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

Water-soluble pyrolysis products as novel urease inhibitors safe for plants and soil fauna

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ABSTRACT. Water-soluble compounds (WS) obtained by the pyrolysis of three lignocellulosic biomasses (larch, poplar and switchgrass) were tested as potential inhibitors of the enzyme urease. Due 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). Oat germination rate, early growth, and development were not affected by any WS sample tested at this lower dose, as well as 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.

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 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 co-formulations 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 di-methyl 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 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 challenging because of their low concentrations in the bio-oil but 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 bio-fuels 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, 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 anti-urease 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 sub-fractions, one soluble in ethyl acetate (WS-EtOAc) and the other one only soluble in water (WS-H_2O). These two sub-fractions 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 for the experiments.

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 grounded 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 the 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 co-axial 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 re-charged with other biomass and subjected to the same procedure until a total of 40-50 g of biomass were 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 sub-fractions 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: small oxygenates (like alcohols and carbonyl compounds i.e. hydroxyacetaldehyde), anhydrosugars/sugars (like levoglucosan), short-chain length carboxylic acids, phenolics and furans (like catechol and derivatives). The unidentifiable compounds were indicated as “unknown”. The quantitative analysis of catechol present in WS, WS-H_2O, and WS-EtOAc samples was 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 PHA 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 ESI for the corresponding volumes of each WS sample).

Soil urease inhibition assay. WS samples were tested at three concentrations of catechol: 0.5, 5, and 50 μg g^{-1} of soil (corresponding to concentrations of catechol of 3, 30, and 300 μM in the spiking solution, respectively) (see Table S1 in ESI 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 of 5 μg g^{-1} of soil were also tested. EtOAc was evaporated under N_2 from WS-EtOAc samples, and then the samples were re-suspended 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 96 μg g^{-1} of soil concentration. The soil urease activity was determined through the quantification of NH_3 produced by using a modified Kandeler and

151 Gerber method,[22] using dried soil samples (see ESI). Soil respiration was tested as an indicator of
152 microbial activity when WS samples were added to the soil (see ESI).

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

158 **Filter paper contact germination test**. Germination tests on cress (*Lepidium sativum* L.) seeds
159 were conducted in Petri dishes according to the procedure described in UNI 11357:2010 (see
160 ESI). Seed germination rate (%), shoot length (cm), and root length (cm) after 72 h were
161 reported.

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

169 **Earthworm reproduction test.** The earthworm *Eisenia andrei* Bouchè, 1972 was used to run a
170 56 days reproductive toxicity test according to the OECD Guideline No 222 (see ESI). The
171 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. Homogeneity of variance was confirmed using Cochran's C test. Whenever ANOVA detected significant differences, the Student-Newman-Keuls (SNK) post-hoc 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).

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 anti-urease 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 anti-urease 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 tpoplar and larch biomass was 2-3 times higher than WS obtained from switchgrass (see Figure S1a in ESI). 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 anti-urease activity of interest for the present work and reduce the presence of compounds that could have an adverse effect towards 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 ESI) 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 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, respectively. Vanillin and 4-hydroxybenzoic acid, belonging to the phenolic aldehydes and phenolic acids classes, were found in all samples, as 2-methylfuran among the furanic compounds.

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.

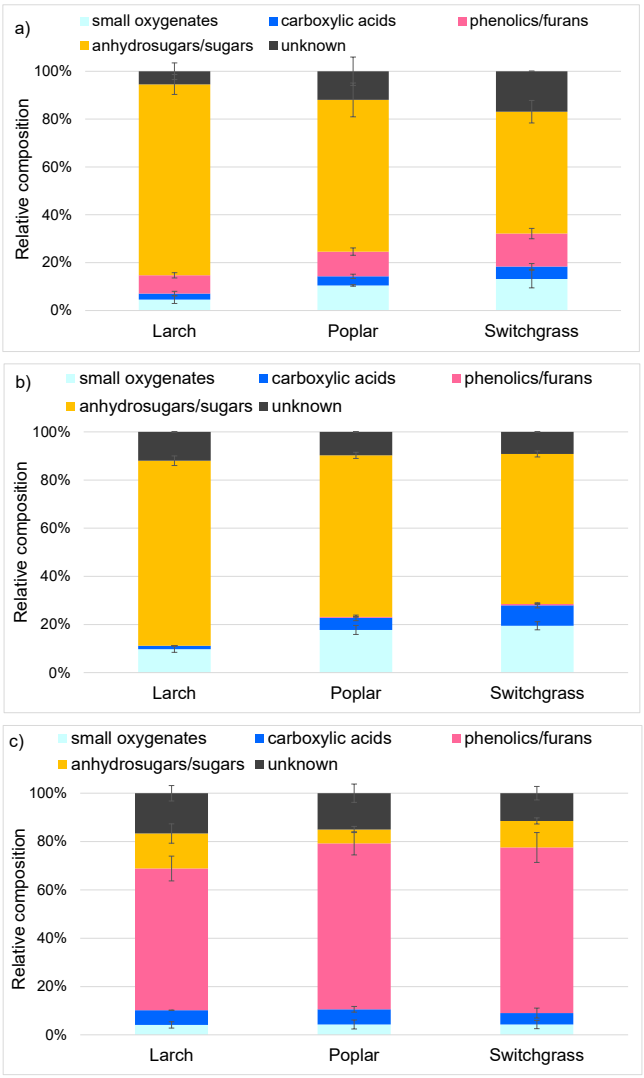


Table 1. Relative abundance of the main GC-MS detectable phenolic compounds found in the WS-EtOAc samples after liquid-liquid separation of WS samples from larch, poplar, and switchgrass biomass.

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

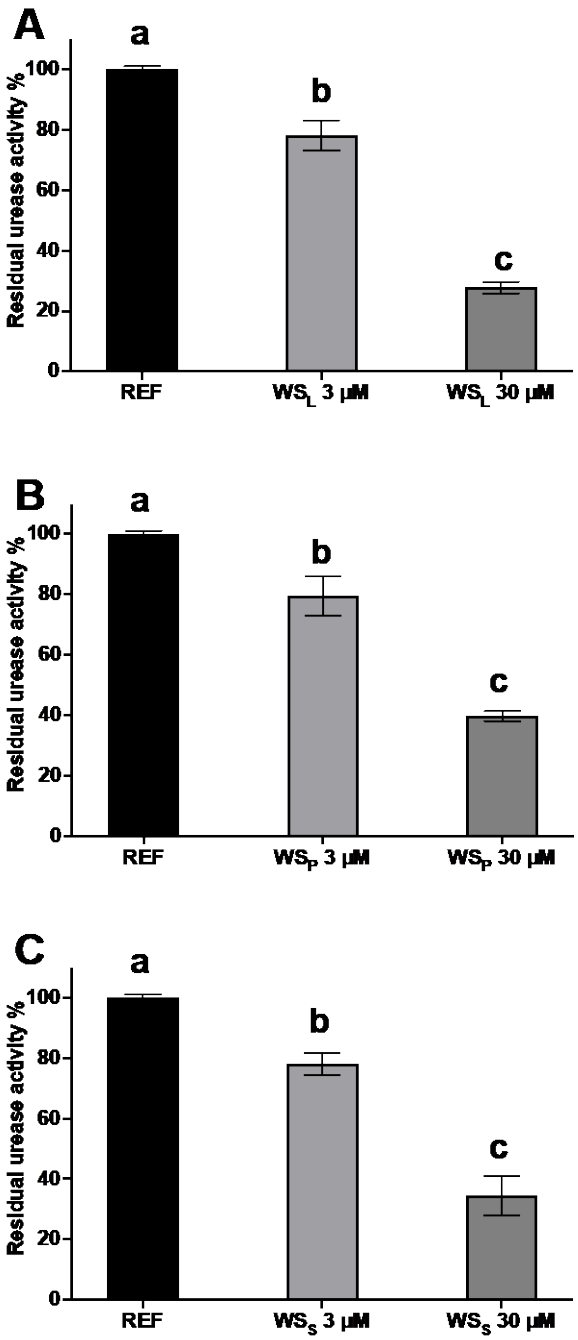
224

225 **Urease inhibition assays**

226 Given the presence of catechol and the pool of phenolic compounds that characterized each WS
227 sample, their capacity to inhibit urease *in vitro* was assessed as urease residual activity measured
228 in the presence of two concentrations of catechol, 3 and 30 μM, kept constant for each WS
229 sample (Figure 2). Catechol is a well-known urease inhibitor,[3,4,6] as well as some of its mono-
230 and di-substituted derivatives that are more active than catechol itself (e.g. 3-methyl catechol,
231 4,5-dimethyl catechol, 4-methyl catechol, and 3,4-dimethyl catechol). For this class of phenolic
232 compounds, a common mode of action has been demonstrated:[4] covalent adduct occurs between
233 the inhibitor and the thiol of a conserved cysteine residue located on a helix-turn-helix motif, the
234 latter flanking the active site cavity and directly involved in the catalytic mechanism through a
235 conformational change from an open to a closed state which in turn triggers the hydrolysis of
236 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 a *ca.* 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 with the anti-urease 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.

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) 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.



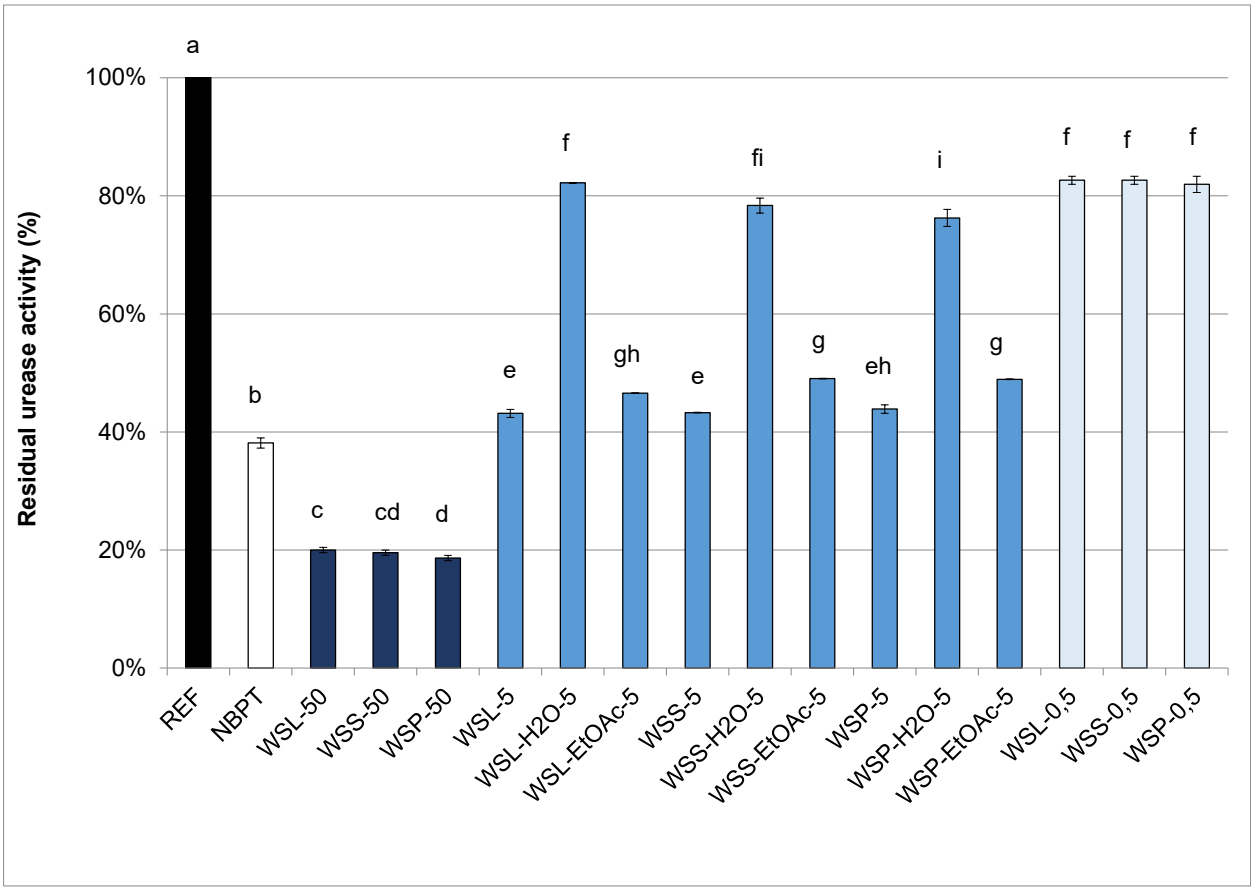
252

253 The inhibition of urease was also tested in a series of *in vivo* assays, by using the enzyme
254 naturally present in agricultural soils (Figure 3). Three concentrations of WS samples were
255 tested, i.e. 0.3, 3, and 30 µM of catechol in the spiking solution, corresponding to catechol
256 concentrations of 0.5, 5, and 50 µg g^{-1} of soil; the two fractions obtained through the liquid-liquid

257 separation of all the WS samples (i.e. WS-H_2O and WS-EtOAc) were also tested at the catechol
258 concentration of 5 μg g^{-1}. The results were compared with the inhibition activity of NBPT tested
259 at a concentration of 96 μg g^{-1}l. The urease activity was decreased by approximately 20% when
260 WS samples were tested at a concentration of catechol of 0.5 μg g^{-1} in comparison to the
261 experiment performed in the absence of WS, while these values increased up to approximately
262 60 and 80% when urease was treated with catechol concentrations of 5 and 50 μg g^{-1},
263 respectively. A dose-dependent mode of action was thus observed and no one of the tested WS
264 samples was statistically different from the other samples tested at the same concentration. The
265 80% inhibition of urease activity obtained with WS samples at a concentration of catechol of 50
266 μg g^{-1} was in line with the value reported by Bremner and Douglas (74% of inhibition),[6]
267 suggesting that catechol was the main inhibitor among the phenolic compounds present in the
268 WS mixtures. WS samples were not toxic for soil microorganisms when tested at a catechol
269 concentration of 5 μg g^{-1} (see Figure S2 in ESI), in line with the literature results,[12,17] indicating
270 that the effects observed were due to an actual inhibition of the enzyme urease rather than a
271 lethal effect on the microorganisms themselves. The inhibition behavior of WS-H_2O and WS-
272 EtOAc samples reflected their content in terms of GC-MS detectable phenolic compounds: the
273 urease activity was decreased by a ca. 50% with all the WS-EtOAc samples, while the inhibition
274 was about 20% when the WS-H_2O samples were tested. It is worth mentioning that, even if the
275 content of GC-MS detectable phenolic compounds in the WS-H_2O samples was negligible
276 (Figure 1c), a certain urease inhibition was observed, ascribable to non-phenolic compounds or
277 to phenolic compounds that are not GC-MS detectable; the inhibition potential of the compounds
278 present in the WS-H_2O samples was also evident from the comparison between WS-EtOAc
279 samples and WS samples: the latter included both the fractions and were more active against

urease than the first. The urease inhibition by NBPT, tested at a concentration of 96 μg g^{-1}, was 62%, slightly (but significantly) higher than the ones obtained with all WS samples tested at a concentration of catechol of 5 μg 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 g^{-1} of soil), and 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.



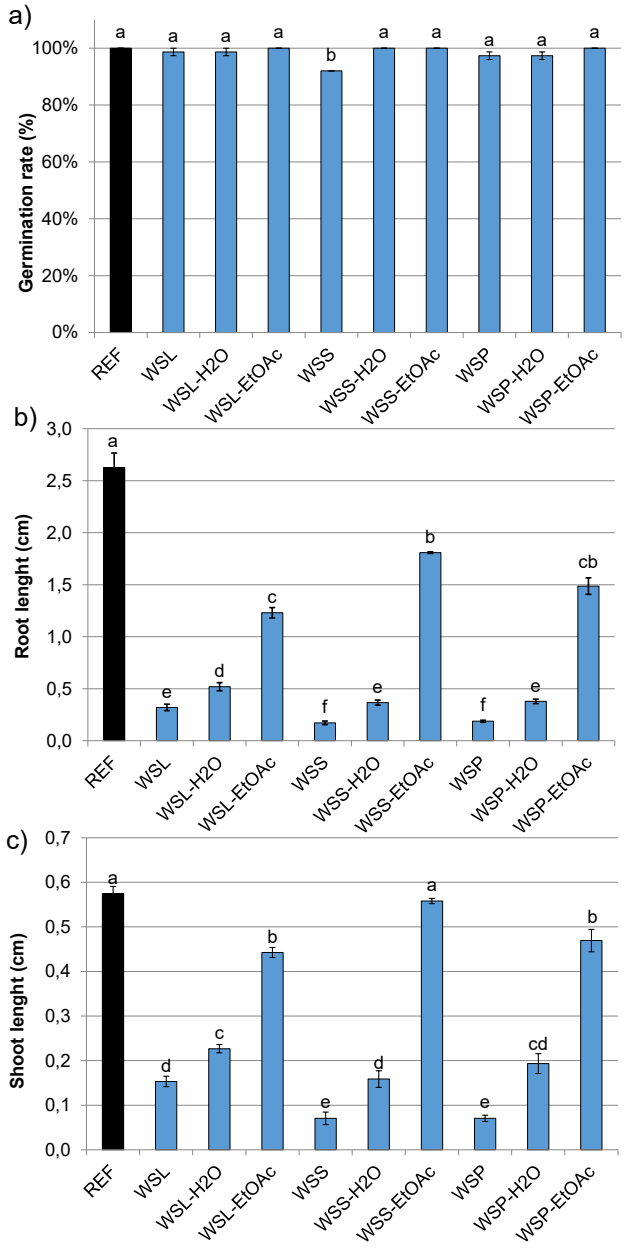
Phytotoxicity assays

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 (Figures 4b and 4c); 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. 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

314 samples, highlighting how low pH values cannot be the sole cause of the phytotoxicity here
315 observed.[27] This hypothesis is in line with the main causes of germination inhibition for various
316 plant seeds exposed to water extracts of biochar identified so far: i) the exposure to solutions
317 with a pH value <5, or ii) the presence of phenolic compounds. Even if PAHs are identified as
318 the main compounds responsible for the phytotoxicity of pyrolysis products, the negligible
319 concentrations here found in WS and WS-EtOAc samples (2-3 ng mL^{-1} for naphthalene and 0.5-
320 0.8 ng mL^{-1} for pyrene, at least 3 orders of magnitude lower than the phytotoxic doses reported
321 in the literature)[28] can exclude their role in the reduced root and shoot growth (see Table S2 in
322 ESI). It is worth mentioning that studies conducted to elucidate the phytotoxicity of water
323 extracts of biochar (i.e. aqueous solutions containing re-condensed pyrolysis liquids)[27]
324 highlighted that the volatile organic compounds present in pyrolysis liquids generally cause
325 delayed seed germination, thus reduced time for growth and reduced shoot and root length, rather
326 than negative effects on seed growth after germination (i.e. reduced shoot and root development
327 is a result of inhibition of germination). The high germination rates and the low root and shoot
328 development found here seem to not follow this hypothesis.

329

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



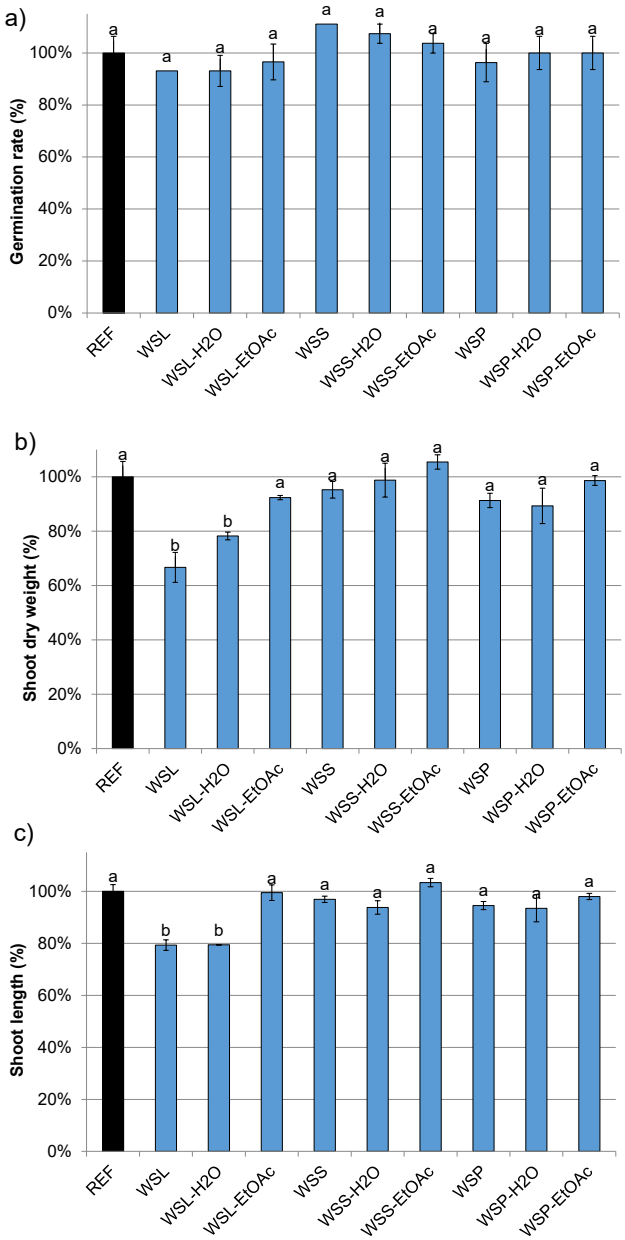
334

335 The effect on seedling emergence and early growth of higher plants was evaluated following
336 exposure to each WS sample and the corresponding WS-H_2O and WS-EtOAc fractions obtained
337 after liquid-liquid separation (Figure 5 and Figure S3 in ESI). The same WS dose used for the
338 soil urease inhibition assay (5 μg g^{-1} of soil) was used, and oat (*Avena sativa*) was chosen as the
339 test species. Independently on the endpoint tested (seed germination rate, shoot length and dry

340 weight, and chlorophyll content), no one of the tested samples gave values statistically different
341 from the control except the WS sample from larch biomass and its WS-H_2O fraction for which a
342 statistically significant 20% reduction of the shoot length and weight was observed after the
343 exposure. The root growth was not affected as well (see Figure S4 in ESI). Thus, in most cases,
344 the doses here applied did not show any phytotoxic effect and, as already noticed by other
345 authors for phenolic acids, these results showed that although the WS samples tested affected
346 germination and seedling growth in Petri dishes, these adverse effects are eliminated or strongly
347 attenuated in soil.[29] This is in line with the use of the so-called "wood vinegar" (i.e. the aqueous
348 liquid produced from slow pyrolysis of hardwood from which the tar is separated by
349 sedimentation) in agriculture as a fertilizer and growth-promoting agent since the 1930s.[30]

350

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



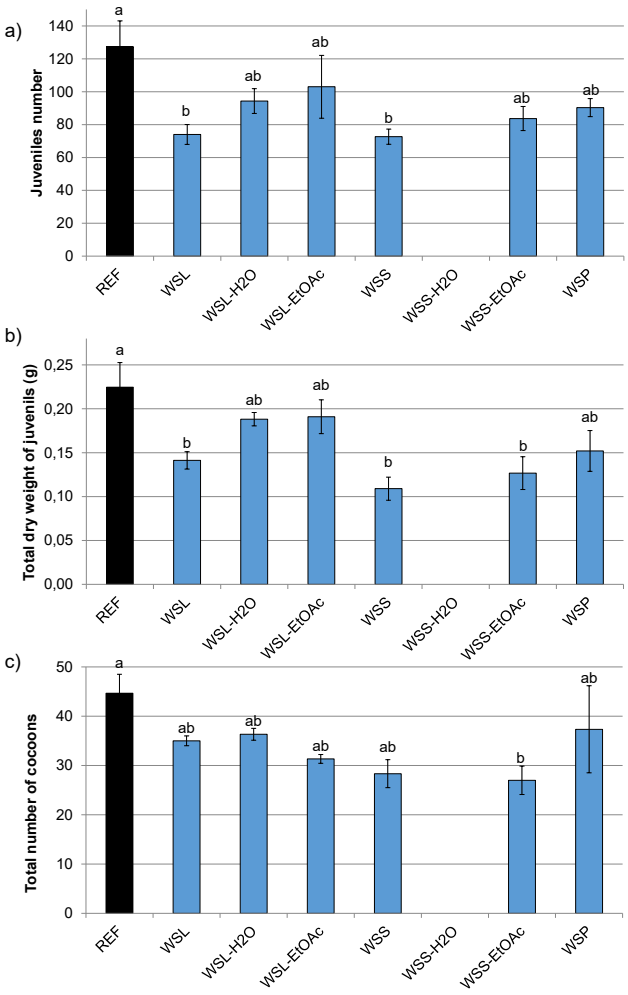
355

356 **Earthworm reproduction test**

357 The effect of WS samples and WS fractions on survival, growth, and reproduction of the
358 earthworm *Eisenia andrei* was assessed by testing the same doses of WS samples used for *A.*
359 *sativa* early growth tests (catechol concentration of 5 μg g^{-1} of soil) (Figure 6). Adult survival
360 was 100% in all treatments, except the WS-H_2O sample from switchgrass 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 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 ESI). 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.



380

381 **Conclusion**

382 The valorization of agricultural lignocellulosic residues for obtaining products that can have a
383 positive effect on agricultural practices themselves perfectly matches the principles of circular
384 economy and waste reduction. Following such an approach, the present study reveals how
385 pyrolysis liquids enriched in phenolic compounds can play a role in agriculture never reported
386 before, opening the possibility of multiple exploitations of pyrolysis products in this field. In
387 spite of having different phenolic profiles that reflect the biomass origin, the pyrolysis liquids
388 here investigated had similar inhibition effects on both soil urease and JBU. The same holds for

the toxicity towards the biological endpoints tested, indicating that pyrolysis liquids with a heterogeneous composition in terms of individual chemical constituents behave homogeneously in terms of anti-urease 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, non-phytotoxic for *A. sativa* early growth, non-toxic 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 anti-urease 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 agricultural crops, soil fauna and different types of soil.

ASSOCIATED CONTENT

Supporting Information. 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; detailed methods for soil urease inhibition assay and ecotoxicity tests.

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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., S.C., A.B. and D.Z. have made substantial contributions to the critical revision of the article for important intellectual content.

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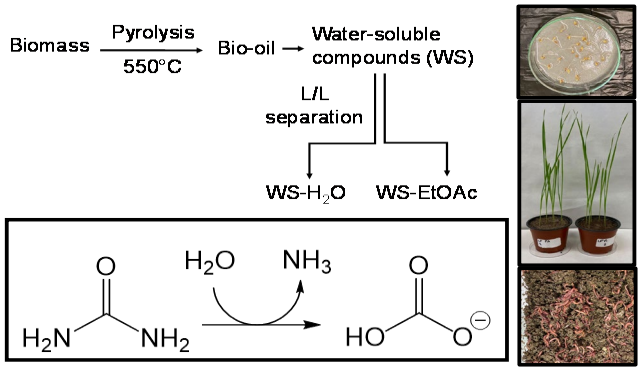

529

530 **SYNOPSIS**. Water-soluble pyrolysis products from lignocellulosic biomass are non-phytotoxic
531 urease inhibitors useful for agricultural applications.

532