Reduction of phytotoxic effect of cadmium heavy metal by biomass of edible fungus, *Armillaria tabescens*

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Abstract

This study aimed to reduce the damage caused by Cd, the heavy metal, on the corn plant with the biomass of the edible mushroom *Armillaria tabescens.* For this purpose, control, Cd application (0.1 mM), Cd + *A. tabescens* (0.01 mM + 10 g biomass) application, and *A. tabescens* (10 g biomass) application were prepared as the experimental setup. Our study was carried out in a hydroponic environment. Firstly, root-stem lengths and wet-dry weight were determined. Lipid peroxidation (LPO) and reactive oxygen species (H_2O_2 and O^{2-}) and antioxidant enzyme (SOD) levels were measured. In addition, changes in protein, chlorophyll, proline, and sugar levels in seedlings were also evaluated. According to our results, in hydroponics medium the damage size was high in the corn plants exposed to Cd heavy metal while it was low in Cd + *A. tabescens* application. In addition, it was seen that *A. tabescens* significantly reduced heavy metal stress in Cd + *A. tabescens* application by considering the changes in chlorophyll content, carotenoid, total sugar, protein, and proline contents. Moreover, it was observed that only *A. tabescens* application contributed to the growth and development of corn plants. Our research findings showed that *A. tabescens* mushrooms can be used to reduce Cd stress. This study is a first in terms of heavy metal removal in hydroponic environment.

Keywords: edible mushroom; heavy metal; hydroponic environment; stress parameters

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Introduction

Increasing population, rapid industrialization, and ever-increasing traffic intensity are among the main reasons contributing to a rise in the amount of heavy metals pollution around the world. Industrial activities cause heavy metal pollution in both groundwaters and soils. Soils contaminated with heavy metals such as Cd, Hg, Pb, and Zn are not suitable to produce plants. Plants grown in these area, accumulating heavy metals in leaves and roots cause many dangerous diseases such as cancer (Tromboni et al., 2015; Jin et al., 2018).

Heavy metals such as iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), and cadmium (Cd) are essential for plant growth and functioning. They catalyze different enzymatic and redox reactions, electron transport, and the main

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component of DNA and RNA metabolism (Miransari, 2011). At high concentrations, heavy metals destroy the permeability and functioning of proteins, plasma structure, and plasma membrane. In addition, heavy metals cause oxidative stresses (Sajedi et al., 2010), adversely affecting plant growth with negative consequences for ecosystem health and the human food chain (Lebeau et al., 2008; Li et al., 2017).

Among heavy metals, Cd is highly toxic for plants, animals, and humans as it has been ranked No. 7 among the top 20 toxicants (Gill et al., 2012). Cd is one of the most important phytotoxic chemicals. Despite its high phytotoxicity, it has high solubility in water and is taken up by plant roots and easily transported to other tissues to enter the food chain thus causing serious problems (neurotoxic, mutagenic, and carcinogenic effects) for human health (Gill et al., 2011; Dalcorso et al., 2013). At high concentrations, Cd causes photosynthesis inhibition in plants, leading to increased lipid peroxidation (Márquez-García et al., 2011). It is also responsible for the formation of reactive oxygen species such as superoxide (O^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH). ROS (reactive oxygen species) accumulation causes cell death (Gill and Tuteja, 2010).

The negative effects caused by heavy metals can be reduced or completely eliminated by using various microorganisms (Latef, 2018). Bioremediation can be employed using rhizobacteria (promoting plant growth), mushrooms, and also using the soil as a filter medium (Lebeau et al., 2008). For this purpose, Pleurotus spp., *Trametes versicolor, Phanerochaete chrysosporium* and various arbuscular mycorrhizal fungi and ecto mycorrhizal fungi are used. In particular, *Armillaria rhizomorph* is capable of binding heavy metals such as Zn, Pb, and Cd and maintaining the balance of heavy metals in the earth (Rigling et al., 2006).

In this context, we aimed to reduce the adverse effects of Cd heavy metal, which is phytotoxin in our study, with *Armiilaria tabescens* (Durkan et al., 2011) which is an edible fungi.

Materials and Methods

Organisms used in the study

In our study, seeds of maize *(Zea mays* L. cv. Arifiye-2) which is the registered variety of Sakarya Corn Research Station were obtained from the related station directorate. The procured seeds were washed briefly with ethanol (96%) and then were subjected to 5 minutes surface sterilization in 5% sodium hypochlorite before planting.

Armillaria tabescens obtained from Abdurrahman Dündar, the Associate Professor at Mardin Artuklu University, was used in our study.

Reproduction of *Armillaria tabescens*

Suspension containing 10^7 spores from Armillaria developed on PDA medium was prepared and PDB was inoculated and allowed to incubate at 30 ℃ 180 rpm for 96 hours.

Cultivation of plants and applications (heavy metal and as treatment A. tabescens)

The maize plant (*Zea mays L*.) was grown for 9 days in a hydroponic medium containing 1/2 Hoagland nutrient solution. On the 9th day, $CdCl_2$ (0.1M) was added in the Hoagland medium and *A.tabescens* was developed at the same time in the PDB medium. Then *A.tabescens* was applied on the plant. The plants were harvested 72 hours after the applications. After measuring root and stem lengths, stems and roots were kept in deep freeze for necessary biochemical analysis.

Root and stem length measurement and determination of dry weight (%)

The plant roots and stems were measured with a ruler (Bozcuk, 1978). A total of 3 plants were taken from each group, dried in an oven at 60 ℃ for 72 hours, and then weighed again to calculate the percentage of dry weight of each application.

Determination of the amount of soluble protein

Protein contents were measured based on Smith et al. (1985) with 0.2 g fresh samples taken from leaves and roots. The results were calculated as mg protein/g fresh tissue.

Determination of total carbohydrate content

Total carbohydrate content was determined by the method determined by Dische (1962). Accordingly, 0.1 g of tissue samples were taken from the leaves of the maize plants and were homogenized in 5 ml of cold 2.5 NHCl. The homogenates were allowed to stand in hot water at 100 ℃ for 3 hours and then cooled in a cold water bath. Solid Na_2CO_3 was added to sample the reaction to stop. When the reaction was stopped, 50 ml of the supernatant was added to 45 ml of distilled water and centrifuged at 15,000 rpm for 15 min. After centrifugation, 1 ml of the supernatant was removed from 50 ml of the solution, Anthrone solution was added, and after cooling, the absorbance changes were recorded at 630 nm.

Determination of the total chlorophyll content

Total chlorophyll content was assayed using the method described by Witham et al. (1971). About 1 g fresh leaf tissue was ground with 10 ml acetone (80%). The optical density of the solution was read at 663 and 645 nm. Total chlorophyll content was expressed as mg g^{-1} FW.

Determination of the proline content

First, 0.5 g of tissue samples taken from the leaves and roots of the maize plant were homogenized in 7.5 ml of 3% sulfosalicylic acid. Homogenates were centrifuged at 6000 rpm for 10 min. Then 2 ml of the supernatant was taken and placed in another tube and 1 ml of cold acetic acid and 1 ml of acid ninhydrin were added in the tube and the tube containing the mixture was kept at 100 ℃ for 1 hour. At the end of the incubation, the tubes were taken in cold water bath and the reaction was stopped. Afterwards, 4 ml of toluene was added to the tubes and vortexed. After waiting for some time, the supernatant was removed and absorbance values were read at 520 nm. Toluene was accepted as a blind sample. The amount of proline was calculated from the standard graph in terms of µg in the tissue (Bates et al., 1973).

Measurements of H_2O_2 content and O^{2-} production rate

Briefly, 0.4 g of plant tissue was homogenized in 4 ml of cold 0.1% TCA and the homogenate was centrifuged at 12,800 x g for 30 minutes; then, 0.5 ml of the resulting supernatant was mixed with 0.5 ml of 10 mM KH_2PO_4 (pH: 7.0) buffer and 1 ml KI solution were added. Absorbance values were measured at 390 nm and the results were calculated as the amount of H_2O_2 per g tissue (µg g^{-1} tissue), as compared to the standard graph (Velikova et al., 2000).

The content of superoxide was measured by the method described by Elstner and Heupel (1976) where 0.5 g of the plant leaves were taken and homogenized in 4 ml of 60 mM potassium phosphate buffer (pH: 7.8). The homogenate was centrifuged at 12500 rpm for 12 minutes and then 0.5 ml of the supernatant was added from 0.5 ml of 60 mM potassium phosphate (pH: 7.8) buffer. The mixture was then stirred by adding 0.1 ml of 10 mM hydroxylamine hydrochloride. The mixture was incubated for 1 hour at 25 ℃. Then, 1 ml of 17 mM sulphonamide and 1 ml of 7 mM α-naphthylamine were added and incubated at room temperature for 20 minutes and then specific absorbance values were determined at 530 nm. Standard graph was prepared using sodium nitrite to calculate the content of superoxide anion. The results were shown in nmol. $min^{-1}g^{-1}$ tissue.

Determination of lipid peroxidation level

First, 0.4 g of fresh leaves for LPO were homogenized in 4 ml of 0.1% TCA (trichloroacetic acid), the homogenate was centrifuged at 12,800 rpm for 30 minutes, then 1 ml of the supernatant was removed from the tube and 1 ml of 0.5% TBA was added and the reaction mixture was incubated in boiling water bath for 30 minutes.

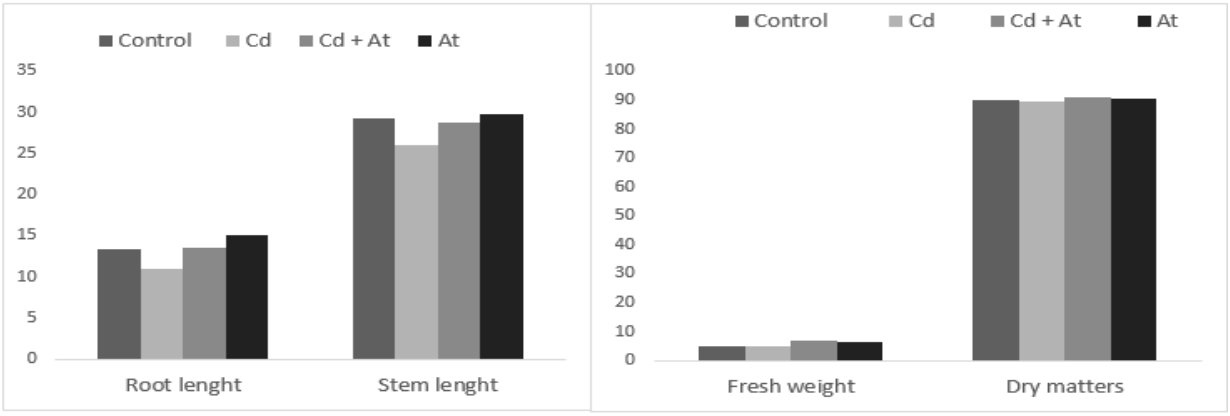

Fig. I. Effects of *A.tabescens* on stem and root length and total dry biomass of Zea mays; A: root and stem length; B: fresh weight and dry matters

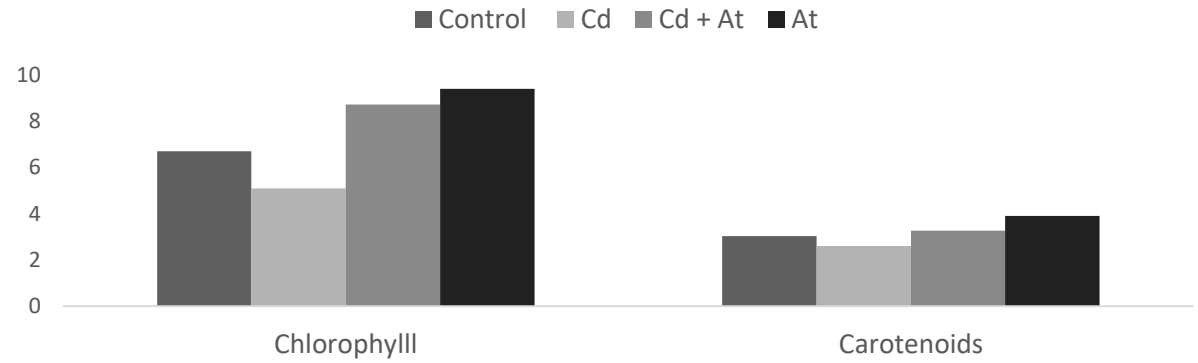

Figure 2. Amount of chlorophyll and carotenoids on Zea mays plants exposed to cadmium stress

The reaction was stopped by removing the tubes in the ice bath. Samples were centrifuged at 12,000 rpm for 5 minutes to remove the supernatant and read the absorbance value for absorbance at 532 nm and non-specific absorbance at 600 nm. For the calculation of lipid peroxidation. The absorbance measured at 532 nm was subtracted from the value determined at 600 nm and calculated in a 1 ml solution of MDA (nmol/µl): [(A532-A600) / 155000] × 10^6)]. Results were given as MDA (nmol g^{-1} tissue) (Jaleel et al., 2007).

Determination of antioxidant enzyme activities

Superoxide dismutase (SOD) activity was determined according to the method of Agarwal and Pandey (2004). The ascorbate peroxidase activity was determined based on the decrease in absorbance at 290 nm (Nakano and Asada, 1981).

Results

Root and stem lengths decreased under Cd treatment in the maize grown in Hoagland compared to control. On the other hand, root and stem lengths increased under Cd + At treatment. Under At application, root and stem lengths were higher than control group. Dry matter amounts were almost the same in control, Cd + At, and At application and higher than Cd application (Fig. I).

The amount of chlorophyll in the corn plant exposed to CD stress decreased significantly compared to the control (5.1). In the A. tabescens application, which was used to reduce heavy metal stress, chlorophyll content increased to 8.71. Carotenoid contents were also similar to those of chlorophyll (Fig. II).

As shown in Fig. III, the amount of protein in the root and stem decreased under Cd application while they increased under Cd + At application. The amount of protein in A.tabescens application alone was higher than that in the

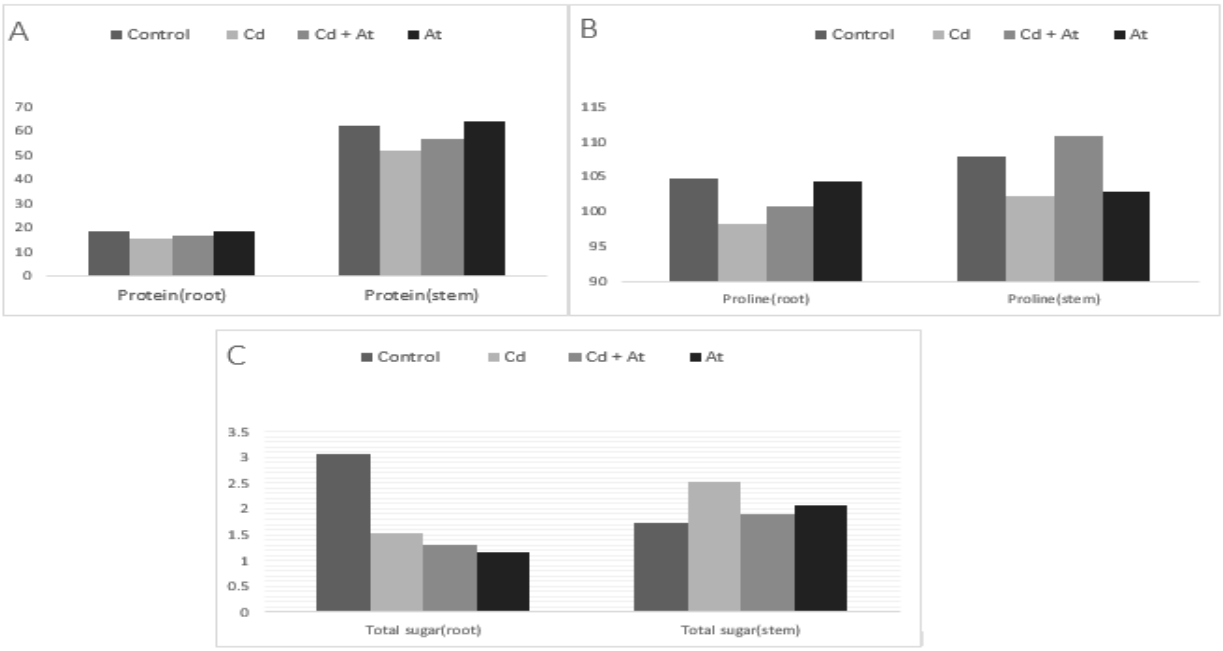

Fig. III. A: total protein contents; B: total proline contents; C: total sugar amounts

Table 1
H_2O_2, O_2^-, and MDA levels in maize plants exposed to cadmium heavy metal stress

Zea mays root	H_2O_2	O_2^-	MDA	SOD
Control	3,55	2,44	0,67	10,6
Cd	4,88	2,56	1,42	15,55
Cd + At	4,03	2,43	0,76	17,58
At	3,09	2	0,56	10,55
Zea mays stem				
Control	3,12	4,23	0,28	10,3
Cd	4,39	5,56	0,4	13,34
Cd + At	3,81	4,73	0,3	15,29
At	2,61	4	0,25	9,37

control group. Furthermore, the proline contents followed similar decrease-increase pattern as the protein's. While the highest sugar content of the roots were measured in the control group, the highest amount of sugar in the stems were measured in Cd application (Fig. III).

Stress indicator, H_2O_2, O_2^-, and MDA under Cd application increased in corn plants under study. Under *A.tabescens* application, H_2O_2, O_2^-, and MDA contents of both roots and stems decreased. In addition, the amount of antioxidant enzyme SOD increased in *A. tabescens* application (Table 1).

Discussion

Cd is one of the heavy metals with high phytotoxicity. Cadmium stress especially affects the roots of plants and prevents plant nutrition, thus preventing plant growth (Qui et al., 2016). Fungi increase the plant root and stem wet weights by keeping heavy metals such as Cd in their bodies (Amir et al., 2013; Hassan at al., 2013; Abeer et al., 2015).

Another negative effect of Cd is that it reduces the amount of chlorophyll in the leaves. Studies show that as the amount of Cd increases, the amount of chlorophyll decreases (Latef, 2018; Nongmaithem et al., 2017). The fungi (arbucides

or non-arteries) applied to reduce heavy metal stress were reported to increase the amount of chlorophyll and carotenoids (Abeer et al., 2015; Wang et al., 2016). Our results are consistent with these studies. Previous studies have shown that the total amount of protein and sugar is increased in most plants with the concentration of heavy metal (Zheng et al., 2009). Our study results differ from the literature as decrease in the amount of protein was detected in Cd application.

In addition, total sugar content decreased in roots under Cd treatment while in stems higher sugar content was measured compared to the other groups. Proline is an amino acid and one of the non-enzymatic defense molecules of plants under various biotic and abiotic stresses. Its role in stressed plants is to suppress root extension and the negative effects of stress (Clemens, 2006; Burritt, 2012). In our study under the treatment involving Cd + At, the root and stem proline levels were higher than in Cd application. A + Tabesens may have tried to decrease Cd stress by increasing the amount of proline in Cd + At administration. In a study Latef (2018) found similar effects of Cd stress on the plants' total protein and total sugar contents.

The greatest indicator of damage caused by environmental stress is the increase in the amount of lipid peroxidation. The increase in H_2O_2 and O_2^- radicals indicates that plants are exposed to various abiotic and biotic stresses. Heavy metal stress, which is one of the abiotic stress factors, causes an increase in MDA, H_2O_2, and O_2^- levels in plants. Many studies to reduce heavy metal damage used mycorrhizal or non-mycorrhizal fungi and significantly reduced the amount of MDA, H_2O_2, and O_2^- (Ling-Zhi et al., 2011; Shan et al., 2012; Irfan et al., 2014). SOD, one of the most important enzymes that plays an active role in detoxification in the antioxidant defense system, provides the cell defense of the plant by breaking down the produced H_2O_2 into water and 0_2^-. In the plants inoculated with fungus the amount of SOD increases to cope with heavy metal and other stress conditions (Bhaduri and Fulekar, 2012; Abeer et al., 2015; Nongmaithem et al., 2017). In this study, the amount of MDA, H_2O_2, and O_2^- increased in Cd application and decreased in A. tabescens application. The amount of SOD increased in Cd + At application while under the At application alone, it was almost the same as the control. Our results confirm the findings of the studies reported by Latef (2013) and Abeer (2015) in that A. tabescensin reduces stress by retaining Cd heavy metal or by assisting the plant's defense system. This study is the first remediation study using fungi in hydroponic environment. The results showed that heavy metal remediation can be done by using capped or moldy fungi such as A tabescens in hydroponic environments and that plants can be grown under reduced stress conditions. In addition, it was determined that not only mycorrhizal fungi but also non-mycorrhizal fungi could contribute to plant growth in evaluating the potential of using biomasses in metal removal studies. This preliminary study is being further developed to determine the effects of the same fungus on other heavy metals and also the potential effects of other fungi for phytoremediation.

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