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Integrated Biophysical Assessment of the Effect of Zinc Oxide Nanoparticles on the Photosynthetic Apparatus of Spinach (*Spinacia oleracea* L.)

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Abstract. *Zinc oxide nanoparticles have been reported as the most common nanostructured materials in agricultural areas and, recently, researchers found that the concentration of zinc oxide nanoparticles in the soils and air is growing, causing concerns in society regarding their possible effects on plant photosynthesis. This study used an integrated biophysical approach that incorporated three different techniques to assess the short-term effect of zinc oxide nanoparticles on the photobiochemical apparatus of spinach (Spinacia oleracea L.): (i) delayed luminescence measurements of isolated thylakoid membranes to determine Photosystem II charge recombination, (ii) Clark-type oxygen electrode measurements for the determination of photosynthetic oxygen evolution from isolated chloroplasts, and (iii) UV-Vis spectrophotometric analysis of acetone-extracted chlorophyll pigments exposed to red, blue, and white LED light for photostability assessment. Zinc oxide nanoparticles treated samples displayed a gradual decrease in the levels of delayed luminescence over 60 min of dark incubation, while control samples remained quite unchanged, implying disturbed charge recombination in P_{680}^{+}/Q_A^{-} pair of PSII (reaction center). It was observed that photosynthetic oxygen production gradually declined in both the control and the treated chloroplast samples, yet the initially high rates were retained in the latter. UV-Vis spectrophotometric analysis of the extracted chlorophyll pigments showed a faster decrease in the main chlorophyll absorption peaks at 437 nm (Soret) and 668 nm (Qy) in ZnO-treated samples, an effect that was most evident under white light.*

Keywords: *environmental nanotoxicology, ZnO nanoparticles, Photosystem II, thylakoid membranes, electron transport, photobleaching, chlorophyll degradation*

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Introduction

Well known that nanoparticles due to smaller sizes interact strongly with biological systems, they have found broad use in agriculture (Mgadi et al., 2024), plant physiology (Singh et al., 2024), biotechnology processes (Vinukonda et al., 2025), medical practices (Haleem et al., 2023), diagnostics, drug delivery (Paluszkiewicz et al., 2021) and therapeutic applications (Yetisgin et al., 2020). Agriculture is the leading segment among industries trying out this potential: using tiny particles for delivery of agrochemicals only in the needed area and at the right time, minimizing losses caused by leaching, drift, hydrolysis, photolysis, and microbial degradation of conventional pesticides and fertilizers, while nanosensors and slow-release nanofertilizers help reduce pollution by better input management (Jainendra et al., 2024). This increased use of engineered nanomaterials has turned zinc oxide nanoparticles (ZnO NPs) into a top-produced, nanostructured material thanks to their catalytic, antimicrobial, and UV-absorbing properties for the agriculture, cosmetics, and consumer goods sectors.

Nevertheless, these very qualities that make ZnO NPs highly useful for farmers have also been the reasons for environmental and human health concerns when they were released. For instance, exposure to high concentrations of ZnO nanoparticles has been reported to significantly suppress seedlings emergence and root development in ryegrass and a subsequent study on the same species found that ZnO NP exposure reduced biomass and caused severe root damage, with root cells becoming highly vacuolated or collapsed, while most nanoparticles remained adhered to the root surface and translocation of free zinc ions to shoots stayed minimal (Rehman et al., 2023). Beyond the root system, ZnO NPs have also been reported to reduce photosynthetic efficiency by disturbing electron transport, altering membrane integrity, and inducing oxidative stress (Chen et al., 2024).

Despite this evidence, most existing studies characterize ZnO NP phytotoxicity at a single organizational level whole-plant biomass, root morphology, or bulk photosynthetic efficiency without resolving which step of the photosynthetic process is primarily affected, or whether ZnO NPs act mainly at the level of Photosystem II (PSII) reaction-center photochemistry, the downstream electron-transport and water-splitting machinery, or the chlorophyll pigment pool itself (Dang et al., 2024). Addressing this gap requires techniques that can isolate and probe each step individually, since PSII, embedded in the thylakoid membrane, depends on a finely tuned sequence of light-driven charge separations, electron transfers, and water-splitting reactions that make it one of the most sensitive targets of nanoparticle-induced stress (Wang et al., 2016).

Three complementary biophysical techniques can meet this need. Delayed luminescence (DL) arises from the recombination of charges separated during PSII photochemistry, providing a sensitive, non-destructive readout of PSII reaction-center function. Oxygen evolution, measured polarographically with a Clark-type electrode, gives a direct measure of PSII's water-splitting and electron-transport capacity, especially with an artificial acceptor such as DCBQ. The absorption spectrum of extracted chlorophyll, with its Soret band near 430–440 nm and Qy band near 660–670 nm, reflects pigment integrity, and its light-dependent decay indexes photo-oxidative degradation.

The present study therefore combines these three approaches to determine how short-term ZnO NP exposure affects the photosynthetic apparatus of spinach (*Spinacia oleracea* L.) at three organizational levels isolated thylakoid membranes, intact chloroplasts, and the free pigment pool under different light qualities asking whether ZnO NPs primarily disturb PSII photochemistry, the electron-transport chain, the pigment pool, or all three simultaneously, and whether this depends on light exposure.

Materials and Methods

Plant Material and ZnO Nanoparticle Suspension

Fresh leaves of spinach *(Spinacia oleracea L.)* were used as the source of thylakoid membranes, chloroplasts, and pigment extracts in all three experiments. Zinc oxide nanoparticles (99.8% purity, 10–30 nm particle size, SkySpring Nanomaterials, Inc.) were used throughout. In each experiment, a ZnO stock suspension was freshly prepared by dispersing 10 mg of ZnO powder in 100 mL of distilled water, followed by ultrasonic sonication (15–20 min) to ensure homogeneous dispersion of the particles before further dilution into the respective assay mixtures.

Oxygen Evolution of Isolated Chloroplasts

Photosynthetic oxygen evolution was measured polarographically using a Clark-type oxygen electrode. 2,6-dichloro-p-benzoquinone (DCBQ) was used as an artificial electron acceptor to assess the efficiency of electron transport independently of downstream (Calvin cycle) limitations. The ZnO-treated reaction mixture consisted of 7.0 mL HEPES buffer (pH 7.0), 70 µL of chloroplast suspension, and 700 µL of the ZnO nanoparticle suspension (100 µg/mL stock), giving a total volume of approximately 7.77 mL and a final ZnO concentration of approximately 9 µg/mL. The control mixture consisted of 5 mL HEPES buffer (pH 7.0) and 50 µL of chloroplast suspension, with no ZnO added. Both mixtures were kept in the dark on ice throughout the incubation period to minimize uncontrolled photochemical or thermal degradation.

At each time point, a 1 mL aliquot was withdrawn from the incubation mixture. Before each measurement, 5 µL of DCBQ was pipetted into the sample; the oxygen evolution was measured by a Clark electrode approximately 20 seconds after this step. The control group was exposed to four time points of measurements (0, 30, 60, and 90 min), while the ZnO-treated group was subjected to six of them (0, 15, 30, 45, 60, and 90 min). Time series obtained from the electrode response were digitized and converted with respect to the time elapsed to analyze the pattern of oxygen production over time.

Delayed Luminescence of Isolated Thylakoid Membranes

Isolated spinach thylakoid membranes were used as the biological material. Two reaction mixtures were prepared in MES buffer (25 mM, pH 6.5): a control mixture consisting of 10 mL buffer containing only the thylakoid membranes, and a treatment mixture consisting of 9 mL buffer plus 1 mL of the ZnO nanoparticle suspension. Two corresponding stock samples were prepared in parallel one containing 30 µL of thylakoid suspension alone (control stock) and one containing 30 µL of thylakoid suspension plus 3 µL of the ZnO suspension. The latter corresponded to a final ZnO concentration of 9.09 mg/L in the treatment sample.

For each measurement, 4 µL of the appropriate stock was placed quartz cuvette and combined with 2 mL of buffer. The sample was kept in the dark for approximately 3 minutes to allow thermal relaxation and to minimize photooxidative processes before recording. Delayed luminescence was then excited with a high-intensity light pulse (1100 units, ~2s) and recorded with a chart-recorder DL apparatus. Measurements were performed at 0, 30, and 60 minutes of incubation for both the control and the ZnO-treated samples, all under dark, ice-cold conditions to minimize spontaneous photooxidative degradation during the incubation period.

Chlorophyll Pigment Extraction and Light-Dependent UV-Vis Absorption Spectroscopy

A ZnO nanoparticle suspension was prepared by dispersing 10 mg of ZnO powder in 100 mL of distilled water and homogenizing the mixture for 15 minutes in an ultrasonic sonicator. The photosynthetic pigment extract was obtained by homogenizing 1 g of fresh spinach leaves in 100 mL of 95% acetone. Homogenization was carried out by mixing it thoroughly and then it was centrifuged at 15, 000 rpm for five minutes keeping the sample at 4 ºC. After that, only the supernatant was used for carrying out the experiments.

To prepare the ZnO-treated samples, 7 mL of the ZnO suspension was added to 35 mL of pigment extract to obtain a homogeneous mixture; control samples consisted solely of the pigment extract. For spectrophotometric analysis, 1 mL aliquots of both the control and ZnO-treated samples were mixed with 2 mL of 95% acetone. Absorption spectra were recorded on a Shimadzu UV-1280 UV-Visible spectrophotometer over the wavelength range of 350–750 nm.

Samples were incubated under white, red, and blue LED light sources at an intensity of 50 µmol $m^{-2}s^{-1}$, and UV-Vis absorption spectra were recorded at successive incubation intervals for both control and ZnO-treated extracts under each of the three light qualities. This design allowed the rate of light-driven chlorophyll pigment degradation to be compared between untreated extracts and extracts exposed to ZnO nanoparticles, separately for red, blue, and white illumination.

Results

ZnO Nanoparticles Accelerate Light-Dependent Bleaching of Extracted Chlorophyll

It is well known that light plays a powerful modulator role in the synthesis of nanoparticles, significantly influencing their morphological and functional properties (Rasulzadeh & Ramazanli, 2025). The absorption spectra of the acetone-extracted pigment fraction showed the two characteristic chlorophyll bands expected for this extraction method: a blue-region (Soret) band near 430–440 nm and a red-region (Q_y) band near 660–670 nm, the latter serving as the principal diagnostic peak for chlorophyll a integrity. Under all three light qualities tested (red, blue, and white LED, 50 µmol m^{-2} s^{-1}), both the control and the ZnO-treated pigment extracts showed a progressive decline in the height of these bands with increasing illumination time, consistent with light-driven bleaching of chlorophyll during incubation. However, the rate of this decline was consistently greater in the ZnO-treated extracts.

Table 1

Chlorophyll Q_y absorption peak (~668 nm) of control and ZnO-treated pigment extracts under red, blue, and white light illumination (0, 30, and 60 min of incubation)

Light group / Time, min.	Red, control	Red, ZnO	Blue, control	Blue, ZnO	White, control	White, ZnO
0 min	0.920	0.869	0.962	0.945	0.633	0.492
30 min	0.717	0.471	0.701	0.535	0.479	0.281
60 min	0.492	0.299	0.532	0.415	0.348	0.187
Decline at 60 min	46.5%	65.6%	44.7%	56.1%	45.0%	62.0%

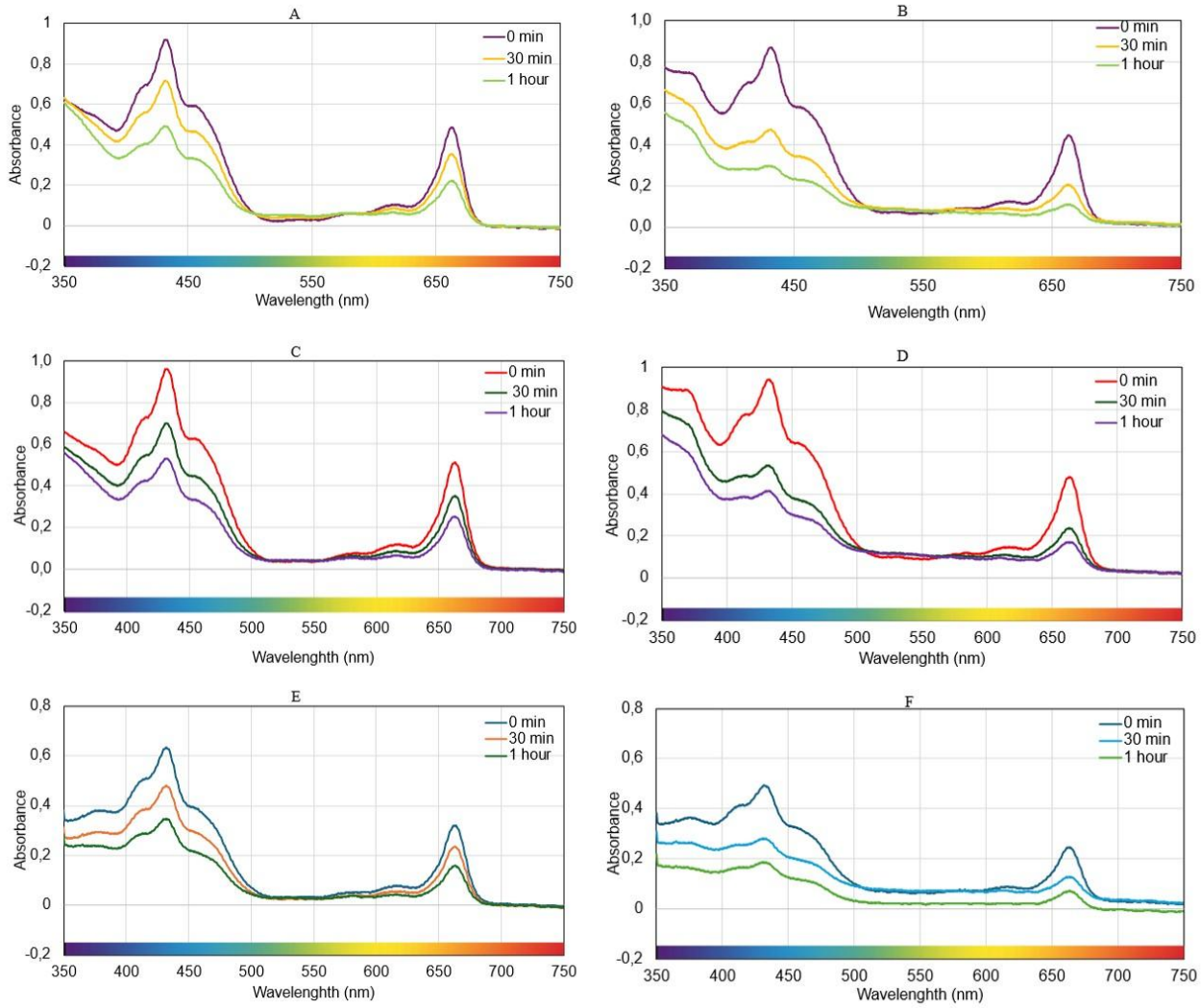


Figure 1

Absorption spectra (350–750 nm) of acetone-extracted chlorophyll, exposed to red, blue, and white LED illumination (50 µmol m^{-2} s^{-1}), for control extracts (A, C, E) and ZnO-treated extracts (B, D, F), recorded at 0 min, 30 min, and 1 hour

Under red light, the Q_y peak of the control extract declined 46.5% over 60 minutes, while the ZnO-treated extract fell from declined 65.6% over the same period despite starting from a similar initial value, the ZnO-treated sample lost pigment absorbance markedly faster. A similar pattern was observed under blue light, where the control declined by 44.7% and the ZnO-treated extract by 56.1%, and under white light, where the control declined by 45.0% and the ZnO-treated extract by 62.0%. The corresponding Soret-band absorbance (~437 nm) followed the same trend in all three light conditions, confirming that the effect was not restricted to a single spectral feature but reflected an overall loss of intact chlorophyll in the extract. White-light illumination produced the most pronounced overall effect (Ramazanli, 2024): the ZnO-treated extract already showed a lower Qy absorbance than the control at the beginning (0.492 vs. 0.633), before any additional incubation time had elapsed, suggesting that under broad-spectrum white light the interaction between ZnO nanoparticles and chlorophyll begins essentially immediately upon mixing, rather than developing gradually as observed under monochromatic red or blue light. By 60 minutes of white-light exposure, the ZnO-treated extract retained only about 30% of the control's initial Qy absorbance, compared with roughly 55% retention in the control itself.

Oxygen Evolution Declines Gradually in Both Control and Zno-Treated Chloroplasts

Photosynthetic oxygen evolution, decreased over time in both the control and the ZnO-treated thylokoid preparations, but the two groups differed in their starting values and in the shape of the decline.

In the control group the amount of oxygen evolved over time was set as 100% at the beginning and then slowly decreased to 90.3% (after 60 and 90 min) that is a relative decrease of 9.7% compared with the period during the experiment. Similarly, in the ZnO-treated group, the oxygen production was considered as 100% at time zero, there was no change in the first 15 min but decreased later to 89.2% at 60 and 90 min, a relative decrease of 10.8% compared to the starting point.

Notably, the ZnO-treated samples maintained a higher absolute oxygen-evolution signal than the control at every measured time point. However, because the two groups started from different baseline activities, the absolute comparison cannot, by itself, be used to conclude that ZnO nanoparticles stimulated PSII activity; it more plausibly reflects batch-to-batch variability in thylokoid preparation combined with a genuine, but proportionally similar, time-dependent decline shared by both groups. The comparable relative rates of decline (9.7% vs. 10.8%) suggest that, at the ZnO concentration used here (approximately 9 µg/mL), electron transport through PSII was not severely disrupted, and that both groups predominantly reflect the natural loss of thylokoid activity with incubation time for example, gradual destabilization of the isolated thylokoid preparation, weakening of membrane integrity, or natural deactivation of enzyme systems outside the thylakoid rather than an acute ZnO-induced inhibition of electron transport. The absence of an additional, faster decline specific to the ZnO group also suggests that the nanoparticle concentration used was not high enough to induce severe oxidative stress in the chloroplast preparation under these short-term, dark, ice-cold conditions (Hsu et al., 2023).

Delayed Luminescence Diminishes Continually in ZnO-Treated Thylakoids

Thylakoid membranes of control plants gave almost the same delayed luminescence signal from 0 to 60 min dark incubation except that intensity even got a little higher (~7%). Such constant DL is an indication that isolation procedure, buffer system (MES) and incubation conditions (dark cold on ice) little change the functionality of the photosystem for the course of the experiment. Hence, any variations found in the group of ZnO can not be explained by deterioration of the thylakoid preparation only. On the other hand, thylakoid membranes exposed to ZnO nanoparticles had consistently lower levels of delayed luminescence (DL) at every time point than the controls, with the DL gradually declining during the incubation period.

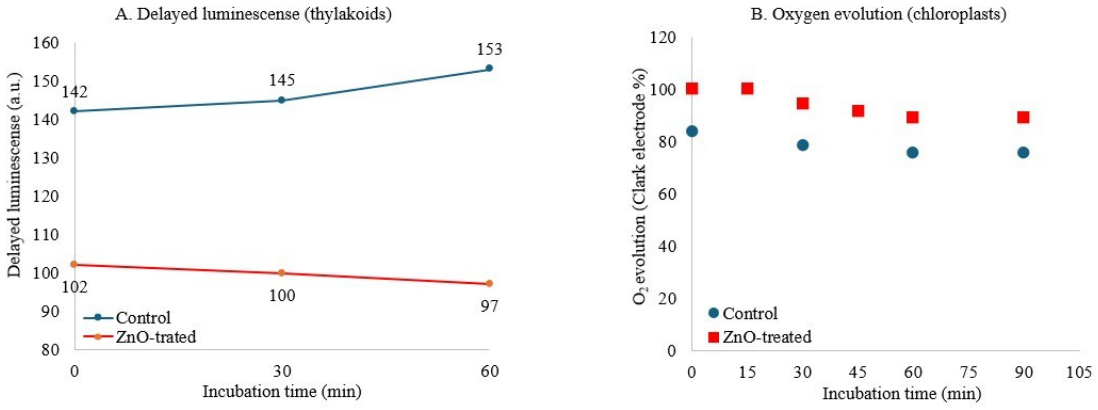


Figure 2

(A) Delayed luminescence of control and ZnO-treated spinach thylakoid membranes over 60 min of dark incubation. (B) Photosynthetic oxygen evolution of control and ZnO-treated isolated chloroplasts, measured with a Clark-type electrode

Relative to the control at the same time point, ZnO-treated samples showed a 28% lower DL amplitude at 0 min, a 31% lower amplitude at 30 min, and a 37% lower amplitude at 60 min a widening gap that indicates a progressive, rather than instantaneous, perturbation of PSII charge recombination by the nanoparticles. Because the experiment was conducted entirely in the dark and on ice, extensive light-driven reactive oxygen species (ROS) generation is unlikely to explain this decline; the pattern is more consistent with a direct physicochemical interaction between ZnO nanoparticles and the thylakoid membrane that develops over the incubation period.

Discussion

The three complementary assays revealed distinct mechanisms of ZnO nanoparticle–photosystem interaction. Delayed luminescence demonstrated gradual, dark-developing impairment of PSII charge recombination, indicating direct physicochemical interactions between nanoparticles and thylakoid membranes rather than light-driven reactive oxygen species (ROS) production. Similarly, these results are consistent with studies that had already explained the ZnO-induced suppression of the photosynthetic efficiency by electron transport and alterations of membrane integrity. But, when it comes to oxygen evolution the control and ZnO samples had similar decrease in relative rates (9.7% vs. 10.8%), indicating the electron transport capacity had not been severely affected by that concentration of ZnO.

This difference could very well explain why some methods can find the electron transport perturbation before it becomes obvious, such as through changes in bulk of the electron transport capacity. Chlorophyll absorption spectroscopy revealed a third mechanism: accelerated photo-oxidative bleaching under illumination. Zinc oxide nanoparticles have a well-documented photocatalytic activity that allows them to generate a significant amount of ROS under different light wavelengths, white. red, blue, and especially white light, thereby triggering the degradation of pigment molecules in unprotected form. The response of photosynthetic membranes to illumination was a major factor and far greater than under the dark condition, which indicates that the light availability is one of the crucial features for the plant toxicity due to ZnO nanoparticles. DL and photooxidation of water methods used different biological samples and buffer systems, so they did not allow for a fair quantitative comparison on this basis. Also, the assay for pigments made use of cell-free extracts hence it was not influenced by intact membrane protection effects or photoprotective mechanisms that are operational in planta.

Conclusion

This study demonstrates that ZnO nanoparticles perturb spinach photosynthesis through at least two distinguishable mechanisms: (1) light-independent impairment of PSII charge recombination, and (2) light-dependent acceleration of chlorophyll photo-oxidation. Delayed luminescence proved most sensitive for detecting subtle reaction-center perturbations, while oxygen-evolution measurements confirmed that bulk electron transport remains relatively intact at tested concentrations. These results show that delayed luminescence and pigment absorption spectroscopy are two powerful methods in complementing each other and allowing for non-invasive assessments of nanoparticle-photosystem interactions in plant systems. The results underline the necessity of including both dark and light conditions in the study of nanomaterial-related plant toxicity.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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