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Coriolus hirsutus Laccase Effect on Atrazine Adsorption and Desorption by Different Types of Soil

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Abstract—Study of adsorption–desorption behavior of herbicide atrazine in soils of different geographical zones in the presence of *Coriolus hirsutus* laccase was performed. Laccase was shown to significantly increase adsorption coefficient and to facilitate irreversible adsorption of atrazine to soil. Supposably, laccase catalyzes oxidative binding of atrazine to soil.

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Growing rates of environmental pollution and, in particular, soil contamination urge to develop new effective biotechnology methods of soil detoxication. Herbicides are among major soil contaminants, as they are extensively used to control weed growth. Incorrect use of herbicides (dosage excess or scheme violation) often leads to negative consequences, that is, contamination of agricultural products, soil, and adjoining environ.

Highly stable herbicides are particularly harmful pollutants. A widely applied simtriazine herbicide, atrazine, is among them [1]. According to the literature data, it is binding to humic acids (HA), which determines atrazine accumulation in soil and the level of atrazine toxicity. Humic acids compose major part of the soil organic matter both in solution and immobilized on minerals [2, 3].

Although the processes of atrazine sorption onto HA are thoroughly studied [4], there is no agreement on the binding mechanism. One of the most widely shared views implies that binding occurs via hydrophobic interactions with charge-transfer complexation [5]. The binding of atrazine results in a lower concentration of the free herbicide in soil and, consequently, in a lower toxicity [6]. However, a change in conditions may lead to desorption and to an increase in mobility and toxicity.

On the other hand, a number of phenol and amine xenobiotics were shown to be capable of irreversible incorporation into HA structure by means of oxidative binding [9], which requires catalysts. In soils this catalytical function is carried out by oxidoreductases: peroxidases, laccases, and polyphenoloxidases [7, 8]. There are few and contradictory data on atrazine oxidative binding to HA. Horseradish peroxidase introduction into soil hasn't increased atrazine adsorption capacity, and in some cases has even led to its decrease [2]. Yet some authors [9] consider oxidative binding of atrazine as one of the possible mechanisms of soil-bound atrazine formation. Effects of other oxidoreductases on atrazine adsorption to soil haven't been studied.

Laccases, among other soil oxidoreductases, possess a number of favorable qualities, that is, broad substrate specificity, wide ranges of thermal and pH stability, and high activity levels in soil during the year [10, 11]. The major producers of laccases in soil are white rot fungi, *Coriolus hirsutus* being one of the representatives. Now laccase-producing basidiomycetes and laccases themselves are widely used in biotechnological utilization of lignin containing wastes and in recultivation of media contaminated by polychlorbiphenyls (PCB), polynuclear aromatic hydrocarbons (PAH), synthetic dyes, and herbicides [12].

Therefore, the study of atrazine adsorption and desorption mechanisms in the presence of laccase is of utmost importance. This kind of investigation would allow to determine whether atrazine is capable of oxidative binding to HA with the participation of laccase and to evaluate the role of the enzyme in simtriazine xenobiotics detoxication. The data of such kind, in turn, will provide grounds for biotechnological recultivation of the contaminated territories.

Thus, the aim of the research is a comparative study of atrazine adsorption and desorption processes in different types of soil in the presence of laccase from basidiomycete *Coriolus hirsutus*.

MATERIALS AND METHODS

Soil samples collection and characterization. To carry out adsorption–desorption experiments, a group of soil samples was collected from three soil zones, including the sod podzolic soil (SP) of Moscow oblast, the grey wooded soil (GW) of Tula oblast, and the black

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one (B) from Kursk oblast. Samples were collected from humus-accumulated horizon at 0–5 cm and stored at room temperature.

The following chemical and physical properties of the soil samples were measured using different analytical methods [13]: acidity, organic carbon contents (C_{org}), and granulometric composition (GMC).

Determination of enzymatic activity. Extracts of the soil samples were obtained according to [14] by 140 mM sodium–phosphate buffer, pH 7.1, extraction in the ratio of 1 : 10 at 25°C for 24 h. Laccase activity in the extracts was measured using PerkinElmer spectrophotometer, United States, in a 1-cm cuvette at 25°C.

Syringaldazine (Sigma, United States) was used as a substrate. Reaction mixture contained 3 ml of 0.1 M syringaldazine in 0.1 M acetate buffer, pH 4.5. Reaction was initiated by introduction of 0.05 ml of the soil extract into the mixture. Absorbance was measured at 530 nm. Activity was measured in standard units, one standard unit being an increment of absorbance in 1 ml of the reaction mixture within 1 min.

Enzyme. The laccase used in the study was from Polyporaceae genus basidiomycete *Coriolus hirsutus* 072 (Wulf. Ex. Fr.) Quel. The strain was kindly provided to us by V.P. Gavrilova (Cultures collection of Komarov Botanical Institute, Russian Academy of Sciences, Saint-Petersburg).

The cells were grown in submerged cultivation conditions. Extracellular laccase was isolated from the culture broth according to a technique developed earlier [15]. The enzyme stock solution, 17.5 g/l in 50 mM potassium–phosphate buffer, pH 5.0, was prepared immediately before use.

Herbicide. We used high purity atrazine (99.97%, Dr. Erhensdorf Ltd., Germany). Atrazine stock solution (1 mg/ml) in methanol (HPLC grade, Sigma) was stored at 4°C in a dark place.

Adsorption–desorption experiments. The initial atrazine concentrations were 1, 2, 3, 5, 8, and 10 mg/l, which made 5, 9, 14, 23, 37, and 47 μM, respectively. The preliminary experiments have shown that incubation time of 24 h was enough for equilibrium attainment.

Adsorption experiments were carried out in centrifuge screw-cap tubes in triplicate. Each tube was first filled with 2 g of soil, and then 10 ml of 50 mM potassium–phosphate buffer solution, pH 5.0, was added. The tubes were incubated at 27°C and stirred on Elmi shaker, Latvia, to achieve equilibrium between the soil and the solution for 24 h. Then atrazine and laccase were introduced into the samples. Laccase concentration made up 3.5 g/l. After another 24 h of stirring, tubes were centrifuged (1200 g, 30 min) to separate the solid and the liquid phases. Supernatant aliquots of 50 μl were filtrated through cellulose filters with permeability limit of 5 kDa (Ultrafree-MC, Amicon, United States). HPLC analysis was used to determine atrazine contents in the samples. The quantity of the bound atrazine was calculated as the difference between initial concentration and concentration in the filtrate.

The remaining solution in the test tubes was removed with a pipette and substituted by potassium–phosphate buffer without atrazine and laccase. Tubes were shaken for 24 h and centrifuged. An aliquot was prepared for HPLC analysis as it has already been described. The assayed atrazine concentration was then used to calculate the quantity of the desorbed atrazine. The procedure has been performed until atrazine concentration reached minimum detection level. Desorption was performed for 7 times.

HPLC analysis for atrazine determination. Atrazine along with its major metabolites (desethylatrazine, desisopropylatrazine, 2-hydroxyatrazine, desethyl-2-hydroxyatrazine, desisopropyl-2-hydroxyatrazine) was determined using HPLC (Coulter System Gold chromatograph, Beckman, United States) according to a technique described in [16] with little modification. A reverse phase C18 4.6 mm × 15 cm column (Ultrasphere ODS, Beckman, United States) was used to separate the samples. Optical density of the outflow was recorded at 210, 225, and 230 nm. Mobile phase consisted of acetonitryl (solution A) linear gradient of 2 to 98% in 1 mM potassium–phosphate buffer, pH 7.0 (solution B). Elution time made up 35 min at 1 ml/min flow. The column was thermostated (30°C).

RESULTS AND DISCUSSION

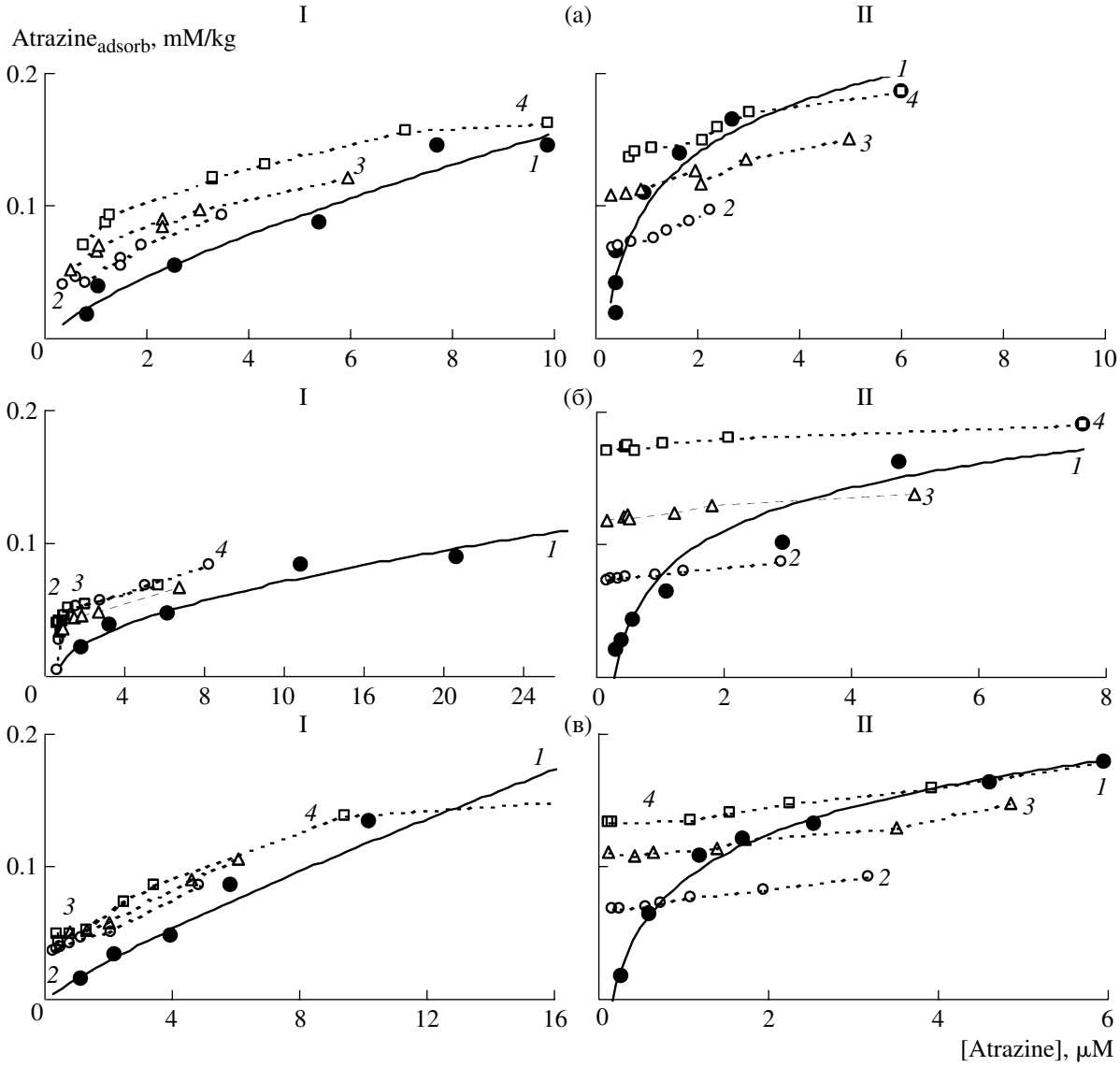
Soil sample characteristics. Physical and chemical properties of soil samples under study varied greatly. The highest acidity (pH 4.7) was characteristic of the sod podzolic soil; values obtained for the black soil (pH 6.7) and for the grey wooded soil (pH 6.6) were similar.

The black soil exhibited highest C_{org} contents (5.8%), while for the sod podzolic and the grey wooded soils, the value reached 3.8 and 2%, respectively. The black soil was also the most abundant in clay fraction (particle size < 0.001 mm). According to grain size analysis, black and grey wooded soil samples were classified as medium loam, and the sod podzolic one—as silty loam.

The samples did not possess any enzymatic activity in consequence of the sample preparation technique used and long storage time. Thus, laccase input alone affected atrazine adsorption–desorption behavior.

Atrazine adsorption to different types of soil with or without laccase. *Coriolus hirsutus* laccase effect on atrazine adsorption–desorption behavior in different types of soil was investigated by the comparison of both adsorption and desorption with or without the enzyme.

Atrazine adsorption–desorption isotherms drawn from the experimental data are presented on the figure. As follows from these data, laccase introduction altered the form of adsorption isotherm. In the absence of laccase (I) isotherms were close to linear. The presence of laccase (II) changed the isotherms character: steep rise



Atrazine (*1*) adsorption and desorption isotherms for atrazine initial concentrations of 23 (*2*), 37 (*3*), and 47 (*4*) μM in the absence of *Coriolus hirsutus* laccase (*I*) and in the presence of it (*II*) for (a) sod podzolic, (b) grey wooded, and (c) black soil samples.

in adsorbed atrazine with the increase of equilibrium concentration in the start was followed by decrease in adsorption rate.

In order to quantify atrazine adsorption, the curves were fitted with the Freundlich isotherm frequently used in description of interaction between xenobiotics and soil [17–19]:

$$\text{Atrazine}_{adsorb} = K_F[\text{Atrazine}]^{n_F},$$

where Atrazine$_{adsorb}$ is the quantity of atrazine adsorbed, the equilibrium concentration of it being equal to [Atrazine]; K_F is Freundlich adsorption coefficient, and n_F is a nonlinearity index of the isotherm. The calculated Freindlich coefficients are given in the table.

Without laccase Freundlich coefficients (K_F) for soils under study varied in the range of 0.81–5.55 in accordance with literature data [2]. The highest K_F value was noted for the black soil, which is probably due to the highest C_{org} content in this soil.

Laccase introduction caused rapid increase in atrazine adsorption (see table). Values of Freundlich coefficient for the studied soils also increased up to 3.13–6.79 and exceeded those obtained in the absence of laccase.

Preliminary experiments have shown that there was no interaction between atrazine and laccase in the absence of soil. On the other hand, in experiments on atrazine adsorption to different types of soil, no atrazine metabolites were detected. Thus, we may suppose that, when both soil particles and laccase are present in the medium, atrazine metabolites arise but then bind to the soil. It should be noted that the maximum increase of Freundlich coefficient (almost 4-fold) was observed for

Freundlich equation and hysteresis coefficients of atrazine adsorption--desorption isotherms for different types of soil with and without laccase

Soil type	Adsorption			Desorption				H
	K_F	n_F	R^2	Atrazine, μM	K_F	n_F	R^2	
In the absence of laccase								
SP	4.50 ± 0.20	0.72 ± 0.03	0.93	23	0.24 ± 0.01	0.22 ± 0.01	0.85	3.3 ± 0.2
				37	0.30 ± 0.02	0.25 ± 0.01	0.90	2.9 ± 0.2
				47	0.57 ± 0.03	0.27 ± 0.02	0.88	2.7 ± 0.1
GW	0.81 ± 0.04	0.56 ± 0.03	0.97	23	0.10 ± 0.01	0.11 ± 0.01	0.90	5.1 ± 0.2
				37	0.10 ± 0.01	0.13 ± 0.01	0.90	4.3 ± 0.2
				47	1.68 ± 0.08	0.52 ± 0.03	0.94	1.1 ± 0.1
B	5.55 ± 0.30	0.83 ± 0.04	0.98	23	0.15 ± 0.01	0.17 ± 0.01	0.99	4.9 ± 0.2
				37	0.29 ± 0.01	0.22 ± 0.01	0.88	3.8 ± 0.2
				47	0.36 ± 0.02	0.23 ± 0.01	0.89	3.6 ± 0.2
In the presence of laccase								
SP	5.80 ± 0.29	0.60 ± 0.03	0.70	23	0.23 ± 0.01	0.150 ± 0.007	0.85	4.0 ± 0.2
				37	0.31 ± 0.02	0.140 ± 0.007	0.98	4.3 ± 0.2
				47	0.36 ± 0.02	0.130 ± 0.007	0.92	4.6 ± 0.2
GW	3.13 ± 0.15	0.56 ± 0.03	0.99	23	0.11 ± 0.01	0.030 ± 0.001	0.85	18.7 ± 0.9
				37	0.15 ± 0.01	0.029 ± 0.001	0.75	18.7 ± 0.9
				47	0.20 ± 0.01	0.029 ± 0.001	0.77	19.3 ± 0.9
B	6.79 ± 0.34	0.66 ± 0.03	0.86	23	0.16 ± 0.01	0.064 ± 0.005	0.88	10.3 ± 0.3
				37	0.19 ± 0.01	0.062 ± 0.004	0.69	10.6 ± 0.4
				47	0.22 ± 0.01	0.060 ± 0.003	0.68	11.0 ± 0.5

the grey wooded soil, containing minimum of organic matter; while for the black and the sod podzolic soils, K_F increase was less pronounced and made up about 1.2. Consequently, the soil constituent which initiates atrazine metabolism in the presence of laccase is inorganic matter, for its surface is inaccessible when C_{org} contents is high (as in case of black and sod podzolic soils) and readily approachable when soil organic matter is low [21].

Atrazine desorption from different types of soil with or without laccase. In desorption experiments in case of initial atrazine concentration 14 μM, the concentrations to be detected were close to the detection limit (2 μM), which made it difficult to explain the data. Thus, here we present only the data obtained for the desorption experiments in which atrazine initial concentration made up 23, 37, and 47 μM.

Dissimilarity between adsorption and desorption isotherms evidences the hysteresis in atrazine adsorption to soil and its partial reversibility both with and without laccase. However, the level of atrazine irreversible adsorption was higher in the presence of laccase, indicating that laccase introduction contributes to the irreversible adsorption of atrazine.

Desorption isotherms were also fitted with Freundlich isotherm. The calculated Freundlich coefficients K_F and indices n_F are given in the table.

Hysteresis was quantified using hysteresis index H [20]:

$$H = n_{Fa}/n_{Fd},$$

where n_{Fa} and n_{Fd} are power indices in Freundlich adsorption and desorption equations, respectively.

Laccase introduction into black and sod podzolic soil samples was found to decrease H value from 1 to 0.56–0.66. Coefficient of determination R^2, when fitting data with Freundlich isotherm, also decreased in these samples. Therefore, atrazine adsorption to soil in the presence of laccase had a more complicated nature rather than a simple distribution between the two phases. The grey wooded soil, for which n_F did not change, made an exception.

Desorption characteristics in the absence of laccase were similar for all kinds of soil investigated, evidenced by the obtained hysteresis indices values. The rise of atrazine initial concentration was accompanied by decrease in hysteresis indices H and simultaneous increase in K_F, indicating the intensification of desorption in samples with high atrazine initial concentration (see table). Presumably, atrazine initial concentration

being low, it occupies the sites where the strongest binding occurs; increase in atrazine concentration leads to its binding to the sites of less affinity for atrazine. Consequently, desorption is more intense in the case of high initial concentrations.

Atrazine *H* values for sod podzolic and black soil types were found to be greater than unity for all experimental concentrations of herbicide, indicating partial reversibility of adsorption and formation of irreversibly bound atrazine. Hysteresis index was rather low and close to unity (*H* 1.1) in case of the maximum atrazine initial concentration (47 μM) in the grey wooded soil, indicating easy and practically complete desorption of adsorbed atrazine at given concentration.

Abnormal behavior of the grey wooded soil is probably due to the specific nature of its organic matter. The sample was collected in field and contained significant amount of lightly decomposed biomass and humic substances, poor in aromatic structures compared to other soils. As hydrophobic interactions are responsible for atrazine binding to humic substances, low amount of aromatics in the organic matter of the soil is probably the reason for almost complete reversibility of atrazine adsorption to the soil in case of the highest atrazine initial concentration.

Introduction of laccase promoted dramatic decrease in the amount of atrazine desorbed. Hysteresis index increased for samples of any initial concentration value (see table). Hysteresis indices for soils under study made up 4.0 to 28.0, exceeding *H* values for adsorption–desorption in the absence of the enzyme 2 to 27 times. The maximum effect on *H* growth was shown for the grey wooded soil in case of the highest atrazine initial concentration 47 μM.

It should be emphasized that, unlike in the absence of laccase, for samples containing the enzyme atrazine, initial concentration practically did not affect hysteresis coefficient values. In contrast to the steady decrease of *H* value with the growing atrazine initial concentration in the absence of laccase, the addition of enzyme in some cases led to a slight increase in *H*. In other words, increase in atrazine concentration in the media did not affect the irreversibility of its adsorption, or led to increase in adsorption. This indicates that atrazine removal from the reaction media is irreversible, that is, there are other binding mechanisms along with physical adsorption. We consider oxidative binding to be the most probable mechanism of atrazine incorporation into the soil organic matter. The possibility of such binding in the presence of oxidoreductases has been shown earlier for a number of xenobiotics [7, 8]. The hypothesis is also supported by the fact that another possible way of atrazine irreversible withdrawal from the media—that is, atrazine decomposition—was discarded, since no atrazine metabolites were detected in the experiments. Therefore, the data obtained shows the possibility of irreversible atrazine binding to different kinds of soil with the participation of laccase as catalyst.

As follows from the experimental data, *Coriolus hirsutus* laccase introduction aids in atrazine binding to soil. In the presence of laccase, the binding presumably occurs by oxidative binding mechanism, evidenced by adsorption and desorption isotherms' characteristic shape, and by increase in atrazine, bound irreversibly. Thus, interaction between atrazine and soil in the presence of laccase results in irreversible binding of the herbicide. Whereas only the so-called "free," or unbound atrazine displays toxicity, the conclusion is that laccase presence in soil contributes to atrazine detoxication in the environ. The data obtained can be useful for designing a biotechnological approach to the detoxication of soils, contaminated by atrazine and other simtriazine herbicides.

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