ISTANBUL UNIVERSITY PRESS

Variation of Response Patterns Associated with an Avirulent Plant Symbiont Directed Defense Gene Expressions in Maize Exposed to Toxic Elements

Necla Pehlivan[1]

[1]Recep Tayyip Erdogan University, Department of Biology, Rize, Turkey

ORCID IDs of the authors: N.P. 0000-0002-2045-8380

ABSTRACT

Objective: Microbe-assisted plant heavy metal (HM) tolerance is gaining momentum over a conventional breeding or transgenic approach being used to generate tolerant varieties capable of completing their life cycle in the metalliferous environments. To withstand toxicity, along with the current anthropogenic pressure, applications of fungi representing the largest group of eukaryotic organisms is considerably rising.

Materials and Methods: The hypothesis that a novel strain, which belongs to the *Trichoderma* genus (TS143), was previously identified as being multi HM-resistant, improves plant HM-tolerance by regulating hydraulic conductance and defense system was tested at a molecular level.

Results: While only a marginal increase in the expression level of 70 kDa chaperon protein (*HSP1*) gene was obtained, peroxidase (*POD1*) and plasma membrane intrinsic aquaporin (*PIP1-5*) genes were found to be upregulated (<2 fold) in the presence of chronic exposure to the HM-mix, (500 mg L^{-1} As, Cd, Cu, Pb, Zn) explaining the vivid metabolic modification underlying the metal stress response by target fungus. Up-regulation of the ROS-scavenging peroxidase and aquaporin genes affirming that the responses of *POD1* (9.44 fold) and *PIP1-5* (3.55 fold) expression may serve as potential sensitive biomarkers for HM-induced cellular toxicity monitoring with TS143 biostimulation.

Conclusion: Determining transcriptional level changes might pave the way for further applied research which would analyze gene level interactions of *Trichoderma*-HMs-plants.

Keywords: Heavy metal tolerance, maize, *Trichoderma*, stress markers, gene expressions

INTRODUCTION

Metal(oid)s, unlike other pollutants, are not biodegradable thus accumulating in soil over time (1). Hence these could readily be transmitted to the environment by two basic routes: the anthropogenic activities such as pesticide and fertilizer use (2) and the natural processes (rock sedimentation and soil erosion, volcanic eruptions, geothermal processes, forest fires, wind, etc.). However, several studies in the past decade have shown that metal(oid) pollution in the environment is mostly of anthropogenic origin (3). Thus, soils contaminated with high concentrations of metal(oid)s driven by anthropogenic input can cause significant yield losses in agricultural areas (4). In these areas, heavy metal(oid)s (HMs) are easily absorbed by plant roots (5) and migrated to the above-ground parts (6) causing dramatic physiological damage in plants (7). Impairments in the cell wall structure, cytoplasmic instability along with enzyme malfunction, decreased reactive oxygen species (ROS) scavenging capacity, nuclear DNA and organelle damage which would ultimately be blocking the photosynthesis are among the most serious

impacts which are the result of the accumulated HM amount/accumulation rate in a specific organ over a certain period of time (8). Particularly, multi-HM exposure such as simultaneous As, Cd, Cu, Pb, and Zn (HM mix) that could create synergistic damaging effects even at much lower concentrations more than a single HM does (9).

Fungi-plant associations can result in some degree of cleansing of the soil from toxic metals (10). It has also been widely reported that one of the most promising species in this regard belongs to the *Trichoderma* genus (11). Species in this genus, which are also commercially available today (12), show a beneficial bio-molecular significance in the plant rhizosphere via nutrient uptake efficiency increase, mycoparasitic activity, antibiosis, and competitiveness against plant pathogens commonly found in the soil (13, 14) thereby providing tolerance to both abiotic and biotic stressors (11, 15). These species have been widely reported to alleviate abiotic stresses through cellular processes such as osmolyte and seconder metabolite synthesis, Na cation elimination, or root improvement (16) by chemical signals after root penetration with the help of cysteine-rich hydrophobin proteins isolated from the fungus itself (17). In fact, it`s even been reported that the volatile metabolites of this micro fungus family do not cause any damage to plants, on the contrary, they develop various protection responses such as preventing excessive formation of ROS by anthocyanin accumulation and increase in the expression of defense genes (18).

Although studies are showing that HMs in soil are more bio-available thanks to the members of this genus (19, 20), the information on the contribution of these genus members to the HM response at the molecular level in plants is still scarce (18), particularly for new *Trichoderma* variants. Thereby, determination of the efficacy of the target fungal strain promoting defense response at the plant gene level give a more informative approach and can pave the way to further mechanistic research paths. In this study, the effect of *Trichoderma* TS143, a new, genetically characterized local isolate with high HM tolerance, which has a high potential for use in the rehabilitation of mine sites and biodiesel production (20) but whose effects on plant transcript dynamics are unknown was investigated at the gene level. The response triggered by TS143 directed regulation/target transcript on maize was targeted for analysis under multi-HM stress.

MATERIALS AND METHODS

Simultaneous TS143 and Multi-HM Exposure

A *Trichoderma* isolate, which is able to grow in HM concentrations between the range of 500-2500 ppm, was used in the experiments. This strain is one of the strains in which we analyzed the efficacy of its metal uptake in plants and proved that it increased the phytoremediation ability of maize plants (20). Based on the dry matter of the soil according to the US EPA method (21), 500g of soil was placed in glass jars then, solutions containing 500 mg L^{-1} As, Cd, Cu, Pb and Zn were added from the top. The solution mix was prepared by readily available

$Na_2HAsO_4 \cdot 7H_2O$, $Cd(NO_3)_2 \cdot 4H_2O$, $Cu(NO_3)_2 \cdot 3H_2O$, $Pb(NO_3)_2$, and $Zn(NO_3)_2 \cdot 6H_2O$ chemicals. After that, the jars were placed in a horizontal shaker at 24±1°C and 75 rpm for 2 days. The soil used in the pot experiments was ready after the solutions were evaporated at 40°C in an oven. TS143 was one of several members of the genus isolated by Dr. Rengin Eltem/Ege University from the soils sampled at a specific site of an active mining area in the Northeastern Black Sea region of Turkey. The strain was applied as a 10^8 colony forming unit (CFU) mL^{-1}/500g soil under in-vitro conditions.

Pot Experiments

Commercially supplied soil was autoclaved under 1.1 atm at 121°C and cooled down under sterile conditions. The pots used were pre-disinfected with bleach, then were rinsed through distilled water. The soil not contaminated with HMs was used as the control group (Control), while uncontaminated soil treated with the isolate only (TS143) was used as the positive control. To determine fungal efficacy, two more groups consisting of the HM cocktail only (HM mix) and As, Cd, Cu, Pb, Zn mix + TS143 (HM mix+TS143) were used to sow maize seeds (*Zea mays* L. *RX9292*) after a regular sterilization process. Six seeds were sown at a 2cm depth in the soil for all pots. After seven days, the seedlings were reduced to two plants in each pot to allow better future growth. The pots were watered every four days for 21 days (without using supportive fertilizers) to provide enough saturation of the soil. After the chronic HM exposure period with and without TS143, shoot samples taken for further use in RNA isolation were washed several times in distilled water then treated with liquid nitrogen and stored at -80°C.

Organ Specific Expression Analysis for Potential Target Genes

To measure changes at the transcriptional level in the marker genes belonging to enzymatic antioxidant and homeostasis mechanisms playing vital roles in free radical detoxification and/or possible water scarcity protection (due to the ionic toxicity under HM exposure), sequence-specific primers were synthesized. Heat shock 70 kDa protein1 (*HSP1*) which is one of the regulatory proteins that protect peroxidase1 (*POD1*) and plasma membrane intrinsic aquaporins 1-5 (*PIP1-5*) as an indicator of possible disruption related to water intake were selected. The maize glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was used as an internal reference for the normalization of the gene expression (22, 23).

RNA Isolation, cDNA Synthesis and Quantitative RT-PCR Analysis

The shoot samples of plants grown in the climate cabinet (Daihan, South Korea) for 21 days under the conditions of 16h light (120 µmol m^2sec^{-1} light intensity), eight hours dark mode at 25°C day/22°C night, and 70% humidity, were powdered in liquid N_2. The RNAs that belonged to the samples were isolated according to Chomczynski (24) using TRIzol®Reagent (AMBION). All solutions during isolation were used after being treated with diethyl pyrocarbonate (DEPC) and autoclaved.

Table 1. Primers of the target and reference genes used in the real time q-PCR experiments

Database entry#	gene_name	amplicon size (bp)	primers (F-R)
GRMZM2G137839	Peroxidase1 ZmPOD1	136	5'-CTGCTGAGTGACCCTGTCTTC-3' 5'-GGATAGGGTCTATTTAAGCATCAG-3'
GRMZM2G310431	Heat Shock 70 kDa Protein1 ZmHSP1	128	5'-CCACCAACACCGTCTTCGAT-3' 5'-TACAATCATGGGTTTGTCACCAG-3'
GRMZM2G081843	Plasma membrane intrinsic aquaporins 1-5 ZmPIP1-5	90	5'-CACGTGGTCATCATCAGGG-3' 5'-CGTATGCTGCATGGTTGCT-3'
GRMZM2G176307	glyceraldehyde 3-phosphate dehydrogenase ZmGAPDH	105	5'-AGCAGGTCGAGCATCTTCG-3' 5'-CTGTAGCCCCACTCGTTGTC-3'

cDNA synthesis (55°C 15s and 95°C 3 min) and qPCR were not performed sequentially, thus an EvaGreen 20X (Biotium, CA, US) and a commercial 2X One-Step RT-PCR mix was used to amplify fragments ranging between 90-136 bp for target genes by using 0.5 µg RNA for each. The expression level of the specific transcripts for each different RNA sample consisting of 3 replicates (biological) for each group was determined. Negative control (No Template Controls-NTC) and non-amplification control groups (No Amplification Controls-NAC, a minus-reverse transcriptase control) was added in the reactions (45X of 15s at 95°C-denaturation, the 30s at 60°C-annealing and 40s at 72°C-extension for the amplification and 5s at 95°C, 1 min at 65°C, continued up to 97°C and 30s at 40°C cooling for melting peak quantification). Respective fluorescence was measured by LightCycler 480 Software (Roche). The Standard Curve Set Efficiency was 2.00. The comparative CT method ($2^{-\Delta\Delta Ct}$) for relative quantitation of gene expression was used (25) in data analysis.

Statistical Analysis
SigmaPlot 13 Software (Systat, CA) was used for T-test analysis to see if there were any biologically significant differences between treatments and the control groups. The significance was determined at 5% confidence level.

RESULTS

The Combined Effects of HM Mix and the Fungus on the Seedling Phenotype
Various HMs can impair almost all stages of the plant life cycle including germination, growth development, and reproduction (22). In this work, the response of soil-grown maize seedlings was studied to tackle the following question: whether *Trichoderma* could be beneficial in terms of diminishing the injurious potential of the metal(oid) cocktail when maize seedlings grown under combined HM mixture conditions by interfering and affecting antagonistically. Compared with the control seedlings, the group of plants belonging to TS143 only/HM mix-free (-HM) exhibited no significant growth difference however, phenotypic compositions were remarkably distinguishable in the HM mix-exposed (+HM) groups with and without fungal species application compared to either control or TS143

only groups (Figure 1, lower panel). While a slight difference in green leaf number (data not shown) was observed on the TS143+HM mix plants, growth was significantly retarded in the +HM only groups indicating plant phenotype and survival was influenced by 15 days of exposure when fungus was absent. Before the experiments (Figure 1, upper panel) no plants, in different application groups, performed significantly better than the other.
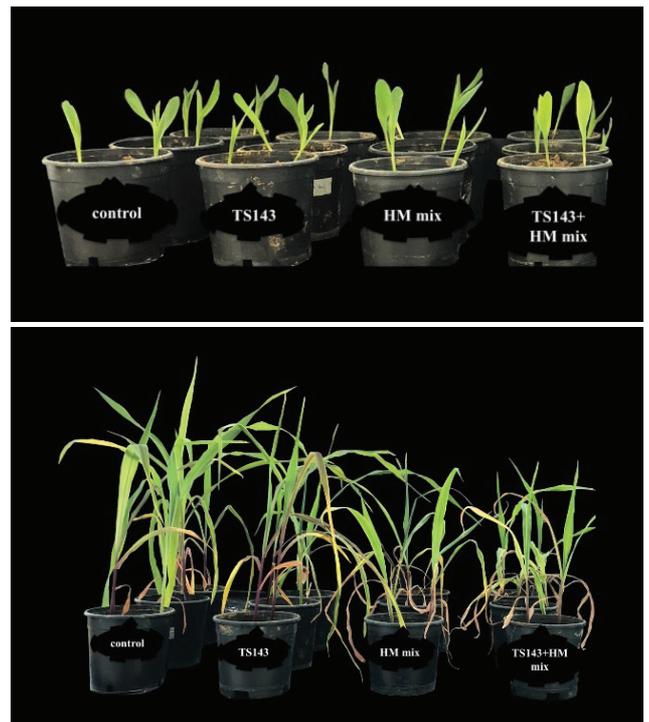

Figure 1. Maize phenotypes after simultaneous *Trichoderma* and chronic multi-HM exposure. Upper panel: Images of seven day old seedlings with no application. Lower panel: *Trichoderma* and multi-HM exposed maize seedlings for 15 days (21 days after sowing). Control: untreated; TS143: *Trichoderma* applied; HM mix: As, Cd, Cu, Pb, Zn-exposed, TS143+HM mix: *Trichoderma*+As, Cd, Cu, Pb, Zn exposed maize seedlings.

HM Cocktail Alters Response Patterns of Plasma Intrinsic Protein, Peroxidase and Heat Shock Defense Genes in Maize

To find evidence for a possible connection and/or interlinking between combined HM stress, TS143 bio- stimulation, and plant response against an oxidative burst caused by ROS, four experimental groups with maize seedlings were prepared to test the protection potential of the target strain under As, Cd, Cu, Pb, Zn-applied conditions. In this context, the mRNA expression profiles of previously identified defense gene expressions reported to be induced by different HMs (34) were analyzed (yet, despite these proteins conferring putative trace element caused stress tolerance, *HSP*, *PIP*, and *POD*-encoding genes cannot be listed as sole protection components for HMs). Activation under HM mix conditions in the gene encoding *ZmPIP1-5* (1.40-fold)*, ZmHSP1* (1.8-fold) and *ZmPOD1* (5.55-fold) respectively showed molecular evidence of cellular damage that overlapped with phenotypic changes (Figure 2). On the other hand, TS143 only gene expressions for all three genes tested were found dramatically lower than the control seedlings. However, HM mix+TS143 induced elevated gene expressions higher than that of the HM mix only conditions not for *ZmHSP1* (0.04-fold difference), but *ZmPIP1-5* and *ZmPOD1* (approximately 4-and 9.5-fold respectively).

TS143 Regulates Maize Plasma Intrinsic Aquaporin 1-5 and Peroxidase1 under Chronic HM Stress

This study found a strong direct (positive) correlation between the *ZmPIP1-5* and *Zmperoxidase1* quantified *mRNA* expression and the phenotypic performance of the TS143 supported maize plants continuously grown in soil with +As, Cd, Cu, Pb, Zn concentrations (Figure 2) as compared to the HM mix only

group, indicating positive effects yielding water uptake aid and triggered cellular defense signaling. Relative expression for *ZmHSP1*, *ZmPIP1-5,* and *ZmPOD1* was calculated (mean±standart error) as 0.08±0.01, 0.35±0.06 and 0.09±0.01 in TS143 only groups respectively, while expression was 1.89±0.2, 1.40±0.2, 5.55±0.3 for HM mix only group and 0.04±0.01, 3.58±0.3 and 9.44±0.6 for TS143+HM mix groups. Almost no change or a complete decline compared to the other two genes, in *ZmHSP1* transcript expression detected below intermediate levels (0.04-fold) which could indicate destructive effects of the heavy metal(oid)s used on the crosstalk between molecular chaperons, protein labeling, and degradation pathways of maize cells. Particularly, *Zmperoxidase1* which was calculated to be expressed at a relatively higher level than that of *ZmPIP1-5* and *ZmPIP1-5* in maize subjected to the same exact regime (TS143+HM mix) shows that an enzymatic antioxidant gene differed markedly, while it remained unchanged under control conditions with almost no difference.

DISCUSSION

Plants yield significant changes in gene expression to counteract HM stress if they are not hyperaccumulators able to complete life cycles without major symptoms due to toxicity (26). However, according to the recent plant HM stress reports, there is still scarce data on gene transcript changes driven by the fungal genus *Trichoderma* that recently emerged as a group of symbionts with immense impact on human welfare (27). The concepts designated to analyze the physiological outcomes of HM cocktails on plants under a fungus effect were even rarer (28) compared to other reports showing the efficacy of different *Trichoderma* genotypes under a single HM element (e.g., Pb (29) or Cu (30). Also, reports mostly focused on biotic stress-triggered gene expression dynamics in plants induced by the genus (31) rather than abiotic factors. In addition, research on the efficacy of newly identified genetic variants isolated from a mining niche might be more valuable in terms of showing the molecular plant protection potential of a novel strain in plant molecular HM tolerance which has not previously been investigated. This background along with evidence showing that the Trichoderma genus has been shown to dissolve oxidative zinc (Zn^{2+}) via ligand complex release (32) and Cd extraction (33), allowed this study to prepare four experimental groups with maize seedlings exposed to HM mix-free (-HM) and HM mix-exposed (+HM) with/without fungal species (Figure 1) to test the protection potential of the target strain.

Given the metal(oid) uptake and/or transport was reported to be more a quantitative trait subject to crosstalk of multiple genes and the regulation of HM transport/tolerance in plants requires more than one physiological pathway (8), of particular interest, the transcriptional expression pattern of an aquaporin gene and analogue mechanisms of one of the enzymatic antioxidants (peroxidase1) and stress proteins, protecting enzyme malfunction (HSP1) were chosen to be analyzed and confirmed by quantitative PCR.
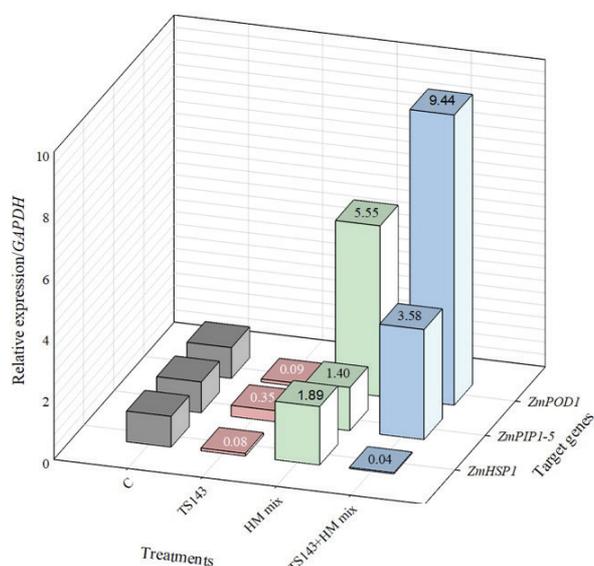

Figure 2. Profiling of representative genes expressing *peroxidase1_ZmPOD1, plasma membrane intrinsic aquaporins 1-5_ ZmPIP1-5* and *heat shock protein1_ZmHSP1* in maize. Data is given as mean (n=3). Control: untreated; TS143: *Trichoderma* applied; HM mix: As, Cd, Cu, Pb, Zn-exposed, TS143+HM mix: *Trichoderma*+As, Cd, Cu, Pb, Zn exposed maize seedlings (for 15 days).

While PODs are one of the major enzymatic antioxidants catalyzing the breakdown of H_2O_2 to scavenge radicals, or sometimes generating H_2O_2 themselves as signal molecules (34), the HSPs are the quality controllers of PODs , assisting conformational changes (35). They belong to multi-gene superfamilies whose proteins are present in the mitochondria or chloroplasts showing induced expression not only by thermo-stress but also by HMs (35). Hence, validation of these related gene expressions in maize seedlings interacting simultaneously with HMs and TS143 was performed. As shown in the Figure 2, the TS143 was found actively mediating HMs up-take and translocation by possibly playing with reduction kinetics of the metal(oid)s or facilitating vacuolar storage in maize seedlings under chronic multi-HM exposure, given the analysis was performed in shoot tissues (translocation). Simultaneous exposure of the HM-mix along with TS143 did not exhibit increased expression in *HSP1*, possibly indicating that the *HSP1* might not be a very sensitive biomarker, would serve for HM-induced toxicity monitoring for the current experimental set up in maize. Only slight distinctive expression levels indicating *HSP1* involvement were obtained for the HM-mix-only group. In this group, given the used dose in the experiments (500 mg L^{-1}) was relatively high, excretion was expected to be decreased and xylem loading would increase gradually which in turn would trigger a rapid involvement of molecular detoxification elements, potentially including the over-expression of HSPs (as confirmed almost 2-fold increase in *ZmHSP1*). The confounding levels of expression (0.04-fold) in the HM mix+TS143 groups could be attributed to an avoidable action in the *HSP1* up-regulation with the help of TS143 protection, given the primary function of this gene is to prevent the damage-induced aggregation of proteins under stress (35). However, this could be valid only if there was not a complete breakdown in the metal regulatory network of the seedlings consisting of HSPs, because the proteins of the HSP gene family were reported to be found at significant levels even in non-stressed plant cells when none of the stress factors were present (36). However, the HM mix-only exposure caused an almost 2-fold increase in *ZmHSP1* (Figure 2) gene of the seedlings, in direct opposition to the control and TS143-applied only groups. *POD1* expression, on the other hand, was significantly induced (p<0.05; t-test) by chronic exposure of HMs+TS143. The 9.44-fold increase might be showing the cytotoxic range for an applied HM dose that is exceedingly difficult to avoid for seedlings. The *POD1* increase rate in the HM-mix exposed-only group was calculated 5.5-fold more (p<0.05; t-test) compared to the control. This data may be grounded on the TS143 colonization induced signaling cascades improving POD-arbitrated redox state by regulating the downstream genes while adjusting the molecular defense machinery. Similar results were exemplified by several other studies (37, 38, 29) proving enhanced antioxidative defense were characterized by higher expressions of *POD* genes.

Beyond generating free radicals that attack cells, HMs also generate ionic stress due to water scarcity (39). The data interpreting that the aquaporin genes of a hyperaccumulator, *Pteris vittata TIP4;1* might be responsible for metal uptake as a metal transporter (40) confirms the functionality and involvement of PIPs in this notion and a lead to analyze maize aquaporin expression change. Relative expression was calculated 2-fold more than HM mix-exposed only seedlings and *PIP1-5* expression reached almost 3.6-fold (p<0.05; t-test) which was more than both the control and TS143- applied only group, following two weeks of application. Up-regulation of maize aquaporin expression (>2 fold) might be pinpointing the contribution of TS143 in the reduction of water scarcity by trapping metals and not permitting translocation from roots to the shoots. Certain studies confirm the current data and reporting that the genus is effective on plant-water relations via co-metabolic exchange by breaking down the robust chemistry of toxicants (14).

CONCLUSION

Besides the fact that the fungi belong to *Trichoderma* genus produce the strongest mycotoxins in the world along with its Aspergillus counterparts (41), this research further affirmed the increased potential in alleviating trace toxic elements which caused cellular injuries in plants (29, 30). A transcriptional per-spective of molecular protection was interpreted in this work by showing selected gene expression dynamics lead by TS143. The particular species used is an isolate whose molecular phylogeny and major physiological effects have recently been character-ized under HM-stress and its effective use was recommended to provide agro-ecological benefits in environmental biotechnol-ogy (20). This research data provides further supports that the target isolate triggers plant molecular antioxidant defense and water dynamics via coordinating aquaporin, peroxidase, and molecular chaperon genes to a certain extent.

Researching the impacts on plant molecular physiology lead by more novel genotypes occupying a wide variety of natural/artifi-cial niches as in the case of TS143, would be useful in either further plant-fungus methods or exploitation and effective use of fungi in the areas of agricultural and environmental biotechnology.

REFERENCES

1. Nriagu JO, Bhattacharya P, Mukherjee AB, Bundschuh J, Zeven-hoven R, Loeppert RH. Arsenic in soil and groundwater: an over-view. Bhattacharya P, Mukherjee AB, Bundschuh J, Zevenhoven R, Loeppert RH, editors. Trace Metals and other Contaminants in the Environment. Elsevier; 2007.p.3-60.

2. Goutam J, Sharma J, Singh R, Sharma D. Fungal-mediated bioreme-diation of heavy metal-polluted environment. In microbial rejuve-nation of polluted environment 2021; 51-76. Springer, Singapore.

Eur J Biol 2021; 80(1): 35-41

Pehlivan. Gen Expression Patterns Linked to TS143 in HM Stressed Maize

3. Xu Y, Shi H, Fei Y, Wang C, Mo L, Shu M. Identification of soil heavy metal sources in a large-scale area affected by industry. Sustainability 2021; 13(2): 1-15.

4. Ma Y, Rajkumar M, Zhang C, Freitas H. Beneficial role of bacterial endophytes in heavy metal phytoremediation. J Environ Manage 2016; 174: 14-25.

5. Pečiulytė D, Repečkienė J, Levinskaitė L, Lugauskas A, Motuzas A, Prosyčevas I. Growth and metal accumulation ability of plants in soil polluted with Cu, Zn and Pb. Ekologija 2006; 1: 48-52.

6. Song Y, Cui J, Zhang H, Wang G, Zhao FJ, Shen Z. Proteomic analysis of copper stress responses in the roots of two rice (*Oryza sativa L.)* varieties differing in Cu tolerance. Plant and soil 2013; 366(1): 647-58.

7. Yan A, Wang Y, Tan SN, Yusof ML, Ghosh S, Chen Z. Phytoremediation: a promising approach for revegetation of heavy metal-polluted land. Front Plant Sci 2020; 30(11): 1-15.

8. Zhang H, Xin L, Zisong X, Yue W, Zhiyuan T, Meijun A, et al. Toxic effects of heavy metals Pb and Cd on mulberry (*Morus alba L.)* seedling leaves: Photosynthetic function and reactive oxygen species (ROS) metabolism responses. Ecotoxicology and Environmental Safety 2020; 195: 1-11.

9. Redha A, Al-Hasan R, Afzal M. Synergistic and concentration-dependent toxicity of multiple heavy metals compared with single heavy metals in *Conocarpus lancifolius*. Environ Sci Pollut Res 2021; 14: 1-5.

10. Skowrońska M, Bielińska EJ, Szymański K, Futa B, Antonkiewicz J, Kołodziej B. An integrated assessment of the long-term impact of municipal sewage sludge on the chemical and biological properties of soil. Catena 2020; 189: 1-6.

11. Druzhinina IS, Kopchinskiy AG, Kubicek CP. The first 100 *Trichoderma* species characterized by molecular data. Mycoscience 2006; 47: 55-64.

12. Sood M, Kapoor D, Kumar V, Sheteiwy MS, Ramakrishnan M, Landi M, et al. *Trichoderma*: the "secrets" of a multitalented biocontrol agent. Plants 2020; 9(6): 1-25.

13. Harman GE, Kubicek PK. *Trichoderma* and *Gliocladium*. Enzymes, biological control and commercial applications (Vol 2) London: Taylor and Francis 1998.

14. Harman GE, Howell CR, Viterbo A, Chet I, Lorito M. *Trichoderma* species-opportunistic, avirulent plant symbionts. Nat Rev Microbiol 2004; 2(1): 43-56.

15. Garbeva PV, Van Veen JA, Van Elsas JD. Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness. Annu Rev Phytopathol 2004; 8(42): 243-70.

16. Contreras-Cornejo HA, Macías-Rodríguez L, Alfaro-Cuevas R, López-Bucio J. *Trichoderma spp.* improve growth of *Arabidopsis* seedlings under salt stress through enhanced root development, osmolite production, and Na$^+$ elimination through root exudates. Mol Plant-Microbe Int 2014; 27: 503-14.

17. Samolski I, Rincon AM, Pinzón LM, Viterbo A, Monte E. The qid74 gene from *Trichoderma harzianum* has a role in root architecture and plant biofertilization. Microbiology 2012; 158(1): 129-38.

18. Kottb M, Gigolashvili T, Großkinsky DK, Piechulla B. *Trichoderma* volatiles effecting Arabidopsis: from inhibition to protection against phytopathogenic fungi. Front Microbiol 2015; 29(6): 1-14.

19. Babu AG, Shim J, Bang KS, Shea PJ, Oh BT. *Trichoderma* virens PDR-28: a heavy metal-tolerant and plant growth-promoting fungus for remediation and bioenergy crop production on mine tailing soil. J Environ Manage 2014; 132: 129-34.

20. Pehlivan N, Gedik K, Eltem R, Terzi E. Dynamic interactions of *Trichoderma harzianum* TS 143 from an old mining site in Turkey for potent metal (oid)s phytoextraction and bioenergy crop farming. J Hazard Mater 2021; 403: 1-10.

21. US EPA. Risk Assessment Guidance for Superfund Volume I: Human Health Evaluation Manual (Part E, Supplemental Guidance for Dermal Risk Assessment)-Interim Guidance. 2001.

22. Zhao L, Hu Q, Huang Y, Keller AA. Response at genetic, metabolic, and physiological levels of maize (*Zea mays*) exposed to a Cu (OH)$_2$ nanopesticide. ACS Sustain Chem Eng 2017; 5(9): 8294-301.

23. Qiao Y, Ren J, Yin L, Liu Y, Deng X, Liu P, et al. Exogenous melatonin alleviates PEG-induced short-term water deficiency in maize by increasing hydraulic conductance. BMC Plant Biol 2020; 20: 1-4.

24. Chomczynski P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques 1993; 15(3): 532-4.

25. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nat Protoc 2008; 3(6): 1101-8.

26. Li JT, Gurajala HK, Wu LH, van der Ent A, Qiu RL, Baker AJ, et al. Hyperaccumulator plants from China: a synthesis of the current state of knowledge. Environ Sci Technol 2018; 52(21): 11980-94.

27. Gorai PS, Barman S, Gond SK, Mandal NC. Chapter 28 – *Trichoderma*. Editor(s): Amaresan N, Senthil M, Kumar KA, Krishna K, Sankaranarayanan A. Beneficial microbes in agro-ecology, Academic Press 2020; 571-591, ISBN 9780128234143.

28. Adams P, De-Leij FAAM, Lynch JM. *Trichoderma harzianum Rifai* mediates growth promotion of crack willow (*Salix fragilis*) saplings in both clean and metal-contaminated soil. Microb Ecol 2007; 54(2): 306-13.

29. Sun X, Sun M, Chao Y, Wang H, Pan H, Yang Q, et al. Alleviation of lead toxicity and phytostimulation in perennial ryegrass by the Pb-resistant fungus *Trichoderma asperellum* SD-5. Funct Plant Biol 2020; 48(3): 333-341.

30. Vargas JT, Rodríguez-Monroy M, Meyer ML, Montes-Belmont R, Sepúlveda-Jiménez G. *Trichoderma asperellum* ameliorates phytotoxic effects of copper in onion (*Allium cepa L.*). Environ Exp Bot 2017; 136: 85-93.

31. Schmidt J, Dotson BR, Schmiderer L, van Tour A, Kumar B, Marttila S, et al. Substrate and plant genotype strongly influence the growth and gene expression response to *Trichoderma afroharzianum* T22 in Sugar Beet. Plants 2020; 9(8): 1-14.

32. Altomare C, Norvell WA, Björkman T, Harman GE. Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus *Trichoderma harzianum Rifai* 1295-22. App Env Microb 1999; 65(7): 2926-33.

33. Cao L, Jiang M, Zeng Z, Du A, Tan H, Liu Y. *Trichoderma atroviride* F6 improves phytoextraction efficiency of mustard (Brassica juncea (L.) Coss. var. foliosa Bailey) in Cd, Ni contaminated soils. Chemosphere 2008; 71(9): 1769-73.

34. Chen S, Yu M, Li H, Wang Y, Lu Z, Zhang Y, et al. SaHsfA4c from *Sedum alfredii* hance enhances cadmium tolerance by regulating ROS-scavenger activities and heat shock proteins expression. Front Plant Sci 2020; 11: 1-14.

35. Zhang K, Ezemaduka AN, Wang Z, Hu H, Shi X, Liu C, et al. A novel mechanism for small heat shock proteins to function as molecular chaperones. Sci Rep 2015; 5(1): 1-8.

36. Vierling E. The roles of heat shock proteins in plants. Annu Rev Plant Physiol 1991; 42(1): 579-620.

37. Mastouri F, Björkman T, Harman GE. *Trichoderma harzianum* enhances antioxidant defense of tomato seedlings and resistance to water deficit. Mol Plant Microbe Interact 2012; 25(9): 1264-71.

Eur J Biol 2021; 80(1): 35-41

Pehlivan. Gen Expression Patterns Linked to TS143 in HM Stressed Maize

38. Zhang S, Gan Y, Xu B. Application of plant-growth-promoting fungi *Trichoderma longibrachiatum* T6 enhances tolerance of wheat to salt stress through improvement of antioxidative defense system and gene expression. Front Plant Sci 2016; 7: 1-11.

39. Rucińska-Sobkowiak R. Water relations in plants subjected to heavy metal stresses. Acta Physiol Plant 2016; 38(11): 1-13.

40. He Z, Yan H, Chen Y, Shen H, Xu W, Zhang H, Shi L, Zhu YG, Ma M. An aquaporin Pv TIP 4; 1 from *Pteris vittata* may mediate arsenite uptake. New Phytol 2016; 209(2): 746-61.

41. Abbott SP. Mycotoxins and indoor molds. Indoor Env Con 2002; 3: 14-24.