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Biosynthesized silver nanoparticles to control fungal infections in indoor environments

Cecilia Deyá and Natalia Bellotti

Centro de Investigación y Desarrollo en Tecnología de Pinturas (CIDEPINT), CIC-CONICET Av. 52 e/121 y 122, B1900AYB, La Plata, Argentina

E-mail: n.bellotti@cidepint.gov.ar

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Abstract

Fungi grow especially in dark and moist areas, deteriorating the indoor environment and causing infections that particularly affect immunosuppressed individuals. Antimicrobial coatings have as principal objective to prevent biofilm formation and infections by incorporation of bioactive additives. In this sense, metallic nanoparticles, such as silver, have proven to be active against different microorganisms specially bacteria. Biosynthesized method is a promising environmentally friendly option to obtain nanoparticles. The aim of this research was assess the employment of plants extracts of *Aloysia triphylla* (cedrón), *Laurelia sempervirens* (laurel) and *Ruta chalepensis* (ruda) to obtain silver nanoparticles to be used as an antimicrobial additive to a waterborne coating formulation. The products obtained were assessed against fungal isolates from biodeteriorated indoor coatings. The fungi were identified by conventional and molecular techniques as *Chaetomium globosum* and *Alternaria alternate*. The results revealed that the coating with silver nanoparticles obtained with *L. sempervirens* extract at 60 °C with a size of 9.8 nm was the most efficient against fungal biofilm development.

Keywords: nanoparticles, antifungal additives, coatings, plant extracts, fungal infections
Classification numbers: 2.04, 4.02, 4.04, 5.18

1. Introduction

Moulds and other biological agents constitute the major sources of indoor air pollution and buildings materials deterioration [1, 2]. Clinical evidence increasingly indicates that highly polluted indoor environment, especially with fungi, increases the risks of health problems, such as allergies, infections and chronic diseases [3, 4]. Besides due to its apical growth that breaks the substrate where they are growing, and the release of great amount of degrading acids and enzymes these microorganisms are considered the most deteriorating to the coatings [5–7]. One of the possible strategies to prevent biodeterioration in indoor environment is the development of antimicrobial coatings [8]. These could be formulated with additives that prevent development of biofilms on surfaces [9, 10].

Consequently, the development of new antifungal additives, more efficient and environmentally friendly, is indispensable for the development of new protective coatings formulations [8, 11, 12]. Among new research lines, the synthesis of metallic nanoparticles is one of the more promissory ones, for the wide application field such as in cloths, detergents, cosmetics, pharmaceutical products and coatings [13–15]. Nanoparticles (NPs) are characterized for having properties depending on their size and shape that are depending on the synthesis methods and conditions [16]. The most frequent methodology for nanoparticles synthesis involved reducing agents such as sodium borohidrure, hydrogen, citrates and solvents

such as tetrahidrofurane and N,N-dimethylformamide [17]. Moreover, stabilizing agents like surfactants must be used to control the size of the particles in the nanorange [18]. The use of aqueous plant extracts in the synthesis of nanoparticles results in an efficient option, low cost and environmentally friendly, rules that are enclosed in the 'green chemistry' principles [17]. This synthesis method offers several possibilities taking into account the great diversity of plans with a wide group of metabolites that includes reducing and stabilizing agents.

In related to the exposition, the present work had as objective the use of aqueous vegetables extracts of *Aloysia triphylla* (cedrón), *Laurelia sempervirens* (laurel) and *Ruta chalepensis* (ruda) for the synthesis of AgNP. The plants were selected taking into account the abundance in la pampa region and references [19]. After the synthesis, the characterization of the NPs was done and the bioactivity evaluated against fungal isolated of the genera *Chaetomium* and *Alternaria*, obtained previously from acrylic indoors biodeteriorated coatings [14]. Molecular analysis of the isolated strains was done to support their identity.

Fungi were selected for their ability to grow on coatings and their negative impact on human health [7, 20]. In this sense *Chaetomium* and *Alternaria* are able to produce a variety of different compounds including mycotoxins, which are toxic to mammals, and enzymes that degrade the cellulosic thickeners employed in waterborne paints formulations [21]. This results in a biodeterioration process and in the decline of indoor environmental quality which affect especially to the urban dwellers [2, 3].

The synthesized NPs were characterized by UV–Vis spectroscopy, transmission electron microscopy (TEM), dynamic light spectroscopy (DLS) and Fourier transformed infrared (FTIR) spectroscopy. The NPs obtained with the laurel extract were selected to evaluate the antifungal activity due to that presented lower size and stability along time. In the following step, acrylic coatings were formulated and prepared employing the synthesized NPs. For last, the resistance to fungal growth on coatings was assessed by plate assay following a procedure similar to the established in ASTM D5590 standard [11, 22].

2. Materials and methods

2.1. Nanoparticles synthesis

The AgNP synthesis was made with three different plant extracts: *A. triphylla* (cedrón), *L. sempervirens* (laurel) and *R. chalepensis* (ruda), once obtained were labeled as NPA, NPL and NPR, respectively. The leaves were recollected, washed three times with distilled water (DW) and dried at 45 °C. The extracts were made by a simple liquid–solid extraction method, 20 g of the dried plant leaves were cut and put in contact with 200 ml of DW at 80 °C for 3 min. Then, were filtered to obtain the extracts and kept in caramel flasks, at 4 °C, until used [23]. The NP synthesis was done mixing 20 ml of the extract with 180 ml of $AgNO_3$ solution at regulated temperature, under constant stirring. The concentration of $AgNO_3$ solution was 10^{-3} M [14, 24]. The temperatures were 26 or 60 °C. The pH of the finals suspensions were regulated to 8 with NH_4OH 50%v/v [23]. The particles in suspension were kept in the fridge at 4 °C. A control setup was also maintained without leaf extract.

2.2. Nanoparticles characterization

UV–Vis spectroscopy was proven to be useful to analyze NPs. The method is based in that the reduction of the metallic ions in nanosize produce, in the case of silver, a yellow solution with an intense band at 400–450 nm, attributed to the collective excitation of the surface electrons (superficial plasmonic absortion) [25]. Spectra were obtained in range of 350–600 nm of samples treated in 1, 45 and 102 d after synthesis in order to know the stability of the particles. As blank a solution of the extract, with the same dilution, was used. The measurements were performed in duplicate in a spectrophotometer (SP 2000 UV).

Dynamic light scattering (DLS) technique was used to confirm particle size distribution. Size distribution measurements were made in triplicate with a Malvern Zetasizer Nano ZS Instruments operating with a He–Ne laser at a wavelength of 633 nm and a detection angle of 90°; all samples were analyzed for 60 s at 25 °C. To confirm shape, samples were diluted with DW and 50 μl of each suspension was placed on a formvar-coated copper grid for TEM. The corresponding equipment was a JEOL JEM-1230 at an accelerating voltage of 100 kV and x-ray diffraction (XRD) using GBC MMA SPELLMAN.

FTIR spectrum of the dried AgNPs was obtained using a Perkin-Elmer Spectrum One Spectrometer.

2.3. Molecular analysis and identification of fungi

Two filamentous fungi were isolated from biodeteriorated indoor coatings in La Plata city (34°54′S and 57°55′W) by classic microbiological techniques as part of a previous work [14]. These fungi were identified based on their micro and macro-morphological characteristics, using standard taxonomic keys and selected considering their characteristics according to bibliographic data [14, 26]. Identification was confirmed by direct sequencing of fungal internal transcribed spacer (ITS) regions using polymerase chain reaction (PCR) (Macrogen, Korea) and the data analysis [27]. The primers used were ITS1 (5′-TCC GAT GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′). The sequences obtained were analyzed by Blast searches using GenBank nucleotide database (www.ncbi.nlm.nih.gov/blast/Blast.cgi) to identify the isolate or reveal the closest known analogue.

2.4. Antifungal activity

AgNPs antifungal activity was evaluated by the agar diffusion method by the used of cylinders that are disposed on plates with rose bengal (RB) based culture medium (5 g protease peptone, 1.0 g glucose, 0.1 g KH_2PO_4, 0.05 g $MgSO_4{\cdot}7H_2O$, 1.5 g agar and DW up to 100 ml) without RB [28, 29]. The spore

suspension was prepared from *C. globosum* and *A. alternate* cultures with 15–30 d of incubation at 28 °C. The inoculums concentration was adjusted to 10^5 spores ml^{-1} employing a neubauer chamber. Plates with 15 ml of RB inoculated with 200 μl of spore suspension were prepared. Three glass cylinders of 6 mm inner diameter were arranged on inoculated RB. Then, 20 μl of the each sample were introduced in the respectively cylinders. The test was performed with AgNPs suspension obtained and the respective controls ($AgNO_3$ 10^{-3} M solution and the leaf extract). Finally, plates were incubated for 48 h at 28 ± 1 °C. Then, zone of inhibition were assess in every case, considering diameters <6 mm without antifungal activity (−), and ⩾6 mm with antifungal activity (+). The procedure was performed for triplicate for each sample and repeated twice.

This assay was carried out only with particles with an absorption band between 400 and 450 nm (nanosize) and stable along time.

2.5. Formulation and preparation of paints

The selected NPs were used to prepare an acrylic water-based paint according to the composition: 5.3% TiO_2, 17.3% $CaCO_3$ (natural), 1.4% $CaCO_3$ (precipitated), 8.1% acrylic resin (1:1), 4.7% additives and 63.2% of DW (% by volume). This formulation corresponds to the control paint and results compatible with a ceiling paint due its high pigment volume concentration (PVC), 85%.

The paints were prepared in a high speed disperser and the NPs were incorporated, from the aqueous suspension in replacement of part or all the DW. This represents an important advantage because the propose NPs synthesis and later paint preparation do not present waste products, responding to the 'green chemistry' principles [30]. The paints were formulated with two concentrations of NPs 3.6 and 5.4 mg in 100 g of paint (NPL1 and NPL2, respectively). The concentrations of the NPs in the paint were chosen according to [14]. As additives, antifoaming, cellulosic thickener, dispersants and surfactants were used (Tyosil Q202, Diransa San Luis; Cellosize 52 000, Dow; sodium hexametaphosphate, triton X-100, Merck; respectively). Besides control paints (C) prepared with the same formulation but without any biocide, another control was made but replacing AgNPs suspension by the leaf extract with the same concentration.

2.6. Coatings bio-resistance test

The obtained paints were applied on glasses with a brush and let cure for 15 d under laboratory conditions. The glasses were cut in pieces 2.5 × 2.5 cm^2 and irradiated with a germicide UV-lamp (Philips, 20 W) for 40 min on each side. Afterwards, three glasses, of each paint sample were place in plates with minimum mineral medium (MM). The MM-agar was prepared dissolving 5 g NaCl, 1 g HK_2PO_4, 1 g $(NH_4)H_2PO_4$, 1 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4$, 3 g KNO_3 and 20 g of agar in DW (up to 1000 ml) [11]. This medium was selected for their poor growing conditions which force the mold to grow on coatings, employing these as the carbon source. The coatings samples were inoculated with 50 μl of the spore suspension (10^5 spores ml^{-1}) of the respectively mold, *C. globosum* and *A. alternata* and incubated at 28 ± 1 °C for 1 month. At the end of the test, the fungal growth was evaluated according to ASTM D5590 standard specification. Consequently, observed growth on specimens are referred to percentage (%) of area covered and has been rating as 0 (none, 0%), 1 (trace of growth, <10%), 2 (light growth, 10–30%), 3 (moderate growth, 30–60%), and 4 (heavy growth, 60–100%).

Table 1. Relationship between visual assessment and ΔE differences G [31].

ΔE	Assessment of colour differences
<0.2	No visible
0.2–0.5	Very slightly
0.5–1.5	Slightly
1.5–3.0	Evident
3.0–6.0	Very evident
6.0–12.0	Big
>12.0	Very big

2.7. Observation by scanning electron microscope (SEM)

Coatings exposed to mold for 1 month by bio-resistance test were observed by SEM. The selected ones were those presented better results and the respective controls. In order to proceed with the observation, the samples were removed from the plates with tweezers. After that, the remains of culture medium were eliminated from the bottom and the samples were cut into squares approximately of 10 mm side. The microscope was a Philips FEI Quanta 200, and the working conditions were low vacuum (10^{-2}–1 torr) due to this the samples not require being dehydrated allowing keep more natural morphology of the fungal biofilm.

2.8. Gloss/color measurements of the coatings

CIElab color parameters were evaluated on experimental coatings and compared with the control coatings without NPs. The CIElab diagram represents the area where all the real colors are placed, the vertical axis L represent the luminosity of the color which varies from 0 to 100 (white). The other two orthogonal axis, a and b, form the chromaticity plane, according to opponents color criteria, the variation magenta–blue green (a) and yellow–blue (b). The change of color (ΔE), was calculated as [14, 31]:

$$\Delta E = \sqrt{(L - L_{NP})^2 + (a - a_{NP})^2 + (b - b_{NP})^2}. \quad (1)$$

Being L, a and b the CIElab parameters of the control panels and L_{NP}, a_{NP} and b_{NP}, the values corresponding to the tested panels. The assessment of color change was carried out using the rating in the table 1 which relates visual appearance and ΔE [31]. The measurements were done employing a By K Gardner gloss-meter.

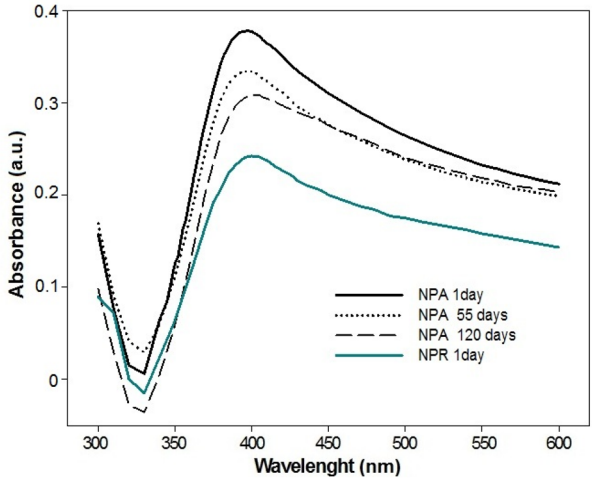

Figure 1. UV–Vis spectra of the silver NPA and NPR, obtained at 60 °C with *A. triphylla* and *R. chalepensis*, respectively.

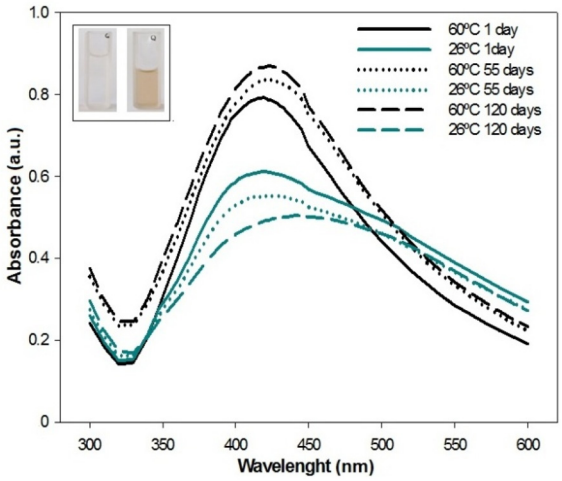

Figure 2. UV–Vis spectra of silver NPL obtained at 60 °C and 26 °C with *L. sempervirens* leaf extract. Inset: image detail aqueous dilutions of leaf extract control and Ag NPL (60 °C) after 1 d of synthesized.

3. Results and discussion

3.1. Characterization of the nanoparticles

Absorption UV–Vis spectra of the NPs suspensions obtained showed how, according to the extract employed to the synthesis, the spectroscopic properties of the particles varied. In figure 1, the spectra of the silver NPA and NPR, prepared at 60 °C with *A. triphylla* and *R. chalepensis* leaf extract, are shown. Both present wide bands with a maximum absorbance at ~400 nm. The wideness of the bands indicates a wide size distribution. NPA tended to agglomerate resulting in decreased absorbance at longer times. The spectra of the NPA obtained at 26 °C were similar to the one obtained at 60 °C (figure 1). The negative absorbance at ~325 nm may indicate that the agglomeration of the NP also includes some extract.

In the case of the silver NPR the spectra after 1 d is similar to the ones obtained with NPA, but, as time passes particles agglomerated and precipitated and therefore the spectra could not be obtained (figure 1). In this case, due to the results, NP was not obtained at 26 °C. According with these results, the NPA and NPR were unstable along time.

In the case of the NPL obtained with *L. sempervirens* leaf extract at 60 °C, the spectra showed that the absorption band, between 400 and 450 nm, was narrower compared with the others and its intensity did not decrease along time, indicating that these NP have smaller size distribution and more stability (figure 2) [25]. On the contrary, when the working temperature was 26 °C, the results were similar to the ones obtained with NPA (figure 1) but the intensity was higher, indicating a major amount of NP.

Taking into account these results, only the NPL obtained at 60 °C were further characterized and tested against mold and incorporated in the paint.

Figure 3(a) shows the size distribution of NPL determined by DLS with an average size of 9.8 nm. In figure 3(b), a TEM image of the same NP is shown. These results confirmed the one obtained by UV–Vis spectroscopy. The image obtained by TEM also showed the quasi-spherical shape of the silver NPL. Besides, it can be seen, in the figure 3(c), that an organic based material is around the particles possibly part of leaf extract.

The silver NPL FTIR spectrum was examined and it can be seen in the figure 4. The band in the 3600–3000 cm^{-1} region would be associated with OH groups present in aromatic or aliphatic alcohols. The peak appeared at ~2930 cm^{-1} corresponds to the stretching of N–C in nitrogenous compounds likes alkaloids [32, 33]. The peaks in 1640, 1550, 1400 cm^{-1}, once again would be pointing the presence of nitrogenous compounds specifically amide groups. The important peak at 1035 cm^{-1} would be related to ether linkages [33]. This analysis provides an idea about how the biomolecules bearing different functionalities are present together with the NP synthesized. Besides, as it was indicated above, the presence of organic compounds would help to stabilized AgNP [17].

3.2. Molecular analysis and identification of fungi

The ITS regions of the ribosomal DNA sequences from isolates strains selected to perform the bioassays were analyzed by a Blastn search of sequences available in National Center for Biotechnology Information (NCBI) database. Identification of fungi based on the closest match of ITS sequences in the GenBank data base resulted as *Chaetomium globosum* and *Alternaria alternate* (isolate EL-17) with the accession numbers KC202936.1 and JQ316450.1, respectively. Similarity to known species, *C. globosum* and *A. alternaria*, was 99 and 95% ($E = 0$), respectively. Morphological analyses supported identification obtained from the NCBI sequence data. These species were selected to carry on the bioassays due to their ability to grow on paint films and negative impact on human health [7, 20]. Sequences obtained have been deposited in GenBank database and the accession numbers KU936228 and KU936229 to *C. globosum* and *A. alternaria* were assigned respectively.

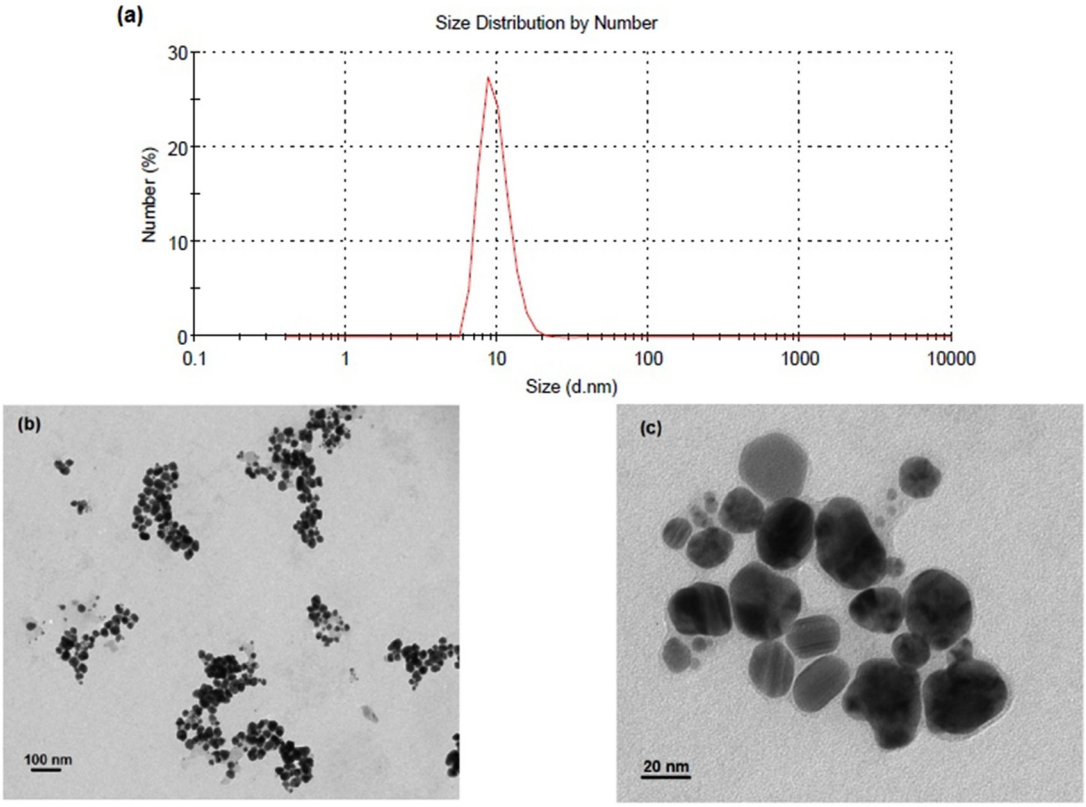

Figure 3. (a) Size distribution (DLS); and (b) and (c) transmission electron microscope (TEM) images of silver NPL (60 °C).

3.3. Antifungal activity

The diffusion test showed that the silver NPL was active against both mold with an inhibition zone diameter of 6 mm which is considered as active (+). The controls with $AgNO_3$ solution were no active (−), inhibition zone diameter less than 6 mm, against *C. globosum* and active in the case of *A. alternate,* with inhibition zone of 6 mm. Controls with *L. sempervirens* leaf extract resulted active against both molds with inhibition zone of 11 mm. Although the *L. sempervirens* leaf extract was active it tends to diffuse which is not appropriate for an antifungal agent to be used in coatings.

The antifungal additive to control biodeterioration of coatings must partition into the phase where its activity is required [1]. Beside, low water solubility helps to reduce the leaching rate when water is present, makes longer lasting coatings. Indoor coatings are exposed to water too when relative humidity abruptly increases which is usual in bathrooms and kitchen [34]. In this sense, the silver NPL showed to be active against the two molds tested without diffusing beyond the area where the colloid was deposited. Therefore, these results were promising from the point of view of the application propose to the synthesized product.

3.4. Coatings bio-resistance test

Silver NPL obtained at 60 °C were selected to elaborate the paints due to the characterizations results which showed that these NP has smaller size distribution, more stability and antifungal activity. To assess the coatings samples performance

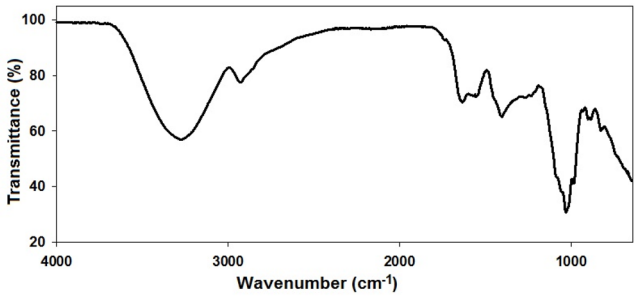

Figure 4. FTIR spectrum of AgNPs synthesized with *L. sempervirens* leaf extract.

exposure to molds during 1 month in bio-resistance test the rating propose by ASTM D5590 was used.

Paints NPL1 and NPL2 correspond with 3.6 and 5.4 mg of silver NPL in 100 g of paint respectively showed that an increase in NP concentration result in an increase in the inhibitory action against both molds. NPL1 presented light growth, rating (R) as 2, with *C. globosum* and moderate ($R = 3$) with *A. alternate*, NPL2 have lower fungal growth with traces ($R = 1$) to light ($R = 2$), respectively, while the control presented heavy growth ($R = 4$) with both molds. In general, formulations with NPL showed to be more active against *C. globosum*. The efficiency of the coatings with NPL against the fungal defacement was confirmed and, compared with the control paints with *L. sempervirens* leaf extract, presented a better performance. Figure 5 shows the plates obtained with the bio-resistance test after 1 month with paint NPL2 and the corresponding controls without biocides.

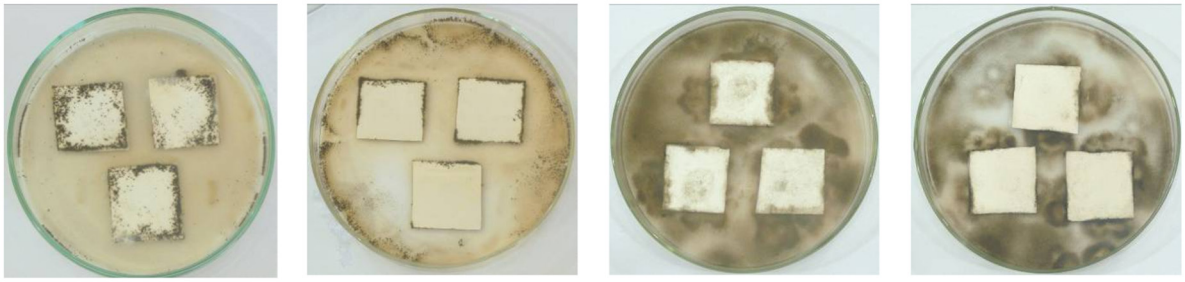

Figure 5. Coatings bio-resistance tests against *C. globosum* (a) control and (b) with NPL2; *A. alternata* (c) control and (d) with NPL2, after 1 month of incubation.

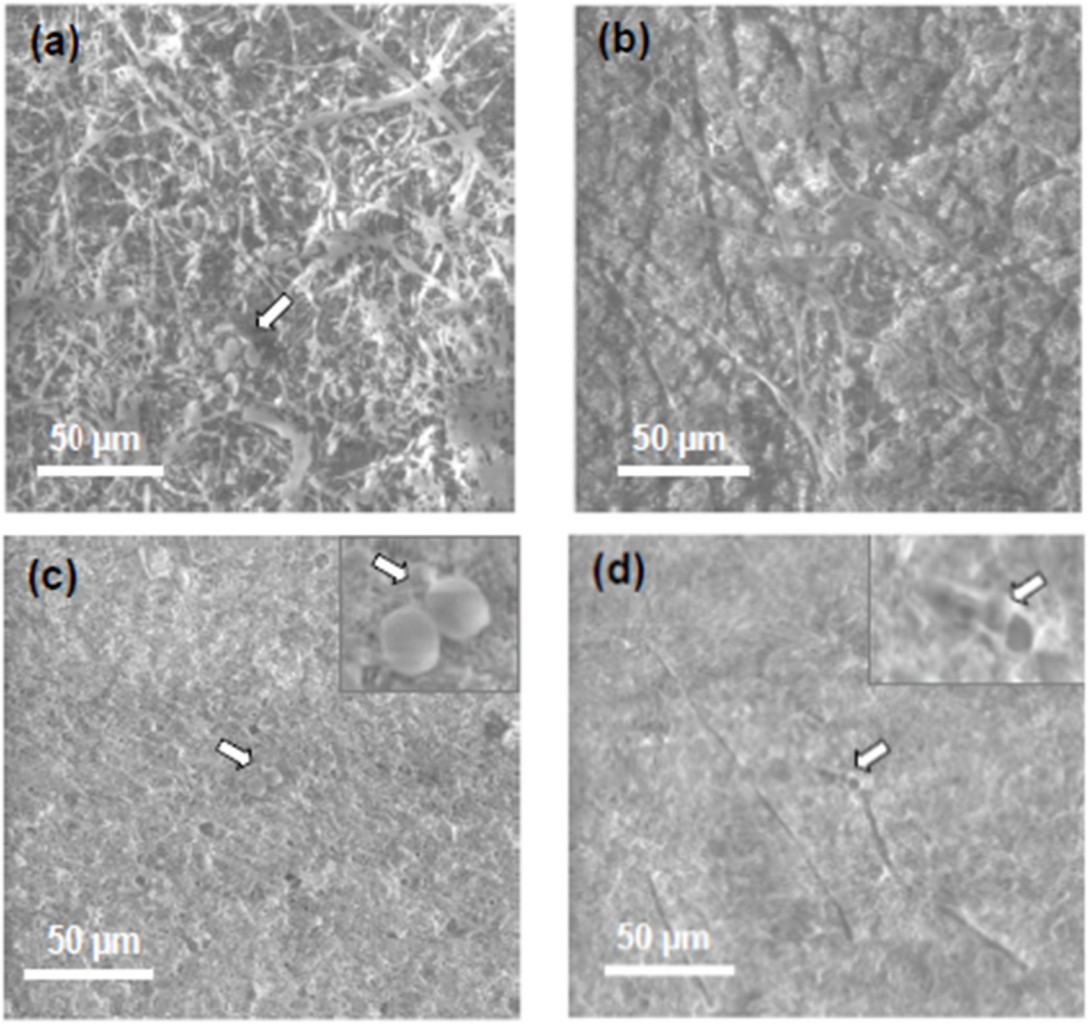

Figure 6. SEM micrograph: (a) and (b) *C. globosum* and *A. alternata*, abundant mycelium on control coatings without biocides; (c) and (d) coating NPL2 inoculated with both molds respectively (1500×).

3.5. Observation by scanning electron microscope (SEM)

Figure 6 shows SEM microphotographs of the coatings resulted more efficient, NPL2 (5.4 mg of AgNPs in100 g of paint), taken after one month of incubation at 28 °C. The control coating without biocide inoculated with *C. globosum*. Figure 6(a), showed heavy growth with an important hyphae network that extends all over the surface, spores can be seen too (detail with the white arrow). Something similar occurs with *A. alternate* (figure 6(b)). These observations contrast with the coatings that have AgNPs in their formulation, where has not been seen spore germination and mycelium development (figures 6(c) and (d)). The spores observed on these last microphotographs are those that were deposited as part of the inoculation to perform the assay (detail with the white arrow).

Energy dispersive x-ray microanalysis system (EDS) used to obtain a semi quantitative surface analysis do not detected the presence of Ag in any coating probably because the low

concentration of Ag is lower than the detection limit of the equipment.

3.6. Gloss and color measurements of the coatings

The assessment of color change using the equation (1) and the table 1 showed that the addition of AgNP to the paints produced a very evident change in color in the case of the paint with less NPL: $\Delta E = 4.66$. In the case of the paint with higher AgNP concentration, the change was big ($\Delta E = 6.33$) [31]. These values agreed with those obtained in a previous work employing similar Ag NP concentration but synthesized with other reducing agents [14]. In the case of the coatings with leaf extract, ΔE varied around 2, being the change evident.

Gloss values were almost constant and around 2.2, a common value for ceiling paints.

4. Conclusions

It was possible to synthesized nanometric silver particles with aqueous plant extracts. The qualities of the obtained particles showed depend on the plant extract used. The procedure employed to follow time stability, UV–Vis spectra, was suitable.

L. sempervirens leaf extract was the most efficient one because narrow size distribution and stable against time nanoparticles were obtained with it, especially when the synthesis is performed at 60 °C. Beside the silver nanoparticles obtained showed to be active against *C. globosum* (KU936228) and *Alternaria alternaria* (KU936229) in the antifungal activity test. Therefore, these nanoparticles were integrated in acrylic indoor paint formulation.

The results obtained from the bio-resistance test showed that the coatings were active against fungal growth compared with the controls. Coating efficiency was increased when the concentration of NPs was higher. The use of NPs suspensions is very suitable from the point of view of paint elaboration process due to the replacement of the formulated water by which is part of the suspension. Beside, NPs incorporation can be easily done.

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