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The Effects of Polyethyleneimine Coating of Gold Nanoparticles on Photosynthesis and Growth of the Moss *Physcomitrium patens*
Efectos del Recubrimiento de Polietilenimina de Nanopartículas de Oro sobre la Fotosíntesis y el Crecimiento del Musgo *Physcomitrium patens*

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SUMMARY

The widespread use of gold nanoparticles (Au-NP) as nanocarriers has led to several studies in animal cells to evaluate their cytotoxic effects. In contrast, little is known about the impact of gold nanoparticles on photosynthesis and plant growth. In this work, we present a study on the physiological and morphological responses of bryophytes to Au-NPs prepared with different contents of polyethyleneimine (PEI) using *Physcomitrium patens* (*P. patens*) as a model system. Results are presented where Au-NPs without PEI induce the generation of larger gametophores. On the other hand, Au-NPs with a high concentration of PEI were found to be phytotoxic. In contrast, Au/PEI-NPs with a medium PEI concentration cause early stress, but plant cells can recover growth, greening and photosynthetic activity under these conditions. Finally, low concentrations of Au/PEI-NPs do not cause adverse effects on *P. patens* in terms of growth, gametophyte development, and photosystem II maximum quantum yield. Toxicity was closely related to the content of PEI. This study reveals for the first time the effects of Au-NPs and Au/PEI-NPs on bryophytes at different growth stages.

Index words: *colloids systems, non-seed plant, metallic nanoclusters, phytotoxicity, stabilizer polymer.*

RESUMEN

El uso generalizado de nanopartículas de oro (Au-NP) como nanotransportadores ha llevado a varios estudios en células animales para evaluar sus efectos citotóxicos. En contraste, se sabe poco sobre el impacto de las nanopartículas de oro en la fotosíntesis y el crecimiento de las plantas. En este trabajo, presentamos un estudio sobre las respuestas fisiológicas y morfológicas de briofitas a Au-NPs preparadas con diferentes contenidos de polietilenimina (PEI) utilizando *Physcomitrium patens* (*P. patens*) como sistema modelo. Se presentan resultados donde Au-NPs sin PEI inducen la generación de gametóforos más grandes. Por otro lado, se encontró que Au-NPs con una alta concentración de PEI eran fitotóxicas. En contraste, Au/PEI-NPs con una concentración media de PEI causan estrés temprano, pero las células vegetales pueden recuperar el crecimiento, el reverdecimiento y la actividad fotosintética en estas condiciones. Finalmente, bajas concentraciones de Au/PEI-NPs no causan efectos adversos sobre *P. patens* en términos de crecimiento, desarrollo de gametofitos y rendimiento cuántico máximo del fotosistema II. La toxicidad estuvo estrechamente relacionada con el contenido de PEI. Este estudio revela por primera vez los efectos de las nanopartículas de oro y de las nanopartículas de oro/PEI sobre briofitas en diferentes etapas de crecimiento.

Palabras clave: *sistemas coloides, planta sin semillas, nanoclusters metálicos, fitotoxicidad, polímero estabilizador.*

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INTRODUCTION

Nanoparticles (NPs) are of great interest from the point of view of their physical, chemical, and morphological properties (González-Grandío *et al*., 2021). However, their impact is more significant when they are applied in different areas, such as biology and medicine. The colloidal noble metal NPs, often composed of copper, silver and gold, exhibit unique characteristics such as Localized Surface Plasmon Resonance (LSPR), sizes on the order of proteins, adjustable sizes, a high surface-to-volume ratio, and surfaces with the ability to react and bind to a huge number of active molecules (An *et al.*, 2022; Kozytskiy *et al.*, 2015; Kretschmer, Mansfeld, Hoeppener, Hager, and Schubert, 2014), which make them excellent candidates for developing new drug nanocarrier systems, optical nanobiosensors, nanosystems for photodynamic therapy, biomarkers (Kozytskiy *et al.*, 2015), new non-viral vectors with the ability to introduce genetic material into tissue or cells (Sanzari, Leone, and Ambrosone, 2019). The NPs' surfaces are frequently covered with a polymer coating in order to accomplish this goal. The layer's role is to improve solubility, stability, and biocompatibility; it also protects against harsh physiological conditions and acts as a binder for biological materials such as proteins, enzymes, plasmids, DNA, and RNA (Bombin *et al.*, 2015). While polymer-coated nanoparticles have made great strides, determining their true environmental impact (toxicity) is still a key obstacle to expanding their use in natural processes like photosynthesis and plant growth.

Polyethyleneimine (PEI) is one of the most used cationic polymers in the industry for the creation of food packaging films (Tian *et al.*, 2012), bioremediation processes (Sun *et al.*, 2021), biomedicine as carriers for nanogels (Zhang *et al.*, 2021a), and artificial skin or injectable fillers (Song *et al.*, 2022). In nanotechnology, PEI is used as a reducing as well as stabilizing agent in the preparation of metallic nanoparticles at moderate temperatures (< 373 K) (Kim, Lee, Lee, Park, and Shin, 2008), which provides versatility in synthetic procedures. This feature would help synthesize Au-NPs by colloidal chemistry methods such as the modified Turkevich technique (Kretschmer *et al.*, 2014). Moreover, its extraordinary characteristics, such as biodegradability, biocompatibility, low toxicity and its ability to interact with biological entities, make PEI unique for advanced biology (Chen, Lv, Sun, Chi, and Qing, 2020).

Therefore, coating metallic nanoparticles with PEI will ensure the preparation of nanosystems with new and improved biological functionalization properties compared to the individual components that compose them. For example, gold nanoparticles coated with polyethyleneimine (Au/PEI-NPs) show synergetic effects that can be exploited in different applications, such as interaction with anionic polyelectrolytes and biological entities (plasmids) (An *et al.*, 2022), cellular uptake, gene delivery/transfection (Lee, Yoon, and Cho, 2013), hydrophilicity, imaging, proton uptake (pH tolerance) (Akinc, Thomas, Klibanov, and Langer, 2005) and currently in the development of new drug nanocarrier systems. Furthermore, due to their cationic nature, reports have suggested that the rapid interaction of Au/PEI-NPs with the surface of cell membranes could induce cellular uptake, which would be beneficial for specific biological applications: for RNA/DNA layering in the Au/PEI-NPs vector, in DNA condensation reactions to create a gene delivery scaffold for biomarker development, and for gene delivery into cells after conjugation with DNA or small interfering RNA (siRNA) (Zhang *et al.*, 2021b).

Despite the significant efforts that have been made in the preparation and application of metallic nanoparticles, the possible damage of Au/PEI-NPs on plants (vascular or non-vascular) still needs to be clarified; in general, information on the effects of nanoparticles on bryophyte cells are still poorly known and deserves attention (Liang *et al.*, 2018; Canivet, Dubot, Garçon, and Denayer, 2015; Canivet, Dubot, and Denayer, 2014).

The objective of this research work was to study the effects of Au-NPs and Au/PEI-NPs prepared with different PEI concentrations on photosynthetic efficiency and growth of the plant *Physcomitrium patens* (*P. patens*), one of the most used models to study evolutionary developmental biology questions, stem cell reprogramming, and the biology of nonvascular plants. We found that Au-NPs showed beneficial effects on gametophore development. However, a toxic effect was also identified, dependent on the concentration of PEI used in the synthesis of Au-NPs. The evaluation of cytotoxic effects of Au-NPs and Au/PEI-NPs on bryophytes is of great interest to contribute to the knowledge in phytonanotechnology.

MATERIALS AND METHODS

Gold Nanoparticles Synthesis and Characterization

For the synthesis of Au-NPs and Au/PEI-NPs, gold salt ($HAuCl_4 \cdot 3H_2O$, ACS reagent) and the 25 kDa branched polymer polyethyleneimine were purchased from Sigma-Aldrich (Chemical Co., St. Louis MO, USA). All reagents were used as received without further purification. Deionized water (> 18 MΩ cm^{-1}) was always used to wash the laboratory material in the synthesis of metallic nanoparticles. Colloidal dispersions of Au-NPs were prepared by

chemical reduction of metal ions in the presence of a polymer, as reported elsewhere. The synthesis proceeded as follows: a solution of metal ions (0.033 mmol in 25 mL of H_2O) was prepared by dissolving crystalline salt of chloroauric acid in water. We considered a control solution of Au-NPs by placing 50 mL of the metal solution and added sodium borohydride and sodium citrate (0.099 mmol) at room temperature for 20 min with constant stirring.

For the preparation of Au/PEI-NPs, 50 mL of the solution of metal ions was placed in a 250 mL capacity glass reactor and subjected to strong stirring in the presence of different concentrations of PEI 0.02, 0.06, 0.12, 0.50 and 1.50% (w/v). The obtained mixture was stirred and then refluxed at 353 K for 2 h using a magnetic hot plate with stirring (500 rpm). During the reaction, a change from yellow to ruby red was observed, indicating the formation of Au/PEI-NPs. At the end of the reaction, the obtained solution was cooled to room temperature for purification by dialysis (50 kDa cut-off) against distilled water to remove the unbound PEI. The dispersions obtained by this method were finally used for application and characterization.

For greater clarity with the treatments, we represent them as follows: Au-NPs (uncoated Au-NPs); Au NPs prepared with different concentrations of PEI: high concentrations Au/PEI-NPs 1.5% (w/v) and Au/PEI-NPs 0.5% (w/v); medium concentration Au/PEI-NPs 0.12% (v/w); and lower concentrations Au/PEI-NPs 0.06% (w/v) and Au/PEI-NPs 0.02% (w/v), respectively.

Characterization Equipment

The optical absorption spectra of the Au-NPs and Au/PEI-NPs colloids were obtained using a UV-Vis-NIR spectrophotometer (Shimadzu UV 3101 PC double beam) at room temperature. Structural characterization of Au-NPs and Au/PEI-NPs was performed by transmission electron microscopy (TEM) on an atomic resolution analytical microscope (JEOL JEM-ARM200F). For TEM observations, a drop (6 µL to 10 µL) of aqueous colloidal solution of Au-NPs and Au/PEI-NPs was placed on a carbon-coated copper microarray and subsequently dried under vacuum. Particle size analysis of TEM images was performed with ImageJ (for each sample, three different regions were measured). Approximately 100 particles were measured for Au-NPs and Au/PEI-NPs colloids, and the values are shown as mean diameters with their respective standard deviation. A Nikon digital camera and an optical microscope (Leica) were used to estimate gametophyte growth and development.

Plant Growth Condition and Exposure to Gold Nanoparticles

Protonemal tissue of *P. patens* (Gransden) was used for routine subculture on a solid $PpNH_4$ medium. Briefly, after 15 days of culture, protonemal tissue was harvested for experiments. The harvested protonema tissue was suspended in sterile soft agar and blended using a homogenizer equipped with a disposable plastic dispersing element. 7µL of the blended tissue was inoculated onto cellophane discs (0.5 cm in diameter) previously placed on new plates of solid $PpNH_4$ medium. The tissues were incubated at 295 K in a culture chamber with a photoperiod of 8 h of light (40 µmol m^{-2} s^{-1}) and 16 h of darkness. The experiment was monitored on days 0, 4, 7, 14, and 21, when the protonema developed into the leafy gametophyte. To incubate the *P. patens* tissues growing on the cellophane discs with the colloidal, Au-NPs and Au/PEI-NPs stock solutions (0.5 mL) were added onto the solid medium.

Morphology Investigation of the Protonema and Leafy Gametophyte

Images of the protonemata and leafy gametophytes observed on different days (0, 4, 7, 14, and 21) were taken by a Nikon digital camera, and an optical microscope (Leica) was used to estimate gametophyte growth and development at 21 days. The phyllids (the leaf-like organs of bryophytes) of the gametophytes were obtained with a tweezer and then investigated by the optical microscope.

PSII of the Protonema and Leafy Gametophyte

Protonemal tissue was collected on days 0, 4, 7, 14, and 21. Then the maximum quantum yield of PSII was measured under dark conditions using a portable fluorometer (FlourPen FP 110, PSI Instruments, Czech Republic) as previously described (Komakech *et al.*, 2020). In dark-adapted samples, QY is equivalent to Fv/Fm. Each sample type was measured in triplicate.

Statistical Analysis

All means were calculated from three independent experiments and are expressed as mean ± standard deviation. Statistical significance analysis was performed using the Student's t-test. Each experimental value was compared with the corresponding control value at each test point. Differences were considered significant if $P < 0.05$.

RESULTS AND DISCUSSION

Characterization of Gold Nanoparticles

In Figure 1 the absorption spectra of prepared colloidal Au-NPs and Au/NPs-NPs dispersions are shown. Absorption maximum in the visible range of the electromagnetic spectrum was observed for the colloidal dispersion, generally assigned to the LSPR of Au nanoparticles. The absorption spectra of the preparation of colloidal Au/PEI-NPs dispersions prepared with different PEI contents reveal the presence of a maximum peak (LSPR) assigned to the presence of Au nanoparticles with nanometer-scale size. The peak position shifted towards lower wavelengths, from 529 to 518 nm. This shift in the position of the LSPR band is generally attributed to the decreased size of the Au/PEI-NPs. On increasing the PEI content in the reactant solution, the absorption intensity decreases due to the absorption of polymer on the nanoparticles; this phenomenon is consistent with previous observations (Kim *et al.*, 2008). PEI-coated Au NPs act favorably on the surface tension, electrostatic and hydrophobic interactions, coordinate bonding, and van der Waals attractive forces that make Au/PEI-NPs thermodynamically stable at the interface (Polte, 2015). The obtained dispersions were stable for months without significant changes in the absorption spectra.

Similarly, TEM micrograph observations also revealed such a trend of size variation in Au colloids. Figure 2 shows TEM micrographs of colloidal Au/PEI-NPs dispersions prepared with different contents of PEI. All TEM micrographs clearly reveal the formation and stability of particles with nanoscale sizes, which are crucial requirements for biological applications.

Based on the histograms in Figure 3 of the synthesized Au-NPs and Au/PEI-NPs, it is detected that the size distribution follows a Gaussian fit with a narrow size distribution for the metallic particles. We can observe that Au/PEI particles are smaller compared to Au metal nanoparticles and that the average size decreases with high PEI content from 15.0 nm to 8.6 nm. The decrease in size can be explained by considering the reduction kinetic of the metal Au ions in the presence of PEI. As the interaction between Au ions and PEI precedes the reduction, the interactive forces between the metal ion and PEI will include electrostatic interactions, hydrogen bonds, and coordinate bonds, which cause the reduction rate of Au ions to be slower; thus, an increase in PEI content in the reagent solution inhibits the growth rate of Au nanoparticles, and consequently, the nanoparticles are smaller.

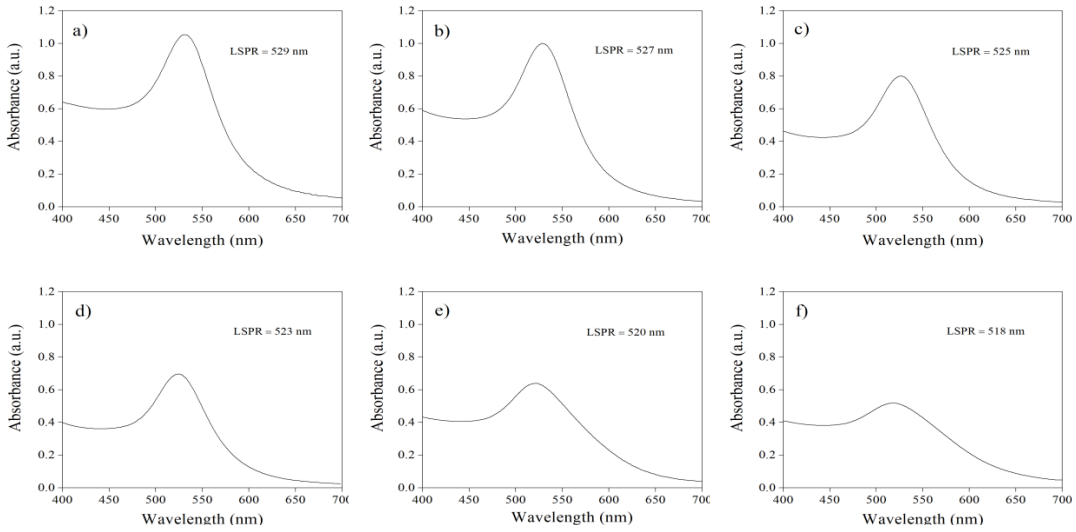

Figure 1. Absorption spectra of colloidal Au/PEI-NPs dispersions prepared with different PEI contents: a) Au, b) 0.02, c) 0.06, d) 0.12, e) 0.5 and f) 1.5% (w/v).

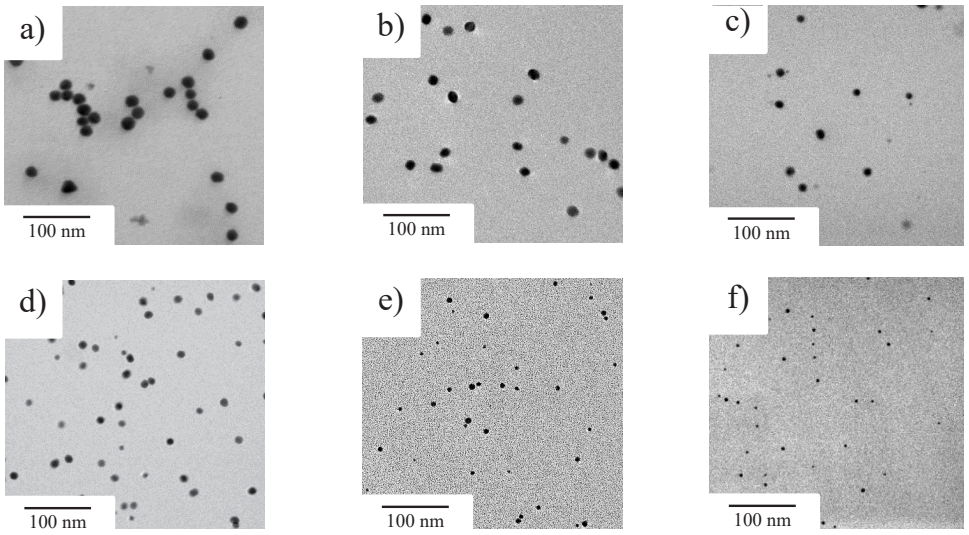

Figure 2. TEM micrographs of Au-NPs synthesized with different PEI contents: a) Au, b) 0.02, c) 0.06, d) 0.12, e) 0.5 and f) 1.5% (w/v).

In this case, our results are comparable to those obtained by Kim *et al*. (2008). By changing the PEI concentration in the synthesis, the size of Au/PEI-NPs can be controlled. As pointed out by many other investigations (Sun, Dong, and Wang, 2005, 2004), the formation of Au-NPs occurs in a single process that only involves heating an aqueous solution of metal precursor and polyethyleneimine. The colloidal chemistry method described here enables the synthesis of Au/PEI-NPs that can be used for biological applications.

Response of *P. patens* Protonema to the Synthesized Au-NPs and Au/PEI-NPs

The life cycle for moss is extremely short (21 days) under ideal conditions of light, water and continuous nutrition. Therefore, during this time, it is possible to observe the effect of the presence of metal nanoparticles and PEI polymer on its phenotype, such as variations in color and gametophore formation (Cove, 2005). Figure 4 shows images of the effects of Au-NPs, different contents of PEI and Au/PEI-NPs on the phenotype during 21 days of *P. patens* moss development. Au-NPs had no negative effect on the protonema colonies during their life cycle since they did not lose their color and their growth was normal, just like the control that was treated with water.

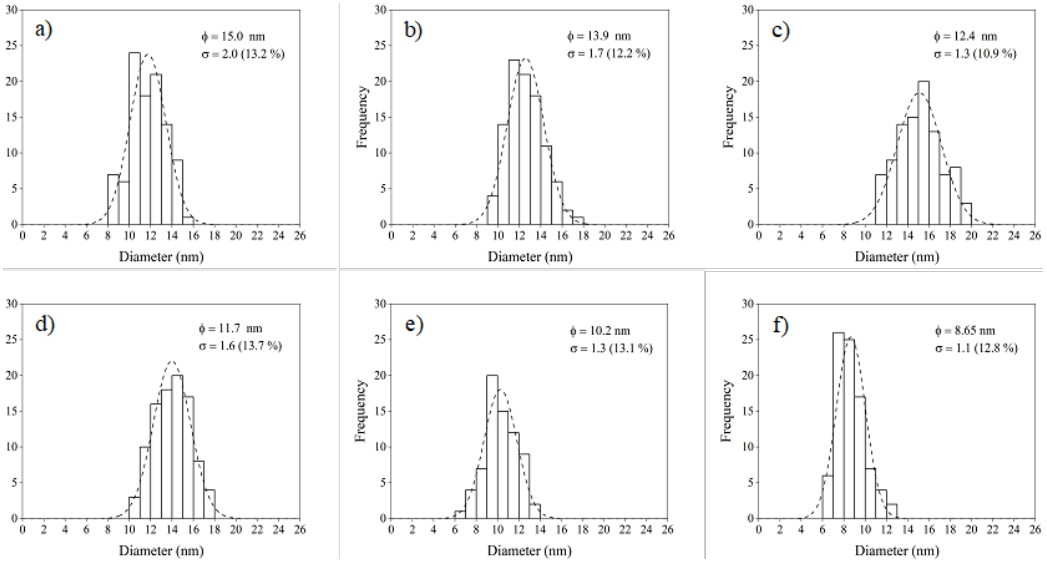

Figure 3. Diameter distribution histograms of Au/PEI-NPs synthesized with different PEI contents: a) Au, b) 0.02, c) 0.06, d) 0.12, e) 0.5 and f) 1.5% (w/v).

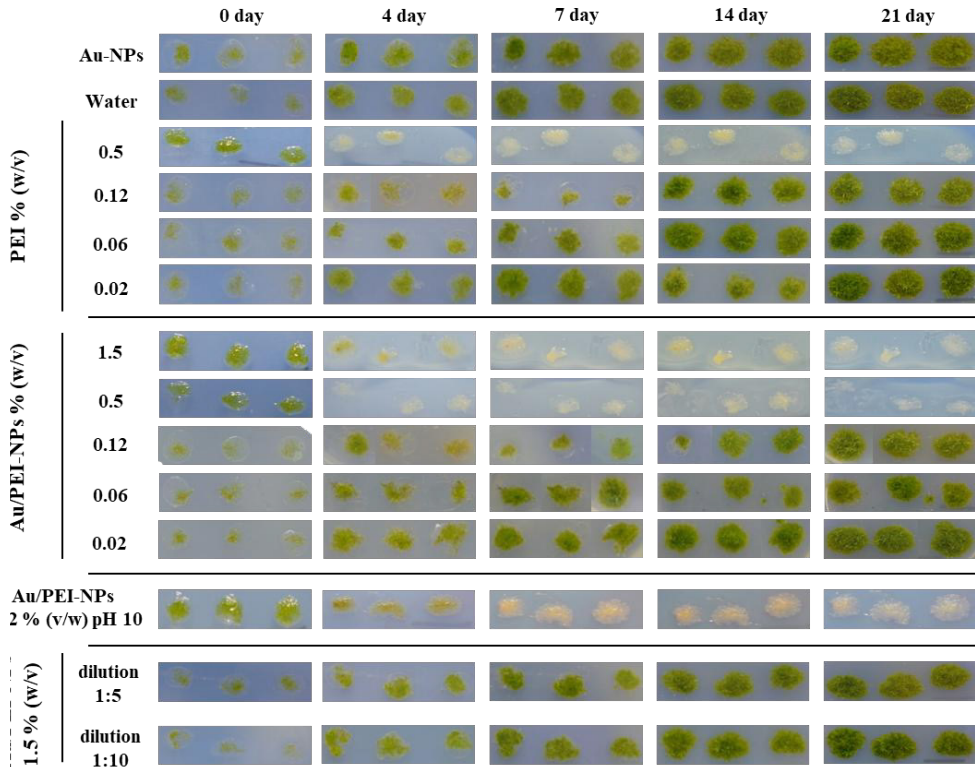

Figure 4. Phytotoxic evaluation of Au-NPs, different PEI contents and Au/PEI-NPs with different PEI contents at pH 7. Phytotoxic evaluation of Au/PEI-NPs at pH 10 and of 1:5 and 1:10 dilutions of Au/PEI-NPs containing 1.5% (w/v) PEI during 21 days of *P. patents* development.

On the other hand, the evaluation of PEI and Au/PEI-NPs treatments prepared at different contents of PEI presented a chlorotic phenotype from day 4 of exposure to high contents of PEI 0.5% (w/v) and PEI 1.5 and 0.5% (w/v) for Au/PEI-NPs, showing a whitish coloration in plants, indicating a loss of photosynthetic capacity (Sun *et al*., 2023), evidencing the phytotoxicity of PEI and PEI present in Au/PEI-NPs. This phenotype is maintained until day 21, with no recovery observed. Meanwhile, at contents of 0.12% (w/v) of PEI and PEI in the Au/PEI-NPs, initial stress was observed on days 4 and 7, revealing a brown color in the tissues; by day 14, the characteristic green coloration was observed until day 21, where its development was completed. However, for low contents of 0.06 and 0.02% (w/v) of PEI and PEI in the Au/PEI-NPs, no negative effects on the phenotype were observed; the plants developed in a similar way to the control treatment (water). All treatments developed at pH 7 are optimal for the good development of *P. patents*.

In order to evaluate the effect of pH 10 resulting from the synthesis of Au/PEI-NPs on the life cycle of *P. patents*, Au/PEI-NPs with a content of 0.12% (w/v) of PEI were used for this treatment. Thus, Figure 4 shows that on day 4, the plant tissue died without recovery and gametophores did not develop. These results contrast with those obtained at pH 7 with the same PEI content, demonstrating that the pH of the colloidal solution causes phytotoxicity (Sanzari, *et al*. 2019).

Finally, 1:5 and 1:10 dilutions of the resulting Au/PEI-NPs dispersion containing 1.5% (w/v) PEI were prepared to monitor the possible decrease of phytotoxicity in *P. patents* with the decrease of PEI content at pH 7. In Figure 4, a null effect of phytotoxicity is observed, as there are no negative effects on color and growth, showing a normal development of *P. patents*. This indicates that PEI concentration affects plant physiology since the polymer tends to agglomerate in the surrounding cell wall, preventing the exchange of nutrients (Schwab, *et al*., 2015). Therefore, it can be hypothesized that the low PEI concentration generated by dilution can eliminate the phytotoxicity effect.

Consequently, the content of PEI in Au/PEI-NPs and pH are essential factors to be taken into account when applying to plant cells. In this case, it was observed that the content of PEI was the most critical factor. Recent reports, presented similar observations, when applying single-walled carbon nanotubes (SWNTs) coated with SWNTs-pristine with adsorbed oligonucleotides and polyethyleneimine-SWNTs (PEI-SWNTs) introduced by leaf filtration

in *Arabidopsis thaliana* (*A. thaliana*), found that high contents of PEI produced an adverse, irreversible response and resulted in cell death. They also found that low concentrations of PEI caused damage to infiltrated leaves over time (González-Grandío *et al*., 2021). Thus, they conclude that PEI exerts extensive transcriptional reprogramming leading to metabolic suppression and programmed cell death in infiltrated areas of *A. thaliana* leaves.

The Impact of Au-NPs and Au/PEI-NPs on the Development of Leafy Gametophyte

At the end of 21 days of *P. patents* moss development at pH 7 in the presence of Au-NPs, PEI at 0.12% (w/v), Au/PEI-NPs containing PEI at 0.12% (w/v), and the control in the presence of water, well-formed gametophores were obtained and observed individually under a microscope (Figure 5). The size, shape, and number of phyllodes that compose a gametophore indicate that the early stress suffered does not affect its formation. Therefore, Au-NPs and Au/PEI-NPs treatments can produce gametophores of up to 2 cm, observable to the naked eye. Usually, an average gametophore of *P. patents* is 5 mm or less, grown in the presence of water (Cove *et al*., 2009). This observation suggests that Au-NPs and Au/PEI-NPs promote the cell division rate of *P. patents*. The level to which nanoparticles act on cell division will be the subject of future studies. It is important to note that, to our knowledge, there are no previous reports analyzing this effect. However, it is tempting to speculate that Au-NPs and Au/PEI-NPs may interact with specific phytohormones or proteins involved in the regulation of cell division, the transduction of specific signals, or the expression of a particular set of genes (Cove *et al*., 2009).

Effects of Au-NPs and Au/PEI-NPs on the Photosynthetic Efficiency

Interestingly, incubating with Au-NPs and Au/PEI-NPs has no adverse effect on the photosynthetic efficiency of PSII (Fv/Fm) of *P. patens* protonema cells. This supports the idea that nanoparticles have excellent potential to be used in plant cells for different biotechnological purposes. However, as was observed in the cell growth experiments, the use of a high content of PEI also negatively affects the photosynthetic efficiency of PSII (Figure 6). A substantial decrease in Fv/Fm values was observed from day 4 of incubation of protonema tissue in 0.5% PEI conditions, and such an effect is permanent, as plant cells cannot recover this capacity, either at day 21, again pointing to the adverse role of high PEI contents. On the other hand, the medium PEI 0.12% content causes a slight decrease in Fv/Fm values, although typical average Fv/Fm values for unstressed conditions are quickly restored with time. No effect on Fv/Fm was interestingly detected for PEI 0.06 and 0.02% (w/v), showing a similar behavior with those seen for water or Au-NPs. These Fv/Fm values found for the different PEI contents are comparable to those obtained when the Au NPs were applied in the PEI functionalized forms. These observations suggest that PEI originated the adverse effect on the photosynthetic efficiency and was not caused by the Au-NPs.

To explore the possibility that pH was playing a role in the negative impact of Fv/Fm, the experiment was conducted at pH 10 Au/PEI-NPs with a PEI content of 0.12% (v/w). Our results suggest that pH does affect the decrease in PSII activity. In contrast, when we applied 1:5 or 1:10 dilutions, Fv/Fm values were positive.

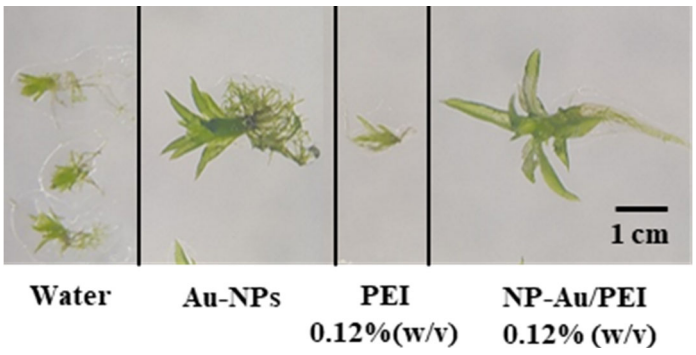

Figure 5. Effect on the growth and development of the leafy gametophyte at different treatments: water, Au-NPs, PEI at 0.12% (w/v) and Au/PEI-NPs containing 0.12% (w/v) PEI.

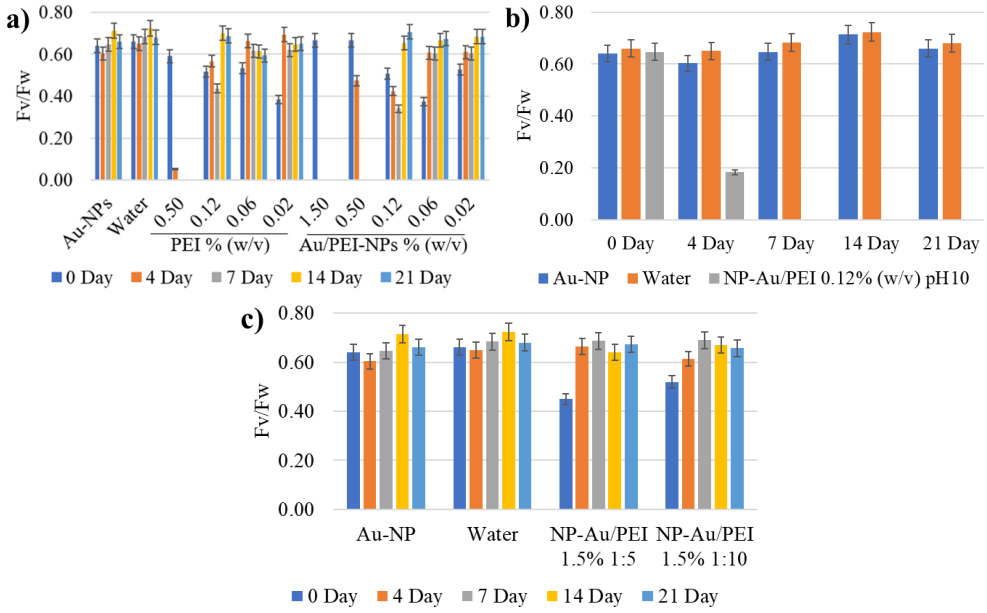

Figure 6. a) Analysis of the maximum quantum yield of PSII by PEI, b) analysis of the effect of the average PEI concentration in PSII at pH 10 and c) analysis of the effect of Au/PEI-NPs dilutions containing 1.5 % (w/v) PEI on the early stress of *P. patents*.

CONCLUSIONS

In this work, gold/polyethyleneimine nanoparticles (Au/PEI-NPs) were prepared at moderate temperature by tetrachloroauric acid (III) reduction using polyethyleneimine (PEI) as a reducing and stabilizing agent. The effect of PEI on the synthesis of Au-NPs in formation and size was studied. The Au/PEI nanoparticles were characterized by UV-Vis spectroscopy and transmission electron microscopy. It was observed that the prepared Au/PEI nanoparticles were stable to agglomeration and were spherical in shape with a size in the nanometer scale. The effect of Au-NPs and Au/PEI-NPs with different PEI content on the growth and development of *P. patens* at the gametophyte stages (protonema and leafy gametophyte) was found to be significant. Au-NPs enhanced the growth of *P. patens*. In contrast, *P. patens* showed different sensitivity to Au/PEI-NPs depending on the content of PEI in the NPs and not on the size. Au/PEI-NPs with different contents of PEI showed different abilities to inhibit the growth of *P. patens*. High contents of PEI were the most phytotoxic to protonema. Medium and low content of PEI showed lower phytotoxicity for protonema. These findings allow us a better understanding of the effects of Au-NPs and PEI on NPs for biological applications, e.g., nanocarriers of molecules such as plasmids or proteins.

ETHICS STATEMENT

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF SUPPORTING DATA

All data generated or analysed during this study are included in this published article.

COMPETING INTEREST

I state that is not potential conflict of interest regarding the publication of this contribution.

FINANCING

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AUTHORS' CONTRIBUTIONS

Validation, Investigation, Writing-original draft, conceived and planned the experiments, carried out the experiments, validated the results of the investigation, contributed to the interpretation of the results, and took the lead in writing the manuscript: Z.O.S. Validation, Investigation, conceived and planned the experiments, carried out the experiments, and contributed to the interpretation of the results: J.A.L., and G.C.N. Conceived and planned the experiments, validated the results of the investigation, and contributed to the interpretation of the results: M.A.V.L. Validated the results of the investigation, and contributed to the interpretation of the results: J.D.R. Validation, Investigation, Writing-original draft, Writing-review & editing, validated the results of the investigation, contributed to the interpretation of the results, and took the lead in writing the manuscript: J.F.S.R.

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