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When sustainable nano-chemistry meets agriculture: lignin nanocapsules for bioactive compounds delivery to plantlets

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ABSTRACT

First developed for biomedical and industrial applications, nanovectors have recently been extended to agriculture. Therefore, innovative plant growing procedures making use of nanoparticles should be adapted to sustainable processes and materials. This work aims at proposing newly synthetized polymeric nanocapsules (NCs) to be used as biocompatible vectors for delivery of bioactive compounds to plants. Nanoparticles were fabricated from lignin, which is the main byproduct of wood processing and is currently a waste material. Lignin can thus find a virtuous fate and be reused in the context of circular economy. Specifically, we loaded lignin NCs with Gibberellic Acid (GA), assessing that stable and reproducible nanoparticles could be obtained in a range of GA content that is relevant for delivery purposes, i.e. 0.5-1.5 mg ml^{-1}. Plain and GA-loaded NCs were characterized by Dynamic Light Scattering (DLS) and Scanning Electron Microscopy (SEM). Assays of cytotoxicity and cargo release were carried out in two model plants, *Eruca vesicaria* and *Solanum lycopersicum*. These experiments, conducted both *in vitro* and *in vivo*, included the investigations of the percentage of germination, the stem and primary root lengths, as well as the fresh and dry weight of treated plants *vs* non-treated ones. Furthermore, NCs were loaded with Fluorol Yellow 088 to track their entrance and accumulation in seeds and seedlings.

INTRODUCTION

Concerns about environmental problems have led to sustainable approaches in new technology and, in particular, in nanoparticle design[1]. Thus, different sustainable practices have been adopted in laboratory protocols, including the use of renewable starting materials, less toxic

solvents and biodegradable compounds[2,3]. These procedures become of fundamental importance when nanoparticles move from laboratory to large-scale manufacturing[4,5] and, particularly, in agricultural processes, the latest frontier of nanoscience applications[6]. Although nanoparticles are still at an early step in agricultural development, it is evident that they are a promising strategy for enhancing the effectiveness of crop production agents (i.e hormones[7], biocides[8], insecticides[9] etc.) while at the same time reducing their environmental impacts[10,11,12].

In this context, among various nanocarriers, polymeric nanocapsules (NCs) have received attention for their ability to trap bioactive components inside or on the shell matrix, protecting them from degradation[13]. Moreover, ad hoc designed polymeric NCs may unite biocompatibility, biodegradability and controlled release[14] to improve shelf life. The preparation of these NCs can be carried out within the frame of solvent/co-solvent engineering for polymer-based nanosystems[15], a versatile procedure which allows different protocols to be used, depending on the chemical properties of the inner core, the shell and the cargo molecules[16].

Several polymers are currently used in plant science for delivery purposes, such as starch, alginate, chitin, albumin, and cellulose[17]. Polymeric β-glucan, obtained by the treatment of crustaceous chitin, possesses itself an antifungal activity[18]. Another promising polymer is alginate, extracted from some brown algae (*Phaeophyceae*), which can be manipulated to obtain NCs for insecticides[19] delivery.

Herein, a procedure based on lignin NCs was devised for delivering bioactive compounds to plants, thus suggesting novel uses for an abundant and biocompatible starting material, which, in addition, represents the main by-product in paper and cellulose manufacturing[20,21]. For these reasons, lignin stands for valuable and sustainable alternative to synthetic polymers[22]. As a possible case study, the selected cargo was Gibberellic Acid (GA3) a plant growth regulator,

widely used in agriculture for enhancing seed germination[23]. Lignin NCs, both empty and GA3-loaded were tested *in vivo* and *in vitro* on the seeds of two model species, *Solanum lycopersicum* L. cv. Ciliegino (tomato) and *Eruca vesicaria* (L.) Cav. subsp. *sativa* (Miller) (arugula), allowing to achieve factual information on NC phytotoxicity and the potential activity of the cargo in an easy and rapid way. Possible effects on germination and seedling development were also evaluated. In the literature the effect of nanovectors on germination and seedlings are rather sparse and discordant[24,25,26]. Some authors observe that carbon nanomaterials and metal nanoparticles have a positive effect on seed germination and seedling growth[27,28,29], while others report that plants treated with nanomaterials show symptoms of toxicity[30,31,32]. The negative effects of nanoparticles on plants depend on several factors, the carriers type, size and concentration[33,34]. In particular, metal nanoparticles of Zn, Au, Al as well as carbon nanotubes, have been used *in vitro* seed germination of tomato[35] and arugula [33] to verify their toxicity by applying different concentrations.

In this work we investigated, for the first time, the penetration and accumulation of lignin NCs into different plant tissues by including a fluorophore in the formulation. Following the fluorophore signal it is possible to shed light on the fate of the NCs, once entered inside the plants, as previously attempted with a different method, that is using X-rays microscopy for tracking CeO_2 in *Solanum lycopersicum*[36].This enabled to track the NCs pathways *in vivo* which is an important issue to understand the action mechanism and the efficacy of the NCs[37].

MATERIALS AND METHODS

Materials

Kraft Lignin and Acetone were purchased by Sigma-Aldrich. Common olive oil was used for nanocapsule preparation. Milli-Q filtered water was obtained from a Millipore system (20 MΩ cm at 25°C). Fluorol Yellow 088 (FY088) and GA3 were provided by Sigma-Aldrich as well as phloroglucinol and toluidine blue. Technovit 7100 resin were purchase by Kulzer, Emgrid Australia. Plant agar was provided by Micropoli (Italy). Tomato and arugula seeds were provided by Blumen.

Preparation of Lignin NCs

Lignin was dissolved in an alkali solution at a concentration of 1% w/v. GA3 was diluted in acetone at three different concentrations 0.5mg/mL, 1mg/mL and 1.5mg/mL. The obtained solutions were mixed with olive oil, at 1:1 (v/v) ratio. 300µL of this oil/acetone solution were added, dropwise and under magnetic stirring, to 3mL of aqueous lignin (pH 10.5). Then, high power sonication was used to emulsify the oil phase in the lignin solution and to facilitate the GA3 encapsulation in the newly formed nanocapsules. For this purpose, a Branson 450 Digital Sonifier was used at the 50% of power (400W), for 100s (Figure 1). Finally, nanocapsules were diluted 1:1 in Milli-Q water.

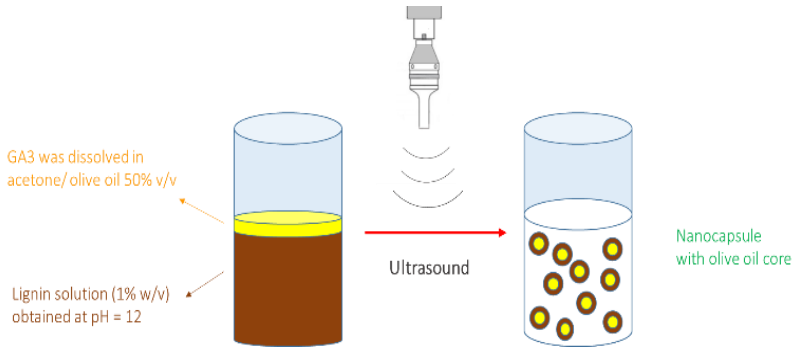

Figure 1: Scheme of nanocapsule preparation.

To track the presence nanocapsules in seeds and roots, a fluorochrome was loaded inside the vector. In particular, FY088 was dissolved in olive oil (0.1% w/v) and then added to acetone, 1:1 (v/v) ratio. After this step, the procedure adopted was the same as that used for GA3 loading.

Dynamic Light Scattering (DLS), Scanning Electron Microscopy (SEM)

DLS measurements were performed on a Malvern Zetasizer Nano ZS (ZEN 1600 model, Malvern Instruments Southborough, MA), equipped with He-Ne 633nm, 4mW laser with backscattering detection. DLS experiments were performed over 11 runs and in duplicate. Samples were diluted 1:250 with MilliQ water before measuring to adjust turbidity.

SEM measurements were performed at the CEME- Centro di Microscopie Elettroniche "Laura Bonzi", CNR Research Area (Florence, Italy). Gaia 3 (Tescan s.r.o, Brno, Czech Republic) FIB-SEM (Focused Ion Beam-Scanning Electron Microscope) Electron beam used for SEM imaging had the voltage of 15kV and operating in high-vacuum and with secondary electron detector. Samples were deposited on a stub, dried in vacuum and then coated with an ultrathin coating of gold to enhance the contrast thanks to the presence of an electrically-conducting material.

Germination experiments

All solutions used for treatments were sterilized under UV rays for 2 hours. Seeds of arugula and tomato were surface-sterilized dipping in ethanol 70% v/v for 2minutes and then in sodium hypochlorite 10% v/v for 15min, washed and soaked in distilled sterile water for one night. Sterilized seeds of tomato and arugula were immersed in the solution of the different treatments for 30min. The treatment included: not-loaded nanocapsules (indicated with NGA0),

nanocapsules loaded with three GA3 cargo concentrations: 0.5mg/mL, 1mg/mL and 1.5mg/mL (indicated with NGA0.5; NGA1.0 and NGA1.5 respectively), only GA3 at the three different concentrations (indicated with GA0.5; GA1.0 and GA1.5 respectively), and Control (MS medium for *in vitro* experiments and water for *in vivo* experiments).

In vitro experiments

For *in vitr*o experiments, sterilized seeds of arugula and tomato were transferred to a small jar (100ml) containing 25ml of agarized medium. Murashige and Skoog[38] medium added with 20g/l sucrose and 7g/l agar, was used for germination test; 1ml liquid layer of several solutions was poured over the solid medium. The MS agarized medium alone was used as the Control. Two jars, with 30 seeds for a jar, were used for each treatment. All *in vitro* seeds germinated were maintained in the growth chamber at 23±1°C with 8/16 photoperiod.

In the preliminary experiment, the concentrations of NGA0.5 and GA0.5 were assessed, while in the second experiment all treatments were applied (NGA3 loaded and not-loaded and GA3 treatments). The seed was considered germinated when the radicle protrusion was present.

At day 5 and day 10, germination percentage of arugula was recorded and for tomato at day 7 and day 14. Growth parameters were evaluated at 14 days, in the tomato experiment.

In vivo experiments

For *in vivo* experiments, after sterilization and pretreatment with the different solutions, 90 seeds for each treatment, were transferred for germination in Ø 90 mm Petri dishes (30 seeds for each) lined with filter paper type Filtrak, moistened with 2 ml of test solution or water. All germination tests were performed in growth chambers at 25°C (12h) and 19°C (12h) in the dark. After

germination, the Petri dishes were opened and maintained in the light/dark (12/12h), at the same temperature, with 60% relative humidity.

Germinated seeds were counted daily, and a seed was considered as germinated at the radicle protrusion. Growth parameters were recorded at the end of the experiment, that was when the treatments with GA_3 encapsulated and no-encapsulated in nanoparticles, reached at least the 85% of seeds germination: 3 days for arugula and at 6 days for tomato plantlets.

Data collection and analysis

Germination percentage (G) was measured according to the equation (1) as described by Al-Ansari and Ksiksi[39]:

$$G = \frac{Number\ of\ germinated\ seeds}{Total\ number\ of\ seeds\ tested} \times 100 \quad (1)$$

At the end of the experiments *in vivo* and *in vitro*, seedling length, with stem and primary root length, secondary roots and fresh weight (FW) of the seedlings were recorded (leaves, stems, roots). Seedlings were dried in a hot oven at 75 °C overnight, and the dry weight (DW) was measured. The data were analyzed one way ANOVA and the differences between treatment means at 5% probability by using the Least Significant Difference (LSD) method. All statistical tests were performed using a statistical software package (Statgraphics Centurion XVI).

Anatomy and ultrastructure

Samples for histological studies were collected after 24h, 48h and 72h from treatment with nanocapsules. Fresh samples were sectioned by Cryo-Cut Microtome (-20°C) to obtain longitudinal and transverse 20-30µm thick sections. To investigate the presence of lignin

nanocapsules in seeds or roots, a saturated solution of phloroglucinol in water with HCl at 20% (v/v) was used to stain the sections.

Samples were fixed in FAA (5:5:90 v/v/v 40% formaldehyde: glacial acetic acid: 70% ethanol) at 5°C[40] and then dehydrated in an ethanol series (70%, 80%, 95% 100% v/v). These samples were then embedded in Technovit 7100 resin and sectioned with a Reichert-Jung Ultracut E microtome to obtain 2.5 µm sections. The sections were mounted on microscope slides and stained in toluidine blue O 0.5% w/v in distilled water with the addition of sodium carbonate to give a pH of 11.1[41] A Leitz DMRB light and fluorescence microscope and a stereomicroscope equipped with a digital camera were used for microscopic examinations.

RESULTS AND DISCUSSION

Structural and morphological characterization of Lignin NCs by DLS and SEM

DLS measurements were performed twice as shown in figure 2. DLS data indicated that NGA0 had a mean size of about 300nm, while NCs loaded with GA3 at the three different concentrations showed an average diameter of about 200-250nm. NGA1 showed the smallest mean size of about 175nm in comparison to NGA0.5 and NGA1.5 of about 240nm and 220nm respectively. More structural details from DLS measurements are reported in Table S1.

The PolyDispersity Index (PDI) of all samples was in the range 0.17-0.38. In particular, NGA0 presented a PDI of about 0.4 indicating that this sample had a broad size distribution (Figure 2A), while the loaded NCs showed a PDI value of about 0.2, suggesting narrower size distribution, as shown in figure 2 (B, C, D). This behaviour suggested that GA3 affected NCs, inducing the formation of a more tightly packed structure in comparison to the empty carriers.

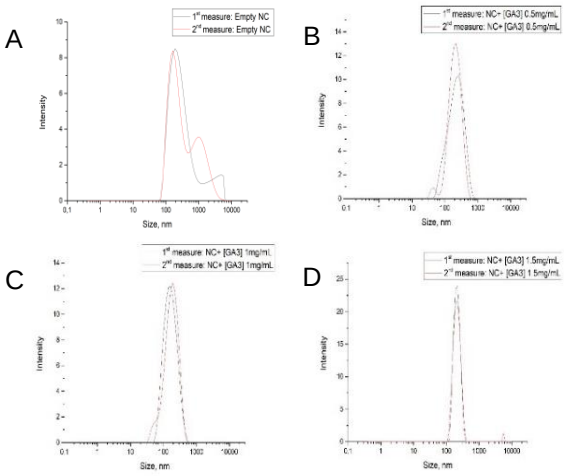

Figure 2: Size distributions of NGA0, NGA0.5, NGA1 and NGA1.5 (A B, C, D) obtained from intensity weighted DLS results.

Figure 3 revealed that both empty and loaded NCs had a spherical shape with a homogeneous surface. The average size of NCs was similar to the mean size obtained by DLS. This indicated that lignin nanocapsules preserved their shape in spite of the dehydration needed for SEM analysis, confirming the stability of these polymeric nanoparticles. A small fraction of NCs had an average diameter of 500-800nm, i.e a size range outside the optimal values for DLS measurements, since it can be affected by small dust particles and/or agglomerates that may be present in samples prepared from natural components. On the other hand, filtering is not a valid option, due to the possibility of modifying the size distribution by particles adsorption and retention in the filter pore.

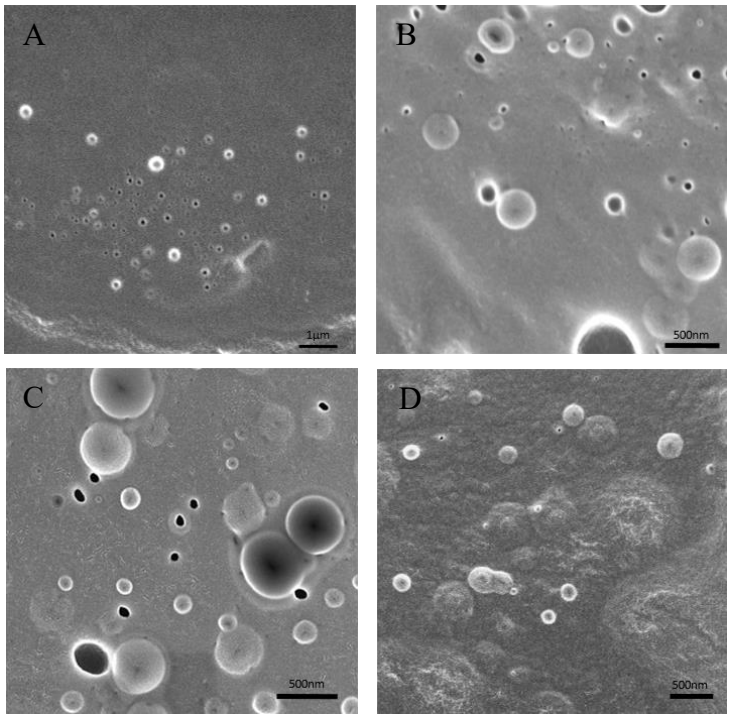

Figure 3: SEM micrograph of (A) NGA0 (MAG 11kx), (B) NGA0.5 (MAG 31.6kx), (C) NGA1 (MAG 36.2kx) and (D) NGA1.5 (MAG 23kx).

Finally, in Figure S2 shows the SANS diagrams of empty and GA3-loaded NCs. Theses curves presented a low q plateau, clearly due to nanostructured objects in solution whose diameter was below ~250 nm for loaded NCs and ~200 nm for plain NCs. These data confirm the presence of an abundant fraction of small particles.

Germination experiments

In vivo experiments

Effects of nanoparticles loaded with GA3 on arugula seeds germination are shown in Figure S3 and Table 1. At the first day, the percentage of germinated seeds treated with only GA3 was slightly higher than this with NGA (Figure S3). After 3 days, GA0.5 showed the greatest

percentage (97%). At the same time, NGA1.5 (93%) display no statistically significant differences with GA1.0 (91%), while it has a higher percentage compared to GA1.5 with 86%, to NGA0 with 78% and to Control with 76%. The latter didn't show a growth increase during the experiments.

However, the percentage of germinated seeds treated with NCs loaded and not-loaded with GA3 resulted greater than the Control (Figure S3 and Table 1).

We further investigated the effects of NCs on the growth and development of seedlings (Table 1). A significant increase in stem and root length occurred in the treatment of NGA1.0 (respectively 2.57 and 2.52cm) with a total length statistically significant compared to the other treatments (5.09cm). GA3 and nanocapsules treatments showed a seedlings total length greater than water. Furthermore, arugula seedlings, grown on NGA0, NGA0.5, NGA1.0 and NGA1.5, exhibited an increase in vegetative biomass, from 28% to 50%, respect to the Control (Table 1; Fresh Weight). Moreover, plantlets, grown on NGA0.5, NGA1 and NGA1.5, exhibited a fresh and dry weight higher compared to GA3 and Control (water). In Figure S4 qualitative seedlings traits of arugula are reported.

Table 1: The effect of Lignin NCs on growth and development of arugula seedlings at the end of the experiment: germination percentage (G), stem, root and total length, fresh weight (FW) and dried weight (DW). The value presented is the mean ± SD and different letters in each column, are significantly different at 5% probability level- using LSD Multiple Range Test (n=30).

Treatments	G(%)	Stem length (cm)	Root length (cm)	Total length (cm)	FW (mg)	DW (mg)
Control	76±1.4e	1.82±0.4c	1.56±0.7d	3.38±1.0c	29±0.02d	2.7±0.004d
NGA0	78±1.6e	2.15±0.5b	2.29±0.9ab	4.44±1.4ab	33±0.03bcd	3.5±0.004b

NGA0.5	90±1.4c	2.17±0.4b	1.89±0.8bcd	4.06±0.9bc	43±0.06a	4.2±0.005a
NGA1.0	88±1.4d	2.57±0.5a	2.52±0.6a	5.09±0.8a	44±0.04a	4.5±0.001a
NGA1.5	93±1.5b	2.19±0.4b	1.63±0.7cd	3.82±1.0bc	37±0.03ab	3.1±0.002c
GA0.5	97±1.3a	2.22±0.4b	2.14±0.7abc	4.36±1.0b	36±0.01bc	2.6±0.004d
GA1.0	91±1.1c	2.20±0.5b	2.21±0.8ab	4.43±1.2ab	31±0.01bcd	2.6±0.003d
GA1.5	86±1.4d	2.16±0.4b	1.80±0.8bcd	3.96±1.2bc	31±0.04cd	2.5±0.002d

Effects of nanoparticles loaded with GA3 on tomato seeds germination are shown in Figure S3 and Table 2. Figure S5 shows the trend of the germination percentage for 6 days after soaking in the treatment solutions. At the second day after soaking, Control and NAG1.0 percentage of seeds germinated were higher than the other treatments (respectively 20% and 11%) and, at the 3rd day, an evident increase in percentage was observed in all treatments (Figure S5). Statistically significant difference in germination percentage was observed at the end of the experiment: values varied from the 95% G of the NGA1.5 to 89% G of NGA0.5 and GA0.5. No differences were recorded among Control, NGA0 and GA1. (92%; 93% and 93% respectively, Table 3).

The total length of seedlings of tomato, ranged from 10.3 (Control) to 7.41cm (NGA1.5), among GA3 treatments, the seedlings grown on GA1.5 were the highest (9.06cm, Table 2). The seedlings treated with NCs presented values of the stem and roots length, comparable to the plants grown on gibberellic acid alone and no positive effect was observed for these parameters.

Table 2: The effect of Lignin NCs on growth and development of tomato seedlings at the end of the experiment: germination percentage (G), stem, root and total length, fresh weight (FW) and dried weight (DW). The value

presented is the mean ± SD and different letters in each column, are significantly different at 5% probability level- using LSD Multiple Range Test (n=30).

Treatments	G (%)	Stem length (cm)	Root length (cm)	Total length (cm)	FW (mg)	DW (mg)
Control	92.00±1.5b	4.20±0.9ab	6.05±1.4a	10.3±2.0a	75±0.04bc	3.6±0.02
NGA0	93.00±1.5b	4.06±0.9b	3.92±1.3cd	7.99±1.5cd	46±0.06d	3.3±0.03
NGA0.5	89.00±1.4c	4.25±1.4ab	4.63±1.1b	8.88±1.5bc	67±0.03c	3.9±0.01
NGA1.0	90.00±1.6c	4.44±1.4ab	4.49±1.3bc	8.93±1.2b	70±0.02bc	5.1±0.03
NGA1.5	95.00±1.7a	4.07±1.0b	3.34±1.2d	7.41±1.4d	92±0.03a	3.9±0.03
GA0.5	89.00±1.7c	4.21±1.3b	4.02±1.4bc	8.23±1.5bcd	63±0.02c	3.3±0.02
GA1.0	93.00±1.8b	4.46±1.1ab	4.47±1.3bc	8.93±1.6b	83±0.04b	3.5±0.03
GA1.5	89.00±1.4c	4.69±0.9a	4.37±1.1bc	9.06±1.1b	88±0.02a	3.9±0.04

Regarding vegetative biomass (FW, Table 2), NGA1.5 and GA1.5 exhibited the highest weight, respectively with 23% and 18% more than the control (Table 2). No statistically significant difference was observed in the dry weight. In Figure S6 qualitative seedlings traits of tomato are reported.

In vitro experiments

All arugula seeds started germination with the radicle protrusion, after 2 days the beginning of the experiment (Figure S7A).

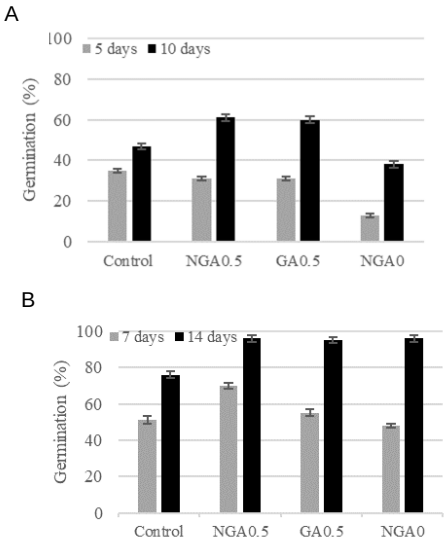

Figure 4: Effect of Lignin NCs loaded and no-loaded with GA3 on *in vitro* seed germination percentage in arugula(A) and tomato (B). Data are presented as means (±SD) of 2 replicates containing 30 seeds.

After 5 days Control arugula seeds without treatment had higher germination (Figure 4A), while it decreased in the presence of nanovectors no-loaded (NGA 0.13%). NGA0.5 and GA0.5 treatments showed the same germination percentage (31%) without significant differences (Figure S7B). At 10 days of culture, the germination in NGA0.5 and GA0.5 (61 and 60% respectively) increased, exceeding the control of approx. 23 %.

As shown in Figure 4B, tomato seedlings, at 7th day NGA0.5, gave a good germination percentage (70%) respect to control and GA0.5 treatments (51% and 55% respectively). NGA0 treatment showed a low germination rate, however, at the end of the experiment (14 days) its germination (95%) was comparable to GA encapsulated and not encapsulated in NCs (96%). These last-mentioned treatments were better than control, which had 76% of germinated seeds at the end of the experiment.

In the second experiment, the effects of the treatments on germination percentage are reported in arugula (Figure 5) and tomato (Figure 6). The application of NGA0.5 confirmed the effectiveness of this treatment (Figure S8A) with 45% of seeds germination after 5 days and with 76% after 10 days in arugula (Figure 5). Also treatment with NGA1.0 after 10 days showed well-development seedlings (S8B). Among treatments with GA3, the GA0.5 concentration gave better results in developed seedling after 5 and 10 days (36 and 70% respectively). Seeds placed on Control medium showed intermediate germination values with 40% at the first recording and 60% at the second one.

In tomato the presence of the NCs, promoted the initial stage of germination (Figure S9A-B). After 7 days (Figure 6), a similar percentage of germinated seeds are recorded, in the presence of NGA0.5 and GA1.0 (67.9 and 68.2 % respectively), taking into account that the first treatment had half gibberellic acid concentration. Applying the same concentration, 0.5mg of GA3, the treatment with NCs showed higher seed germinated (Figure S9C). While the application of NGA1.5 had the lowest germination (40%).

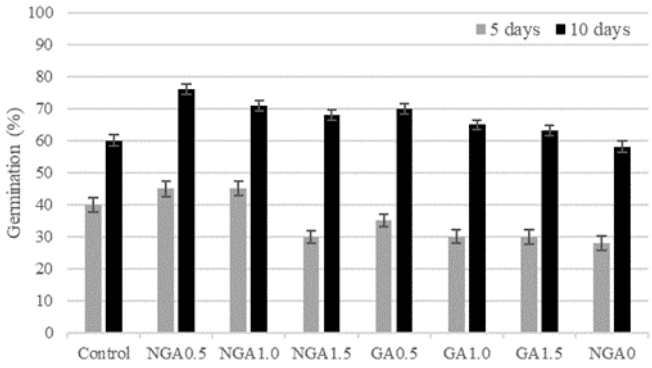

Figure 5: Effect of Lignin NCs loaded and no-loaded with different GA3 concentrations on *in vitro* seed germination percentage in arugula. Data are presented as means (±SD) of 2 replicates containing 30 seeds.

At the end of the experiment (14 days), there were no marked differences between seeds that were placed on Control, GA1.0, GA1.5, and NGA0 treatments, with about 90% of germination, while was evident a slowing in seeds germination on the presence of NCs loaded with GA3.

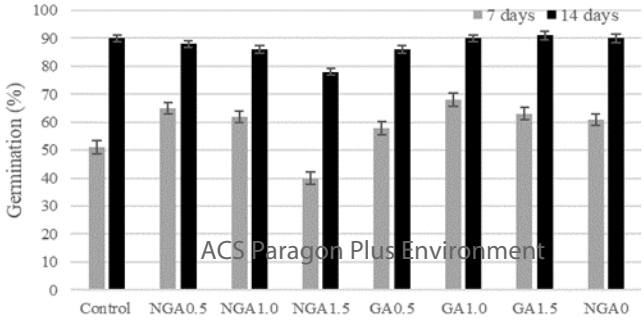

Figure 6: Effect of Lignin NCs loaded and no-loaded with different GA3 concentrations on *in vitro* seed germination percentage in tomato. Data are presented as means (±SD) of 2 replicates containing 30 seeds.

Tomato seedling with developed cotyledons and root system were recognized (Figure S9F and S9G) as fully germinated and growth parameters were evaluated (Table 3). The total length seedling (stem length plus primary root length) developed on different treatments was in the range 8.6-9.9cm. The higher value of seedling length was recorded in presence of NGA0.5, where the stem length was 4.9cm similar at GA1.0 and GA1.5 treatments, but with the principal root length significantly longer (5.0cm) respect to ones.

Table 3: The effect of Lignin NCs on parameters of tomato seedling growth after 14 days of *in vitro* germination. The value presented is the mean ± SD and different letters in each column, are significantly different at 5% probability level-using LSD Multiple Range Test (n=30).

Treatments	Stem length (cm)	Primary root (cm)	Secondary roots (n)	Seedling length(cm)	FW (mg)	DW(mg)
Control	3.6±0.1d	5.0±0.3a	3.3±0.4d	8.6±0.4d	23.2±0.2e	1.3±0.08
NG0	4.4±0.1b	4.9±0.1b	3.9±0.3cd	9.3±0.3b	29.1±0.1c	1.0±0.07
NGA0.5	4.9±0.1ab	5.0±0.2a	4.8±0.2a	9.9±0.4a	39.5±0.4a	1.9±0.09
NGA1.0	4.0±0.08c	5.3±0.3a	3.0±0.3d	9.3±0.3b	25.6±0.2de	1.8±0.07
NGA1.5	4.0±0.07c	5.4±0.2b	2.4±0.2e	9.4±0.2b	23.4±0.1e	1.0±0.06
GA0.5	4.5±0.09b	4.8±0.2b	4.4±0.6b	9.3±0.2b	36.1±0.5b	1.3±0.09
GA1.0	5.0±0.2a	4.8±0.1b	3.9±0.4c	9.8±0.2a	24.8±0.2e	1.8±0.08
GA1.5	5.0±0.1a	3.9±0.1c	4.1±0.3bc	8.9±0.3c	28.2±0.1cd	1.3±0.06

The major lengths of primary root were recorded in NCs not-loaded, NCs loaded with GA3 and Control, while the effects of GA3 concentrations were evident in the stem lengths (4.5- 5.0cm). The bioactive gibberellins (GAs), indeed, are plant hormones that promote stem elongation and leaf growth. In some species, GAs also induce seed germination and modulate flowering time and the development of flowers, fruits, and seeds[42] .

The GA0.5 concentration loaded and not-loaded influenced and promoted the development of secondary roots.

The efficacy of NGA0.5 was appreciable in most of the parameters examined and it was similar as results obtained with GA1.0, whereas in this case, the concentration of GA3 was double.

All treatments applied to tomato exhibited an increase in FW of total biomass respect to the Control; the higher biomass production was in presence of NGA0.5 (39.5mg). DW was not affected significantly by the different treatments applied on the seeds, though the treatment NGA0.5 and NGA1.0 had higher values for this parameter.

Anatomy and ultrastructure

In order to investigate NCs permeation and accumulation in living plant tissues, a first study was conducted in tomato seeds after 72h of treatment with NGA0.5. Sections were stained with phloroglucinol, to reveal lignin. Considering that lignin is scarcely present in the endosperm of seeds, it was possible to highlight only the polymer matrix of NCs. In particular, Figure 7A shows light red spherical objects (indicated by arrows) in the endosperm in comparison with the Control (Figure 7B). This represented the first proof that NCs may penetrate the external tegument of the seeds, reaching the internal tissues.

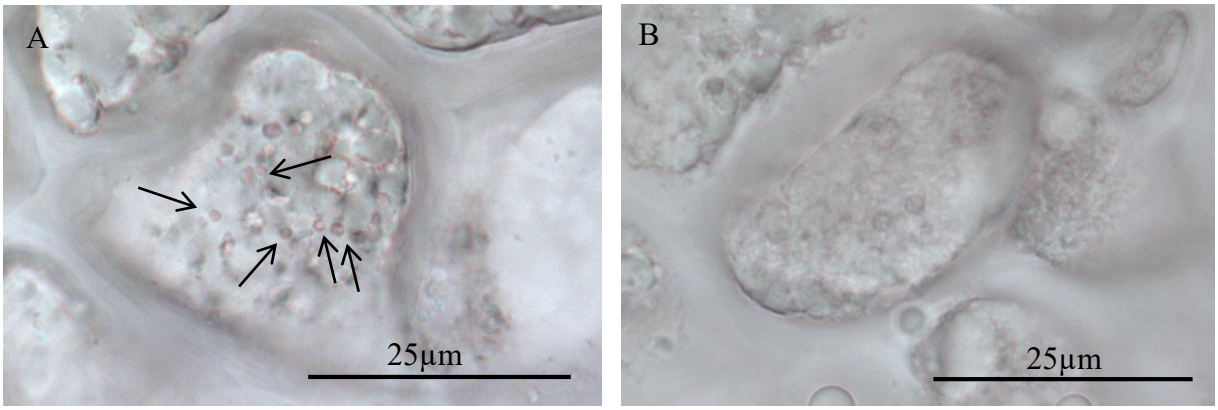

Figure 7: Endosperm of tomato seeds after a 72 h treatment with (A) NGA0.5 (arrows) and (B) Control.

A more consistent study to validate the NCs entrance and pathways into the vascular tissues was done using a fluorochrome (FY088) loaded into the vesicles. Figure S10 shows that the fluorochrome was successfully loaded in the NCs within the inner core and stabilized by the lignin matrix.

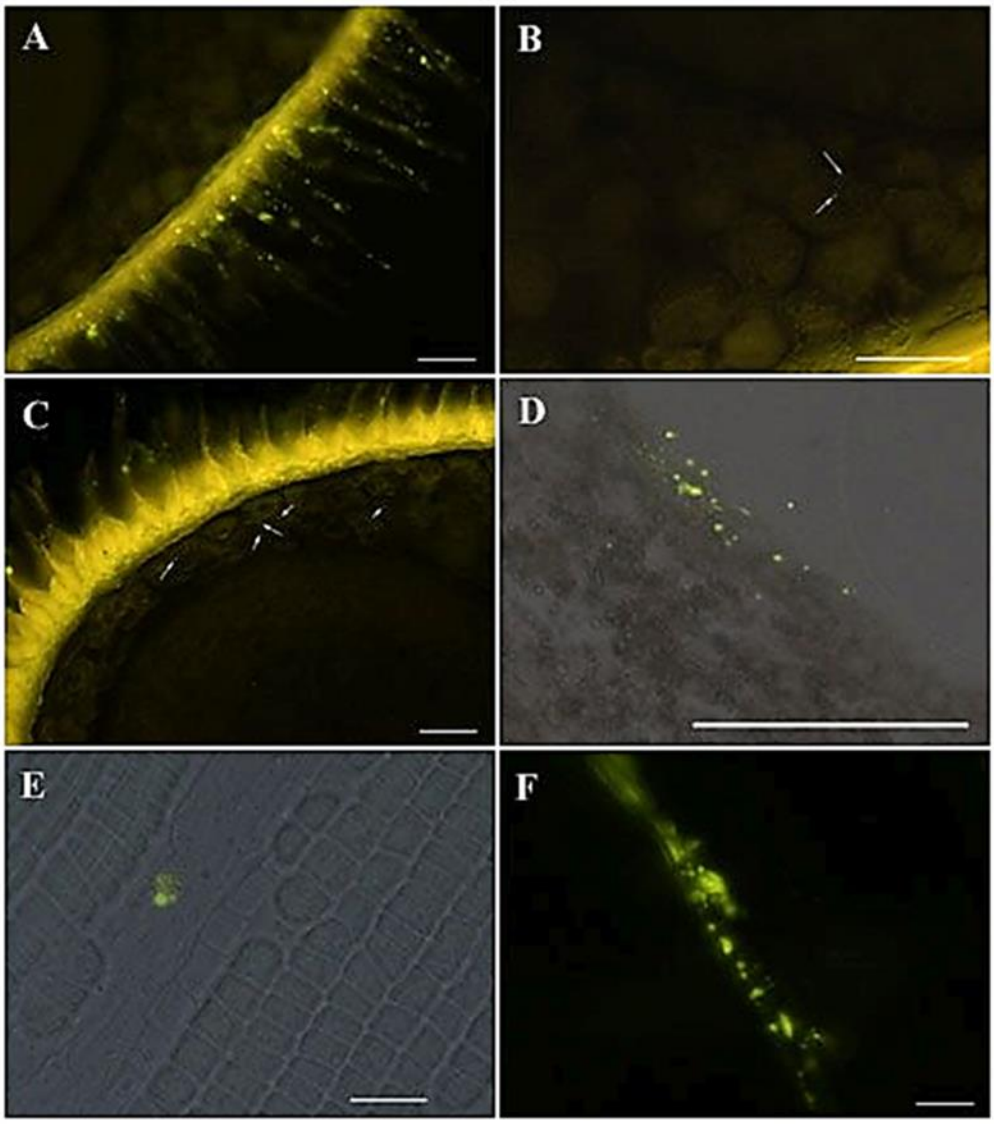

Figure 8: Seed coat (A) and endosperm (B) of tomato seeds after a 24h treatment in NCs loaded with FY088; Seed coat (C) and root (D) of tomato after a 48h treatment in NCs loaded with FY088; Xilem vessels (E, F) tomato root after a 72h treatment in NCs loaded with FY088. Scale bar: A, B, C, F =50µm; D=100µm; E= 200µm.

NCs loaded with the fluorochrome were used to treat tomato seeds, following the same procedure used for *in vivo* experiments. After 24h, seeds were sectioned to assess the NCs

permeation. Figure 8A and B show the seed coat and endosperm, respectively. The intense yellow colour corresponded to the accumulation of NCs on the seed coat and hairs. This suggested that NCs were able to cross the innermost part of the seed coat.

After 48h, NCs were still present in the seed coat (Figure 8C) and had permeated the epidermis of the root which had just germinated (Figure 8D). In particular, in roots, NCs had crossed the external layer and were found in the cortex layers underneath the rizodhermis, suggesting that it is easier for NCs to penetrate the root than the seed coat.

As shown in figures 8E and F, after 72h NCs were able to enter the root tissues, and, through the cortical cells and the endodermis, to reach the xylem vessels of the vascular system.

CONCLUSIONS

Polymers are efficient matrices for the entrapment, transport and controlled release of bioactive compounds. In plants, natural polymers constitute promising sustainable options due to their biocompatible and biodegradable nature. These materials are also by-products and can be reintroduced in the production cycle following the criteria of circular economy.

Here we proposed the formulation of lignin-based nanocapsules with size 200-500nm, which are stable and easily reproducible at different scales. This could open a range of possibilities in agriculture. Lignin nanocapsules had no adverse effects *in vivo* and *in vitro*, on arugula and tomato, at lignin concentration up to 1% w/v. Indeed, all the seeds treated with nanocapsules germinated and the seedlings showed tolerance without symptoms of toxicity. Anatomy studies with fluorochrome loaded NCs evidenced the capacity of lignin nanoparticles to penetrate the tissues of seedlings. . To explaine the above findings, we hypothesize that the presence of many hydrophilic groups in the lignin chemical structure, which are localized on the external surface of

nanocapsules in aqueous environment, could positively affect the processes related to plant germination and growth by increasing water availability, as reported for other engineered nanoparticles[36]. Finally, the lignin NCs prepared in this study proved to be able to pass the seed tegument and penetrate the rizhodermis of the seedling eventually reaching the vascular system.

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SUPPORTING INFORMATION

Average diameter of nanocapsules and their PolyDispersity Index; SANS diagrams of empty Nanocapsules and Nanocapsules loaded with GA 3mg/mL; Germination percentage of E. vesicaria seeds in vivo; Seedlings of 3-day-old arugula growing on GA3 and Lignin NCs loaded with GA3; Germination percentage of *S. lycopersicum* seeds in vivo; Seedlings of 6-day-old tomato growing on GA3 and Lignin NCs loaded with GA3; Radicle protrusion of *Eruca vesicaria*, after 2 days from the beginning of experiment and development of seedlings treated with NGA0.5 and GA0.5 after 5 days in the first experiment; Development in *E. vesicaria* seedlings treated with Control and NGA0.5 after 10 days in the second experiment and in *E. vesicaria* seedling treated with NGA1.0 after 10 days of culture; In vitro seed germination *of S. lycopersicum*; Lignin nanocapsules loaded with FY088.

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Lignin based nanocapsules are sustainable carriers for agrochemicals delivery which can harmlessly enter germinating plantlets.

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