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

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Methods

Soil urease inhibition assay. The method of Kandeler and Gerber,[1] adopted by the Italian legislation,[2] was followed for determining the urease activity in soil samples. Fresh soil sample (5 g) was mixed with deionized water (5 mL) at 37˚C for 10 min in a flask, then urea was added (2.5 mL at a concentration of 0.08 M) and the mixture was stirred in an orbital shaker for 10 min. The flasks were incubated in a water bath at 37˚C for 24 h, and then KCl (50 mL at a concentration of 1 M in 0.01 M of HCl solution) was added to each flask to stop the enzymatic reaction. The samples were furtherly shaken for 1 h. Ammonia release was instead determined by Kjeldahl distillation, following the method XIV.4 described by the Italian legislation[3] and adapted according to the Unit Distillation Kjeldahl (UDK) 126A from Velp Scientific ® laboratory instrument handbook. The whole content of each flask was transferred to Kjeldahl glass tubes and NaOH (25 mL at 32% concentration) was added before starting the distillation. Ammonia produced during the distillation was collected into an H_3BO_3 aqueous solution (25 mL at a concentration of 0.6 M) and titrated with

H_2SO_4 (0.05M) using an alcoholic solution of bromocresol green and methyl red dyes as the indicator.

Soil respirometry assay. The microbial activity of soil added with WS samples was measured by soil manometric respirometry assay following the literature procedure with slight modifications.[4] Deionized water and WS samples (total volume of 74.4 mL) were added to dried soil samples (126 g) to have a catechol concentration of 5 μg g^{-1} soil, and charged in respirometry sensor BOD bottles (Velp Scientific ®, IT). Evolved CO_2 was absorbed by KOH positioned in the neck of each bottle so that a decrease in gas pressure was observed (CO_2 absorption and O_2 consumption) and expressed as mgO_2 L^{-1}; such value was then converted into mg O_2 g^{-1} of soil to indicate the soil respiration. The test was run for 11 days at 20±2˚C. Each WS sample was tested in triplicate, run in parallel with a blank containing only soil and water (74.4 mL).

Filter paper contact germination test. Germination tests were conducted according to the procedure described in UNI 11357:2010.[5] Fifty seeds of cress (*Lepidium sativum* L.) were incubated with WS samples and WS fractions in distilled water (5 mL) on sterilized cellulose filter paper (Whatman no. 1) placed in a Petri dish sealed with paraffin film. The chosen concentration of each WS sample was tested in quadruplicate (see Table S1 for the volume of each WS sample). Similarly, a control was

prepared with just distilled water. All Petri dishes were incubated at 25±2 ˚C, for 72 h in the dark. After 72 h of exposure, visible root development (used as the operational definition of seed germination), shoot length, and root length were used as endpoints. Seed germination rates were reported as percentages (%). Shoot length and root length data were reported in cm.

Plant emergence and early growth test. The effects of WS samples and WS fractions on the emergence and early growth of higher plants were tested according to ISO 11269-2:2012.[6] Ten seeds of oat (*Avena sativa* L.) were planted in pots containing 200 g of artificial soil (dry weight) wetted with 60 mL of a solution of each WS sample or just distilled water for the control (see Table S1 for the volume of each WS sample). Each condition was tested in quadruplicate. The nominal concentration of catechol referred to dry soil was 5 μg g^{-1}. The artificial soil used for the test consisted of 10% ground sphagnum peat (< 0.5 mm), 20% kaolinite clay (> 50% kaolinite), 70% quartz sand (> 50% of grains measuring 0.05–0.2 mm), all measured on a dry weight basis. All pots were incubated at 20±1˚C, 16/8 h light/dark cycle, illumination 7000-9500 lx. Each pot was watered with distilled water on alternate days. Fertilizer was not added to the soil, to avoid interference with any possible growth inhibition or stimulation caused by WS samples. After the emergence assessment within each pot (about three days after sowing), the seedlings were thinned to give a total of five evenly-spaced specimens in each pot. After 14 days from the thinning, the following

endpoints were evaluated: shoot length, biomass (mass of the five shoots in each pot after drying at 60˚C for 48 h), and visible damage (chlorosis, necrosis, wilting, deformations). The effect on roots was documented only photographically, due to difficulty in separation from soil and obtaining reliable measurements.

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.[7] *E. andrei* was purchased from a local worm farmer (Lombricoltura Compagnoni, Mandello del Lario (LC), Italy) and the earthworms were maintained for several weeks in a mixture of soil and sphagnum peat and under the same environmental conditions used for the experiment (20±1 ˚C, 16/8 h dark/light cycle, illumination 400–800 lx). Adult earthworms with a well-developed clitellum and of similar size were selected for the test. Groups of 10 individuals were formed, weighed, and randomly assigned to experimental glass containers (20×12 cm, height 8 cm) filled with 500 g of artificial soil (see previous paragraph) previously wetted with 200 mL of a solution of each WS sample ((see Table S1 for the volume of each WS sample). The nominal concentration of catechol referred to dry soil was 5 μg g^{-1}. Containers were then closed with transparent perforated lids and placed in the controlled temperature chamber. The earthworms were fed weekly with 2 g of oat flour per container. Each WS sample and a control condition were tested in triplicate. On day 28, all adult worms present in

each container were removed, counted, and weighed. The containers, with the cocoons laid during the previous 28 days, were incubated for a further 28 days. Oat flour was provided once, at the beginning of this second phase. At the end of the experiment (day 56) effects on reproduction were assessed by determining the number and weight of juvenile earthworms and the number of both hatched and unhatched cocoons. An additional container was prepared for each treatment and processed as described above, except that soil samples for pH measurement were taken on days 1, 14, 28, 35, and 56. The biological endpoints were not measured in these additional replicates.

Figure S1. a) Percentage on biomass weight input basis of char, water-insoluble tar (i.e. pyrolytic lignin, PL), and water-soluble fraction (WS) obtained by pyrolysis of larch, poplar, and switchgrass (the gas amount was calculated by difference); b) percentage on WS weight input basis of the two fractions (WS-EtOAc and WS-H_2O) obtained by liquid-liquid separation of WS samples.

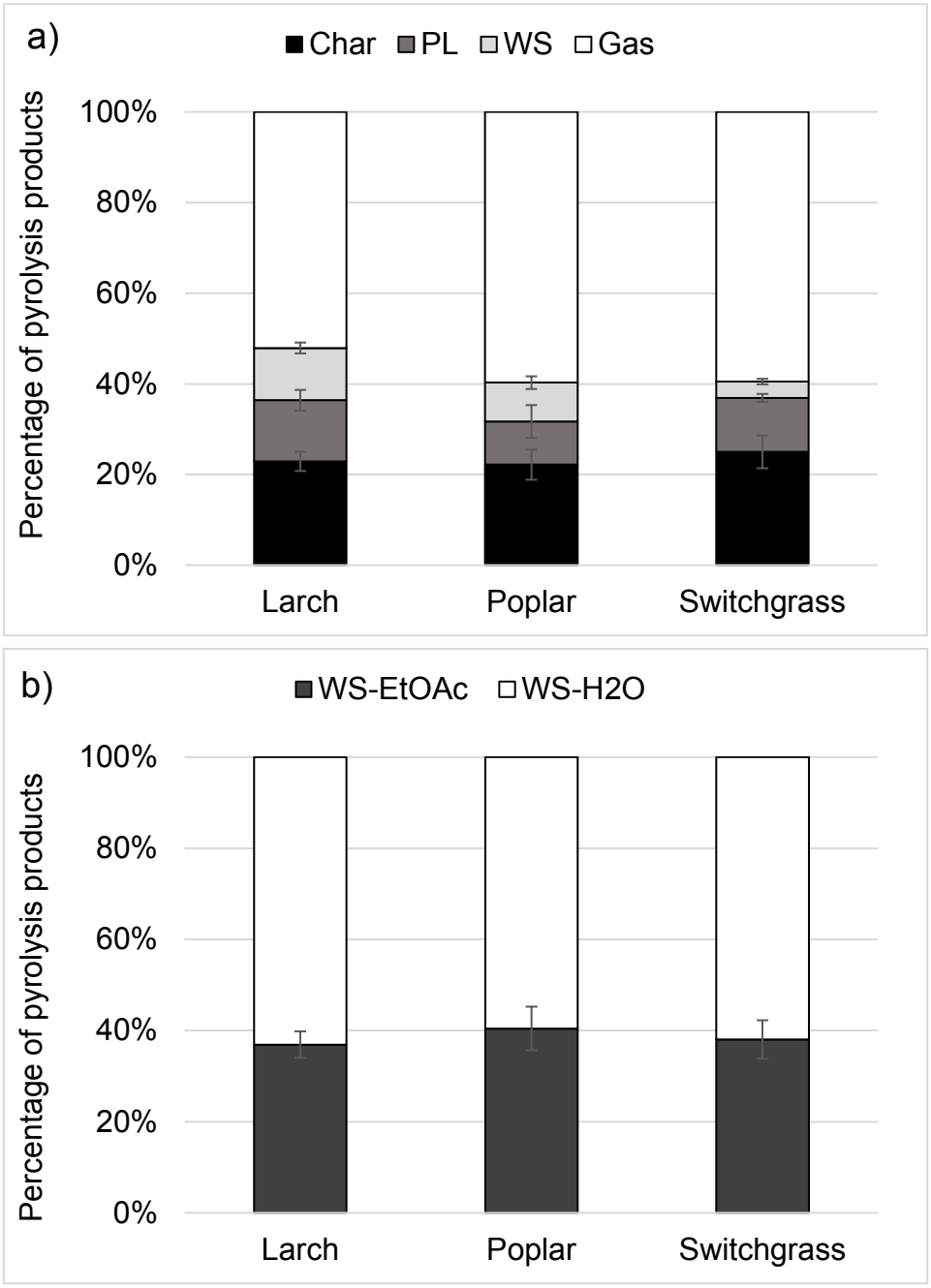

Figure S2. Soil respiration (mg O_2 g^{-1} soil) assessment in the presence of WS samples (catechol concentration of 5 μg g^{-1} soil).

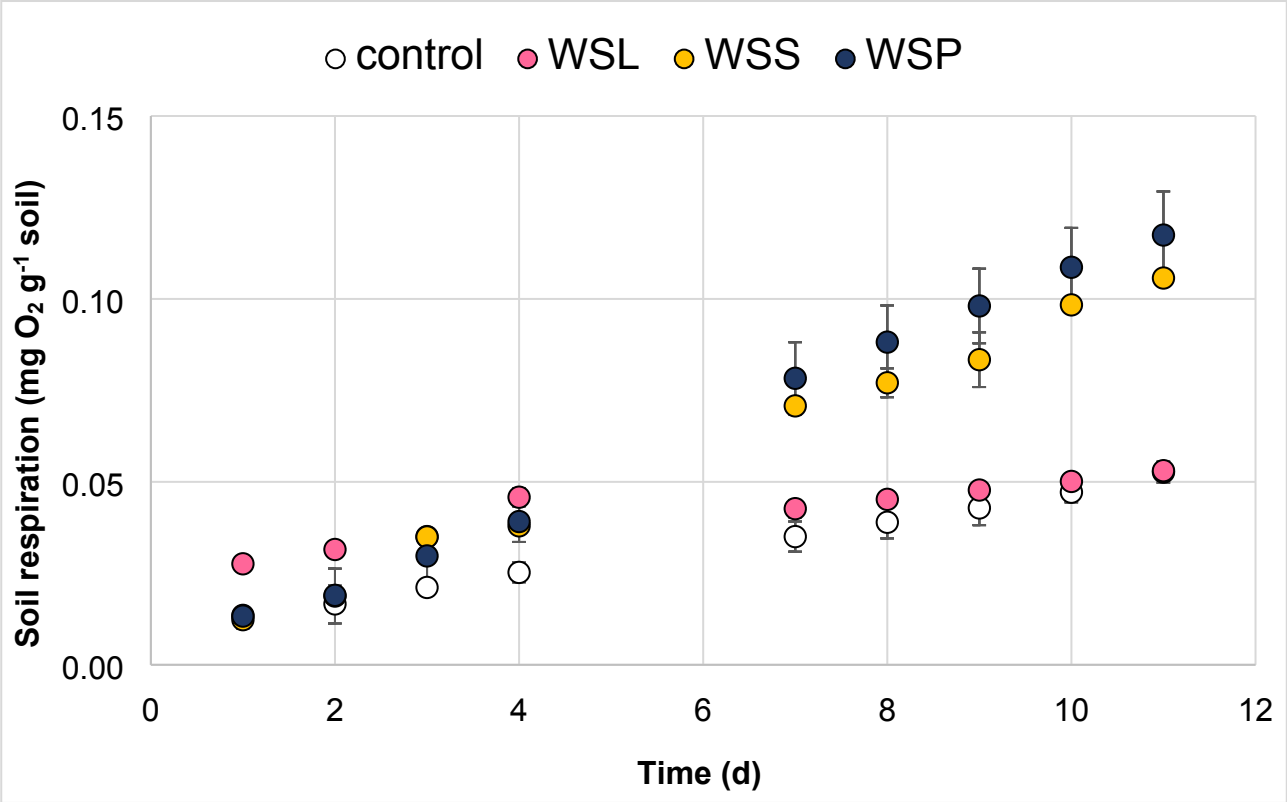

Figure S3. Chlorophyll content in *Avena sativa* leaves after treatment with WS samples and their WS-H_2O and WS-EtOAc fractions. Values were reported as mean ± standard error (n = 4). The

treatments were not significantly different from each other.

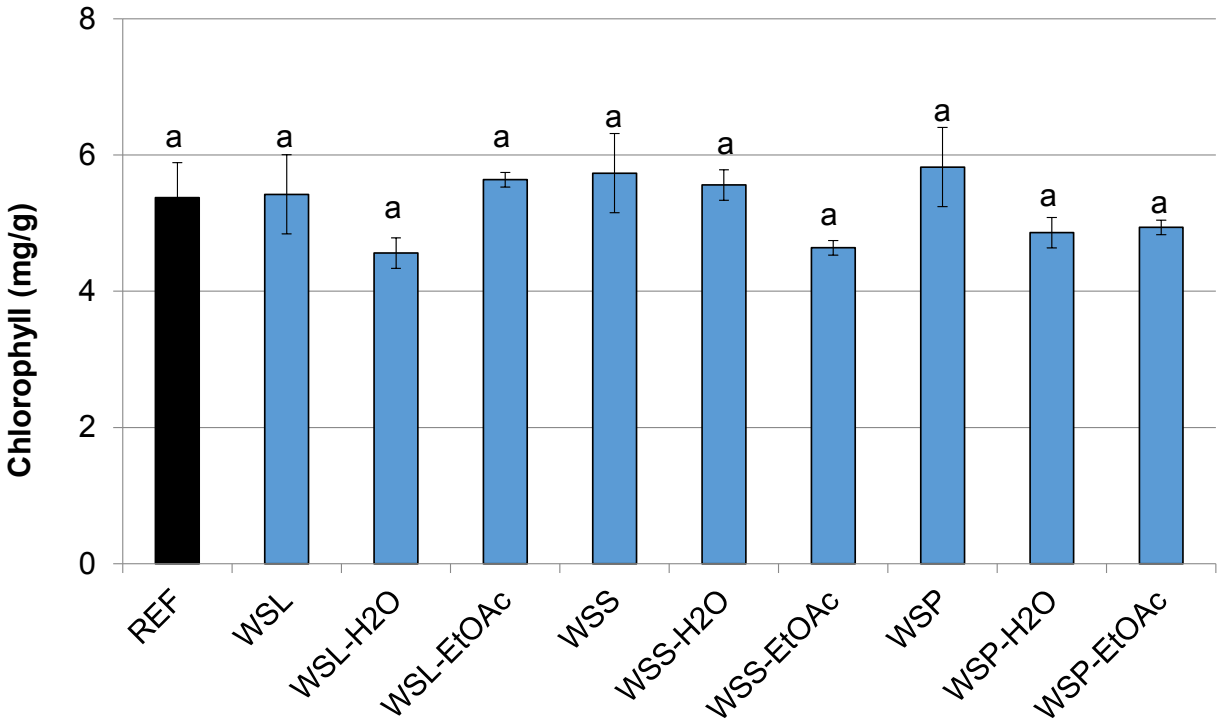

Figure S4. Visual comparison of the effect of WS samples and their WS-H_2O and WS-EtOAc fractions on the development of roots and shoots of *Avena sativa*.

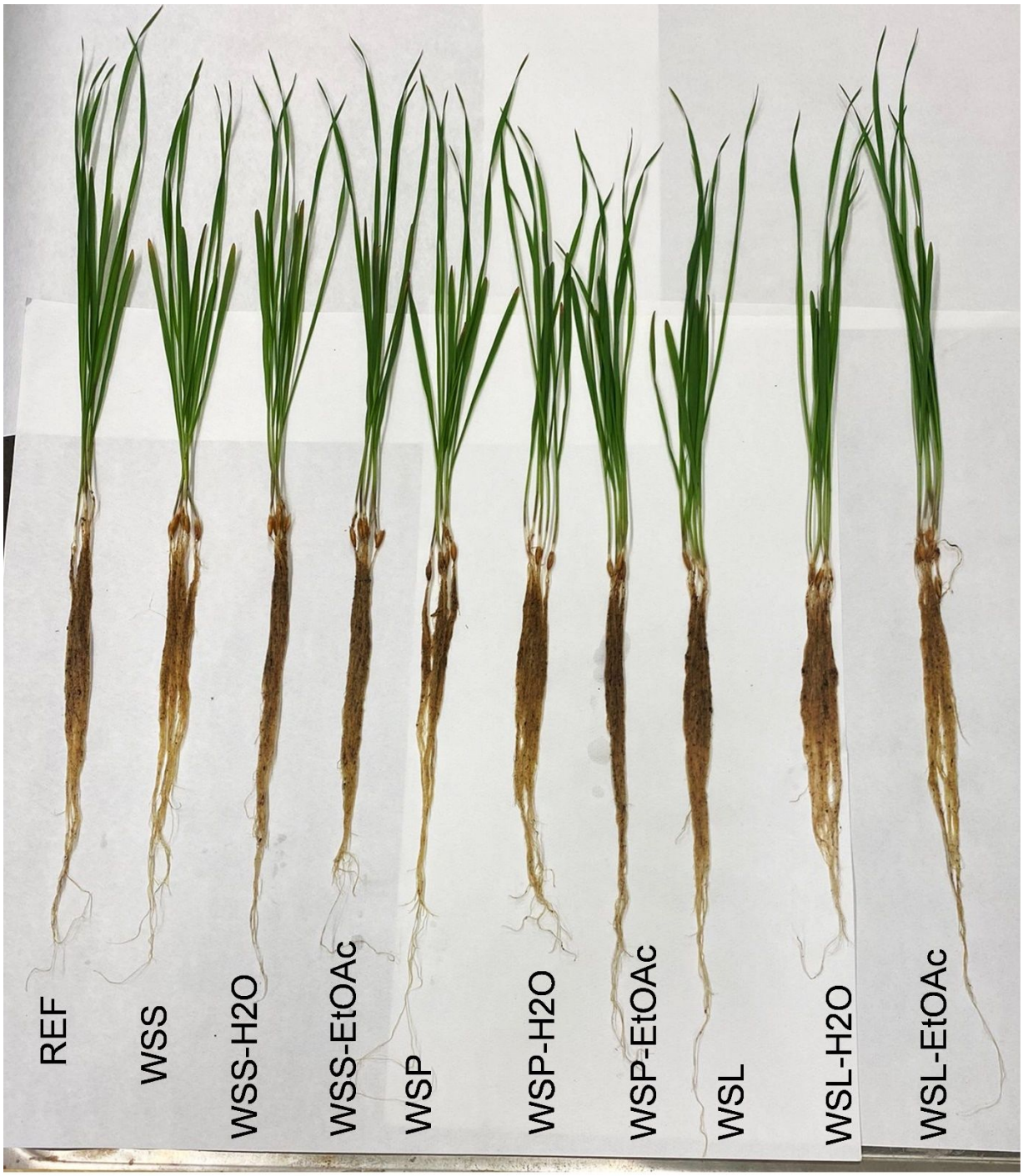
REF
WSS
WSS-H2O
WSS-EtOAc
WSP
WSP-H2O
WSP-EtOAc
WSL
WSL-H2O
WSL-EtOAc

Figure S5. Effect of WS samples and their WS-H_2O and WS-EtOAc fractions applied into the soil on the percentage of hatched cocoons of the earthworm *Eisenia andrei*. 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.

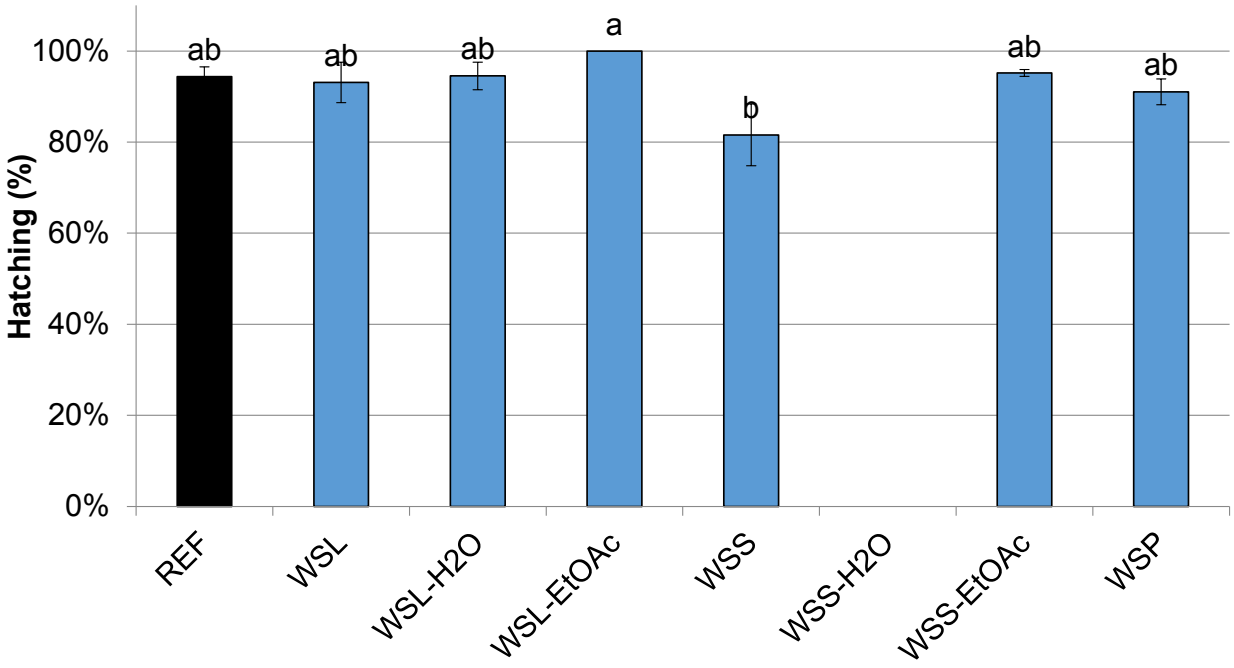

Table S1. a) Characteristics of the WS samples tested (total concentration of water-soluble compounds and catechol), and b) WS volumes used in each test (the volumes of WS-EtOAc and WS-H_2O fractions were the same as the corresponding WS sample).

a)

Biomass	Conc. (mg_{WS} mL^{-1})	Conc. catechol (μg mL^{-1})	Conc. catechol (μg mg_{WS}^{-1})
Larch	145	841	5.8
Poplar	81	437	5.4
Switchgrass	29	226	7.8

b)

Biomass	Volume of WS (mL)				
	In vitro urease	Soil urease	*L. sativum* germination	*A. sativa* germination	*E. andrei* toxicity

	assay[a]	assay[b]	test[c]	test [d]	test[e]
Larch	0.04	0.03	0.02	1.2	3
Poplar	0.08	0.06	0.04	2.3	5.7
Switchgrass	0.18	0.11	0.07	4.4	11.1

[a] WS volume added to 10 mL of test solution, catechol concentration of 30 μM; 10-times lower volume was used for the catechol concentration of 3 μM; [b] WS volume added to 7.5 mL of test solution (5 g of soil), catechol concentration of 30 μM (i.e. 5 μg g^{-1} of soil); 10-times lower volume was used for the catechol concentration of 0.5 μg g^{-1} of soil; 10-times higher volume was used for the catechol concentration of 50 μg g^{-1} of soil; [c] WS volume added to 5 mL of test solution, catechol concentration of 30 μM; [d] WS volume added to 200 g of soil, catechol concentration of 5 μg g^{-1} of soil; [e] WS volume added to 500 g of soil, catechol concentration of 5 μg g^{-1} of soil.

Table S2. Polycyclic aromatic hydrocarbon (PHA) amount (μg g^{-1}) detected in WS and WS-EtOAc samples. Acenaphthylene, acenaphthene, fluorene, and dibenzo[*a,h*]anthracene were not detected in any sample (n.d.: not detected).

PHA	**WS**			**WS-EtOAc**		
	Larch	Poplar	Switchgrass	Larch	Poplar	Switchgrass
Naphthalene	4.90	2.95	4.35	2.19	2.03	1.98

Phenantrene	1.10	0.62	0.98	0.69	0.37	0.55
Anthracene	n.d.	n.d.	0.08	n.d.	n.d.	n.d.
Fluoranthene	0.34	0.18	0.22	0.13	0.14	0.18
Pyrene	1.38	0.78	1.16	0.68	0.50	0.57
Crysene	0.16	n.d.	0.15	n.d.	n.d.	n.d.
Benzo[*a*]anthracene	0.14	n.d.	0.12	n.d.	n.d.	n.d.
Benzo[*b*]fluoranthene	n.d.	n.d.	0.16	n.d.	n.d.	n.d
Benzo[*k*]fluoranthene	0.56	0.45	0.44	n.d.	n.d.	n.d
Benzo[*a*]pyrene	n.d.	n.d.	0.08	n.d.	n.d.	n.d.
Indeno[1,2,3-*cd*]pyrene	n.d.	2.03	0.09	n.d.	n.d.	n.d.
Benzo[*ghi*]perylene	n.d.	n.d.	0.08	n.d.	n.d.	n.d.
Total	*8.58*	*7.01*	*7.91*	*3.69*	*3.04*	*3.28*

References

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4. Pitombo, L. M., Ramos, J. C., Quevedo, H. D., do Carmo, K. P., Paiva, J. M. F., Pereira, E. A., do Carmo, J. B. 2018. Methodology for soil respirometric assays: Step-by-step and guidelines to measure fluxes of trace gases using microcosms. MethodsX, 5, 656-668.

5. UNI 11357: 2010. Water Quality - Determination of the inhibition of the seed germination and root elongation of *Cucumis sativus* L. (cucumber), Lepidium *sativum* L. (watercress), *Sorghum saccharatum* Moench (sorghum) - Short - Chronic Toxicity Test. Ente Nazionale Italiano di Unificazione (UNI).

6. ISO 11269-2:2012 Soil quality - Determination of the effects of pollutants on soil flora - Part 2: Effects of contaminated soil on the emergence and early growth of higher plants.

7. OECD Guidelines for the Testing of Chemicals, Section 2 - Effects on Biotic Systems, Test No. 222: Earthworm Reproduction Test (*Eisenia fetida*/*Eisenia andrei*).