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Research Report

Laccase-induced decontamination and humification mechanisms of estrogen in water–crop matrices

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Abstract

Enzymatic humification plays a crucial biogeochemical role in eliminating steroidal estrogens and expanding organic carbon stocks. Estrogenic contaminants in agroecosystems can be taken up and acropetally translocated by crops, but the roles of laccase-triggered rhizospheric humification (L-TRH) in pollutant dissipation and plant uptake remain poorly understood. In this study, the laccase-induced decontamination and humification mechanisms of 17β-estradiol (E2) in water–crop media were investigated by performing greenhouse pot experiments with maize seedlings (*Zea mays* L.). The results demonstrated that L-TRH effectively dissipated E2 in the rhizosphere solution and achieved the kinetic constants of E2 dissipation at 10 and 50 μM by 8.05 and 2.75 times as much as the treatments without laccase addition, respectively. The copolymerization of E2 and root exudates (i.e. phenols and amino acids) consolidated by L-TRH produced a larger amount of humified precipitates with the richly functional carbon architectures. The growth parameters and photosynthetic pigment levels of maize seedlings were greatly impeded after a 120-h exposure to 50 μM E2, but L-TRH motivated the detoxication process and thus mitigated the phytotoxicity and bioavailability of E2. The tested E2 contents in the maize tissues initially increased sharply with the cultivation time but decreased steadily. Compared with the treatment without laccase addition, the uptake and accumulation of E2 in the maize tissues were obviously diminished by L-TRH. E2 oligomers such as dimer, trimer, and tetramer recognized in the rhizosphere solution were also detected in the root tissues but not in the shoots, demonstrating that the acropetal translocation of E2 oligomers was interrupted. These results highlight a promising strategy for decontaminating estrogenic pollutants, boosting rhizospheric humification, and realizing low-carbon emissions, which would be beneficial for agroenvironmental bioremediation and sustainability.

Keywords: steroidal estrogens, laccase, polymerization, humification products, maize uptake

Significance Statement

Enzymatic polymerization and humification are widely applied for transforming estrogenic contaminants in environments. Yet, the effects of laccase-triggered rhizospheric humification (L-TRH) on contaminant dissipation and crop uptake remain largely unexplored. In this study, maize seedlings are selected to investigate the decontamination and humification of 17β-estradiol (E2) in L-TRH by performing a greenhouse pot test. L-TRH allows the maize seedlings to extenuate the risks of E2 contamination by modulating polymerization between E2 and certain root exudates in the rhizosphere microenvironments, resulting in the generation of a large number of humified products and a reduction in carbon emissions. Our results unveil the decontamination and humification mechanisms of E2 in water–maize media by L-TRH and imply the implication potential of L-TRH in ensuring agrifood safety and human health.

Introduction

17β-Estradiol (E2), a representative natural steroidal estrogen, has been classified as group 1 carcinogen by the World Health Organization because of its ubiquitous presence in ecosystems and strong endocrine-disrupting effects (1, 2). Previous studies have disclosed that E2 at <3.67 nM can negatively affect organism (male fish) growth, reproduction, genetics, and metabolism, leading to dysfunction of the endocrine system (3, 4). In the past three decades, the annual growth rate of global E2 emissions was about 10%, with >90% from animal waste (5). Up to now, E2 has been

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found in agricultural fields around the world as it is continuously released into agroecosystems by stormwater runoff, sewage irrigation, and manure return (6). The prolonged exposure of plants to E2 is attracting attention for agrifood safety in the uptake, accumulation, and acropetal translocation of E2 by crops (7, 8). Therefore, the remediation of E2-polluted water, soil, and crop matrices is essential to evade ecological risks and ensure public health.

Remediation of E2-contaminated media is usually achieved by physicochemical adsorption, advanced oxidation, and microbial metabolism (9, 10). Although these techniques can govern E2 and eventually reduce its estrogenic activity to a certain extent, the long period and low efficiency, or the high cost and complicated operation, are the key drawbacks to restrict their practical applications (11). Fortunately, enzyme catalysis has generated considerable interest from the research community in E2 decontamination and humification because of its simplicity, operability, low specificity, and environmental friendliness (12). Laccase, a green and accessible multicopper oxidase (active center, T1-Cu, T2-Cu, and a pair of T3-Cu), is universally produced by a wide range of fungi, bacteria, or higher plants. Compared with other bioenzymes (i.e. tyrosinase and peroxidase), laccase can fully accomplish elimination and humification of E2 without H_2O_2 in complex environmental media by forming various polymeric products (13). Given that H_2O is the only by-product, laccase is beneficial for its in situ bioremediation, especially for phenolic pollutants (14). Nonetheless, information about the role of laccase-triggered rhizospheric humification (L-TRH) in treating E2-contaminated water–crop media is very limited.

Therefore, it is highly desirable to explore the process of polymerization between E2 and root exudates, as well as the uptake and acropetal translocation of E2 by crops involved in L-TRH. Root exudates, such as phenolic compounds, amino acids, and sugars, have been reported to account for 2–14% of net primary production (15) and thus can play a vital role in modulating rhizosphere microenvironment, laccase activity, E2 migration, and plant metabolism. So far, information about the impacts of root exudates and even simulants on laccase-induced E2 dissipation is still lacking. Our previous studies found that laccase effectively elicited the polymerization of E2 and small phenols to form various humified precipitates (13). In particular, these precipitates could be considered biostimulants that had positive effects on crop growth and development (16, 17). Unfortunately, no information is available on the mechanisms of E2 decontamination and humification in L-TRH by greenhouse pot experiments. Consequently, a deeper understanding of L-TRH remains a great challenge for the bioremediation and humification of E2 in water–crop matrices.

In this work, maize seedlings (*Zea mays* L.) were selected as test crops and exposed to two concentrations of E2 (10 and 50 μM) in hydroponic greenhouses. The objectives of this work were to: (i) investigate the effect of L-TRH on the dissipation kinetics of E2 in the rhizosphere solution of maize seedlings; (ii) identify the enzymatic products of E2 and characterize the physicochemical properties of the humified precipitates; (iii) assess the phytotoxicity caused by E2 and its humified products to maize seedlings; (iv) explore the uptake and acropetal translocation of E2 in the maize tissues during L-TRH; and (v) elucidate the decontamination and humification mechanisms of L-TRH in E2-polluted water–maize systems. As a result, our work would provide a multifunctional method for controlling steroidal estrogens, sequestrating organic carbon, and producing green crops in agroecosystems, which would help achieve agroenvironmental bioremediation and sustainability.

Results and discussion

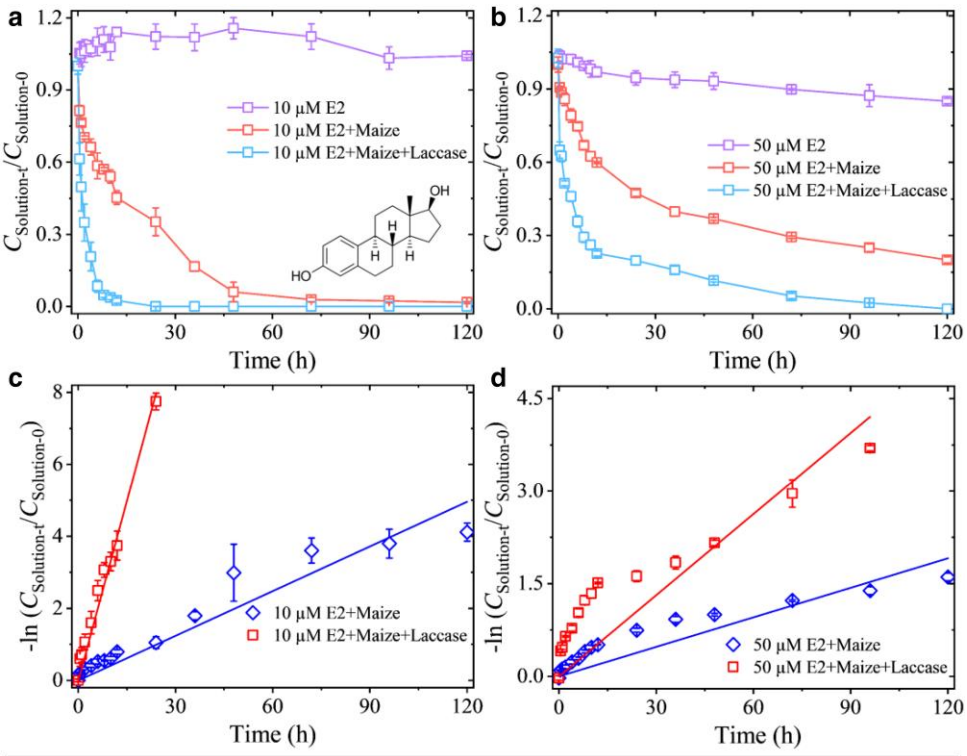
E2 dissipation kinetics in the rhizosphere solution by L-TRH

L-TRH played a crucial role in the decontamination process of E2. As shown in Fig. 1a and b, compared with the treatments without laccase addition, the dissipated amounts of E2 in the rhizosphere solution for 10 and 50 μM exposures within 12 h by L-TRH increased from 54.7 to 97.5% and 40.1 to 77.3%, respectively. E2 of 100% was dissipated in solution by L-TRH after 24 and 120 h of cultivation at two concentrations, respectively. Such a high dissipation efficiency was beneficial for mitigating the uptake and acropetal translocation of E2 by maize seedlings. A pseudo-first-order kinetic model was applied to understand the dissipation process of E2 in the rhizosphere solution (Fig. 1c and d). The values of the rate constant (k) were determined by using linear regression ($R^2 = 0.8729$–0.9883), and then the half-life values ($t_{1/2}$) were calculated. For the treatments without laccase addition, the $t_{1/2}$ values of E2 dissipation in solution increased from 16.8 to 43.6 h when exposure to E2 increased from 10 to 50 μM. In particular, the $t_{1/2}$ values of E2 dissipation by L-TRH observably decreased (2.1 and 25.8 h) at two concentrations. Coexisting E2 and root exudates kept the efficiency of targeting dissipation in L-TRH. To further explore the process of E2 dissipation in the rhizosphere solution by L-TRH, three key routes to dissipate E2 were reasonably proposed.

In route I, maize roots directly released a variety of laccase isozymes to assist E2 oxidation and conversion in L-TRH (18). As displayed in Fig. S1, the activity of laccase in the rhizosphere solution presented an upward trend. After a 120-h exposure to 10 and 50 μM E2, the laccase activities secreted from the roots into the solution amounted to 0.02 and 0.03 U mL^{-1}, respectively. This might be attributed to the induced expression of the laccase gene when the primary roots of the maize were subjected to E2 stress (19). In 10- and 50-μM E2 treatment groups with laccase addition (0.13 U mL^{-1}), the laccase activities in solution after 120-h cultivation increased to amounts of 0.22 and 0.24 U mL^{-1}, respectively. Presumably, the addition of external laccase and E2 exposure collectively stimulated the production and secretion of laccase by the maize roots and/or rhizosphere microorganisms. Thus, the laccase released from the maize roots and/or rhizosphere microorganisms was responsible for aiding the oxidation and humification of E2.

In route II, L-TRH expedited E2 oxidation and polymerization in the rhizosphere solution, thereby enhancing E2 dissipation. The roots of maize exuded large amounts of phenols, amino acids, and sugars into the solution (Fig. S2), which might be involved in E2 conversion and organic carbon sequestration during L-TRH (20). The humification process of E2 and certain root exudates in the rhizosphere solution was consolidated by adding laccase, which was capable of producing highly complex E2 polymers with O-containing hydrophilic groups (13, 21). As mentioned previously, L-TRH not only eliminated E2 (Fig. 1) but also reduced the contents of root exudates (Fig. S2). These results imply that the polymerization and humification of E2 and certain root exudates were boosted by L-TRH.

In route III, E2 was taken up and acropetally translocated by maize seedlings and thus accelerated its dissipation in the rhizosphere solution during L-TRH. Root uptake, translocation, and metabolism of E2 in the maize tissues had been reported previously (22). The concentration of E2 decreased rapidly in solution at the preliminary stage of exposure to maize seedlings but increased observably in the root and shoot tissues. Consequently, L-TRH was able to decontaminate E2-contaminated water–crop media,



e

Treatments	k (h^{-1})	$t_{1/2}$ (h)	R^2
10 μM E2+Maize	0.0413	16.78	0.9514
10 μM E2+Maize+Laccase	0.3324	2.09	0.9883
50 μM E2+Maize	0.0159	43.59	0.9067
50 μM E2+Maize+Laccase	0.0438	25.83	0.8729

Fig. 1. The kinetic model of E2 dissipation in the rhizosphere solution by L-TRH. a) 10 μM E2 dissipation. b) 50 μM E2 dissipation. c) Pseudo-first-order rate plots for 10 μM E2 dissipation. d) Pseudo-first-order rate plots for 50 μM E2 dissipation. e) The corresponding kinetic constants. $C_{Solution\text{-}t}$ is the initial concentration of E2 in the rhizosphere solution, and $C_{Solution\text{-}t}$ is the residual concentration of E2 in the rhizosphere solution at a given time t. Error bars represent SD values ($n = 5$).

which could be used to store organic carbon and alleviate E2 uptake by maize seedlings. Routes II and III of the above-mentioned assumptions will be confirmed below.

Intermediate products of E2 in solution by L-TRH

High-resolution mass spectrometry (HRMS) was applied to detect the main intermediate products of E2. A total of eight intermediates were tentatively recognized in solution after a 10 μM E2 exposure to maize seedlings in L-TRH (Fig. S3 and Table S1). These intermediates were also identified in the treatment without laccase addition, but none of them was detected in the blank control (the treatment without laccase and E2 addition). Dehydrogenation, hydroxylation, and polymerization of E2 were found in the rhizosphere solution. Intermediate products P1 (estrone [E1], $m/z = 269.1552$) and P2 (estriol [E3], $m/z = 287.1648$) were the typical dehydrogenation and hydroxylation products of E2, respectively. These two processes were recognized as the key steps for E2 oxidation and decomposition. The oxidoreductases released from higher plants, such as laccase and 17β-hydroxysteroid dehydrogenase, were anticipated to induce dehydrogenation and hydroxylation of E2 (22, 23). The intermediate products P3 (E2 dimer, $m/z = 541.3312$), P4 (E2 trimer, $m/z = 811.4943$), and P5 (E2 tetramer, $m/z = 1{,}081.6563$) were identified as self-polymerized products. This is because laccase evoked self-polymerization of E2 directly from the solution with O_2 supply. However, no products larger than the tetramer were detected, owing to the reduced methanol solubility with the increase in the molecular weight (MW) of polymeric products. It is noted that some small root exudates of maize, such as vanillic acid (Vaa), caffeic acid (Caa), and leucine (Leu), could also be catalytically polymerized with E2 to produce Vaa-E2 (P6, $m/z = 437.1960$), Caa-E2 (P7, $m/z = 449.1980$), and Leu-E2 (P8, $m/z = 400.2498$) codimers. As suggested in route II, the formation of self-/copolymeric products elicited by laccase-triggered one-electron oxidation was recognized as an important humification process for the decontamination and detoxification of E2.

The relative abundances of E2 key intermediates could be used as an indicator for the qualitative determination of products. In the rhizosphere solution, the abundances of E2 and its four representative intermediates (E1, E2 dimer, E2 trimer, and E2 tetramer) relative to the initial E2 area over time are shown in Fig. S4. The relative abundance of E2 decreased more rapidly in L-TRH compared with the treatment without laccase addition (Fig. S4a), which is consistent with Fig. 1a. In the treatment without laccase addition, the relative abundance of E1 increased initially and peaked in 4 h because of E2 oxidation, but decreased from the solution gradually (Fig. S4b). A similar phenomenon was also found

for the relative abundances of the E2 dimer, trimer, and tetramer within 12 h (Fig. S4c–e), implying that E1 and E2 oligomers were further converted into the unidentified intermediates. Moreover, the uptake and transport of maize roots could also cause a decrease in the relative abundances of E2 intermediate products. In particular, as the degree of polymerization increased, the relative abundances of the polymerization products decreased more slowly. This is because the formed E2 dimer was further transformed into trimer, tetramer, oligomer, and polymer by radical-regulated covalent binding (24). In L-TRH, the relative abundance values of the E2 oligomers were lower than those with the treatment without laccase addition, but the variations of their contents were much faster. These results suggest that E2 was principally converted into oligomers and polymers after adding laccase, and only a small amount of E2 was taken up by the roots of maize and then transported into the shoots.

Characteristics of the humified precipitates

The humified precipitates of E2 and root exudates generated by L-TRH were characterized by using scanning electron microscopy–energy-dispersive X-ray spectrometer (SEM–EDS), Fourier transform infrared spectroscopy (FTIR), ^{13}C-NMR, and Cs-STEM. The yield of humified precipitates after adding laccase was 3.55 times higher than that of the treatment without laccase addition. Figure S5 shows the morphologies and elemental compositions of the precipitates formed in L-TRH. Compared with the treatment without laccase addition, the humified precipitates formed after adding laccase had a rougher surface and a larger particle size. In the treatment groups without and with laccase addition, the C, O, N, and S element ratios were 53.9:15.6:30.0:0.5 and 54.0:17.2:28.1:0.7, respectively. It implies that there was a higher C/N ratio and a lower C/O ratio in L-TRH. However, the relevant conclusions need to be cautiously validated. A higher C/N ratio had a faster and more stable degree of humification, thus expediting the production of precipitates (25). The C/O ratio reflected the hydrophilicity of precipitates, and a lower C/O ratio manifested a higher content of O-containing hydrophilic groups. Furthermore, EDS elemental mappings reveal that the C, O, N, and S elements were homogeneously distributed in the humified precipitates.

The FTIR and ^{13}C-NMR spectra of the two humified precipitates are displayed in Fig. S6. The wide stretching vibration band of O–H was observed at ∼3,420 cm^{-1}, which was attributed to the large number of phenolic –OH and –COOH groups in the humified precipitates (Fig. S6a). The structures of functional groups in the humified precipitates had the most significant changes in the peak areas of 600–1,800 cm^{-1}, in which the aromatic C=C and conjugated carbonyl C=O were stretched at ∼1,650 cm^{-1}. The absorption band at about 1,520 cm^{-1} corresponded to the ribbon structures of carboxylic C–O and C–OH. In addition, the absorption peaks at 1,241 and 1,237 cm^{-1} indicated that the phenolic –OH and the aromatic part were connected by aryl ether bonds, which caused the stretching vibration of C–O–C. The absorption bands at 1,057 and 1,055 cm^{-1} were considered to be C–O stretching of the polysaccharide. Thus, the molecular skeletons of both precipitates presented the phenolic –OH, –COOH, and ether linkage, regardless of with and without laccase addition. Figure S6b reveals the main carbon components of the two humified precipitates recognized by ^{13}C-NMR spectra. The peaks at 15–45 ppm could be attributed to methylene C, which was part of the hydrocarbon skeleton. Relatively, high intense peaks at 52 and 72 ppm suggest that the skeleton could contain more O-based functionality like –OH, as previously reported (26). Peaks at 110–140 ppm clearly manifest the presence of a phenyl ring. The peaks that appeared in the region of 165–185 ppm indicate the presence of aliphatic –COOH in the humified precipitates. Compared with the treatment without laccase addition, the humified precipitates produced by adding laccase had more aromatic C and O-containing functional groups.

The microscopic distribution of the four organic carbon species, i.e. aromatic C, phenolic C, aliphatic C, and carboxyl C, in the humified precipitates was further characterized using Cs-STEM coupled with high-angle annular dark field (HAADF)-STEM imaging and electron energy loss spectroscopy (EELS) elemental mapping (27, 28). As shown in Fig. 2a, the four organic carbon signals are thinly dispersed over the humified precipitates for the treatment without laccase addition. However, the carbon signals were powerfully localized in the specific regions of the humified precipitates by adding laccase, with a higher carbon intensity (Fig. 2b). It is because L-TRH enhanced the sequestration of organic carbon in the rhizosphere solution to produce more complex and close-knit precipitates. Overall, the four organic carbon signals correlated well with each other, suggesting that they were evenly distributed within the two humified precipitates at the nanoscale, but the dominant chemical composition demonstrated a slight variation. These are consistent with the FTIR and ^{13}C-NMR results, confirming that L-TRH improved the polymerization of E2 and certain root exudates by binding of C–C and C–O, consequently producing more complex humified precipitates with the abundant O-containing components (route II).

Effect of L-TRH on crop growth and biochemical parameters

The uptake and accumulation of steroidal estrogens in crops generally resulted in oxidative damage and eventually led to inhibition of plant growth and even cell death. After a 10-day exposure of maize seedlings to E2, the growth parameters of the roots and shoots of maize were determined in different treatment groups (Fig. 3). As described in Fig. 3a, 10-μM E2 exposure mildly promoted the growth of maize seedlings, but 50-μM E2 exposure greatly restrained the maize growth and even caused yellowing of the leaves, which visually reflected the plant effect of E2. Interestingly, the effect of E2 on plant height, root length, and fresh weight (FW) was effectively reduced by L-TRH (Fig. 3b–e). For example, compared with the control samples without laccase addition, the plant height and root length of maize seedlings in L-TRH after 50-μM E2 exposure increased from 20.3 to 23.0 cm and 8.8 to 11.6 cm, respectively ($P < 0.05$); the FW of the shoots and roots increased from 0.47 to 0.61 g plant^{-1} and 0.22 to 0.30 g plant^{-1}, respectively ($P < 0.05$). These results indicate that the intermediate products of E2 formed by L-TRH had a less toxicity than their parent compounds.

Plant photosynthesis converts light energy into chemical energy stored in carbohydrates (29). To further define the effect of L-TRH on the biochemical parameters of maize seedlings, the contents of photosynthetic pigments (i.e. chlorophyll a [C_a], chlorophyll b [C_b], and chlorophyll a + b [C_{a+b}]) were determined in the maize leaves. As shown in Fig. S7, compared with the planted control, the contents of C_a, C_b, and C_{a+b} increased slightly in maize leaves after 10-μM E2 exposure for 120 h ($P > 0.05$). In particular, the contents of photosynthetic pigments under the stress of 50 μM E2 were greatly lower than that of maize treatment without E2 ($P < 0.01$), but their contents clearly increased after adding laccase ($P < 0.01$). These results are in accord with Fig. 3,

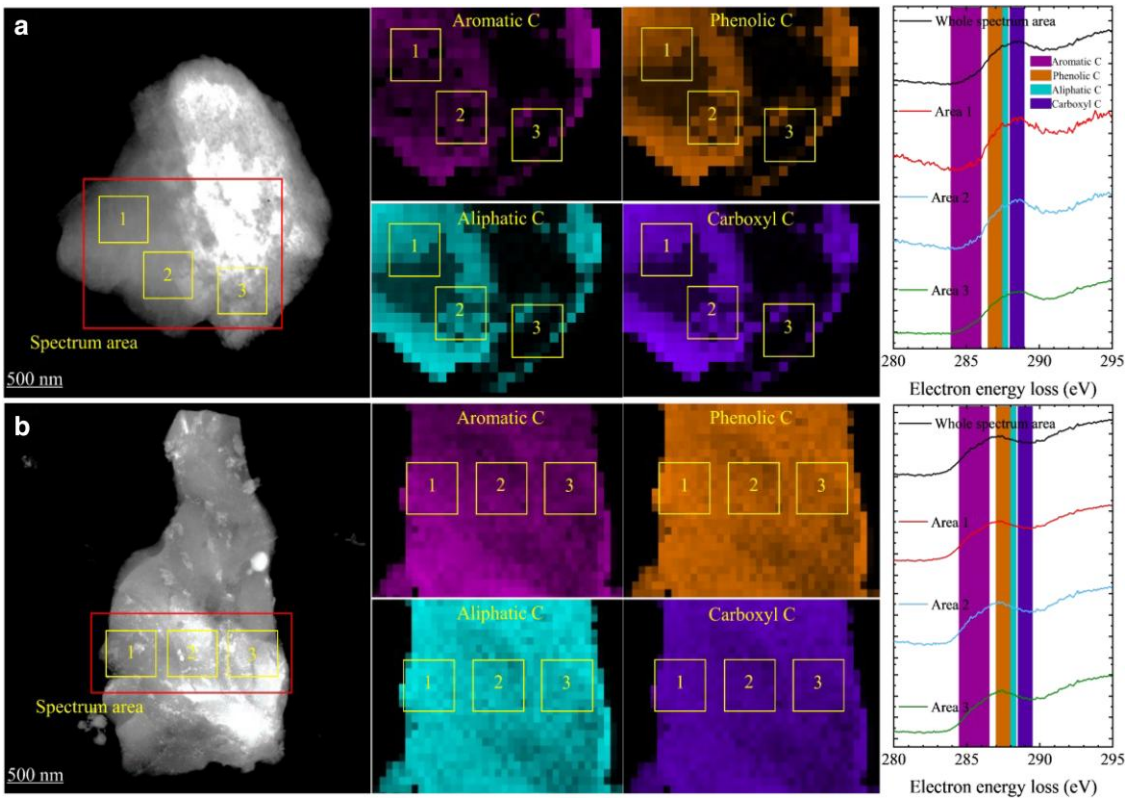


Fig. 2. HAADF images, EELS mappings, and EELS spectra of the whole and selected areas for the humified precipitates. EELS spectra were performed to obtain the relative abundances of four different carbon structures (i.e. aromatic, phenolic, aliphatic, and carboxyl C). a) The formed precipitates without laccase addition. b) The formed precipitates with laccase addition.

demonstrating that the growth and biochemical parameters of maize seedlings were closely related to the exposure dose of E2 in water–maize media. Fortunately, L-TRH effectively eliminated the negative effects of E2 on the growth and development of maize seedlings by rapidly forming polymers with low toxicity and even nontoxicity.

L-TRH reduced E2 uptake and acropetal translocation in maize tissues

E2 was rapidly taken up by maize roots and acropetally transported through transpirational pull, but L-TRH mitigated the uptake and acropetal translocation of E2 by maize seedlings. As shown in Fig. 4a and b, the concentrations of E2 in the maize tissues (C_{Root} and C_{Shoot}) increased initially during the 10-μM E2 exposure period, peaked at 14.53 and 3.11 μmol kg^{-1} (FW) for roots and shoots, respectively, but then gradually decreased to 1.73 and 0.79 μmol kg^{-1} for 120-h cultivation, respectively. Notably, the maximum concentrations of E2 in the roots and shoots by L-TRH were 9.27 and 1.73 μmol kg^{-1}, respectively. After 120-h cultivation, the concentrations of E2 in the roots and shoots by L-TRH lowered to 0.46 and 0.14 μmol kg^{-1}, respectively. It is because E2 was effectively dissipated from the rhizosphere solution by L-TRH, alleviating the root uptake potential (route III). Similarly, the concentration of E2 in the maize tissues also presented an initial increase and then a decrease trend after 50-μM E2 exposure, but the maximum concentrations of E2 in the maize tissues were much higher than those for 10-μM E2 exposure (Fig. 4c and d). Lower accumulation values imply fewer E2 present in the maize tissues and lower risk of crop contamination. The accumulated amounts of E2 in the maize tissues (A_{Root} and A_{Shoot}) were calculated and are displayed in Fig. 4e–h. The accumulation of E2 in the maize shoots was much lower than that in the roots, regardless of with and without laccase addition, indicating that the roots were the dominant sink of E2 present in maize. It is because the log K_{ow} value was 3.94 for E2 (>3.5), which could limit its acropetal translocation (8). After a 120-h exposure to 10 μM E2, the accumulated amounts of E2 in the roots and shoots with adding laccase were 0.17 and 0.06 nmol $plant^{-1}$, respectively, lower than those of the treatment without laccase addition by 74.2 and 50.0%, respectively. These results reveal that E2 accumulation was obviously limited in maize seedlings by L-TRH, only about 0.06% of the total exposure dose to E2. This value was largely correlated with the high hydrophobicity and the short $t_{1/2}$ of E2. The E2 accumulation in crops at both high and low concentrations shared a similar trend. Consequently, L-TRH alleviated the uptake and acropetal translocation of E2 in the maize tissues, and then reduced the risks associated with pollution by E2 in crops.

The key intermediates of generated from E2 conversion in the rhizosphere solution by L-TRH also were absorbed by the maize roots, and certain small products were acropetally translocated. In our work, relatively low-toxic E1, E3, and E2 oligomers were found in the maize roots during L-TRH, but only E1 and E3 were identified in the shoots (Table S2). A similar result was also observed for the treatment without laccase addition. It could be attributed to the higher MW and hydrophobicity of E2 oligomers, which were easier to interact with the organic components in the roots, such as the root lipids (30), but more difficult to transport into shoots. This observation warrants further investigations. Notably, the recognized E1 and E3 in the maize tissues also were able to be produced by plant enzyme-mediated E2 oxidation and metabolism in vivo (22). The relative abundances of E2, E1, and

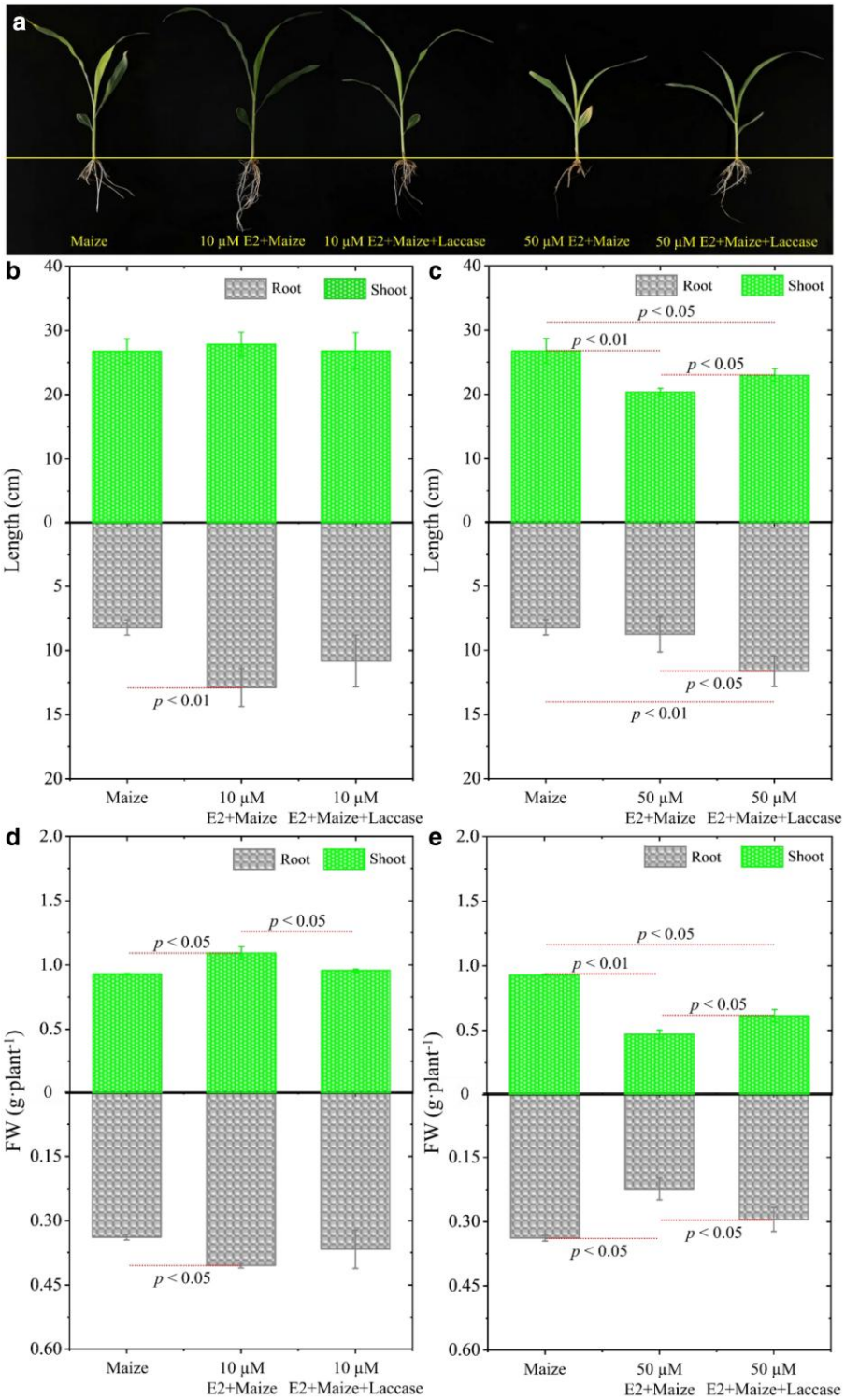


Fig. 3. The growth parameters of maize seedlings during L-TRH according to two initial concentrations of E2 exposure. a) Photographical images of maize seedlings in different treatment groups. b) The plant height and root length of maize seedlings after 10 μM E2 exposure. c) The plant height and root length of maize seedlings after 50 μM E2 exposure. d) The FW of maize seedlings after 10 μM E2 exposure. e) The FW of maize seedlings after 50 μM E2 exposure. Error bars represent SD values ($n = 5$).

E2 oligomers in the roots of maize are shown in Fig. S8. The trend of variation in E2 abundance was proven to coincide with the variation of E2 content in roots (Fig. 4). With an increase in culture time, the relative abundances of E2 dimer, trimer, and tetramer in roots increased initially but then decreased, implying that E2 oligomers might be mainly converted into glycoconjugates, nonextractable residues, and highly polar copolymers in maize roots (31). To the best of our knowledge, this was the first report about the detection of E2 oligomers in maize roots.

With the results and analyses above, the mechanisms of decontamination and humification boosted by L-TRH in E2-polluted water–maize systems are proposed and illustrated in Fig. 5. Taking

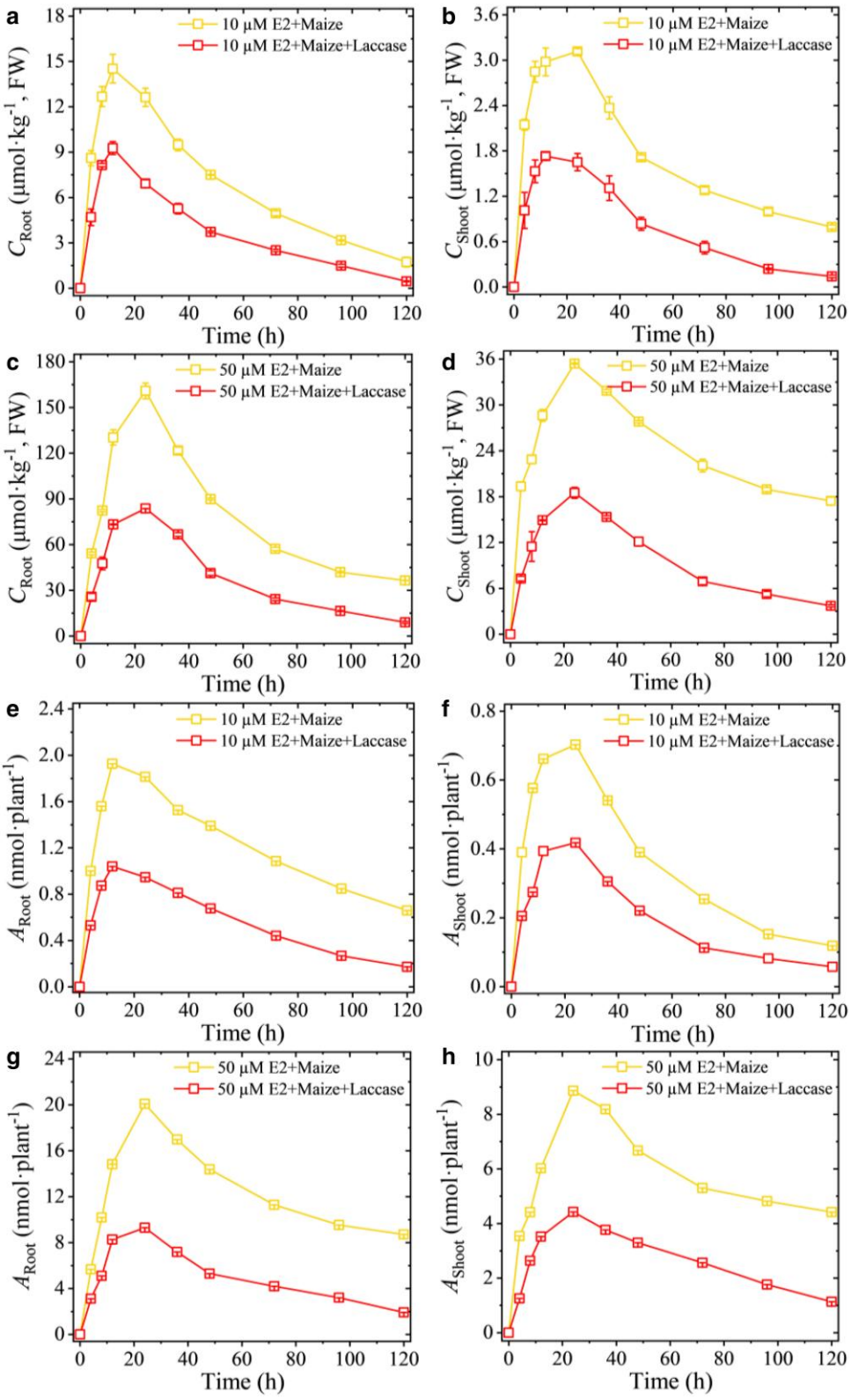


Fig. 4. Time-dependent concentration and accumulation of E2 in maize tissues after exposure to two initial spikes in culture solution. a) The concentration of E2 in maize roots during the 10 μM E2 exposure, and b) the corresponding concentration in maize shoots. c) The concentration of E2 in maize roots during the 50 μM E2 exposure, and d) the corresponding concentration in maize shoots. e) The accumulation of E2 in maize roots during the 10 μM E2 exposure, and f) the corresponding accumulation in maize shoots. g) The accumulation of E2 in maize roots during the 50 μM E2 exposure, and h) the corresponding accumulation in maize shoots. C_{Root} and C_{Shoot} (μmol·kg^{-1}, FW) are the concentrations of E2 in maize roots and shoots, respectively; A_{Root} and A_{Shoot} (nmol·plant^{-1}, FW) are the accumulations of E2 in maize roots and shoots, respectively. Error bars represent SD values (n = 5).

into account the high redox potential (400–800 mV) of laccase at the T1-Cu site, E2 and certain root exudates, such as phenols, polyphenols, and amino acids in the rhizosphere solution, were fleetly oxidized by capturing electrons and generating radicals,

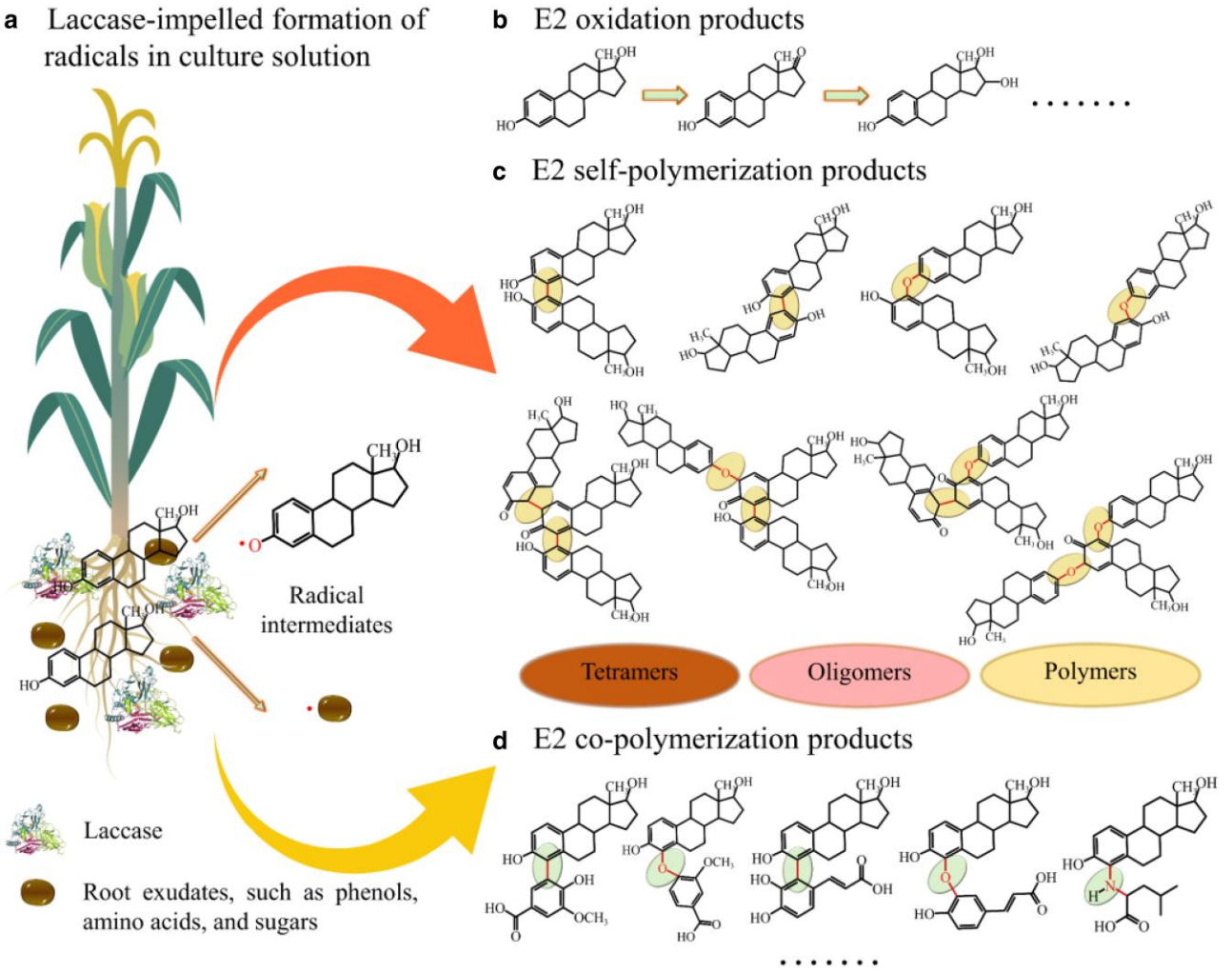


Fig. 5. The mechanisms of E2 decontamination and humification propelled by L-TRH in water-maize systems. a) Laccase-impelled formation of radicals in culture solution. b) E2 oxidation products. c) E2 self-polymerization products. d) E2 co-polymerization products.

which further underwent various oxidation and polymerization reactions (17). On the one hand, low-toxic E2 oxidation products, such as E1 and E3, were generated by dehydrogenation and oxygenation, which also caused adverse effects on agroecosystems (22). Both could be further oxidized and decomposed to produce small molecule compounds via enzyme-induced plant metabolism. On the other hand, the created radicals were covalently bound to form oligomers, polymers, and very complex precipitates, by continuous polymerization of C–C, C–O, and/or C–N bonds. Simultaneously, four electrons transferred into the T2/T3-Cu cluster via a cysteine and histidine bridge were used for reducing O_2 into H_2O (14). In comparison with the oxidation and self-polymerization products, deeper copolymerization was verified under L-TRH, resulting in the thorough elimination of phytotoxicity. L-TRH not only boosted E2 decontamination and detoxification in water–crop matrices but also contributed to the sequestration of organic carbon and the security of agrifood. These advantages are universally ignored in the traditional strategies, in which the decomposition and mineralization of organic pollutants in the rhizosphere microenvironments are involved.

Environmental implications

Maize is one of the most widely grown crops in the world, playing an increasing and diverse role in agrifood systems (32). Steroidal estrogens can transfer from agroenvironments to maize roots and become accumulated in shoots (7, 22). According to the mass-balance model, estrogenic contaminants were extensively metabolized by functional enzymes in maize, but some of them still existed in crop tissues for a long time, posing threats to agrifood safety and human health (Figs. 1 and 4). Laccase, as a green catalyst, has a great potential for consolidating the polymerization and humification of estrogens from the rhizosphere microenvironments (16, 17), thereby intercepting the uptake and transport of pollutants by roots and weakening their metabolic stress in crop bodies. Considering the complexity and diversity of the rhizosphere microenvironments, the decontamination and humification mechanisms of estrogenic pollutants induced by L-TRH remain unexplored. In our work, L-TRH allowed the maize seedlings to alleviate the risks of E2 contamination by propelling polymerization between E2 and certain root exudates in the rhizosphere solution. As a result, strongly hydrophobic oligomers, polymers, and eventually humified precipitates were largely formed. Significantly, the anoxic zones in the rhizosphere could cause an unfavorable effect for laccase-induced E2 decontamination and humification (33); thus, an external O_2 supply would be more beneficial in L-TRH.

After a 10-day cultivation, the mass distribution of ^{14}C-radioactivity (^{14}C-E2 polymeric products) in the humified precipitates was accurately identified to evaluate the humification degree of E2. ^{14}C-activity in the humified precipitates produced

by adding laccase was 2.32 times higher than that of the control samples without laccase addition, implying that L-TRH visibly enhanced the polymerization of E2 and root exudates and thus lowered carbon emissions. These polymeric products were $>10^7$ times less water soluble than E2 and became fantastically less bioavailability and transferability than their parent compound (24). Furthermore, the heterogeneous precipitates with highly complex architectures were very stable, avoiding the re-release of steroidal structures into the rhizosphere microenvironments. This experimental study of L-TRH in E2-contaminated water–maize systems demonstrates that, L-TRH, as a high-efficiency, low-energy-consuming, green, and sustainable strategy, was able to decontaminate estrogenic contaminants and accelerate organic carbon humification. To improve the understanding of L-TRH, rhizosphere microorganisms and other bioenzymes released by the roots associated with E2 conversion need to be further explored. In addition, future studies should focus on tracking the utilization of the humified products by maize seedlings and propelling the practical application of L-TRH in water/soil-crop systems at sites contaminated with steroidal estrogens.

Conclusion

For the first time, the mechanisms of decontamination and humification propelled by L-TRH in E2-polluted water–crop media were reported with greenhouse pot experiments in this work. L-TRH rapidly dissipated E2 in the rhizosphere solution for growing maize seedlings. Compared with the treatment groups without laccase addition, the $t_{1/2}$ values of E2 dissipation at 10 and 50 μM in L-TRH decreased from 16.8 to 2.1 h and 43.6 to 25.8 h, respectively. This was mainly because L-TRH consolidated the polymerization of E2 and root exudates in the rhizosphere solution, consequently generating a large amount of E2-humified precipitates with typical acid functional groups. The formation of precipitates not only reduced the phytotoxicity and bioavailability of E2 but also alleviated the uptake, accumulation, and acropetal translocation of E2 by the maize seedlings. In particular, E2 oligomers such as dimer, trimer, and tetramer could also be taken up by root tissues, but these oligomers were difficult to reach shoots. Our work presents a simple and green approach for managing steroidal estrogens and stocking organic carbon in rhizosphere microenvironments, which would be conducive to agroenvironmental bioremediation and sustainability.

Materials and methods

Biochemical materials

Maize seeds were provided by Anhui Academy of Agriculture Sciences, Hefei, China. E2 (>98%; octanol-water partition coefficient, log $K_{ow} = 3.94$), 2,6-dimethoxyphenol (2,6-DMP, 99%), and *Trametes versicolor* laccase (≥0.5 U mg^{-1}) were purchased from Sigma-Aldrich Co. (China). [4-^{14}C]-radiolabeled E2 (>99% by TLC, the radiolabeled carbon was located in position 4 of the A-ring) was purchased from American Radiolabeled Chemicals Inc. (USA). Chromatographic methanol and acetonitrile were supplied by Fisher Scientific Inc. (USA). Other chemical reagents were analytical grade and acquired from Aladdin Co. (China), unless otherwise specified. Murashige and Skoog (MS) medium (including vitamins) for the pot culture experiments was obtained from MDBio Inc. (China; www.mdbio.com.cn). All MS media were sterilized at 121 °C for 30 min with an autoclave.

Pot culture experiments

A series of pot culture experiments were carried out to investigate the role of L-TRH in controlling E2-contaminated water–maize media. Hydroponic tests were selected to overcome the effect of soil inhomogeneity and microbial activity, consequently focusing on the rhizospheric humification of E2, as well as its uptake and transportation in the maize tissues. The sterilized maize seeds (3% H_2O_2, v:v) with similar size and plumpness were chosen and germinated in the dark at 28 °C for 3 days. The geminated seedlings were then transferred into MS media and cultivated in a growth chamber with a 14-h white light (25 °C)/10-h darkness (20 °C) cycle at 65% relative humidity for 7 days. Maize seedlings with similar size (~9.0 cm in height) were selected for the pot culture experiments. To investigate the dose-dependent effects of E2 on maize seedlings, four treatment groups were established at two concentrations of E2: (i) 10 μM E2 + Maize seedlings; (ii) 10 μM E2 + Maize seedlings + Laccase; (iii) 50 μM E2 + Maize seedlings; and (iv) 50 μM E2 + Maize seedlings + Laccase.

Each group contained 120 mL of MS culture solution in a sterile glass reactor, planted with three maize seedlings, which was individually spiked with 10 and 50 μM E2. In the uptake kinetic tests, a low level of E2 was set at 10 μM, which is close to the detected concentration in an actual manure-polluted site in Anhui, China; the selected 50 μM E2 exceeded its environmentally relevant level, as the growth of plants was consistently inhibited at >36.7 μM of E2. Simultaneously, 0.13 U mL^{-1} of laccase was added in the reactor to activate rhizospheric humification. The glass reactor was adequately wrapped with aluminum foil to minimize photolysis and volatilization of E2. The reactors were placed in the controlled growth chamber, and the maize seedlings exposed to E2 were harvested at intervals of 4, 8, 12, 24, 36, 48, 72, 96, and 120 h. Roots were rinsed with methanol and deionized water in depth and dried on tissue paper, and maize seedlings were separated into roots and shoots. Subsequently, the average length of the root, the height of the plant, and the FW were measured from 10 randomly selected maize seedlings. The root and shoot samples were homogenized and then stored at −20 °C before analysis. The planted (the treatment without laccase and E2 addition) and unplanted blank controls (no maize seedlings and laccase) were also established. All treatment groups were prepared in quintuplicate.

Determination of laccase activity and root exudates

The laccase activity in the rhizosphere solution was measured with the enzyme-specific substrate 2,6-DMP. The details are described in supplementary material. The contents of root exudates, such as total phenols, amino acids, and sugars, were spectroscopically analyzed (UV-2550, Shimadzu Co., Japan), as also listed in supplementary material.

Biochemical parameters of maize seedlings

Biochemical parameters, such as C_a, C_b, and C_{a+b} in the leaves of maize, were determined by colorimetric assay (see supplementary material for details).

E2 quantification and product identification

The rhizosphere solution and crop samples were collected at a predetermined time interval. For the analysis of E2 in solution, the extraction and quantification procedures are provided in detail in supplementary material. For the analysis of E2 in the maize tissues, an aliquot of plant samples was extracted in 5 mL of

acetone and hexane (1:1, v:v) by ultrasonication (Elmasonic S100; Elma Co., Germany) for 30 min, followed by centrifugation at 4,500 r min^{-1} for 15 min. The collected supernatants were stored in the refrigerator at 4 °C. Such an extraction procedure was thrice repeated. Subsequently, all supernatants were merged and filtered through a column with silica gel and Na_2SO_4. Finally, the supernatants were concentrated to dry by N_2 and then redissolved in 1 mL of methanol. The E2 concentration was quantified with high-performance liquid chromatography (Waters, Milford, MA, USA), as mentioned in supplementary material. The E2 recovery in plant samples was in the range of 92.51–98.26% ($n = 5$), demonstrating the precision of this detection method.

Different from the E2 quantification, the E2 intermediate products in the rhizosphere solution were ultrasonically extracted by equal volumes of ethyl acetate, and then concentrated, purified, and analyzed using an LTQ-Orbitrap HRMS instrument (Thermo Fisher Scientific Inc., Germany). Control samples included parallel cultivation with the treatment without laccase or E2 addition for comparison. For HRMS measurement, total ionization chromatography was screened from m/z 50 to 1,500 (mass accuracy < 5 ppm). The obtained experimental data were analyzed with Xcalibur software (Thermo Fisher Scientific Inc.). More details about the sample extraction and analysis method are summarized in supplementary material.

Collection and characterization methods of humified precipitates

A larger-scale pot experiment was conducted to collect the humified precipitates in L-TRH. After a 10-day cultivation, the planted solutions were filtered and then acidified to pH 1.5 by adding 1 M HCl. The humified precipitates were collected, followed by freeze-drying, grounding, and sieving (100 mesh) to characterize their physicochemical properties. The micromorphology and elemental composition of humified precipitates were determined using SEM (Regulus 8100; Hitachi Co., Japan) equipped with an EDS (Bruker Co., Germany). The chemical bonding in the humified precipitates was analyzed between 4,000 and 400 cm^{-1} by FTIR spectroscopy (Nexus-870; Nicolet Co., USA). Solid-state ^{13}C nuclear magnetic resonance spectroscopy (^{13}C-NMR, Avance III; Bruker Co.) was used to examine the chemical structures of the humified precipitates. The microscopic distribution of the nanoscale organic carbon in the humified precipitates was also determined by spherical aberration–corrected scanning transmission electron microscopy (Cs-STEM, Titan Cubed Themis G2300, FEI, USA) coupled with HAADF-STEM imaging and EELS elemental mapping.

Determination of ^{14}C-radioactivity in the humified precipitates

In L-TRH, the culture solution was spiked with [4-^{14}C]-radiolabeled E2 and then planted with maize seedlings. The humified precipitates were collected and combusted in a biological oxidizer. The produced $^{14}CO_2$ was detected by liquid scintillation counting (LS-6500; Backman Coulter Co., USA). The details are given in supplementary material.

Supplementary Material

Supplementary material is available at *PNAS Nexus* online.

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Author Contributions

K.S., L.-Z.D., and M.-H.C. conceived and planned the experiments and carried out the relative experiments. K.S., Y.-B.S., G.-D.F., and H.-Q.Y. analyzed the various characterization methods. K.S., Y.-B.S., S.-Y.L., and H.-Q.Y. contributed to the planning and coordination of the project. K.S. wrote the initial draft of the manuscript and further modified it by H.-Q.Y. All authors contributed to the discussion of the results and the manuscript.

Data Availability

All data are included in the article and supplementary material.

References

1 La Merrill MA, *et al.* 2020. Consensus on the key characteristics of endocrine-disrupting chemicals as a basis for hazard identification. *Nat Rev Endocrinol.* 16(1):45–57.
2 Walker DM, Gore AC. 2011. Transgenerational neuroendocrine disruption of reproduction. *Nat Rev Endocrinol.* 7(4):197–207.
3 Berninger JP, *et al.* 2019. Predictive analysis using chemical-gene interaction networks consistent with observed endocrine activity and mutagenicity of US streams. *Environ Sci Technol.* 53(15): 8611–8620.
4 Gore AC, *et al.* 2015. EDC-2: the Endocrine Society's second scientific statement on endocrine-disrupting chemicals. *Endocr Rev.* 36(6):E1–E150.
5 Zhang QQ, Xing C, Cai YY, Yan XT, Ying GG. 2021. How much do human and livestock actually contribute to steroids emission and surface water pollution from past to the future: a global research. *Sci Total Environ.* 772:145558.
6 Masoner JR, *et al.* 2023. Contaminant exposure and transport from three potential reuse waters within a single watershed. *Environ Sci Technol.* 57(3):1353–1365.
7 Adeel M, *et al.* 2023. Estrogens in plants and emerging risks to human health. *Environ Int.* 178:107985.
8 Chen X, *et al.* 2022. Uptake and transport of steroid estrogens in soil-plant systems and their dissipation in rhizosphere: influence factors and mechanisms. *J Hazard Mater.* 428:128171.
9 Cai J, *et al.* 2021. Accurate removal of trace 17β-estradiol and estrogenic activity in blended systems under a photoelectrocatalytic circulating flow. *Environ Sci Technol.* 55(18):12585–12595.
10 Ismanto A, *et al.* 2022. Endocrine disrupting chemicals (EDCs) in environmental matrices: occurrence, fate, health impact, physio-chemical and bioremediation technology. *Environ Pollut.* 302:119061.
11 Bilal M, Barceló D, Iqbal HMN. 2021. Occurrence, environmental fate, ecological issues, and redefining of endocrine disruptive estrogens in water resources. *Sci Total Environ.* 800:149635.
12 Chen M, Waigi MG, Li S, Sun K, Si Y. 2019. Fungal laccase-mediated humification of estrogens in aquatic ecosystems. *Water Res.* 166:115040.
13 Liu Q, *et al.* 2021. Fungal laccase-triggered 17β-estradiol humification kinetics and mechanisms in the presence of humic precursors. *J Hazard Mater.* 412:125197.

14 Sun K, Li S, Si Y, Huang Q. 2021. Advances in laccase-triggered anabolism for biotechnology applications. *Crit Rev Biotechnol.* 41(7):969–993.
15 Pausch J, Kuzyakov Y. 2018. Carbon input by roots into the soil: quantification of rhizodeposition from root to ecosystem scale. *Glob Chang Biol.* 24(1):1–12.
16 Chu H, Li S, Sun K, Si Y, Gao Y. 2022. Exolaccase-boosted humification for agricultural applications. *iScience*. 25(9):104885.
17 Li S, *et al.* 2022. Insights into the applications of extracellular laccase-aided humification in livestock manure composting. *Environ Sci Technol.* 56(12):7412–7425.
18 Xie T, Liu Z, Wang G. 2020. Structural basis for monolignol oxidation by a maize laccase. *Nat Plants*. 6:231–237.
19 Liang M, Haroldsen V, Cai X, Wu Y. 2006. Expression of a putative laccase gene, *ZmLAC1*, in maize primary roots under stress. *Plant Cell Environ.* 29(5):746–753.
20 McLaughlin S, Zhalnina K, Kosina S, Northen TR, Sasse J. 2023. The core metabolome and root exudation dynamics of three phylogenetically distinct plant species. *Nat Commun.* 14:1649.
21 Wang GD, Li QJ, Luo B, Chen XY. 2004. *Ex planta* phytoremediation of trichlorophenol and phenolic allelochemicals via an engineered secretory laccase. *Nat Biotechnol.* 22:893–897.
22 Card ML, Schnoor JL, Chin YP. 2013. Transformation of natural and synthetic estrogens by maize seedlings. *Environ Sci Technol.* 47(10):5101–5108.
23 Bhardwaj P, *et al.* 2022. Laccase-assisted degradation of emerging recalcitrant compounds—a review. *Bioresour Technol.* 364:128031.
24 Qin C, *et al.* 2015. Surface catalyzed oxidative oligomerization of 17β-estradiol by Fe^{3+}-saturated montmorillonite. *Environ Sci Technol.* 49(2):956–964.
25 Ma R, *et al.* 2022. Effects of oxygen levels on maturity, humification, and odor emissions during chicken manure composting. *J Clean Prod.* 369:133326.
26 Sundarapandiyan S, *et al.* 2017. Photocatalytic degradation of highly refractive phenolic polymer-mechanistic insights as revealed by electron spin resonance (ESR) and solid-state ^{13}C NMR spectroscopy. *Chem Eng J.* 313:1112–1121.
27 Lu Y, *et al.* 2022. Nano and sub-nano scale distribution of organic carbon species on soil particles revealed by Cs-STEM EELS. *Chem Geol.* 613:121168.
28 Possinger AR, *et al.* 2020. Organo-organic and organo-mineral interfaces in soil at the nanometer scale. *Nat Commun.* 11(1): 6103.
29 Therby-Vale R, Lacombe B, Rhee SY, Nussaume L, Rouached H. 2022. Mineral nutrient signaling controls photosynthesis: focus on iron deficiency-induced chlorosis. *Trends Plant Sci.* 27(5): 502–509.
30 Miller EL, Nason SL, Karthikeyan KG, Pedersen JA. 2016. Root uptake of pharmaceuticals and personal care product ingredients. *Environ Sci Technol.* 50(2):525–541.
31 Schmidt B, Schuphan I. 2002. Metabolism of the environmental estrogen bisphenol A by plant cell suspension cultures. *Chemosphere*. 49(1):51–59.
32 Tanumihardjo SA, *et al.* 2020. Maize agro-food systems to ensure food and nutrition security in reference to the sustainable development goals. *Glob Food Sec.* 25:100327.
33 Huber D, *et al.* 2016. Influence of oxygen and mediators on laccase-catalyzed polymerization of lignosulfonate. *ACS Sustain Chem Eng*. 4(10):5303–5310.