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Research Article

Water-Soluble Pyrolysis Products as Novel Urease Inhibitors Safe for Plants and Soil Fauna

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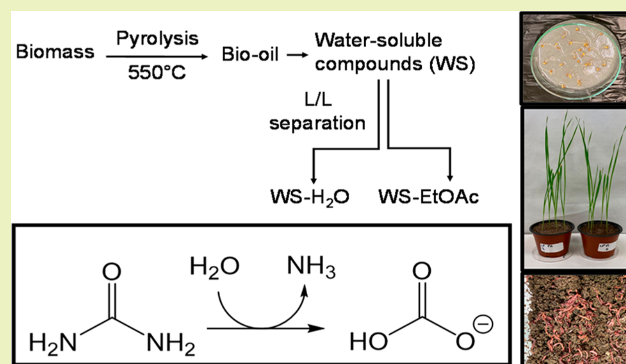
ABSTRACT: Water-soluble compounds (WS) obtained from the pyrolysis of three lignocellulosic biomasses (larch, poplar, and switchgrass) were tested as potential inhibitors of the enzyme urease. Thanks to the presence of an array of phenolic compounds like catechol, methoxy/hydroxy phenols, phenolic acids, and phenolic aldehydes, all the WS samples tested at a catechol concentration of 30 μM inhibited the activity of jack bean urease (JBU) by 60%−70% and by 80% that of urease naturally present in the soil. A 10 times lower dose of WS samples (catechol concentration of 3 μM) inhibited the activity of JBU by 20%, while that of soil urease by 50%, in line with the known inhibition of *N*-(*n*-butyl) thiophosphoric triamide (NBPT). The germination rate, early growth, and development of oat were not affected by any WS sample tested at this lower dose, as well as the cress germination rate, while the development of cress roots and shoots was lower than the control presumably because of the low pH of the tested WS solutions. Earthworm survival was not significantly affected by any WS sample tested, but an effect was observed on the ability of the eggs to develop into viable newborns.



KEYWORDS: *Lignocellulosic biomass, pyrolysis, ammonia reduction, antiurease activity, soil, phytotoxicity, earthworms*

INTRODUCTION

Soil urease is the enzyme responsible for the accelerated hydrolysis of urea-based fertilizers used in agriculture and the consequent formation of ammonia (NH_3); it has been estimated that the release of NH_3 accounts for 14% of N applied worldwide, with peaks of 40% in more humid environments like the tropics.[1] NH_4^+-containing secondary aerosol can be formed when NH_3 losses occur in the atmosphere, and this aerosol is the major fraction of PM2.5 aerosol. Urea hydrolysis catalyzed by soil urease can also affect the soil compartment through the formation of ammonium carbonate which may temporarily cause a local increase of pH value in the area surrounding urea granules that can cause damage to germinating seedlings and young plants.[2] Moreover, when NH_3 losses become relevant, more fertilizer is needed to achieve high crop yields implying significant economic issues. The use of urease inhibitors has become a widespread practice to reduce and mitigate the entity of this phenomenon: several synthetic compounds have proven a significant urease inhibition activity, but only *N*-(*n*-butyl) thiophosphoric triamide (NBPT) and two derivatives (*N*-(*n*-propyl) thiophosphoric triamide, NPPT, and *N*-(2-nitrophenyl) phosphoric triamide, 2-NPT) are commercially used worldwide as coformulations with urea.[1] These compounds have a structural analogy with urea and are capable of temporarily blocking soil ureases by binding to the Ni(II) ions in the active site of the enzyme, decreasing the urea hydrolysis rate. Other inhibitors containing a phenolic scaffold, such as catechol and its mono- and dimethyl derivatives and hydroquinones, have been proven to inhibit urease by binding to a conserved cysteine residue located onto a mobile helix-turn-helix motif in the active site cavity.[3−5] Catechol, in particular, is one of the simplest molecules bearing a phenolic structure identified as a powerful inhibitor of soil urease, capable of inhibiting more than 70% of the activity even at low concentrations.[6] Even if several phenolic compounds have marked inhibitory effects on urease activity in the soil, other natural macrostructures containing many phenolic moieties like lignin, tannins, and humic acids seem not to behave likewise, presumably because of their lower water solubility than single phenolic units.[6] In particular, the polyphenolic structure of lignin is chemically very stable and,

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therefore, difficult to transform or to be structurally modified without the application of harsh reaction conditions, like high temperatures (i.e., pyrolysis) or the use of strong bases (i.e., the Kraft process). Pyrolysis is one of the most investigated technologies for directly liquefying lignocellulosic material to a crude bio-oil enriched in a mixture of compounds derived from cellulose, hemicellulose, and lignin, like anhydrosugars, furans, phenols, and carboxylic acids. Separating such a variety of molecules into single chemicals or chemical classes is a challenging task because of their low concentrations in the bio-oil but it would be highly desirable in a biorefinery approach. The main application of crude bio-oils, as unseparated mixtures of chemicals with different moieties, is in the field of biofuels, but given the abundance of oxygen-containing functional groups, an upgrading (e.g., by hydrodeoxygenation or zeolite cracking) is mandatory to improve their stability over time and the heating value.[7] Another use of bio-oils is as a carbon source for fermentative processes,[8−10] but also in this case, an upgrading (e.g., by liquid−liquid extraction or adsorption on activated carbon) for detoxifying the mixture leaving just fermentable compounds like sugars/anhydrosugars is required.[8−10] Furans and phenols are known to be toxic, so bio-oils from various lignocellulosic feedstock have been also used against various biological targets (e.g., crustacea, algae, weeds, insects, nematodes, bacteria, and cells) as pesticides.[11−17] In the present paper, we aimed at increasing the knowledge on the biological properties of bio-oils from lignocellulosic feedstock against urease, a target that was never investigated before, by exploiting the known antiurease activity of phenolic compounds that are abundant in bio-oils of lignin-rich biomass. To this purpose, the bio-oils obtained from the pyrolysis of three lignocellulosic biomass (switchgrass, larch, and poplar) were fractionated into water-soluble fractions and water-insoluble tars. The fractions containing the water-soluble pyrolysis products (WS) were further separated by liquid−liquid separation into two subfractions, one soluble in ethyl acetate (WS-EtOAc) and the other one only soluble in water (WS-H_2O). These two subfractions and the whole WS samples were then tested against urease, plants, and soil earthworms. The intent was to prepare novel formulations useful for agricultural purposes that should not damage plant germination and growth or adversely affect soil fauna.

■ MATERIALS AND METHODS

Chemicals, Biomass, and Soil. All chemicals and solvents were purchased from Sigma-Aldrich and used without any further purification. Jack bean urease (*Canavalia ensiformis*, JBU) Type C-3, powder, ≥600 units mg^{-1} solid, was used in the *in vitro* urease inhibition assay.

Panicum virgatum (switchgrass) and *Populus alba* (poplar) biomass were grown at the Experimental Farm of the University of Bologna (Bologna, Italy). *Larix europaea* (larch) was purchased from Legnami Larese s.r.l. (Ravenna, Italy). Before pyrolysis experiments, the samples were dried at 60 °C for 48 h. Switchgrass biomass was ground in a hammer mill to pass a 1 mm screen, while poplar and larch biomass were cut into pieces of about 3 cm^2.

A surface soil sample for the soil urease assay (0−20 cm) was collected from an apple orchard located in Ravenna, Italy. The soil, classified as Udifluventic Haplustept,[18] displayed the following characteristics: silty clay loam texture, pH 8.5, electrical conductivity (EC) 0.17 dS m^{-1}, $CaCO_3$ tot. 203 g kg^{-1}, total organic carbon (TOC) 11.3 g kg^{-1}, and total nitrogen (TN) 1.3 g kg^{-1}.[19] After removing plant roots, debris, and visible fauna, the soil sample was air-dried in the dark at room temperature, then crushed with a mortar, sieved (<2 mm), and stored in polyethylene bags at 4 °C.

Pyrolysis and Pyrolysis Product Characterization. Biomass was subjected to bench-scale pyrolysis using an apparatus consisting of a sliding sample carrier placed in a heated quartz tube connected to ice traps and a settling chamber. The quartz tube was heated by a cylindrical coaxial furnace and purged by 1.5 L min^{-1} N_2 flow. The biomass sample (5−6 g for each pyrolysis) was moved into the heated zone of the quartz tube and heated for 20 min at 550 °C (measured temperature) under N_2 flow. The resulting char was collected and ground to powder in a mortar, and then, the sliding sample carrier was recharged with other biomass and subjected to the same procedure until a total of 40−50 g of biomass was pyrolyzed. Bio-oil produced from such a series of pyrolysis was collected in an ice trap with 50 mL of water. The component of the bio-oil soluble in water (water-soluble pyrolysis products) was hereafter called WS (indicated as WS_L, WS_P, and WS_S from larch, poplar, and switchgrass biomass in Figures 2−6), while the water-insoluble part (tar or pyrolytic lignin) was hereafter called PL. PL was recovered after washing with acetone all the apparatus (the trap and the quartz tube) and then evaporating acetone. The concentration of WS in water was determined by sampling aliquots of 0.1 mL and then drying them under nitrogen. The liquid−liquid separation of WS (10 mL) was performed with ethyl acetate (10 mL, two times): the resulting two subfractions were hereafter called WS-H_2O and WS-EtOAc. The qualitative profiles of WS, WS-H_2O, and WS-EtOAc samples were determined by GC-MS analysis after drying under nitrogen each sample (0.1 mL) and silylation (60 min at 70 °C with 0.1 mL acetonitrile, 0.08 mL bis(trimethylsilyl)trifluoroacetamide containing 1% of trimethylchlorosilane, and 0.04 mL of pyridine).[17] Compounds were identified by comparison with the NIST database and grouped into four categories: (i) small oxygenates (like alcohols and carbonyl compounds, i.e., hydroxyacetaldehyde), (ii) anhydrosugars and sugars (like levoglucosan), (iii) short-chain length carboxylic acids, and (iv) phenolics and furans (like catechol and derivatives). The unidentifiable compounds were indicated as "unknown". The quantitative analyses of catechol present in WS, WS-H_2O, and WS-EtOAc samples were performed by GC-MS analysis[17] using a calibration curve prepared with silylated catechol (0.67−67 μg mL^{-1}). The concentration of catechol in each sample was used to determine the amount of WS samples to be tested in the urease assays and in the ecotoxicity tests. The analysis of polycyclic aromatic hydrocarbons (PAH) was performed on WS and WS-EtOAc samples according to the literature, by using a deuterated PAH standard mix (acenaphthene-d10 was utilized to quantify naphthalene, acenaphthylene, acenaphthene, and fluorene; phenanthrene-d10 to quantify phenanthrene, anthracene, fluoranthene, and pyrene; chrysene-d12 to quantify the remaining PAHs).[20]

***In Vitro* Urease Inhibition Assay.** The activity of *Canavalia ensiformis* (jack bean) urease (JBU) in the absence and the presence of WS samples was determined by using the pH-STAT method in 2 mM HEPES buffer at pH 7.5, also containing 2 mM EDTA, following an already reported protocol in which a preincubation time of 2 h was adopted.[21] WS samples were tested at two doses, corresponding to two concentrations of catechol (3 or 30 μM) (see Table S1 in the Supporting Information (SI) for the corresponding volumes of each WS sample).

Soil Urease Inhibition Assay. WS samples were tested at three concentrations of catechol in soil: 0.5, 5, and 50 μg g^{-1} (corresponding to concentrations of catechol of 3, 30, and 300 μM in the spiking solution, respectively) (see Table S1 in SI for the volumes of each WS sample). The WS-EtOAc and residual WS-H_2O fractions coming from the liquid−liquid separation of aliquots of WS samples corresponding to a concentration of catechol in soil of 5 μg g^{-1} were also tested. EtOAc was evaporated under N_2 from WS-EtOAc samples, and then, the samples were resuspended in the same amount of water as the initial WS sample before use. Therefore, we obtained three different WS samples: the initial one (WS), the WS-EtOAc fraction, and the residual WS-H_2O fraction. NBPT (*N*-(*n*-butyl) thiophosphoric triamide) was tested as the reference urease inhibitor at a concentration of 96 μg g^{-1} in soil. The soil urease

activity was determined through the quantification of NH_3 produced by using a modified Kandeler and Gerber method,[22] using dried soil samples (see SI). Soil respiration was tested as an indicator of microbial activity when WS samples were added to the soil (see SI).

Eco-Toxicity Tests. A single dose of WS samples was tested in all the toxicity tests, corresponding to a final concentration of catechol of 30 mM in the case of the filter paper contact germination test, or 5 μg g^{-1} of soil in the cases of plant emergence and early growth test and earthworm reproduction test (see Table S1 in SI for the corresponding volumes of each WS sample). WS-H_2O and WS-EtOAc fractions were prepared and tested as described above.

Filter Paper Contact Germination Test. Germination tests on cress (*Lepidium sativum* L.) seeds were conducted in Petri dishes according to the procedure described in UNI 11357:2010 (see SI). Seed germination rate (%), shoot length (cm), and root length (cm) after 72 h were reported.

Plant Emergence and Early Growth Test. The emergence and early growth of oats (*Avena sativa* L.) were tested according to ISO 11269-2:2012 (see SI). Five endpoints were evaluated at the end of the test: (i) seed germination rate, reported as a percentage (%) relative to the control (distilled water), (ii) shoot length and (iii) shoot weight (mass of the five shoots in each pot after drying at 60 °C for 48 h), reported as percentages (%) relative to the control (distilled water), (iv) chlorophyll content (mg g^{-1}, after extraction with acetone and spectrophotometric analysis at 750 and 665 nm),[23,24] and (v) visible damages (chlorosis, necrosis, wilting, deformations).

Earthworm Reproduction Test. The earthworm *Eisenia andrei* Bouchè 1972, was used to run a 56 days reproductive toxicity test according to the OECD Guideline No 222 (see SI). The effects on survival, growth, and reproduction were assessed by determining the number and weight of adults, the number and weight of juvenile earthworms, and the number of both hatched and unhatched cocoons at the end of the test.

Statistical Analysis. Differences among treatments (different WS samples and WS fractions, NBPT, and control) were tested by one-way analysis of variance (ANOVA) performed on untransformed data. The homogeneity of variance was confirmed using Cochran's C test. Whenever ANOVA detected significant differences, the Student−Newman−Keuls (SNK) posthoc pairwise comparison test was performed. Treatments not significantly different from each other according to the SNK test were marked with the same letter in the figures. Differences were considered significant for $p < 0.05$. All tests were carried out using Statistica 10 (Statsoft, Tulsa, OK, USA).

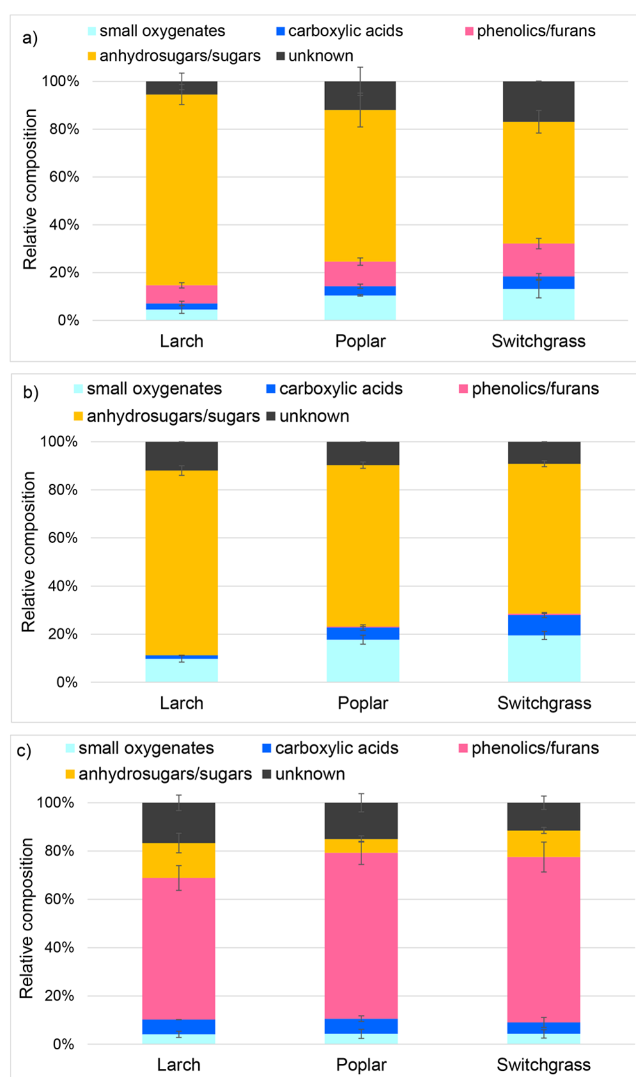

Figure 1. Relative composition (%) of the GC-MS detectable compounds found in (a) WS samples before the liquid−liquid separation, (b) WS-H_2O, and (c) WS-EtOAc samples.

RESULTS AND DISCUSSION

Characterization and Fractionation of Pyrolysis Products. In the present work, poplar, larch, and switchgrass biomass were used to prepare the corresponding bio-oils to be tested as antiurease formulations; the three types of lignocellulosic biomass belong to the classes of hardwood, softwood, and herbaceous biomass, respectively, known to have different lignin compositions (e.g., different monolignol ratios) and therefore potential precursors of phenolic compound mixtures with different antiurease effects. The intermediate pyrolysis conditions here applied gave similar amounts of char and PL, independent of the type of biomass treated, while the amount of WS obtained from poplar and larch biomass was 2−3 times higher than WS obtained from switchgrass (see Figure S1a in SI). The relative composition of WS samples from the three biomasses was largely dominated by anhydrosugars, like levoglucosan, and in minor amounts by sugars (Figure 1a), reaching 80% of the total GC-MS detectable compounds in the case of WS sample from larch, while the furanic derivatives and the phenolic compounds ranged between 8% and 14%. Catechol was the main compound identified in the class of aromatic compounds; its concentration was 5.8, 5.4, and 7.8 μg mg^{-1} in WS samples from larch, poplar, and switchgrass biomass, respectively.

Since all WS samples were acid (pH 3.5−3.7) due to the presence of short-chain length carboxylic acids, like acetic and glycolic acid,[25] and such an acidity could negatively impact seed germination or earthworm survival and reproduction, a liquid−liquid separation was applied to enrich the samples in those phenolic compounds with a potential antiurease activity of interest for the present work and reduce the presence of compounds that could have an adverse effect toward other biological targets like plants and soil invertebrates. The liquid−liquid separation of all the WS samples with ethyl acetate gave three fractions soluble in ethyl acetate (WS-EtOAc) that corresponded to about 40% of each WS (see Figure S1b in SI) and contained considerable amounts of low-molecular-weight phenolic components (Figure 1c): phenols, catechols, and guaiacols covered 60%−70% of the relative distribution of the GC-MS detectable compounds, while their presence in the WS-H_2O samples was below 1% (Figure 1b). In turn, WS-H_2O samples were enriched in anhydrosugars and sugars (70%−80%) and small oxygenated compounds like hydroxyacetaldehyde (10%−20%). Catechol and phenolic compounds with

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methoxy and hydroxylic groups were the main components of the three WS-EtOAc samples (Table 1), representing 90%, 50%, and 66% of all the GC-MS detectable aromatic compounds found in the WS-EtOAc samples from larch, poplar, and switchgrass biomass, respectively. Vanillin and 4-hydroxybenzoic acid, belonging to the classes of phenolic aldehydes and phenolic acids, were found in all samples, as 2-methylfuran among the furanic compounds.

Table 1. Relative Abundance of the Main GC-MS Detectable Phenolic Compounds Found in WS-EtOAc Samples after Liquid−Liquid Separation of WS Samples from Larch, Poplar, and Switchgrass Biomass

Compound	Relative abundance (%)		
	Larch	Poplar	Switchgrass
2-methoxy-4-propenylphenol	0.9	–	0.8
2,5-dihydroxybenzyl alcohol	1.2	1.3	1.8
4-hydroxytoluene	1.5	2.0	2.6
2-hydroxytoluene	1.0	–	0.8
phenol	1.3	2.4	2.6
3,4-dihydroxybenzyl alcohol	1.8	1.1	0.9
2-methoxyphenol	4.3	2.1	4.7
1,3,5-trihydroxybenzene	8.0	13.2	11.2
2-(2-hydroxyethyl)phenol	11.1	6.3	5.4
4-(2-hydroxyethyl)phenol	–	7.4	–
3,5-dihydroxytoluene	24.4	–	19.5
catechol	33.4	15.1	15.3
Total methoxy/hydroxy phenols	88.7	50.9	65.5
vanillin	2.2	1.9	1.8
3,5-dimethoxy-4-hydroxybenzaldehyde	–	4.4	–
4-hydroxybenzaldehyde	–	–	4.6
Total phenolic aldehydes	2.2	6.2	6.3
4-hydroxybenzoic acid	0.9	6.0	1.5
3,5-dimethoxy-4-hydroxycinnamic acid	0.3	–	–
vanillic acid	–	0.5	–
benzoic	–	0.6	–
syringic acid	–	0.9	–
3,4-dihydroxyhydrocinnamic acid	–	1.0	–
4-hydroxyhydrocinnamic acid	–	–	0.6
3-methyl-2-hydroxybenzoic acid	–	–	0.6
Total phenolic acids	1.2	9.0	2.6
2-methylfuran	5.7	5.3	9.3
3-methyl-2-furoic acid	1.7	–	–
Total furans	7.4	5.3	9.3
unknown	0.5	28.5	16.1

Urease Inhibition Assays. Given the presence of catechol and the pool of phenolic compounds that characterized each WS sample, their capacity to inhibit urease *in vitro* was assessed as urease residual activity measured in the presence of two concentrations of catechol, 3 and 30 μM, kept constant for each WS sample (Figure 2). Catechol is a well-known urease inhibitor,[3,4,6] as well as some of its mono- and disubstituted derivatives that are more active than catechol itself (e.g., 3-methyl catechol, 4,5-dimethyl catechol, 4-methyl catechol, and 3,4-dimethyl catechol). For this class of phenolic compounds, a common mode of action has been demonstrated:[4] covalent adduct occurs between the inhibitor and the thiol of a conserved cysteine residue located on a helix-turn-helix motif, the latter flanking the active site cavity and directly involved in the catalytic mechanism through a conformational change from an open to a closed state which in turn triggers the hydrolysis of urea. The formation of such adduct results in the block of the helix-turn-helix motif in the open state, thus hampering the hydrolytic event to occur. In all three enzyme−WS mixtures, urease activity was strongly decreased in a concentration-dependent manner. In particular, urease activity was decreased by about 20% when WS samples were tested at a catechol concentration of 3 μM in comparison to the experiment performed in the absence of WS, while these values increased up to 60%−70% when urease was treated with the highest concentration of catechol (30 μM). These results were in line

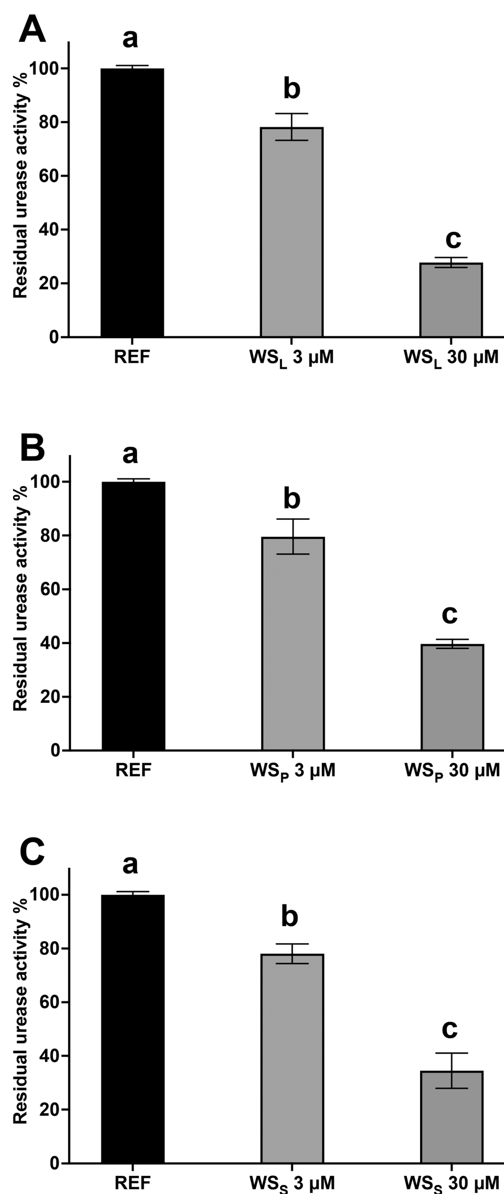

Figure 2. Residual percentage activity of urease after preincubation of 2 h, referred to 100% (control) in the presence of two doses of WS samples from larch (A), poplar (B), and switchgrass (C) biomass corresponding to 3 and 30 μM of catechol. Values were reported as mean ± standard error (n = 3). Treatments marked with different letters (a, b, and c) were significantly different from each other ($p < 0.05$).

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with the antiurease activity of a variety of catechol derivatives tested at 30 μM, highlighting how the pool of phenolic compounds found here in each WS sample positively contributed to the inhibition of the enzyme with their different moieties in different positions of the aromatic ring.

The inhibition of urease was also tested in a series of *in vivo* assays, by using the enzyme naturally present in agricultural soils (Figure 3). Three concentrations of WS samples were tested, i.e., 0.3, 3, and 30 μM of catechol in the spiking solution, corresponding to catechol concentrations of 0.5, 5, and 50 $\mu g\ g^{-1}$ of soil; the two fractions obtained through the liquid−liquid separation of all the WS samples (i.e., WS-H_2O and WS-EtOAc) were also tested at the catechol concentration of 5 $\mu g\ g^{-1}$. The results were compared with the inhibition activity of NBPT tested at a concentration of 96 $\mu g\ g^{-1}$. The urease activity was decreased by approximately 20% when WS samples were tested at a concentration of catechol of 0.5 $\mu g\ g^{-1}$ in comparison to the experiment performed in the absence of WS, while these values increased up to approximately 60% and 80% when urease was treated with catechol concentrations of 5 and 50 $\mu g\ g^{-1}$, respectively. A dose-dependent mode of action was thus observed, and not one of the tested WS samples was statistically different from the other samples tested at the same concentration. The 80% inhibition of urease activity obtained with WS samples at a concentration of catechol of 50 $\mu g\ g^{-1}$ was in line with the value reported by Bremner and Douglas (74% of inhibition),[6] suggesting that catechol was the main inhibitor among the phenolic compounds present in the WS mixtures. WS samples were not toxic for soil microorganisms when tested at a catechol concentration of 5 $\mu g\ g^{-1}$ (see Figure S2 in SI), in line with the literature results,[12,17] indicating that the effects observed were due to an actual inhibition of the enzyme urease rather than a lethal effect on the soil microorganisms themselves. The inhibition behavior of WS-H_2O and WS-EtOAc samples reflected their content in terms of GC-MS detectable phenolic compounds: the urease activity was decreased by about 50% with all the WS-EtOAc samples, while the inhibition was about 20% when the WS-H_2O samples were tested. It is worth mentioning that, even if the content of GC-MS detectable phenolic compounds in the WS-H_2O samples was negligible (Figure 1c), a certain urease inhibition was observed, ascribable to nonphenolic compounds or to phenolic compounds that are not GC-MS detectable. The inhibition potential of the compounds present in the WS-H_2O samples was also evident from the comparison between WS-EtOAc samples and WS samples: the latter included both fractions and were more active against urease than the first. The urease inhibition by NBPT, tested at a concentration of 96 $\mu g\ g^{-1}$, was 62%, slightly (but significantly) higher than the ones obtained with all WS samples tested at a concentration of catechol of 5 $\mu g\ g^{-1}$ (57 ± 0.4% on average).

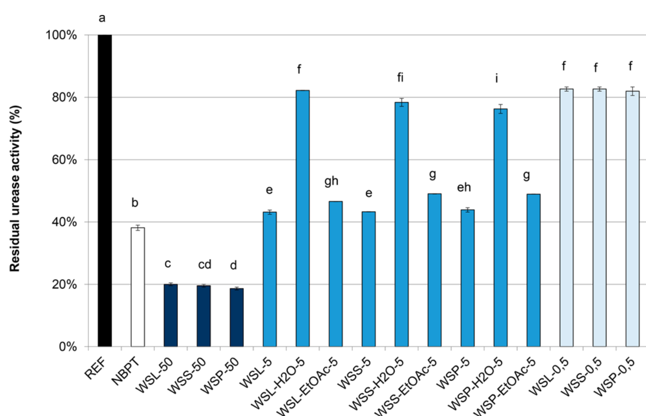

Figure 3. Residual percentage activity of soil urease referred to 100% (control) in the presence of three doses of WS samples (corresponding to 0.5, 5, and 50 μg of catechol per g of soil), their WS-H_2O and WS-EtOAc fractions, and NBPT. Values were reported as mean ± standard error (n = 4). Treatments marked with the same letter (a−h) were not significantly different from each other (p > 0.05).

Phytotoxicity Assays. The impact on cress (*Lepidium sativum*) seed germination was determined by testing the effect of each WS sample and the corresponding WS-H_2O and WS-EtOAc fractions obtained after liquid−liquid separation. The same WS concentration used for the *in vitro* urease inhibition assay corresponding to a catechol concentration of 30 μM was used (Figure 4). Neither WS samples nor their fractions influenced the germination rate (Figure 4a), except for the WS sample from switchgrass that gave a germination rate of 92%, which was significantly lower than the control and the other treatments. This result was in line with the data obtained after exposure of *Carum carvi* seeds to a concentration of slow pyrolysis liquids of 5%.[12] On the other hand, both root and shoot lengths were significantly lower than the control with all samples tested with the exception of the shoot length obtained after the treatment with the WS-EtOAc fraction from switchgrass biomass (Figure 4b,c); the effect measured after the treatment with WS samples was the most intense among the tested treatments, with an inhibition of 90% and 70%−80% of the root and shoot development, respectively. All the WS-H_2O samples decreased root length by 80%, while the shoots were 60%−70% shorter than the control. The WS-EtOAc samples were the least toxic samples tested, both on root and shoot growth: the root lengths were 30%−40% lower than the control values while the shoot lengths were just 20% shorter or not significantly different from the control, as in the case of WS-EtOAc sample from switchgrass biomass. A possible explanation for these observations can rely on the presence of short-chain carboxylic acids, known to be phytotoxic,[12] which can be responsible for the lower pH values of WS and WS-H_2O solutions measured at the beginning of the test (3.5 ± 0.1 and 3.7 ± 0.1, respectively) than the ones of WS-EtOAc solutions (4.4 ± 0.1). The stronger effect of WS samples on the tested seeds could be a joint effect of organic acids and phenolic compounds,[26] the latter not present in the WS-H_2O samples, highlighting how low pH values cannot be the sole cause of the phytotoxicity here observed.[27] This hypothesis is in line with the main causes of germination inhibition for various plant seeds exposed to water extracts of biochar identified so far: (i) the exposure to solutions with a pH value <5 or (ii) the presence of phenolic compounds. Even if PAHs are identified as the main compounds responsible for the phytotoxicity of pyrolysis products, the negligible concentrations here found in WS and WS-EtOAc samples (2−3 ng mL^{-1} for naphthalene and 0.5−0.8 ng mL^{-1} for pyrene, at least 3 orders of magnitude lower than the phytotoxic doses reported in the literature)[28] can exclude their role in the reduced root and shoot growth (see Table S2 in SI). It is worth mentioning that studies conducted to elucidate the phytotoxicity of water extracts of biochar (i.e., aqueous solutions containing recondensed pyrolysis liquids)[27] highlighted that the volatile organic compounds present in pyrolysis liquids

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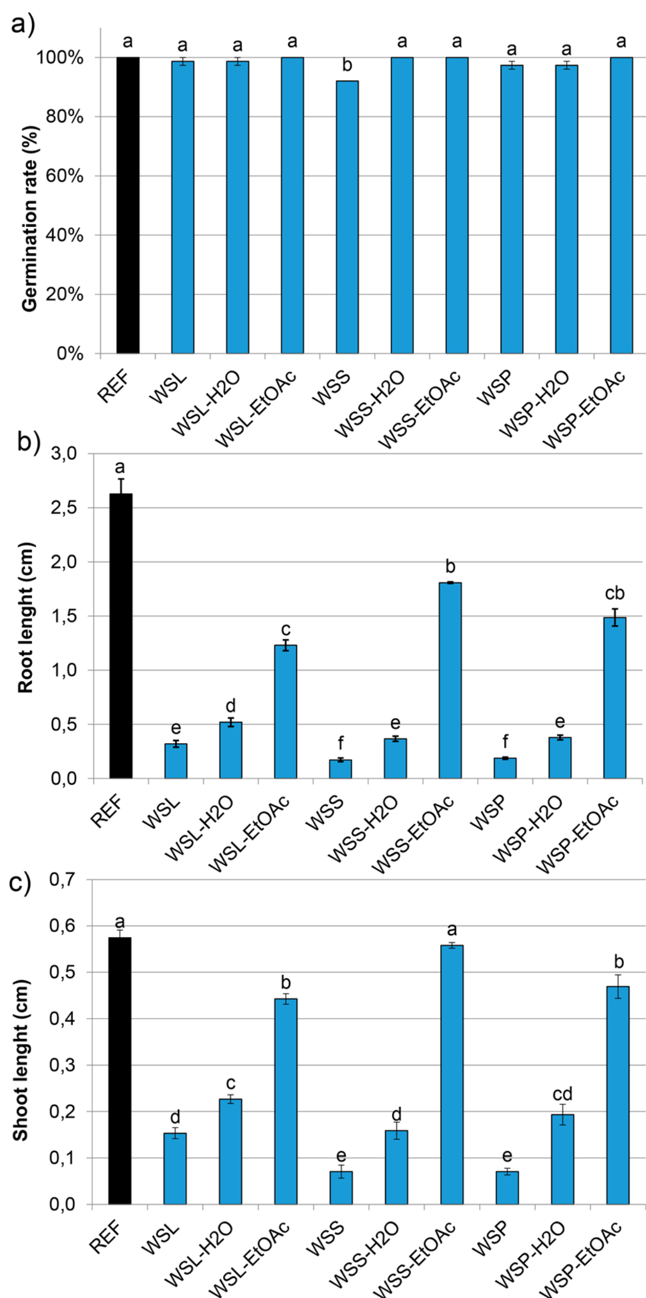

Figure 4. Effect of WS samples and their WS-H_2O and WS-EtOAc fractions on *Lepidium sativum* germination in a filter paper contact test, expressed as (a) seed germination rate, (b) root length, and (c) shoot length. Values were reported as mean ± standard error ($n = 4$). Treatments marked with the same letter (a–f) were not significantly different from each other ($p > 0.05$).

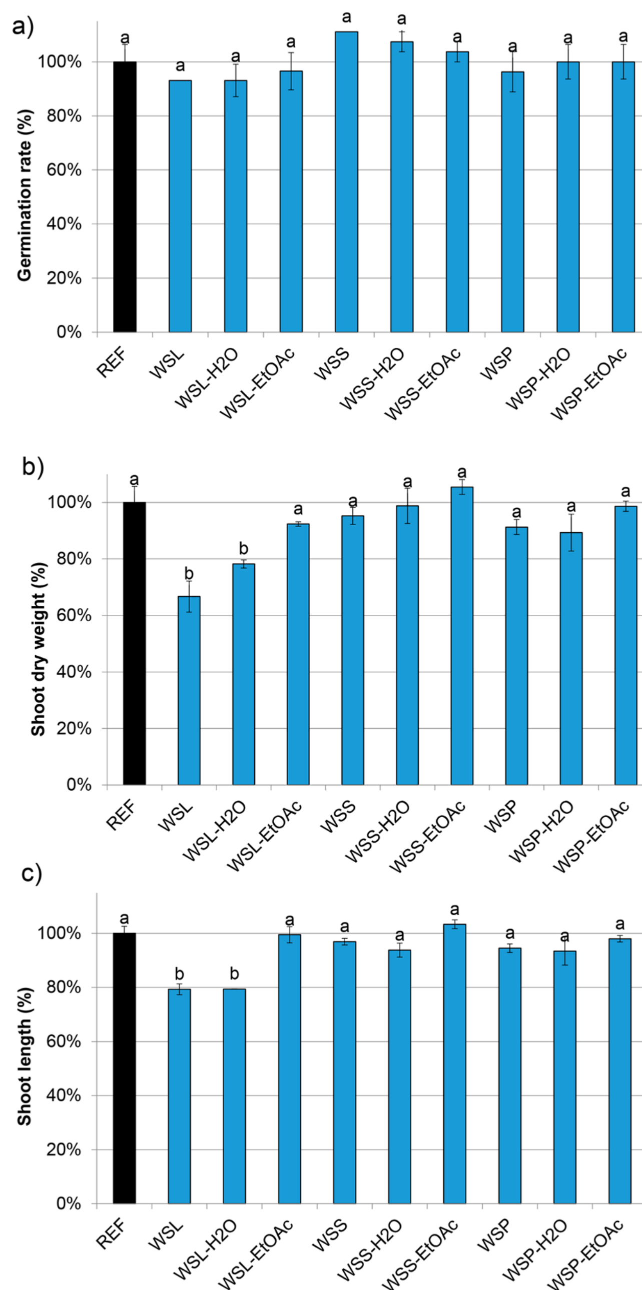

Figure 5. Effect of WS samples and their WS-H_2O and WS-EtOAc fractions on early growth of *Avena sativa*, expressed as (a) seed germination rate, (b) shoot length, and (c) root length. Values were reported as mean ± standard error ($n = 4$). Treatments marked with the same letter (a or b) were not significantly different from each other ($p > 0.05$).

generally cause delayed seed germination, thus reduced time for growth and reduced shoot and root length, rather than negative effects on seed growth after germination (i.e., reduced shoot and root development is a result of inhibition of germination). The high germination rates and the low root and shoot development found here seem to not follow this hypothesis.

The effect on seedling emergence and early growth of higher plants was evaluated following exposure to each WS sample and the corresponding WS-H_2O and WS-EtOAc fractions obtained after liquid–liquid separation (Figure 5 and Figure S3 in SI). The same WS dose used for the soil urease inhibition assay (5 μg g^{-1} of soil) was used, and oat (*Avena sativa*) was chosen as the test species. Independently on the endpoint tested (seed germination rate, shoot length and dry weight, and chlorophyll content), not one of the tested samples gave values statistically different from the control except the WS sample from larch biomass and its WS-H_2O fraction for which a statistically significant 20% reduction of the shoot length and weight was observed after the exposure. The root growth was not affected as well (see Figure S4 in SI). Thus, in most cases, the doses here applied did not show any phytotoxic effect, and as already noticed by other authors for phenolic acids, these results showed that although the WS samples tested affected

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germination and seedling growth in Petri dishes, these adverse effects are eliminated or strongly attenuated in soil.[29] This is in line with the use of the so-called "wood vinegar" (i.e., the aqueous liquid produced from slow pyrolysis of hardwood from which the tar is separated by sedimentation) in agriculture as a fertilizer and growth-promoting agent since the 1930s.[30]

Earthworm Reproduction Test. The effect of WS samples and WS fractions on survival, growth, and reproduction of the earthworm *Eisenia andrei* was assessed by testing the same doses of WS samples used for *A. sativa* early growth tests (catechol concentration of 5 μg g^{-1} of soil) (Figure 6). Adult survival was 100% in all treatments, except the WS-H_2O sample from switchgrass biomass where dead worms laying at the soil surface were observed since the first days and where no individuals survived to the end of the exposure. The initial mean live weight of individual adults was 575 mg and increased by 26% by the end of the exposure, without statistically significant differences among treatments. Even if slightly lower, the total number of laid cocoons was not significantly different from the control in any treatment where the adults survived; the null value for the WS-H_2O sample from switchgrass biomass was a direct consequence of the complete mortality of the parent adults (Figure 6c). The same holds for the percentage of hatched (empty) cocoons (Figure S5 in SI). A reduction in the number and total dry weight of juveniles recovered on day 56 was observed for all the treatments even if the observed values were significantly different from the control only for WS samples from larch and switchgrass biomass, and WS-EtOAc fraction from switchgrass biomass, due to the variability within treatments (Figures 6a and 6b).

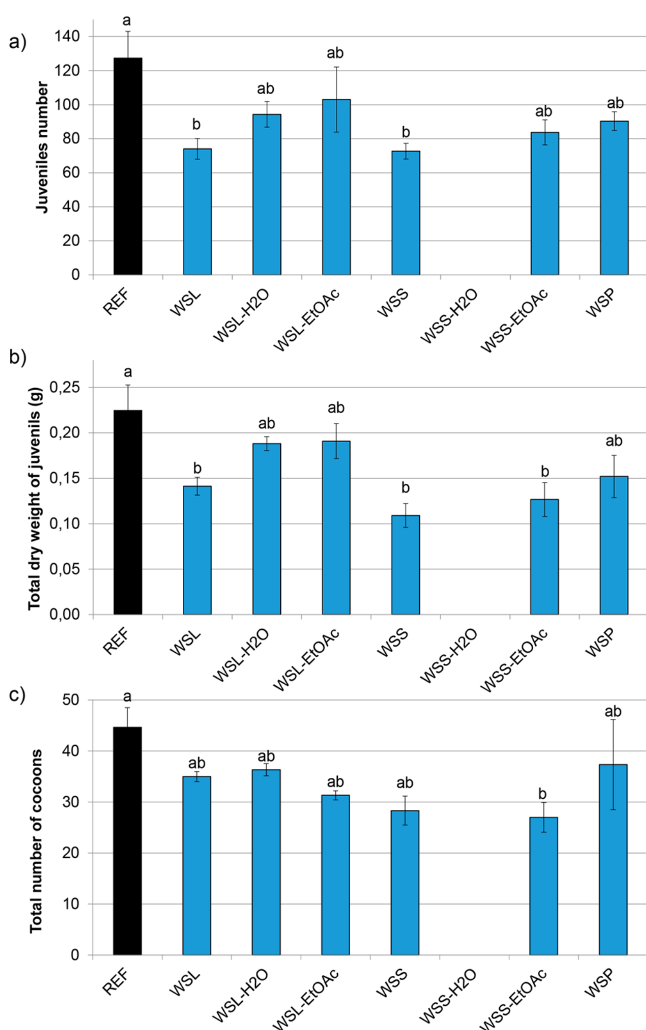

Figure 6. Effect of WS samples and their WS-H_2O and WS-EtOAc fractions applied into the soil on survival, growth, and reproduction of the earthworm *Eisenia andrei*: (a) the number of live juveniles at the end of the experiment, (b) the total dry weight of juveniles at the end of the experiment, (c) the total number of laid cocoons. Values are reported as mean ± standard error ($n = 3$). Treatments marked with the same letter (a or b) are not significantly different from each other ($p > 0.05$).

CONCLUSION

The valorization of agricultural lignocellulosic residues for obtaining products that can have a positive effect on agricultural practices themselves perfectly matches the principles of circular economy and waste reduction. Following such an approach, the present study reveals how pyrolysis liquids enriched in phenolic compounds can play a role in agriculture never reported before, opening the possibility of multiple exploitations of pyrolysis products in this field. Despite having different phenolic profiles that reflect the biomass origin, the pyrolysis liquids here investigated had similar inhibition effects on both soil urease and JBU. The same holds for the toxicity toward the biological endpoints tested, indicating that pyrolysis liquids with a heterogeneous composition in terms of individual chemical constituents behave homogeneously in terms of antiurease and (phyto)toxic activity. In particular, a dose of water-soluble pyrolysis products corresponding to a catechol concentration of 5 μg g^{-1} of soil was effective in inhibiting soil urease and was nonphytotoxic for *A. sativa* early growth and nontoxic for earthworm survival and reproduction; this was true for all the biomass tested, especially for the ethyl acetate fraction obtained after liquid−liquid separation of water-soluble pyrolysis products. These findings suggest that a variety of lignocellulosic waste and residues could be exploited for producing antiurease formulations useful for agricultural purposes. Finally, given the water solubility of the pyrolysis products here tested, modes of application similar to NBPT in the field could be adopted, like a direct addition to the soil or as a liquid formulation that coats urea granules for a more homogeneous cover and efficacy; future studies will be dedicated to investigating the best application mode in the field and the effects of pyrolysis products on the real environment, including relevant crops, soil fauna, and different types of soil.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.3c02162.

Mass balance of pyrolysis products, PAH analysis and quantification, catechol concentration in each WS sample, volume of WS samples used in each test, soil respirometry assay, chlorophyll analysis and photographs of *A. sativa* after exposure to WS samples and WS fractions, hatched cocoons of *E. andrei* after exposure to

WS samples and WS fractions, and detailed methods for soil urease inhibition assay and ecotoxicity tests (PDF)

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Author Contributions

The manuscript was written through the contributions of all authors. All authors have approved the final version of the manuscript. C.S., L.M., and A.P. have made substantial contributions to the conception and design of the study. E.G., L.M., and A.R. have made substantial contributions to the acquisition, collection, and assembly of data. C.S., E.G., L.M., and A.P. have made substantial contributions to the analysis and interpretation of data. C.S. and L.M. have made substantial contributions to the drafting of the article. P.G., C.S., A.B., and D.Z. have made substantial contributions to the critical revision of the article for important intellectual content.

Notes

The authors declare no competing financial interest.

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