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Making agriculture more sustainable: an environmentally friendly approach to the synthesis of lignin@Cu pesticides

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ABSTRACT

Despite its high chemical value, most of lignin is nowadays burnt as low value fuel. It is therefore important to find innovative applications for its use. Copper compounds are used as plants protection products for more than 50 different diseases in viticulture, arable crops, hops and horticulture, and they have been using for more than 100 years. Minimization of copper in agriculture has become a fundamental issue due to its negative environmental impact. Here we present a series of hybrid organic-inorganic materials (lignin@Cu), deriving from the combination of lignin with brochantite, $Cu_4(OH)_6SO_4$. Optimization of the synthetic procedures has allowed to isolate lignin-based materials containing different percentages of copper, where the brochantite crystals are featured by different morphologies and dimensions. A more environmentally safe synthesis of lignin@Cu materials by mechanochemistry is also investigated, which reduces the amount of water used and makes easier and faster the isolation of the final materials. Tests on strawberry and tomato plants in greenhouse have highlighted a significative efficacy of the lignin@Cu materials against different pathogens at a copper content much

lower than the one of copper-based commercial pesticides. A crystal morphology-activity correlation is also traced out. The synergic activity of lignin and copper ions can be used to reduce the copper content for efficient pathogen control. Moreover, the mechanochemical approach ensures a greener synthetic approach, in a perspective of a more sustainable agriculture.

KEYWORDS: Lignin, Copper, Brochantite, Mechanochemistry, Plant protection products, Cu-based antimicrobials, $Cu_4(OH)_6SO_4$

INTRODUCTION

Sustainability is one of the keywords for a better future. There is an urgent need to find out more sustainable practices in all the human activities related with industrial and agricultural productions. The Food and Agricultural Organization of the United Nations (FAO) has declared 2020 as the International Year of Plant Healthy, to underline the need to protect plant health to end hunger, protect the environment and boost economic

development (http://www.fao.org/plant-health-2020/about/en/). The same organization estimates that 40% of food crops is lost, each year, due to plant pests and diseases.[1] Furthermore, the emerging economies and developing countries have raised almost three-fold the annual value of trade agricultural products over the past decade, and a raising of about 60% of the agricultural production by 2050 is estimated to be necessary to satisfy the continuously increasing food demand. The need to assure an adequate production must deal with the equally fundamental need to develop environmentally friendly crop-protection practices. Copper-containing pesticides have been using since 1885 (Bordeaux mixture)[2] and since then many effective copper-based antimicrobial products have been developed.[3-4-5-6] Although not yet fully understood and dependent on several factors (physical form, Cu oxidation state, form of application, pH among others),[7] the antibacterial activity of Cu is based on two main mechanisms. When the metal is delivered as Cu^{2+} the membrane depolarization mode of action prevails[8], while in the presence of Cu-nanoparticles the generation of Reactive Oxygen Species (ROS) seems dominant[9]. The effectiveness of Cu-based compounds has led to their extensive use to control foliar pathogens worldwide, especially in organic farming, where the use of many

conventional pesticides is forbidden. However, there are important concerns about their long-term sustainability owing to the accumulation of the metal into the soil with consequent damages to the microbiota, long-term phytotoxicity and potential food and groundwater contamination.[3] In response to this alert, the European Community has recently lowered the annual maximum copper limit, from 6 Kg/ha to 4 Kg/ha.[10] However, copper-based pesticides still represent one of the few alternatives for the efficient manage of bacterial and fungal diseases on crops. A possible perspective for a more sustainable crop protection is therefore to develop new Cu-based compounds with high antimicrobial activity and low copper content.

Lignin is a natural, abundant and biodegradable biopolymer,[11] usually considered as a waste of the paper pulp manufactury.[12] Nowadays most of lignin is burnt to produce steam and energy, although the development of high value applications of lignin is a field of intense research.[13] For example, lignin has been used to encapsulate bioactive molecules[14-15-16] and as carrier.[17] Although depending on several aspects, such as wood species, the way of production and the way of fractionation, the capacity of lignin to inhibit

the growth of several microorganisms, such as *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Candida albicans*, is known.[18-19] Its antimicrobial activity is ascribable to the presence of polyphenolic structures that causes cell membrane damages and lysis of bacteria, with consequent cell content release.[20] The antimicrobial activity of lignin nanoparticles has also been reported.[20-21-22-23] Moreover, lignin materials functionalized with metal-based antimicrobial agents such as silver,[24-25] Cu_2O[26] or silver-gold[27] nanoparticles have recently been published.

On the basis of these premises, we recently prepared an innovative hybrid organic-inorganic material[28] composed by lignin and brochantite $Cu_4(OH)_6SO_4$. In that paper we showed that lignin exerts a control over the crystallization process of the Cu-containing phase, avoiding the formation of oxides and leading to the exclusive formation of brochantite. Moreover, the optimization of the synthetic procedure allowed to isolate different materials with a fine control of the amount of copper loaded and of the dimension/shape of the brochantite crystals. The importance of the particle shape/dimension on the activity of Cu-based pesticides is well known and related with the

adhesion and permanence of the product on the leaf surface.[29] Tests *in vitro* against a panel of pathogens of agronomical interest highlighted the synergistic effect of lignin and brochantite allowing a significant lowering of the Cu-content with respect to commercially available Cu-based pesticides.

The sustainability of a product derives also from the procedure followed for its production. In this regard, mechanochemistry is a well-established synthetic procedure for making solid compounds through a solvent-free approach. Mechanochemistry has been applied for the preparation of slow-release-fertilizers,[30–32] co-crystals of agronomical interest,[33–36] to enhance the solubility of phosphates,[37] as well as for the synthesis of organic insecticides.[38]

In this work we provide a new and simpler approach for the synthesis of lignin@Cu materials based on mechanochemistry that, compared with the wet procedure, has undoubted practical advantages that make easier a potential scale-up of the process in view of large-scale production.

Finally, the correlation between the dimensions/shape of the brochantite crystals and the antimicrobial activity of the corresponding lignin@Cu materials will be traced out on the base of *in vivo* tests against *P. infestans* carried out on tomato plants. The results collected in greenhouse against several pathogens on tomato and strawberry will be also addressed, in order to highlight a broad-spectrum activity and confirm the effectiveness of lignin@Cu as crop-protective agents with a reduced copper content.

EXPERIMENTAL SECTION

Materials and methods. The technical lignin employed (Kraft lignin) in this study is referred to as HMW and the Cu-containing materials (lignin@Cu) will be referred to as HMW@Cu (HMW@CuX%_w or HMW@CuX%_m; w = wet, m = mechanochemistry, X = 3-20%).

HMW (BioPiva 100®; *Pinus taeda*, M_w = 4400-5000 g/mol, M_n = 1200-1300 g/mol) lignin was kindly provided by UPM-Kymmene Oyj (Helsinki, Finland) and Green Innovation GmbH (Innsbruck, Austria). $CuSO_4 \cdot 5H_2O$ and NaOH 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.

Mechanochemical syntheses were conducted by means of a planetary ball mill Retsch PM100 using an 80 mL agate jar and 5 spheres of the same material with a diameter of 10 mm.

General procedure for the preparation of HMW@Cu.

Wet procedure. HMW@CuX%_w (X = 3, 6, 10, 15, 20) were obtained according to the experimental protocol previously described,[28] with slight modifications. Briefly, HMW was suspended in distilled water and added of $CuSO_4 \cdot 5H_2O$ at room temperature. pH was adjusted to 7 by adding NaOH 1 M dropwise. Stirring was carried on for 2 hours at room temperature. The brown solid was filtered and washed with water, then it was first air dried and then left at 80 °C overnight. The dried material was grinded for 2 minutes at 300 rpm in order to obtain a brown powder. Different lignin/$CuSO_4 \cdot 5H_2O$ mass ratios were applied, ranging from 1g/0.12 g to 1g/1.20 g; all the experiments were performed at least twice. The experimental details are reported in **Table S1**.

Mechanochemical synthesis. HMW@CuX%_m (X = 3, 6, 10 20) were synthesized adjusting the amount of $CuSO_4 \cdot 5H_2O$ introduced in the jar with respect to the amount of lignin, keeping constant the molar ratio between copper salt and NaOH ($CuSO_4 \cdot 5H_2O$/NaOH=1/2). For the experimental details see **Table S2**. Here, the procedure for the preparation of 1g of HMW@Cu10%_m is detailed. An 80 ml agate jar was charged with 0.6 g of solid lignin and 0.3 g of $CuSO_4 \cdot 5H_2O$, which were pre-ground for 2 minutes. Subsequently, 0.08 g of NaOH were added. In order to assure a homogeneous wetting of the solid mixture, 2 mL of distilled water were added to the mixture. The grinding lasted 1 hour with an inversion time of 30 minutes. The resulting paste was washed with 50 ml of water, dried overnight at 60°C using a heating plate, and then grinded 2 minutes at 300 rpm.

Experimental details on the optimization of the selective syntheses of brochantite over posnjakite are reported in **Table S3**, by using HMW@Cu10%_m as reference.

Sample characterization. Copper content of the lignin@Cu materials was measured by ICP-AES analysis (Inductively Coupled Plasma – Atomic Emission Spectroscopy) by means of a JY 2501 of the HORIBA Jobin Yvon, ULTIMA2 model, following the procedure

already reported (see Supporting Information for instrument and sample preparation details).[28]

The mineral phase was identified by X-ray powder diffraction analysis (XRPD). Data were collected on a Thermo Scientific ARL X'TRA diffractometer in theta−theta Bragg−Brentano geometry with CuKα radiation. All experimental data were compared against the cell parameters reported in literature.[39] XRPD data were collected on HMW@Cu10%_m milled at different time scale, namely 15, 30 and 60 minutes, in Bragg−Brentano (BB) geometry with CuKα radiation on a Rigaku Smartlab XE diffractometer equipped with a solid-state Hypix3000 2D detector. To increase the limit of detection (LoD) of any crystalline impurity, data were collected with 5° Soller slits and variable vertical slits, which guarantee the same volume of sample under the beam along the measurement. Data were then normalized to the counting time. To evaluate the content of the materials, Pawley refinements were performed against cell parameters reported in literature.[40]

XRPD analysis on HMW@Cu10%_w after formulation was performed on a Rigaku SmartLab XE diffractometer equipped with a CBO parabolic mirror (parallel beam, CuKα)

and a 2D HyPix3000 solid state detector. Data were collected overnight in the 3-80 2Θ (deg) range in transmission geometry. The sample was loaded into a 1.0 mm glass capillary and aligned against the beam position (vertical direction) before data acquisition. Transmission Electron Microscopy (TEM) characterizations were carried out using a FEI TECNAI F20ST microscope operating at 200 kV and equipped with EDAX PV9761-SUTW Energy Dispersive X-ray Spectrometer (EDS). Scanning Transmission (STEM) pictures were recorded using a High Angle Annular Dark Field (HAADF) detector: in this imaging mode, the intensity I of an image point is proportional to $Z^{1.7}t$, where Z is the mean atomic number and t is the thickness of the specimen. The specimens were prepared by grinding the powders in isopropyl alcohol. The solution was subsequently sonicated for 15 min and drop casted on holey carbon film heated at 50 °C.

Tests on crops in greenhouse. Tests were conducted at Soc. Agr. AGOFLOR S.C.- via delle Messi, 101-64014 Martinsicuro (TE)-ITALY and at Centro Ricerche Agronomiche ed Ambientali, Res Agraria srl, via A. Canova 19/2, 64018 Tortoreto Lido (TE)-ITALY (climatic chambers). HMW@Cu3%_w, HMW@Cu10%_w, and HMW@Cu20%_w were appropriately formulated in order to have 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 (**Table S4-S7**).

Three different trials were performed on different crops against various pathogens (**Table 1**).

Trial 1. The tests were conducted in greenhouse conditions (54 plots, 10 plants/plot) by using Four Seasons variety of strawberry. The following parameters were evaluated: percentage of attacked fruits in the field and after storage (efficacy).

Table 1. Target diseases and crops analyzed after foliar application of tested compounds.

Trial	Tested compound	Crop	Pathogen
1	HMWCu10%_w	Strawberry (*Fragaria sp.*) Variety: Four Seasons	*Botrytis cinerea*
2	HMWCu10%_w	Tomato (*Solanum lycopersicum*) Variety: Optima	*Pseudomonas syringae, Xanthomonas campestris, Xanthomonas arboricola fragrari, Botritis cinerea*

3	HMWCu3%_w HMWCu10%_w HMWCu20%_w	Tomato (*Solanum lycopersicum*) Variety: Optima	*Pseudomonas infestans*

A good level of disease was assessed on the trial area; disease's level further increased after artificial inoculation performed before the third application and at the last assessment it was possible to observe about 20% of disease's diffusion on fruits of untreated plots. The efficacy and selectivity assessments on *B. cinerea* were made at application B, 7 days after application C and at 3 days of storage, *i.e.* 3 days after the second assessment and 10 days after application C (**Table S5**). Iprodione (Rovral WG, Basf Italia) and *Bacillus amyloliquefaciens* (Amylo-X WG, Biogard) were used as reference.

Trial 2. The tests were conducted in climatic chamber with all abiotic parameters under control, by using Optima variety of tomato and sterile soil. The following parameters were evaluated: percentage of leaves attacked on 60 leaves per plot (incidence), percentage of area attacked on 60 leaves per plot (severity). Two different dosages were tested for HMWCu10%_w, 3 L/ha and 10 L/ha. The first signs of diseases appeared 7 days after the first application on untreated plots, with an incidence of 10% for *Pseudomonas*

syringae tomato, *Xanthomonas campestris*, *Xanthomonas arboricola fragari* and with an incidence of about 2% for *Botrytis cinerea* in average. A high level of attack by all the different pathogens occurred further by 2 artificial inoculations. Commercial copper(II) hydroxide was used as reference (Coprantol Hi Bio 2.0, Syngenta).

The efficacy and severity assessments on *P. syringae tomato, X. campestris, X. arboricola fragrari, B. cinerea* were carried out as follow: 15 days after application A, 8 days after application B, 7 days after application C (**Tables S6**).

Trial 3. The trial was carried out in climatic chamber with all abiotic parameters under control, by using Optima variety of tomato and sterile soil. 11 plants were used for treatment. The products were applied in foliar method in three times at different phenological phases and after artificial inoculation, 3 days before first application. The efficacy (number of attacked leaves) and severity (% of attack on leaves) assessments on *P. infestans* were carried out as follow: 7 days after application A, 7 days after application B, 7 days after application C (**Table S7**).

Statistical analyses were applied to the incidence and severity values compared to the untreated control. For each assessment date the homogeneity of variance was tested by

Bartlett's test. For all trials, phytotoxicity symptoms were assessed at every visit to the trial site. No phytotoxicity was observed on any visit on any plot treated with HMW@Cu3%_w, HMW@Cu10%_w, and HMW@Cu20%_w.

RESULTS AND DISCUSSION

Synthesis of lignin@Cu. For the preparation of lignin@Cu materials two different approaches were followed. In the wet-procedure lignin was vigorously stirred in a $CuSO_4$ water solution while NaOH was added dropwise.[28] The weight ratio between lignin and $CuSO_4 \bullet 5H_2O$ was adjusted based on the desired final Cu-content, while a Cu^{2+}/OH^- = 1:2 molar ratio was kept constant for all the experiments. XRPD analysis confirmed that complete conversion to brochantite can be obtained in 2 hours, drastically reducing the time previously adopted (24h).[28]

The final material was collected through a rather long and tedious filtration step and then dried overnight at 80 °C.

In the mechanochemical procedure, lignin and $CuSO_4 \bullet 5H_2O$ were pre-mixed in an agate jar by means of a planetary ball-mill. The mixture was then added of the desired amount of a NaOH solution. As for the wet-procedure, the mass ratio between lignin and $CuSO_4 \bullet 5H_2O$ was adjusted on the base of the desired Cu-content, keeping constant the Cu^{2+}/OH^- = 1:2 molar ratio. An additional small volume of water was added to assure the homogeneous milling of the solid mixture (see below). The collection of the final product was much easier if compared with the wet procedure. The two synthetic approaches are schematically reported in **Figure 1**.

The copper content was determined by ICP-AES analysis, while the crystallographic nature of the mineral phase was determined by XRPD and further confirmed by ED-TEM analyses (*vide infra*). Both procedures led to the complete upload of the desired amount of copper, thus ensuring a fine control on the metal content in the isolated materials (see Supporting Information for details). By wet procedure we isolated five different materials containing 3, 6, 10, 15 and 20% w/w of copper (HMW@CuX%_w, X = 3, 6, 10, 15, 20) (**Table S1**). By mechanochemistry, four different materials corresponding to 3, 6, 10 and 20% w/w were isolated (HMW@CuX%_m, X = 3, 6, 10, 20) (**Table S2**).

Optimization of the mechanochemical procedure. If under wet conditions the inorganic phase was exclusively brochantite ($Cu_4(OH)_6SO_4$), in the case of mechanochemistry the nature of the Cu-containing phase was dependent on the amount of water added to the solid mixture. In this regard, the optimization of the mechanochemical synthesis was conducted using lignin@Cu10%_m as a reference. Initially, lignin, $CuSO_4 \cdot 5H_2O$ and NaOH were neatly ground for 1, 2 and 4 hours (**Table S3**), with no water added. Under these conditions, posnjakite ($Cu_4(OH)_6SO_4 \cdot H_2O$) was found as exclusive crystalline Cu-containing phase (**Figure S1a**).

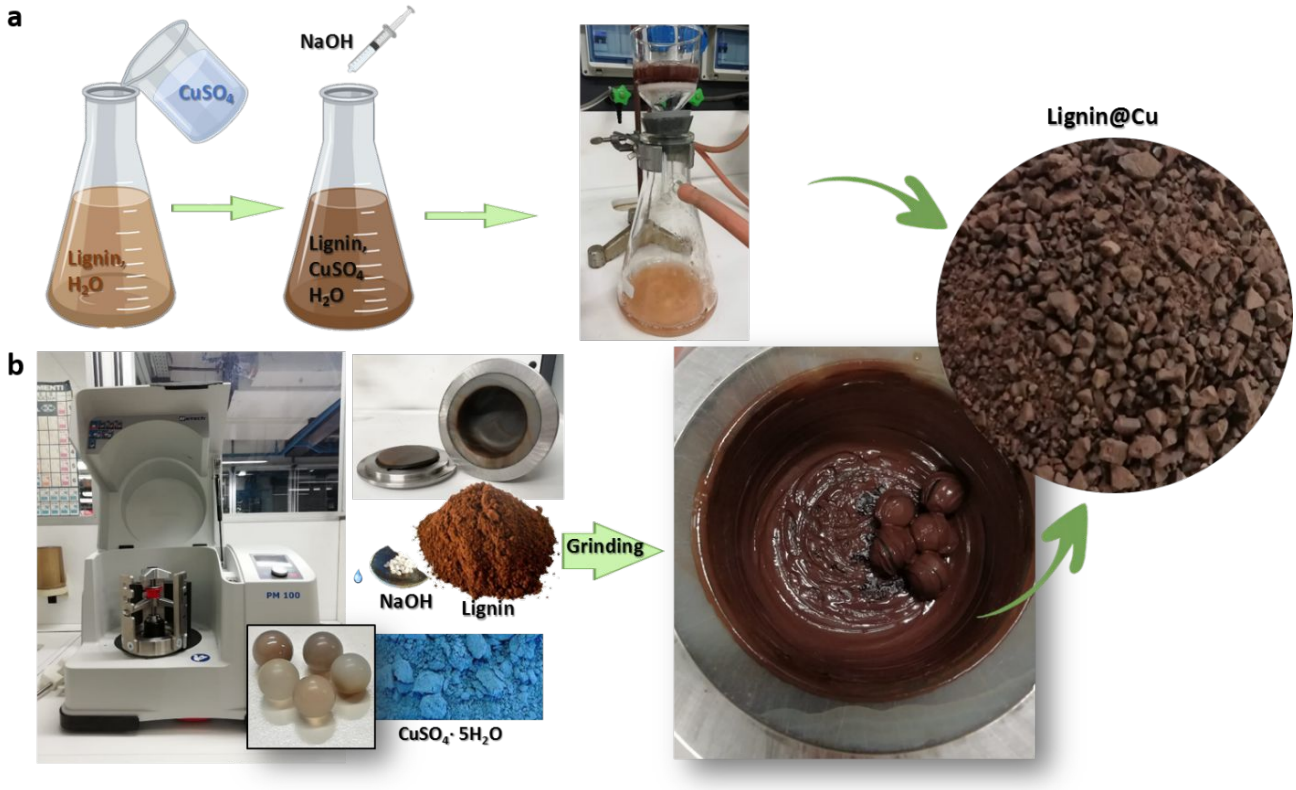


Figure 1. Comparison between the two synthetic pathways followed to obtain the hybrid material lignin@Cu: a) wet procedure, b) mechanochemistry.

The ICP-AES evidenced the complete upload of copper already after one hour of milling. No transition from posnjakite to brochantite was observed even when grinding was prolonged up to 4 hours. Brochantite is described as thermodynamically more stable than posnjakite, and conversion of posnjakite into brochantite is described when the first is left in contact with the mother liquors at room temperature.[38-39] The syntheses were then repeated with an increasing amount of water with respect to the total weight of the solid reactants (**Table S3**). Liquid assisted grinding (LAG) procedure with the addition of 2 ml of water resulted in pure brochantite as unique Cu-containing crystalline phase (**Figure S1c**). In the case of lower amounts of water, a mixture of posnjakite and brochantite was instead obtained (**Figure S1b**). In conclusion, the use of water resulted to be crucial to trigger the transition from posnjakite to brochantite: by monitoring LAG synthesis of HMW@Cu10%_m as function of time (**Figure 2**), a mixture of posnjakite and brochantite

was detected after 15 and 30 mins respectively; after 1h of milling, a full conversion into brochantite was reached.

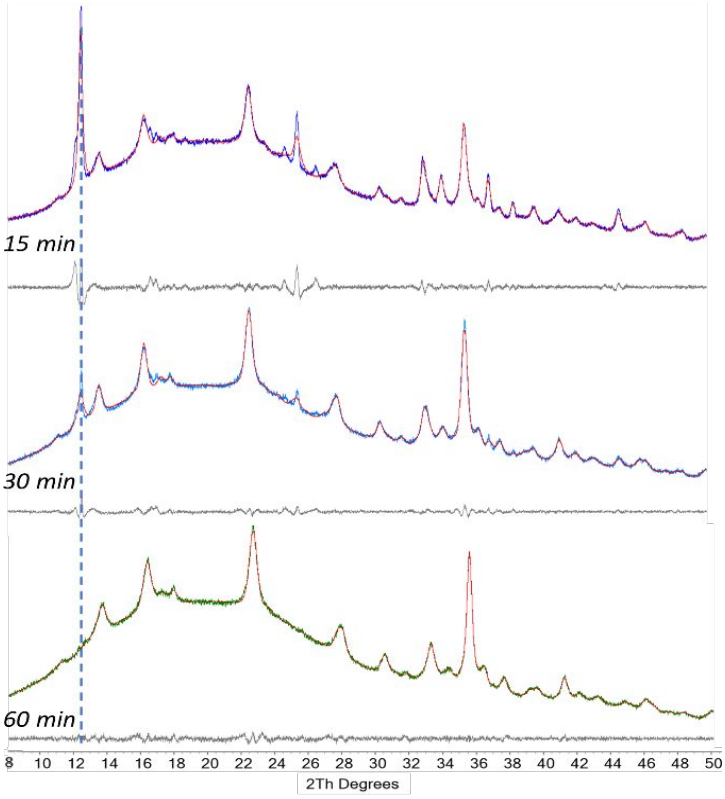


Figure 2. Pawley fit (red lines) against experimental data of HMW@Cu10%_m obtained according to mechanochemical protocol at different grinding times; 15 minutes (top, blue lines), 30 minutes (middle, cyan line) and 60 minutes (bottom, green line). Vertical blue dashed line represents the position of the (010) reflection of posnjakite (minority phase)

that progressively decreases as function of time in favour of brochantite (majority phase). Grey lines represent the difference between the experimental and calculated patterns.

Based on these results, the synthesis of HMW@Cu3%_m, HMW@Cu6%_m and HMW@Cu20%_m were performed by LAG with the addition of at least 2 ml of water lasting the milling for 1 hour. In all cases brochantite was detected as exclusive mineral phase and no trace of Cu(I)-containing specie was found.

Once optimized the syntheses of the materials, we started to investigate, by an in-depth TEM analysis, the possible correlation between copper content and shape/dimension of brochantite crystals, comparing the results collected with the two synthetic approaches. The results are discussed in the following section.

TEM analysis of lignin@Cu materials. The samples isolated by the wet and mechanochemistry procedures were analysed by TEM microscopy. In all cases, SAED (selected area electron diffraction) patterns evidenced the presence of the major reflections of the brochantite and the EDS analyses conducted on several crystals exhibited a semi-quantitative S/Cu ratio close to ¼ in agreement with the brochantite

composition. Hence, all samples corresponded to a physical mixture of crystalline brochantite and amorphous lignin. The morphological analysis of the crystalline entities revealed that HMW@Cu3%_w contained nanometric spherical crystals with a diameter spanning from 2 to 20 nm, as already evidenced in our previous work[28] (**Figure 3a** and **Table 2**). In HMW@Cu6%_w, beside the spherical entities just mentioned, small sticks with a length spanning from 50 to 150 nm and a thickness of 10-20 nm were found (**Figure 3c**). In the other HMW@CuX%_w samples (X=10 or 20) with higher Cu-contents the crystals appeared exclusively as sticks (**Figure 3e-g**).

The morphological analysis conducted on the samples obtained by mechanochemistry evidenced a rather different situation. Nanospheres were not found, not even in HMW@Cu3%_m (**Figure 3b**). Here in fact the crystals appeared as sticks with a length of 40-150 nm and a thickness of 10-20 nm, being comparable with the ones found in HMW@Cu10%_w (**Figure 3e**). The increase of the Cu-content again affects more the length than the thickness of the crystals (**Table 2)**.

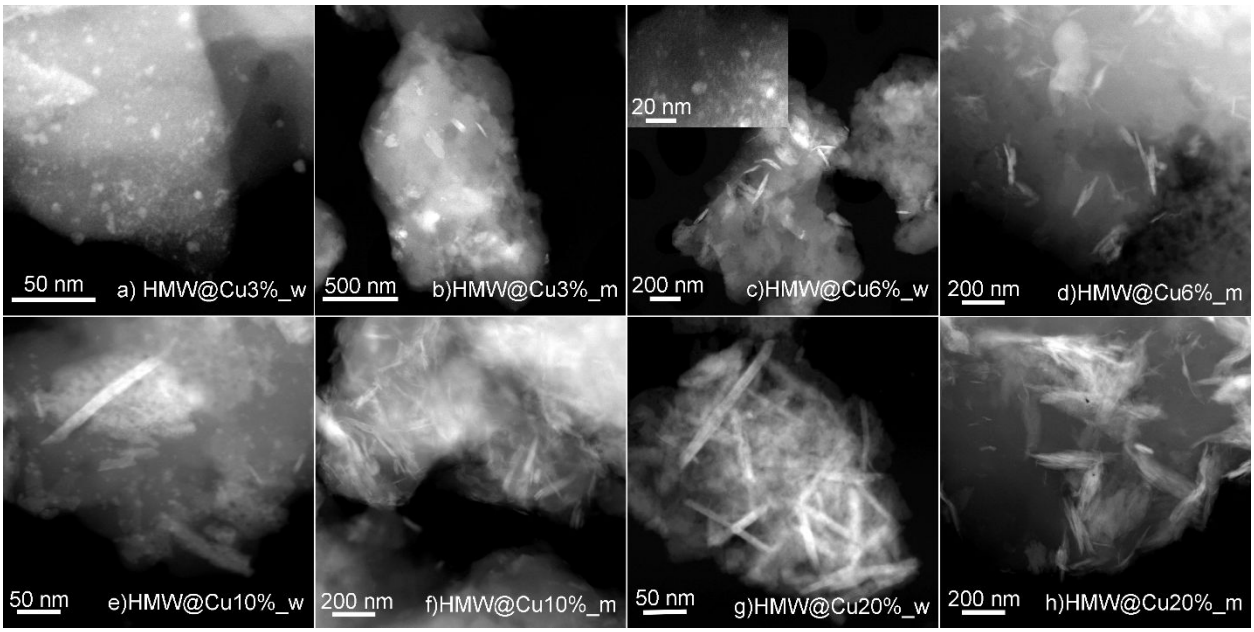


Figure 3. STEM-HAADF images of lignin@Cu hybrid materials. The brochantite crystals appear brighter than the lignin matrix. Comparison between aqueous and mechanochemical synthesis and correlation between size of crystals and copper concentration.

Table 2. Morphological details for HMWCuX%_w and HMW@CuX%_m as determined by TEM analysis.

Synthetic procedure	Sample	Morphology	Dimensions (nm)

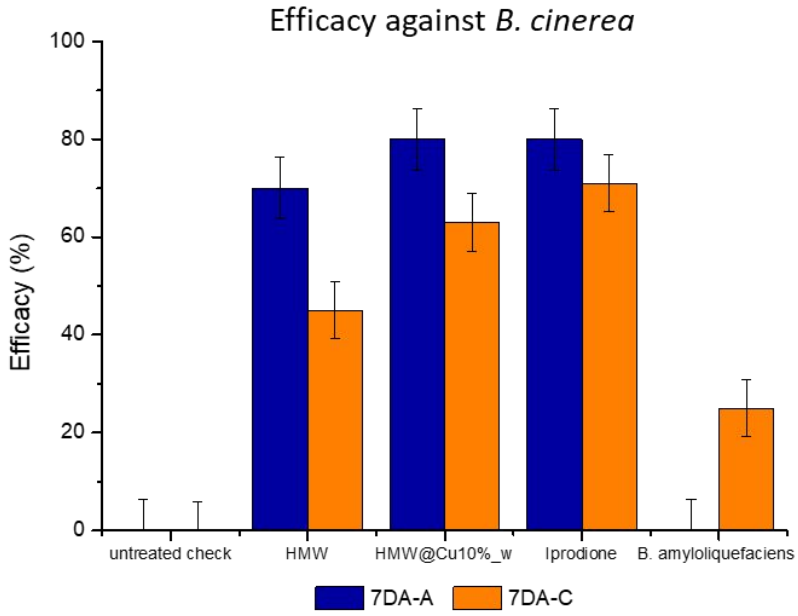
			Thickness/Length
Wet	HMW@Cu3%_w	Spheres	2-20
	HMW@Cu6%_w	Spheres Sticks	2-10 10-20/50-150
	HMW@Cu10%_w	Sticks	10-30/50-200
	HMW@Cu15%_w	Sticks	10-30/100-250
	HMW@Cu20%_w	Sticks	10-20/40-150
Mechanochemistry	HMW@Cu3%_m	Sticks	10-20/40-150
	HMW@Cu6%_m	Sticks	8-40/30-200
	HMW@Cu10%_m	Sticks	10-50/60-300
	HMW@Cu20%_m	Sticks	10-50/60-300

Tests on crops in greenhouse. Preliminary tests have highlighted a very promising potential for lignin@Cu hybrid materials as crop-protective agents, with very good results against *Rhizoctonia solani* on tomato plants[28]. Therefore, we decided to broaden our

investigation to strawberry by using HMW@Cu10%_w, which was appropriately formulated in order to have a sprayable suspension. The integrity of the active phase after formulation was verified by XRPD analysis (**Figure S2**). TEM analysis conducted on the same formulate evidenced the integrity of the crystals of brochantite, whose dimensions were comparable to those found in the pristine material (**Figure S3**). A local variety of strawberries (*Four Seasons*) was chosen, and efficacy of HMW@Cu10%_w was evaluated against *B. cinerea,* a well-known worldwide diffused plant pathogen (Trial 1). As a reference, two different products were used: iprodione, a contact chemical fungicide currently applied to a wide variety of crops, and *Bacillus amyloliquefaciens*, a biopesticide used as a biocontrol bacteria. Three applications of tested compounds with 7 days of interval were made (applications A-C), according to the schedule detailed in the Supporting Information. Assessments on % attacked fruits were made at application B, *i.e.* 7 days after application A, and 7 days after application C. Moreover, we evaluated the efficacy of the treatments over 3 days of storage: a correct management of the post-harvest phase is in fact crucial to keep the fruit's quality intact. Results of the trial are collected in **Table S8** and depicted in **Figure 4**. The untreated check was set at 0%. In

terms of efficiency, HMW@Cu10%_w evidenced a particularly good performance on the control of disease diffusion and intensity on fruits in comparison with the untreated plot (**Figure 4A**). HMW@Cu10%_w has a behavior analogous to that of the antifungal reference iprodione, while it is much more performing than the biopesticide *B. amyloliquefaciens*. These results are confirmed when assessing the disease's diffusion on fruits at 3 days of storage: HMW@Cu10%_w allows a very good control of the disease, as can be inferred by comparison with the untreated check and with both lignin alone and the commercial references (**Figure 4B**). Looking at these encouraging results, we tested two different dosages of HMW@Cu10%_w, corresponding to 3 L/ha and 10 L/ha, against various pathogens (*Pseudomonas syringae tomato*, *Xanthomonas campestris*, *Xanthomonas arboricola fragari, B. cinerea*) on tomato plants, by using copper hydroxide, a commercial copper-based pesticide, as reference. Based on the previously collected *in-vitro* results that showed a generally lower activity of pure HMW and copper sulphate (in term of metal content) with respect to HMW@CuX%_w (X = 2, 6 and 10) against the same pathogens,[28] we included only commercial pesticides as bare control (iprodione, B.

amyloliquefaciens or copper hydroxide). Data are collected in **Table S9** and illustrated in **Figure 5** and **Figure 6**.



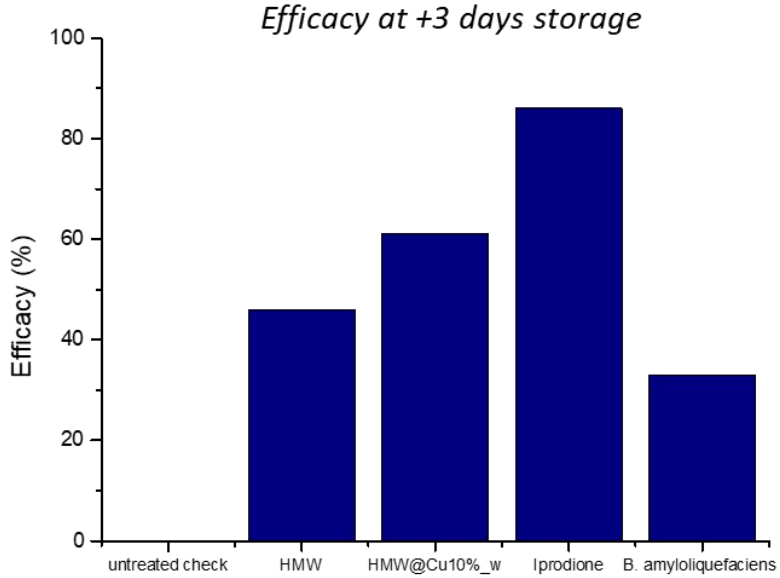


Figure 4. A) Efficacy of treatments against *B. cinerea* on strawberries (variety: *Four Seasons*), with untreated check set as 0%. 7DA-A: 7 days after application A; 7DA-C: 7 days after application C. B) Efficacy of treatments against *B. cinerea* at +3 days of storage, 10 days after application C. Untreated check set as 0%.

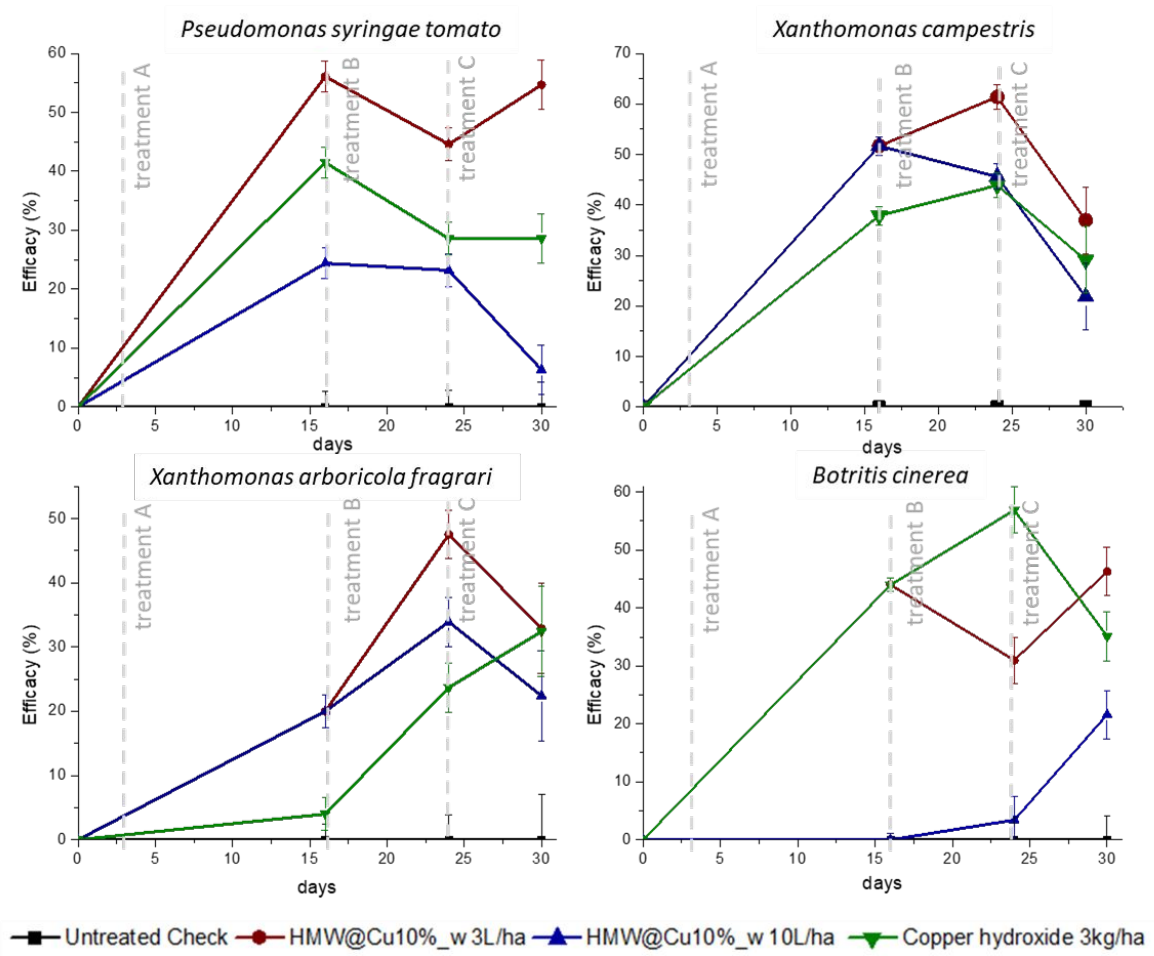

Pseudomonas syringae tomato
Xanthomonas campestris
Xanthomonas arboricola fragrari
Botritis cinerea
treatment A
treatment B
treatment C
Efficacy (%)
days
Untreated Check
HMW@Cu10%_w 3L/ha
HMW@Cu10%_w 10L/ha
Copper hydroxide 3kg/ha

Figure 5. Efficacy on tomato plant of the tested compounds against various pathogens expressed as percentage of attacked leaves on 60 leaves per plot at scheduled assessments, with untreated check set as 0%. Day 0 corresponds to inoculum of the pathogen.

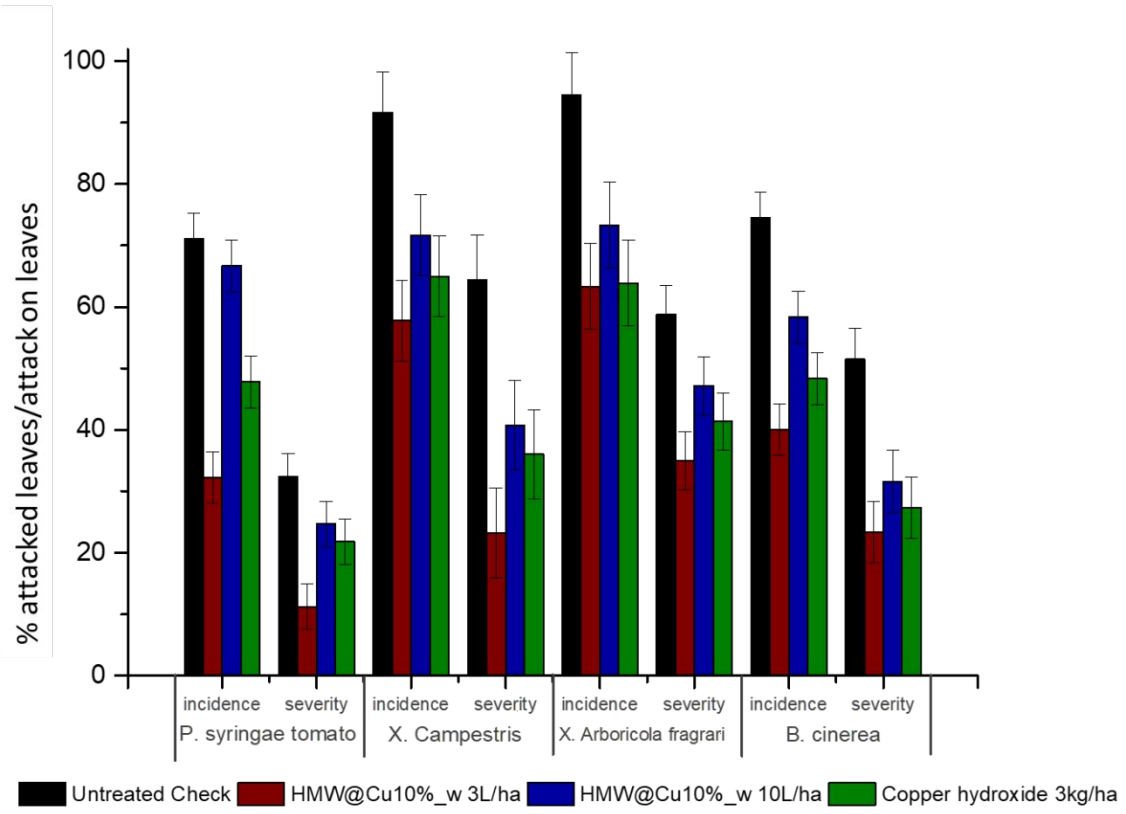


Figure 6. Percentage of attacked leaves (incidence) and of attack on leaves (severity) in tomato plants on day 30 after first inoculum for HMW@Cu10%_w in two different applications (3 L/ha, 10L/ha) against *P. syringae tomato*, *X. campestris*, *X. arboricola fragrari* and *B. cinerea*.

HMW@Cu10%_w has an excellent disease control for all the pathogens tested, as can be seen from the comparison with the untreated control in **Figure 5**. Unexpectedly, in all cases, a better performance is confirmed for the dosage of 3 L/ha when compared to 10 L/ha. As can be seen in **Figure 5**, HMW@Cu10%_w with a dosage of 3 L/ha presents, over time, better results with respect to commercial copper hydroxide and importantly, such results are obtained with a much lower copper content. The final copper concentration *per* hectare was in fact 30 g for HMW@Cu10%_w, while it was much higher for the commercial product, i.e. 600 g/ha. In **Figure 6** it is illustrated the behaviour of the tested compounds in term of incidence and severity of the pest on leaves (percentage of attacked leaves and percentage of attacked leave area on 60 leaves per plot, respectively) on day 30 after inoculum of the disease. For all the tested pathogens, HMW@Cu10%_w at the dosage of 3 L/ha shows better results than copper hydroxide in term of incidence and comparable results in term of severity. Again, the newly developed hybrid material can ensure a good control of the disease at low copper content, significantly lower than the one required by the reference pesticides.

Finally, we wanted to investigate the influence of the morphology of crystals of brochantite on activity.[42] We therefore evaluated the behaviour of HMW@CuX%_w with different copper content, i.e. with different morphology and dimensions of the crystals of the inorganic phase, towards *Phytophthora infestans* in tomato plant. Three different copper percentages were tested: 3, 10 and 20% (HMW@Cu3%_w, HMW@Cu10%_w and HMW@Cu20%_w, respectively). The final copper concentration was set identical for the three formulations (30 g/ha of metal), so that the quantity of copper dispensed to the plants was the same for the three experiments. The number of affected leaves and the percentage of attack on leaves were assessed, and data are reported in **Table S10** and **Figure 7**.

As can be inferred from **Figure 7A**, HMW@Cu10%_w and HMW@Cu20%_w assure a similar good control of the disease with respect to the untreated check, while HMW@Cu3%_w has lower activity. Looking at both incidence and severity on day 24 after inoculum (**Figure 7B**) it is possible to trace a dependence of the activity on the percentage of copper contained in the sample: HMW@Cu3%_w has the worst profile, while HMW@Cu20%_w gives the best results.

This trend seems to suggest that better results can be achieved with greater sticks of brochantite crystals typical of HMW@Cu20%_w, rather than with small, spherical crystals featuring HMW@Cu3%_w. A possible explanation could come from a longer persistence of the active material on leave due to a slower dissolution or higher adhesion,[43,44] the last being facilitated by the larger surface of the crystalline faces. In fact, copper pesticides are mainly insoluble and once applied, provide a protective film on leaf surface.

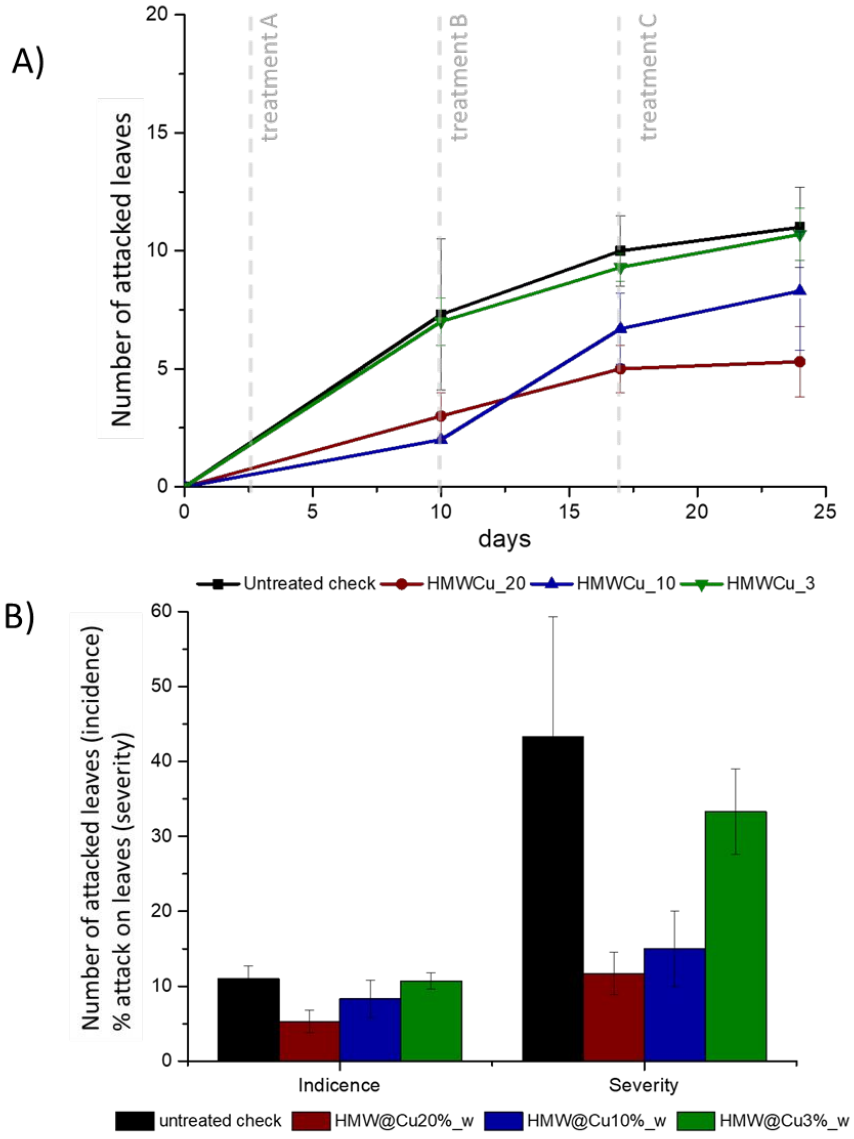


Figure 7. A) Number of attacked leaves on 11 plants at scheduled assessments. Day 0 corresponds to inoculum of the pathogen. B) Number of attacked leaves (incidence) and percentage of attack on leaves (severity) for tested compounds against *P. infestans* on tomato plants on day 24 after inoculum of the pathogen.

This layer acts as a Cu^{2+} reservoir that assures a prolonged metal release. This in turn leads to a better activity profile over time, as evidenced by the fact that dependence of disease control over the copper content is more evident at the last assessment, 24 days after inoculum (**Figure 7B**).

CONCLUSIONS

This work represents a contribution to one of the most essential subjects for a better future, the development of more sustainable agricultural practices. In this context, the development of effective green pesticides featured by a low environmental impact is a challenge of paramount importance. In this paper, we describe the valorization of lignin, an underutilized waste of the paper and bioethanol industry, in combination with Cu^{2+}, one of the oldest and most frequently applied antibacterial and antifungal agents. To limit environmental and toxicological risks, it is desirable to achieve effective disease control with reduced metal content. The combination of lignin with *in situ* grown nanocrystals of brochantite leads to hybrid materials that have great potential for the control of

microorganisms of agronomical interest, as evidenced by the *in vivo* tests described in this paper. The amount of copper necessary to have a good control over the pathogens is about 20 times lower than that usually employed with commercially available copper-based pesticides. The optimization of the material has been based on the experimental finding that the crystal morphology of the copper containing phase is dependent on the amount of copper loaded into lignin. Hence, the materials containing crystals with a stick shape and a length of about 10-30 nm are more effective than those containing spherical crystals of 2-10 nm, both in terms of incidence and severity of the infection. A possible explanation could come from the higher adhesion on leaf expected for the stick shape crystals, which assure a slower and then more prolonged copper release over time. In view of a possible scale-up of the synthesis, we have investigated the possibility of isolating the same materials by mechanochemical synthesis by means of a planetary ball-mill. The morphological control of the copper-containing crystalline phase is still possible, although with a lower degree with respect to the wet procedure. Since mechanochemistry is considered a greener technique with respect to conventional solution syntheses, here we have demonstrated the possibility of obtaining, through a sustainable synthetic

approach, effective pesticides deriving from the recovery of a waste and featured by a reduced amount of heavy metal. This research must be considered a preliminary approach to the development of a greener Cu-based pesticides. In fact, although the cytotoxic profile of lignin[45] and copper[46-47] are known, studies aimed at elucidating the cytotoxicity and mechanism of action of lignin@Cu materials are necessary. These are currently under way in our laboratories.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available online free of charge.

ICP-AES experimental details, XRPD, TEM images of formulate and statistical dimensional analysis, UV-Vis spectra, experimental conditions for tests in greenhouse, biological results (PDF).

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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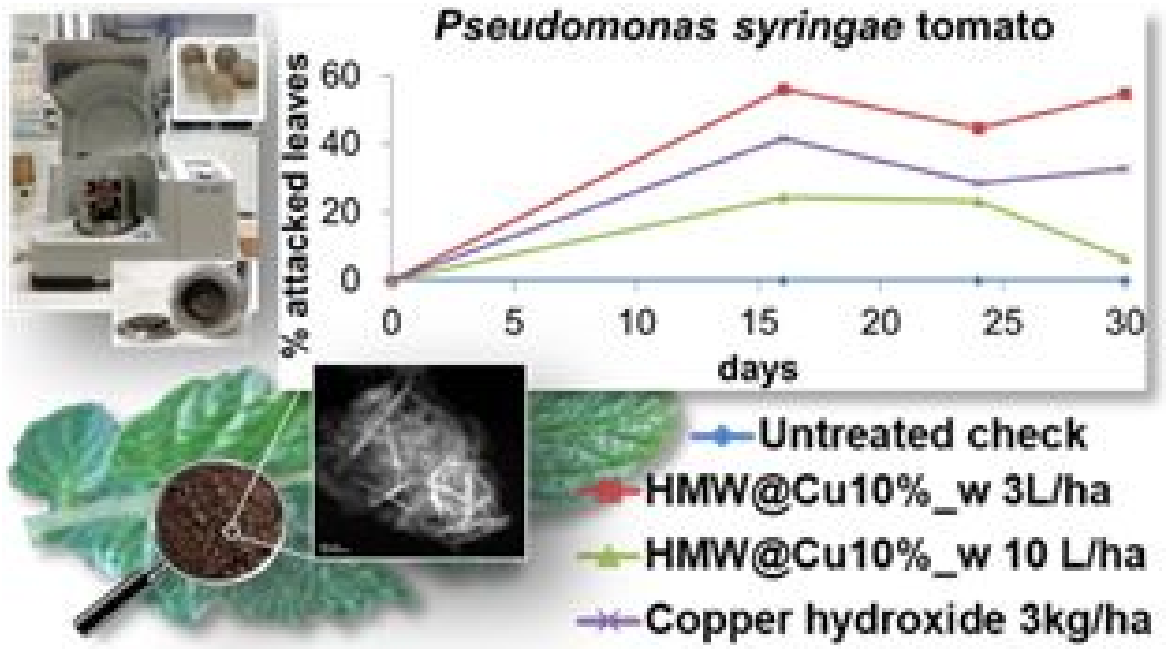

Preparation of lignin@Cu efficient pesticides with low Cu-content by solution methods and mechanochemical synthesis