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Enrico Battiston, Livio Antonielli, Stefano Di Marco, Florence Fontaine, Laura Mugnai. Innovative Delivery of Cu(II) Ions by a Nanostructured Hydroxyapatite: Potential Application in Planta to Enhance the Sustainable Control of *Plasmopara viticola*. *Phytopathology*, 2018, 109 (5), pp.748-759. <10.1094/PHYTO-02-18-0033-R>. <hal-02944551>

HAL Id: hal-02944551

<https://hal.science/hal-02944551v1>

Submitted on 27 Oct 2021

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Innovative Delivery of Cu(II) Ions by a Nanostructured Hydroxyapatite: Potential Application in Planta to Enhance the Sustainable Control of *Plasmopara viticola*

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Accepted for publication 5 December 2018.

ABSTRACT

Downy mildew caused by *Plasmopara viticola* is probably the most serious disease affecting grapevine (*Vitis vinifera*), and it is capable of causing consistent yield losses. In organic viticulture, the only acceptable and effective means to control the disease is by applications of copper-based fungicides. However, the use of copper in agriculture is expected to be further restricted by European countries because of its critical ecotoxicological and phytotoxicological profile. Research on ways to reduce the effective amounts of copper by developing innovative formulations as well as optimization of the distribution and persistence of copper-based pesticides for downy mildew control seems to be a promising approach. This research investigated the delivery properties of biomimetic synthetic hydroxyapatite (HA) to enhance the biological activity of Cu(II) ions. To this aim, four Cu(II) compounds were formulated with the innovative HA component and applied in an in vitro antifungal assay against *Botrytis cinerea*, a common grapevine pathogen suitable for in vitro activity tests, and finally, in in planta efficacy assays against *P. viticola* under greenhouse conditions. The in vitro results

highlighted a different inhibition activity for each Cu(II) compound and indicated a different interaction between the cupric compounds and HA, potentially related to different delivery mechanisms of Cu(II) from HA. Under greenhouse conditions, additional findings on the biological activity of the applied formulations were gained, especially on the efficacy of various concentrations of HA in the formulations, the influence of dose variation of the formulation and the treatment efficiency, and the persistence under rain-washing effect. This study revealed promising findings on the formulation based on the HA particles and the soluble Cu(II) compound, which resulted in reduced disease severity and incidence in all of the experimental conditions, including the lower Cu(II) dosage and the rain-washing effect. This suggests that coformulation of the three insoluble Cu(II) compounds with HA might significantly enhance the adsorption and release of Cu(II) ions by HA particles.

Keywords: drug delivery, smart pesticides, sustainability.

Grapevine (*Vitis vinifera* L.) is the most widely cultivated and economically important fruit crop (Vivier and Pretorius 2000). Grapevine, grown both for table grape and wine production, is also one of the crops that requires the highest fungicide input for disease control (Pertot et al. 2017). The large amounts of fungicides used are especially linked to the susceptibility of most grapevine cultivars to downy and powdery mildews in climatic conditions favorable to these diseases (Gomès and Coutos-Thévenot 2009). Downy mildew, caused by the oomycete pathogen *Plasmopara viticola* (Berk. & M.A. Curt.) Berl. & de Toni 1888, is probably the most serious grapevine disease, especially in grape-growing regions with relatively cool and wet spring seasons (Kassemeyer et al. 2015). In optimal weather conditions (relative humidity [RH]: 95 to 100%; temperature: 20 to 24°C) for the pathogen and with no protective treatment, downy mildew can cause huge losses (Agrios 2005). For this reason, in such climates, disease control becomes crucial to avoid yield losses.

Chemical control is the most effective option currently used to control downy mildew on grapes (Selim 2013). In the last decades, fungicides based on different chemical families (strobilurins, dithiocarbamates, phenylamides, phthalimides, or cupric compounds) have been developed and are applied by vine growers

according to a calendar schedule as protection against the disease (Jermi 2012). However, other than the environmental impact, the emergence of resistant strains of *P. viticola* is one of the major critical aspects related to the chemical control of downy mildew as a direct consequence of the continual use of synthetic fungicides (Chen et al. 2007), especially single-site fungicides owing to their specific mode of action. Resistant strains were also detected in an experiment under controlled conditions after the application of a mixture based on multisite synthetic fungicides (Samoucha and Gisi 1987).

Because copper was extensively used for the control of oomycete pathogens (Heitefuss 2000), no resistant strains to cupric fungicides were reported in *P. viticola*. This observation is related to the nonspecific mode of action of copper-containing fungicides. The wide activity spectrum of copper is based on the complexes that copper forms with membrane enzymes with sulphydryl, hydroxyl, amino, or carboxyl groups, which lead to enzyme inactivation (Selim 2013). However, unlike some systemic fungicides, copper needs to be applied before zoospores germinate to ensure efficient disease control. Based on these properties, because copper was discovered to be effective against downy mildew by Millet in the 19th century (Gessler et al. 2011), different cupric salts have been applied over the years in preventive control strategies according to the meteorological conditions favorable for downy mildew infection (Gisi 2002).

Applications of copper-based fungicides are still the most reliable means to protect grapevines against downy mildew in organic viticulture (Berkelmann-Löhnertz et al. 2012). However, concerns have been expressed about the use of copper for plant protection, mainly owing to the critical ecotoxicological profile of

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The author(s) declare no conflict of interest.

cupric fungicides, which is well described in the literature (Flemming and Trevors 1989). Repeated applications of copper for downy mildew control may be responsible for several phytotoxic effects, such as burning of young shoots and leaves (Claus 1979). The main effect of high copper concentrations on plant cells is an oxidative stress caused by the increased concentration of reactive oxygen species, which can severely damage the cell membranes, causing them to rupture and resulting in leakage of the cell contents (Apel and Hirt 2004; Yruela 2005). Among the side effects of copper, its accumulation in agricultural soils is considered the most controversial. Being a heavy metal, copper is not degraded in soil, and the long-term use of cupric fungicides in organic agriculture is responsible for extensive copper accumulation in soils (Komárek et al. 2010; Rusjan et al. 2007). Increased copper concentrations negatively affect the concentration and diversity of the microbial communities in vineyard soils (Lejon et al. 2008), and consequently, the reduction of microbial activity and changes in microbial populations can lead to lower mineralization rates of organic pesticides (Jacobson et al. 2007). In addition, the excessive accumulation of copper in the soil profile may pollute the groundwater (Robinson et al. 2006).

In view of the side effects of copper, European regulations are limiting the amount of copper applied in vineyards (European Commission 2018). In Spain, Italy, and France, up to 6 kg ha⁻¹ per year or 30 kg ha⁻¹ per 5 years of copper is allowed for crop protection as established in Regulation (European Commission) 354/2014 (European Commission 2014). In some other European countries (e.g., The Netherlands and Denmark), the use of copper in agriculture is forbidden, whereas in other countries, there are additional restrictions (e.g., 3 kg ha⁻¹ per year in Germany). In 2018, the European Food Safety Authority reviewed the maximum residue levels of copper currently established at the European level for food commodities, including wine grapes, because the exposure risk for consumers was found to be slightly higher than the tolerable threshold (European Food Safety Authority 2018).

Nevertheless, the use of copper is still tolerated, especially in organic viticulture, considering its exclusive property as a wide-spectrum fungicide and the lack of efficient alternative treatments for control of grapevine downy mildew. In view of the legislative restrictions, studies have been aimed at reducing the effective amount of copper used for the control of *P. viticola* in organic viticulture, revealing the potential for efficient control at 200 to 400 g of Cu ha⁻¹ per application with 12 to 14 applications per year (Cabús et al. 2017). This study confirmed previous results related to the control of downy mildew in vineyards applying a maximum of 3 kg of Cu ha⁻¹ per year using concentrations of 50 to 400 g of Cu ha⁻¹ according to the phenological phase (Hoffman et al. 2008).

Research oriented toward the development of innovative formulations of copper seems to be a promising approach to enhancing the control of downy mildew and minimizing the side effects of copper. In a comparative trial performed in a greenhouse and vineyard (Dagostin et al. 2011), two novel copper-based formulations showed positive results. The first, based on copper gluconate, was particularly effective in the vineyard, and the second one, based on copper peptidate, provided high levels of disease control in both conditions but caused phytotoxicity in treated plants. The possibility to optimize the distribution and persistence in planta of copper-based pesticides has also been considered by many studies in nanotechnology. In particular, the development of slow-release systems for pesticides has opened new possibilities to reduce the amount of the active substance applied, leading to efficient plant protection and disease control (Madhuri et al. 2010; Nair et al. 2010). Promising results were achieved by the engineered nanoparticles (NPs), some of which were formulated with copper (Cu NPs) (Lee et al. 2008). Nonetheless, numerous authors have reported the need for further evaluations of such NPs on the cytotoxicity and genotoxicity within the plant tissues (Jo and Kim 2009; Lin and Xing 2008).

This research arises from the need to improve the distribution and persistence of copper on grapevine leaves by modifying its structure through a specific and achievable formulation with a biocompatible material. In that respect, the aggregation and stability of synthetic nanostructured particles of hydroxyapatite (HA) and four Cu(II) compounds were previously investigated, revealing promising results (Battiston et al. 2018). Indeed, HA has been studied extensively and applied successfully in the medical field as a biocompatible and biomimetic material thanks to its unique drug delivery properties, which are active on metal ions as well as both organic and inorganic compounds (Kim et al. 1998; Roveri and Iafisco 2010; Suzuki et al. 1993).

The aim of this study was to assay the activity, under controlled conditions, of formulations based on HA particles and four different Cu(II) compounds against *P. viticola*. More specifically, the work was focused on (i) the antifungal activity of each Cu(II) compound formulated with HA and tested on reference fungal pathogens in vitro and in vivo; (ii) the efficacy of various concentrations of HA in the formulations; (iii) the influence of dosage variation of the treatments [at lower Cu(II) concentrations than the dosages recommended for cupric fungicides]; and (iv) the evaluation of protective treatments based on the same formulations under rain-washing effects.

MATERIALS AND METHODS

Functionalization of HA with Cu(II). A water suspension containing 30% wt wt⁻¹ of nanostructured HA (Ndg Natural Development Group Srl) was prepared according to a patented process of synthesis (Roveri et al. 2016). The technical powder of four Cu(II) compounds (Cnr-Ibimet) was prepared to functionalize HA: copper sulfate pentahydrate (CuSPHy), tribasic copper sulfate (CuTBS), copper oxychloride (CuOxCl), and copper hydroxide (CuHyOx). Functionalization of the products was carried out by maintaining the HA slurry with the respective Cu(II) compound and water complex. The various Cu(II) compounds were presolubilized or suspended in water and then added to the HA slurry with slow agitation for 4 h. Formulations were prepared based on two approaches for two different efficacy assays (EAs): (i) varying both the HA and Cu(II) percentage (Table 1) and (ii) varying the HA percentage and maintaining a predefined Cu(II) concentration (Table 2). The formulations were analyzed by inductively coupled plasma optical emission spectrometry (Arcos-Spectro; Ametek) to verify the copper concentration.

TABLE 1. Samples based on hydroxyapatite (HA) and Cu(II) compounds and related formulations prepared according to the variation of both the HA and Cu(II) % wt wt^{-1z}

Samples	HA % wt wt ⁻¹	Cu(II) compound		pH
		% wt wt ⁻¹	Cu(II) % wt wt ⁻¹	
HA-3	3	0.0	0.0	7.88
HA-6	6	0.0	0.0	10.35
CuSPHy-0	0	21.0	5.2	5.57
CuSPHy-3	3	21.0	5.2	5.70
CuSPHy-6	6	16.0	4.0	6.05
CuTBS-0	0	9.6	5.2	7.52
CuTBS-3	3	9.6	5.2	7.79
CuTBS-6	6	7.4	4.0	7.35
CuOxCl-0	0	9.5	5.2	7.61
CuOxCl-3	3	9.5	5.2	7.80
CuOxCl-6	6	7.0	3.8	7.31
CuHyOx-0	0	8.5	5.2	7.65
CuHyOx-3	3	8.5	5.2	7.80
CuHyOx-6	6	6.5	4.4	7.19

^z pH was measured at 20°C on samples after dilution with distilled water (pH 7.50). CuHyOx, copper hydroxide; CuOxCl, copper oxychloride; CuSPHy, copper sulfate pentahydrate; CuTBS, tribasic copper sulfate.

Copper doses. In the samples (Tables 1 and 2), the Cu(II) concentration was established at $5.25 \pm 0.04\%$ wt wt⁻¹ for the formulations up to 5.4% wt wt⁻¹ of HA and $4.15 \pm 0.30\%$ wt wt⁻¹ for the formulations with 6% wt wt⁻¹ of HA owing to the Cu(II) compound stability in the formulation with HA.

The amount of product applied (or treatment dose) for each sample was varied so that the final amount of Cu(II) applied in planta in each replicate was standardized among all treatments. According to a previous study, which analyzed the effective amount of Cu(II) on treated leaves in downy mildew control assays (Cabús et al. 2017), samples were applied in planta by spraying solutions/suspensions with the following empirically derived concentrations of Cu(II): 0.01, 0.025, 0.05, and 0.075% wt wt⁻¹ (Table 3). Such concentrations were also adopted according to the aim of each assay. Otherwise, considering the irrelevant antimicrobial activity of the pure HA described in the literature (Groza et al. 2016; Kim et al. 1998), in the preliminary in vitro antifungal assay, the samples (Table 1) were applied at higher concentrations than in planta. More specifically, the solid growth media were dosed with Cu(II) at concentrations of 0.05, 0.1, and 0.2% wt wt⁻¹.

Preliminary in vitro antifungal assay. For a preliminary understanding of the putative antifungal activity of HA formulated with each Cu(II) compound, the samples (Table 1) were applied in vitro for the mycelial growth inhibition test (Aqueveque et al. 2016). A strain of *Botrytis cinerea* Pers. was chosen as the representative grapevine pathogen, which is often tested for in vitro antifungal assays alongside in vivo control assays on *P. viticola* (Hao et al. 2017). Malt extract agar (MEA), composed of malt extract 20 g liter⁻¹ (Liofilchem Srl), agar 15 g liter⁻¹ (Liofilchem Srl), and distilled water, was amended with three different dosages (Table 1) of three Cu(II) concentrations (0.05, 0.1, and 0.2% wt wt⁻¹) combined with the HA concentration related to the sample. Petri dishes (9-cm diameter), each containing 15 ml of medium, were inoculated by transferring 7-mm-diameter mycelial discs taken from the periphery of 5- to 7-day-old cultures of *B. cinerea* and placing them mycelium side down at the center of the plate. Two replicates of treated and untreated control plates were incubated at 25°C in darkness and repeated in three independent experimental

runs. Control plates were plain MEA. Growth inhibition percentage (GI%) was calculated daily until the fifth day postinoculation as follows:

$$GI\% = \frac{(DC-DO)}{DC} \times 100$$

where DC is the diameter of mycelial growth in control plates and DO is the diameter of mycelial growth in treated plates.

In planta antifungal assay. Experiments in controlled conditions were performed in an experimental greenhouse at Fondazione Edmund Mach (San Michele all'Adige, Trento, Italy). Four consecutive EAs against *P. viticola* were carried out to evaluate (EA1) the performance of each Cu(II) compound formulated with HA, (EA2 and EA3) the efficacy of various concentrations of HA in the formulations based on the two most effective Cu(II) compounds and applied in planta at various dosages, and (EA4) the efficacy and stability of the most effective formulations under a rain-washing effect. The same protocol was applied for all of the EAs, applying a rain fastness test in EA4. Each EA was repeated in three independent experimental runs.

Plant material. One-year-old grapevine plants (*V. vinifera* 'Chardonnay' grafted on Kober 5BB rootstock) were grown in individual 2.5-liter pots of peat-rich, prefertilized (nitrogen, phosphorus, potassium: 14:16:18) standard soil (Topfsubstrat D450; Stender) under natural light (15 h each day) at temperatures ranging from 18 to 28°C. Six potted vines with at least one to two shoots and 8 to 10 developed leaves were prepared for each treatment: two potted vines were biological replicates for each treatment repeated in three independent experimental runs for each of the EAs.

Preventive treatments and inoculation. Distilled water was used as the control treatment alongside the standard treatments based on cupric or organic commercial fungicides (Table 4). In laboratory conditions (temperature $20 \pm 1^\circ\text{C}$, RH $65 \pm 5\%$, and artificial light), reference fungicides were diluted according to the recommended dosage, whereas each experimental sample was dissolved or suspended in a specific volume of distilled water according to the Cu(II) concentration investigated in planta in each EA. The pH of the diluted samples and distilled water (Table 1) was measured with a pH meter (pH 538; WTW GmbH). Each treatment was performed by spraying 30 ml of diluted formulation on the plants with a garden spray pump (Sprayer 0.6 L; Leroy Merlin), taking care to cover homogeneously both the adaxial and abaxial leaf surfaces. Before inoculation, plants were left to dry at room temperature. Inoculum of *P. viticola* was prepared immediately before inoculation by washing grapevine leaves bearing freshly sporulating lesions with cold (4°C) distilled water. The concentration of the inoculum suspension was adjusted to 5×10^5 sporangia ml⁻¹ based on sporangial counts estimated with a hemocytometer. Six hours posttreatment, the inoculum suspension was sprayed on potted vines, taking care to cover the abaxial surface of leaves. The control plants were not inoculated. Plants were transferred to a dark growth chamber for 16 h at $20 \pm 1^\circ\text{C}$ and RH of 80 to 99%, and then, they were kept in the greenhouse for 7 days (incubation period) at $25 \pm 1^\circ\text{C}$ with an RH of 60% and natural light. After the incubation period, plants were placed again in a dark growth chamber for 16 h at $20 \pm 1^\circ\text{C}$ with an RH of 80 to 99% to promote *P. viticola* sporulation.

Rain fastness test. In EA4, rain fastness was simulated 4 h after the treatments. Potted vines were subjected to 0 or 30 mm of simulated rain (30 mm h⁻¹) applied by means of 14 sprinklers positioned 2.2 m above the soil (Fig. 1) that were protected from wind and regulated to produce drops similar in size to raindrops (0.3 to 2.5 mm). After the simulated rainfall, plants were left to dry for 4 h and then inoculated with the pathogen as previously described. Results recorded on commercial fungicides and the positive control

TABLE 2. Samples based on hydroxyapatite (HA) and two Cu(II) compounds (copper sulfate pentahydrate [CuSPHy] and tribasic copper sulfate [CuTBS]) and related formulations prepared varying the HA (% wt wt⁻¹) and maintaining a predefined Cu(II) concentration (5.2% wt wt⁻¹)^z

Samples	Cu(II) compound		
	HA % wt wt ⁻¹	% wt wt ⁻¹	Cu(II) % wt wt ⁻¹
EA2			
CuSPHy-0.6	0.6	21.0	5.2
CuSPHy-1.2	1.2	21.0	5.2
CuSPHy-2.4	2.4	21.0	5.2
CuSPHy-3.6	3.6	21.0	5.2
CuSPHy-5.4	5.4	21.0	5.2
EA3			
CuTBS-0.6	0.6	9.6	5.2
CuTBS-1.2	1.2	9.6	5.2
CuTBS-2.4	2.4	9.6	5.2
CuTBS-3.6	3.6	9.6	5.2
CuTBS-5.4	5.4	9.6	5.2

^z EA, efficacy assay.

TABLE 3. Cu(II) concentrations applied in planta by the experimental treatments per each efficacy assay (EA)

Efficacy assay	Cu(II) % wt wt ⁻¹
EA1	0.025
EA2	0.050, 0.025, 0.010
EA3	0.050, 0.025, 0.010
EA4	0.075

were analyzed separately from results recorded on experimental treatments to avoid any misinterpretation of the data.

Assessment of disease severity and incidence. The treatment activity was evaluated based on two parameters: disease incidence (percentage of leaves with oil spot symptoms and/or visible sporulation) and disease severity (percentage of leaf area covered by sporulating lesions). According to the European and Mediterranean Plant Protection Organisation (EPPO) standard scale (European and Mediterranean Plant Protection Organization 2004), both parameters were visually estimated using continuous values of percentage (from 1 to 100%).

Environmental scanning electron microscopy. In EA1, the distribution of the treatments on the plant foliage and the structural features of the particles suspended in the tested formulations (based on HA 3%) were assessed by direct analyses in planta with an environmental scanning electron microscope (ESEM; Fei Quanta 200 ESEM; FEI Corporation) operating in low-vacuum mode at 25 kV without pretreatment of the samples. X-ray (EDS) microanalysis by an energy-dispersive system coupled to the ESEM (EDAX; software EDAX Genesis; AMETEK) was used to investigate the composition of the particles and the distribution of the related elements on the leaf surface, which are (namely) Ca, P, Cu, S, and Cl. Observations were performed on the leaves of treated potted vines 8 days posttreatment. Foliar samples were collected from each replicate from all of the investigated conditions by counting from the basal part of the primary shoot and sampling the fourth leaf. Leaves were cut into small portions (10 × 10 mm) and transferred to a conductive adhesive carbon mounted on aluminum stubs.

Statistical analysis. Statistical analysis was performed using R Statistical Software. In the *in vitro* assay, the measurements were performed on two replicates repeated in three independent experimental runs. Analysis of variance (ANOVA) was performed to determine the significance of differences ($P \leq 0.05$) between treatment mean values of GI%. Experiments under greenhouse conditions were designed as randomized complete blocks with two replicates per treatment repeated in three independent experimental runs for each EA. Preliminary analyses of disease severity and incidence on leaves were conducted in separate experiments, and homogeneity of variance was checked before ANOVA. Data were then combined and considered in the same regression model for which homoscedasticity was again tested via a “residuals versus

fitted values” plot, and distribution fitting was evaluated using the bootstrapping method implemented in the `fitdistrplus::descdist` R function. Data on disease severity and incidence on leaves were analyzed with ANOVA. When differences were found ($P \leq 0.05$), Tukey’s honestly significant difference (HSD) test based on least square means (lsmeans R package) was applied to detect differences between the various levels of the factor (Lenth 2016). In order to not affect the data interpretation, contrasts between reference treatments (the positive control and the commercial fungicides) were performed separately from the contrasts between the experimental treatments, as the reference treatments do not express the factors and the factor levels expressed by the experimental treatments. Results on commercial fungicides were also plotted (`ggplot2` R package) to evaluate the effects of these products on severity of



Fig. 1. Rain fastness simulator (FEM, San Michele all’Adige, Italy) equipped with 14 sprinklers positioned 2.2 m above the soil and regulated to produce drops similar in size to raindrops (0.3 to 2.5 mm).

TABLE 4. Reference treatment results on the mean disease severity (percentages) and mean disease incidence (percentages) detected in potted grapevines (cultivar Chardonnay) in each efficacy assay (EA)^z

Active ingredient % wt wt ⁻¹	Treatment	Dosage % wt wt ⁻¹	Disease severity (%) ± SE	Disease incidence (%) ± SE
EA1				
Water	Positive control	–	35.4 ± 10.6	96.4 ± 3.6
Dimetomorf 50	Quantum (Adama)	0.05	5.5 ± 1.0	72.6 ± 6.6
CuHyOx 42.37	Coprantol WG (Syngenta)	0.20	6.6 ± 1.3	66.6 ± 7.2
EA2				
Water	Positive control	–	60.9 ± 7.4	100.0 ± 0.0
Dimetomorf 50	Quantum (Adama)	0.05	2.4 ± 0.5	20.0 ± 8.4
CuSPHy 49.6	Bordo Flow (Manica)	0.40	4.6 ± 0.6	63.1 ± 10.4
EA3				
Water	Positive control	–	38.7 ± 11.7	86.2 ± 8.5
Dimetomorf 50	Quantum (Adama)	0.05	1.7 ± 0.4	28.0 ± 5.5
CuTBS 28	Tri Base (Nufarm)	0.33	6.5 ± 2.0	61.3 ± 10.8
EA4				
0-mm rain				
Water	Positive control	–	55.7 ± 7.9	100.0 ± 0.0
Dimetomorf 50	Quantum (Adama)	0.05	2.8 ± 0.8	44.4 ± 10.3
CuSPHy 49.6	Bordo Flow (Manica)	0.60	1.2 ± 0.5	20.0 ± 7.0
CuTBS 28	Tri Base (Nufarm)	0.49	2.4 ± 0.3	40.8 ± 3.4
50-mm rain				
Water	Positive control	–	54.9 ± 2.9	100.0 ± 0.0
Dimetomorf 50	Quantum (Adama)	0.05	5.6 ± 0.7	63.5 ± 9.6
CuSPHy 49.6	Bordo Flow (Manica)	0.60	5.3 ± 1.9	65.9 ± 11.7
CuTBS 28	Tri Base (Nufarm)	0.49	12.6 ± 0.6	97.8 ± 1.3

^z CuHyOx, copper hydroxide; CuSPHy, copper sulfate pentahydrate; CuTBS, tribasic copper sulfate; SE, standard error.

downy mildew and determine the relationship between the amount of the active ingredient applied and the efficacy of the treatment.

RESULTS

Preliminary in vitro antifungal assay. The activities of three concentrations of formulations based on Cu(II) compounds and HA on mycelial growth of *B. cinerea* are shown in Figure 2. All of the Cu(II) compounds in different concentrations inhibited the growth of *B. cinerea*. CuSPHy was the most effective for inhibiting mycelial growth at all applied concentrations of Cu(II) (0.05, 0.1, and 0.2% wt wt⁻¹). Mycelial growth of *B. cinerea* was inhibited by CuTBS at nearly 65% by the lower concentration and 87% by the higher concentration of Cu(II). CuHyOx showed the lowest activity against pathogen growth at 0.05 and 0.1% Cu(II), with no increase in inhibition at the highest concentration of Cu(II). The concentration of CuOxCl affected its activity, because the lowest concentration of Cu(II) showed the lowest inhibition of the pathogen (GI% = 10), which significantly increased (GI% = 70) at 0.2% Cu(II). HA had a variable effect on the Cu(II) compounds depending on its concentration. At 3% HA, CuSPHy was still the most effective

inhibitor of *B. cinerea* growth, even when the concentration of Cu(II) was reduced. At the lowest concentration of Cu(II), the same level of HA had an opposite effect on CuHyOx and CuOxCl, reducing the inhibitory activity of the former and increasing the growth inhibition of the latter. CuTBS showed a similar inhibition of the corresponding pure Cu(II) compound, whereas the activity of CuHyOx was reduced by 3% HA, especially at the higher amount of Cu(II). Different results were reported by 6% HA. Such a level reduced the inhibitory activity of the lowest concentration of CuSPHy, whereas on the other Cu(II) compounds, it increased the GI%, especially for CuOxCl and CuHyOx at 0.2% Cu(II). The GI% index differed significantly ($P \leq 0.05$) between the different Cu(II) compounds, the applied Cu(II) amounts, and the percentage of HA.

In planta EA. *Effect of Cu(II) compounds formulated with HA.* The greenhouse trial EA1 investigated the efficacy of four Cu(II) compounds formulated with two percentages of HA in the control of the grapevine pathogen *P. viticola*. Results of disease severity are shown in Figure 3. Data of treatments based on (i) water as positive control, (ii) organic fungicide, (iii) cupric fungicide, and (iv) 3% HA are presented separately (Fig. 3A) and compared with the results of the experimental treatments (Fig. 3B). The positive

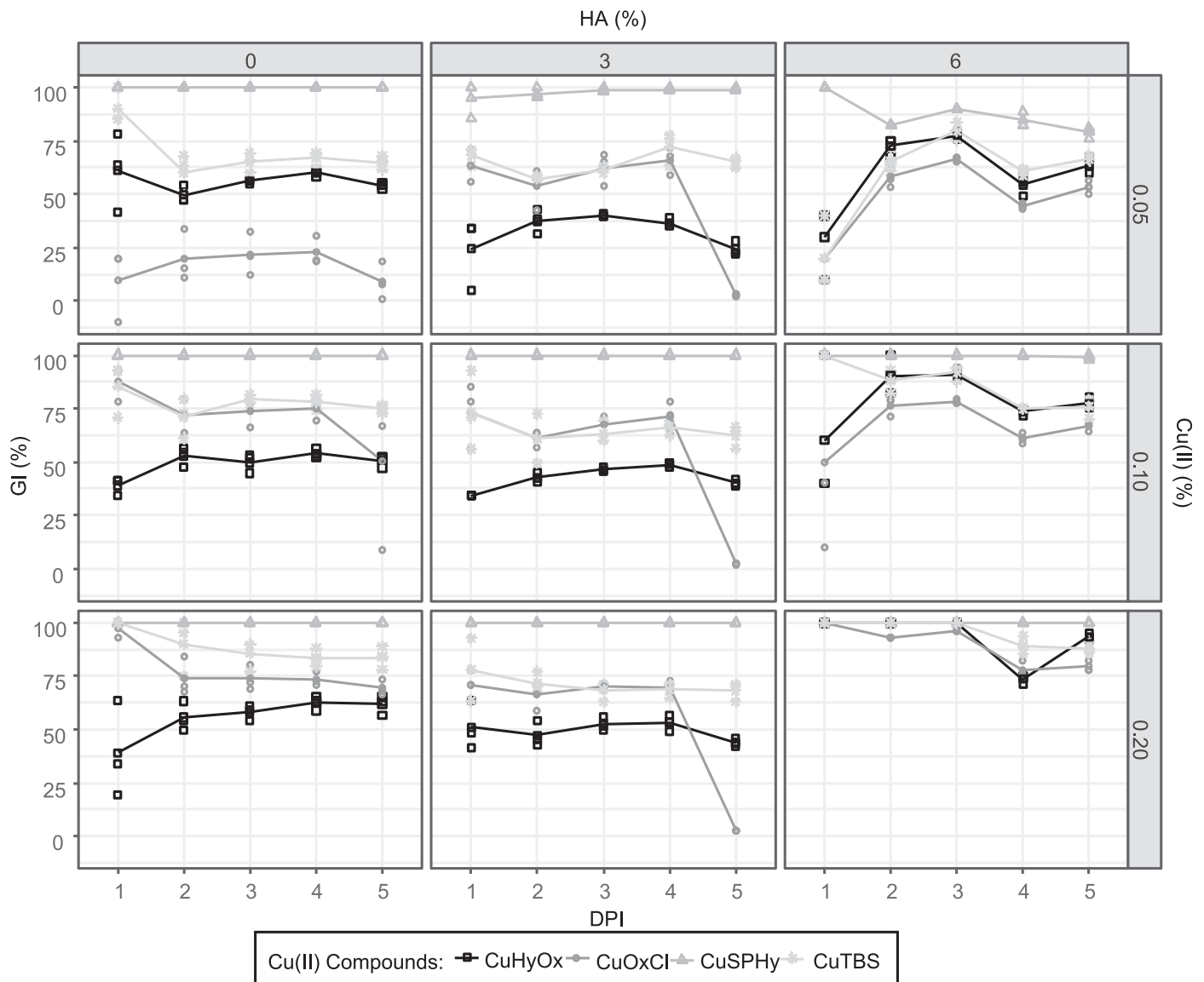


Fig. 2. Effects on mycelial growth of *Botrytis cinerea* of different Cu(II) concentrations from four Cu(II) compounds formulated with different hydroxyapatite (HA) concentrations. Petri dishes were incubated at the temperature of 25°C for 5 days in darkness. HA (%) indicates the HA concentration in the applied formulation, and Cu(II) (%) indicates the Cu(II) concentration in the applied formulation. CuHyOx, copper hydroxide; CuOxCl, copper oxychloride; CuSPHy, copper sulfate pentahydrate; CuTBS, tribasic copper sulfate; DPI, days postinoculation; GI (%), growth inhibition percentage of *B. cinerea*.

control showed various degrees of disease severity, with a maximum value of nearly 75% and a mean of 35%. Mean disease severity in the plants treated with the organic fungicide and the cupric fungicide was significantly lower than the positive control, indicating good disease control ($P = 0.0129$ and $P = 0.0163$, respectively). No infected leaves were seen in the noninoculated control plants. The treatment based on 3% HA did not have any effect on *P. viticola*, resulting in a disease severity that was not

significantly different from the positive control. The effects owing to Cu(II) compound and HA were both found to be significantly different from each other ($P \leq 0.05$). In Table 5, the contrasts (Tukey's HSD test) within the factor Cu(II) compound and the factor HA percentage are reported for disease severity and incidence. Considering the pure Cu(II) compounds, the lowest disease severity of 5% was reported by CuSPHy, which was significantly different from CuHyOx ($P = 0.0189$), CuTBS ($P = 0.0001$), and CuOxCl ($P < 0.0001$). The applied Cu(II) concentration ($0.025\% \text{ wt wt}^{-1}$) was not effective for the other pure compounds. Concerning the formulations based on 3% HA, CuTBS resulted in a significant reduction ($P = 0.0040$) in disease severity compared with CuOxCl, whereas at 6% HA, the effect was slighter reduced. CuOxCl responded positively to the formulation with HA, showing a significant difference in disease severity at 6% HA, but it did not correspond to an acceptable disease control. The apparent efficiency of the formulation based on CuHyOx with 3% HA was not significant and did not increase at 6% HA. Concerning the disease incidence, the most useful results were observed with CuSPHy and 3% HA, in which 52% of the leaves were symptomatic, followed by CuTBS and 3 and 6% HA with ~70% disease incidence. In all other treatments, disease incidences were >80%, and the positive control had 100% disease incidence (data not shown). A consistent phytotoxic effect was reported on all of the leaves treated with CuSPHy, but this was not observed with the same compound at 3 and 6% HA. No phytotoxic signs were visible on plants in the control, commercial fungicide treatments (Table 4), or any other treatments.

Effect of HA concentration and applied amount of Cu(II). The variability of the factor HA percentage in the formulation and the effect of the factor dosage percentage were studied in greenhouse trials EA2 and EA3 by applying treatments based on CuSPHy and CuTBS, which were the two most effective Cu(II) compounds in EA1. In Table 4, results of the control and the comparison treatments are shown. No symptoms were detected in the negative control. Symptoms were more severe and diffused in EA2 (61 and 100%, respectively) than in EA3 (39 and 86%, respectively), and in both experiments, the respective differences were significant. The treatment dimetomorf was highly efficient in reducing both disease severity and incidence. Figure 4 shows the disease severity (percentage) detected in EA2 (Fig. 4A) and EA3 (Fig. 4B). Considering the formulations based on CuSPHy (Fig. 4A) in comparison with the CuSPHy fungicide (Table 4), good disease control was reported at all of the levels of HA and at 0.05% Cu(II),

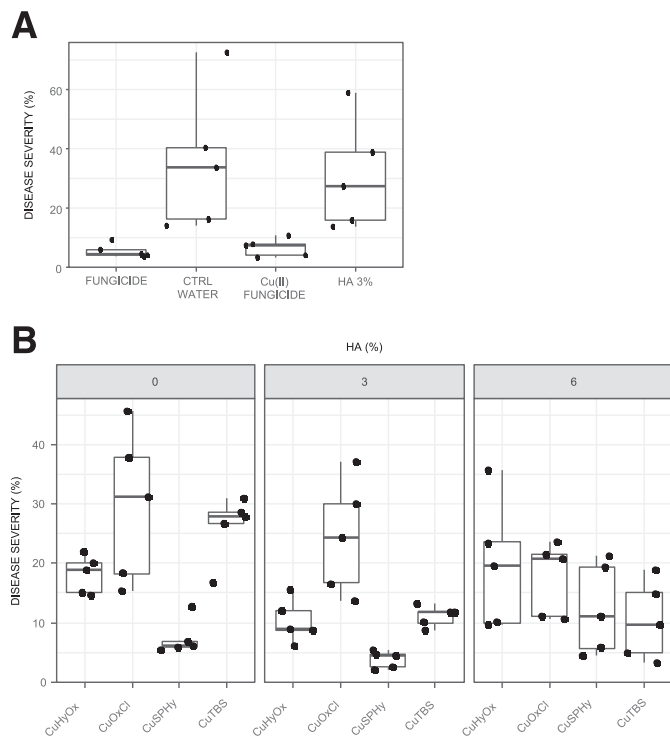


Fig. 3. Results on disease severity (percentages) detected in potted grapevines (cultivar Chardonnay) treated with water (positive control), dimetomorf, cupric fungicide, or 3% hydroxyapatite (HA) and comparison with the experimental treatments. **A**, Commercial fungicides and the positive control are presented and analyzed separately from the experimental treatments to not affect the data interpretation. **B**, Experimental treatments. CuHyOx, copper hydroxide; CuOxCl, copper oxychloride; CuSPHy, copper sulfate pentahydrate; CuTBS, tribasic copper sulfate; HA, hydroxyapatite.

TABLE 5. Contrasts in pairs (Tukey's honestly significant difference (HSD) test) within the factor copper compound and the factor hydroxyapatite (percentage) showing the significant differences on disease severity and incidence (efficacy assay 1)^z

Disease incidence	Disease severity											
	CuHyOx			CuOxCl			CuSPHy			CuTBS		
	0	3	6	0	3	6	0	3	6	0	3	6
CuHyOx												
0	–	n.s.	n.s.	*	–	–	*	–	–	n.s.	–	–
3	n.s.	–	*	–	*	–	–	n.s.	–	–	n.s.	–
6	n.s.	n.s.	–	–	–	n.s.	–	–	n.s.	–	–	*
CuOxCl												
0	n.s.	–	–	–	n.s.	*	n.s.	–	–	*	–	–
3	–	n.s.	–	n.s.	–	n.s.	–	*	–	–	*	–
6	–	–	n.s.	n.s.	n.s.	–	–	–	n.s.	–	–	n.s.
CuSPHy												
0	n.s.	–	–	n.s.	–	–	–	n.s.	n.s.	*	–	–
3	–	*	–	–	*	–	*	–	n.s.	–	n.s.	–
6	–	–	n.s.	–	–	n.s.	n.s.	*	–	–	–	n.s.
CuTBS												
0	n.s.	–	–	n.s.	–	–	n.s.	–	–	–	*	*
3	–	n.s.	–	–	*	–	–	*	–	n.s.	–	n.s.
6	–	–	n.s.	–	–	n.s.	–	–	n.s.	n.s.	n.s.	–

^z CuHyOx, copper hydroxide; CuOxCl, copper oxychloride; CuSPHy, copper sulfate pentahydrate; CuTBS, tribasic copper sulfate; n.s., not significant difference at $P \leq 0.05$ (Tukey's HSD test). * indicates significant difference at $P \leq 0.05$.

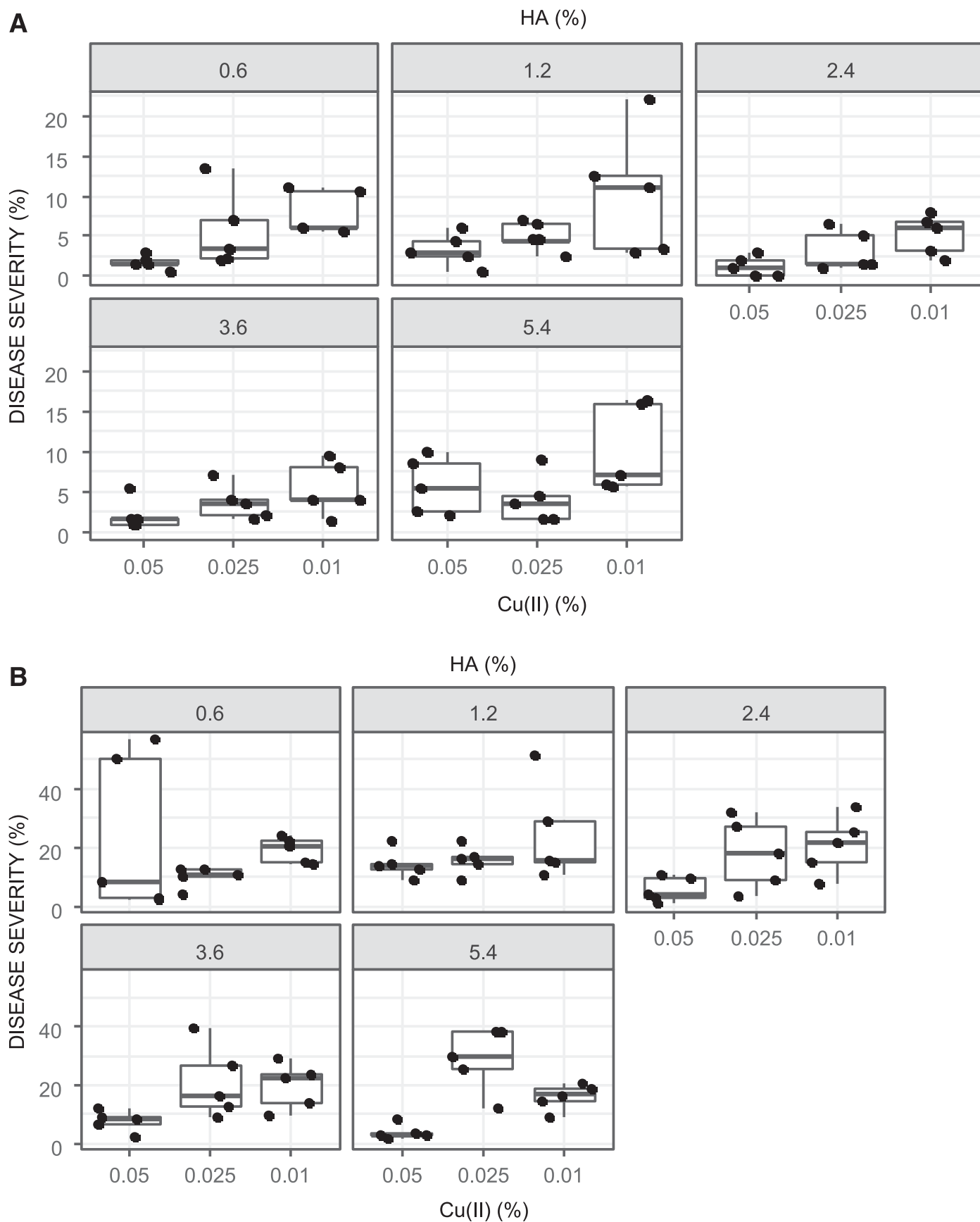


Fig. 4. Results on disease severity (percentages) detected in potted grapevines (cultivar Chardonnay). **A**, Results of copper sulfate pentahydrate-based treatments. **B**, Results of tribasic copper sulfate-based treatments. Both Cu(II) compounds were formulated with five different percentages of hydroxyapatite (HA) and applied in planta in three different dosages corresponding to decreasing amounts of Cu(II).

but it had a wider distribution of disease severity values at 5.4% HA and the lowest mean severity between 2.4 and 3.6% HA. At lower levels of the factor dosage percentage, the same formulations also had a wider distribution of the results in disease severity, which is close to the commercial fungicide (4.6%) at 0.025% Cu(II), whereas at 0.01% Cu(II), it shows higher mean values. A high phytotoxic effect was detected on the grapevine leaves treated with the CuSPHy formulations at 0.6% HA and less at 1.2% HA, with major intensity at the higher Cu(II) dosage. Overall, the CuSPHy treatments resulting in lower disease severity, and the most stable results, even at lower Cu(II) percentage, were based on 2.4 and 3.6% HA. In the parallel experiment EA3, the CuTBS fungicide (Table 4) was effective in controlling *P. viticola*. For CuTBS formulations applied at 0.05% Cu(II), the factor HA percentage (from 0.6 to 5.4%) resulted in reduced distribution of disease severity values, showing good disease control compared with the 2.4% HA. The contrast was observed in the same treatments applied at 0.025% Cu(II), but the trend was not observed at the lowest dosage of Cu(II), where disease severities were nearly 20% for all HA percentages. No signs of phytotoxicity were detected on the leaves treated with CuTBS-based formulations at all Cu(II) percentages. In both experiments EA2 and EA3, the factor dosage percentage was significant ($P \leq 0.05$), whereas the factor HA percentage was also significant as seen in EA2. Comparisons of means by Tukey's HSD test within the factor HA percentage and the factor dosage percentage ($a = 0.05\% \text{ wt wt}^{-1}$, $b = 0.025\% \text{ wt wt}^{-1}$, and $c = 0.01\% \text{ wt wt}^{-1}$) are presented in Table 6, highlighting the significant differences in disease severity in both trials. Unlike EA1, few contrasts in EA2 and EA3 were significant for both factors. However, based on the disease severity, the most effective formulations were found at 2.4 to 3.6% HA for CuSPHy and 3.6 to 5.4% HA for CuTBS applied at 0.5% Cu(II). The same results, in terms of tendency and significance, were observed based on disease incidence recorded in both trials (data not shown).

Treatments efficacy and stability under a rain-washing effect. Based on the previous results, which revealed the optimal HA level for CuSPHy and CuTBS, both Cu(II) compounds formulated with 3% HA were applied in a greenhouse rain fastness test (EA4) to study their efficacy and stability in comparison with the pure Cu(II) compounds and the reference treatments (Table 4). No significant

differences were detected in both positive controls (potted vines washed by the simulated rain and unwashed), which had 100% disease incidence and nearly 55% disease severity. The rain-washing effect was not detected on leaves treated with organic fungicide, which was highly effective under both conditions. CuSPHy fungicide (Table 4) showed the best performance in reducing disease incidence and severity and provided good disease control, even after the simulated rain. CuTBS fungicide (Table 4) lost efficacy in the simulated rain condition, showing approximately the same degree of downy mildew infection as the positive control (Fig. 5A). The treatment based on CuSPHy revealed the highest control activity, reducing both severity and incidence, with low level of variability in the results under both conditions (Fig. 5B). However, the treatment resulted in severe phytotoxicity. CuSPHy formulated with HA did not show any phytotoxicity, and despite the nonsignificant difference from the results reported by the same pure compound, disease severity was <5% in both conditions (Fig. 5B). Treatment based on CuTBS was significantly less effective than CuSPHy. The differences in disease severity and incidence were much greater in the simulated rain test. Under such conditions, the formulation based on CuTBS and HA did not give a significant reduction in severity or incidence of *P. viticola* compared with the CuTBS-based treatment, revealing a similar protection to the CuTBS fungicide. Pairwise comparisons by Tukey's HSD test within the factor Cu(II) compound and the factor rain-washing effect were highly significant for both factors.

ESEM. The in planta ESEM observations showed aggregation of the HA particles (Fig. 6). Distribution of the particles on the leaf surfaces of treated potted vines was studied 8 days posttreatment (EA1). Particles based on pure HA revealed the Ca/P ratio of HA (i.e., 1.67). These particles were aggregated in spotted areas and were not regularly distributed over the leaf surface (Fig. 6A). A similar observation was noted on the CuSPHy-HA-based particles, which were distributed on the leaf surface and tended to be concentrated in one point (Fig. 6B). The HA-CuTBS-based treatment showed a more homogeneous and dispersed distribution of particles on the leaf than the HA-CuSPHy treatment (Fig. 6C). The same observation was seen for HA-CuOxCl- and HA-CuHyOx-based treatments in Figure 6D and E, highlighting, in the first case, the joint presence of particles and round patchy

TABLE 6. Contrasts in pairs (Tukey's honestly significant difference (HSD) test) within the factor hydroxyapatite (percentage) and the factor dosage percentage ($a = 0.05\%$, $b = 0.025\% \text{ wt wt}^{-1}$, $c = 0.01\% \text{ wt wt}^{-1}$) showing the significant differences on disease severity (efficacy assays 2 and 3)^z

CuTBS	CuSPHy														
	0.6			1.2			2.4			3.6			5.4		
	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
0.6															
a	–	n.s.	*	n.s.	–	–	n.s.	–	–	n.s.	–	–	n.s.	–	–
b	*	–	n.s.	–	n.s.	–	–	n.s.	–	–	n.s.	–	–	n.s.	–
c	n.s.	n.s.	–	–	–	n.s.	–	–	n.s.	–	–	n.s.	–	–	n.s.
1.2															
a	n.s.	–	–	–	n.s.	*	n.s.	–	–	n.s.	–	–	n.s.	–	–
b	–	n.s.	–	n.s.	n.s.	–	n.s.	–	–	n.s.	–	–	n.s.	–	–
c	–	–	n.s.	n.s.	n.s.	–	–	*	–	–	*	–	–	–	n.s.
2.4															
a	*	–	–	n.s.	–	–	–	n.s.	n.s.	n.s.	–	–	*	–	–
b	–	n.s.	–	–	n.s.	–	n.s.	n.s.	–	n.s.	–	–	–	n.s.	–
c	–	–	n.s.	–	–	n.s.	*	n.s.	–	–	–	n.s.	–	–	*
3.6															
a	*	–	–	n.s.	–	–	n.s.	–	–	–	n.s.	n.s.	n.s.	–	–
b	–	n.s.	–	–	n.s.	–	–	n.s.	–	n.s.	–	n.s.	–	n.s.	–
c	–	–	n.s.	–	–	n.s.	–	–	n.s.	n.s.	n.s.	–	–	–	*
5.4															
a	*	–	–	n.s.	–	–	n.s.	–	–	n.s.	–	–	–	n.s.	*
b	–	*	–	–	n.s.	–	–	n.s.	–	–	n.s.	–	*	–	*
c	–	–	n.s.	–	–	n.s.	–	–	n.s.	–	–	n.s.	n.s.	n.s.	–

^z CuSPHy, copper sulfate pentahydrate; CuTBS, tribasic copper sulfate; n.s., not significant difference at $P \leq 0.05$ (Tukey's HSD test). * indicates significant difference at $P \leq 0.05$.

clusters of the same and in the second one, the higher density of micrometric aggregates. In the leaf samples treated with distilled water (positive control), a particulate distributed on the leaf surface was detected. Elemental analysis of this particulate did not reveal any signal corresponding to copper, calcium, or phosphorous, indicating that the particulate was not attributable to the applied substances (Fig. 6F).

DISCUSSION

The main aim of this research was to evaluate the application in planta of an innovative tool to enhance the biological activity of Cu(II) ions for the sustainable control of the grapevine pathogen *P. viticola*. The research highlighted the delivery properties of the biomimetic synthetic HA, which was shown to enhance the biological activity of Cu(II) ions. Clearly promising results were shown by applying HA in the final in planta EAs against *P. viticola* under greenhouse conditions, especially on the soluble Cu(II) salt (CuSPHy), reducing both disease severity and disease incidence in all of the experimental conditions. The treatment also showed a high efficacy and persistence under rain-washing effect.

Despite the higher practical significance and efficiency of the in planta assays in screening fungicides (Varo et al. 2017), significant and remarkable findings on the antifungal activity of the investigated substances were revealed by the preliminary in vitro assay. Being susceptible to broad-spectrum treatments based on copper (Jacometti et al. 2010), *B. cinerea* was also studied in previous laboratory trials performed to evaluate mycelial growth inhibition by innovative antimicrobial particles (Ouda 2014). In general, different grades of inhibition by each Cu(II) compound were highlighted according to their applied dosage. CuSPHy was highly efficient in inhibiting mycelial growth of *B. cinerea* at all of the applied concentrations, possibly because of the high solubility of this Cu(II) salt, which dissolves in solution the highest concentration of Cu(II). The lower antifungal activity reported by the other Cu(II) salts, such as CuTBS, CuOxCl, and CuHyOx, is a result of their lower solubility, because such compounds tend to remain in suspension, releasing slowly and in certain conditions, the Cu(II) ions (Gessler et al. 2011). The insignificant antimicrobial activity reported in the literature for particles based on HA (Groza et al. 2016; Kim et al. 1998) could explain the contradictory role shown by the HA formulation on each Cu(II) compound. With CuSPHy, the most effective Cu(II) salt, the highest concentration of

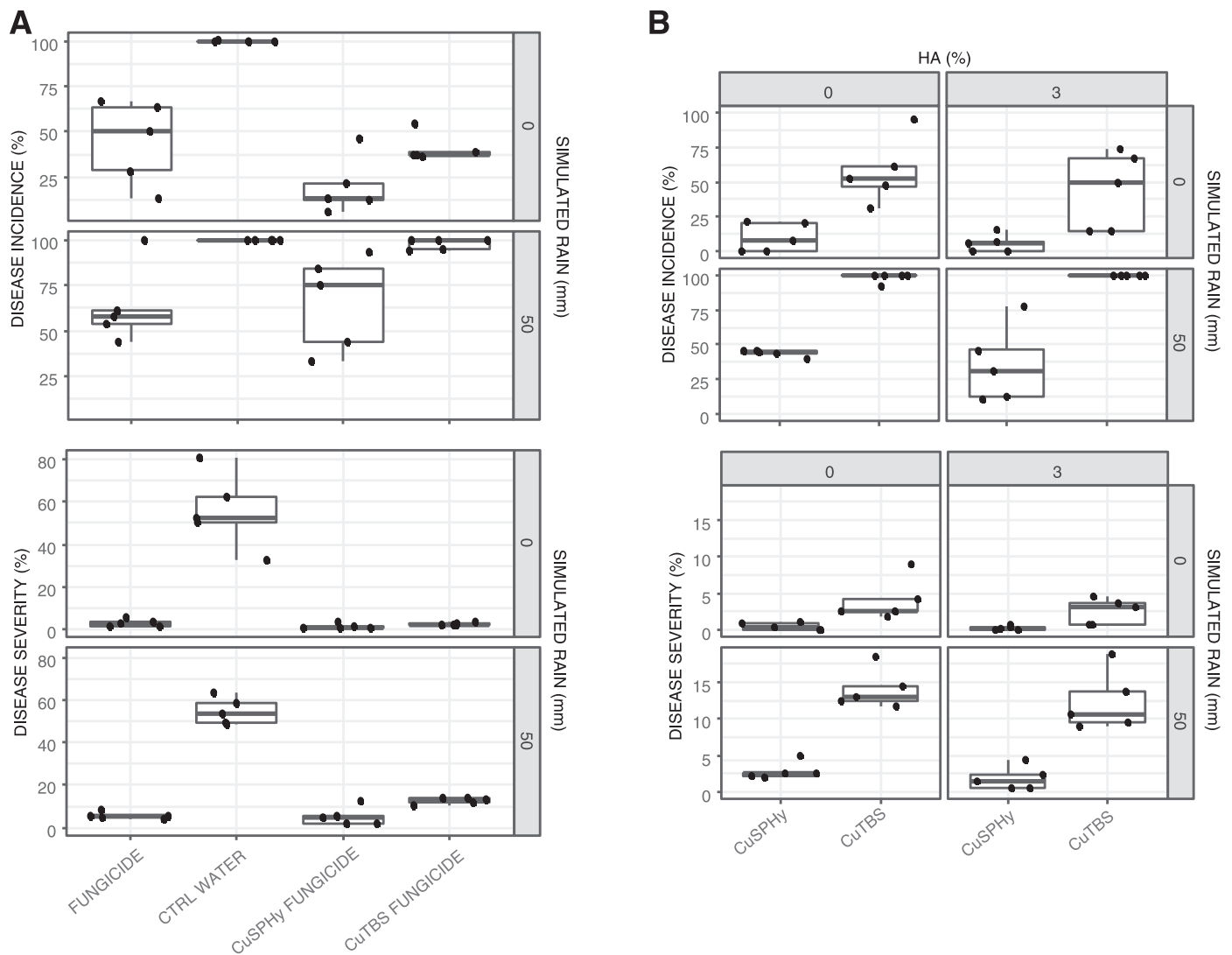


Fig. 5. Results of the rain fastness test on disease severity (percentages) and disease incidence (percentages) detected on leaves of potted grapevine (cultivar Chardonnay) under greenhouse conditions. Plants were treated and exposed to 0 or 50 mm of simulated rain before inoculation with *Plasmopara viticola* (5×10^5 sporangia ml^{-1}). **A**, Commercial fungicides and the positive control are presented and analyzed separately from the experimental treatments to not affect the data interpretation. **B**, Experimental treatments. CuSPHy, copper sulfate pentahydrate; CuTBS, tribasic copper sulfate; HA, hydroxyapatite.

HA (6%) significantly reduced the inhibitory activity of CuSPHY when applied at the lowest concentration of Cu(II) (0.05%). This was thought to be related to the excess of HA, with no antimicrobial activity, compared with the Cu(II) ions in solution. In the same condition, the opposite effect was reported with CuHyOx. In general, this effect was shown on all of the Cu(II) compounds applied at 0.2% Cu(II), in which the 6% HA enhanced significantly the inhibition of *B. cinerea*. These observations have suggested to investigate the delivery role potentially played by HA in planta, in relation to each Cu(II) salt and to the concentration ratio between HA and Cu(II) ions into the formulation.

Under greenhouse conditions, promising findings on the biological activity of formulations based on Cu(II) and nanostructured particles of HA were gained from these experiments. The formulations previously applied in vitro were sprayed in the trial EA1 onto the foliage of potted vines at the same Cu(II) ions concentration (0.025%) of the cupric fungicide in comparison according to early greenhouse EAs, which defined such amount as the minimal quantity of copper ions able to acceptably prevent downy mildew (Dagostin et al. 2011). In this condition, CuSPHY formulated with 3% HA reduced significantly both disease incidence and severity, whereas with a higher HA content (6%), the HA-CuSPHY formulation lost protective activity against *P. viticola*, confirming the tendency detected in the growth inhibition of *B. cinerea* by the same formulations. The non-significant biological activity shown by the same concentration of HA confirms that the high efficiency of the HA-CuSPHY formulation is not a consequence of any fungitoxic activity of the

HA-based particles. However, very good disease control was also achieved by the treatment based on the pure CuSPHY, but this resulted in severe phytotoxicity on the treated leaves. As described by Gessler et al. (2011), CuSPHY is the main constituent of Bordeaux mixture, which is a cupric fungicide developed by neutralizing and fixing the highly soluble and phytotoxic Cu(II) ions of this copper salt. It is known in the literature for being the most ancient and efficient fungicide applied for the control of *P. viticola* (Romanazzi et al. 2016). Among the nonsoluble Cu(II) compounds, in the EA1, there was no evidence of a significant effect on controlling the disease compared with the cupric fungicide (based on CuHyOx). However, in the experimental treatments, the formulation with HA revealed the same tendencies shown in vitro, such as the reduced variability of the results (CuOxCl) and the general increase in treatment efficiency (CuTBS), except for CuHyOx, which showed the lowest disease severity at 3% HA. Likewise, the low efficiency reported by the treatments based on the pure nonsoluble Cu(II) compounds is certainly related to the application of the pure technical compounds without any coformulate that is used in the commercial products.

Electron microscopy was useful for studying the distribution of particles and their aggregation in planta, because the application of HA arises from its property of generating nanostructured biologically active coatings (Roveri and Iafisco 2010). Such aggregated particles were detected on the leaf samples treated with HA. A similar configuration was observed on leaves treated with HA-CuSPHY. However, treatments based on the insoluble Cu(II) compounds and HA seemed to be distributed in smaller particle

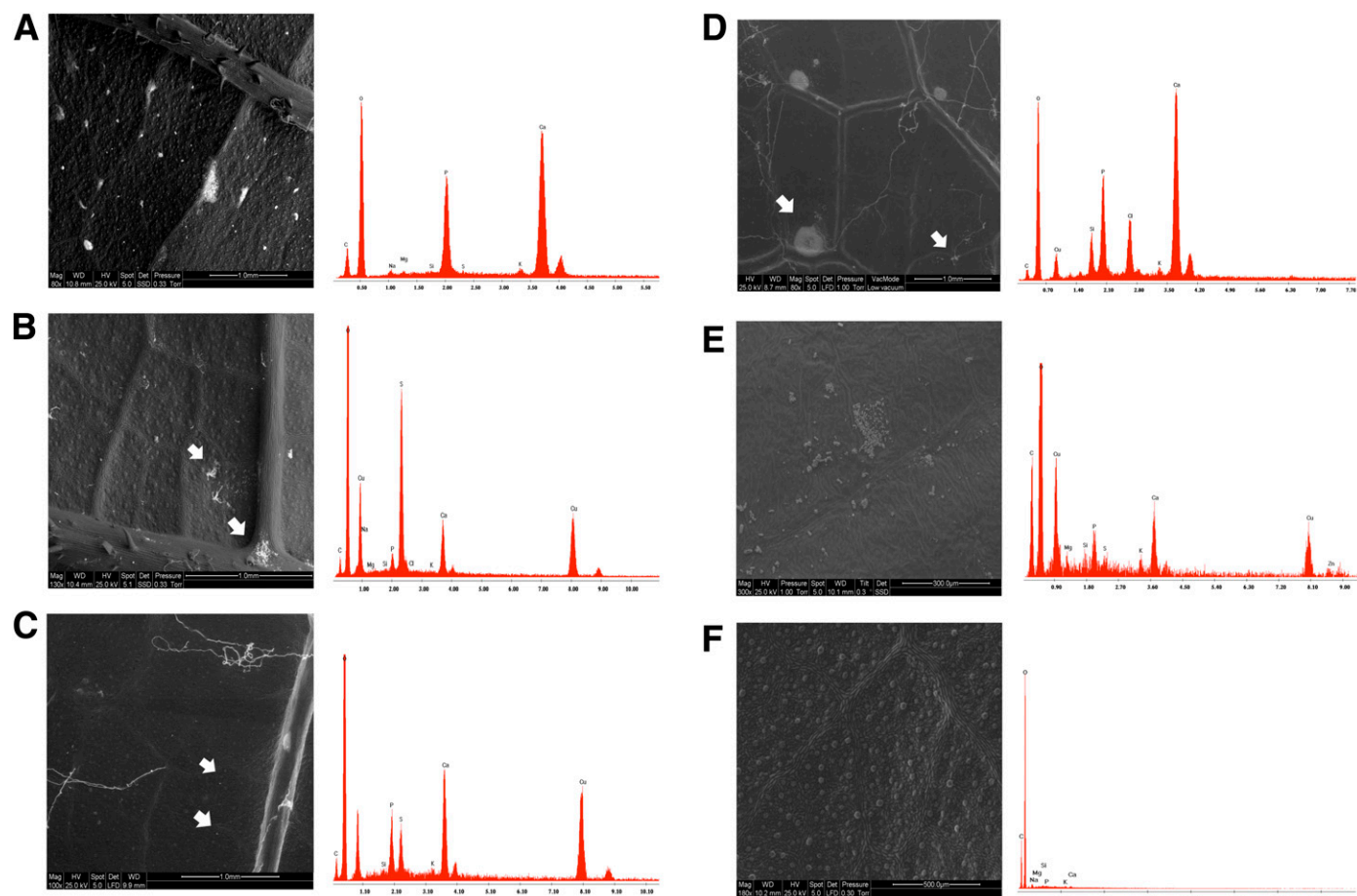


Fig. 6. Environmental scanning electron microscope images paired to the related X-ray spectra of elements and related to the formulations applied on the leaf surface of *Vitis vinifera*. **A**, Pure suspension of hydroxyapatite (HA; scale bar = 1 mm). **B**, Formulation of copper sulfate pentahydrate (scale bar = 1 mm). **C**, Formulation of HA-tribasic copper sulfate (scale bar = 1 mm). **D**, Formulation of copper oxychloride (scale bar = 1 mm). **E**, Formulation of HA-copper hydroxide (scale bar = 0.3 mm). **F**, Distilled water in control leaf sample (scale bar = 0.5 mm).

aggregations, showing different aggregates compared with the HA clusters. The diverse particle conformation and distribution reflect the different results shown in the greenhouse trial EA1. This aspect highlights the importance of spreading and delivering of Cu(II) ions on the leaf surface with respect to the control of early fungal infection and their agents, the *P. viticola* zoospores (Jürges et al. 2009; Kiefer et al. 2002). Furthermore, the variable response of treatments in EA1 may be explained by the effect of the irregular aggregation of particles and therefore, their irregular size, which have a direct impact on the droplet size generated during the spraying application of the protective substance, thus influencing significantly the treatment efficiency (Ferguson et al. 2016).

The formulation HA-CuSPHy was further investigated as a potential innovative cupric fungicide compared with the formulation HA-CuTBS based on the insoluble Cu(II) compound. In both formulations, the efficacy of various concentrations of HA was studied in relation to Cu(II) concentrations. In general, the results (EA2) confirmed that the most effective CuSPHy formulation [containing 5.2% Cu(II)] is based on HA varying between 2.4 and 3.6% as seen in the greenhouse trial EA1. This encouraging result was shown by the overdosage [0.05% Cu(II)] and unexpectedly, underdosage treatments [0.01% Cu(II)]. This latter result opens up interesting perspectives in comparison with early studies aimed at investigating the efficacy of reduced copper dosage against *P. viticola* (Cabús et al. 2017; Dagostin et al. 2011; Heibertshausen et al. 2007; Hoffman et al. 2008) and considering probable future restrictions on the use of copper in agriculture. Otherwise, the findings collected on the formulations based on CuTBS (EA3) indicate a dissimilar activity of the factor HA percentage on the insoluble copper salt. In fact, despite a generally lower efficacy of CuTBS formulations than the ones based on CuSPHy, the less variable results in disease severity were reported at the highest percentage of HA and the over- and underdosage of Cu(II) ions. This observation suggests a deeper investigation into the methods and materials applicable to the optimal coformulation between the suspension of HA nanostructured particles and the suspensions of the insoluble Cu(II) compound, such as CuTBS.

In greenhouse experiments carried out with rain-washing simulation, it was possible to evaluate the stability and persistence on grapevine leaves of the formulations based on HA-CuSPHy and HA-CuTBS. In contrast to the study by Dagostin et al. (2010), the cupric treatments were applied at higher dosage of Cu(II) ions (0.075%) and had a higher amount of simulated rain (50 mm) received by the potted vines to highlight in this condition the potential performance of the less-efficient formulation based on CuTBS. However, despite the high solubility in water of CuSPHy, the formulations based on this salt strongly reduced the disease severity and incidence in both conditions (0 and 50 mm). Excluding the positive control represented by the pure CuSPHy treatment, because of the severe and diffused phytotoxicity on the treated leaves, the remarkable control achieved by the HA-CuSPHy without any side effect on the plants was confirmed, thus indicating a significant interaction between the Cu(II) ions and the HA nanostructured particles. This result may become even more promising considering the strong impact of climate change on the downy mildew management. In fact, Salinari et al. (2006) predicted the need for at least two more fungicide sprays to control *P. viticola* epidemics under the most negative climate change scenario. In this perspective, the possibility to enhance the persistence of copper under a rain-washing effect may significantly contribute to disease control in future.

The significant reduction in control activity under rain-washing effect by the HA-CuTBS treatment was further correlated to a nonoptimal coformulation method considering the similar tendency reported by the pure CuTBS treatment and in comparison with the lower disease severity shown by the CuTBS fungicide. In this respect, the relevance of the formulation process on cupric fungicide efficacy has already been commented on in the literature,

especially under environmental conditions, such as weather conditions and plant growth, that can influence strongly the product efficiency (Dagostin et al. 2011).

In conclusion, this study indicates the possibility of enhanced biological activity in vitro and in planta of Cu(II) ions by modifying their distribution, persistence, and delivery on the leaf surface through a formulation with nanostructured particles of HA. This forms a biocompatible material able to generate biologically active coatings on the treated surface. In this context, a deeper study of functional models might encourage research on other interesting applications of agricultural tools also based on organic and inorganic compounds that are applicable for sustainable plant protection purposes.

ACKNOWLEDGMENTS

We thank I. Pertot, D. Angeli, and O. Giovannini from the Department of Sustainable Agro-Ecosystems and Bioresources of the Fondazione Edmund Mach (San Michele all'Adige, Italy) for technical and logistical support in performing the greenhouse trials; C. Comparini and M. Santoro from the Department of Agrifood Production and Environmental Sciences of the University of Firenze for managing the in vitro assay and A. Simoni from the Department of Agricultural Sciences of the University of Bologna for giving the technical support in performing the samples preparation and characterization; and A. Phillips for providing a careful review of this paper.

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