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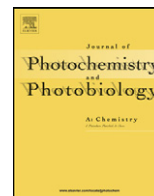Artigos e Materiais de Revistas Científicas - IFSC/FCM

2011-12

In vivo observation of chlorophyll fluorescence quenching induced by gold nanoparticles

In vivo observation of chlorophyll fluorescence quenching induced by gold nanoparticles

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ABSTRACT

To our knowledge, the present work reports the first in vivo observation of chlorophyll *a* fluorescence quenching induced by gold nanoparticles. Laser-induced fluorescence spectroscopy was used to collect in vivo chlorophyll *a* fluorescence, using a portable optical fiber-based spectrofluorimeter. Fluorescence quenching was observed for all plants submitted to the gold nanoparticle treatment, and both excitation wavelengths, 405 nm and 532 nm, were capable of detecting interactions between gold nanoparticles and plants. Our results also suggest that gold nanoparticles were able to translocate and accumulate in the soybean plants after seed inoculation.

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1. Introduction

Chlorophyll fluorescence has been used as an accurate and non-destructive probe of photosynthetic efficiency, which can directly or indirectly reflect the impacts of environmental factors and changes in the physiological state of the plants [1]. The photosynthetic efficiency of many plants decreases when they are subjected to stress conditions [2,3]. Therefore, chlorophyll fluorescence has been used as a standard method to investigate the chlorophyll content in plants, identify plant class, and detect plant stresses caused by nutrient deficiency, polluting agents, etc. [4].

Ultraviolet and visible (UV–Vis) light absorbed by green leaves can induce two distinct regions of fluorescence, emissions in the range of wavelengths between 400 nm and 600 nm (blue/green fluorescence) and between 600 nm and 800 nm (red/far-red fluorescence). The blue/green fluorescence is associated with several leaf fluorophores such as hydroxycinnamic acids, flavonols, isoflavones, flavanones, and phenolic acids; while, in vivo, the red/far-red fluorescence is produced only by chlorophyll *a* (Chl*a*) [5]. In the red/far-red region, most of the fluorescence, with maxima at 685 nm and 735 nm, is emitted by Chl*a* in photosystem II (PSII) at room temperature. Nevertheless, a small fluorescence contribution from photosystem I (PSI) in the range between 710 nm and 720 nm has been reported [5].

Engineered nanoparticles (ENPs) have recently received much attention because of their industrial applications. A large number of ENPs are already used in a wide range of consumer products; more than 60% are in the field of health and fitness, including cosmetics and personal-care products. Paints, coatings, textiles, electronics, pharmaceuticals, environmental remediation, food, and food packaging are other important applications [6]. An exponential growth in the development, manufacture, and use of nanomaterials over the past decade has followed the development of several beneficial applications in a diverse range of products and areas, including health and medicine, food production, energy, and environment [7–9]. However, the production, use, and disposal of nanomaterials will inevitably lead to their release into the atmosphere, water, and soil, and there are still uncertainties about the fate, behavior, impacts, and toxicity of release into the environment [7–13]. In this context, plants will not be able to avoid the environmental stress that may be induced by the increased release of ENPs into the biosphere. Although a few papers have reported the impact of ENPs on plants in recent years, many questions about the behavior and fate of ENPs in plants remain unanswered [14]. Both positive and negative effects have been reported, and the impact of ENPs on plants varies, depending on the composition, concentration, size, and physical and chemical properties of ENPs and plant species [15].

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The efficiency in the production of chemical energy in the photo-synthetic system can be strongly altered in the presence of metallic nanoparticles [16]. Two competing effects could induce changes in the efficiency of a photosystem: the increase in light absorption by chlorophyll molecules, due to plasmon resonance (enhanced local field near the metal surface [17]); and energy transfer from chlorophyll to metallic nanoparticles, by inducing a decrease in the quantum yield of the photosynthetic system. Recently, Barazzouk et al. reported that the emission intensity of Chl*a* could be quenched by gold nanoparticles in a solution medium [18]. This quenching has been attributed to the process of photoinduced electron transfer from excited Chl*a* to gold nanoparticles. The main aim of the present study was to investigate which effect gold nanoparticles can induce in the Chl*a* fluorescence emitted by leaves, in an in vivo analysis.

2. Materials and methods

2.1. Soybean preparation

Soybean [(*Glycine max* L.) Merr.] seeds of the variety "BRS 245 RR", with a germination percentage of 92%, were used in this study. 200 g of soybean seeds was treated with 0.6 ml of fungicide (Derosal Plus®, carbendazim, and thiram-200 ml commercial formulation 100 kg^{-1} seeds) and 0.5 g of inoculant (Biomax 7.2×10^9 cfu/g). The seeds were sown in pots with a volume of 3.26 dm^3 containing 2500 cm^3 of Rhodic Eutrudox soil. The soil in each pot was previously fertilized with 25 g of a 0–20–20 NPK commercial fertilizer formulation. Four soybean seeds were sowed per pot. Two weeks after germination, two plants were left in each pot. The pots were watered with a sufficient volume of tap water to maintain the soil at 100% of field capacity. The plants were grown in a greenhouse at room temperature.

2.2. Chlorophyll extraction

The chlorophyll extraction was conducted according to the protocol proposed by Richards and Thompson [19]. 3 g of soybean leaves was added to 30 ml of methanol P.A. and macerated for 2 min. After that, the mixture was stirred cold for 20 min and then centrifuged at 2000 rpm for 5 min. For the absorption and fluorescence measurements, 1 ml of the chlorophyll extract was diluted in 10 ml of methanol P.A., obtaining a chlorophyll concentration of 5.46 μM.

2.3. Gold nanoparticles

Gold colloids with three different diameters: 5 nm, 10 nm, and 20 nm were used in the present experiment. All gold colloids contain approximately 0.01% HAuCl$_4$ suspended in 0.01% tannic acid with 0.04% trisodium citrate, 0.26 mM potassium carbonate, and 0.02% sodium azide as a preservative. The nanoparticles were purchased from Sigma–Aldrich.

2.4. Fluorescence measurements

Fluorescence emission spectra of the chlorophyll extract were measured using a fluorescence spectrophotometer (Cary Eclipse, Varian). A portable optical fiber-based spectrofluorimeter (MM'Optics) was used to measure chlorophyll fluorescence in leaves, specifically in the cotyledon, unifoliate leaf, and central leaflet of the first fully expanded trifoliate leaf, as shown in Fig. 1. The portable system is composed of (i) two excitation sources (at 405 nm and at 532 nm); (ii) one monochromator; and (iii) one type Y optical fiber used to drive the excitation and emission lights for the fluorescence measurements in plants. In both cases, the molecules were excited at two wavelengths (405 nm and 532 nm),
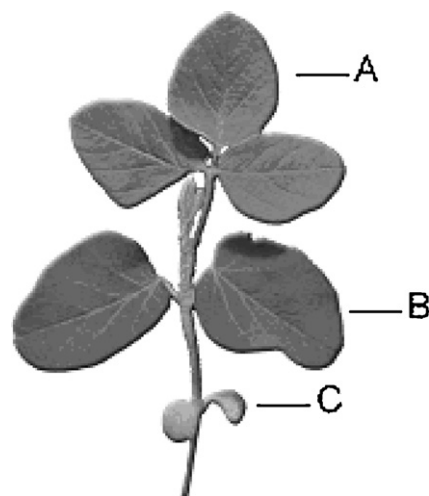

Fig. 1. Soybean leaves: trifoliate (A), unifoliate (B) and cotyledon (C).

and the fluorescence spectra were collected from 600 to 800 nm. Absorption was measured by means of a UV–Vis absorption spectrophotometer (Cary 50, Varian), and the absorption spectra were collected from 490 to 610 nm. Quartz cuvettes of 1-cm path length were used to make absorption and fluorescence measurements in the chlorophyll solution. All measurements were carried out at room temperature.

2.5. In vivo analysis

The fluorescence analyses were carried out directly in the leaves. The plants were divided into two groups: the control group, where the seeds received the normal preparation as described in Section 2.1; and the group where GNPs were added to the solution that was applied to the seeds before planting. 2.26 ml of colloidal gold nanoparticles (5 nm) was diluted in 2.74 ml of milli-Q water. Forty plants were studied in this experiment; 20 plants were used as controls, and 20 plants were treated with GNPs. For each group the average spectrum over 20 plants was taken to analyze the fluorescence behavior. The fluorescence signal was collected from cotyledon leaves, unifoliate leaves, and trifoliate leaves from the upper surface of the leaves.

A second experiment was carried out, where the solution of nanoparticles was applied directly on the leaf surface by means of a syringe. The chlorophyll fluorescence of the central leaflet of the first trifoliate leaf of 40 soybean plants was used for this experiment. Twenty plants were used as a control, and the other 20 plants were treated with GNPs. 200 μl of the GNPs solution, at a gold concentration of 33.86 mM, was deposited directly on the bottom surface of the central leaflet of the first trifoliate leaf, after the development of the third trifoliate leaf. The same amount of solution, free of nanoparticles (milli-Q water), was deposited directly on the bottom surface of the central leaflet of the first trifoliate leaf of the control plants. The fluorescence signal was collected from the upper surface of the central leaflet of the first trifoliate leaf.

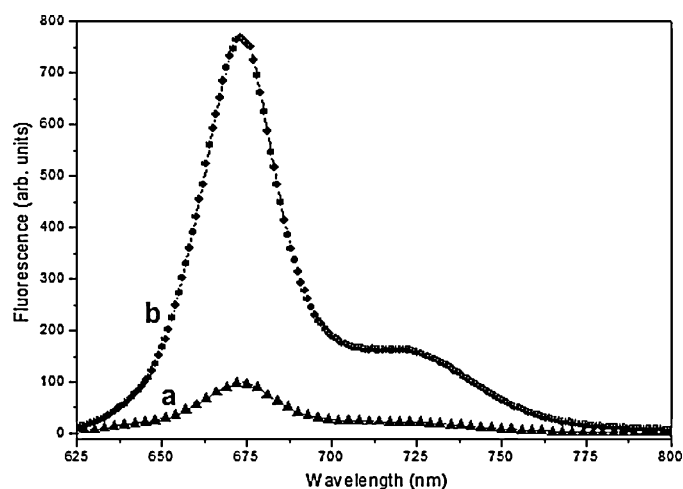## 3. Results and discussion

3.1. Chlorophyll extract

Aiming to characterize the interaction between gold nanoparticles and chlorophyll molecules, we started this study by analyzing the interaction between the chlorophyll extract and gold nanoparticles. As shown in Fig. 2, the 538-nm absorption band that is characteristic of the surface plasmon band of gold nanoparticles
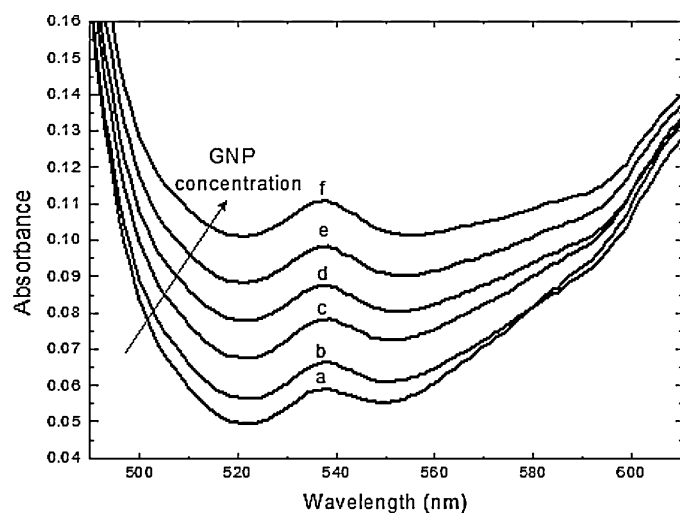
Fig. 2. Increase of the absorbance at plasmon resonance in the chlorophyll extract induced by nanoparticles of diameter 5 nm. GNP concentration at: (a) zero, (b) 3.6 μM, (c) 7.2 μM, (d) 10.6 μM, (e) 14.0 μM and (f) 17.3 μM.

Fig. 4. Fluorescence spectra of chlorophyll molecules in methanol solution. Excitation light at: (a) 532 nm and (b) 405 nm.

increases as a function of nanoparticle concentration. This effect is a result of the resonance plasmon absorption induced by gold nanoparticle surfaces; the cooperative oscillation of free electrons in the nanostructure is resonant with green light [20]. A linear increase was observed for 5-nm, 10-nm, and 20-nm sized GNPs, as shown in Fig. 3. However, the 20-nm GNPs showed the lowest absorption effect as a function of the GNP concentration, and an angular coefficient (β) of 1.1×10^{-3} was obtained by fitting the experimental data. The 5-nm and 10-nm GNPs showed a similar angular coefficient of approximately 3.0×10^{-3}. Nevertheless, it is important to mention that the addition of nanoparticles in the extract did not change the chlorophyll absorbance; the absorption of chlorophyll extract containing GNPs was the sum of chlorophyll and nanoparticle absorptions.

Fluorescence measurements of these same solutions of chlorophyll and GNPs were collected. Fig. 4 shows the typical fluorescence spectra of chlorophyll molecules when excited at 405 nm and

532 nm. Two emission bands were observed between 625 and 800 nm, the red and far-red bands with the maximum at 673 and 723 nm, respectively. Although the results showed that chlorophyll fluorescence induced by light at 405 nm was 8 times more intense than the fluorescence induced by light at 532 nm, both spectra had a similar shape. The observed difference in the fluorescence intensity may be probably due to the highest absorbance of the samples at 405 nm. Our results revealed that chlorophyll absorption at 405 nm was about 10 times higher than absorption at 532 nm (data not shown). Analyzing the F_{673}/F_{723} ratio, we see that the fluorescence intensity at 673 nm was 4.54 times more intense than the fluorescence intensity at 723 nm, for both excitation wavelengths. This ratio is calculated on the basis of the relationship between the peak fluorescence intensity at 673 to 723 nm.

When GNPs were added to the chlorophyll extract, there was no change in the fluorescence spectrum shape. However, the results showed that the GNPs induced a fluorescence quenching of the chlorophyll molecules. The fluorescence quenching was observed for both excitation wavelengths and for all GNP sizes. Table 1
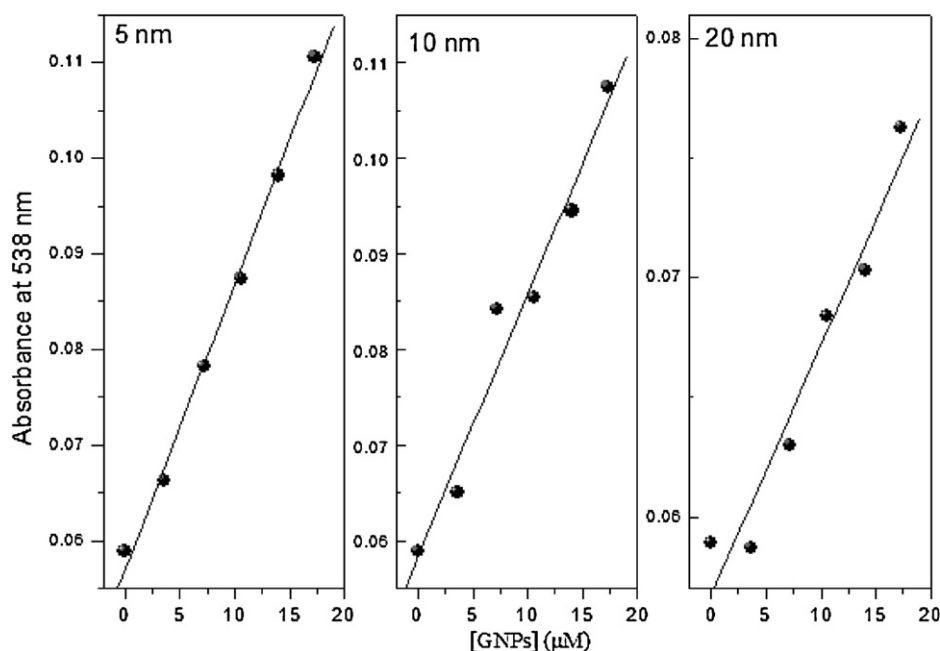
Fig. 3. Absorbance of the plasmon resonance at 538 nm in the chlorophyll extract as a function of GNP concentration.

Table 1
Chlorophyll fluorescence suppression as a function of GNP concentration and size, when excited at 405 nm and 532 nm.

[GNPs] (μM)		0.0	3.6	7.2	10.6	14.0	17.3
Fluorescence suppression (%)							
	5 nm	0.00	1.10	2.21	4.70	6.87	8.95
Excitation at 405 nm	10 nm	0.00	1.25	3.54	5.74	6.90	8.45
	20 nm	0.00	0.90	2.85	5.65	5.77	6.78
	5 nm	0.00	1.00	2.26	6.44	7.76	10.36
Excitation at 532 nm	10 nm	0.00	0.58	2.91	4.35	5.88	7.02
	20 nm	0.00	0.00	0.46	0.95	1.00	2.20

shows the percentage of fluorescence suppression as a function of GNP concentration. The suppression of chlorophyll fluorescence was linear, as a function of the GNP concentration, and the highest suppression rate was observed in the solutions of 5-nm GNPs for both excitation wavelengths. Fig. 5 shows that the plot of F_0/F versus [GNPs] is linear in the nanoparticle concentration range studied, revealing that the quenching follows the Stern–Volmer relation $F_0/F = 1 + K_{sv}[Q]$, where F_0 and F are the fluorescence intensities of chlorophyll in absence and presence of quencher Q (i.e., gold nanoparticles), respectively. K_{sv} is the Stern–Volmer quenching constant. From the slope of the plot, we can observe that there was a slight decrease in the quenching constant as a function of nanoparticle size when chlorophyll was excited at 405 nm. However, a higher decrease was determined when fluorescence analysis was performed by excitation at 532 nm where K_{sv} induced by nanoparticles of diameter 5 nm was 6 times higher than that of diameter 20 nm. The fluorescence quenching of a chlorophyll solution induced by 8 nm GNPs was recently reported by Barazzouk et al., who attributed this effect mainly to the process of photoinduced electron transfer from excited chlorophyll to GNPs [18], although the process of energy transfer was not ruled out because of a small overlap between absorption of GNPs and fluorescence of chlorophyll. They showed that the difference between the oxidation potential of excited chlorophyll molecules and the Fermi level of gold and the strong binding between chlorophyll and GNPs provide favorable conditions for electron transfer to occur. Therefore, instead of the excited electrons of chlorophyll molecules returning to the ground state emitting light radiation, the excited electrons are transferred to the GNPs, resulting in a decrease of the fluorescence signal. Thus, the highest suppression observed for the 5-nm GNPs solution was due to the fact that for the same concentration of GNPs, the samples prepared with 5-nm GNPs had the largest surface area. As a consequence, the largest number of chlorophyll molecules could be adsorbed on the surface of the nanoparticles, inducing the largest number of electron transfers from the excited chlorophyll to the GNPs.

3.2. In vivo analysis

Gold nanoparticles of 5 nm diameter were used to carry out the in vivo analysis. This size was chosen for two main reasons: (1) the 5 nm-sized GNPs induced the highest suppression rate of chlorophyll fluorescence in the extract analysis; (2) the smallest size of nanoparticles could induce better penetration and translocation of the nanoparticles in the plants. From our results, there was no observed alteration in the germination process and in the initial stage of plant growth induced by nanoparticles. Nevertheless, the results showed that the chlorophyll fluorescence bands in vivo were shifted 12 nm to the red when compared with the chlorophyll extract emission, and that the GNPs also induced a quenching of chlorophyll fluorescence in the plants. The quenching was observed for all plants submitted to the nanoparticle treatment and for both excitation wavelengths, 405 nm and 532 nm, as shown in Figs. 6 and 7, respectively. Despite the observed suppression, both groups (plants submitted to nano particles and control plants) had the same value of the F_{685}/F_{735} ratio. The ratio of about
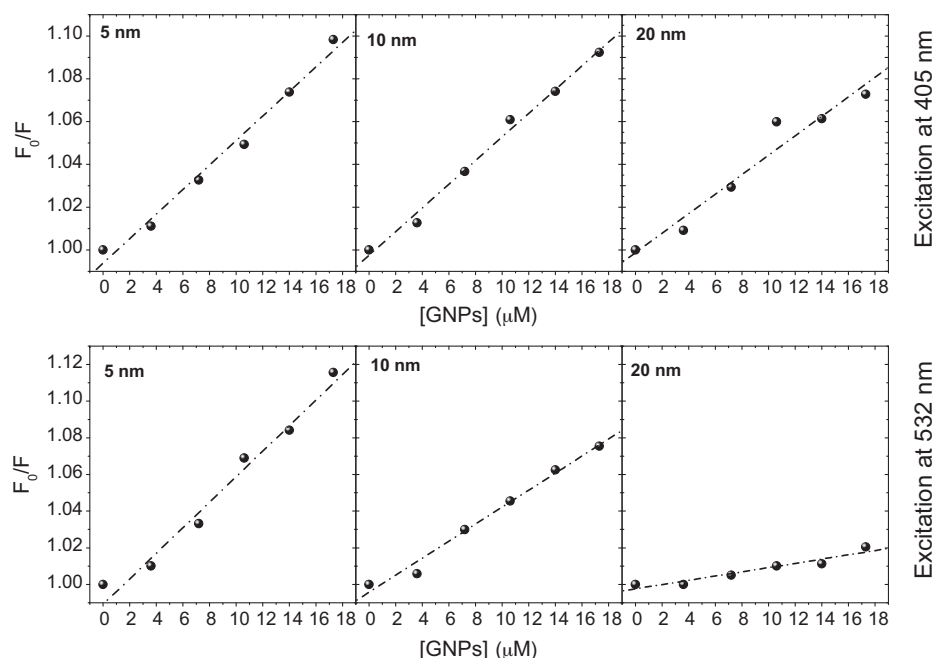
Fig. 5. F_0/F ratio at 673 nm as a function of GNP concentration for 5 nm, 10 nm, and 20 nm nanoparticle diameters, respectively. F_0 and F are the fluorescence intensities of chlorophyll in absence and presence of gold nanoparticles. Excitation wavelength at 405 nm (above) and 532 nm (below).
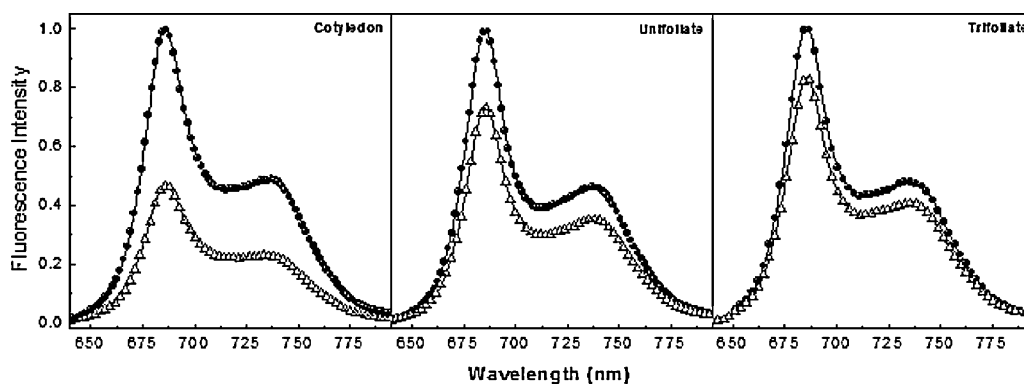
Fig. 6. Soybean plant fluorescence by excitation at 405 nm: control plants (●) and plants submitted to nanoparticle treatment (△).

2.05 was determined for the leaves excited at 405 nm, and 1.08 for the leaves excited at 532 nm. We can also observe that the overall shape of the fluorescence spectrum depends on the wavelength of the excitation light. Marcassa et al. reported a similar dependence in their experiments, where chlorophyll fluorescence of orange trees (*Citrus aurantium* L.) was obtained with excitation light at 443 nm and 532 nm [21].

The cotyledon leaves showed the highest fluorescence intensity suppression; the fluorescence quenching was 53% and 57% for excitation at 405 nm and 532 nm, respectively. The unifoliate leaves showed a fluorescence suppression of 26% and 44% for excitation at 405 nm and 532 nm, respectively. The lowest fluorescence suppression was observed for trifoliate leaves, where the fluorescence intensity of the leaves submitted to GNPs showed a reduction of 17% and 6% for excitation at 405 nm and 532 nm, respectively. These results suggest that the GNPs were able to penetrate into the seeds through the seed coats and translocate from seeds to leaves in soybean plants. A recent study showed that carbon nanotubes are able to penetrate the seed coat of tomato seeds [22]. Another study showed that pumpkin plants (*Cucurbita maxima*) grown in an aqueous medium containing iron oxide nanoparticles were able to absorb, translocate, and accumulate the nanoparticles in plant tissues [23]. The study showed that about 0.6% of the nanoparticles supplied were accumulated in the leaves, and 45.4% of the nanoparticles were detected in the roots. Lin et al. also showed that fullerene C70 could be easily taken up by roots and transported to shoots, in an experiment using rice plants (*Oryza sativa*) [24].

In the present study, the highest suppression detected in the cotyledon leaves revealed that the GNPs concentration was highest in these leaves. This result was expected, because cotyledon leaves originate from the seed, and the nanoparticles were deposited directly on the seed. In contrast, the lowest fluorescence quenching was observed for the trifoliate leaves, the tallest leaves and the last leaves to sprout. The fluorescence quenching could be attributed to the presence of GNPs in the leaves, because, as observed in the previous results, the excited electron of chlorophyll molecules can be transferred to GNPs, inducing the increase of non-photochemical quenching in plants. However, in the in vivo analysis, other mechanisms could be involved in the suppression of the fluorescence. For example, the presence of nanoparticles can inhibit the photosynthetic electron transport, inducing a dissipation of energy through non-photochemical processes. Changes in photosynthetic activity induced by ENPs in green algae (*Chlamydomonas reinhardtii*) were recently shown by Saison et al. They showed that copper oxide nanoparticles covered with polystyrene (core–shell type) induced cellular aggregation processes, and had a deteriorative effect on chlorophyll by inducing the photoinhibition of photosystem II. This inhibition of photosynthetic electron transport induced a strong dissipation of energy by non-photochemical processes [25]. In plants, reductions in chlorophyll fluorescence can also be associated with damage to reaction centers and the efficiency of electron transport in photosystem II [26]. In fact, the reorganization of the photosynthetic machinery, including changes in the size of the antenna complex, adjustments in chlorophyll and protein contents, and in the range of alternative energy-dissipation pathways have been reported as responses to stressors [27]. However, the same value of the F_{685}/F_{735} ratio obtained for the plants submitted to nanoparticle treatment and the control plants suggest that the effect of chlorophyll excited-electron transfer to gold nanoparticles was probably the dominant process in the chlorophyll quenching. This assumption stems from the expectation that environmental stresses can modify the chlorophyll concentration, and it is well known that the F_{685}/F_{735} ratio is a good indicator of the chlorophyll content and can be used as a non-destructive measure of
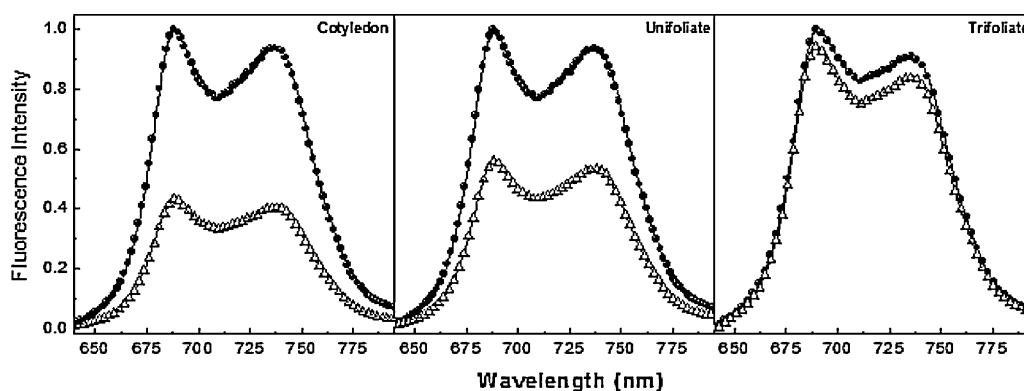

Fig. 7. Soybean plant fluorescence by excitation at 532 nm: control plants (●) and plants submitted to nanoparticle treatment (△).

chlorophyll concentration [4]. In fact, most environmental stresses are expected to slowly modify the chlorophyll concentration [6]. In a recent report, our group showed that the F_{685}/F_{735} can be used to detect water stress in soybean plants and to distinguish transgenic from conventional plants [28].

The results of an additional analysis, where nanoparticles were deposited directly on the leaf surface, showed that the presence of GNPs on leaves induced a suppression of 22% and 19% in the fluorescence intensity emitted by leaves when excited at 405 nm and 532 nm, respectively. These results confirm that the presence of nanoparticles on leaves can be responsible for the chlorophyll fluorescence suppression. As the nanoparticles were deposited on the bottom surface of the leaves and the fluorescence measurements were carried out on the upper surface of the leaves, there was no contribution of the shadow effect (by blocking the light absorption) to the fluorescence quenching. Therefore, the results suggest that the GNPs were able to penetrate the leaf surface, possibly through the stomatal aperture, and to interact with the photosynthetic apparatus. This indication is supported by recent studies which have been reported that nanoparticles can be taken up by plants via the stomatal pores [29,30].

4. Conclusions

The data revealed that gold nanoparticles induce quenching of chlorophyll fluorescence, and that the quenching depends on the particle size and concentration. This phenomenon is mainly attributed to the effect of photoinduced electron transfer from excited chlorophyll molecules to gold nanoparticles, resulting in a decreased chlorophyll fluorescence signal. The largest fluorescence suppression effect was induced by gold nanoparticles sized 5 nm. This effect was due to the largest surface area available for molecular adsorption on nanoparticles, for sample solutions with the same concentration of GNPs. Because the available nanoparticle surface area also depends on the concentration of the nanoparticles, the degree of chlorophyll fluorescence suppression was dependent on the GNPs concentration.

This study has provided the first in vivo observation of chlorophyll fluorescence quenching induced by gold nanoparticles. The results showed that laser-induced fluorescence spectroscopy can be used to investigate the alterations in the physiological response of plants induced by gold nanoparticles, and that both excitation wavelengths, 405 nm and 532 nm, were able to detect the presence of the gold nanoparticles inside the plants. Despite nanoparticles have been deposited on the seeds, the interaction between nanoparticles and the supramolecular light-harvesting complex of photosystem II was observed in the later stages of plant development. The results suggest that the GNPs were able to penetrate into the seeds and translocate to the leaves. The data also suggest that different concentrations of nanoparticles were accumulated in the cotyledon, unifoliate, and trifoliate leaves. The highest nanoparticle concentration was observed in the cotyledon leaves, and the lowest accumulation of nanoparticles was detected in the trifoliate leaves. Nevertheless, in spite of these advances, it is evident that further investigations must be conducted to clarify the processes of penetration, translocation, and accumulation of nanoparticles in plants.

Acknowledgments

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