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Polyamine and Laccase Production under Cadmium Stress in *Trametes (Coriolus) versicolor* and *Funalia trogii*

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Abstract
In the present study the effect of increasing the concentrations of the heavy metal cadmium (Cd) (0.1-200 ppm) on the free polyamines (PAs) level and laccase activity was studied in the white rot fungi *Trametes (Coriolus) versicolor* and *Funalia trogii* at 6 and 15 days.
Spermidine (Spd) was the most abundant PA in the two white rot fungi, while Spermine (Spm), and Putrescine (Put) were found only in trace amounts or were not determined. The highest Spd levels (1952.05 μg/DW and 2391.54 μg/DW) were observed in 1 ppm Cd at 15 days of incubation in *T. versicolor* and *F. trogii* respectively. The laccase activity of both species, especially in the 15-day cultures, showed a significant increase compared to the control. The highest increase was evident at 100 ppm Cd where the laccase activity was over 19 fold higher than that of the control sample in the 15 day culture of *T. versicolor*. The dry biomass in the 0.1 and 1 ppm Cd concentrations showed a significant increase in *T. versicolor* in the six and 15 day incubation periods. In all cases, the dry biomass in both the six and 15 day cultures of *F. trogii* showed a significant increase compared to the control cultures.
The findings gathered through this study show that Cd can amplify free Spd levels and increase laccase activity in *T. versicolor* and *F. trogii*. This increase under Cd stress suggests their possible role in combating the adverse effect of heavy metal stress.
Keywords: Cadmium, *Funalia trogii*, laccase, polyamines, *Trametes (Coriolus) versicolor*.

Kadmiyum Stresi Altındaki *Trametes (Coriolus) versicolor* ve *Funalia trogii* 'de Poliamin ve Lakkaz Üretimi

Özet
Bu çalışmada, artan konsantrasyondaki kadmiyum (Cd) (0.1–200 mg/L) uygulamasının, ağır metal cevabında indikatör olan serbest poliaminlerin düzeyine ve lakkaz aktivitelerine olan etkisi beyaz çürükçül funguslardan *Trametes (Coriolus) versicolor* and *Funalia trogii* ' de 6. ve 15. günlerde araştırıldı.
Spermidin (Spd) her iki beyaz çürükçül fungusta da en yoğun bulunurken, Spermin (Spm) ve Putresin (Put) sadece eser düzeyde bulundu ya da belirlenemedi. En yüksek Spd düzeyi (1952.05 μg/DW; 2391.54 μg/DW) 1 ppm Cd uygulanan 15 günlük inkübasyon süresindeki sırasıyla *T. versicolor* and *F. trogii* ' de tespit edildi. Her iki türdeki lakkaz aktivitesi özellikle 15 günlük kültürlerde kendi kontrollarına göre önemli düzeyde artış gösterdi. Lakkaz aktivitesinde en yüksek artış, 100 ppm Cd uygulanan 15 günlük *T. versicolor* kültürlerinde kontrol grubuna göre 19 kat daha yüksek bulunarak elde edildi. Kuru ağırlık 0.1 ve 1 ppm Cd uygulanan 6 ve 15 günlük *T. versicolor* kültürlerinde belirgin bir artış göstermiştir. 6 ve 15 günlük *F. trogii* kültürlerinde bütün uygulama gruplarında elde edilen kuru ağırlık kontrol grubu ile kıyaslandığında belirgin bir artışa neden olmuştur.
Bu çalışma sonucu, kadmiyum uygulanan *T. versicolor* ve *F. trogii* kültürlerinde serbest Spd düzeyi ve lakkaz aktivitesinde artış olduğunu gösterdi. Cd stresi altındaki *T. versicolor* ve *F. trogii* 'de serbest Spd düzeyi ve lakkaz aktivitesindeki artış ağır metal stresi ile mücadelede rol oynadığı fikrini verdi.
Anahtar Kelimeler: *Funalia trogii*, kadmiyum, lakkaz, poliaminler, *Trametes (Coriolus) versicolor*

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INTRODUCTION

The naturally occurring polyamines (PAs) Spermidine (Spd) and Spermine (Spm) and the diamine Putrescine (Put) are small polycationic molecules found ubiquitously in all organisms and implicated in several biological processes. There have been some studies regarding the potential function of PAs, which mainly focused on metabolism and catabolism concerning the protection from specific biotic and abiotic stresses

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(Mutlu and Bozcuk 2007, Groppa and Benavides 2008).

In fungi, several reports have demonstrated the occurrence of changes in the PA metabolism associated with growth and developmental events. During certain transformation practices such as spore germination, dimorphism, and sporulation, PAs are required (Ruiz-Herrera 1994). When *Ustilago maydis* (Guevara-Olvera et al. 2000) and *Yarrowia lipolytica* (Jimenez-Bremont et al. 2001) are taken into consideration, for example, a level of PA concentration which is greater than that needed to maintain vegetative development has to be present in order to support dimorphic transition. The PA metabolism of phytopathogenic fungi has attracted a great deal of attention due to the demonstration that specific inhibition of PA biosynthesis can lead to the control of fungal plant diseases (Walters and Mackintosh 1997, Garriz et al. 2008).

The physiological responses, found in fungi, against metal contamination implicate certain defence systems, both active and passive, which enable them to withstand or decrease metal toxicity (Gadd 2001, Baldrian 2003). The toxic effects of free metal ions could be decreased by the binding of the metal to the cell walls and their sequestration by binding to thiol peptides or organic acids and complexation in the vacuoles (Gadd and Sayer 2000). These defence mechanisms are not well understood in white rot fungi.

As to the metabolism of the fungi, we see that certain heavy metals are crucial; however, there is no evident biological role. In order for the fungi to grow, some metals are required, such as manganese, iron, copper, zinc, and nickel. Those that have no crucial role, in general terms, are chromium, cadmium, mercury, lead, and silver (Gadd 1993). Both essential and non-essential heavy metals are toxic for fungi, when present in excess (Brennan and Schiestel 1996). Cadmium (Cd) is considered to be one of the most dangerous heavy metals and its toxic effect on several organisms is well known (Baldrian 2003, Benavides et al. 2005, Güner et al. 2012, Vural 2013). In our study Cd was chosen because it binds to the sulfhydryl groups of protein, which can lead to the inactivation of enzymes. Although the PAs have been studied extensively in the cadmium stress response of higher plants (Groppa and Benavides 2008, Zacchini et al. 2011), their role in white rot fungi under heavy metal stress has been neglected.

Some extracellular ligninolytic enzymes; namely, laccases and various peroxidases, are produced by white rot fungi. Such enzymes facilitate to the biodegradation process of lignin and organic xenobiotics (Kahraman and Yesilada 2001, Yıldırım and Abdunnasir 2010). The PA's functions are a significant part in general fungal stress responses, which happen due to the changes of the Cd treatment (Fink-Boots et al. 1999, Jarosz-Wilkolazka et al. 2006). There have been many recent studies regarding the oxidative stress defense mechanisms; however, only few of which are likely functioning in the adaptive stress responses seen among white rot fungi.

The aim of this paper was to investigate the effect of Cd on the formation of potential heavy metal response indicators such as different free PA content and laccase activity in two species of the white rot fungi *T. versicolor* and *F. trogii*.

MATERIALS AND METHODS

Organisms

The white rot fungi *Trametes (Coriolus) versicolor* ATCC 200801 and *Funalia trogii* ATCC 200800 were used in this study. They were maintained at 4°C after sub culturing at 30ºC, every 2-3 weeks, on Sabouraud dextrose agar (SDA).

Inoculum Preparation

The fungi were cultured at 30ºC on slant SDA. After 1 week, conidial suspensions were prepared according to Kahraman and Yesilada (2001) and used to cultivate the inoculum. Then 5 mL of each suspension was transferred into a 250 mL flask with 100 mL of Sabouraud dextrose broth (SDB). After a 5-day incubation, cultures were homogenized (Kinetic Polytron Homogenizer) and used to inoculate the fresh media.

Growth Media and Culture Setting Preparation

The homogenate, 2 mL, was transferred into 250 mL Erlenmeyer flaks containing 50 mL of Stock Basal Medium (SBM). The Cd was added in the form of $CdCl_2$ to the SBM at concentrations of 0.1, 1, 10, 50, 100, and 200 ppm respectively. The growth medium consisted of (in g/L of distilled water); KH_2PO_4, 0.2, $CaCl_2.2H_2O$, 0.1, $MgSO_4.7H_2O$, 0.05, $NH_4H_2PO_4$, 0.5, $FeSO4.7H_2O$, 0.035, Glucose, 2, and Yeast Extract, 1. The control and cadmium treatment cultures were then incubated at 30°C with agitation (150 rpm) for six and 15 days.

After six and 15 days of incubation, the pellets were harvested and homogenized for the PAs assay.

Extraction, Characterization, and Quantification of Free PAs

The free PA determination was accomplished using the method of Smith and Davies (1985) with modifications. The Mycelial cultures were extracted using 5% (v/v) cold $HClO_4$ at a ratio of 100 ppm $HClO_4$ and centrifuged at 10,000xg/10 min. The supernatant containing the free PAs was frozen and stored at -20°C. Then 200 μL aliquots of the supernatant were added to 200 μL of saturated sodium carbonate and 400 μl of dansyl chloride in acetone (10 ppm) in a reaction vial. The mixture was incubated in a thermal reaction block at 60°C for 1 h in the dark. Then 100 μl of proline was added to the mixture to remove the excess dansyl chloride. After 0.5 h, the PAs were extracted in 500 μl of toluene with vigorous vortexing for 30 s causing the mixture to separate into two phases. The lower aqueous phase was removed with a 1 mL syringe and discarded. The organic phase, containing the PAs, was completely dried under an air stream and all PA samples were treated in the same way. The PA residue was dissolved in 1 mL of methanol and passed through a 0.2 μm pore size syringe filter, and analyzed immediately or stored at -20°C.

HPLC Analysis

The samples were injected into a fixed 20 μl loop for loading onto a reversed phase C_{18} column. The samples were eluted from the column with a programmed water and methanol (v/v) solvent gradient, changing from 60% to 95% in 23 min at a flow rate of 1 mL/min. The elution was completed in 30 min (Smith and Davies, 1985). The column was washed with 100% methanol for 5 min before the next sample was injected. Retention times of the different dansylated PAs were as follows: 15 min Put, 22 min Spd, and 29 min Spm. The fluorescence detector was set to an excitation wavelength of 365 nm and an emission wavelength of 510 nm for the dansyl polyamines. The eluent peaks with their areas and retention times were recorded by an attached integrator.

Laccase Activity

Laccase (E.C.1.10.3.2) activity in the growth medium was determined spectrophotometrically by monitoring the increase in absorbance at 420 nm. One enzymatic unit was defined as the amount of enzyme that oxidized 1 μmol of ABTS (2, 2 azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) per minute (Roy-Archand and Archibald 1991).

Determination of fungal dry weights (Dry biomass)

The fungal dry weights were established by refining the ingredients of each flask. Pre-weighed Whatman No: 1 filter paper was used in this process and it was dried at 50°C to a fixed weight. The results were explained as g of biomass per 50 mL of culture.

Statistics

The experiments were performed with three replicates. The results are expressed as the means of the three replicates used for each experiment (± standard error). The difference between the results was studied, and followed by a comparison with least significant differences (LSD) at the level of 5%.

RESULTS AND DISCUSSION

Effect of Cd on the Endogenous PA Content of Ttwo White Rot Fungi

In the present study, the content of free PAs was studied in white rot fungi (*T. versicolor* and *F. trogii*) exposed to Cd.

Free Spd was found as the major PA in *T. versicolor* and *F. trogii*, whereas, Spm, and Put were present only in trace form or were not determined in the six and 15 day cultures. The free Spd levels in both of the control cultures were found to be higher in the six than the 15 day cultures. The content of the free Spd changed significantly due to the Cd treatment (Figure 1 and 2). The highest Spd levels (1952.05 μg/DW and 2391.54 μg/DW) were observed in 1 ppm Cd at 15 days of incubation in *T. versicolor* and *F. trogii*, respectively ($P<0.05$). In the case of the 1 ppm Cd treatment, the Spd concentrations in *T. versicolor* were 111.3 and in *F. trogii* 95.58 times higher were measured when compared to the control in the 15 day incubation period.

These findings are consistent with those of other similar studies. For example, Pitkin and Davis (1990) reported that Spd were commonly the most abundant PA; however, lower levels of Put and Spm than Spd were found in *Neurospora crassa*. Biondi et al. (1993) and Walters et al. (1997) also observed similar trends in *Ophiostoma ulmi* and *Aspergillus fumigatus*, respectively. Mutlu et al. (2010) reported that Spd was the most abundant PA in the two white rot fungi, *T. versicolor* and *F. trogii*. These studies showed that Spd is generally considered to be the major PA and an essential metabolite in fungi, with Put and Spm being usually present at 10% or less than that of the Spd pool.

In our experiment, the obtained data showed a positive relationship between the Spd titre and heavy metal tolerance except in low cadmium concentrations (0.1 and 1 ppm). This finding is accepted as an adaptive reaction taken by the fungus against stress. Groppa et al. (2007) and Karina et al. (2005) also support this view in plant systems. Compared with higher plants, little information is available on the mechanisms of desiccation tolerance in white rot fungi.

Furthermore, the level of free Spd was much higher in the six day periods in *F. trogii* than *T. versicolor*, whereas, the level of free Spd appeared to be higher in the 15 day period for *T. versicolor* than *F. trogii*. Moreover, the free Spd level in *T. versicolor* grown in 10, 50, and 100 ppm Cd stress for 15 days was 48.73, 6.40, and 9.74 fold higher than the *F. trogii*. The modifications in the PA content stemming from the heavy metals, stated in the study in question, revolve only around long-term exposure.

Laccase Activity in the Presence of Cd

The function of laccase found in general stress responses in various organisms has been stated in certain reports (Jarosz-Wilkoiazka et al. 2002, Mayer and Staples 2002). In this work, increasing the concentration of Cd in the culture medium up to a certain value resulted in a considerable increase in laccase activity ($P<0.05$).

The highest increase was observed at 100 ppm Cd where the laccase activity was 19.40-fold higher than that of the control sample in 15 the day culture of *T. versicolor*. The addition of Cd at 100 ppm at six and 15 day to the cultures of *F. trogii*, induced more than 3.28 and 6.51-fold higher enzyme activity than the control, 28.90±1.29 and 23.75±3.61 mU/mL, respectively. As shown in Table 1, the laccase activity in both species in the15 day cultures is much higher than in the six day cultures. Lower activity was found in Cd at 200 ppm, whereas, in Cd at 100 ppm, the enzyme activity was even higher than in the control. These results are very similar to the findings concerning the increase in soil containing 10-100 ppm Cd and the slight decrease at 500 ppm Cd by Baldrian et al. (2000) and Kumarasamy et al. (2009) in *Pleurotus ostreatus* and *Ganoderma lucidum*, respectively.

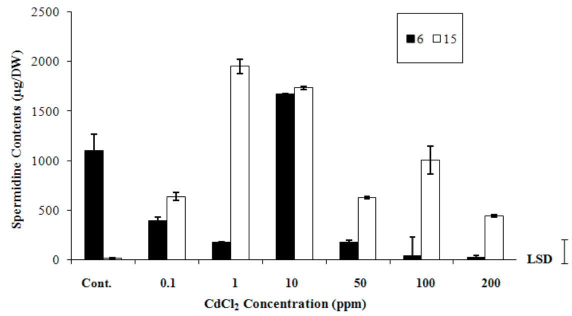

Fig 1. The influence of the various $CdCl_2$ concentrations on the free Spd content in the cultures of *Trametes (Coriolus) versicolor* in the 6 and 15 day cultures.

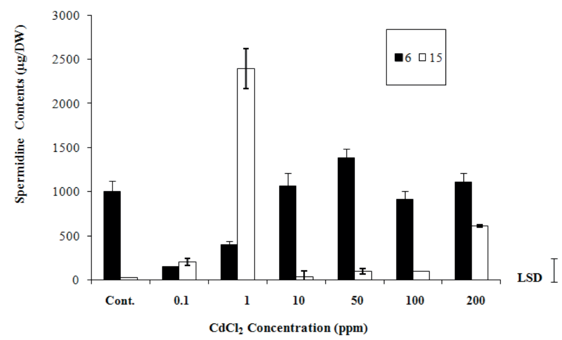

Fig 2. The influence of various $CdCl_2$ concentrations on the free Spd content in the cultures of *Funalia trogii* in the 6 and 15 day cultures.

Table 1. The production of extracellular laccase and dry biomass in the *Trametes (Coriolus) versicolor* and *Funalia trogii* cultures.

		Laccase Activity (mU/mL)		Dry Biomass (mg/50 mL)	
	Treatment	Days		Days	
Species	$CdCl_2$ (mgL^{-1})	6	15	6	15
T.versicolor	Cont. (SBM)	0.05±0.01	1.56±0.43	33.50±5.16	77.37±7.22
	0.1	1.80±0.52	4.16±0.57	77.60±1.07	106.33±11.56
	1	1.02±0.01	3.62±0.79	87.07±9.22	91.77±5.09
	10	3.92±0.822	6.88±0.42	44.13±8.70	77.23±11.81
	50	28.67±5.52	14.87±2.24	17.47±2.46	70.37±11.13
	100	1.92±0.602	30.27±1.54	11.67±0.75	21.05±3.25
	200	6.17±1.05	0.49±0.01	11.80±0.80	13.77±2.15
F. trogii	Cont. (SBM)	8.80±2.39	3.65±0.51	4.56±0.56	5.65±0.43
	0.1	2.34±0.79	14.25±0.49	49.43±2.96	40.5±5.68
	1	5.57±0.96	14.63±2.40	46.17±9.87	44.63±4.91
	10	4.86±1.40	11.80±0.58	34.17±3.10	38.60±2.16
	50	3.76±0.79	18.20±1.23	38.5±3.45	44.83±6.67
	100	28.90±1.29	23.75±3.61	30.93±2.81	51.40±7.32
	200	5.09±1.89	4.68±1.57	19.67±1.97	45.63±9.67
	$LSD_{0.05}$	7.56		12.93	

Generally, extracellular laccase activities were higher in *F. trogii* than *T. versicolor*, irrespective of whether Cd was present or not. Baldrian (2003) suggested, different degrees of heavy metal tolerance can be detected among different species of white rot fungi. This variance concerning heavy metal tolerance can, at the same time, be seen within a single species. *Trametes versicolor*, one of the white rot species analyzed by Palmans et al. (1995), was found to be resistant to the metals: Cd, Zn, Hg, Co, Cr, Mo, Ni, Pb, and Sn. On the other hand, *Lentinus* sp., *Pholiota nameko*, *Pleurotus* sp., and *Pycnoporus sanguineus* turned out to be obviously less resistant when compared to those aforementioned.

Trametes pubescens was used during the analysis to analyze the induction of laccase by other metals, which can induce oxidative stress. The findings showed that it was only Mn and Cu that increased the laccase formations. Ag, Cd, Hg, and Zn did not, on the other hand, play an efficient role in increasing the laccase formation (Galhaup and Haltrich 2001). Moreover, an addition of 1-5mM Cd did enhance the laccase activity in *Pleurotus ostreatus* (Galhaup and Haltrich 2001).

The results show that the presence of cadmium in the environment plays an important role in the regulation of extracellular laccase enzyme activities. Different species of white rot fungi differ in the degrees of their heavy metal tolerance.

The enhanced activity of laccase under heavy metal presence also suggests an important role in the fungal adaptation processes. White rot fungi could also be used to remove heavy metals from aqueous solutions by adsorbing the metals into the mycelium and to biodegrade various organic xenobiotics by extracellular laccase. The presence of harmful compounds in the medium which might appear lethal to other organisms stimulates the detoxification abilities of this fungal group by increasing the extracellular discharge of ligninolytic enzymes, especially laccase. This offers extremely broad possibilities for the biotechnological use of white rot fungi.

Cd and Growth Relation (as dry biomass)

Dry biomass in 0.1 and 1 ppm Cd concentrations showed a significant increase in *T. versicolor* in the six and 15 day incubation periods ($P<0.05$). Only the highest Cd concentrations (100 and 200 ppm) used in this work inhibited the dry biomass development of *T. versicolor*. At the end of the experiment, the dry mass of mycelium in 200 ppm Cd was only 35.22% and 17.80% of the control value in the six and 15 day cultures, respectively (Table 1). In a study conducted by Jarosz-Wilkołazka et al. (2006), an increase in Cd concentration did evidently lead to a decrease in the fungal dry weights of *Abortiporus biennis*. It was pronounced by Falih (1997) that, in an agitated condition, high levels of heavy metal concentration lowered the growth rate of *Phanerochaete chrysosporium*. Besides, when copper was added to the growth medium of *Pleurotus ostreatus*, a decrease in mycelial growth was observed (Baldrian and Gabriel 2002).

In all cases the dry biomass in both the six and 15 day cultures of *F. trogii* showed a significant increase compared to the control cultures ($P<0.05$). The findings concerning the increase in dry biomass levels as a result of Cd application are very similar to those of Baldrian and Gabriel (2002) in *Pleurotus ostreatus*.

CONCLUSION

The accumulation of free Spd under Cd stress, observed in this work, may indicate its potential role in overcoming the adverse effect of heavy metal stress. Nevertheless, in order for the function of PAs to be explained in a clearer way, we need a wider range of expansive studies regarding the fundamental mechanism of PA accumulation. These processes and active defence mechanisms of fungi against metal toxicity are not understood comprehensively; therefore, we need more related studies. What is deduced from the results is that the available information cannot, for the present time, provide an overall explanation regarding the effects of PAs on fungal development in an environment with heavy metals. These results may imply that the heavy metal affects the PA metabolism in different ways, depending on the species, strength of stress and duration of the treatment.

The enhanced activity of laccase in stress situations may also suggest that it has a role in the fungal adaptation processes. When the effect of heavy metals on the activity of laccase enzyme is taken into consideration, we can clearly see that it is also apparent in the biotechnological processes changing with their activity. There is a difference between the white rot fungi species and strains in terms of their laccase producing functions.

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