

## Formation of Bound Residues of 8-Hydroxybentazon by Oxidoreductive Catalysts in Soil

JONG-SOO KIM, JONG-WOO PARK, SUNG-EUN LEE, AND JANG-EOK KIM\*

Department of Agricultural Chemistry, Kyungpook National University, 1370 Sankyuck-Dong, Buk-Gu, Daegu 702-701, Korea

This study was performed to determine which oxidoreductive catalysts were most efficient in catalyzing the binding of 8-hydroxybentazon to soil humic substances. 8-Hydroxybentazon was completely transformed by an oxidoreductive enzyme, laccase of *Myceliophthora thermophila*, at pH 3.0–7.0 within 30 min. When abiotic catalysts, manganese(IV), iron(III), and aluminum oxides were used in the same pH range, 8-hydroxybentazon was completely transformed only by manganese(IV) oxide ( $\delta$ -MnO<sub>2</sub>), but a relatively small amount of 8-hydroxybentazon was transformed by iron(III) oxide and aluminum oxide. The adsorption of 8-hydroxybentazon in the soil showed an H-type and coincided well with the Langmuir isotherm. To better understand the factors involved in the rapid and strong binding of 8-hydroxybentazon with soil humic substances, 8-hydroxybentazon transformation by oxidoreductive catalysts was studied in various soil conditions: air-dried, preincubated, sterilized, and iron(III) oxide and manganese(IV) oxide free. 8-Hydroxybentazon was completely transformed within 24 h in the decreasing order of preincubated, air-dried, and sterilized soils. However, little transformation was observed in the iron(III) oxide and manganese(IV) oxide free soils. These results suggest that the major catalyst responsible for the rapid and strong binding of 8-hydroxybentazon to soil humic substances is a metal oxide, manganese(IV) oxide, not a soil oxidoreductive enzyme.

**KEYWORDS:** Bentazon; hydroxybentazon; oxidoreductive catalyst; laccase; metal oxide; oxidative coupling

### INTRODUCTION

To predict potential hazards to the soil environment by pesticides and other xenobiotics, studies on their behavior are very important. The adsorption process is an important mode of interaction between the soil and pesticides and is controlled by physicochemical properties, which include molecular size and shape, acidity or basicity, polarity and charge, water solubility, lipophilicity of the pesticide, and properties of the soil (1).

After the pesticides enter the soil, various interactions between the soil solid phase and pesticides occur. The formation of covalent bonds between pesticides and/or their metabolites, which structurally resemble phenolic and aniline compounds, and soil components is often mediated by biotic or abiotic catalysts and results in stable and mostly irreversible incorporation into soil organic matter (2). It has been considered that covalent bonds of organic contaminants to soil components are representative bound residues of pesticide and/or their metabolites and detoxification mechanism of hazardous chemicals. The definition of bound residue suggested by Führ et al. (3) and accepted by the International Union of Pure and Applied Chemistry (IUPAC) is "Bound residue is a compound in soil, plants or animals which persists in the matrix in the form of

the parent or its metabolites after extraction. The extraction method must not substantially change the compounds themselves or the structure of the matrix".

The herbicide bentazon (3-isopropyl-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide) has relatively high water solubility, is stable to hydrolysis, and is nonvolatile (4). It has been investigated whether the hydroxylation of bentazon on the phenyl ring in the 6- or 8-position (also products of bentazon metabolism in plants) is the initial step of bentazon degradation (5). These metabolites have not been found in soil after the application of bentazon due to a more rapid disappearance than formation (6). The major factor of hydroxybentazon disappearance is the transformation of hydroxybentazon by biotic or abiotic oxidoreductive catalysts. Then, the transformed chemicals were covalently bound to the soil organic matter (7).

Our studies were performed to elucidate which oxidoreductive catalysts are most influential in the transformation of hydroxybentazon. In addition, we want to explain the important role of metal oxides in the formation of bound residue of various phenolic compounds in the soil environment.

### MATERIALS AND METHODS

**Chemicals.** The herbicide bentazon and its metabolite 8-hydroxybentazon were obtained from BASF. Catechol was purchased from Aldrich Chemical Co. Guaiacol and salicylic acid were purchased from Sigma Chemical Co.

\* Corresponding author (telephone 82-53-950-5720; fax 82-53-953-7233; e-mail jekim@knu.ac.kr).

**Oxidoreductive Catalysts.** The laccase isolated from *Myceliophthora thermophila* was obtained from Novo Nordisk. One unit of laccase activity was defined as the amount of enzyme that caused changes in optical density of 1.0 per minute at 468 nm of the substrate 2,6-dimethoxyphenol. Birnessite ( $\delta$ -MnO<sub>2</sub>) was prepared according to the method of McKenzie (8). Iron(III) oxide (Fe<sub>2</sub>O<sub>3</sub>) and aluminum oxide (Al<sub>2</sub>O<sub>3</sub>) were purchased from Hayashi Chemical Co.

**Preparation of Soils.** Soil from the upland field of the Agricultural Experiment Station at Kyungpook National University (Daegu, Korea) was used in this study. Soil was collected from the top 0–15 cm, air-dried, passed through a 2.0 mm sieve, and stored below 4 °C before use. The characteristics of this soil are shown as the content of organic carbon (1.0%), pH [H<sub>2</sub>O (1:5)], 7.3, CEC (13.2 cmol<sup>+</sup>/kg of air-dried soil), and soil texture (light clay). To get the organic matter removed soil, it was oxidized with 6% hydrogen peroxide and washed with methanol and distilled water several times. The sterilized soil was prepared by autoclaving at 121 °C for 30 min three times during 24 h. To activate soil microorganisms, the soil was preincubated for 14 days at a moisture level of 50% of the maximum water holding capacity in an incubator at 28 °C. The soil was extracted with sodium dithionite–citrate solution to remove selectively iron(III) oxide and manganese(IV) oxide according to the Holmgren method (9).

**Transformation Reactions.** 8-Hydroxybentazon (0.1 mM) was dissolved in a citrate–phosphate buffer at different pH values, and 10 mL of it was incubated for laccase (6.6 unit/mL) or metal oxides (1 mg/mL). The incubation without enzyme or metal oxides served as a control for estimating the change in the transformation of 8-hydroxybentazon. After 24 h of incubation with metal oxides and 30 min with laccase, then the reaction mixture was filtered through a 0.45  $\mu$ m nylon membrane. Finally, the filtrate was analyzed using high-performance liquid chromatography (HPLC). Three hundred milligrams of air-dried, preincubated, sterilized, and iron(III) oxide and manganese(IV) oxide free soils was incubated with 5 mL of 8-hydroxybentazon (0.1 mM) solution in a 25 mL centrifuge tube with a Teflon-lined cap to estimate the catalytic effects on the transformation of 8-hydroxybentazon at specific time intervals. The soil slurry was centrifuged at 15000g for 10 min. Two milliliters of the supernatant was taken and filtered through a 0.45  $\mu$ m nylon filter, and then the filtrate was analyzed using HPLC.

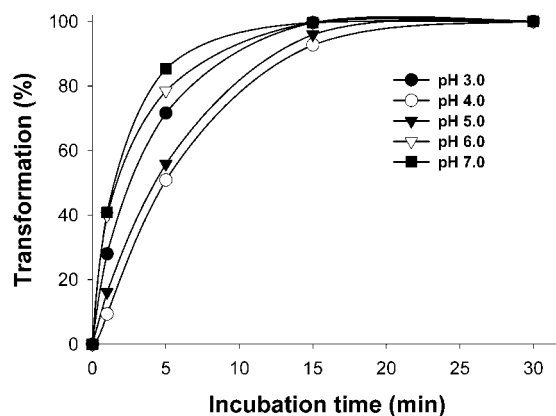
**Extraction of 8-Hydroxybentazon.** 8-Hydroxybentazon was added to air-dried soil at a rate of 10 mmol/kg of air-dried soil and placed in a 25 mL centrifuge tube with a Teflon-lined cap. One gram of soil sample was incubated at 28 °C for 24 h and kept in darkness until extraction. 8-Hydroxybentazon was extracted with 5 mL of various solvents [methanol, 0.5 M KH<sub>2</sub>PO<sub>4</sub>, methanol/KH<sub>2</sub>PO<sub>4</sub> (5:5, v/v), and acidified methanol] and methods (vigorous shaking or microwave). A ProLabo microwave equipment (Sovwave 100) was used. The soil samples were inserted into a quartz extraction cell. Microwave extraction was performed in 5 mL of previous mentioned solvents under 120 W of irradiation power for 20 min. The soil slurry was centrifuged at 15000g for 10 min. Two milliliters of the supernatant was taken and filtered through a 0.45  $\mu$ m nylon filter, and then the filtrate was analyzed using HPLC.

**Adsorption of 8-Hydroxybentazon.** Amounts of 300 mg of natural (air-dried) or oxidized soil were placed in a 25 mL centrifuge tube with a Teflon-lined cap. Five milliliters of an aqueous solution of 8-hydroxybentazon at concentrations of 0.01, 0.02, 0.1, 0.2, 0.5, and 1 mM was added to each tube. The tube containing soils and the aqueous solutions was shaken for 24 h using a mechanical shaker at room temperature. After shaking, the suspension was centrifuged at 15000g for 10 min. Two milliliters of the supernatant was taken and filtered through a 0.45  $\mu$ m nylon filter, and then the filtrate was analyzed using HPLC. The adsorption experiment data were examined using the Langmuir and Freundlich equations. The Langmuir and Freundlich equations can be described as follows:

$$X = X_m K C_{eq} / (1 + K C_{eq}) \quad \text{Langmuir equation}$$

$$X = K C_{eq}^{1/n} \quad \text{Freundlich equation}$$

$X$  is the amount of pesticide adsorbed per unit weight of the soil (mg/kg),  $X_m$  is the maximum amount of pesticide adsorbed (mg/kg),  $C_{eq}$  is



**Figure 1.** Time course of 8-hydroxybentazon transformation by the laccase of *M. thermophila* as a function of pH.

**Table 1.** Transformation of 8-Hydroxybentazon after 24 h of Incubation with Various Metal Oxides as a Function of pH

metal oxide	8-hydroxybentazon transformed at pH			
	3.0	5.0	7.0	7.8
Al <sub>2</sub> O <sub>3</sub>	4.1	3.9	0	3.2
Fe <sub>2</sub> O <sub>3</sub>	18.1	9.6	4.9	9.5
MnO <sub>2</sub> <sup>a</sup>	100	100	100	100

<sup>a</sup> Birnessite.

the equilibrium concentration of the pesticide (mg/L),  $K$  is an affinity parameter, and  $1/n$  is an empirical parameter.

**High-Performance Liquid Chromatography.** 8-Hydroxybentazon was quantified with a Shimadzu LC-10A HPLC system using a 4.0 mm  $\times$  15 cm reversed phase column (Inertsil phenyl-3, 5  $\mu$ m, MetaChem) with UV detection at 254 nm. The mobile phase set at a flow rate of 1.0 mL/min was a mixture of an aqueous component, A (1% acetic acid in Milli-Q water), and methanol, B ([A]/[B] = 45:55, v/v).

## RESULTS

To understand the transformation of 8-hydroxybentazon by *M. thermophila* laccase, it was incubated with laccase at different incubation times and pH ranges (Figure 1). In a time course experiment, transformation of 8-hydroxybentazon by laccase was 71.6, 55.8, and 85.4% after 5 min at pH 3.0, 5.0, and 7.0, respectively. However, 8-hydroxybentazon was completely transformed by laccase within 30 min regardless of the pH range. It was observed that the color of the reaction mixture turned yellow under the acidic pH conditions and dark brown under the neutral pH conditions.

Table 1 shows the transformation of 8-hydroxybentazon by aluminum, iron(III), and manganese(IV) oxides (birnessite) as a function of pH. In the case of manganese(IV) oxide the transformation of 8-hydroxybentazon was 100% in all pH ranges. The transformation of 8-hydroxybentazon by iron(III) oxide showed 18% transformation at pH 3.0, whereas <10% was transformed at other pH values. The catalytic effect of aluminum oxide on the transformation of 8-hydroxybentazon was much lower, <5%.

The recovery studies of 8-hydroxybentazon in soil were conducted. After 24 h of aging, 8-hydroxybentazon was not extracted with various solvents such as methanol, 0.5 M KH<sub>2</sub>PO<sub>4</sub>, methanol/KH<sub>2</sub>PO<sub>4</sub> (5:5, v/v), and acidified methanol. Extraction methods included shaking and microwave extraction. To observe the adsorption characteristics of 8-hydroxybentazon, an adsorption experiment was conducted with oxidized and

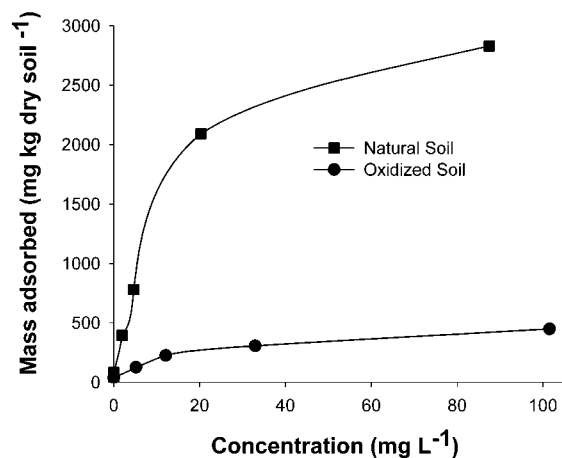


Figure 2. Adsorption isotherms of 8-hydroxybentazon on oxidized and natural soils.

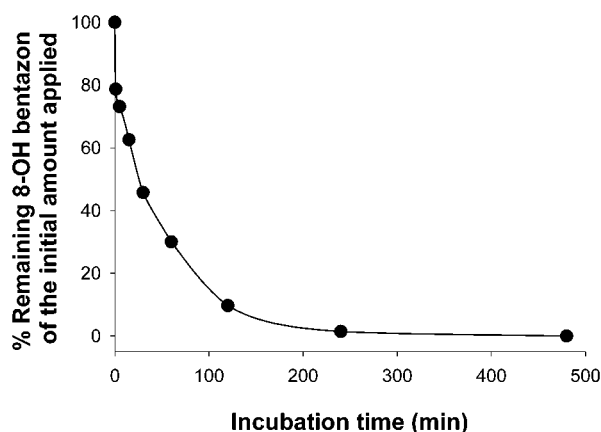


Figure 3. Time course of 8-hydroxybentazon transformation by the Bok-hyen soil.

Table 2. Langmuir and Freundlich Constants for the Adsorption of 8-Hydroxybentazon on Oxidized and Natural Soils

soil	Langmuir equation			Freundlich equation		
	$X_m$	$K$	$r^2$	$K$	$1/n$	$r^2$
oxidized soil	526	0.063	0.99	72	0.52	0.94
natural soil	3,448	0.055	0.99	316	0.41	0.97

nonoxidized soil (air-dried soil). The adsorption isotherm of 8-hydroxybentazon coincides with the H-type (Figure 2). As shown in Table 2, the relationship between the concentrations of 8-hydroxybentazon in aqueous solution and soils at equilibrium time theoretically coincided well with the Langmuir equation ( $R^2 = 0.9943$ ). The maximum adsorption amounts of pesticide ( $X_m$ ) from the Langmuir equation were 526 and 3448 in oxidized and nonoxidized soils, respectively.

The complete disappearance of 8-hydroxybentazon in soil took place after 8 h of incubation (Figure 3). To elucidate the reason for such a fast transformation of 8-hydroxybentazon in soil, the soil was prepared differently: sterilized soil, preincubated soil, iron(III) oxide and manganese(IV) oxide free soil, and air-dried soil. Figure 4 shows that the transformation of 8-hydroxybentazon was completed after 24 h in the sterilized soil, although the transformation rate was slower than that of the preincubated soil. However, traces of 8-hydroxybentazon transformations were observed in the iron(III) oxide and manganese(IV) oxide free soil during 24 h of incubation.

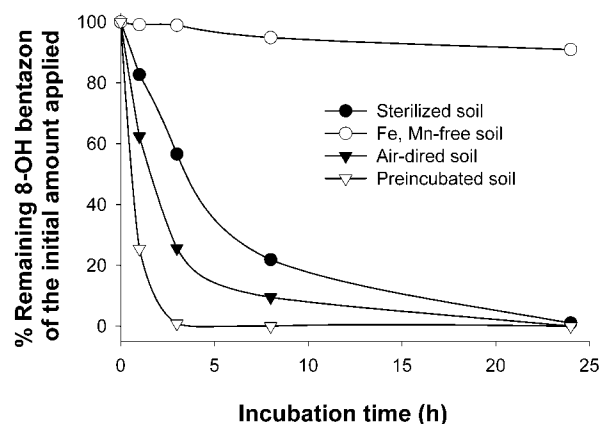


Figure 4. Transformation of 8-hydroxybentazon by differently conditioned Bok-hyen soils.

## DISCUSSION

The catalysts involved in the oxidation of naturally occurring phenolic compounds are oxidoreductive enzymes and metal oxides in soil (10, 11). Laccases (EC 1.10.3.2) are multi-copper-containing enzymes that catalyze the oxidation of phenols and aromatic amines coupled with a reduction of molecular oxygen (12). These enzymes are involved in the depolymerization of lignin, which results in a variety of phenols. In addition, these compounds are utilized as nutrients for microorganisms or repolymerized to humic materials by laccase (13).

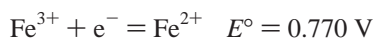
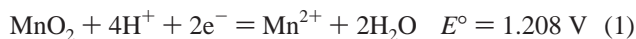
Berry and Boyd (14) showed that the activity of horseradish peroxidase [HRP; a phenol peroxidase (EC 1.11.1.7)] on phenolic compounds is highly dependent on the properties of functional groups in phenolic ring. It is well-known that electron-donating groups (hydroxy, methoxy, and amino) are attached with phenol rings. These phenolic compounds are more easily oxidized than phenol. On the other hand, phenolic compounds with attached electron-withdrawing groups (nitro and carboxylic) are unfavorable in terms of their oxidation reactions (15). The formation of radical species in a hydroxyl group is the rate-determining step for the transformation of phenolic compounds by oxidoreductive enzymes. Formation of these phenolic radicals can be stabilized by electron-donating groups. However, the electron-deficient transition state was highly unstable due to the electron-withdrawing group in phenol.

In the study for our transformation rate, it was shown that catechol (hydroxyl group) disappeared quickly and guaiacol (methoxyl group) was also rapidly transformed. However, salicylic acid (carboxylic group) was not transformed at any pH condition by the purified laccase from *M. thermophila* (data not shown). The laccase from *Polyporus pinsitus* also showed 100% in the 6- and 8-hydroxybentazon transformations (16). These results indicate that the major concern of phenolic compound oxidation mediated by laccase is not substrate specificity but chemical properties. Our study indicates that the oxidation of 8-hydroxybentazon yields a stable radical due to the electron-releasing capacity of the bulky side chain in bentazon, so we can predict that 8-hydroxybentazon would be easily transformed by laccase in a soil environment.

In addition, the transformation of phenolic compounds was mediated by various metal oxides in the soil environment (17, 18). Manganese(IV) oxide was a very effective catalyst with respect to the transformation of 8-hydroxybentazon in all pH ranges. However, 8-hydroxybentazon was only slightly transformed by iron(III) oxide at pH 3.0. At other pH values, little 8-hydroxybentazon transformation was observed (Table 1). The mechanism of transformation can be described by the reduction



of Fe(III) and Mn(IV) coupled with the oxidation of 8-hydroxybentazon. The catalytic effects of manganese(IV) oxide are more powerful in the transformation of 8-hydroxybentazon than iron(III) oxide due to the difference in the standard reduction potentials as indicated by the  $E^\circ$  value in eq 1.



When a pH value is below the zero point charge (ZPC) of metal oxides, negatively charged compounds can be adsorbed by a charge transfer with a metal oxide (19). However, the catalytic effects of aluminum oxide on the transformation of 8-hydroxybentazon were very slight even under very low pH conditions. These results indicate that the interaction between 8-hydroxybentazon and metal oxides by charge transfer had a minor effect on the disappearance of 8-hydroxybentazon.

As bentazon has a high water solubility and small  $\text{p}K_a$  (3.3) it does not readily adsorb on soil components except in very acidic soils or with high anion exchange capacity. In our pre-experiments, the adsorbed amounts of bentazon decreased with increasing soil pH (data not shown). However, mobility studies of bentazon by field lysimeter experiments have shown minor leaching (20, 21). Wagner et al. (22) showed that after 48 days of treatment of a soil with bentazon, ~60% was detected as nonextractable form, <20% was detected as mineralization, and only 10% was detected as the parent compound. Kim et al. (23) also reported that after 56 days of incubation with [ $^{14}\text{C}$ ]bentazon, the bound residue of bentazon was detected at ~70% of initial application, and an almost bound residue fraction was identified in soil organic matter. Kim et al. (24) suggested the following possible mechanism of bound bentazon with soil organic matter: Bentazon can be transformed by oxidative coupling in the presence of humic substances in soil, and there can be identified reaction products of bentazon with catechol by laccase. Several researchers showed that the formation of 6- or 8-hydroxybentazon, a metabolite of bentazon by microorganisms in an aerobic condition, could be explained as another mechanism of bound residue of bentazon in soil (6).

The adsorption isotherm of 8-hydroxybentazon to soils was adequately described as H-type (Figure 2). The H-type isotherm, suggested by McBride (25), reflects a very strong adsorbate-adsorbent interaction through a short-range electrostatic or covalent bond (chemisorption). For example, bipyridylum herbicides, paraquat and diquat, having divalent positive charges, were very strongly bound by cation exchange on smectites (26). However, because of the anion character, 8-hydroxybentazon could not be readily adsorbed by ionic interaction with soil components. Lerch et al. (27) reported that adsorption of hydroxyatrazine, a metabolite of atrazine, to soils occurs by two simultaneous mechanisms: hydrophobic interaction and cation exchange.

However, a significant portion of bound hydroxyatrazine residue could be extracted with an effective extractant, 0.5 M  $\text{KH}_2\text{PO}_4$  (pH 7.5)/ $\text{CH}_3\text{CN}$  (3:1 v/v). The differences in the binding mechanisms of hydroxyatrazine and hydroxybentazon might be due to the chemical properties of the two pesticide metabolites. First, the triazine ring (hydroxyatrazine) has a higher oxidation state than the benzene ring (hydroxybentazon), so the oxidation reaction is relatively more favorable in hydroxybentazon than in hydroxyatrazine. Second, hydroxyatrazine (atrazine  $\text{p}K_a = 1.7$ , weak base) can be readily and strongly adsorbed into soil components by a cation exchange and hydrophobic interaction. Therefore, strongly adsorbed hy-

droxyatrazine is not readily attacked by oxidative catalysts. However, a large portion of hydroxybentazon (bentazon  $\text{p}K_a = 3.3$ , weak acid) is liable to oxidoreductive catalysts.

Adsorption of 8-hydroxybentazon to soil is adequately described by the Langmuir equation ( $R^2 > 0.9943$ ). When the soil organic matter was removed, the maximum amount adsorbed ( $X_m$ ) significantly decreased from 3448 to 526  $\mu\text{g/g}$  of soil (Table 2). These results indicate that soil organic matter is very closely associated with adsorption of 8-hydroxybentazon in soil. We do not know exactly whether 8-hydroxybentazon was coupling with soil organic matter or self-polymerized. However, Lehmann et al. (27) showed the extractability of ferulic acid by two methods: HPLC and  $^{14}\text{C}$  tracing. From the results, ferulic acid recovery by HPLC was approximately half of that by the  $^{14}\text{C}$  technique. This indicates that the extractable  $^{14}\text{C}$  had been incorporated into dimers or trimers of ferulic acid following the oxidative coupling. Judging from Lehmann's results, we think that 8-hydroxybentazon can not only be adsorbed to soil organic components but also form dimers or trimers through cross-coupling in the adsorption experiment of our study.

In our study, we determined that 8-hydroxybentazon could be transformed by biotic (laccase) and abiotic [metal oxide, especially manganese(IV) oxide] oxidoreductive catalysts. Although sterilized soil reduces the transformation rate of 8-hydroxybentazon compared to preincubated soil, it still completely transformed within 24 h. Nonetheless, in iron(III) oxide and manganese(IV) oxide free soils, only a little 8-hydroxybentazon was transformed. Kanuber et al. (29) showed that 8-hydroxybentazon incubated for 32 days was not extracted with methanol from natural soils. The same trends are also shown even in sterilized soil. Lehmann et al. (27) showed that the recovery of naturally occurring phenols significantly increased in iron(III) oxide and manganese(IV) oxide free soils. The increase in recovery could be explained by the fact that phenolic compounds were not transformed by the iron(III) oxide and manganese(IV) oxide.

In conclusion, even if 8-hydroxybentazon can be transformed by both laccase and metal oxides, the major catalysts responsible for the rapid and strong binding to soil humic substances of 8-hydroxybentazon are metal oxides, especially manganese(IV) oxide, not soil oxidoreductive enzymes in the soil environment.

For a more detailed study on the bound residue of bentazon, it is necessary to know the hydroxylation rate of bentazon and the method of quantitative analysis of hydroxybentazon in a soil matrix. Our further study will characterize the binding mechanism of hydroxybentazon to humic substances by metal oxides and laccase.

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