceptualization of this notion both will make the PTEs contaminated marginal lands valuable by turning these lands back into the agriculture. It also will provide the valorization of the maize biomass in the green industry. Similarly, the potentials of castor bean and wheat together with maize has been investigated by other researchers (Olivares et al., 2013; Van Ginneken et al., 2007; Babu et al., 2014) as a source of both phytoremediation and bioenergy production prototypes in their works. However, there is no such designated concept from Turkey's abandoned mining fields in this regard.

Effective phytoremediation of plants depends on the physicochemical properties (pH, oxidation-reduction potential (ORP), organic matter, etc.) in the rhizosphere as well as on their self-physiology. It is being known PTEs bioavailability is controlled by these specific properties (Gedik et al., 2017). On the other hand, some chelating agents (e.g. EDTA) could externally be added to the soil to increase bioavailability and PTEs uptake of plants in this process. However, these chemical additives may cause an increase in the management cost, or even a contamination risk of groundwater may emerge then (Van Ginneken et al., 2007; Meers et al., 2010). Instead, biological agents (microorganisms), which persistently exist on their plant hosts, are better candidates to integrate continuity and sustainability in removing PTEs from the soil. These agents forming synergistic links with their living hosts are more cost-efficient than other chemical applications, as well as they are ecologically friendly and further contribute to the development of plants (Pehlivan et al., 2017).

Beneficial relationships between fungi and plants may result in a certain degree of soil PTEs detoxification. One of the best examples of these fungi is the *Trichoderma* genus (Druzhinina et al., 2005). For instance, in the *Eruca sativa* grown in the copper-treated soil was reported to be increased the metal amount by *Trichoderma harzianum* inoculation up to 4.69% (Al-Rajhi 2013). In this context, we aimed to investigate the contribution extent of our novel *Trichoderma harzianum* strain TS 143 on the PTEs uptake capacity of maize plants together with the potential usage of the contaminated maize biomass in biofuel production.

2. Material and Methods

2.1. Culture of fungal isolates and mycelial growth index (MGI-U)

Isolation of *Trichoderma* species from the soil samples taken from active and abandoned (passive) two mining sites of Cu, Pb, Zn companies located in the Eastern Black Sea Region of Turkey was performed (Fig. 1). Sampled soil kept at 4 °C till fungal isolation. Wheat bran and 5% malt sprout were used in the solid-state fermentation production (Sargin et al., 2013) of *Trichoderma* isolates, while 10 g L^{-1} glucose, 5 g L^{-1} malt and 2.5 g L^{-1} yeast extracts were used in the production by liquid culture fermentation. Malt Yeast Growth media (100 mL) were inoculated with 1.0×10^8 spores mL^{-1} of solutions prepared from 6 days active fungal cultures. After the incubation period, the fungal biomass in the flasks was blended for 3 min and 1/100 of sample dilution was set with sterile Tween 80 solution. As for the case of solid culture, 1 g fresh fungal culture sample dried at 40-45 °C after four days of incubation at 30 °C transferred into the same volume of Tween 80. The count of micro propagules (conidia, chlamydospores, and mycelium fragments) in the samples was determined as CFU mL^{-1}. The counts were estimated by pour plate method with DG-18 agar medium after four days of incubation at 30 °C (Sargin et al., 2013). Micro propagules obtained from solid and liquid culture fermentations were used as inoculants for further experiments.

All of the isolates were screened for PTEs tolerance test according to the agar dilution method. Experiments were conducted triplicate, each petri plate containing different concentrations of PTEs (500-3000 mg L^{-1}). PTEs were inoculated with agar plugs from young mycelia. The plates were evaluated for the fungal growth on days 3, 5, 7. Following formula was used to calculate the percentage inhibition of radial growth: (PIRG %) PIRG (%) = ((R1- R2)/R1)×100% Where; R1 refers to the radial growth of mycelium in control plates R2: indicates the radial growth of the mycelium inoculated with the fungus. PDA plates without PTEs were used as controls. (Govarthanan et al., 2018).

2.2. Minimal inhibitory concentration (MIC-U) and microstructure analysis

The MIC values of As, Cd, Cu, Pb, and Zn against TS 143 were recorded at 28 °C by following the agar dilution methods of Clinical and Laboratory Standards Institute (CLSI, 2018). A total of 15 mL of 90 mm PDA per petri dish was used for the analysis. The concentration of PTEs was tested at least six different concentrations. Agar plates without the metal(oid) ions yet with TS 143 were used as controls. The plates were kept incubated at 28 °C for 7 to 10 days.

Sample preparation for scanning electron microscopy (SEM) analysis was performed with an auto Au-Pd coater (Sputter Coater 108, Cressington), enabling conductive coating of the TS 143. Micro-structure visualization was performed by a SEM instrument (Quanta FEG 250, FEI).

2.3. Molecular evidence

Amplification of the barcode gene, sequencing, and bioinformatic analysis

After a quick low g centrifugation of the target strain grown in PDB, obtained fungal cells were homogenized in liquid nitrogen. Nucleic acid was isolated with a Genomic DNA purification Kit (Thermo Scientific), and purity/quantity of 1 mL was checked by a Nanodrop 2000C UV-Vis spectrophotometer (Thermo Scientific). 0.7% agarose gel electrophoresis in Tris/Acetate/EDTA buffer (1X) at 120 V was applied for UV visualization. Translation elongation factor1 alpha (*tef-1α*) barcode fragment (0.8 kb) (O'Donnell et al., 1998) was amplified with EF1: 5'-ATGGGTAAGGARGACAAGAC-3' and EF2: 5'-GGARGTACCAGTSATCATGTT-3' by T100™ Thermal Cycler (Bio-Rad, USA) at 95 °C 12 min for initial activation then 32X of 95 °C for 15 s, 53 °C for 15 s, 72 °C for 60 s with a final extension of 72 °C for 4 min Cycling mixture (20 µl) containing 5X HOT FIREPol® Blend Master Mix supplemented with 12.5 mM $MgCl_2$ and 1% DMSO was used along with 10 pmol EF1 and EF2 oligos and 0.1 ng of gDNA template. After PCR products were visualized on a UV-transilluminator (Wealtec), target amplicons were diluted and purified with MAGBIO HighPrep™ PCR Clean-up System (AC-60005) to be sequenced in both + and - senses. Amplification primers served as sequencing primers as well. Sanger sequencing was applied using BigDye Terminator v3.1 Kit (Applied Biosystems) on an ABI 3730XL DNA instrument (Applied Biosystems). Bioinformatic analysis was implemented by manually trimmed, and

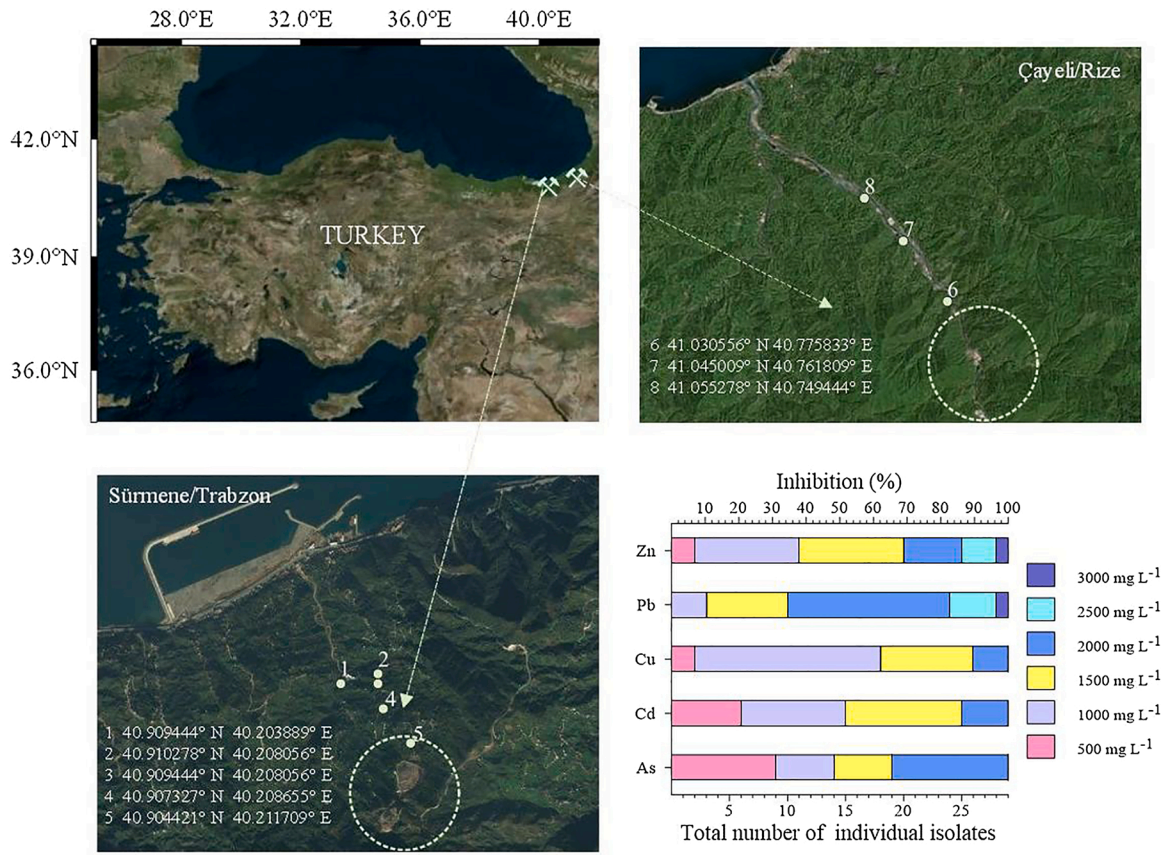

Fig. 1. Sampling locations of fungal isolates and inhibition (%) measurements (lower right) of mycelial growth index (MGI-U) of all sampled fungi (29) under various PTEs stress.

assembled raw sequence reads using IUPAC codes via 4Peaks v 1.8 software. Comparisons that are homologous to the target sequence were analyzed using the other deposited sequences publicly available in the repository (NCBI, http://www.ncbi.nlm.nih.gov) by basic local alignment (BLAST). We aligned 12 sequence hits for homolog protein sequences with Clustal O (1.2.4) multiple sequence alignment tool (https://www.ebi.ac.uk/Tools/msa/clustalo/), yet used 7 of them to view evolutionary relationship via the neighbor-joining method, which calculates distance matrix. Mega X was used to infer evolutionary relationships (Stetcher et al., 2020).

2.4. Preparation of contaminated soil and characterization

To test the bioremediation efficiency range of the TS 143, we spiked the commercial soil at 3 different concentrations of As, Cd, Cu, Pb, and Zn as medium (200 mg kg^{-1}), high (500 mg kg^{-1}) and the extreme (1000 mg kg^{-1}) to mimic the natural soil characteristics of the abandoned mining area in-vitro. Because our preliminary data had shown that Cu (201.2-514.2 mg kg^{-1}), Pb (41.42-70.06 mg kg^{-1}) and Zn (160.7-225.5 mg kg^{-1}) was higher in the agricultural area adjacent to the passive open mining pit where the target strain was isolated from, than the declared limits (100 mg, 60 mg, and 200 mg kg^{-1} respectively (UNEP, 2013; Toth et al., 2016). pH and oxidation and reduction potential (ORP mV) in commercially supplied soil samples which kept in RT (24 °C) at a ratio of 1:2.5 soil/dH_2O (w/v) for 24 hours was measured with Orion 5- Star (Thermo Scientific) multi-parameter measuring device (DeLaune et al., 2013). After the soil was dried at RT and sieved through a 2 mm sieving mesh, total organic carbon (1.14%) and texture analyses (10% sand, 90% silt + clay) were performed. Then the dry matter was determined by taking a certain quantity (25 g) from the soil kept at 105 °C for 24 hours. Based on the amount of dry matter, 500 g of soil was placed in 2 L glass jars, then solutions containing 1 L of 200, 500, and 1000 mg L^{-1} As, Cd, Cu, Pb, and Zn was added. $Cu(NO_3)_2{\cdot}3H_2O$, $Pb(NO_3)_2$, Zn $(NO_3)_2{\cdot}6H_2O$, Na_2HAsO_4 $7H_2O$, and $Cd(NO_3)_2{\cdot}4H_2O$ was used for solution preparations. The jars were shaken at 24 ± 1 °C and 75 rpm for 48 hours on a horizontal shaker, and then the solutions were evaporated at 40 °C in an oven (US EPA, 2001). PTEs concentrations in all soil groups after spiking was given in Table 1.

2.5. Mycoremediation assay of PTEs contaminated soil

5 mL of 10^8 CFU mL^{-1} spore suspension prepared from fresh TS 143 culture was inoculated in the potato dextrose broth (PDB) at 1/7 strength and cultured in a horizontal shaker for 28 °C for 15 days. For control, a series was cultured in the same conditions without TS 143. At the end of the incubation period, the pH of the samples was recorded, then samples were centrifuged, the supernatant was filtered. PTEs

Table 1
Potentially toxic elements concentrations (mg kg^{-1} mean ± standard deviation (SD)/dry weight) of soils used in the treatments

	Control*	Medium*	High*
Cu	12.09 ± 1.54	196.30 ± 8.16	505.67 ± 14.35
Pb	2.38 ± 0.76	194.14 ± 10.74	397.04 ± 18.30
As	1.83 ± 0.47	189.04 ± 7.36	376.63 ± 19.71
Cd	1.89 ± 0.24	194.71 ± 11.08	476.14 ± 24.94
Zn	115.21 ± 9.54	320.08 ± 21.05	595.89 ± 34.76

* control: no spiking, medium: soil spiked with 200 mg L^{-1} of As, Cd, Cu, Pb, and Zn solution, high: soil spiked with 500 mg L^{-1} of As, Cd, Cu, Pb, and Zn solution extreme: soil spiked with 1000 mg L^{-1} As, Cd, Cu, Pb and Zn solution. The extreme group was excluded from the analyses since plants could not be able to tolerate 1000 mg L^{-1} and died.

concentrations were determined by ICP-MS (Agilent, 7800).

2.6. PTEs removal by TS 143

To determine the PTEs accumulation of TS 143, solutions containing 1 mL of 10^8 CFU mL^{-1} fungal spores, 100 mg L^{-1} As, Cd, Cu, Pb, Zn, and 25 mg L^{-1} As, Cd, Cu, Pb and Zn in 50 mL of 1/7 diluted PDB was prepared and kept in a horizontal shaker at 28 °C for 5-7 days. After the incubation period, the samples were dried at 105 °C, and the fungus quantities (g) were determined after filtered through the 47 mm diameter, 0.45 µm pore size filters previously dried overnight at 60 °C. The concentration of the filtered solutions was determined by ICP-MS (Agilent, 7800). The absorbed-adsorbed amount was calculated by subtracting the final concentration from the first concentration of the solutions.

2.7. Plant materials, growth conditions for PTEs uptake

Maize seeds (*Zea mays L.* RX9292) sterilized with 70% EtOH, and 15% sodium hypochlorite with several subsequent washes of distilled H_2O were germinated in 9 mm petri dishes for two days before pot experiments. After two days of germination, seeds were transferred to the potting mix at a depth of 2 cm in 2-L pots (2 seed per pot), seedlings were grown ten days in a climate test chamber with 70% humidity and 16 h light/8 h darkness of long-day period at 25 °C/23 °C program cycle under regular irrigation, then PTEs stress and TS 143 was applied. All soil samples (1 kg per pot) were autoclaved at 1.1 bar at 121 °C and cooled down under sterile conditions before added to the pots. The pots also were disinfected with bleach and rinsed through dH_2O before use. Commercially supplied and uninoculated and non-contaminated soil was used as the negative control, while TS 143 inoculated and non-contaminated soil was used as the positive control. The soil was spiked at three different concentrations (200, 500, and 1000 mg L^{-1}) of As, Cd, Cu, Pb, and Zn are inoculated with TS 143 while the other 3 groups were not inoculated. 10^8 spores g^{-1} of TS 143 were applied in the soil, where plants were transferred ten days after planting (DAP). Since the extreme PTEs group (spiked with 1000 mg L^{-1}) could not be able to show a robust growth until the end of the experimental period, we discarded this group of treatments for further physio-chemical analysis. Right after the finalization of the experimental period of 4 weeks, plant materials were sampled for biochemical analysis, immediately placed and stored at –80 °C till use.

2.7.1. Phytoextraction and growth markers

2.7.1.1. Plant water status measurements. Relative water content (RWC) of the maize leaves were calculated based on the formula of RWC = [(FW–DW)/(TW-DW)] ×100 (FW: fresh weight, TW: turgid weight, DW: dry weight). TW and DW were recorded after o/n rehydration of the leaf samples in a 15 mL conical tube filled with deionized H_2O and after two days of oven drying at 65 °C, respectively. RWC was calculated as a % per TS 143 and/or PTEs treated or uninoculated maize plants.

2.7.1.2. Membrane stability index (MSI) analysis. The fully expanded leaves of maize plants were sampled and used for MSI quantification. The leaf segments were rinsed quickly with deionized H_2O then, placed in a 50 mL conical falcon tube filled with 15 mL of deionized H_2O, initial electrical conductivity (C1) was recorded after incubation in a water container at 42 °C for 30 min. A final measurement of electrolyte leakage (C2) was conducted after autoclaving the samples for 5 min at 121 °C (Sairam et al., 1997).

2.7.1.3. Plant pigment quantitation. Pigment content was quantified for control, and PTEs stressed leaves using the methanol extraction method of Porra et al. (1989). Briefly, TS 143 and/or PTEs treated or uninoculated maize leaf samples (100 mg) were incubated in methanol for 24 h at 4 °C. An mL of each supernatant was spectrophotometrically read at 652 nm and 665.2 nm for obtaining chlorophyll *a* and *b* concentrations. Calculations were based on the amount of chlorophyll a (µg mL^{-1}) = $16.29A_{665.2}$-$8.54A_{652.0}$ and the amount of chlorophyll b (µg mL^{-1}) = $30.66A_{652}$-$13.58A_{665.2}$ formulas with a molar extinction coefficient of the chlorophyll *a* and *b* and given as total pigment amount (*a*+*b*).

2.7.1.4. Carbohydrate and protein assays. The phenol sulphuric acid method, which dehydrates glucose to hydroxymethylfurfural, was used to quantify total carbohydrate quantities of the maize samples. A hundred mg of samples were hydrolyzed in a water bath for three h with 2.5 N HCl and neutralized with solid Na_2CO_3. The absorption of the green-colored reaction mix containing phenol and 96% sulphuric acid was spectrophotometrically read at 490 nm. The total amount was represented as % carbohydrate in each sample. Protein quantitation was performed via the basic principle of protein molecules-dye reagent binding (Bradford 1976). Eight dilutions of bovine serum albumin (Sigma) containing a range of 10 to 100 µg protein quantities were used as protein standards. Concentrations of the total protein in each sample were measured via the spectrophotometric absorbance of the protein-dye complex at 595 nm.

2.7.1.5. PTEs determinations. Soil and maize root and shoot tissues were dried in an oven (<40 °C) then crushed in a mortar for sample homogeneity. 0.5 g of samples were transferred into glass tubes (75 mL) and digested with repeated additions of pure HNO_3 (Trace metal grade 65%) and H_2O_2 (Trace metal grade ≥%30) (5:2) at 120 °C in a thermo-block and kept for 2 hours. Then, the tubes were kept at a similar temperature in the block until the amount of the reaction solution decreased to ~ 1.5 mL. After the tubes brought to RT, samples were diluted to 50 mL with ultra-pure H_2O. The solutions were filtered (0.45 µm pore size PTFE filters) and stored at 4 °C until analysis. The whole process was applied similarly for only 3 blank tubes of HNO_3 and H_2O_2 without a sample (soil, maize shoot or root) for each set for control purposes of detecting contamination (EPA 1996).

ICP-MS (Agilent, 7800) was used to determine the concentrations of all samples (digested soil, plant tissues, and solutions). In every 15 samples, a blank sample, and scandium (Sc) was used as the internal standards for interference detection (US EPA, 1994). Cu, Pb, Zn, As and Cd concentrations in soil and maize shoot and root tissues were calculated as mg kg^{-1} using the formula below:

$$\text{PTEs concentrations}\left(\text{mg kg}^{-1}\right) = \frac{\left(\textit{Detected elements concentration}\left(mg\ L^{-1}\right) - \left(\frac{Blank1+....Blank(n)}{n}\right)\right) x\ \textit{sample volume}\left(50\ mL\right)}{\textit{digested sample weight}\ (g)} \quad (1)$$

Confirmation of ICP-MS measurements were tested using the reference solutions (SCP Science, USA). Verification of the digestion results for the integrity check of the process was tested using certified reference

Table 2
Recovery of the certified reference materials

	Plant			Soil		
	Certified value*	measured value*	Recovery rate (%)	Certified value*	measured value*	Recovery rate (%)
As	0.85	0.89	104.71			
Cd	0.56	0.52	92.85	72	67.71	94.04
Cu	7.03	6.98	99.29	130.6	127.14	97.35
Pb	40.9	42.87	104.82	858	886.1	103.28
Zn	100.6	104.93	104.30	1063	1037.09	97.56

* The results were given as mg kg^{-1} dry weight.

materials (CRM: lichen BCR-482, soil BCR-143R) (US EPA, 1996). The recoveries of Cu, Pb, Cd, As and Zn were calculated according to the values obtained in the measurements for CRMs. Recovery values for PTEs were ranged between the 93-105% (Table 2).

2.8. Statistical Analysis

T-test was performed by SigmaPlot 13 (Systat Software, San Jose, CA) to evaluate whether significant differences exist between the un-inoculated and TS 143 inoculated groups. The significance level was set at 5%.

3. Results and Discussions

3.1. Identification and genetic analysis of the PTEs-tolerant strain

The isolates (29) were obtained around the agricultural soils of an active and passive Cu, Pb, Zn mining sites in the Eastern Black Sea coast of Turkey. Among the strains elected at PTEs tolerance level (Fig. 1), the one showing excellent tolerance capacity and showing more growth/sporulation than other strains was selected and further subjected to species identification. Identification data was visualized in Fig. 2. Right after pre-taxonomic identification with microscopic visualization to see microbial-structure, mycelial growth index, and colony morphology (Fig. 2), molecular identification was conducted by DNA-based methods. The genetic relationship was analyzed via *tef-1α* gene, which suggested to have better resolution than the ITS barcode of rDNA for *Trichoderma* species diversification (Al-Sadi et al., 2015). After PCR-mediated detection and sequencing, blastn search showed that the top hit was *Trichoderma harzianum* culture-collection BMCC:LU1367 *tef-1α* gene, partial cds. (GenBank Accession: KJ871187.1) which was found covering 100% the entire length of our input sequence with 1369 base (max. score) and matches the 99.6% of the base calls. Sequence reads consisting partial genomic sequence of *tef-1α* gene from the fungus named TS 143, was deposited in the GenBank public repository via BankIt (https://www.ncbi.nlm.nih.gov/WebSub/) under accession number: MT508831 (https://www.ncbi.nlm.nih.gov/nuccore/MT508831.1/).The seven representative *tef-1α* protein sequences from different *Trichoderma* species were aligned with CLUSTAL O (1.2.4) multiple sequence alignment tool (//www.ebi.ac.uk/). Then, the cladogram was generated based on the evolutionary distances of K2P nucleotide substitution model (Kimura 1980), which was computed

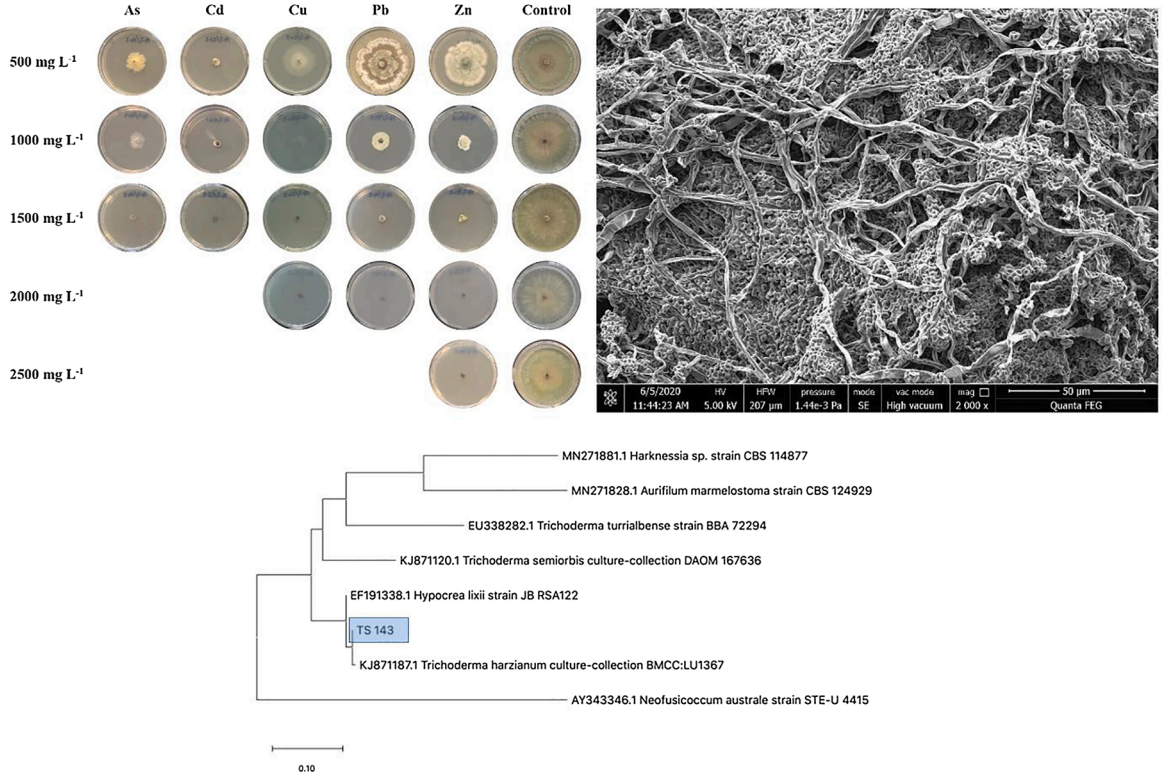

Fig. 2. Identification and evolutionary relationships of taxa, Upper left panel: Colony morphology analysis of TS 143 under different potentially toxic elements concentrations showing mycelial growth of the target strain on day 7, Upper right panel: Microbial mechanism showing the cellular structure, Lower panel: Phylogenetic history of TS 143. Construction was represented as a neighbour-joining tree of *tef-1αs.* The analysis was performed based on the compared sequences using Mega X.

using the maximum composite likelihood of MEGA X (Stecher et al., 2020), free software on the web (Fig. 2).

Mycelial growth index (MGI-U) test indicated that despite showing a decreased mycelial growth compared to the controls, 68%, 79%, 93%, 100% and 93% of the strains was capable of growing on the medium containing 500 mg L^{-1} of As, Cd, Cu, Pb, and Zn respectively. 34%, 13%, 10%, 65%, and 31% of the strains were able to grow up to the 2000 mg L^{-1} of As, Cd, Cu, Pb, and Zn, respectively. While five strains were still alive up to 2500 mg L^{-1} Pb, four strains showed growth up to 2500 mg L^{-1} of Zn. Furthermore, a strain for Pb and three strains for Zn displayed resistance to 3000 mg L^{-1}. We think that Cu, Pb, and Zn growth data is particularly relevant here because, sampling area, the agricultural land around active and abandoned mining areas of all fungi is affluent in Cu, Pb, and Zn.

High PTEs concentrations could easily trigger the development of stress tolerance strategies in fungi (Coelho et al., 2020). Among many strategies against PTEs such as spore production, reduced energy and/or nutrient use or lowering growth to make fungi live longer under harsh environmental conditions (Ayangbenro and Babalola, 2017) here in our work, we observed decreased mycelial growth rate. MIC-U (minimal inhibitory concentrations) was considered as the lowest concentration that metal(oid) ions visibly inhibited the fungal growth. In this context, the doses of 5 PTE ions were tested against TS 143. Relatively high TS 143 survival rates were detected, which follows: As 1400 mg L^{-1}, Cd 1200 mg L^{-1}, Cu 2000 mg L^{-1}, Pb 2100 mg L^{-1}, Zn 3000 mg L^{-1}.

3.2. Mycoremediation of PTEs-contaminated soil

The impact of the fungus on As Cd, Cu, Pb, and Zn mobility in soil, mycoremediation was investigated. Bioleached PTEs amount by fungal spores of TS 143 was given in Fig. 3. PTEs towards inoculant was determined to be more bioavailable than the groups that are not inoculated with the fungus. In inoculated groups, bioleached PTEs were lined up as follows: Cd > Pb > Cu > Zn > As. Pb and Cu bioleaching processes were more efficient when TS 143 existed in the soil. On the other hand, in the uninoculated group, while the highest bioleached PTEs were determined as Cd (5.85%), the lowest one was As (1.36%). In the same group, TS 143 was capable of removing PTEs in the order of Cd > Zn > Pb > Cu > As. However, when we compare the bioleaching process in percentage, no difference was detected between the two groups (TS 143 inoculated and uninoculated) in the soils spiked at various concentrations of PTEs (Fig. 3A). Arsenic remarkably, retained in the soil in both TS 143 inoculated and uninoculated groups. This might be explained with its persistence, chemical structure and/or lower availability in stable fractions due to the residual metal(oid), especially beyond phase 1 (Zhang et al., 2018). Some other actors also might hinder the contact between the inoculant and PTEs, which subsequently interrupt mobility/solubilization (Xia et al., 2013). Amongst factors such as employed concentrations or sorption kinetics of PTE ions, PTEs contact period, redox potential and organic matter of soil used etc., since the variable that most affects PTEs bioavailability in the soil is the pH, we determined pH change which decreased from 5.6 to 5 towards TS 143. Redox potential was increased in TS 143 inoculated group at 5-13% range compared to uninoculated groups. This shows the efficacy of PTEs removal in the bioleaching process with TS 143 fungal activity, which would help promote effective phytoextraction of PTEs. Higher PTEs biosorption in response to lower pH was marked by several other reports (Say et al., 2003; Zhang et al., 2018; Chang et al., 2019). One of the very early works regarding that is Huang et al. (1990). They found that the Cu (II) biosorption of *Saccharomyces* was pH-dependent and max. biosorption being observed was at a pH range of 5.0-7.0.

3.3. PTEs removal by TS 143

Inoculant assisted phyto-bioremediation of PTEs are more ecologically safe compared to physicochemical technologies in contaminated soil remediation (Mello et al., 2019). However, effective mitigation of PTEs toxicity is the key parameter for a soil organism in this process (Franchi et al., 2017). A fungus, for example, with its extensive hyphal network, can easily modify the valance of root chemicals, thus affect the accumulation and transport rate of PTEs along with benefits such as improvement in plant biomass. To elucidate whether our fungal inoculant is advantageous in this regard, PTEs removal capacity of TS 143, which was inoculated into two different concentrations (25 and 100 mg L^{-1}) of As, Cd, Cu, Pb and Zn solutions was investigated (Fig. 3B). Whereas dry biomass of TS 143 (0.99 ± 0.12 g L^{-1}) was recorded higher than that of control (0.94 ± 0.11 g L^{-1}) in the group where PTEs concentrations were 100 mg L^{-1}, dry biomass of TS 143 was found to be 0.87 ± 17 g dry weight L^{-1} in 25 mg L^{-1}. While the low concentration (25 mg L^{-1}) of PTEs had a positive effect on the growth of TS 143, a proportional decrease in growth was observed in parallel with the higher concentration (100 mg L^{-1}) attributable to concentration-dependent slowing down in growth. Our other isolates showed a similar growth trend as well in the inhibition (%) of MGI- U and colony morphology (Fig. 1 and

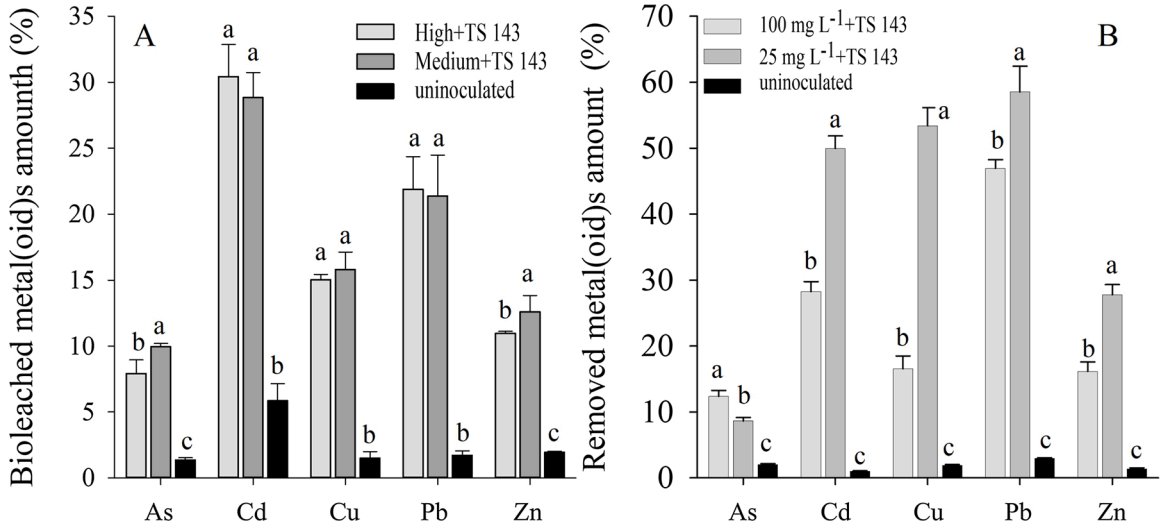

Fig. 3. Mycoremediation capacity of TS 143. A. Bioleached amount from uninoculated (no spiking), medium and high (200 and 500 mg L^{-1} respectively) PTEs spiked soil into the aqueous solution, B. PTEs removal capacity of live TS 143 biomass. Results were given as mean ± standard deviation (n = 3). T-test was used to compare TS 143 inoculated and uninoculated groups. Different letters (a, b) show significant differences ($p < 0.05$).

Fig. 2). This dynamic in fungus growth was corroborated by similar case studies up to date (López Errasquín and Vázquez, 2003; Babu et al., 2014; Ayad et al., 2018).

PTEs removal capacity of TS 143 was examined in two different concentrations and determined to vary between 8.64% and 58% (Fig. 3B). Removed PTEs amount was listed as Pb > Cd > Cu > Zn > As. Thus, except for As, TS 143 had higher PTEs removal capacity in 25 mg L^{-1} solution. The reduction of PTEs removal capacity in TS 143 at high concentrations of PTEs might be related to the toxicity rate, growth inhibition, and subsequent biomass reduction, which we also found that biomass decreased at higher PTE concentrations. However, PTEs removing capacity or boosting phytoextraction varies greatly across fungus genotypes having a different genetic background. Thus, the sequencing of more isolates from various niches can shed light on the information of the diversification of both new *Trichoderma spp.* and their valuable gene loci and proteins. Several other works have also described the effective roles of fungi in PTEs removal. For instance, Sun et al. (2017) have reported around 46% increase of Pb accumulation in *Solanaceae* family, and Li et al. (2017) reported viable remediation of Cd-resistant *Aspergillus* in *Bermudagrass*. On the other hand, to both promote PTEs mobilization and bioavailability and subsequently phytoextraction, external chemical additives are being applied as an option for some cases. Even though sometimes these chemical agents such as ammonium thiosulfate, EDTA or potassium di-hydrogen phosphate might serve here as mobilizing agents (Franchi et al., 2017), this is not favorable for both high cost-effectiveness and different and further chemical contaminations.

3.4. Plant growth and PTEs uptake

Since the chlorophyll intactness is a marker of the effectiveness of the plants` photosynthetic apparatus, and thus the accumulation of sugar and polysaccharide, we investigated whether TS 143 inoculation contributes to the efficacy of photosynthesis in maize plants by determining the chlorophyll concentration in the samples. The total chlorophyll amount was measured higher in the control group, which was also showed higher biomass compared to other experimental groups. In parallel with the increase in the strength of the contamination, the intactness of chlorophylls decreased proportionally. However, TS 143 inoculation boosted the chlorophyll amount by 43% and 32%, respectively, compared to the medium and high PTEs treated only groups that were not inoculated with the fungus. The groups spiked with PTEs and inoculated with TS 143 had 33% and 28% more chlorophyll compared to the medium and high applied PTEs groups only that were not inoculated with the fungus. (Fig. 4A)

Plants cultivated for biofuel production as a renewable energy source are required to have high carbohydrate content (Babu et al., 2014). Maize has long been known as an existing sustainable energy source, among other valuable bioethanol feedstocks such as sorghum, switchgrass, or kudzu (Sage et al., 2009). Since high carbohydrates content through cellulose breakdown is a desirable trait for a fermentable C4 plant such as maize, we determined the total carbohydrate of our maize samples (Fig. 4B). We have found 33.50% carbohydrate increase in medium and 28.65% increase in high PTEs groups of TS 143 inoculated maize plants under contaminated conditions. PTEs negatively affected the protein content; however, 44% and 22% increase were calculated in both TS 143 inoculated and medium and high PTEs contaminated groups, respectively (Fig. 4C). Enhanced MSI and RWC also were found in the presence of TS 143 compared to uninoculated groups (Fig. 4D and 4E). Fresh weights (FWs) of roots and shoots of maize plants in the groups that are contaminated with medium and high concentrations of PTEs and TS 143 inoculated are given in Fig. 4F. In the control group, both root and shoot FWs decreased by 23% and 32%, respectively, in TS 143 inoculated maize plants, compared to the uninoculated ones (Supp. Fig. 1). However, in the PTEs contamination groups, an increase was recorded in both root and shoot FWs. This increment was found as 45% and 44%, respectively, for the root FW in the medium and high PTE groups of TS 143 inoculated plants. As for shoot FWs, this ratio was determined as 30% and 12%, respectively, for the medium and high PTEs groups (Fig. 4F). Interestingly, we observed a much better potential of TS 143 towards higher PTEs stress conditions rather than the medium one. We observed that the higher the PTEs dose, the better the

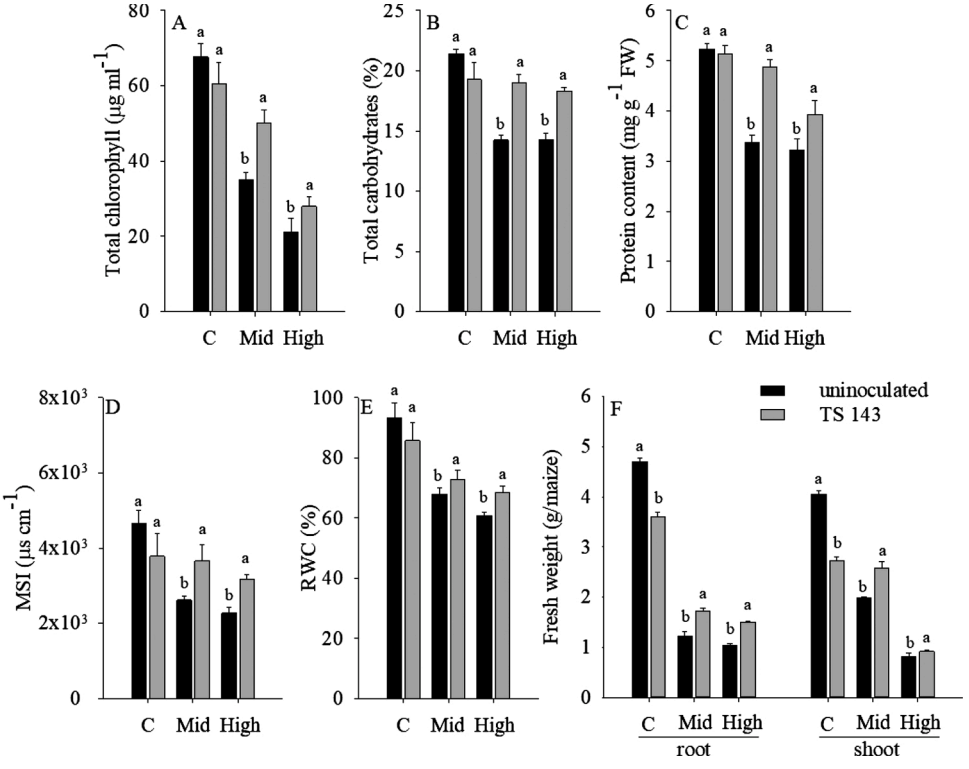

Fig. 4. Metabolic profile data regarding decreased phytotoxicity lead by TS 143. A. total chlorophyll, B. total carbohydrates, C. total protein, D. membrane stability index, E. relative water content, F. fresh weight. C: no spiking (control), Mid (medium): soil spiked with 200 mg L^{-1} As, Cd, Cu, Pb, and Zn solution, High: soil spiked with 500 mg L^{-1} As, Cd, Cu, Pb, and Zn solution. Results were given as mean ± standard deviation (n = 3). T-test was used to compare TS 143 inoculated and uninoculated groups. Different letters (a, b) show significant differences ($p < 0.05$).

effect, and TS 143 could not show any better performance when there was no stress. This data suggest that the TS 143 indeed can act better in a PTEs concentration-dependent way under stress.

The fungal cells contain a polysaccharide called chitin, which has great potential to remove heavy metals from the environments. Amongst all the tested elements here (As, Cd, Cu, Pb and Zn) even though especially Cu and Zn are the essential micronutrients to sustain mainly enzymatic activities of plants (White and Brown, 2010), excessive concentrations of these might be toxic to other biomolecules in the cell as well. Because *Trichoderma* genus can tolerate and/or detoxify excessive PTEs by forming precipitations or crystal complexes, transform-compartmentalize-sequester and/or transporting PTEs by intra-extracellular efflux or biosorption to the cell wall (Cao et al., 2008; Bareen et al., 2012), all our biochemical data concerning maize growth and development under PTEs stress, remarkably showing that eased phytotoxicity could be attributed to the excellent bio adsorbent capacity

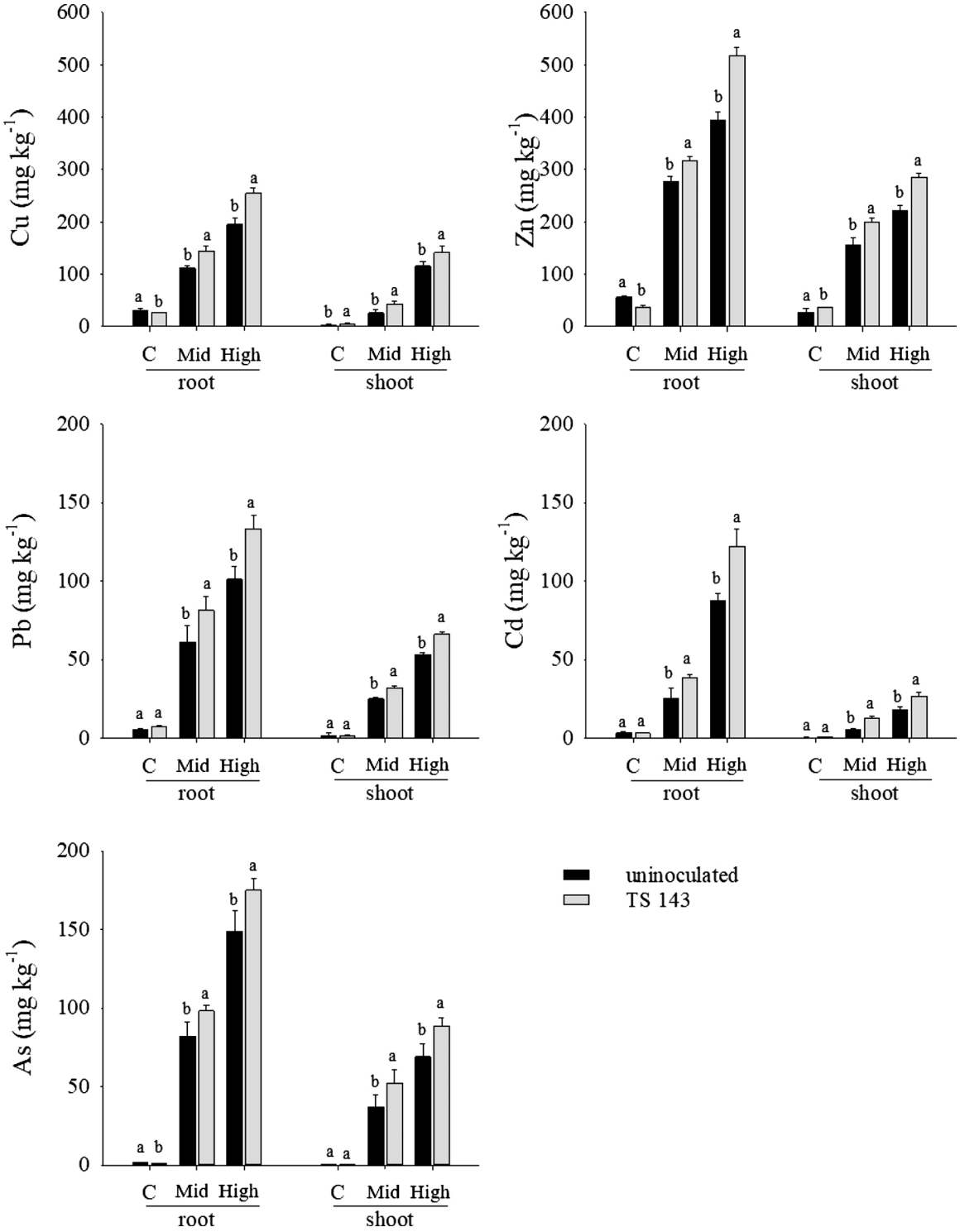

Fig. 5. Potentially toxic elements (PTEs) concentration detected in different maize organs towards TS 143 inoculation. C: no spiking (control), Mid (medium): soil spiked with 200 mg L^{-1} As, Cd, Cu, Pb, and Zn solution, High: soil spiked with 500 mg L^{-1} As, Cd, Cu, Pb, and Zn solution. Results were given as mean ± standard deviation (n = 3). T-test was used to compare TS 143 inoculated and uninoculated groups. Different letters (a, b) show significant differences ($p < 0.05$).

of TS 143. Different studies reported similar stimulation upon *Trichoderma* application for plants grown in PTEs contaminated soil and pointed up that fungus dramatically link the soil to the plant in the rhizosphere (Bareen et al., 2012; Babu et al., 2014) along with establishing better plant water uptake.

PTE concentrations of maize plants grown in the groups that were inoculated with and without TS 143 were given in Fig. 5. PTE concentrations, except for Pb, were detected higher in the roots of the control plants without contamination and inoculation, compared to the inoculated plants. However, PTE concentrations were significantly increased in all the plants that were exposed to the medium and high PTEs and inoculated with TS 143, in both roots and shoot tissues ($p < 0.05$). These results were in parallel with the biomass increase that, with the increasing biomass, maize plants were able to make better PTEs uptake. While these increases in the root tissues of medium PTE groups was lined up in the order of Cd (52%)> Cu (29%) > Pb (28%) > As (20%)> Zn (14%), in high PTE groups the order was Cd (60%) > Pb (36%) > Cu (39%) > Zn (35%) > As (24%). As for the shoot tissues, concentrations was detected in the order of Cd (138%) > Cu (65%) > As (40%) > Pb (28%) > Zn (27%) and Cd (71%)> Cu (37%) > As (37%) > Pb (33%) > Zn (32%) for the respective medium and high groups. However, obtained concentration rates, given the reasonable annual amounts of PTEs removal per hectare, it is clear that this technology is only valid for moderately contaminated soils and cannot be equivalent to the conventional soil remediation in heavily contaminated lands (Meers et al., 2005). On the other hand, the tested maize is a metal(oid) tolerator (PTEs in root higher than in shoot, bioconcentration factor (BCF) < 1 which expresses the net uptake and retention of PTEs in plant tissue from the soil), therefore the BCF would be much lower if we grow the plant at the real contaminated mining area compare to artificial condition. However, given our experimental period, if maize is grown for longer periods, (i.e. for the entire growing season till yielding the grain), then it is likely that the BCF factor could be equal to or greater than 1.

4. Conclusion

Twenty-nine novel indigenous fungal strains were isolated, and the characterization of the most promising PTEs-tolerant strain highlighted with its great potential in boosting the phytoremediation in our designated microcosm-scale phytoextraction process. No study has been reported dealing with the environmental risks of passive mining sites on the agricultural lands of Turkey using this concept by an eco-safe fungal agent via maize plants. Several growth parameters and soil/plant characteristics were examined along with the two different doses of PTEs uptake ability in maize. The applied target strain mostly tolerated all the PTEs at higher levels, further facilitated plant growth and development simultaneously by showing multiple robust growth traits such as photosynthetic pigment ultrastructure intactness, cell membrane stability, RWC and the total carbohydrate/protein contents especially towards PTEs stress in a concentration-independent way. We also suggested the biofuel crop production potential of the contaminated biomass of maize inoculated with TS 143 in the context of a microbiological-phytoremediation program. Considering its ratio among petroleum products (diesel and gasoline which has high amounts of PTEs such as vanadium and nickel) used today, biofuel obtained from contaminated maize will contain negligible amount of PTEs.

Higher plant biomass, especially along with higher carbohydrate contents under PTEs, is remarkable for maize plants in pursuit of eco-safe agriculture and sustainable biofuel production using above-ground plant biomass over and over again. Instead of fossil fuels associated with the greenhouse gas emissions, maize valorization, even though high PTEs in the plant is not good for their post-harvest treatments and some crucial questions emerges regarding the PTEs content of the subsequent product (e.g biogas, biofuels) cannot be neglected (Gomez et al., 2012), represents a co-benefit for the environment. On the other hand, when fungal biomass modified by acid treatment in an elution medium (e.g. HCl-which disrupts formed chelates between the fungal hyphae and adsorbed PTE ions to reverse the process into desorption), can be used consecutively with only a slight decrease in their biosorption capacity. Thus, this type of fungal bio-sorbent not only serves for a natural cleanup strategy but also a cost-effective and sustainable PTEs remover from agricultural systems.

CRediT authorship contribution statement

Necla Pehlivan: Conceptualization, Project administration, Funding acquisition, Resources, Investigation, Formal analysis, Methodology, Data curation, Software, Writing - original draft, Writing - review & editing, Visualization, Supervision, Validation. **Kenan Gedik:** Project administration, Funding acquisition, Methodology, Validation, Investigation, Resources, Data curation, Writing - review & editing, Supervision. **Rengin Eltem:** Methodology, Formal analysis, Validation, Resources, Investigation, Data curation. **Ertugrul Terzi:** Investigation, Resources, Formal analysis, Data curation.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2020.123609.

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