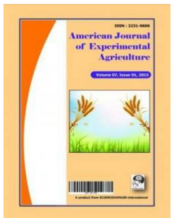

American Journal of Experimental Agriculture
10(3): 1-9, 2016, Article no.AJEA.20479
ISSN: 2231-0606

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Arabidopsis thaliana Reaction in Response to Cadmium and *Trichoderma* Fungi Additive into the Soil

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Authors' contributions

This work was carried out in collaboration between all authors. Author MM designed the study, wrote the protocol, wrote the first draft of the manuscript and performed the statistical analysis. Author EH reviewed the experimental design and all drafts of the manuscript. Author JK managed the analyses of the study and identified the plants. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJEA/2016/20479
Editor(s):
(1) Lixiang Cao, Department of Biotechnology, Sun Yat-sen University, Guangzhou, P. R. China.
(2) Anonymous.
Reviewers:
(1) Atef Mahmoud Mahmoud Attia, National Research Centre, Egypt.
(2) Klára Kosová, Crop Research Institute, Prague, Czech Republic.
Complete Peer review History: http://sciencedomain.org/review-history/11824

Original Research Article

Received 29th July 2015
Accepted 4th September 2015
Published 15th October 2015

ABSTRACT

The aim of this study was to determine the effect of the substrate inoculation (silty soil) using *Trichoderma* genus fungi on cadmium phyto-availability and process of photosynthesis disturbances at *Arabidopsis thaliana*. The two-factor experiment was established, in which the 1st variable factor was the presence or absence of *Trichoderma* genus fungi in the substrate, while the 2nd order factor was the growing amounts of cadmium in the soil. During the vegetation, measurements of chlorophyll fluorescence parameters were carried out. After the experiment complete, the cadmium content was determined in aboveground parts of *Arabidopsis thaliana*.
The cadmium doses incorporated in the silty soil (2, 10, 30 mg Cd/kg DM soil) did not cause any significant disturbances within the photosynthetic system of *Arabidopsis thaliana*. Only Fv/Fm

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parameter values showed some deterioration in the photochemical efficiency of PSII. Cadmium content in the aboveground parts of plants increased along with the soil contamination by this metal, both with and without addition of *Trichoderma* genus fungi. However, plants growing on soils with lower doses of cadmium (2 and 10 mg Cd/kg) with *Trichoderma* genus fungi addition, contained less of the element than those with no fungi addition.

Keywords: Arabidopsis thaliana; chlorophyll fluorescence; Trichoderma; cadmium.

1. INTRODUCTION

Plants of natural ecosystems and agro-ecosystems are subject to exposure to a number of adverse factors (cold, drought, heavy metals, etc.), referred to as environmental stress that disrupt physiological processes. The photosynthesis process is particularly vulnerable to the environmental stress and changes in the photosynthetic system of plants can be illustrated by measuring the chlorophyll fluorescence parameters [1,2,3].

One of the main factors negatively affecting the natural environment are heavy metals [4]. Industry, energy production, and transport are the most significant sources of metals emitted into the atmosphere. Of these metals, cadmium is characterized by high toxicity and high capacity to bio-accumulate within plants [5]. Accumulation of cadmium in soils and plants is determined by the type of geological subsoil [5,6,7] application of wastes for fertilizing and intensive mineral NPK nutrition [5,8]. Dust emitted into the atmosphere as a result of natural processes and human activities is also the source of cadmium.

Changes in soil properties through liming [7,8,9,10] fertilization using phosphorus compounds or enrichment in organic matter also reduce the phyto-availability of heavy metals [7,11,12]. Another way to reduce the bio-accumulation of ballast metals and negative effects of their influence on plants is the use of mycorrhizal inoculation. In recent years, *Trichoderma* genus fungi invoke a lot of interest in the world of science. These microorganisms are among the most studied and applied means for biological plant protection. Its mechanism of action is based on the colonization of roots, the support for plant growth, and the protection against stress factors such as pathogenic organisms [13,14].

The aim of this study was to determine the effect of the subsoil inoculation with *Trichoderma* genus fungi on cadmium phyto-availability and process of photosynthesis at *Arabidopsis thaliana* – thale cress – that is a model organism of a small size and high resistance.

2. MATERIALS AND METHODS

2.1 Experimental Design

The two-factor pot experiment was carried out in the Laboratory of Plant Physiology, University of Rzeszow in Poland. The experiment was established by means of a randomized split-plot method in 4 replications. The first variable factor was the presence (+T) or absence (-T) of *Trichoderma* genus fungi applied into the soil in the form of a granular formulation Trianum-G in the quantity recommended by the manufacturer (Koppert BV), i.e. the first application of 750 g/m^3, while the second 375 g/m^3 subsoil. Another variable factor consisted of cadmium doses applied into the soil in amounts of 0, 2, 10, 30 mg Cd/kg DM soil. At the same time, they were the experimental objects: 0, A, B, C (Table 1). Thale cress (*Arabidopsis thaliana*) was the test species.

2.2 Plant Material and Growth Conditions

Slightly acidic soil of pH_{KCl} 5.57 (hydrolytic acidity 4.5 cmol(+)/kg) and granulometric composition of silty soil (clay content 8%), was used as a subsoil for experiment. The soils contained an average of 7.1 g/kg DM of organic carbon, and 1.12 g/kg DM of nitrogen. Total content of cadmium was 0.703 mg/kg DM.

Arabidopsis plants were grown in pots containing 1 kg of soil previously dried and sieved through a sieve with a diameter of 0.5 mm mesh.

Uniform basic nutrition was applied for all objects by incorporating water solutions of the following salts into the subsoil: 0.126 g KNO_3, 0.118 g $Ca(NO_3)_2$, 0.123 g $MgSO_4$, and 0.085 g KH_2PO_4 per 1 kg of soil [15]. Cadmium was incorporated in the form of water solution of $3CdSO_4 \cdot 8H_2O$.

Table 1. The experimental scheme

Object	Soil with *Trichoderma* genus fungi addition (+T)	Soil without *Trichoderma* genus fungi addition (-T)
	Cadmium dose [mg/kg DM soil]	
Control 0	0	0
A	2	2
B	10	10
C	30	30

Growth density of the test species (*Arabidopsis thaliana*) was 3 plants per pot. Soil moisture was kept stable at 40% in the first phase of experiment and 50% in the second phase. Plants were grown for 46 days maintaining the temperature at the level of 22/18℃, at photoperiod 16/8 h (day/night).

2.3 Chlorophyll Fluorescence

During plant vegetation period, disturbances of photosynthesis process were monitored by measurements of chlorophyll fluorescence parameters using fluorometer HANSATECH. Following parameters were determined: F_0 – zero fluorescence of objects adapted to darkness; F_M – maximum fluorescence; F_V – variable fluorescence $F_V = F_M - F_0$; F_V/F_0 – maximum efficiency of water splitting at the donor side of PSII and F_V/F_M – maximum photochemical efficiency of PSII.

All measurements were made in four replicates, while a single replicate consisted of 10 measurements.

2.4 Chemical Analysis

After completion of the pot experiment, the plant material was dried at 75℃ and grinded. Cadmium content in aboveground parts of *Arabidopsis thaliana* was determined by means of atomic absorption spectrometry (AAS, using Hitachi Z-2000 Japan) device after digestion in a microwave system in concentrated HNO_3 with addition 30% H_2O_2 in Berghoff apparatus [16].

Biological accumulation coefficients (BAC) for cadmium as a ratio of its content in shoots to soil, was also calculated.

2.5 Statistical Analysis

The experiment was established by means of a randomized split-plot method in 4 replications. The results were statistically processed using bi-factor variance analysis (ANOVA) and the Kruskal-Wallis test using Statistica 10 software (StatSoft Inc.).

3. RESULTS

Measured results of chlorophyll fluorescence parameters are illustrated in Fig. 1. Values of parameter F_0 at plants growing on the subsoil without *Trichoderma* addition (-T) are within the range 0.161–0.174 AU, while at plants grown on the subsoil with *Trichoderma* (+T) – within the range 0.153–0.181 AU. Both in one (+T) and the other (-T) experimental variant, value of F_0 decreased along with the increase of cadmium concentration in the soil.

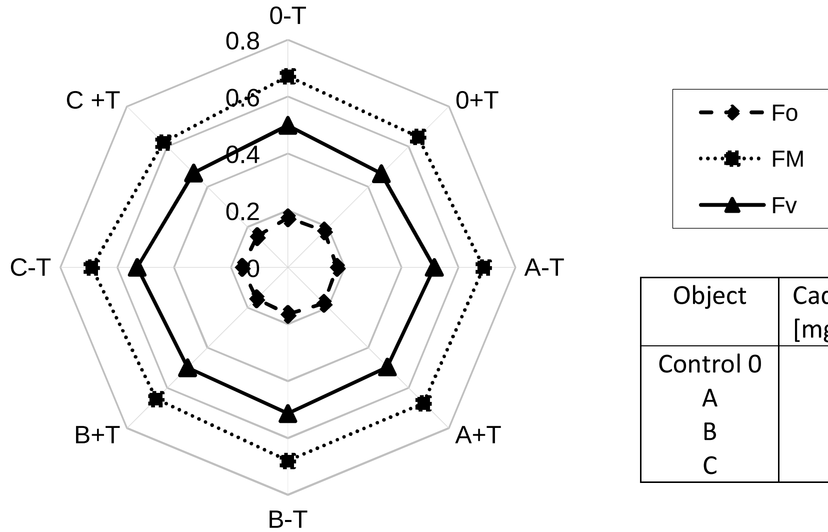
Value of the maximum fluorescence indicator F_M depends on many factors, among others the type of saturating light and chlorophyll content in a tested tissue. The F_M values achieved in the experiment vary from 0.672 to 0.690 AU for variant (-T) as well as from 0.647 to 0.621 AU for variant (+T). Addition of *Trichoderma* genus fungi resulted in the decrease of F_M parameter value at every level of cadmium content in the soil.

Variable fluorescence F_V is a parameter, low values of which prove a poor PSII activity and losses of excitation energy in the form of emitted heat. Fv value is reduced by the impact of environmental stress (high temp., frost, etc.), that cause damage to the thylakoids [17]. In present experiment, F_V parameter oscillated from 0.498 to 0.529 AU at plants grown on the subsoil without *Trichoderma* and from 0.466 to 0.498 AU at plants under the influence of *Trichoderma* fungi. For every levels of cadmium concentration in the subsoil, Fv reached higher values for variant, to which Trianum G preparation was not added.

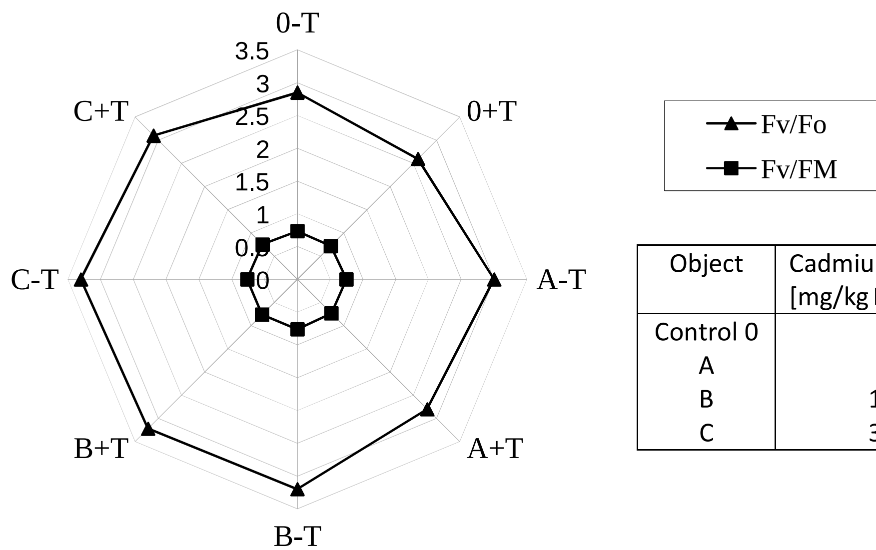
Parameter Fv/F_0 characterizes the maximum efficiency of water splitting at the donor side of PSII system. In the preset experiment, the Fv/F_0 parameter is in the range 2.85–3.28 AU (-T) and 2.60–3.24 AU (+T). The subsoil inoculation using

Trichoderma genus fungi has no effect on Fv/F_0 parameter value.

Maximum photochemical efficiency of PSII, expressed by Fv/F_M ratio, is important parameter that is often used in studies upon plant's physiological status. For majority of plants under no stress conditions and at the stage of full development, this parameter value equals to 0.83 [18]. In present experiment, the Fv/F_M parameter takes the values below 0.80 for plants at every level of cadmium dose applied to the soil, including the level 0 (control).



Object	Cadmium dose [mg/kg DM soil]
Control 0	0
A	2
B	10
C	30



Object	Cadmium dose [mg/kg DM soil]
Control 0	0
A	2
B	10
C	30

Fig. 1. Radar graphs of measured physiological traits in leaves of *Arabidopsis thaliana* depending on addition of *Trichoderma* fungi and cadmium doses (-T – subsoil without *Trichoderma* addition; +T – subsoil with *Trichoderma* addition)

The cadmium rates (0, 2, 10, 30 mg kg^{-1} DM soil) used for the silty soil and corresponded to 0, I, III, and V degree of soil contamination (along with application of *Trichoderma* genus fungi or their absence) differentiated cadmium contents in the aboveground parts of thale cress (*Arabidopsis thaliana*) (Fig. 2).

Cadmium concentration in aboveground parts of *Arbidopsis thaliana* arose along with its content increase in the subsoil, and in variant (-T) it ranged from 0.50 to 48.03 mg kg^{-1} DM, whereas in variant (+T) – from 0.33 to 54.06 mg kg^{-1} DM (Fig. 2). It was also observed that at 0, I, and III degree of cadmium contamination (objects 0, A, and B), content of this element in plants was lower in the presence of *Trichoderma* genus fungi by 34%, 16%, and 10%, respectively. Addition of *Trichoderma* fungi into the subsoil, at the highest dose of cadmium, caused a slight increase in the cadmium content in the aboveground parts of thale cress.

When applying cadmium at rates 2 and 10 mg kg^{-1} subsoil, the BAC coefficients reach lower values for variant (+T) as compared to (–T), and at dose 30 mg kg^{-1}, the numbers are adverse (Table 2). On soils contaminated to a lesser degree, *Trichoderma* genus fungi somehow reduced the transport of cadmium from the subsoil to the aboveground parts of thale cress plants, while this type of ability was not observed on soils extremely polluted due to this element.

4. DISCUSSION

Detection and analysis of chlorophyll fluorescence parameters can be used as a precise tool for studying the photosynthesis under stress conditions, e.g. F_0 indicator provides information on the excitation energy losses during its transfer from the energy antenna to the PSII reaction center. Under the influence of stress factors, i.e. high temperatures, salinity, value of zero fluorescence usually increased. Research performed by Balakhnina et al. [19] revealed that applied cadmium doses did not affect the F_0 indicator, that remained at level comparable to the control. In turn, Vassilev et al. [20], when studying the cadmium impact on barley plants, recorded statistically significant decrease of zero fluorescence, which could result from lowering the contents of photosynthetic pigments in leaves.

Present experiment proved a statistically significant ($p=0.01$) decrease in F_0 value along with the increase of cadmium content in the subsoil (bi-factorial ANOVA), while addition of *Trichoderma* genus fungi into the subsoil had no considerable effects on zero fluorescence value ($p=0.79$).

Values of individual fluorescence parameters are interrelated and changing one will affect the others. In consequence to the loss of the excitation energy in the photosynthetic pigment molecules within the energy antennas, reduction in the maximum fluorescence F_M and variable fluorescence F_V values occurs, hence parameter F_0 affects parameters F_M and F_V. Value of maximum fluorescence parameter F_M depends on many factors, among others type of saturating light and chlorophyll content in tested tissue. In the case of plants exposed to stress factors, the F_M value decreases [21].

Experiment involving cadmium as the tress factor revealed that the influence of varied cadmium dose within the subsoil on F_M value was statistically insignificant ($p=0.71$). Presence of *Trichoderma* fungi contributed the remarkable decrease in F_M parameter value ($p=0.04$) only at plants grown on the subsoil with the highest cadmium rate applied (30 mg kg^{-1}).

Exposure of plants to cadmium ions usually makes the decrease in variable fluorescence Fv [21] as well as Fv/F_0 values. In present experiment, influence of increasing cadmium dose in the subsoil on Fv appeared statistically insignificant ($p=0.51$), while presence of *Trichoderma* genus fungi caused statistically significant decrease in variable fluorescence

Table 2. Values of Biological Accumulation Coefficient (BAC) at *Arabidopsis thaliana* plants grown on silty soil contaminated with cadmium to various extents with (+T) or without presence (-T) of *Trichoderma* genus fungi

	Cadmium dose into the subsoil [mg/kg DM]/Object							
	Control 0		2/A		10/B		30/C	
	-T	+T	-T	+T	-T	+T	-T	+T
BAC	0.71	0.47	2.48	2.09	1.80	1.61	1.60	1.80

value (p=0.01). Parameter Fv/F_0 considerably increased due to increased cadmium doses within the subsoil (Kruskal-Wallis test p=0.00). Parameter Fv/F_0 is remarkably sensitive towards various stress factors [22].

The Kruskal-Wallis test also revealed that growing extent of soil contamination due to cadmium caused highly significant increase in Fv/F_M parameter (p=0.00), although all achieved results of Fv/F_M were below 0.80 level. The optimum value of Fv/F_M at plants not exposed to stress should range from 0.80 to 0.83. Decrease of this value indicates worsening of the photochemical capacity of plant photosynthetic apparatus [23].

Decreased value of Fv/F_M in control object can result from the aging of leaves (sixth day of *Arabidopsis thaliana* growth after sowing), because aging processes lead disruption of chloroplast membrane structure, which directly affects the decrease of PSII efficiency. In turn, lower values of Fv/F_M in the case of other cadmium concentration levels in the subsoil can be associated with delayed kinetics of plant growth due to cadmium and launching the protective mechanisms in response to stress due to this metal. Liu et al. [24] observed reducing the ratio Fv/F_M in conditions of cadmium intoxication.

The *Trichoderma* genus fungi are well-known that have abilities to symbiosis with plant's roots – mycorrhiza. Action of mycorrhizal fungi towards uptake and accumulation of cadmium depends among others on fungus species, plant species, as well as metal concentration in the subsoil. The present experiment showed a diminished cadmium accumulation in aboveground parts of *Arabidopsis thaliana* at the levels of I and III degree of soil contamination due to cadmium, which corresponded to rates: 2 and 10 mg Cd kg^{-1} DM soil. No doubt, it was the result of the protective influence of *Trichoderma,* because mycorrhizal fungi protect plants from heavy metal penetration from the environment by means of secreting substances that bind metals, adsorption enormous amounts of metals on the wall surface, or detoxification within own cells [25].

Belleghem et al. [26], in studies upon sub-cellular cadmium localization in roots and leaves of *Arabidopsis thaliana*, showed that the tolerance mechanism of thale cress consists in excluding cadmium from shoots ("excluder strategy"). This strategy includes blocking the metal in roots (acting as a biological barrier) and transport from leaves back to the roots. The root's function as the barrier was confirmed among others by studies of Dauthieu et al. [27], who observed that amount of cadmium being the subject of allocation from roots to shoots at *Arabidopsis thaliana* is decreased along with its concentration increase in medium.

Biological Accumulation Coefficient (BAC) was calculated as ratio of heavy metal in shoots to that in soil [28]. Calculated values of BAC under condition of subsoil inoculation with *Trichoderma* fungi decrease with the increase of cadmium concentration in the subsoil (Table 2), which indicates the decrease of cadmium transported from the soil to the shoots (with exception of object C - variant +T).

Table 3. Values of physiological traits in leaves of *Arabidopsis thaliana* plants grown on silty soil contaminated with cadmium to various extens with (+T) or without presence (-T) of *Trichoderma* genus fungi

	Cadmium dose into the subsoil [mg/kg DM]/Object							
	Control 0		2/A		10/B		30/C	
	-T	+T	-T	+T	-T	+T	-T	+T
F_0	0.17 a	0.18 a	0.17 a	0.18 a	0.15 b	0.14 b	0.15 b	0.14 b
F_M	0.67 a	0.65 a	0.69 a	0.67 a	0.68 a	0.65 a	0.69 a	0.62 b
F_V	0.50 a	0.46 b	0.51 a	0.49 b	0.51 a	0.50 a	0.53 a	0.47 b
F_V/F_0	2.85 a	2.60 a	3.00 b	2.80 a	3.20 b	3.22 b	3.30 b	3.10 b
Fv/F_M	0.74 a	0.72 a	0.73 a	0.73 a	0.76 b	0.76 b	0.76 b	0.76 b

Different letters show significant difference (ANOVA and Kruskal-Wallis test p< 0.05)

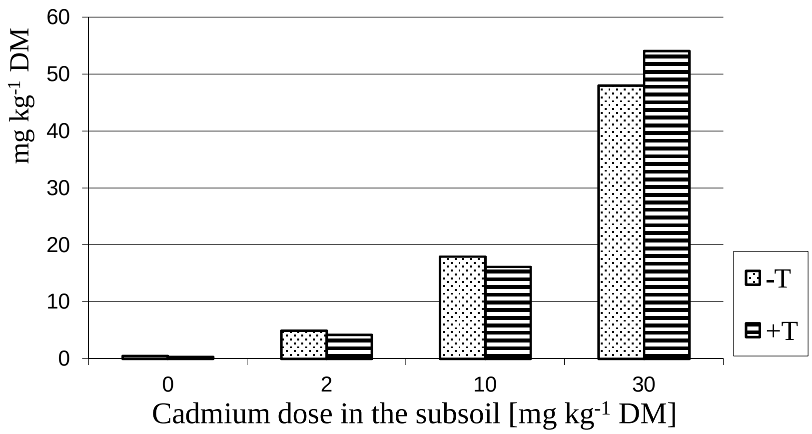


Fig. 2. Cadmium content in the aboveground parts of *Arabidopsis thaliana* [mg kg^{-1} DM] depending on cadmium dose in the subsoil (-T – subsoil without *Trichoderma* addition; +T – subsoil with *Trichoderma* addition)

5. CONCLUSION

Changes in majority of analyzed parameters of chlorophyll fluorescence (F_0, F_M, Fv, Fv/F_0) indicates the lack of disturbances of photosynthetic system at *Arabidopsis thaliana* due to cadmium doses applied to the silty soil. Only values of Fv/F_M parameter indicate some deterioration in the photochemical efficiency of PSII. No doubt it was the result of launching the resistance mechanisms at the plant in a response to the cadmium stress. Cadmium content in aboveground parts of *Arabidopsis thaliana* increased along with the increase of soil contamination degree due to this metal, both with and without addition of *Trichoderma* genus fungi. The *Arabidopsis thaliana* plants growing on the subsoil with lower cadmium rates (0, 2, and 10 mg kg^{-1}) with *Trichoderma* genus fungi addition contained less cadmium than those grown without addition of these microorganisms. The adverse plant's reaction was observed on the subsoil very strongly contaminated with cadmium (30 mg kg^{-1}). It was found on a base of Biological Accumulation Coefficient values (BAC) that silty soil inoculation using *Trichoderma* genus fungi affected the reduction of cadmium transport to aboveground parts of thale cress only from soils that were less contaminated due to this metal. The preset study indicates the possibility to apply *Trichoderma* genus fungi to reduce the cadmium contamination of thale cress grown on soils that are slightly polluted with cadmium (up to 10 mg kg^{-1}).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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