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A Green Approach to Copper-Containing Pesticides: Antimicrobial and Antifungal Activity of Brochantite Supported on Lignin for the Development of Biobased Plant Protection Products

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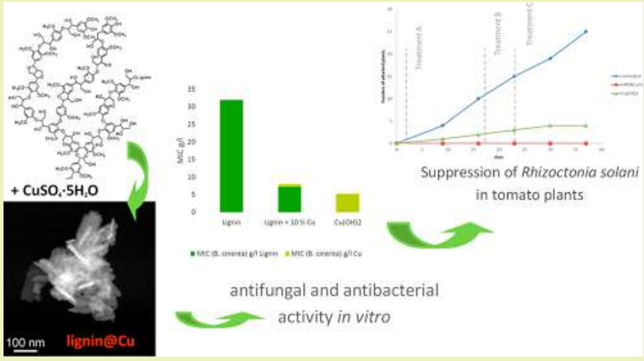
Supporting Information



ABSTRACT: After cellulose, lignin is the most abundant plant-derived polymer in nature. It provides mechanical support to plants, but it has also a defense role against pests and diseases, thanks to antioxidant, bactericidal, and antifungal properties, deriving from its polyphenolic nature. Huge quantities of technical lignins are obtained during several industrial processes and they actually represent a waste of paper pulp and bioethanol industry. Although in the last decades many efforts have been directed to obtain lignin valorization in several fields and for diverse applications, this biobased polymer is still largely underutilized. In particular, very little is known about the possibility to exploit its antioxidant, antifungal, and antibacterial properties in the agronomical field. On the other hand, pest control is often achieved by using copper-based pesticides, but environmental and health issues urge for novel solutions implying reduced copper content. We here describe novel hybrid organic−inorganic materials obtained by combining copper(II) salts with two types of technical lignins. Cu-containing materials (lignin@Cu) have been characterized by different techniques, including X-ray powder diffraction and transmission electron spectroscopy analyses, revealing nanocrystals of brochantite ($Cu_4SO_4(OH)_6$) grown in the lignin matrix. Lignin@Cu was tested for its antifungal and antibacterial profile against a vast panel of pathogens of agronomical interest. Furthermore, preliminary tests on crops in a greenhouse were performed: lignin@Cu had better performances than a commercial pesticide based on copper(II) hydroxide on tomato plants against *Rhizoctonia solani*, indicating a great potential of these materials as plant protection products.

KEYWORDS: *Lignin, Copper, Copper-containing antimicrobials, Plant protection products, Brochantite*

INTRODUCTION

Lignin accounts for 15−30% of lignocellulosic biomass. It is a complex, cross-linked, polyphenolic polymer that provides mechanical support to leaf blades and stems, imparting strength and rigidity to plants. It has also a defense role against physical/chemical stress, pests and diseases, since it has an antioxidant, bactericidal, and antifungal effect.[1] In plants, lignin is produced by oxidative coupling of three phenolic alcohols/acids (coniferyl, p-coumaryl, and synapyl) by the enzyme peroxidase, leading to an amorphous three-dimensional polymer made of methoxylated phenylpropanoid units (Figure 1).[2]

Various types of technical lignins are obtained in large amounts (over 70 million tons per year) as a byproduct of the paper pulp manufacturing process and bioethanol industry. Traditionally, industrial processes that exploit plant polysaccharides burn lignin to obtain energy.[3] Only about 5% of lignin is used in low-value commercial applications and actually it remains the most poorly utilized among lignocellulosic biopolymers.[4] The advent of biorefineries that transform plant-based raw materials (biomasses) in second-generation biofuels generates great quantities of lignin, exceeding the amount necessary to sustain the process by its combustion.[5,6] Moreover, an increase in lignin production is expected within 2030 as a consequence of many governmental plans that aim to support biofuel production.[7,8] Therefore, in the past decade

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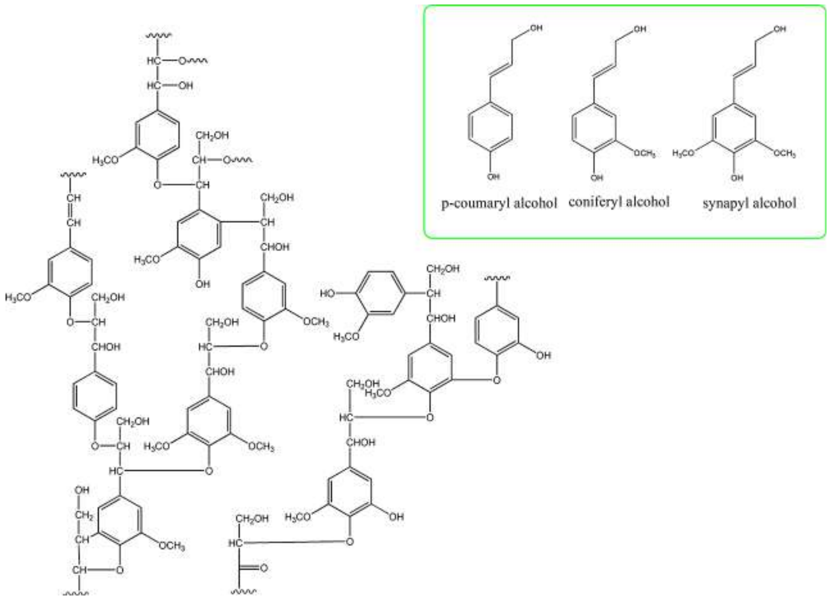


Figure 1. Schematic representation of softwood lignin structure. In the inset, typical lignin building blocks are illustrated.

much has been done in lignin valorization research and development.[9−11] Most efforts have been devoted to obtain high-value products, in particular renewable lignin-based polymers, such as low-cost carbon fibers, elastomers, composites, polymeric foams, and membranes.[2,12,13] There is great interest in catalytic lignin depolymerization to generate low-weight aromatic compounds that can serve as biobased alternatives to petroleum-derived aromatics, including phenol and vanillin.[14−16] Even if lignin valorization has been investigated in many directions, very little has been done regarding zootechnical or agrochemical applications. The research, in this sense, has principally been devoted to use lignin as a nontoxic, low cost, and biodegradable matrix, often in the form of nanocapsules, for the controlled release of active molecules.[17−19]

The polyphenolic nature confers very interesting biological properties to technical lignins. It is well-known, in fact, that natural polyphenols possess anti-inflammatory and anticarcinogenic properties and that they are effective antioxidants.[20] Several *in vitro* and *in vivo* studies on various commercial lignins have demonstrated their antimicrobial properties.[21] Moreover, various investigations have suggested that lignins can be applied to stabilize food and feedstuff because of their antioxidant, antifungal, and antiparasitic properties.[22] Recently, the use of lignin in association with silver nanoparticles has evidenced an active role of lignin in the achievement of good biocidal action against both Gram-positive and Gram-negative bacterial strains.[23,24] Hence, the use in agriculture of technical lignins and lignin-derived compounds is an innovative approach for their valorization. The direct control of pathogens by using synthetic pesticides is still the most effective way to intervene, but it is well-known that their extensive use generates long-term residues in food and in the environment.[25] Concerns on food safety and environmental health, combined with the global issue of emerging resistant pest strains, make it urgent to develop novel crop-protective agents. In this scenario, the exploitation of bioactive natural sources to obtain new agents with novel modes of actions may represent an innovative, effective strategy to minimize at the same time bacterial and fungal proliferation and the use of harmful pesticides. Many natural products and their chemical analogues have been successfully developed as crop-protective agents[26] and lignin or lignin-based derivatives could indeed represent ideal candidates for innovative solutions in this field.

On the other hand, inorganic substances, such as copper sulfate or hydroxide, have been long used for their capacity of inhibiting the development of molds and bacteria. Copper is reported to have a very wide activity spectrum, but copper toxicity and the possibility of its bioaccumulation have led to the introduction of threshold concentration limits.[27]

With these considerations in mind, we pursued the idea to produce antibacterial and antifungal lignin-based materials, seeking to synergistically combine lignin with copper(II) ion, to obtain crop protection with reduced risks for health and environment. The use of plant-derived materials provides an opportunity to limit synthetic preservatives and pesticides, reducing at the same time the amount of copper necessary to limit pathogen growth. This, in turn, means to transform an industrial byproduct into a valuable one.

Here we present the preparation and full characterization of novel hybrid organic−inorganic materials obtained by combining copper(II) salts with two technical lignins with different grade of polymerization, referred to as high molecular weight, HMW (M_w = 4400−5000 g/mol, M_n = 1200−1300 g/mol) and medium molecular weight, MMW (M_w = 800−1500 g/mol, M_n = 300−700 g/mol). Hereinafter, the Cu-containing materials (lignin@Cu) will be referred to as HMW@Cu (HMWCu_X%, X = 2, 6, 10, 14, 17) and MMW@Cu (MMWCu_X%, X = 2, 4, 10, 14, 18), respectively. HMW@Cu and MMW@Cu were tested for their antifungal and antibacterial profile against a vast panel of pathogens of agronomical interest. Preliminary tests on crops in greenhouse were also performed and HMWCu_10% showed a very promising activity against *Rhizoctonia solani* on tomato plants.

■ EXPERIMENTAL SECTION

Materials and Methods. HMW (BioPiva 100; *Pinus taeda*, M_w 4400−5000 g/mol, M_n 1200−1300 g/mol) and MMW (BCD oligomer; *Fagus sylvatica*, M_w 800−1500 g/mol, M_n 300−700 g/mol) lignins were kindly provided by UPM-Kymmene Oyj (Helsinki, Finland) and Green Innovation GmbH (Innsbruck, Austria). $Cu(CH_3COO)_2{\cdot}H_2O$, $Cu(NO_3)_2{\cdot}2H_2O$, $CuCl_2{\cdot}2H_2O$, $CuSO_4{\cdot}5H_2O$, NaOH, DMF, HNO_3 65%, H_2O_2 10% , H_3PO_3 85%, acetic acid, and sodium acetate, were purchased from Sigma-Aldrich and used with no further purification. pH was measured using a Crison pHmeter basic 20 equipped with an Ag/AgCl electrode.

General Procedure for the Preparation of HMW@Cu and MMW@Cu. Lignin (HMW or MMW) was suspended in distilled water (10 mL) to which was added $CuSO_4{\cdot}5H_2O$ dissolved in 5 mL of water, under stirring at room temperature. The pH was raised from 3.5 to 7 with NaOH 1 M, added dropwise: the mixture became darker and thicker. Stirring was carried on for 24 h at room temperature; a slight pH decrease to about 5.5−6 was observed at the end of the stirring time. The brown solid was filtered and washed with water, then it was dried in the air and at 80 °C overnight. Different lignin/$CuSO_4{\cdot}5H_2O$ mass ratios were applied, ranging from 1:0.12 g to 1:1.20 g; all the experiments were performed at least twice. The experimental details are reported in Table 1 (HMW) and Table 2 (MMW), together with the amount of copper in the final material determined by inductively coupled plasma atomic emission spectrometry analysis (ICP-AES). The percentage value reported in the codes of the first column refers to the copper content (weight percent). The copper upload resulted almost quantitative.

Table 1. Copper Content in HMW@Cu, Corresponding to Different HMW/$CuSO_4{\cdot}5H_2O$ Mass Ratios Employed

code	HMW/$CuSO_4{\cdot}5H_2O$ mass ratio (g/g)	recovered solid (g)	Cu % mean ± SD
HMWCu_2%	1/0.12	0.95	2.3 ± 0.1
HMWCu_6%	1/0.25	0.97	5.6 ± 0.1
HMWCu_10%	1/0.50	1.06	9.8 ± 0.3
HMWCu_14%	1/0.80	1.23	14.3 ± 0.2
HMWCu_17%	1/1.12	1.41	17.3 ± 0.9

Table 2. Copper Content in MMW@Cu, Corresponding to Different MMW/$CuSO_4{\cdot}5H_2O$ Mass Ratios Employed

code	MMW/$CuSO_4{\cdot}5H_2O$ mass ratio (g/g)	recovered solid (g)	Cu % mean ± SD
MMWCu_2%	1/0.12	0.96	2.2 ± 0.2
MMWCu_4%	1/0.20	0.97	4.1 ± 0.2
MMWCu_10%	1/0.50	1.12	9.7 ± 0.4
MMWCu_14%	1/0.80	1.42	14.4 ± 0.9
MMWCu_18%	1/1.12	1.23	17.9 ± 0.3

The lignin@Cu materials obtained are powders stable at room temperature for a prolonged time, and can be stored for at least one year without sign of alteration.

Sample Characterization. Copper content was measured by ICP-AES analysis. Briefly, 100 mg of solid sample were suspended in 5 mL of HNO_3 65% and 1 mL of H_2O_2 30%, then digested in a *Milestone* microwave MLS-1200 MEGA (digestion sequence: 1 min at 250 W, 1 min at 0 W, 5 min at 250 W, 5 min at 400 W, 5 min at 650 W, 5 min of cooling). The solutions were diluted to 50 mL with bidistilled water and analyzed by using an emission spectrometer JY 2501 with coupled plasma induction in the radial configuration of the HORIBA Jobin Yvon (Kyoto, Japan), ULTIMA2 model. Instrumental features: monochromator model JY 2501; focal length, 1 m; resolution, 5 pm; nitrogen flow, 2 L/min. ICP source: nebulizer Meinhard, cyclonic spraying chamber; argon flow, 12 L/min; wavelengths range, 160−785 nm; optical bench temperature, 32 °C. The wavelength used for quantitative analysis was chosen by examining the emission line with greater relative intensity, ensuring that there was no spectral interference with the argon emission lines. Acquisition parameters: wavelength Cu (nm), 224.700; voltage (V), 580; gain, 100. The quantitative analysis was performed after the acquisition of a calibration line using standard solutions in HNO_3 10%, to simulate the final acidity of the samples; the concentration range of the standards varied from 1 mg/L to 100 mg/L. Data acquisitions and processing were performed using the ICP JY v 5.2 software (Jobin Yvon).

The mineral phase was identified by X-ray powder diffraction analysis (XRPD) performed using a Thermo X'TRA diffractometer with Cu Kα radiation equipped with a Si(Li) solid-state detector; the parameters were range of acquisition of 2θ, 5−70° and a step of 0.05° with 2 s per step. Infrared (IR) spectra were recorded with a Nicolet 5PCFT-IR spectrophotometer equipped with ATR accessory (diamond), range 4000−400 cm^{-1}.

Microscopy. Selected area diffraction (SAED) and convention transmission imaging mode were carried out using a Philips TECNAI F20ST transmission electron microscope (TEM) operating at 200 kV and equipped with EDAX PV9761/II energy dispersive X-ray spectrometer (EDS). In the scanning transmission imaging mode (STEM) a high angle annular dark field detector (HAADF) is used in order to have a compositional contrast (Z-contrast). The specimens were prepared by grinding the powders in isopropyl alcohol and drop casting on holey carbon film.

Copper Release in Water at Various pH. Aliquots of 250 mg of HMWCu_17% and MMWCu_18% were stirred for 24 h at room temperature in 25 mL of distilled water, sodium hydroxide aqueous solution (pH = 10), or buffered solution (acetate buffer for pH = 4 and 5, phosphate/phthalate buffer for pH = 7). Then, the suspended solids were filtered away to collect the filtrates, which were analyzed by ICP-AES. Results are summarized in Table 3.

***In Vitro* Antibacterial and Antifungal Studies.** *In vitro* assays were performed in accordance to CLSI guidelines (CLSI-2015-M07-A10), properly modified in order to be applied to yeasts and fungi.[28]

Preparation of the Inoculum. For the bacteria, from three to five colonies, that were well isolated and characterized by the same morphology, were selected from a plate with agarized medium. Colonies were transferred to a falcon containing 5 mL of growth-specific medium and incubated at 35 ± 2 °C for bacterial growth. After 24 h, 20% glycerol was added to the cell suspension and then the Cell Bank was prepared, characterized by a microbial concentration $\geq 10^5$ CFU/mL evaluated by plating and counting on solid medium. Table S1 shows the microorganisms with the specific growth media and conditions.

For the yeasts and fungi, from three to five colonies characterized by the same morphology were selected from a plate with agarized medium. Colonies were transferred to a falcon containing 5 mL of growth-specific medium and incubated at 30 ± 2 °C for fungi and yeasts growth. After 72 h, 20% glycerol was added to the cell suspension, and then the Cell Bank was prepared, characterized by a microbial concentration $\geq 10^4$ CFU/mL evaluated by plating and counting on solid medium. For yeasts, the Cell Bank was plated on agarized specific medium for sporulation; after 72 h the spores were collected through the addition of physiological + tween80 0.1% and 20% of glycerol was added to obtain the Cell Bank (spores), characterized by spore concentration $\geq 10^4$ CFU/mL evaluated by plating and counting on solid medium. Table S2 shows the microorganisms with related specific growth media and conditions.

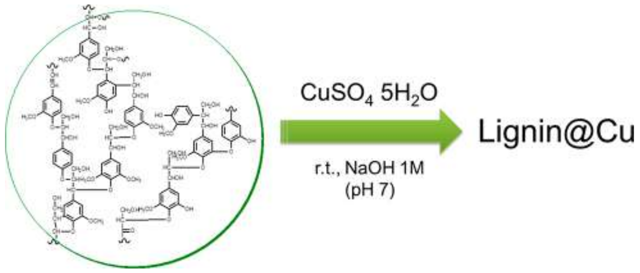
Agar dilution assay was performed as an alternative to broth microdilution assay in order to avoid solubility problems. Tested compounds were incorporated at different concentrations into the agar medium (specific for each microorganism) at 55 ± 5 °C, and after solidification of the medium, the inoculum (10^4 CFU/mL for the fungi and 10^5 CFU/mL for the bacteria) was applied to the agar surface. Concentrations of tested compounds were obtained directly by weight and not by serial dilutions. Positive controls of activity were used: Miconazole (against fungi) and Ceftriaxone (against bacteria). All the tests were carried out in triplicate. Minimum inhibitory concentration (MIC, g/L) of tested compounds were determined for each pathogen, and results are summarized in Tables 4 and 5.

Tests on Crops in Greenhouse. Trials were performed on "*Cuore di Ponente*", an Italian local variety of tomato. Tests were conducted at Soc. Agr. AGOFLOR S.C., via delle Messi, 101-64014 Martinsicuro (TE), ITALY (Lat. 42°51′24.98″ N; Long. 13°54′53.32′E ; Alt. 5 mt a.s.l.). The trial was located in an area of the Abruzzo Region (Italy), with conditioned environmental-climatic conditions of the disease *Rhizoctonia solani*, which was investigated in the trial. HMWCu_10% was appropriately formulated to obtain a stable and sprayable suspension. The final concentration of copper in the formulation was 10 g/L. Experimental conditions, crop details, and application schedules are detailed in the Supporting Information (Tables S3−S5). The number of attacked plants (dead) on 10 plants per plot and 30 plants per replication were evaluated. The efficacy and selectivity assessments on *Rhizoctonia solani* were carried out as follow: 7 and 14 days after application A, 7 days after application B, 7 and 14 days after application C (see Supporting Information for details). Phytotoxicity symptoms were assessed at every visit to the trial site. No phytotoxicity was observed on any visit on any plot treated with lignin@Cu.

RESULTS AND DISCUSSION

Copper(II) sulfate is an active fungicide but it is highly water-soluble and it is therefore easily washed off, thus representing a serious danger for the environment. Copper(II) hydroxide and copper(II) oxide, which are sparingly soluble, represent an alternative, as they adhere to fruits and leaves and are practically fully retained during rain. Physical contact between spores and copper particles is essential to obtain plant protection and, in this respect, the particles should be as small as possible to favor the fungicidal action.[29] As outlined in the Introduction, we would explore the possibility to have a stable upload of copper into lignin, in order to have a convenient source of copper in terms of solubility, but with lower copper use thanks to the antibacterial and antioxidant nature of lignin. We used two different types of lignins, HMW and MMW,[30] with diverse grades of polymerization, in order to verify if differences in antibacterial and antifungal activity could be observed as a function of the different distribution of the polyphenolic chain and lignin source.

Scheme 1. Reaction Conditions for the Preparation of the Lignin@Cu Material



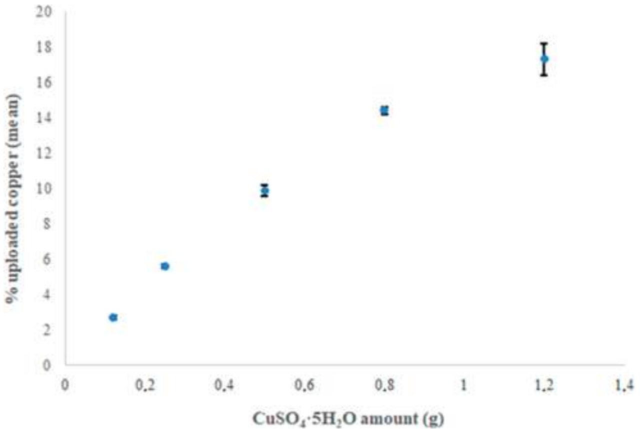


Figure 2. Proportionality between the amount of $Cu(SO_4)_2{\cdot}5H_2O$ added to HMW and the % of uploaded copper.

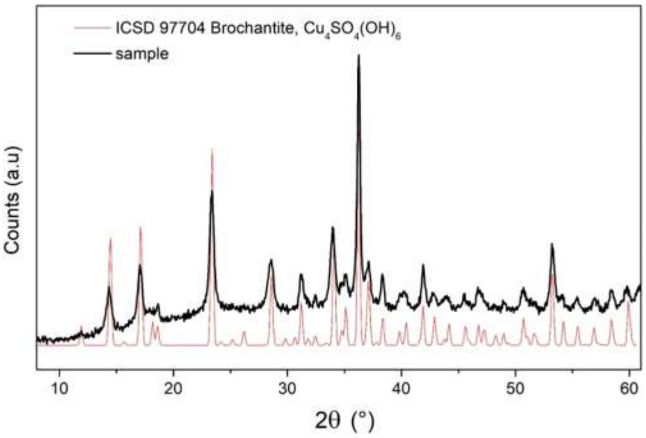


Figure 3. Overimposition of the XRPD plots of a representative HMW@Cu sample (black line) and of brochantite (red lines).[31]

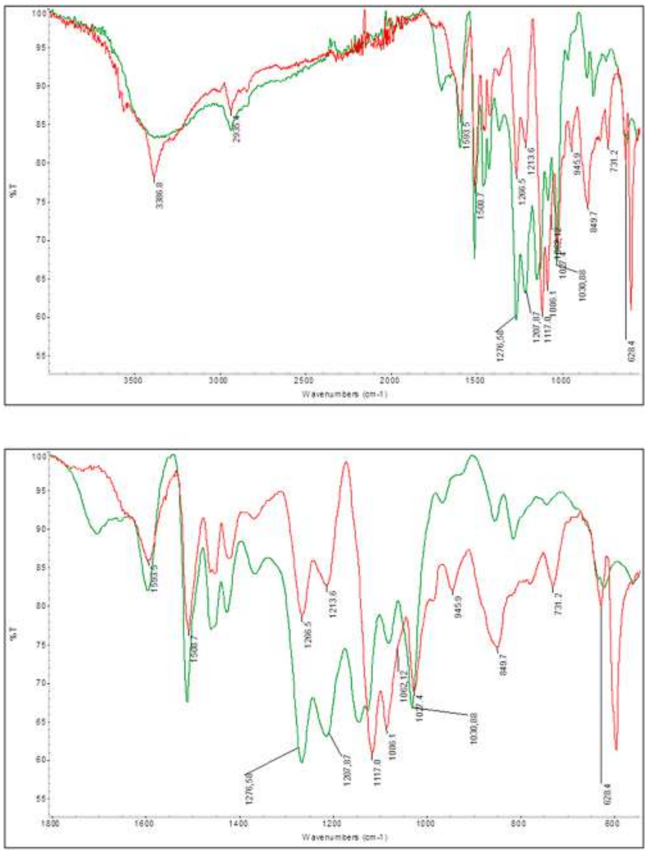


Figure 4. Comparison between FT-IR spectra of HMW (green) and HMWCu_10% (red); entire wavenumber range (top) and zoom (bottom).

Preliminary attempts were made by mixing HMW and various copper salts in distilled water. $Cu(CH_3COO)_2{\cdot}H_2O$, $Cu(NO_3)_2{\cdot}3H_2O$, $CuSO_4{\cdot}5H_2O$, and $CuCl_2{\cdot}2H_2O$ in different mass ratios (from 1:0.5 g to 1:5 g) were tested. The mixtures were stirred for 24 h at room temperature, then the solids were recovered, dried, and analyzed by ICP-AES. In these conditions, no upload of the metal was observed.

To define the optimal uploading conditions, $CuSO_4{\cdot}5H_2O$ was selected as a copper source, and the effect of temperature

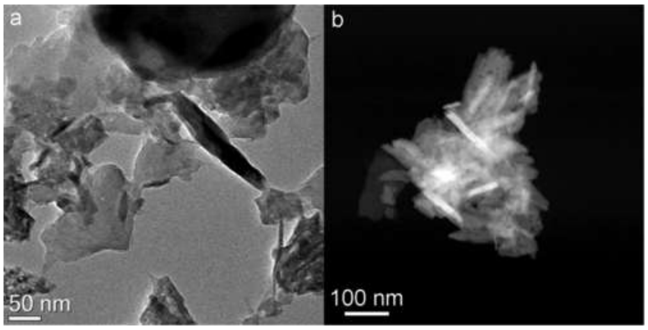


Figure 5. TEM images in *bright field* (left) and HAADF-*STEM* mode (right) of HMWCu_14%. Brochantite appears brighter in the HAADF images.

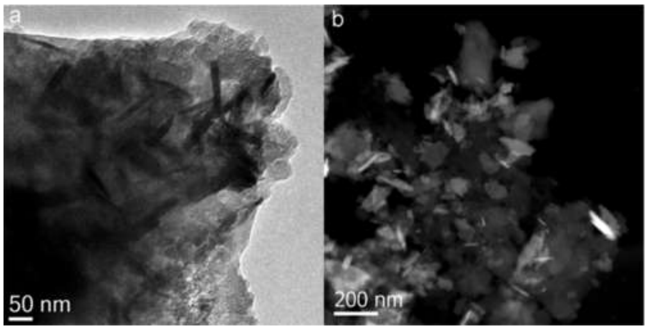


Figure 6. TEM images in *bright field* (left) and HAADF-*STEM* mode (right) of MMWCu_14%. The brochantite crystals appear brighter in the HAADF images.

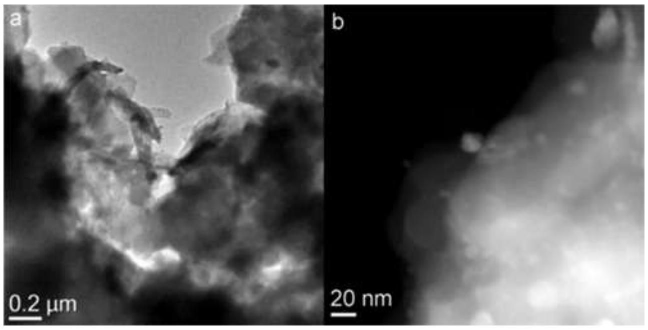


Figure 7. TEM images in bright field (left) and HAADF-STEM mode (right) of HMWCu_2%.

Table 3. Copper Release as a Function of pH

	sample	% release
pH 4	HMWCu_17%	91
	MMWCu_18%	89
pH 5	HMWCu-17%	93
	MMWCu_18%	80
pH 7	HMWCu_17%	0.1
	MMWCu_18%	0.1
pH 10	HMWCu_17%	0.1
	MMWCu_18%	0.0
distilled water	HMWCu_17%	0.3
	MMWCu_18%	0.1

was investigated. The mixture of the copper salt and lignin was heated up to 80 °C overnight, but again ICP-AES analysis revealed a negligible Cu-upload.

The pH of the starting HMW/Cu(II) mixture (about 3.5) was increased to 5 and to 7 in two separate experiments, by dropwise addition of NaOH 1 M. In these cases, ICP-AES analysis revealed the presence of copper in the samples, with the highest copper upload at pH 7 (Scheme 1). Hence, this pH was selected to explore the upload of copper at different HMW/Cu(II) mass ratios. The molar amount of NaOH needed to reach the desired pH turned out to be about twice *vs* $CuSO_4 \cdot 5H_2O$.

As reported in Table 1, the upload of copper in all the cases is almost quantitative, and the metal content in the final material turned out to be dependent on the starting lignin/Cu-salt mass ratio. This allows a fine-tuning of the final percentage of copper loaded on the lignin, as can be inferred from Figure 2.

The same preparation protocol and the same weight ratios were applied to MMW. ICP-AES analysis revealed percentages of upload highly similar to the previous ones, suggesting that the process is independent from the starting lignin (Table 2 and Figure S1).

HMW@Cu and MMW@Cu materials were further analyzed by X-ray powder diffraction analysis (XRPD). Lignin is amorphous and exhibits a broad diffuse scattering in the XRPD diffractogram. When copper is present in a small amount (up to 5%), no diffraction pattern appeared. In all the samples containing higher percentages of metal, irrespective to the type of lignin and amount of uploaded copper, the same diffractogram appeared (Figures S2−S4), which was ascribed to the copper basic sulfate brochantite (Figure 3), $Cu_4SO_4(OH)_6$.[31]

The formation of brochantite from $CuSO_4$ in alkaline solution agrees with the literature data.[32−34] However, considering the heterogeneous conditions under which the HMW@Cu and MMW@Cu materials are prepared, the exclusive formation of brochantite as the Cu-containing phase is of particular value. FT-IR analysis of lignin@Cu reveals the presence of absorption bands relative to both lignin and $Cu_4SO_4(OH)_6$. Strong absorptions at about 1112 and 1083 cm^{-1} indicate the presence of the sulfate anion of brochantite, as well as that of the −OH groups with absorption bands at about 3300 cm^{-1} (Figure 4).[35] The IR spectra of the lignin@Cu materials are all similar (Figures S6 and S7).

To get insights on the morphology and the nature of the lignin-copper material, TEM analyses were performed on two samples of HMW@Cu (HMWCu_2%, where brochantite could not be detected by means of XRPD, and HMWCu_14%) and one sample of MMW@Cu (MMWCu_14%). The images were collected in both *bright-field* and HAADF-STEM modes. At low magnification all the samples appeared composed of sponge-like granules. In both HMWCu_14% and MMWCu_14%, the images at higher magnification revealed the presence of well crystallized particles with elongated morphology blended with a granular material (Figure 5 and 6). SAED patterns can be indexed as brochantite, and the EDS analyses performed on several fragments exhibit a Cu/S cationic ratio close to 4. The sample thus can be described as a physical mixture of lignin granules and nanometric sticks of brochantite, strongly joined together.

The electron beam of the microscope never caused the separation of the two phases, while recrystallization phenomena were occasionally observed. In particular, in HMWCu_2%, the nanometric crystals of brochantite could not grow: the images showed the presence of nanometric spheres scattered

Table 4. Minimum Inhibitory Concentration (MIC, g/l) of Tested Compounds against Food-Related Pathogens[a]

Cpd	*E. coli*	*S. enteridis*	*P. aeruginosa*	*S. aureus*	*C. jejuni*	*C. albicans*
	MIC (g/l)					
HMW	16	32	16	4	16	32
HMWCu_2%	8 (0.20)	16 (0.42)	16 (0.42)	4 (0.10)	16 (0.42)	8 (0.20)
HMWCu_6%	4 (0.22)	8 (0.45)	16 (0.90)	2 (0.11)	8 (0.45)	2 (0.11)
HMWCu_10%	2 (0.20)	4 (0.40)	4 (0.40)	1 (0.10)	4 (0.40)	2 (0.20)
MMW	>32	32	32	16	>32	>32
MMWCu_2%	8 (0.18)	32 (0.70)	>32	4 (0.09)	16 (0.35)	8 (0.18)
MMWCu_4%	4 (0.16)	16 (0.64)	32 (1.2)	4 (0.16)	8 (0.32)	8 (0.32)
MMWCu_10%	4 (0.37)	8 (0.74)	16 (1.48)	4 (0.37)	8 (0.74)	4 (0.37)
$Cu(OH)_2$	0.5 (0.32)	1 (0.65)	2 (1.30)	0.5 (0.32)	<0.25 (<0.16)	2 (1.30)

[a] Copper content (g/L) is given in parentheses. MIC (g/L) lower than copper(II) hydroxide in term of copper content are highlighted in red.

Table 5. Minimum Inhibitory Concentration (MIC, g/L) of Tested Compounds against Bacteria and Fungi of Agronomical Interest[a]

Cpd	*E. amylovora*	*P. syringae*	*X. campestris*	*X. arboricola*	*R. solani*	*M. laxa*	*A. solani*	*F. solani*	*B. cinerea*	*S. tritici*
	MIC (g/l)									
HMW	16	32	4	16	>32	32	>32	>32	>32	32
HMWCu_2%	8 (0.21)	16 (0.42)	4 (0.10)	4 (0.10)	8 (0.21)	16 (0.42)	32 (0.83)	16 (0.42)	32 (0.83)	16 (0.42)
HMWCu_6%	8 (0.45)	8 (0.45)	2 (0.11)	2 (0.11)	4 (0.22)	8 (0.45)	16 (0.90)	16 (0.90)	16 (0.90)	16 (0.90)
HMWCu_10%	4 (0.40)	4 (0.40)	2 (0.20)	2 (0.20)	4 (0.40)	8 (0.80)	8 (0.80)	8 (0.80)	8 (0.80)	8 (0.80)
MMW	32	16	32	8	>32	16	>32	>32	>32	32
MMWCu_2%	32 (0.7)	32 (0.70)	2 (0.04)	8 (0.18)	16 (0.35)	16 (0.35)	>32	32 (0.7)	8 (0.18)	32 (0.7)
MMWCu_4%	8 (0.32)	16 (0.64)	2 (0.08)	4 (0.16)	16 (0.64)	8 (0.32)	>32	16 (0.64)	8 (0.32)	16 (0.64)
MMWCu_10%	4 (0.37)	8 (0.74)	2 (0.18)	4 (0.37)	8 (0.74)	8 (0.74)	8 (0.74)	8 (0.74)	8 (0.74)	8 (0.74)
$Cu(OH)_2$	1 (0.65)	0.5 (0.32)	0.5 (0.32)	<0.25 (<0.16)	0.5 (0.32)	1 (0.65)	8 (5.2)	16 (10.4)	8 (5.2)	1 (0.65)

[a] Copper content (g/L) is given in parentheses. MIC (g/L) lower than copper(II) hydroxide in term of copper content are highlighted in red.

Table 6. Number of Attacked Plants (Dead) on 30 Plants[a]

	7 DA A	14 DA A	7 DA B	7 DA C	14 DA C
untreated	4	10	15	19	25
HMWCu_10%	0	0	0	0	0
$Cu(OH)_2$	1	2	3	4	4

[a] DA A, days after treatment A; DA B, days after treatment B; DA C, days after treatment C.

on the granules of lignin (Figure 7). The two phases are clearly distinguishable in STEM mode. The spheres, with a diameter of about 10 nm, were confirmed to be composed of brochantite.

The behavior of the system in the absence of lignin was also investigated: $CuSO_4 \cdot 5H_2O$ was solubilized in water and treated with 2 equiv of NaOH 1 M added dropwise, without adding lignin. The pH of the resulting mixture was strongly alkaline, about 12.5. The XRPD analysis revealed the formation of both CuO (tenorite) and brochantite (Figure S5).[33,36] Note that CuO was never detected in the lignin@Cu samples.

Copper Release in Water. Lignin@Cu samples released copper only at acidic pH (Table 3), in agreement with the known solubility of brochantite in acids.[37] At neutral, basic or slightly acidic pH (distilled water, pH 6−6.5) the copper release is instead negligible.

***In Vitro* Antibacterial and Antifungal Activity.** HMW, MMW, and related HMW@Cu and MMW@Cu materials were tested for their antifungal and antibacterial activity toward a vast panel of food-related and agronomical pathogens. The results are collected in Table 4 and Table 5. Miconazole (against bacteria) and Ceftriaxone (against fungi) were used as positive control, while copper(II) hydroxide, normally used as commercial fungicide on crops, was considered for comparison as a copper-containing antibacterial and antifungal agent. Copper content (g/L) is given in parentheses. Considering food-related bacteria (Table 4) it is possible to notice that all tested compounds, comprising the two lignins HMW and MMW, have good activity against both Gram-positive and Gram-negative pathogens, thus confirming the potential of lignins and lignin-derived compounds to be used as biobased

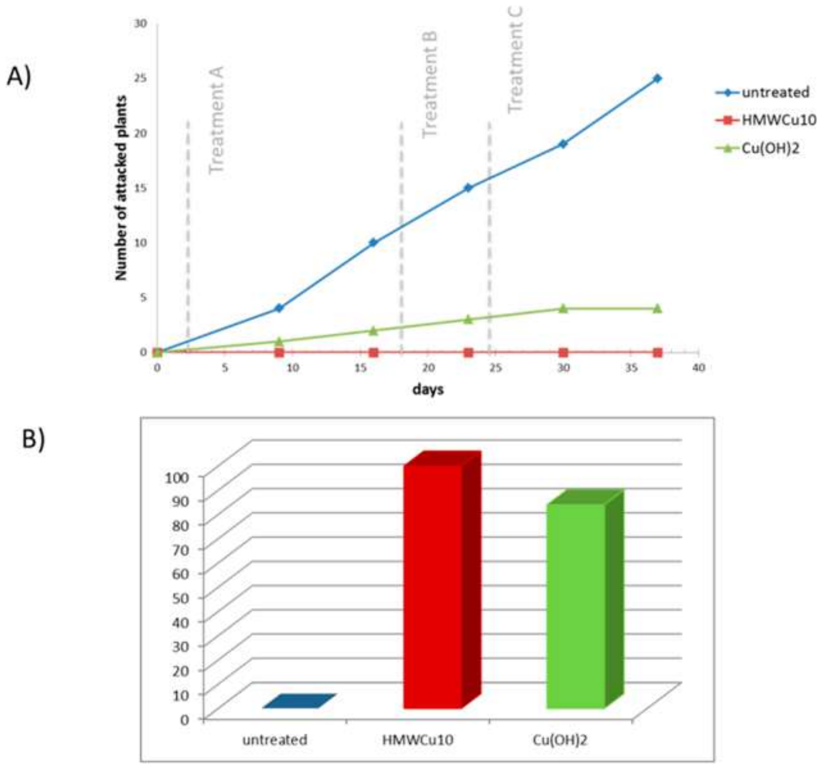


Figure 8. (A) Number of attacked plants (dead) on 30 plants at scheduled assessments. Day 0 corresponds to inoculum of the pathogen. (B) Efficacy of treatments against *Rhizoctonia s.* in term of incidence % on 35 days after first treatment with tested compounds.

broad spectrum antibacterial and antifungal agents. Generally, HMW and HMW@Cu highlight a better profile *vs* MMW and MMW@Cu. This trend is maintained also with phytopathogen bacteria (Table 5), while against fungi the activity is more diversified, confirming, in any case, a broad-spectrum activity of the tested compounds.

It is worth noting that, with very few exceptions, an improvement in activity is observed passing from lignin to MMW@Cu and HMW@Cu. In both cases, in fact, lower MICs are observed for Cu-containing materials vs the corresponding free lignin. In Table 4 it can be noticed that for HMW@Cu compounds the activity is driven by copper content, since the MICs of the HMW@Cu at different Cu percentages correspond to the same copper concentration (given in parentheses), resulting in lower MIC for higher copper-containing species. The nanocrystalline form of brochantite evidenced by TEM analysis (Figures 5–7) could be an explanation of the reduced amount of copper necessary to obtain bacterial inhibition. Moreover, the presence of nanosticks of brochantite in HMWCu_10% and MMW@Cu_10% allows a diminished quantity of lignin necessary to obtain inhibition. The same considerations can be done, at a lower extent, for MMW@Cu compounds, in which probably MMWCu_10% presents a copper concentration higher than necessary to ensure activity. These considerations can also be applied to most of the data reported in Table 5. Therefore, the synergic activity of lignin could be useful to lower the copper concentration employed maintaining at the same time good activity, exploiting the use of natural, low cost, renewable polyphenols.

Interesting is the observation that, with the exceptions of *C. jejuni* and *X. arboricola* (Table 4 and Table 5, respectively), in all the cases the HMW@Cu and MMW@Cu compounds show activity at copper concentration levels equal or lower than commercial copper hydroxide. With HMW@Cu, in particular, MICs corresponding to lower copper content are always observed when compared to commercial copper hydroxide. Particularly interesting are the results obtained with *P. aeruginosa* and *C. albicans* (Table 4): HMWCu_2% presents in fact a MIC with a 3-fold (against *P. aeruginosa*) and 6-fold (against *C. albicans*) lower copper content if compared to copper hydroxide. Even more striking are the results obtained with the fungi *A. solani*, *F. solani*, and *B. cinerea* (Table 5): in these cases, HMWCu_2% presents MIC with a copper content which is more than 6-fold (against *A. solani* and *B. cinerea*) and up to 25-fold (against *F. solani*) lower than $Cu(OH)_2$.

Tests on Crops in Greenhouse. To evaluate possible applications in the agronomical field, we performed tests on crops in the greenhouse. A local variety of Italian tomato (*Cuore di Ponente*) was chosen, and the efficacy of HMWCu_10% was evaluated against *Rhizoctonia solani,* a plant pathogen with a wide host range and worldwide distribution. HMWCu_10% was appropriately formulated so as to obtain a stable suspension that can be applied directly on the plants (final copper concentration, 10 g/L). The place for the trial was selected among those better suitable for the cultivation of the tomato in Italy and where the climatic-environmental conditions were favorable to the setting in of the pathogen. A first application of the tested compound was made 24/48 h after artificial inoculation of the pathogen (application A). Then other 2 applications followed, about 14 days after the first application (application B) and 7 days after the second application (application C), at about 24/48 h after a second artificial inoculation. The first signs of disease appeared 8 days after application A. As a reference, a commercial product composed of copper hydroxide was employed. Results are illustrated in Table 6 and Figure 8.

HMWCu_10% had a very good performance on the control of the diffusion and intensity of the disease if compared to the untreated control (Figure 8). In particular, the percentage of efficacy was effectively superior to the reference commercial product. It is worth noting that the final copper concentration *per* hectare was 30 g for lignin@Cu, while it was much higher for the commercial product, that is, 600 g/ha (see Table S5).

These results clearly indicate that lignin@Cu lead to a drastic diminishing of the concentration of copper employed for pest control.

It is interesting to note that *in vitro* data are collected at buffered pH-neutral for bacteria, in the 5−6.5 range of pH for yeasts and fungi (Tables S1 and S2). While therefore we can expect an almost quantitative release of copper at pH 5, in all other cases the lignin@Cu materials exert their action by contact, in the same way as the copper hydroxide we used as control, which presents solubility similar to brochantite.[38] Different is the situation in the field, where the compounds are sprayed on the leaves: in this case, a slight acidic pH determined by environmental conditions could ensure a slow release, which represents an important advantage.

■ CONCLUSIONS

Valorization of an industrial waste and reduction of the use of copper to obtain pest control represent ambitious goals in the field of green economy. Our study was aimed to give a contribution to this effort, with the idea of combining lignin, an underutilized byproduct of the pulp industry, with copper, the most widely employed metal in agriculture for crop protection. We have here presented a class of hybrid organic−inorganic materials, lignin@Cu, based on lignin: this versatile biobased material has been successfully uploaded with brochantite, which synergistically enforces the antiphytopathogenic profile of the same lignin. Lignin@Cu at various copper contents have demonstrated very promising activity against a broad panel of bacteria and fungi of agronomical interest. Moreover, preliminary results obtained with lignin@Cu in greenhouse on tomato plants infected with *Rhizoctonia s.* highlight that it is indeed possible to reduce the quantity of copper used in agriculture, encouraging the efforts to explore the features of a biobased material such as lignin for the development of plant protection products of a new generation. Tests are actually ongoing in a greenhouse on a variety of crops infected with different pathogens, in order to evaluate the possibility of exploiting lignin@Cu hybrid material as a biobased pesticide with reduced copper content.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.8b05135.

> XRPD, IR spectra, experimental conditions for *in vitro* test on bacteria and fungi, experimental conditions for tests in a greenhouse (PDF)

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Notes

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