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This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

*Published Version:*

Samori' C., Mazzei L., Guidi E., Buscaroli A., Pasteris A., Rombola' A., et al. (2023). Water-Soluble Pyrolysis Products as Novel Urease Inhibitors Safe for Plants and Soil Fauna. ACS SUSTAINABLE CHEMISTRY & ENGINEERING, 11(24), 9216-9224 [10.1021/acssuschemeng.3c02162].

*Availability:*

This version is available at: <https://hdl.handle.net/11585/954139> since: 2024-06-07

*Published:*

DOI: <http://doi.org/10.1021/acssuschemeng.3c02162>

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(Article begins on next page)

# 1 Water-soluble pyrolysis products as novel urease 2 inhibitors safe for plants and soil fauna

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12  
13 **KEYWORDS.** Lignocellulosic biomass; pyrolysis; ammonia reduction; anti-urease activity;  
14 soil; phytotoxicity; earthworms.

15 **ABSTRACT.** Water-soluble compounds (WS) obtained by the pyrolysis of three lignocellulosic  
16 biomasses (larch, poplar and switchgrass) were tested as potential inhibitors of the enzyme urease.  
17 Due to the presence of an array of phenolic compounds like catechol, methoxy/hydroxy phenols,  
18 phenolic acids and phenolic aldehydes, all the WS samples tested at a catechol concentration of 30  
19  $\mu\text{M}$  inhibited the activity of jack bean urease (JBU) by 60-70%, and by 80% that of urease naturally

20 present in the soil. A 10-times lower dose of WS samples (catechol concentration of 3  $\mu\text{M}$ )  
21 inhibited the activity of JBU by 20%, while that of soil urease by 50%, in line with the known  
22 inhibition of N-(n-butyl) thiophosphoric triamide (NBPT). Oat germination rate, early growth, and  
23 development were not affected by any WS sample tested at this lower dose, as well as cress  
24 germination rate, while the development of cress roots and shoots was lower than the control  
25 presumably because of the low pH of the tested WS solutions. Earthworm survival was not  
26 significantly affected by any WS sample tested, but an effect was observed on the ability of the  
27 eggs to develop into viable newborns.

28

## 29 INTRODUCTION

30 Soil urease is the enzyme responsible for the accelerated hydrolysis of urea-based fertilizers  
31 used in agriculture and the consequent formation of ammonia ( $\text{NH}_3$ ); it has been estimated that  
32 the release of  $\text{NH}_3$  accounts for 14% of N applied worldwide, with peaks of 40% in more humid  
33 environments like tropics.<sup>1</sup>  $\text{NH}_4^+$ -containing secondary aerosol can be formed when  $\text{NH}_3$  losses  
34 occur in the atmosphere, and this aerosol is the major fraction of PM<sub>2.5</sub> aerosol. Urea hydrolysis  
35 catalyzed by soil urease can also affect the soil compartment through the formation of  
36 ammonium carbonate which may temporarily cause a local increase of pH value in the area  
37 surrounding urea granules that can cause damage to germinating seedlings and young plants.<sup>2</sup>  
38 Moreover, when  $\text{NH}_3$  losses become relevant, more fertilizer is needed to achieve high crop  
39 yields implying significant economic issues. The use of urease inhibitors has become a  
40 widespread practice to reduce and mitigate the entity of this phenomenon: several synthetic  
41 compounds have proven a significant urease inhibition activity, but only N-(n-butyl)

42 thiophosphoric triamide (NBPT) and two derivatives (N-(n-propyl) thiophosphoric triamide,  
43 NPPT, and N-(2-nitrophenyl) phosphoric triamide, 2-NPT) are commercially used worldwide as  
44 co-formulations with urea.<sup>1</sup> These compounds have a structural analogy with urea and are  
45 capable of temporarily blocking soil ureases by binding to the Ni(II) ions in the active site of the  
46 enzyme, decreasing the urea hydrolysis rate. Other inhibitors containing a phenolic scaffold,  
47 such as catechol and its mono- and di-methyl derivatives, and hydroquinones, have been proven  
48 to inhibit urease by binding to a conserved cysteine residue located onto a mobile helix-turn-  
49 helix motif in the active site cavity.<sup>3-5</sup> Catechol, in particular, is one of the simplest molecules  
50 bearing a phenolic structure identified as a powerful inhibitor of soil urease, capable of inhibiting  
51 more than 70% of the activity even at low concentrations.<sup>6</sup> Even if several phenolic compounds  
52 have marked inhibitory effects on urease activity in the soil, other natural macrostructures  
53 containing many phenolic moieties like lignin, tannins, and humic acids seem not to behave  
54 likewise, presumably because of their lower water solubility than single phenolic units.<sup>6</sup> In  
55 particular, the polyphenolic structure of lignin is chemically very stable, and therefore, difficult  
56 to transform or to be structurally modified without the application of harsh reaction conditions,  
57 like high temperatures (i.e. pyrolysis) or the use of strong bases (i.e. the Kraft process). Pyrolysis  
58 is one of the most investigated technologies for directly liquefying lignocellulosic material to a  
59 crude bio-oil enriched in a mixture of compounds derived from cellulose, hemicellulose and  
60 lignin, like anhydrosugars, furans, phenols, and carboxylic acids. Separating such a variety of  
61 molecules into single chemicals or chemical classes is challenging because of their low  
62 concentrations in the bio-oil but highly desirable in a biorefinery approach. The main application  
63 of crude bio-oils, as unseparated mixtures of chemicals with different moieties, is in the field of  
64 bio-fuels but, given the abundance of oxygen-containing functional groups, an upgrading (e.g. by

65 hydrodeoxygenation or zeolite cracking) is mandatory to improve their stability over time and  
66 the heating value.<sup>7</sup> Another use of bio-oils is as a carbon source for fermentative processes<sup>8-10</sup>  
67 but, also in this case, an upgrading (e.g. by liquid-liquid extraction or adsorption on activated  
68 carbon) for detoxifying the mixture leaving just fermentable compounds like  
69 sugars/anhydrosugars is required.<sup>8-10</sup> Furans and phenols are known to be toxic, so bio-oils from  
70 various lignocellulosic feedstock have been also used against various biological targets (e.g.  
71 crustacea, algae, weeds, insects, nematodes, bacteria, cells) as pesticides.<sup>11-17</sup> In the present  
72 paper, we aimed at increasing the knowledge on the biological properties of bio-oils from  
73 lignocellulosic feedstock against urease, a target that was never investigated before, by  
74 exploiting the known anti-urease activity of phenolic compounds that are abundant in bio-oils of  
75 lignin-rich biomass. To this purpose, the bio-oils obtained from the pyrolysis of three  
76 lignocellulosic biomass (switchgrass, larch, and poplar) were fractionated into water-soluble  
77 fractions and water-insoluble tars. The fractions containing the water-soluble pyrolysis products  
78 (WS) were further separated by liquid-liquid separation into two sub-fractions, one soluble in  
79 ethyl acetate (WS-EtOAc) and the other one only soluble in water (WS-H<sub>2</sub>O). These two sub-  
80 fractions and the whole WS samples were then tested against urease, plants, and soil earthworms.  
81 The intent was to prepare novel formulations useful for agricultural purposes that should not  
82 damage plant germination and growth or adversely affect soil fauna.

83

## 84 **MATERIALS AND METHODS**

85 **Chemicals, biomass, and soil.** All chemicals and solvents were purchased from Sigma Aldrich  
86 and used without any further purification. Jack bean urease (*Canavalia ensiformis*, JBU) Type C-  
87 3, powder,  $\geq 600$  units  $\text{mg}^{-1}$  solid, was used for the experiments.

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

93 A surface soil sample for the soil urease assay (0-20 cm) was collected from an apple orchard  
94 located in Ravenna (Italy). The soil, classified as Udifluventic Haplustept,<sup>18</sup> displayed the  
95 following characteristics: silty clay loam texture, pH 8.5, electrical conductivity (EC)  $0.17\text{ dS m}^{-1}$ ,  
96  $\text{CaCO}_3$  tot.  $203\text{ g kg}^{-1}$ , total organic carbon (TOC)  $11.3\text{ g kg}^{-1}$ , and total nitrogen (TN)  $1.3\text{ g kg}^{-1}$ .<sup>19</sup>  
97 After removing plant roots, debris, and the visible fauna, the soil sample was air-dried in the  
98 dark at room temperature, then crushed with a mortar, sieved ( $< 2\text{ mm}$ ) and stored in  
99 polyethylene bags at  $4^{\circ}\text{C}$ .

100

101 **Pyrolysis and pyrolysis product characterization.** Biomass was subjected to bench-scale  
102 pyrolysis using an apparatus consisting of a sliding sample carrier placed in a heated quartz tube  
103 connected to ice traps and a settling chamber. The quartz tube was heated by a cylindrical co-  
104 axial furnace and purged by  $1.5\text{ L min}^{-1}$   $\text{N}_2$  flow. The biomass sample (5-6 g for each pyrolysis)  
105 was moved into the heated zone of the quartz tube and heated for 20 min at  $550^{\circ}\text{C}$  (measured  
106 temperature) under  $\text{N}_2$  flow. The resulting char was collected and ground to powder in a mortar,

107 and then the sliding sample carrier was re-charged with other biomass and subjected to the same  
108 procedure until a total of 40-50 g of biomass were pyrolyzed. Bio-oil produced from such a  
109 series of pyrolysis was collected in an ice trap with 50 mL of water. The component of the bio-  
110 oil soluble in water (water-soluble pyrolysis products) was hereafter called WS (indicated as  
111  $WS_L$ ,  $WS_P$ , and  $WS_S$  from larch, poplar, and switchgrass biomass in Figures 2-6), while the  
112 water-insoluble part (tar or pyrolytic lignin) was hereafter called PL. PL was recovered after  
113 washing with acetone all the apparatus (the trap and the quartz tube), and then evaporating  
114 acetone. The concentration of WS in water was determined by sampling aliquots of 0.1 mL and  
115 then drying them under nitrogen. The liquid-liquid separation of WS (10 mL) was performed  
116 with ethyl acetate (10 mL, two times): the resulting two sub-fractions were hereafter called WS-  
117  $H_2O$  and WS-EtOAc. The qualitative profiles of WS, WS- $H_2O$ , and WS-EtOAc samples were  
118 determined by GC-MS analysis after drying under nitrogen each sample (0.1 mL) and silylation  
119 (60 min at 70°C with 0.1 mL acetonitrile, 0.08 mL bis(trimethylsilyl)trifluoroacetamide  
120 containing 1% of trimethylchlorosilane, and 0.04 mL of pyridine).<sup>17</sup> Compounds were identified  
121 by comparison with the NIST database and grouped into four categories: small oxygenates (like  
122 alcohols and carbonyl compounds i.e. hydroxyacetaldehyde), anhydrosugars/sugars (like  
123 levoglucosan), short-chain length carboxylic acids, phenolics and furans (like catechol and  
124 derivatives). The unidentifiable compounds were indicated as “unknown”. The quantitative  
125 analysis of catechol present in WS, WS- $H_2O$ , and WS-EtOAc samples was performed by GC-  
126 MS analysis<sup>17</sup> using a calibration curve prepared with silylated catechol (0.67-67  $\mu\text{g mL}^{-1}$ ). The  
127 concentration of catechol in each sample was used to determine the amount of WS samples to be  
128 tested in the urease assays and in the ecotoxicity tests. The analysis of polycyclic aromatic  
129 hydrocarbons (PAH) was performed on WS and WS-EtOAc samples according to the literature,

130 by using a deuterated PHA standard mix (acenaphthene-d10 was utilized to quantify  
131 naphthalene, acenaphthylene, acenaphthene, and fluorene; phenanthrene-d10 to quantify  
132 phenanthrene, anthracene, fluoranthene, and pyrene; chrysene-d12 to quantify the remaining  
133 PAHs).<sup>20</sup>

134 ***In vitro* urease inhibition assay.** The activity of *Canavalia ensiformis* (jack bean) urease (JBU)  
135 in the absence and the presence of WS samples was determined by using the pH-STAT method  
136 in 2 mM HEPES buffer at pH 7.5, also containing 2 mM EDTA, following an already reported  
137 protocol in which a preincubation time of 2 h was adopted.<sup>21</sup> WS samples were tested at two  
138 doses, corresponding to two concentrations of catechol (3 or 30  $\mu\text{M}$ ) (see Table S1 in ESI for the  
139 corresponding volumes of each WS sample).

140 **Soil urease inhibition assay.** WS samples were tested at three concentrations of catechol: 0.5, 5,  
141 and 50  $\mu\text{g g}^{-1}$  of soil (corresponding to concentrations of catechol of 3, 30, and 300  $\mu\text{M}$  in the  
142 spiking solution, respectively) (see Table S1 in ESI for the volumes of each WS sample). The  
143 WS-EtOAc and residual WS-H<sub>2</sub>O fractions coming from the liquid-liquid separation of aliquots  
144 of WS samples corresponding to a concentration of catechol of 5  $\mu\text{g g}^{-1}$  of soil were also tested.  
145 EtOAc was evaporated under N<sub>2</sub> from WS-EtOAc samples, and then the samples were re-  
146 suspended in the same amount of water as the initial WS sample before use. Therefore, we  
147 obtained three different WS samples: the initial one (WS), the WS-EtOAc fraction, and the  
148 residual WS-H<sub>2</sub>O fraction. NBPT (N-(n-butyl) thiophosphoric triamide) was tested as the  
149 reference urease inhibitor at 96  $\mu\text{g g}^{-1}$  of soil concentration. The soil urease activity was  
150 determined through the quantification of NH<sub>3</sub> produced by using a modified Kandeler and



151 Gerber method,<sup>22</sup> 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  $\mu\text{g g}^{-1}$  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<sub>2</sub>O 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 ( $\text{mg g}^{-1}$ , after extraction with acetone and spectrophotometric analysis at 750  
168 and 665 nm),<sup>23,24</sup> 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

172 weight of adults, the number and weight of juvenile earthworms, and the number of both hatched  
173 and unhatched cocoons at the end of the test.

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

181

## 182 **RESULTS AND DISCUSSION**

### 183 **Characterization and fractionation of pyrolysis products**

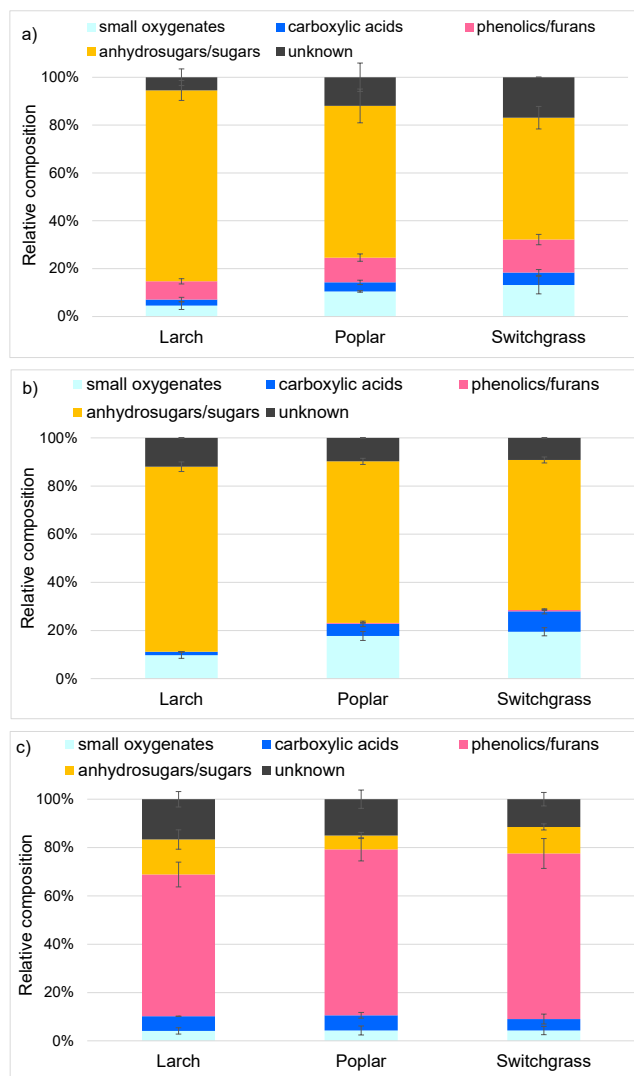
184 In the present work, poplar, larch, and switchgrass biomass were used to prepare the  
185 corresponding bio-oils to be tested as anti-urease formulations; the three types of lignocellulosic  
186 biomass belong to the classes of hardwood, softwood, and herbaceous biomass, respectively,  
187 known to have different lignin compositions (e.g. different monolignol ratios) and therefore  
188 potential precursors of phenolic compound mixtures with different anti-urease effects. The  
189 intermediate pyrolysis conditions here applied gave similar amounts of char and PL, independent  
190 of the type of biomass treated, while the amount of WS obtained from poplar and larch biomass  
191 was 2-3 times higher than WS obtained from switchgrass (see Figure S1a in ESI). The relative  
192 composition of WS samples from the three biomasses was largely dominated by anhydrosugars,  
193 like levoglucosan, and in minor amounts by sugars (Figure 1a), reaching 80% of the total GC-

194 MS detectable compounds in the case of WS sample from larch, while the furanic derivatives  
195 and the phenolic compounds ranged between 8 and 14%. Catechol was the main compound  
196 identified in the class of aromatic compounds; its concentration was 5.8, 5.4, and 7.8  $\mu\text{g mg}^{-1}$  in  
197 WS samples from larch, poplar, and switchgrass biomass, respectively.

198 Since all WS samples were acid (pH 3.5-3.7) due to the presence of short-chain length  
199 carboxylic acids, like acetic and glycolic acid,<sup>25</sup> and such an acidity could negatively impact seed  
200 germination or earthworm survival and reproduction, a liquid-liquid separation was applied to  
201 enrich the samples in those phenolic compounds with a potential anti-urease activity of interest  
202 for the present work and reduce the presence of compounds that could have an adverse effect  
203 towards other biological targets like plants and soil invertebrates. The liquid-liquid separation of  
204 all the WS samples with ethyl acetate gave three fractions soluble in ethyl acetate (WS-EtOAc)  
205 that corresponded to about 40% of each WS (see Figure S1b in ESI) and contained considerable  
206 amounts of low-molecular-weight phenolic components (Figure 1c): phenols, catechols, and  
207 guaiacols covered 60-70% of the relative distribution of the GC-MS detectable compounds,  
208 while their presence in the WS-H<sub>2</sub>O samples was below 1% (Figure 1b). In turn, WS-H<sub>2</sub>O  
209 samples were enriched in anhydrosugars and sugars (70-80%) and small oxygenated compounds  
210 like hydroxyacetaldehyde (10-20%). Catechol and phenolic compounds with methoxy and  
211 hydroxylic groups were the main components of the three WS-EtOAc samples (Table 1),  
212 representing 90, 50 and 66% of all the GC-MS detectable aromatic compounds found in the WS-  
213 EtOAc samples from larch, poplar, and switchgrass, respectively. Vanillin and 4-hydroxybenzoic  
214 acid, belonging to the phenolic aldehydes and phenolic acids classes, were found in all samples,  
215 as 2-methylfuran among the furanic compounds.

216

217 **Figure 1.** Relative composition (%) of the GC-MS detectable compounds found in a) WS  
218 samples before the liquid-liquid separation, b) WS-H<sub>2</sub>O, and c) WS-EtOAc samples.



219

220

221 **Table 1.** Relative abundance of the main GC-MS detectable phenolic compounds found in the  
222 WS-EtOAc samples after liquid-liquid separation of WS samples from larch, poplar, and  
223 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
<i>Total methoxy/hydroxy phenols</i>	<i>88.7</i>	<i>50.9</i>	<i>65.5</i>
vanillin	2.2	1.9	1.8
3,5-dimethoxy-4-hydroxybenzaldehyde	-	4.4	-
4-hydroxybenzaldehyde	-	-	4.6
<i>Total phenolic aldehydes</i>	<i>2.2</i>	<i>6.2</i>	<i>6.3</i>
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
<i>Total phenolic acids</i>	<i>1.2</i>	<i>9.0</i>	<i>2.6</i>
2-methylfuran	5.7	5.3	9.3
3-methyl-2-furoic acid	1.7	-	-
<i>Total furans</i>	<i>7.4</i>	<i>5.3</i>	<i>9.3</i>
unknown	0.5	28.5	16.1

224

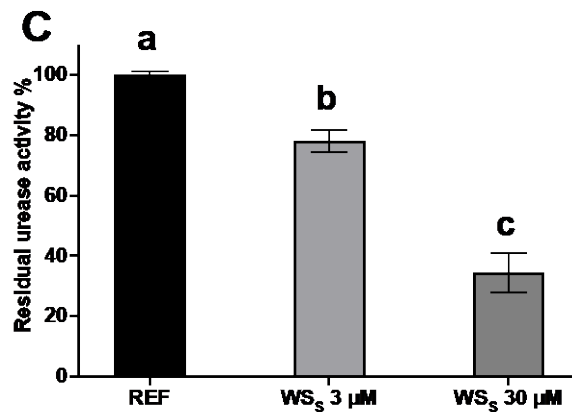
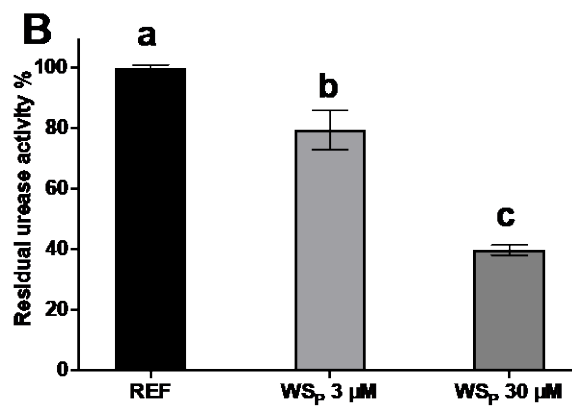
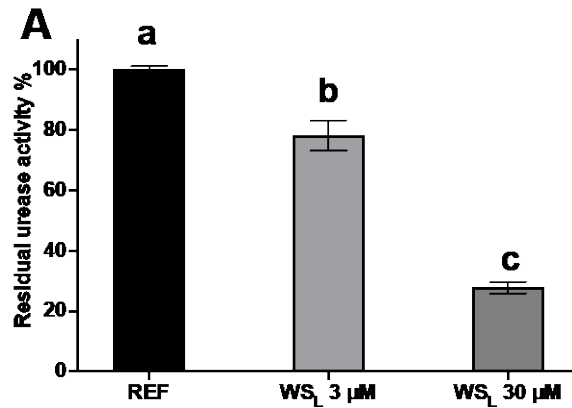
## 225 Urease inhibition assays

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  $\mu$ M, kept constant for each WS  
 229 sample (Figure 2). Catechol is a well-known urease inhibitor,<sup>3,4,6</sup> 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:<sup>4</sup> 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

237 state, thus hampering the hydrolytic event to occur. In all three enzyme-WS mixtures, urease  
238 activity was strongly decreased in a concentration-dependent manner. In particular, urease  
239 activity was decreased by a *ca.* 20 % when WS samples were tested at a catechol concentration  
240 of 3  $\mu$ M in comparison to the experiment performed in the absence of WS, while these values  
241 increased up to 60-70 % when urease was treated with the highest concentration of catechol (30  
242  $\mu$ M). These results were in line with the anti-urease activity of a variety of catechol derivatives  
243 tested at 30  $\mu$ M, highlighting how the pool of phenolic compounds found here in each WS  
244 sample positively contributed to the inhibition of the enzyme with their different moieties in  
245 different positions of the aromatic ring.

246

247 **Figure 2.** Residual percentage activity of urease after preincubation of 2 h, referred to 100 %  
248 (control) in the presence of two doses of WS samples from larch (A), poplar (B), and switchgrass  
249 (C) corresponding to 3 and 30  $\mu$ M of catechol. Values were reported as mean  $\pm$  standard error (n  
250 = 3). Treatments marked with different letters (a, b, and c) were significantly different from each  
251 other.



252

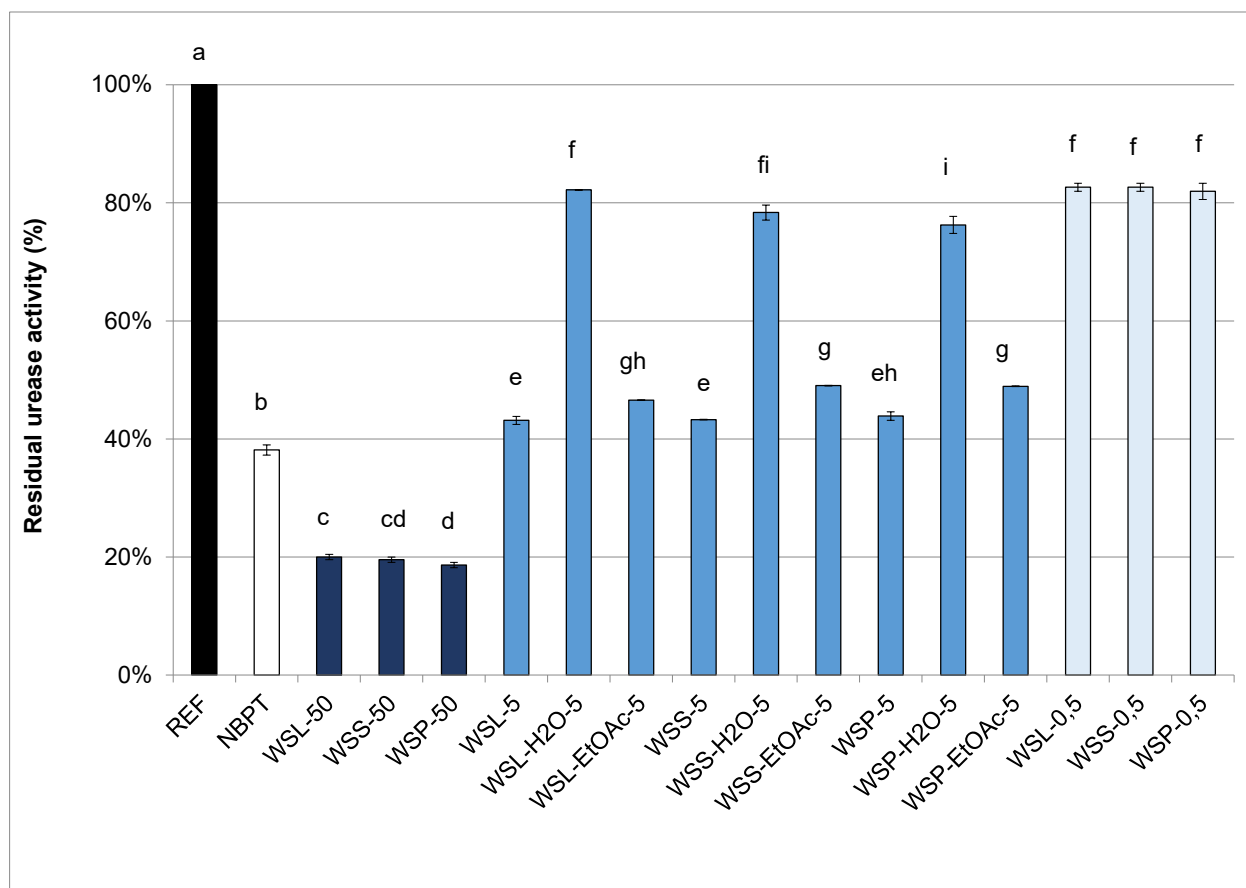
253 The inhibition of urease was also tested in a series of *in vivo* assays, by using the enzyme  
 254 naturally present in agricultural soils (Figure 3). Three concentrations of WS samples were  
 255 tested, i.e. 0.3, 3, and 30 μM of catechol in the spiking solution, corresponding to catechol  
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



257 separation of all the WS samples (i.e. WS-H<sub>2</sub>O and WS-EtOAc) were also tested at the catechol  
258 concentration of 5 µg g<sup>-1</sup>. The results were compared with the inhibition activity of NBPT tested  
259 at a concentration of 96 µg g<sup>-1</sup>. The urease activity was decreased by approximately 20% when  
260 WS samples were tested at a concentration of catechol of 0.5 µg g<sup>-1</sup> 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<sup>-1</sup>,  
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<sup>-1</sup> was in line with the value reported by Bremner and Douglas (74% of inhibition),<sup>6</sup>  
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<sup>-1</sup> (see Figure S2 in ESI), in line with the literature results,<sup>12,17</sup> 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<sub>2</sub>O 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<sub>2</sub>O samples were tested. It is worth mentioning that, even if the  
275 content of GC-MS detectable phenolic compounds in the WS-H<sub>2</sub>O 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<sub>2</sub>O 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

280 urease than the first. The urease inhibition by NBPT, tested at a concentration of 96  $\mu\text{g g}^{-1}$ , was  
 281 62%, slightly (but significantly) higher than the ones obtained with all WS samples tested at a  
 282 concentration of catechol of 5  $\mu\text{g g}^{-1}$  ( $57\pm 0.4\%$  on average).

283  
 284 **Figure 3.** Residual percentage activity of soil urease referred to 100% (control) in the presence  
 285 of three doses of WS samples (corresponding to 0.5, 5, and 50  $\mu\text{g}$  of catechol  $\text{g}^{-1}$  of soil), and  
 286 their WS-H<sub>2</sub>O and WS-EtOAc fractions, and NBPT. Values were reported as mean  $\pm$  standard  
 287 error (n = 4). Treatments marked with the same letter (a-h) were not significantly different from  
 288 each other.



289

290

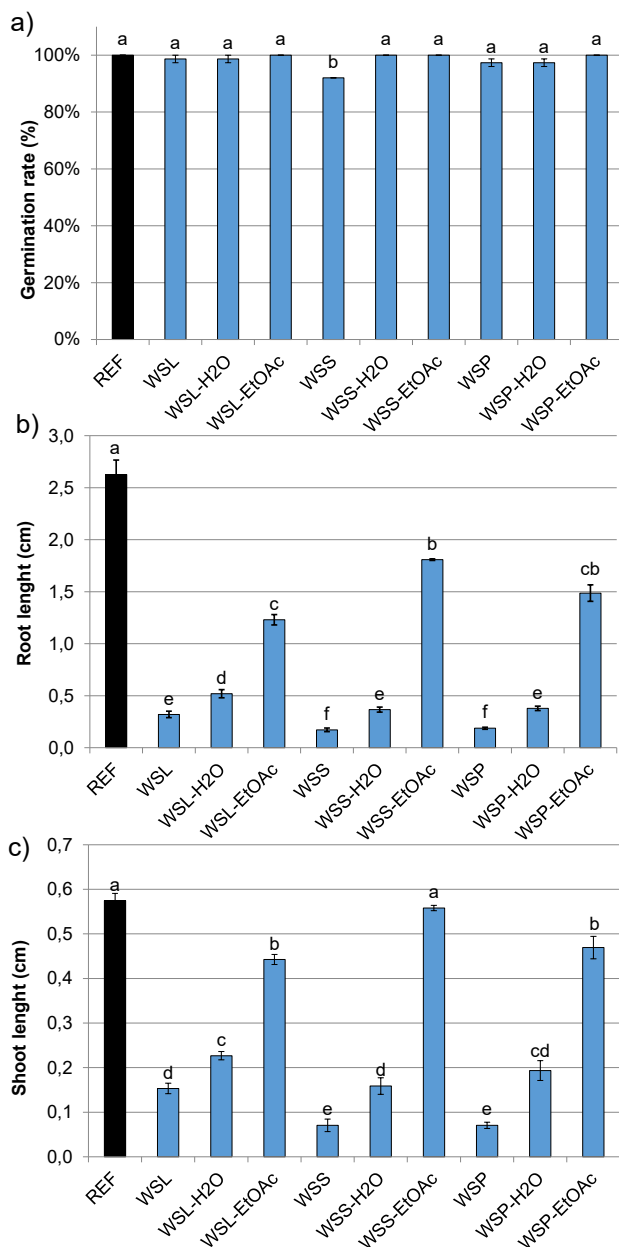
## 291 **Phytotoxicity assays**

292 The impact on cress (*Lepidium sativum*) seed germination was determined by testing the effect  
293 of each WS sample and the corresponding WS-H<sub>2</sub>O and WS-EtOAc fractions obtained after  
294 liquid-liquid separation. The same WS concentration used for the *in vitro* urease inhibition assay  
295 corresponding to a catechol concentration of 30 μM was used (Figure 4). Neither WS samples  
296 nor their fractions influenced the germination rate (Figure 4a), except for the WS sample from  
297 switchgrass that gave a germination rate of 92%, which was significantly lower than the control  
298 and the other treatments. This result was in line with the data obtained after exposure of *Carum*  
299 *carvi* seeds to a concentration of slow pyrolysis liquids of 5%.<sup>12</sup> On the other hand, both root and  
300 shoot lengths were significantly lower than the control with all samples tested with the exception  
301 of the shoot length obtained after the treatment with the WS-EtOAc fraction from switchgrass  
302 (Figures 4b and 4c); the effect measured after the treatment with WS samples was the most  
303 intense among the tested treatments, with an inhibition of 90 and 70-80% of the root and shoot  
304 development, respectively. All the WS-H<sub>2</sub>O samples decreased root length by 80%, while the  
305 shoots were 60-70% shorter than the control. The WS-EtOAc samples were the least toxic  
306 samples tested, both on root and shoot growth: the root lengths were 30-40% lower than the  
307 control values while the shoot lengths were just 20% shorter or not significantly different from  
308 the control, as in the case of WS-EtOAc sample from switchgrass. A possible explanation for  
309 these observations can rely on the presence of short-chain carboxylic acids, known to be  
310 phytotoxic,<sup>12</sup> which can be responsible for the lower pH values of WS and WS-H<sub>2</sub>O solutions  
311 measured at the beginning of the test (3.5±0.1 and 3.7±0.1, respectively) than the ones of WS-  
312 EtOAc solutions (4.4±0.1). The stronger effect of WS samples on the tested seeds could be a  
313 joint effect of organic acids and phenolic compounds,<sup>26</sup> the latter not present in the WS-H<sub>2</sub>O

314 samples, highlighting how low pH values cannot be the sole cause of the phytotoxicity here  
315 observed.<sup>27</sup> 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<sup>-1</sup> for naphthalene and 0.5-  
320 0.8 ng mL<sup>-1</sup> for pyrene, at least 3 orders of magnitude lower than the phytotoxic doses reported  
321 in the literature)<sup>28</sup> 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)<sup>27</sup>  
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<sub>2</sub>O 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.



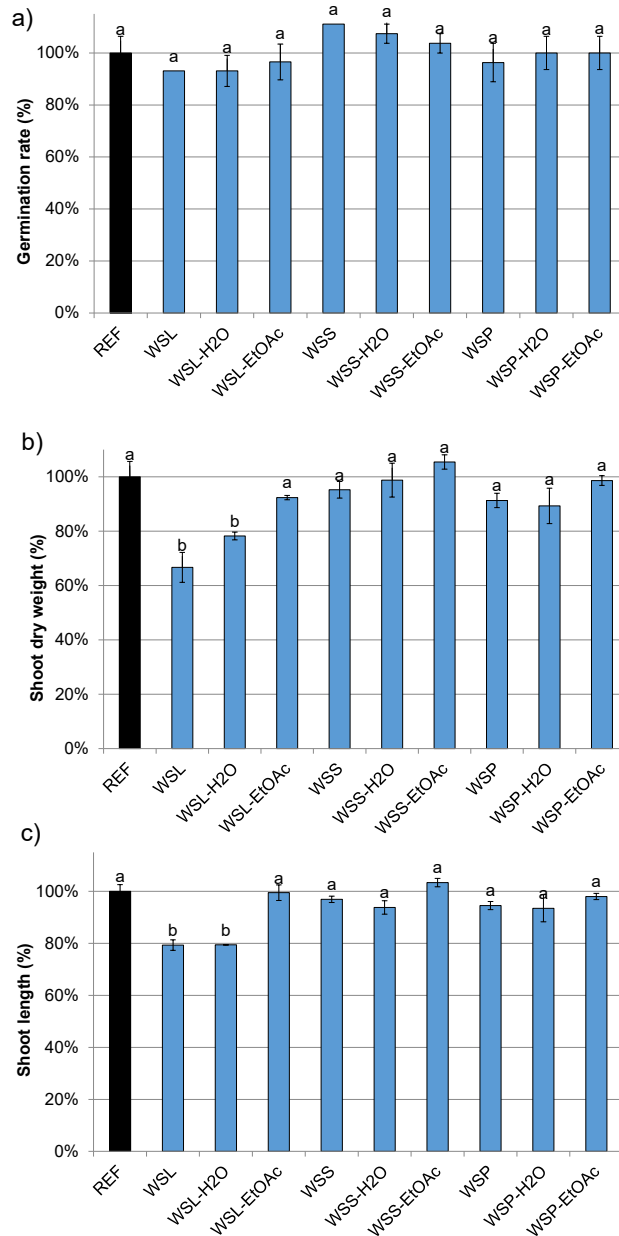
334

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<sub>2</sub>O 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<sup>-1</sup> 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<sub>2</sub>O 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.<sup>29</sup> 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.<sup>30</sup>

350

351 **Figure 5.** Effect of WS samples and their WS-H<sub>2</sub>O 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  $\mu\text{g g}^{-1}$  of soil) (Figure 6). Adult survival  
 360 was 100% in all treatments, except the WS-H<sub>2</sub>O sample from switchgrass where dead worms

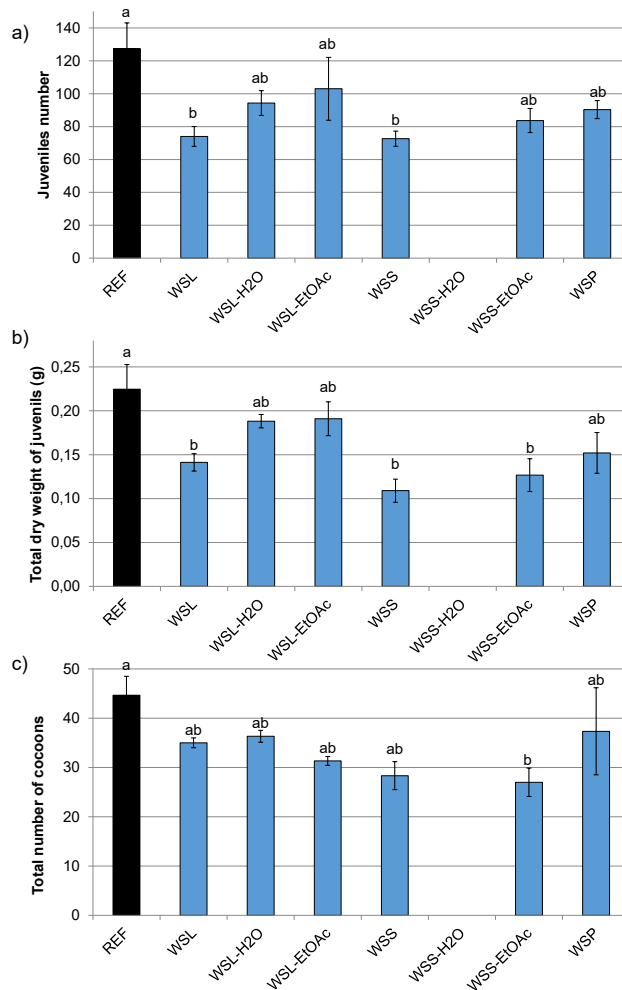
361 laying at the soil surface were observed since the first days and where no individuals survived to  
362 the end of the exposure. The initial mean live weight of individual adults was 575 mg and  
363 increased by 26% by the end of the exposure, without statistically significant differences among  
364 treatments. Even if slightly lower, the total number of laid cocoons was not significantly  
365 different from the control in any treatment where the adults survived; the null value for the WS-  
366 H<sub>2</sub>O sample was a direct consequence of the complete mortality of the parent adults (Figure 6c).  
367 The same holds for the percentage of hatched (empty) cocoons (Figure S5 in ESI). A reduction  
368 in the number and total dry weight of juveniles recovered on day 56 was observed for all the  
369 treatments even if the observed values were significantly different from the control only for WS  
370 samples from larch and switchgrass, biomass, and WS-EtOAc fraction from switchgrass  
371 biomass, due to the variability within treatments (Figures 6a and 6b).

372

373 **Figure 6.** Effect of WS samples and their WS-H<sub>2</sub>O and WS-EtOAc fractions applied into the soil  
374 on survival, growth, and reproduction of the earthworm *Eisenia andrei*: a) the number of live  
375 juveniles at the end of the experiment, b) the total dry weight of juveniles at the end of the  
376 experiment; c) the total number of laid cocoons. Values are reported as mean  $\pm$  standard error (n  
377 = 3). Treatments marked with the same letter (a or b) are not significantly different from each  
378 other.

379





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

389 the toxicity towards the biological endpoints tested, indicating that pyrolysis liquids with a  
390 heterogeneous composition in terms of individual chemical constituents behave homogeneously  
391 in terms of anti-urease and (phyto)toxic activity. In particular, a dose of water-soluble pyrolysis  
392 products corresponding to a catechol concentration of 5  $\mu\text{g g}^{-1}$  of soil was effective in inhibiting  
393 soil urease, non-phytotoxic for *A. sativa* early growth, non-toxic for earthworm survival and  
394 reproduction; this was true for all the biomass tested, especially for the ethyl acetate fraction  
395 obtained after liquid-liquid separation of water-soluble pyrolysis products. These findings  
396 suggest that a variety of lignocellulosic waste and residues could be exploited for producing anti-  
397 urease formulations useful for agricultural purposes. Finally, given the water solubility of the  
398 pyrolysis products here tested, modes of application similar to NBPT in the field could be  
399 adopted, like a direct addition to the soil or as a liquid formulation that coats urea granules for a  
400 more homogeneous cover and efficacy; future studies will be dedicated to investigating the best  
401 application mode in the field and the effects of pyrolysis products on the real environment,  
402 including relevant agricultural crops, soil fauna and different types of soil.

403

#### 404 **ASSOCIATED CONTENT**

405 **Supporting Information.** Mass balance of pyrolysis products; PAH analysis and  
406 quantification; catechol concentration in each WS sample; volume of WS samples used in each  
407 test; soil respirometry assay; chlorophyll analysis and photographs of *A. sativa* after exposure to  
408 WS samples and WS fractions; hatched cocoons of *E. andrei* after exposure to WS samples and  
409 WS fractions; detailed methods for soil urease inhibition assay and ecotoxicity tests.

410

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414

415 **Author Contributions**

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

423

424 **ACKNOWLEDGMENT**

425 We thank Regione Emilia-Romagna (Bando “Alte competenze per la ricerca e il trasferimento  
426 tecnologico” – POR FSE 2014/2020, Obiettivo tematico 10) for E.G. fellowship. This study was,  
427 in part, conducted within the research project PE11 - 3A-ITALY Made in Italy Circolare e  
428 Sostenibile -theme 11 “Made in Italy Circolare e Sostenibile” funded by European Union Next-  
429 Generation National Recovery and Resilience Plan (NRRP), (mission 04, component 2,  
430 investment 1.3—D.D. 341, 15/03/2022, Award Number: PE00000004).

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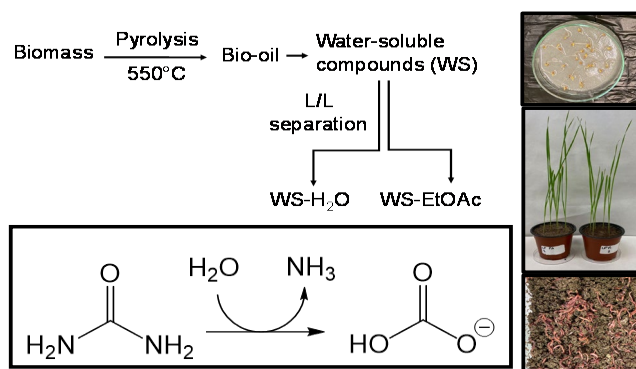
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524

525

526 For Table of Contents Use Only

527 **TABLE OF CONTENT**



528

529

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

532