BioNanoScience
https://doi.org/10.1007/s12668-023-01244-5

Enhancing the Efficacy of Natural Repellents Against Grapevine Pathogens by Tannins-Lignin-Mixed Nanovectors

Sara Falsini[1] · Tommaso Nieri[1,2] · Silvia Schiff[1] · Alessio Papini[1] · Maria Cristina Salvatici[3] · Giuseppe Carella[2] · Laura Mugnai[2] · Cristina Gonnelli[1] · Sandra Ristori[4]

Accepted: 7 November 2023

Abstract
The severe use of conventional pesticides has led to rethinking agriculture protocols for crop protection. In this context, attention has been given to nanopesticides, i.e., formulations containing nanosized particles to deliver poorly soluble bioactive compounds. The aim of this work was to design and prepare nanoparticles from biopolymers such as lignin and tannins to allow the encapsulation and transport of neem oil and capsaicin against three grapevine phytopathogenic fungi: *Verticillium dahliae*, *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum*. Tannins from grape seeds were chosen as adjuvants for the lignin matrix forming the nanocapsules to improve compatibility between the nanovectors and the target since tannins are intrinsic components found in vine plants. Neem oil was used as dispersant for the non-polar bioactive substance capsaicin against pathogenic fungi and for its own antimicrobial properties. The size and structure of the particles in these new formulations were characterized prior to in vitro tests. Scanning electron microscopy (SEM) showed that submicrom globular structures constituted the most abundant population. From dynamic light scattering (DLS), it was found that the average diameter in solution was in the range 250–300 nm for loaded vectors and zeta potential (ZP) showed that all the scattering objects had a negative surface charge (in the range from − 52 to − 37 mV). Small angle X-ray scattering (SAXS) was used to get finer insight into the structural properties of plain and loaded aggregates by fitting the intensity diagrams with a superposition of different contributions, which depended on the specific formulation, in agreement with the SEM pictures taken on the solid obtained from solvent evaporated samples. Regarding the antifungal activity, the most promising results were obtained against the fungi *P. minimum*. In this case, the advantage obtained by administration through nanocapsules was a dramatic reduction in the amount of both neem oil and capsaicin needed for the treatment. The antifungal effect was suggested to stem out from the synergistic activity of the two bioactive compounds.

Keywords Grapeseed tannins · Lignin · Nanocapsules · Fungal assays · Capsaicin · Neem oil

1 Introduction

Over the latest decades, the intensive use of pesticides in agriculture has ensured abundant yields to satisfy the food requests from an ever-increasing world population [1], and at the same time, it has created new risks to the environment and human health [2, 3]. Actually, almost 70–80% of the pesticide persist in soils long enough to pollute groundwaters, thus contaminating the food chain and provoking adverse effects due to their bioaccumulation [4]. Moreover, pesticide persistence in the environment can widen the number of natural competitors [5], worsening the situation and requesting more pesticides to obtain the desired pest control [6]. Among the first synthetic pesticides introduced in the 1940s, persistent organic pollutants (POPs) [7] were banned

✉ Sara Falsini
sara.falsini@unifi.it

1 Department of Biology, University of Florence, via Micheli 1-3, 50121 Florence, Italy

2 Department of Agricultural, Food, Environmental and Forestry Science and Technology (DAGRI), Plant pathology and Entomology section, University of Florence, P.le delle Cascine, 28, 50144 Florence, Italy

3 Institute of Chemistry of OrganoMetallic Compounds, ICCOM-CNR and Consorzio INSTM, Via Madonna del Piano, 10 – 50019, Sesto F.no, Florence, Italy

4 Department of Chemistry "Ugo Schiff" & CSGI, University of Florence, 50019 Sesto Fiorentino, Fi, Italy

Published online: 23 November 2023

in 2001 [8], because of their accumulation in food, general toxicity, and the possibility to be transported for long distances through water and air (e.g., by winds or migratory species) [9, 10]. Alongside POPs, the use of copper-based antimicrobial compounds (CBAC), though particularly effective against pathogens of grapevine, has been progressively limited to specific quantities by the European Commission [11, 12]. New classes of pesticides such as pyrethroids [13], carbamates, and phenyl pyrazoles have also been used, generating a misconception about these compounds, which are sometimes considered not toxic and not persistent in the environment [5].

In present times, researchers are studying innovative technologies to avoid side effects and ecosystem destabilization while ensuring a good quality of the final product [14]. In particular, nanotechnology has gained attention as a potential tool for crop protection in agriculture. Nanopesticides usually consist of active principles vehiculated by nanocarriers [15–19]. Different formulations have been designed ad hoc to enhance the efficacy of the cargo molecules, reduce the dose administered, and promote controlled release [20–22]. In this context, eco-friendly preparations and handling procedures should be also considered, such as using renewable starting materials, less harmful solvents, and compounds that can be naturally decomposed [23]. All such instances gain further importance in transferring nanoparticles from the lab to large-scale production, where nanotechnology represents the cutting edge of applied research. The most promising nanosystems for crop protection are based on molecules from plants as biopesticides and natural polymers as carriers. Among natural biopesticides, plant secondary metabolites are gaining attention since they play a key role in the defense mechanism against natural enemies such as fungi, bacteria, and insects [24]. Secondary metabolites can be classified into different groups according to their chemical nature: phenols (such as tannins, lignin), terpenes (sesquiterpenes, diterpenes, triterpenes), and nitrogen compounds (alkaloids). In this latter group, capsaicin, an alkaloid extracted from chili peppers, has turned out to be a fruit-landing deterrent against hemipteran insects (*Ceratitis capitata*) [25] and an excellent antifungal agent [26]. In particular, Vuerich et al. [27] demonstrated that chili pepper pod extract exerted antifungal properties against some of the major Oomycete pathogens for grapevine including *Botrytis cinerea* Pers., *Phyllosticta ampelicida* (syn. *Guignardia bidwellii*), and *Plasmopara viticola*. Essential oils, containing a mixture of secondary metabolites, generally monoterpenes and sesquiterpenes, also exert an effect in plant protection from parasitises attack [28]. It is well known that neem oil, extracted from *Azadirachta indica* seeds, and containing more than three hundred biologically active molecules [29], is effective against pests [30]. Moreover, it has shown improved antifungal properties against Ascomycete fungi [31] when encapsulated in nanoformulations. For these properties, neem oil can also be used to preserve food in more sustainable packaging based on natural biopolymers, such as chitosan, starch, and pectin [29].

The present work shows enhanced antifungal effect of neem oil when encapsulated in the hydrophobic core of nanoparticles prepared from mixed lignin and tannins. Capsaicin was also added as a natural repellent to these formulations for improving their efficacy. Antifungal assays were conducted against three fungal pathogens that can infect several hosts, such *Verticillium dahliae*, or that typically infect grapevine, i.e., *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum*.

2 Materials and Methods

2.1 Experimental Materials

Kraft lignin, capsaicin, and acetone were purchased by Sigma-Aldrich. Grape seed tannins and neem oil were kindly supplied by Natural-mente srl (http://www.natural-mentesrl.it). Potato dextrose agar and the agar powder were purchased by Liofilchem. Pindarus 25 WDG and Idrorame Flow were purchased by Chimiberg.

2.2 Preparation of NCs

The powder of lignin (L) and grape seed tannins (T) were dissolved separately or in combination (as shown in Figure 1) in KOH solution at pH 11.5. This pH value was selected to overcome the low solubility of lignin in water at pH minor or equal to 7 (Falsini et al., 2019; Agustin et al., 2019). Briefly, 150 µL of neem oil was added to 150 µL of a capsaicin solution in acetone at a concentration of 22 mg/mL and this mixed solution was added to 3 mL of polymers aqueous solutions. The oil phase was emulsified in the solution by high-power sonication. The pure lignin-tannin particles were also sonicated and treated, as the loaded formulations, to serve as reference systems. A Branson 450 Digital Sonifer was used at 95% of power, for 3 min, 1 s of pulse on and 0.5 s of pulse off (5 cycles for each sample). The initial and final pH of the nanoformulations was measured by BioClass XS pH 8+ DHS pHmeter (http://www.bioclass.it).

The acronyms of the samples investigated in this study are listed in Table 1, where composition and starting/final pH are reported.

2.3 Dynamic Light Scattering (DLS) and Zeta Potential (ZP)

The average diameter of the obtained nanoparticles was measured by DLS on a Malvern Zetasizer Nano ZS (ZEN

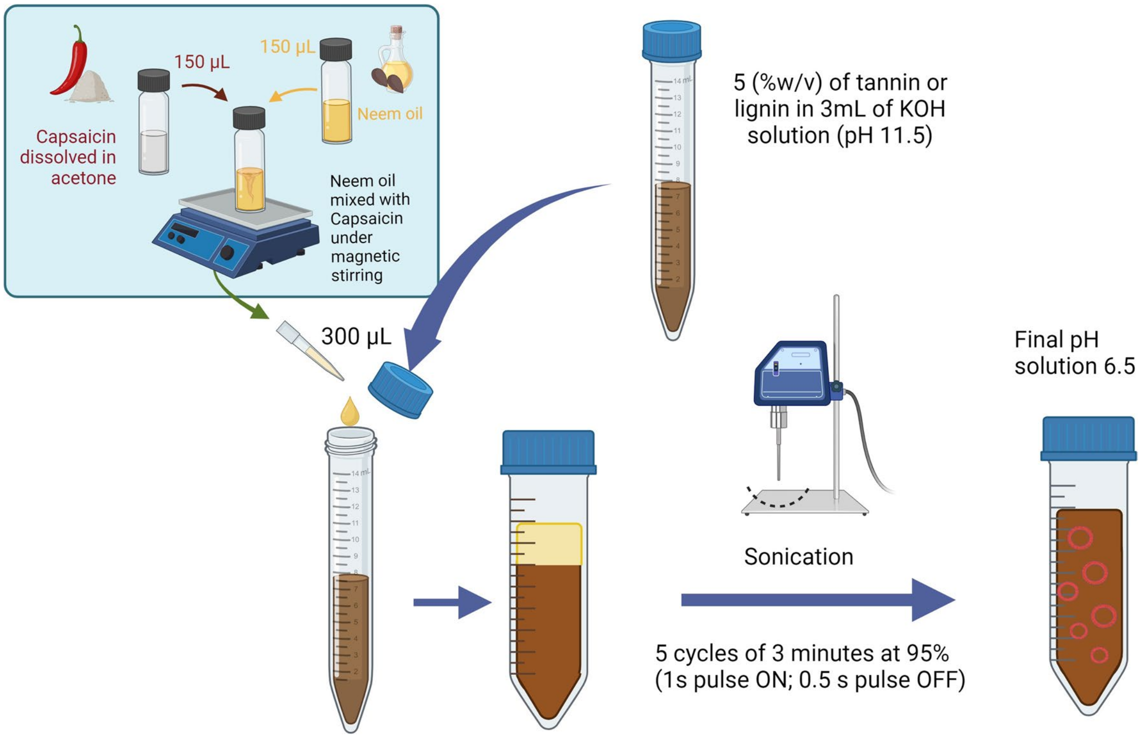

Fig. 1 Technical sketch of the preparation procedure of nanocapsules

Table 1 Samples name and composition, solution pH used for dissolving lignin and grape seed tannins, and final pH of the obtained formulations. *L*, lignin; *T*, tannin; *NCs*, nanocapsules; *N*, neem oil; *CAP*, capsaicin

Samples	Names	Grape seed tannins concentration (w/v %)	Lignin concentration (w/v %)	Neem oil (w/v %)	Capsaicin (w/v %)	pH of the starting KOH solution	pH of the final formulation
Empty vectors	Empty 5L	—	5	—	-	11.5	6.4
	Empty 5T	5	—	—	-	11.5	6.5
Lignin NCs	NCs 5L_N	—	5	4.5	-	11.5	6.0
	NCs 5L_NCAP	—	5	4.5	0.1	11.5	6.0
Mixed NCs	NCs 4L_1T_N	1	4	4.5	-	11.5	5.6
	NCs 4L_1T_N_CAP	1	4	4.5	0.1	11.5	5.6
	NCs 1L_1T_N	2.5	2.5	4.5	-	11.5	5.5
	NCs 1L_1T_N_CAP	2.5	2.5	4.5	0.1	11.5	5.4
Tannins NCs	NCs 5T_N	5	—	4.5	-	11.5	4.8
	NCs 5T_N_CAP	5	—	4.5	0.1	11.5	4.9

1600 model, Malvern Instruments Southborough, MA), equipped with He-Ne 633 with backscattering detection. DLS analysis was performed over 11 runs and in duplicate for each formulation. Samples were diluted at 1:200 with Milli-Q water before measuring to adjust optical density.

Zeta potential measurements were performed with Zetasizer PRO Red Label (Malvern Panalytical Co., Ltd., Malvern, UK). Samples were diluted at 1:100 with MilliQ water. Each reported ZP value was averaged over 3 runs.

2.4 Scanning Electron Microscopy (SEM)

SEM measurements were performed Gaia 3 (Tescan s.r.o, Brno, Czech Republic) and FIB-SEM (Focused Ion Beam-Scanning Electron Microscope). Electron beam used for SEM imaging had the voltage of 10 kV and operating in high-vacuum and with secondary electron detector at the CEME-Centro di Microscopie Elettroniche "Laura Bonzi," CNR Research Area (Florence, Italy). Samples were deposited on a stub, dried in a vacuum, and then coated with an ultrathin coating of gold to enhance the contrast thanks to the presence of an electrically conducting material.

2.5 Small Angle X-ray Scattering (SAXS)

SAXS experiments were performed at the high brilliance SAXS beamline ID02 of the European synchrotron radiation facility (ESRF), Grenoble, France. The wavelength of the incident photons was $\lambda = 1$ Å. The scattering vector q was in the range 0.216×10^{-4}–$0.75\ nm^{-1}$, with a sample-detector distance ranging from 1 to 30 m.

The scattering vector q is defined:

$$q = \frac{4\pi}{\lambda} \sin\frac{\theta}{2}$$

where θ is the scattering angle and λ the X-ray wavelength (= 0.1 nm)

Samples were diluted at 1:10 in Milli Q water and they were placed in quartz capillaries of 1.5-mm diameter. SAXS measurements were performed at room temperature.

The SAXS intensity profile obtained was fitted using SAS view 5.0.5 (https://www.sasview.org/) [32]. The different contributions were superimposed using Origin PRO (http://www.originlab.com/origin) [33].

2.6 Antifungal Activity

The fungal species chosen to test the activity of the nanoformulations are deposited in the collection of DAGRI-Plant pathology and entomology section (University of Florence). These strains have been isolated from grapevine and are associated with different symptoms (Table 2). In particular, three strains of different fungal species *Verticillium dahliae*, *Phaeomoniella chlamydospora*, and *Phaeoacremonium minimum* were used to perform conidial germination assays, following the procedures described by Jaspers [34] and Del Frari et al. [35] with minor modifications.

The fungal colonies were grown on potato dextrose agar (PDA) until they covered the plate surface. Then, colonies were flooded with 2-mL sterile deionized water and conidia were collected using a sterile inoculation loop. The number of conidia in the stock suspension was counted with a Burker chamber and diluted to give 1×10^5 conidia/mL concentration. Conidia suspensions, after lowering the temperature to 30 °C, were mixed with PDA in order to have a final concentration of $1x10^6$ conidia/mL in the growth medium. Nanovectors were administered in drops to the agar surface after medium solidification, to avoid overheating of the natural compounds used in these experiments. The natural compounds alone and empty nanocapsules were also administered, for comparison, at different dilutions, as it is shown in Table 3. Each antifungal assay was performed in triplicate.

The toxicity test was conducted by maintaining the fungi at 25 °C until they covered all the plate surfaces, alternating 12 h of darkness and 12 h of cool fluorescent light for 18 days. The antifungal efficacy was evaluated as the size and intensity of the no-growth halo formed around the application area and each experiment was repeated twice to check reproducibility.

2.7 Statistical Analysis

The antifungal assay was conducted in triplicate for each treatment tested. One-way ANOVA was used to check the significance of differences (at least p-value < 0.05) among

Table 3 Dilution in water of fungicides, natural compounds, and nanoformulations used in toxicity tests

Samples	Compounds	Dilution
Fungicides	Pindarus 25 WDG	0.4% w/v
	Idrorame Flow	0.4% w/v
Natural compounds	Neem oil	78%v/v
	Capsaicin	0.44 %w/v
Empty vectors	Empty 5L	As such, 1:2; 1:4; 1:10 1:20
	Empty 5T	As such, 1:2; 1:4; 1:10
Lignin NCs	NCs 5L_N	As such, 1:2; 1:4; 1:10
	NCs 5L__N_CAP	As such, 1:2; 1:4; 1:10
Mixed NCs	NCs 1L_1T_N	As such, 1:2; 1:4; 1:10
	NCs 1L_1T_N_CAP	As such, 1:2; 1:4; 1:10
	NCs 4L_1T_N	As such, 1:2; 1:4; 1:10
	NCs 4L_1T_N_CAP	As such, 1:2; 1:4; 1:10
Tannin NCs	NCs 5T_N	As such, 1:2; 1:4; 1:10
	NCs 5T_N_CAP	As such, 1:2; 1:4; 1:10

Table 2 Fungal species used to test the antifungal activity

Fungal species	Strain*	Host	Symptom/disease
Verticillium dahliae	PVFi12938Vd	Eggplant	Verticillium wilt
Phaeomoniella chlamydospora	CBS 229.95	Grapevine	Esca complex
Phaeoacremonium minimum	CBS 631.94	Grapevine	Esca complex

*PVFi: DAGRI, Patologia vegetale Florence, Italy; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands means, using GraphPad Prism 7 GraphPad Software, San Diego, CA, USA). An Honestly Significant Differences (HSD)-Tukey test was run for post hoc comparisons to determine if there are any differences in the Inhibition Zone Diameter (IZD) of the mycelium among treatments after conidial germination.

3 Results

3.1 DLS and ZP analysis

The average diameter and polydispersity index of the lignin/tannin nanoparticles prepared in this work are reported in Table 4. The size of empty and neem containing NCs was between 280 nm and 1 µm while the size of NCs loaded with capsaicin was lower, i.e., in the range 265–300 nm, showing that capsaicin molecules were able to increase the local curvature of the particles, which in turn reduced their overall size. Specifically, the size of sample NCs 5T_N, which encapsulated neem oil in tannins polymer was much larger than its capsaicin-loaded counterpart (sample NCs 5T_N_CAP). The very large diameter measured for particles present in the NCs 5T_N formulation indicated that these aggregates did not possess a typical capsule-like morphology, but rather were elongated, flexible particles. In line with these results, lower polydispersity index was obtained for capsaicin-loaded systems (0.20 versus 0.31).

The surface charge of nanoaggregates was negative in all the formulations investigated, varying from – 52 mV (pure lignin NCs) to – 37 mV (pure tannin NCs) with intermediate values for mixed compositions. The presence of capsaicin did not alter significantly the ZP results. From these data, we can infer that plain and loaded nanovectors possessed substantially the same stability and compatibility toward plants.

Table 4 Average diameter and polydispersity index of the nanoparticles

Samples	Names	Zeta average, nm	PDI
Empty vectors	Empty 5L	770 ± 50	0.86
	Empty 5T	950 ± 50	0.87
Lignin NCs	NCs 5L_N	320 ± 20	0.15
	NCs 5L_N_CAP	310 ± 15	0.18
Mixed NCs	NCs 1L_1T _N	280 ± 10	0.11
	NCs 4L_1T_N	305 ± 15	0.09
	NCs 1L_1T _N_CAP	265 ± 10	0.13
	NCs 4L_1T_N_CAP	275 ± 10	0.15
Tannins NCs	NCs 5T_N	1000 ± 50	0.31
	NCs 5T_N_CAP	300 ± 20	0.20

3.2 SEM Micrographs

Figure 2 shows representative SEM images of pure lignin (NCs 5L_N_CAP, panel A) and tannins (NCs 5T_N_CAP, panel B) nanoformulations. Globular objects with size distribution were detected as the typical polymeric aggregates formed in these systems. Similar micrographs were also obtained for samples containing mixed nanoparticles without capsaicin. The most abundant NCs fraction had a diameter of 100–200 nm which was in the range of DLS measurements. It should be noted here that DLS has the advantage of analyzing scattering objects in solutions, i.e.,

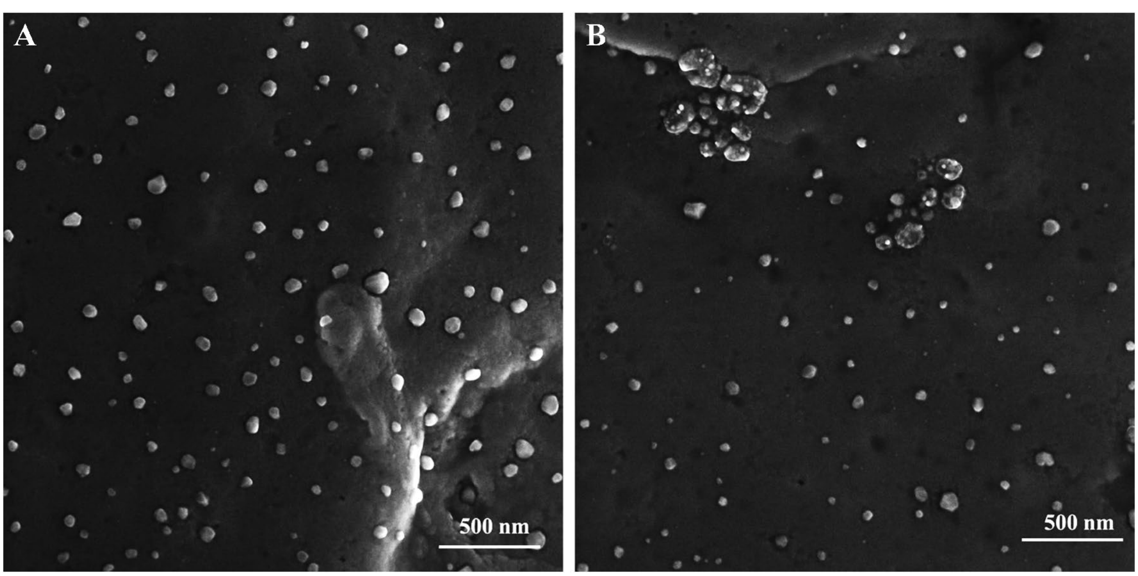

Fig. 2 SEM micrograph of pure lignin (**A**) and tannins (**B**) formulations, NCs 5L_N_CAP and NCs 5T_N_CAP respectively

in their unaltered environment, while Electron microscope reports the morphology of dried samples. Thus, in the case of DLS, transient aggregates due to Brownian motion can be evidenced to the expense of very small particles.

3.3 SAXS Investigations

Figure 3 A reports the SAXS intensity curves of lignin, tannin, and mixed lignin-tannin formulations encapsulating neem oil, while Figure 3B shows the curves of systems containing both neem oil and capsaicin. All profiles indicated that a plurality of scattering objects, i.e., different aggregates, was present in these samples as it was also confirmed by fitting (vide infra). This characteristic was well in line with the complexity of the present formulations and with the analysis of similar polymeric nanosystems made from lignin [36–38]. The main features observed in the SAXS curves were (i) a q^{-3} or q^{-4} decay below q 0.01 nm^{-1}; (ii) a shoulder at intermediate q ($0.01 < q < 0.1$); and (iii) a large bump at high q which was peculiar of formulations containing capsaicin and large amounts of tannins (Figure 3A). Noteworthy, the shoulder at intermediate q could be correlated with nanosystems showing the highest antifungal efficacy. On the basis of fitting (Figure 4A and B), this shoulder was attributed to hollow spheres with 60–90-nm radius and 9–10-nm thickness. We assumed that the hydrophobic neem oil was

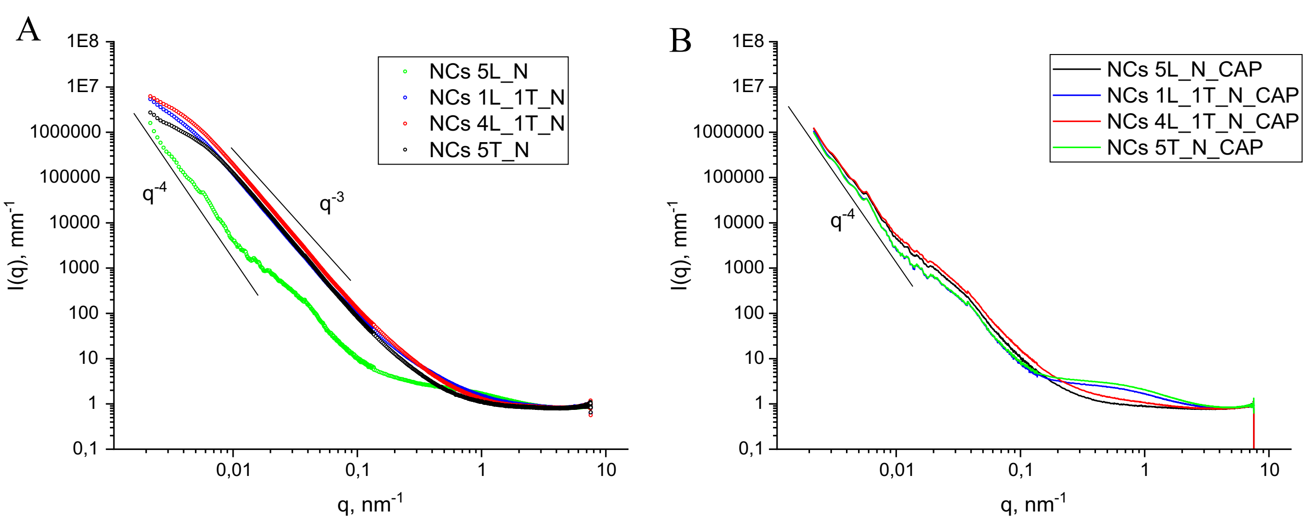

Fig. 3 SAXS intensity diagrams obtained from NCs encapsulating neem oil (**A**) and nanoformulations containing both the essential oil and capsaicin (**B**). Black line: q^{-4} decay due to large (≥ 1 μm) scattering objects

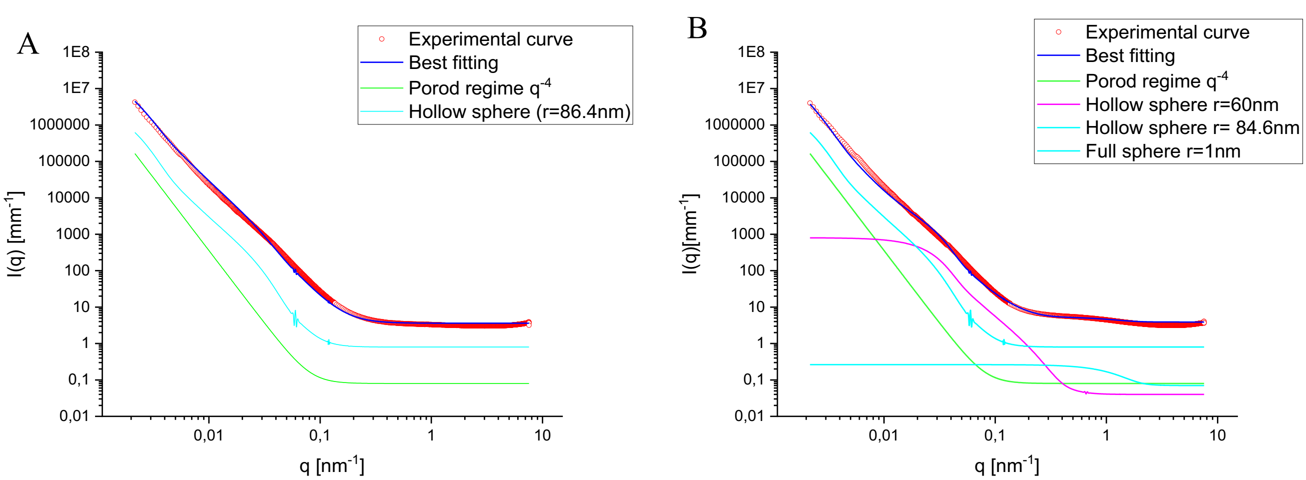

Fig. 4 SAXS intensity diagrams of NCs_5L_N_CAP (**A**) and NCs_5T_N_CAP (**B**). Red circles: experimental data; continuous blue lines: best-fitting obtained with the contributions listed in Table 5. The calculated contributions to the best fit were vertically translated for the sake of clarity

segregated from the water environment and located inside cavities surrounded by a lignin corona. Smaller spheres ($r \approx 1$ nm) were generally detected in samples with mixed composition and were not correlated with the formulation efficacy. They were interpreted as due to micelles or small polymer coils coexisting with larger structures. Such larger objects, whose dimensions exceeded the investigated q range were responsible for the q^{-4} and q^{-3} decays [39–41].

In particular, the q^{-4} power law was attributed to the tails of SAXS profiles due to particles with 500–600 nm size (Porod regime). In Figure 3A, the SAXS diagram of NCs 5L_N differs from the other formulations containing neem oil only and shows the characteristic profile of the well-performing systems as previously discussed.

Figure 4 shows the experimental and fitted data for NCs_5L_N_CAP (Figure 4A) and NCs 5T_N_CAP (Figure 4B). In NCs 5L_N_CAP, the contribution of the Porod regime is predominant over the hollow spheres. The fitting of NCs 5T SAXS profile showed the presence of one further population of hollow spheres and full spheres at high q ($r \approx 1$ nm). The structural properties and their normalized contributions of different populations are listed in Table 5.

Table 5 Normalized contributions of different terms in the best fits obtained with the unified phenomenological model in the SASview package

Sample	Power law contribution q^{-4} G_1	Hollow sphere (H_1) $r \approx 90$ nm thickness = 9 nm	Hollow sphere (H_2) $r \approx 60$ nm thickness = 10 nm	Full sphere (F) $r \approx 1$nm
NCs 5L_N_CAP	0.6	0.4	-	-
NCs 5T_N_CAP	0.41	0.15	0.12	0.32

I(q) for NCs 5L_N_CAP = $((G_1*0.6 + H_1*0.4)*4) - 0.3$

I(q) for NCs 5T_N_CAP= $((G_1*0.45 + H_1*0.15 + H_2*0.12+F*0.32)*0.15) - 0.3$

3.4 Antifungal Assays

Nanoformulation efficacy against the three species listed in Table 2 was evaluated on the basis of the area developed around the drop where fungal mycelium growth was inhibited. The dimension of the IZD increases proportionally to the antifungal efficacy of the administered compounds. The antifungal activity was monitored for 18 days, considering that conidia germinated in 3/4 days and mycelium expanded covering the Petri dishes depending on the sensitivity to the conventional antifungal products and nanoformulations.

The antifungal activities of (i) two commercial fungicides, i.e., Pindarus 25 WDG and Idrorame Flow; (ii) natural compounds such as neem oil, capsaicin, and the two biopolymers lignin and tannin; (iii) the nanoformulations on selected fungal pathogens (*V. dahliae*, *P. minimum*, and *P. chlamydospora*) were showed in Table 6. Pindarus 25 WDG tebuconazole, inhibited the growth of all three selected fungi at a concentration higher than 0.04%w/v, revealing its potentiality as wide spectrum antifungal agent. Although we expected a similar trend for Idrorame Flow, this commercial product exerted an antifungal activity only against *P. chlamydospora*. Regarding the natural compounds, neem oil showed antifungal activity diluted in water emulsion (78% w/v) only on *P. minimum* without impairing *V. dahliae* and *P. chlamydospora* growth. Capsaicin exerted its activity against *V. dahliae* and *P. chlamydospora* at a concentration

Table 6 In vitro antifungal activity of commercial products, pure neem oil, capsaicin, empty nanoaggregates, and NCs (see Table 1 for sample names); *NI*, no inhibition zone around the drop

Samples	Names	*V. dahliae*	*P. minimum*	*P. chlamydospora*
Fungicides	Pindarus 25 WDG	Active (≥ 0.04 % w/v)	Active (≥ 0.04 % w/v)	Active (≥ 0.04% w/v)
	Idrorame Flow	NI	NI	Active (0.4 % w/v)
Natural fungicides	Neem oil	NI	Water emulsion 78% w/v	NI
	Capsaicin	≥2.2 %w/v	≥4.4 %w/v	≥ 2.2 %w/v
Empty nanovector	Empty 5L	NI	NI	NI
	Empty 5T	NI	NI	NI
Lignin NCs	NCs 5L_N	NI	Active [Neem] ≥ 0.45 % w/v	NI
	NCs 5L_N_CAP	NI	Active [Neem] ≥ 0.45 % w/v	NI
Mixed NCs	NCs 1L_1T_N	NI	Active [Neem] ≥ 0.45 % w/v	NI
	NCs 4L_1T_N	NI	Active [Neem] ≥ 1.5 % w/v	NI
	NCs 1L_1T__N_CAP	NI	Active [Neem] ≥ 0.45 % w/v	NI
	NCs 4L_1T_N_CAP	NI	Active [Neem] ≥ 0.45 % w/v	NI
Tannins NCs	NCs 5T_N	NI	NI	NI
	NCs 5T_N_CAP	NI	Active [Neem] ≥ 0.45 % w/v	NI

≥ 2.2% w/v while on *P. minimum* in doses ≥ 4.4% w/v. Both grape seed tannins and lignin administered as empty nanoparticles (samples Empty_5L and Empty 5T) did not show any antifungal activity.

Among the tested fungi, *P. minimum* was revealed to be the most sensitive to the marketable products and the different nanoformulations tested (Figure 5), compared to the water control (Figure 5D). Although Idrorame Flow did not show any significant antifungal activity against this fungus, Pindarus 25 WDG reduced the *P. minimum* growth with an IZD of 2.68 cm ± 0.2 (Figure 5C). Among the nanoformulations, the effect of capsaicin (Figure 5D) was significantly enhanced by both NCs 4L_1T_N_CAP and NCs_5T_N_CAP producing the IZD of 5.1 cm ± 0.3 and 4.8 cm ± 0.5, respectively. In particular, NCs 4L_1T_N_CAP significantly increased both capsaicin and neem oil antifungal activity enlarging the inhibition of the pure natural compounds alone (i.e., capsaicin and neem oil). We can assert that properly vehiculated capsaicin and neem oil exerted a synergic effect against fungi. NCs 5L_N_CAP (Figure 5F) produced a significantly larger inhibitory zone at 0.56 % w/v of neem oil and 0.0125%w/v of capsaicin, i.e., at a concentration one hundred fifty times lower compared to the treatment with the neem oil alone and comparable to the effect promoted by capsaicin at a concentration of 4.4%w/v (Figure 6). The inhibition area induced by NCs 5L_N_CAP had a diameter of 3.98 cm ± 0.23, which is more than double with respect to the inhibition area induced by the pure neem oil (IZD: 1.62 cm ± 0.2). A similar trend was observed for NCs 1L_1T_N_CAP which induced the IZDs of dimensions 4.52 cm ± 0.6 and 4.4 ± 0.3 at the following concentration of neem oil 1.13% w/v and 0.56% w/v, and capsaicin 0.025% w/v and 0.0125% w/v. This indicated that the efficacy of combined capsaicin and neem oil as antifungal agents were boosted by times, respectively, through their combined administration in the form of nanocapsules.

4 Conclusions

In this work, we prepared biopolymer-based nanoformulations to vehiculate neem oil and capsaicin [42] against three fungal pathogens that typically affect plants of commercial interest, such as vegetables or grapes. Tannins and lignin are both polyphenolic compounds; therefore, in the design of mixed formulations, we assumed that compatibility between

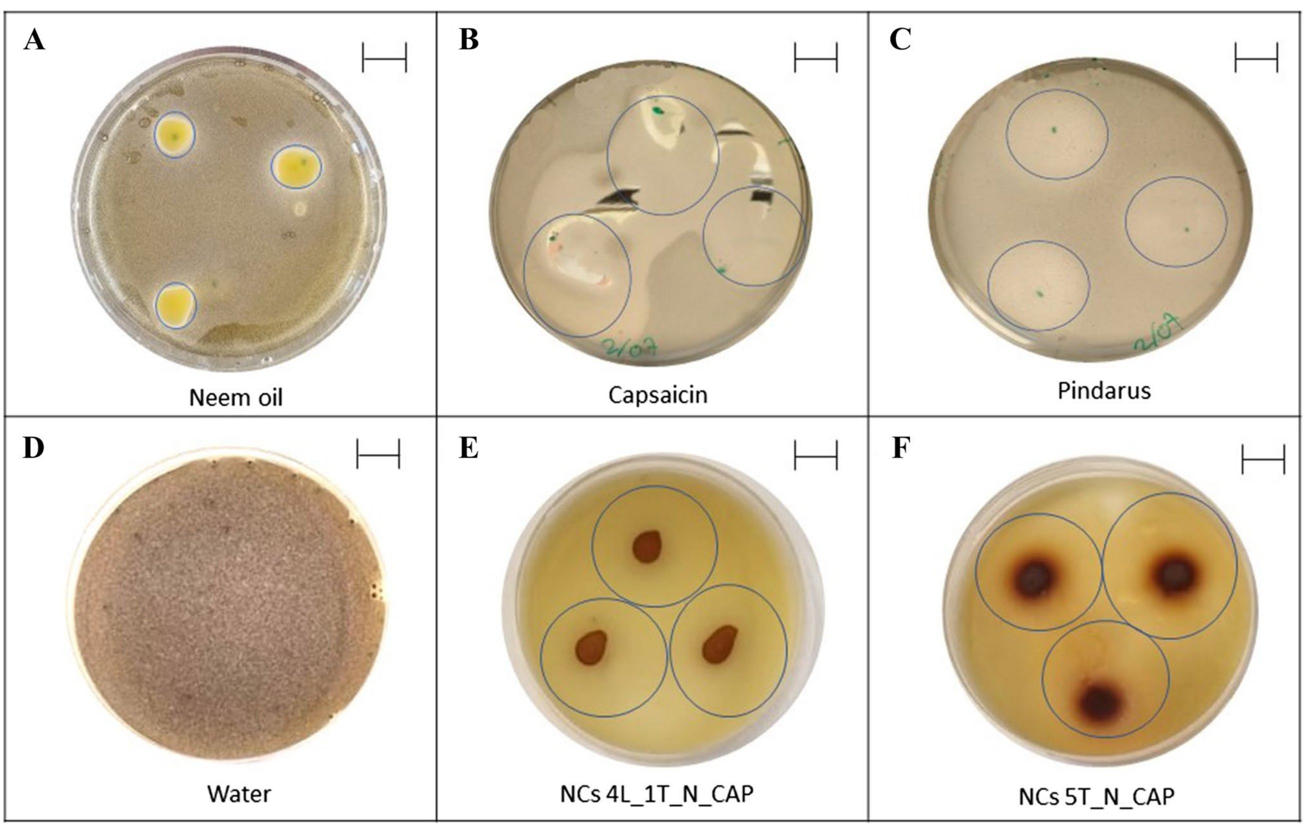

Fig. 5 Effect on *P. minimum* growth exerted by **A** Neem administered as 78% w/v water emulsion; **B** Capsaicin; **C** Pindarus 25 WDG (0.04% w/v); **D** water; **E** NCs_4L_1T_N_CAP (Neem conc = 2.3%w/v; CAP conc = 0.5%w/v); **F** NCs_5T_N_CAP (Neem conc. = 4.5% w/v; CAP conc = 0.1% w/v). Scale bar: 1 cm

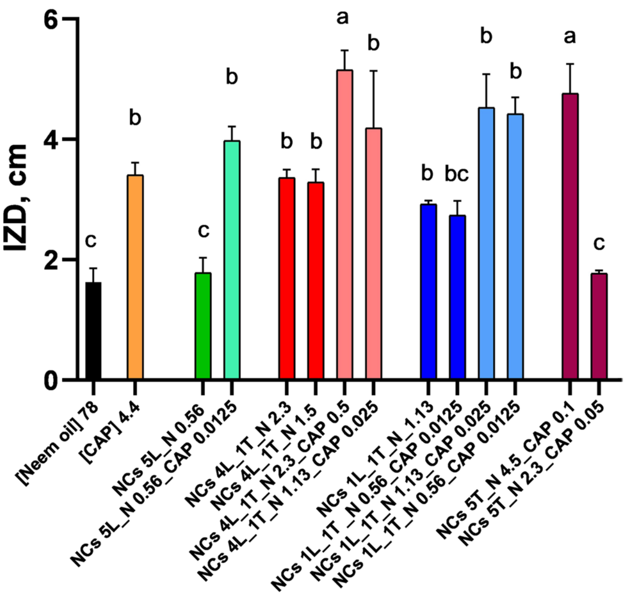

Fig. 6 Inhibition zone diameter (cm) of *P. minimum* exposed to neem oil (78 w/v) or capsaicin (4.4% w/v) compared to the other formulations. Letters close to the histogram indicate the significant differences among the treatments according to Tukey's test (at least p-value < 0.05)

these molecules should be good. The obtained results confirmed this hypothesis, in that none of the samples prepared showed signs of phase separation or even partial miscibility. In-depth physico-chemical characterization of the obtained nanocapsules was performed by DLS, ZP, SEM, and SAXS. DLS showed that nanocapsules have average dimensions of 265–300 nm, which is a suitable range in drug delivery, either for improving the release of active compounds and conferring stability to the formulation [43]. In particular, stability was indicated by the negative surface charge measured by zeta potential. In the formulations containing capsaicin, the mean diameter was slightly smaller with respect to NCs loaded only with neem oil. This suggested that in the present conditions (i.e., nanoformulations made by mixing tannins with lignin), capsaicin favored the formation of more tightly packed aggregates. DLS data were in agreement with SEM and SAXS, that provided additional information on the structure and size of particles in the formulation. Specifically, SEM and SAXS revealed that more than one population contributed to the overall scattered intensity.

The principal aim of this work was to set up and fully characterize biopolymer-based nanoformulations, given the relevance that this approach is gaining in the search for low-impact pesticides [44], even more so following the request of reducing the impact of chemicals on the environment following the Farm to Fork Strategy of the European Union. The suitability and efficacy in improving the antifungal activity of a substance were tested selecting natural substances, as capsaicin and neem oil, known to have some efficacy in disease control and to have positive characteristics for environmental impact [45]. The potential of biopolymer-based nanoformulations was explored and confirmed on two types of pathogens, involved in Esca complex, a wood disease of grapevine on which a lot of research is concentrated at present, in searching sustainable control tools, and on a wide spectrum pathogen of relevance on many vegetable crops, as a good example of useful application of a low impact tool for disease control. The antifungal assays performed in this study allowed to show that the formulation containing smaller particles, among which, NCs_5T _N_CAP, exerted the highest fungal effect against *P. minimum*. We can thus infer that the larger interface provided by very small particles (diameter < ca. 100nm) plays a critical role in the contact and exchange between capsaicin and pathogen organisms.

These results open the road to a new approach for improving consistently the antifungal efficacy of active compounds with low environmental impact. The method set up and evaluated in this research can be applied to other natural substances, allowing future applications to disease control not only in grapevine but also in other crops where a strong reduction of synthetic pesticides is more and more needed for the safety of the environment and the consumer health.

Acknowledgements This work was supported by the University of Florence (Fondi di Ateneo 2021–2022). We acknowledge the European Synchrotron Radiation Facility (ESRF) for beamtime provision. In particular, we would like to thank Dr. Theyencheri Narayanan and Dr. Michael Szutcki for their assistance and support in using the ID02 beamline.

Code Availability Not applicable.

Author Contribution Sara Falsini: conceptualization, methodology, formal analysis, investigation, data curation, writing—review and editing; Tommaso Nieri: methodology, formal analysis, writing—review and editing; Silvia Schiff: methodology, formal analysis, writing—review and editing; Alessio Papini: resources, writing—review and editing; Giuseppe Carella: methodology, formal analysis, writing—review and editing; Maria Cristina Salvatici: methodology, resources, writing, and results discussion; Laura Mugnai: methodology, resources, supervision, project administration, funding acquisition; Cristina Gonnelli: conceptualization, methodology, writing—original draft, writing—review and editing, supervision, project administration; Sandra Ristori: conceptualization, methodology, resources, writing—review and editing, supervision, project administration, funding acquisition.

Funding Open access funding provided by Università degli Studi di Firenze within the CRUI-CARE Agreement.

Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics Approval The authors declare that their study is compliant with ethical standards.

Consent to Participate All the authors listed consent to participate.

Consent for Publication We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. All authors have approved the final manuscript and agreed with its submission to Agronomy for Sustainable Development.

Competing Interests The authors declare no competing interests.

References

1. Godfray, H. C. J. (2014). The challenge of feeding 9–10 billion people equitably and sustainably. *The Journal of Agricultural Science, 152*, 2–8.
2. Wilson, C., & Tisdell, C. (2001). Why farmers continue to use pesticides despite environmental, health and sustainability costs. *Ecological Economics, 39*, 449–462.
3. Struelens, Q. F., Rivera, M., Alem Zabalaga, M., Ccanto, R., Quispe Tarqui, R., Mina, D., Carpio, C., Mantilla, M. R. Y., Osorio, M., Roman, S., Muñoz, D., & Dangles, O. (2022). Pesticide misuse among small Andean farmers stems from pervasive misinformation by retailers. *PLOS Sustain Transform, 1*(6), e0000017. https://doi.org/10.1371/journal.pstr.0000017
4. The Stockholm Convention (2021) *Persistent Organic Pollutants*, opened for signature May 23, 2001, UN Doc. UNEP/POPS/CONF/4, App. II (2001), reprinted in 40 ILM 532 (2001).
5. Nguyen Dang Giang, C., Le DB, N. V. H., Hoang, T. L., Tran, T. V., Huynh, T. P., Nguyen, T. Q., et al. (2022). Assessment of pesticide use and pesticide residues in vegetables from two provinces in Central Vietnam. *PLoS One, 17*(6), e0269789. https://doi.org/10.1371/journal.pone.0269789
6. Liu, B., Fan, Y., Li, H., Zhao, W., Luo, S., Wang, H., Guan, B., Li, Q., Yue, J., Dong, Z., Wang, Y., & Jiang, L. (2021). Control the entire journey of pesticide application on superhydrophobic plant surface by dynamic covalent trimeric surfactant coacervation. *Advanced Functional Materials, 31*, 2006606. https://doi.org/10.1002/adfm.202006606
7. Meeker, J.D., & Boas, M. (2011) Pesticides and thyroid hormones. In: O. N. Jerome (Ed) *Encyclopedia of environmental health* (pp. 428–437.). Burlington: Elsevier.
8. United Nations Environment Programme (2010). *United nations environment programme - annual report 2009: seizing the green opportunity*. https://wedocs.unep.org/20.500.11822/7824
9. Zhang, Y., Qi, S., Xing, X., Yang, D., Devi, N. L., Qu, C., Liu, H.-X., Zhang, J.-Q., & Zeng, F.-M. C. (2018). 21 - Legacies of organochlorine pesticides (OCPs) in soil of China—A review, and cases in Southwest and Southeast China. In B. De Vivo, H. E. Belkin, & A. Lima (Eds.), *Environmental Geochemistry, edition no second* (pp. 543–565). Elsevier.
10. Zhao, Y., & Chen, Y.-P. (2023). Coming ecological risks of organochlorine pesticides and novel brominated flame retardants in the Yellow River Basin. *Science of the Total Environment, 857*, 159296.
11. Lamichhane, J. R., Osdaghi, E., Behlau, F., Köhl, J., Jones, J. B., & Aubertot, J.-N. (2018). Thirteen decades of antimicrobial copper compounds applied in agriculture. A review. *Agronomy for Sustainable Development, 38*, 28.
12. European Commission. Commission Implementing Regulation (EU) 2021/1165 of 15 July 2021 Authorising Certain Products and Substances for use in Organic Production and Establishing Their Lists. 2021. http://data.europa.eu/eli/reg_impl/2021/1165/oj
13. Thatheyus, A. J., & Gnana Selvam, A. D. (2013). Synthetic Pyrethroids: Toxicity and Biodegradation. *Applied Ecology and Environmental Sciences, 1*(3), 33–36.
14. Kookana, R. S., Boxall, A. B. A., Reeves, P. T., Ashauer, R., Beulke, S., Chaudhry, Q., Cornelis, G., Fernandes, T. F., Gan, J., Kah, M., Lynch, I., Ranville, J., Sinclair, C., Spurgeon, D., Tiede, K., & Van den Brink, P. J. (2014). Nanopesticides: Guiding principles for regulatory evaluation of environmental risks. *Journal of Agricultural and Food Chemistry, 62*, 4227–4240. https://doi.org/10.1021/jf500232f
15. Clemente, I., Menicucci, F., Colzi, I., Sbraci, L., Benelli, C., Giordano, C., Gonnelli, C., Ristori, S., & Petruccelli, R. (2018). Unconventional and sustainable nanovectors for phytohormone delivery: Insights on Olea europaea. *ACS Sustainable Chemistry & Engineering, 6*(11), 15022–15031.
16. Falsini, S., Clemente, I., Papini, A., Tani, C., Schiff, S., Salvatici, M. C., Petruccelli, R., Benelli, C., Giordano, C., Gonnelli, C., & Ristori, S. (2019). When sustainable nanochemistry meets agriculture: Lignin nanocapsules for bioactive compound delivery to plantlets. *ACS Sustainable Chemistry & Engineering, 7*(24), 19935–19942.
17. Sipponen, M. H., Lange, H., Crestini, C., Henn, A., & Sterberg, M. (2019). Lignin for nano- and microscaled carrier systems: Applications, trends, and challenges. *ChemSusChem, 12*, 2039–2054.
18. Machado, T. O., Beckers, S. J., Fischer, J., Müller, B., Sayer, C., de Araujo, P. H. H., Landfester, K., & Wurm, F. R. (2020). Bio-based lignin nanocarriers loaded with fungicides as a versatile platform for drug delivery in plants. *Biomacromolecules, 21*(7), 2755–2763. https://doi.org/10.1021/acs.biomac.0c00487
19. Gigli, M., Fellet, G., Pilotto, L., Sgarzi, M., Marchiol, L., & Crestini, C. (2022). Lignin-based nano-enabled agriculture: A mini-review. *Frontiers in Plant Science, 13*. https://doi.org/10.3389/fpls.2022.976410
20. Huang, S., Wang, L., Liu, L., Hou, Y., & Li, L. (2015). Nanotechnology in agriculture, livestock, and aquaculture in China. A review Agron. *Sustainable Development, 35*, 369–400. https://doi.org/10.1007/s13593-014-0274-x
21. Gahukar, R. T., & Das, R. K. (2020). Plant-derived nanopesticides for agricultural pest control: Challenges and prospects. *Nanotechnology for Environmental Engineering., 5*, 3. https://doi.org/10.1007/s41204-020-0066-2
22. Machado, T. O., Grabo, J., Sayer, C., Pedro, H. H., de Araújo, P. H. H., Michel, L., Ehrenhard, M. L., & Wurm, F. R. (2022). Biopolymer-based nanocarriers for sustained release of agrochemicals: A review on materials and social science perspectives for a sustainable future of agri- and horticulture. *Advances in Colloid and Interface Science, 303*, 102645.
23. Yousef, H. A., Fahmy, H. M., Arafa, F. N., Abd Allah, M. Y., Tawfik, Y. M., El Halwany, K. K., El-Ashmanty, B. A., Al-anany, F. S., Mohamed, M. A., & Bassily, M. E. (2023). Nanotechnology in pest management: Advantages, applications, and challenges. *International Journal of Tropical Insect Science, 43*, 1387–1399. https://doi.org/10.1007/s42690-023-01053-z

24. Jones, W. P., & Kinghorn, A. D. (2005). Extraction of Plant Secondary Metabolites. *Natural Products Isolation, 20*(2), 323–351.
25. Falsini, S., Rosi, M. C., Ravegnini, E., Schiff, S., Gonnelli, C., Papini, A., Adessi, A., Urciuoli, S., & Ristori, S. (2023). Nanoformulations with exopolysaccharides from cyanobacteria algae: Enhancing the efficacy of bioactive molecules in the Mediterranean fruit fly control. *Environmental Science and Pollution Research*. https://doi.org/10.1007/s11356-023-28180-x
26. Martini, R., Serrano, L., Barbosa, S., et al. (2014). Antifungal cellulose by capsaicin grafting. *Cellulose, 21*, 1909–1919. https://doi.org/10.1007/s10570-014-0219-1
27. Vuerich, M., Petrussa, E., Filippi, A., Cluzet, S., Fonayet, J. V., Sepulcri, A., Piani, B., Ermacora, P., & Braidot, E. (2023). Antifungal activity of chili pepper extract with potential for the control of some major pathogens in grapevine. *Pest Management Science*. https://doi.org/10.1002/ps.7435
28. Tiku, A. R. (2018). Antimicrobial compounds and their role in plant defense. In A. Singh & I. Singh (Eds.), *Molecular aspects of plant-pathogen interaction*. Springer. https://doi.org/10.1007/978-981-10-7371-7_13
29. Kumar, S., Singh, N., Devi, L. S., Kumar, S., Kamle, M., Kumar, P., & Mukherjee, A. (2022). Neem oil and its nanoemulsion in sustainable food preservation and packaging: Current status and future prospects. *Journal of Agriculture and Food Research, 7*, 100254. https://doi.org/10.1016/j.jafr.2021.100254
30. Campos, E. V. R., de Oliveira, J. L., Pascoli, M., de Lima, R., & Fraceto, L. F. (2016). Neem oil and crop protection: From now to the future. *Frontiers in Plant Science, 7*, 1494. https://doi.org/10.3389/fpls.2016.01494
31. Falsini, S., Nieri, T., Paolini, A., Schiff, S., Papini, A., Mugnai, L., Gonnelli, C., & Ristori, S. (2023). Tannins-lignin mixed nanoformulations for improving the potential of neem oil as fungicide agent. *Environmental Science and Pollution Research International, 30*(13), 39131–39141. https://doi.org/10.1007/s11356-022-24991-6
32. SasView Sas View for small angle scattering analysis. https://www.sasview.org/. Accessed April 1, 2023
33. Origin(Pro). (2022). *Version 2022*. Northampton, MA: OriginLab Corporation. USA. http://www.originlab.com/origin. Accessed 10.07.23.
34. Jaspers, M. V. (2001). Effect of fungicides, *in vitro*, on germination and growth of *Phaeomoniella chlamydospora*. *Phytopathologia Mediterranea, 40*, S453–S458.
35. Del Frari, G., Gobbi, A., Aggerbeck, M. R., Oliveira, H., Hansen, L. H., & Ferreira, R. B. (2019). Fungicides and the grapevine wood mycobiome: A case study on tracheomycotic ascomycete *Phaeomoniella chlamydospora* reveals potential for two novel control strategies. *Frontiers in Plant Science, 10*, 1405.
36. Salentinig, S., & Schubert, M. (2017). Softwood lignin self-assembly for nanomaterial design. *Biomacromolecules., 18*, 2649–2653.
37. Agustin, M. B., Penttila, P. A., Lahtinen, M., & Mikkonen, K. S. (2019). Rapid and direct preparation of lignin nanoparticles from alkaline pulping liquor by mild ultrasonication. *ACS Sustainable Chemistry & Engineering, 7*, 19925–19934.
38. Sipponen, M. H., Henn, A., Penttilää, P., & Österberg, M. (2020). Lignin-fatty acid hybrid nanocapsules for scalable thermal energy storage in phase-change materials. *Chemical Engineering Journal, 393*, 124711.
39. Lindner, P. & Zemb, Th. (2002). *Neutron, X-Rays and Light. Scattering methods applied to soft condensed matter*. Amsterdam: Elsevier.
40. Cerar, J., Jamnik, A., & Tomi, M. (2015). Testing classical approach to polymer solutions on SAXS Data of λ-Carrageenan, κ-Carrageenanand Methylcellulose Systems. *Acta Chimica Slovenica, 62*, 498–508.
41. Kwok, J. J., Park, K. S., Patel, B. B., Dilmurat, R., Beljonne, D., Zuo, X., Lee, B., & Diao, Y. (2022). Understanding solution state conformation and aggregate structure of conjugated polymers via Small Angle X-ray Scattering. *Macromolecules, 55*, 4353–4366.
42. Li, Y., Bai, P., Wei, L., Kang, R., Chen, L., Zhang, M., Tan, E. K., & Liu, W. (2020). Capsaicin functions as Drosophila ovipositional repellent and causes intestinal dysplasia. *Scientific Reports, 10*, 9963. https://doi.org/10.1038/s41598-020-66900-2
43. Singh, R. P., Handa, R., & Manchanda, G. (2021). Nanoparticles in sustainable agriculture: An emerging opportunity. *Journal of Controlled Release, 329*, 1234–1248. https://doi.org/10.1016/j.jconrel.2020.10.051
44. Prasad, R., Bhattacharyya, A., & Nguyen, Q. D. (2017). Nanotechnology in sustainable agriculture: Recent developments, challenges, and perspectives. *Frontiers in Microbiology, 20*(8), 1014. https://doi.org/10.3389/fmicb.2017.01014
45. Ayilara, M. S., Adeleke, B. S., Akinola, S. A., Fayose, C. A., Adeyemi, U. T., Gbadegesin, L. A., Omole, R. K., Johnson, R. M., Uthman, Q. O., & Babalola, O. O. (2023). Biopesticides as a promising alternative to synthetic pesticides: A case for microbial pesticides, phytopesticides, and nanobiopesticides. *Frontiers in Microbiology, 14*, 1040901. https://doi.org/10.3389/fmicb.2023.1040901