Coupling *Nicotiana tabaccum* Transgenic Plants with *Rhizophagus irregularis* for Phytoremediation of Heavy Metal Polluted Areas

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Several studies have shown that hairy roots (HRs) can increase the phytoextraction of organic and inorganic pollutants. In addition, microorganisms colonizing the rhizosphere of hairy roots have demonstrated the efficacy of HRs either in removing or in stabilizing of pollutants. This growth chamber study aimed to determine the effect of colonization by the arbuscular mycorrhizal (AM) fungus (Rhizophagus irregularis) on the HRs of tobacco (Nicotiana tabaccum L. cv. Virginia gold) under heavy metals (Cu, Pb and Zn) conditions. Seedlings of N. tabacum were grown in contaminated substrate without infection caused by Agrobacterium rhizogenes expressed in the form of HRs disease, a second one with infection, and the third treatment contained AM fungus beside the A. rhizogenes infection. In the order they have been described, these treatments were coded with C, CT and CTM. The experiment demonstrated that HRs mutants resisted better in terms of higher biomass content in the contaminated soil than the normal plants. Even more, the transgenic plants also had a very strong interaction with mycorrhizal fungi. Thus, soil respiration, biomass and some biochemical variables such as assimilating pigments, protein content and lipid peroxidation indicate a decrease in stress due to the presence of heavy metals in the CTM treatment. Furthermore, CTM treatment significantly alleviated the concentration of toxic elements in plants, compared with the CT treatment, in which a significant increase was registered when compared to treatment C.

Keywords: Agrobacterium rhizogenes, Hairy roots, Arbuscular mycorrhizal fungi, Heavy metals, Phytoremediation

Environmental contamination is a great worldwide problem in areas of intense industry that is related to human activities, such as non-ferrous industry, mining, smelting, intensive agriculture, city wastes, etc., and leads to their bioaccumulation into the food chain. As a result, a comprehensive literature on remediation methodologies and a range of tools have been proposed by many authors in order to support decision making in contaminated land remediation with both organic [e.g.1] and inorganic pollutants [e.g. 2]. However, different technologies can open new gateways in remediation research. The natural ability of plants to remove and/or stabilize the potentially toxic elements, has gained increasing attention in the recent years, thus the most used strategy being phytoremediation, a cost effective and environmentally - friendly technique [3, 4]. Furthermore, the application of myco-phytoremediation [5-7] and/or plant transformation by genetic engineering methods [8-10] lead to the development of an appropriate plant system for environmental cleanup. The obtaining of HRs cultures from various plant species, mainly dicotyledonous, by infecting them with *A. rhizogenes*, are not new studies. However, such studies are nowadays more often conducted in order to use HRs as research tools to accumulation, and/or removal of different pollutants. This is possible because genetic engineering methods have led to the development of specific plant mechanisms, able to cope with pollutants detoxification and various other mechanisms such as absorption, transformation, conjugation or compartmentation of pollutant in the vacuolar compartment and/or wall cell [11]. HRs of new species of plants are continuously study, research is in progress even for species recalcitrant to transformation, through different strategies. Various techniques are being used based for instance, on transformation mediated by sonication in monocotyledons as reported by [12]. Exhaustive advanced molecular biology techniques were carried out based on the genetic transformation of plant cells by *Agrobacterium* strains, as well as/not to mention host plant genomics as reported by many authors [13, 14, 9]. Few studies considering the AMF - assisted HRs remediation were reported [15, 16, 14]. Because it is considered a notably competent research tool for phytoremediation there is a progressive trend [9, 17, 10] and it requires more attention. In this study, an experiment was conducted to determine the effect of colonization by the AMF (*Rhizophagus irregularis*) on the HRs of tobacco (*Nicotiana tabaccum* L. cv. Virginia gold) under heavy metals conditions.

Experimental part

Materials and methods

Location, description of the polluted area and experimental design

A growth chamber experiment was performed using heavy metal polluted soil sampled from Pantelimon area located in the eastern part of Bucharest, Romania (geographical coordinates: latitude: 44.4538, longitude: 26.22244° 27'14" North, 26°13'19" East, in WGS84 system).

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Table 1
GENERAL PHYSICOCHEMICAL CHARACTERISTICS OF INVESTIGATED SOILS (C - CONTAMINATED SOIL AND R - NON-CONTAMINATED, REFERENCE SOILS, COMPOSITE SAMPLE) BEFORE THE EXPERIMENT

Variables /units	pH (H_20)	EC µS x cm^{-1}	$N-NH_4^+$	$N-NO_3$	$N-NO_2$	$P-PO_4^{3-}$
			µg x g^{-1} d.w.			
R	7.34	69.8	41.98	22.48	0.202	84.32
C	5.85	161.1	30.29	19.15	0.083	15.65
Variables /units	**C_{org} %**	**Cd**	**Co**	**Cu**	**Pb**	**Zn**
			µg x g^{-1} d.w.			
R	7.4	1.249	23.05	32.08	11.80	111.7
C	3.59	2.127	15.66	341.2	1726	537.1
Accepted level*	-	2	10-75	30-100	2-60	17-125

**The accepted level in soil for plants which can be used for human was reported by [24]*

The source of pollution consists of two factories *Acumulatorul* and *Neferal* with activities in the production of batteries and non-ferrous industry since 1932. After 1995, the industrial activity in this area has been limited to ecological recovery of non-ferrous metals from wastes, such as lead from used batteries, thus minimizing the pollution. So far, only studies carried out by [18, 19] and [20] were published about this area. These authors reported that 1,748 ha have been polluted with Pb (max. 784 mg x kg^{-1}), 1,314 ha with Cu (max. 180 mg x kg^{-1}) and 874 ha with Zn (max. 380 mg x kg^{-1}). Concentrations of metals increased by 70% Cu, 45% Pb and 30% Zn from 1980 to 1995. More recent researches were reported by [21, 22]. According to our studies about 50 ha near the two factories were found to be highly contaminated and required measures for ecological rehabilitation.

The polluted soil classified by [21] in Chernisols, Luvisols (the dominant class), and Hydrosols, was moderately acidic, with a clayey texture, having small humus contents (1.8 up to 3.7%), total nitrogen (0.130 up to 0.163%) and small amounts of soluble phosphorus (11 up to15 mg x kg^{-1}) [23] and Corg (2 up to 3.6%) [22]. In our investigations this contaminated soil was characterized by high concentrations of Pb, Cu and Zn as can be seen in table 1 along with other variables in comparison with the physicochemical characteristics of a non-contaminated soil.

After a preliminary test of germination using 13 plant sp. (data not showed) we opted for *Nicotiana tabacum* L. cv. Virginia gold and *Helianthus annuus* L. *cv Arena PR* selected for the most vigorous development and their germination degree, but also for the potential degree of mycorrhization and the performance of this plant sp. in forming HRs. Before planting the one seedling per pot in non-contaminated (reference) and contaminated soils, these were sterilized by autoclaving (2.2 bar, 121°C × 30 min × 2 time) and weighed in 400 ml polyethylene pots with four replications for each, of the following established treatments: 1) non contaminated soil, and 2) contaminated soil mixed with clay, both treatments containing normal tobacco seedlings, 3) non contaminated soil, 4) contaminated soil mixed with clay, and 5) contaminated soil mixed with clay and inoculated with 10% according to [25] commercial product AMF 510 (160 spores per g soil) *Rhizophagus irregularis* sequestered in expanded clay (2 to 4 mm particle size), all three of the latter treatments were applied to transgenic tobacco seedlings. In the order in which they were described, the five treatments were coded with R, C, RT, CT and CTM. This experiment was conducted in a growth chamber with controlled conditions: 70% relative humidity and light/dark cycle regime of 16 h light / 22°C and 8 h night / 16°C with Sylvania cool-white incandescent lamps, 11 W m^{-2}. Watering was performed once per day to maintain the soil moisture content.

In these experiments, the identification of an effective strategy for *N. tabacum* and *H. annuus* for genetic transformation was attempted, using the DNA fragment-T located in the Ri (root inducing) plasmid of soil bacterium *Agrobacterium rhizogenes*. This bacterium has *oncogenes* role responsible for inducing of neoplasia known as *hairy roots* (HRs) common by numerous dicotyledonous. The bacterial strains used for transformation were *A. rhizogenes* 8916 and *A. rhizogenes* 9402 (hyper virulent strain). Both are wild Ri plasmid strains and were obtained from the research center INRA, from Dr. D. Tepfer, Versailles, France. The genetic transformation method was described in [16]. It can also be noticed that even if the two species have used the same bacterial strains, the HRs phenotype was different. HRs crop was achieved in a shorter time for the *N. tabacum* sp. (about 2 - 4 weeks) while at *H. annuus* sp. the time was greater with 3-6 weeks. Another trend characterized as negative aspect on *H. annuus* was the browning one, at the same time with the weakening of the germplasm resistance from fungus attack, which caused loss in a significant percentage of them. Due to these aspects it was decided to work only with *N. tabacum* sp. The efficiency of *A. rhizogenes*-mediated genetic transformation was studied based on molecular analysis (data not shown) and in vitro genotypic and phenotypic marker genes used in this system of the transgeneza, based on visual analysis of HRs phenotypic characteristic.

Plant sampling and processing

After the growing procedure, the harvesting was done in 60 days after the planting, wet-weighed, and separated for analyses into roots, shoots (shoots plus leaves) and flowers. All roots were briefly washed with tap water and finally all plant parts were washed with distilled water. After the plant material was lyophilized, the dry biomass was measured, then ground using a stainless-steel mill equipped with cooling system and later kept at -45 °C to the analysis. The concentration of elements was determined from plant samples after HNO_3 (65% suprapure from Merck) microwave digestions, and the analyses were performed using mass spectrometry with inductively coupled plasma ICP-MS, Perkin-Elmer ELAN DRC-e 6000, with axial field technology. Standard solutions were prepared by diluting 10µg x mL^{-1} multielement solution (Multielement ICP Calibration Standard 3, matrix 5% HNO3, Perkin Elmer Pure Plus). The analyzed elements were Cd,

Co, Cu, Pb and Zn. Also, plant variables were assessed such as total proteins [26], lipid peroxidation (LP) in terms of the malondialdehyde (MDA) content [27], and assimilating pigments [28]. All methods were described in [29].

Soil sampling and processing

After harvesting the plants, soil respiration was measured according to [30] and then soil pH, electrical conductivity (EC), mineral N content ($N\text{-}NH_4^+$, $N\text{-}NO_3^-$, $N\text{-}NO_2^-$), phosphorus content in available form for plants ($P\text{-}PO_4^{3-}$) and the pseudo-total content of metals after *aqua regia* (suprapure acids from Merck) microwave pressure-assisted digestions according to [31]. These analyses were performed using the same ICP-MS as described in section 2.2. The quality assurance and quality control criteria were satisfied by checking the standard reference material CRM 142 R for trace elements in a light sandy soil. The differences were of no more than 5%.

Statistical analysis

Data were subjected to analysis of variance (one-way ANOVA) and when significant

differences were detected, averages were compared by Tukey test ($p<0.05$). Tukey's method takes into consideration all pairwise possible differences of means at the same time using R software version 3.3.2 [32].

Results and discussions

After harvesting the plants, soil was physicochemical characterized and the measured variables presented in table 2. It can be seen that soils *p*H belongs to the neutral class (≤ 7) for R and RT treatments, and acidic class (5 up to 6.5) for C, CT and CTM treatments, according to [33] classification. EC registered low values for all treatments corresponding to the desalted soil class (0-2 mS x cm^{-1}) according to [34]. Concerning the dissolved inorganic nitrogen - DIN computed as a sum of $N\text{-}NH_4^+$, $N\text{-}NO_3^-$, $N\text{-}NO_2^-$, there was a sufficiency content (≥ 50 µg x g^{-1}) for the development of plants, according to [35] for all treatments. The available phosphorus content ($P\text{-}PO_4^{3-}$) registered sufficiency in R and RT treatments, but presented a deficiency (< 60 µg x g^{-1}) in treatments with contaminated soil, according to [36]. One-way ANOVA analysis of soil respiration score shows that the effect of treatments was significant, $F(4, 15) = 148.79$, $p < 0.005$. Post hoc comparison using the Tukey HSD test shows that there are significant differences between all soil types, except R against CTM. We bring to attention that, when the statistical analysis was performed, there was no separation made between plant type and soil type factors. The statistical differences where obtained considering both factors simultaneously. We conclude that the effects of HRs (RT and CT treatments) were positive, and what's more, the inoculation of AM fungi (on CTM treatment) had a spectacular positive effect on soil respiration, the value of this variable reaching that of the R soil. However, it cannot be said the same about the Corg, where the effects of either HRs or AMF, were insignificant, the Corg percentage in the R soil being roughly double that of the C soil. Regarding the heavy metal content in the soil, there was a statistically significant effect between treatments (Cu, $F(4,15) = 9.35$, $p<0.$ 0.005; Pb, $F(4,15) = 79.46$, $p<0.$ 0.00; Zn, $F(4,15) = 73.63.35$, $p<0.$ 0.00; Co, $F(4,15) = 34.04$, $p<0.$ 0.00). However, the Cd content of the soil did not differ significant between treatments, $F(4,15) = 1.28$, $p>0.3$.

The genetic transformation of plants via Ri plasmid of *A. rhizogenes* seems to be relatively straightforward for improving the metal accumulation traits [37, 38]. It is well known that the uptake of metals and their distribution in various plant parts demonstrate the capacity of plants to remove heavy metals from soil. In the case of HRs, they can be used for various plant species to test their capacity to extract and/or sequester metals [39, 11]. For instance, [40] reported in two cultures of *Solanum nigrum* HRs an accumulation of Zn up to 98 and 90% respectively within 15-18 days of the culture period. On the other hand, [41] reported that HRs of *A. bertolonii* can hyperaccumulate Ni. Also, higher Cd accumulation was found by *N. tabacum* HRs as reported [42]. In current study, all plant parts from CT treatment accumulated significantly higher concentrations of heavy metals (excepting Cd and Zn)

Table 2

GENERAL PHYSICOCHEMICAL CHARACTERISTICS (min. AND max. VALUES) OF INVESTIGATED SOILS AFTER PLANT HARVESTING. WITH EC IS NOTED ELECTRICAL CONDUCTIVITY, DIN DISSOLVED INORGANIC NITROGEN, Sr SOIL RESPIRATION, (n=4)

Variable /units	pH (H_20)	EC µS x cm^{-1}	$N\text{-}NH_4^+$	$N\text{-}NO_3^-$	$N\text{-}NO_2^-$	DIN	$P\text{-}PO_4^{3-}$
			µg x g^{-1} d.w.*				
R	6.78-7.06	124.1-176	51.33-64.32	26.69-36.51	0.15-0.22	72.23-99.0	52.04-60.11
RT	6.93-7.11	158.9-169.5	59.93-66.56	29.66-35.57	0.17-023	98.82-102.1	61.11-73.47
C	5.59-5.74	134.2-252	43.94-52.87	10.51-13.36	0.051-0.073	54.5-63.85	7.09-15.33
CT	5.56-5.95	100.4-194.7	48.12-54.57	10.19-13.89	0.05-0.09	58.38-68.55	7.39-16.52
CTM	5.98-6.28	82.4-159.4	58.53-69.58	10.53-17.79	0.043-0.058	69.37-87.41	9.07-15.75
Variable /units	**Sr mg CO_2 x g^{-1} d.w. x 2 h**	**C_{org} %**	**Cd**	**Co**	**Cu**	**Pb**	**Zn**
			µg x g^{-1} d.w.				
R	6.86-7.11	8.33-8.94	1.0.28-13.19	20.37-24.3	30.35-32.08	10.17-12.18	98.8-111.7
RT	8.73-9.74	8.61-9.25	0.7-1.418	21.33-22.33	27.68-31.68	9.9-12.54	85.5-109.4
C	2.58-3.89	4.56-4.67	1.44-2.288	13.44-16.95	208.9-523.5	1389-1937	455.6-591.1
CT	3.68-4.43	4.58-4.95	1.087-2.149	13.21-15.86	205.7-412.0	1196-1516	415.3-560.7
CTM	6.59-6.99	4.47-5.28	0.806-3.441	13.68-16.68	254.4-576.8	1181-1823	373.0-530.2

** d.w. means dry weight*

compared with plants from C treatment. To show the statistical differences between heavy metal content in plants, only aboveground plant parts (shoots and leaves) were compared (roots and flowers have a similar pattern). Using the analysis of variance and Tukey multiple comparisons of means, all pairwise possible differences of means, occurring at any one time simultaneously, were taken into consideration, as can be seen in table 3. It is also well documented that no HRs plant - AMF symbiosis enhances the phytoremediation of contaminated substrates and reduces the concentration of toxic compounds [46, 47]. The variation of heavy metal content in aboveground plant parts between the three treatments can be seen in Figure 1. In case the mean values do not overlap, there are more arguments to sustain that there is a real effect of the treatment on plant development. The black line on the graphs points out this aspect. The score of heavy metal content in aboveground plant parts from the CTM treatment are detached from others, these being significantly higher in the case of Cu and lower for the other elements. As in our results [15] reported an alleviation of Cd in Ri T-DNA - transformed *Daucus carota* roots/AMF associations. The same authors performed similar studies using HRs/AMF associations in *Pteris vittata* and found a higher biomass production, compared to the non-inoculated treatments.

In the case of CT treatment, HRs mutants induced an increase in stress expressed in terms of lower protein and higher LP contents. What's remarkable is the increase of chlorophyll content in the CT treatment. These results obtained on genetically modified plants without inoculation with AMF are for particular growth conditions and development stages. However, we emphasize that they are in agreement with a more vigorous plant development. Currently, there are many examples in literature about HRs alterations in the secondary metabolism of plants, probably by increasing the production of antioxidant enzymes. For instance, an enhance in SOD and POX activities in *N. tabacum* HRs infected with *A. rhizogenes* was reported by [43]. Also, [44] reported higher concentrations of glutathione (GSH) and oxidized GSH in roots and/or leaves of *Populus tremula*. On the other hand, HRs plants can increase the biomass production and consequently lead to an induction of cell wall ligniûcation and enhanced H_2O_2 accumulation by *Nicotiana tabacum* [45].

In a similar study, inoculation of *N. tabacum* HRs with *Rhizophagus irregularis* led to efficiently protection of roots against phenol-induced oxidative damage [16]. In our study, the question of using *Rhizophagus irregularis*

Table 3

ANALYSIS OF VARIANCE AND TUKEY MULTIPLE COMPARISONS OF MEANS CONCENTRATION OF HEAVY METALS IN PLANTS

Analysis of Variance													
	df	SS	MS	F	P	SS	MS	F	P	SS	MS	F	P
Elements		Pb				Co				Cu			
Plant type	2	864	432	14	0.001	11.45	5.78	9.07	0.007	122.7	61.3	16.7	0.0009
Residuals	9	276	30.2	-	-	5.68	0.63	-	-	33.	3.68	-	-
Tukey multiple comparisons of means													
Treatments	diff	lwr	upr	P *adj*		diff	lwr	upr	P *adj*	diff	lwr	upr	P *adj*
CT-C	8.3	-2.6	19.2	0.139		0.9	-0.64	2.5	0.27	-0.21	-4.	3.5	0.98
CTM-C	**-12.**	**-23.**	**-1.3**	**0.029**		-1.44	-3.0	0.12	0.07	**6.6**	**2.9**	**10.4**	**0.002**
CTM-CT	**-20.**	**-31.**	**-9.7**	**0.001**		**-2.3**	**-3.9**	**-0.8**	**0.005**	**6.9**	**3.1**	**10.6**	**0.001**

Note: bold values indicate statistical significant differences

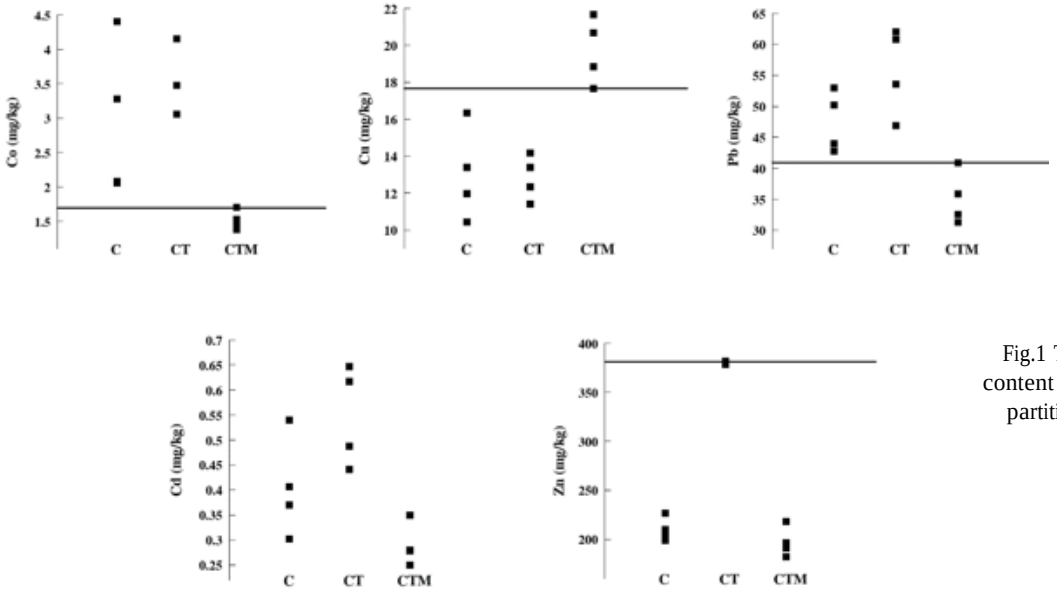

Fig.1 The score of heavy metal content in aboveground plant parts partitioned on C, CT and CTM treatments

Table 4

ANALYSIS OF VARIANCE AND TUKEY MULTIPLE COMPARISONS OF MEANS CONCENTRATION OF CHLOROPHYLL A, PROTEIN, LIPID PEROXIDATION AND BIOMASS IN PLANTS FOR C, CT AND CTM TREATMENTS

Analysis of Variance																			
Variables	Chl a					Protein					Lipid peroxidation (LP)					Biomass			
	df	SS	MS	F	P	SS	MS	F	P		SS	MS	F	P		df	SS	MS	F	P
Plant type	2	122.7	61.3	16.7	0.0009	6920	3460	144.9	1.4e-7		0.128	0.061	155.9	1.0e-7		2	8.6	4.3	16.5	0.0009
Residuals	9	33.0	3.6	-	-	214.8	23.9	-	-		0.0003	3.9e-4	-	-		9	2.34	0.26		
Tukey multiple comparisons of means																				
Treatments	diff	lwr	upr	P *adj*		diff	lwr	upr	P *adj*		diff	lwr	upr	P *adj*		diff	diff	lwr	upr	adj
CT-C	0.21	-3.57	4.	0.987		-33	-42	-23	1.e-5		0.144	0.104	0.183	7.e-6			0.04	-0.96	1.0	0.99
CTM-C	**6.8**	**3.1**	**10.6**	**0.001**		**25.**	**15.8**	**35.**	**0.0001**		**-0.103**	**-0.142**	**-0.063**	**0.0001**			**1.8**	**0.8**	**2.8**	**0.001**
CTM-CT	**6.6.**	**2.9.**	**10.4**	**0.002**		**58.**	**49.**	**68.**	**1.e-7**		**-0.247**	**-0.286**	**-0.208**	**1,e-7**			**1.77**	**0.76**	**2.78**	**0.002**

Note: bold values indicate statistical significant differences

Fig. 2. The score of biochemical variables in aboveground plant parts partitioned on C, CT and CTM treatments

inoculation together with *N. tabacum* HRs was whether, or not, the AMF symbiosis can decrease adverse effects due to the presence of heavy metals. To point out these aspects, statistical differences between treatments for the biomass production (sum of all plant parts) were highlighted. Other mentioned biochemical variables (only for the aboveground plant parts, the others having similar patterns) and treatments were compared using analysis of variance and Tukey multiple comparisons of means, in the same way as for physicochemical variables (table 4). Between treatments, there are statistical significant differences for all presented variables. It can be observed in table 4 and on graphs depicted in figure 2 that the biomass production, chlorophyll a, and protein content were significantly higher in CTM treatment, while oxidative damage like lipid peroxidation (LP) measured as malondyaldehide (MDA) content was significantly lower. These results could be attributed to the beneficial contribution of AMF in host roots under stress conditions. Similar results were published by [16] who found lower levels of MDA in transgenic HR cultures, in association with *G. intraradices*.

Conclusions

As expected, using HRs of *Nicotiana tabaccum* L. soil respiration, biomass production and chlorophyll a, were higher than in the normal plants. Despite these results, it cannot be concluded that there was a less stress in the CT treatment, due to the lower protein content and higher lipid peroxidation content. This response of the plants could be attributed to a significantly higher content of heavy metals. However, the colonization by the AMF on the HRs mutants under heavy metals stress led to the highest biomass production. All other measured biochemical variables indicate a decrease in stress in the CTM treatment. In addition, CTM treatment significantly alleviated the concentration of some toxic elements in plants, while the CT treatment registered a significant increase compared to treatment C. Although little is known about the behavior of HRs when infected with AMF in heavy metal stress conditions, it can be concluded that the colonization by the AMF (*Rhizophagus irregularis*) of HRs mutants provide a promising tool in the field of phytoremediation. However, future research on a wide range of plant species is needed.

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