

Original Research Paper

Exploring the Synergistic Effects of Agitation on the Interaction Between Nanoparticles of Polyethylene and Benzophenone in Microalgae *Tetraselmis* sp.

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Abstract: Microalgae play a crucial role in the aquatic food chain, climate change mitigation and economic applications. Several factors, such as light, temperature, nutrients, agitation and pollutants, can significantly influence their growth. In SPIE of its importance, there is a lack of research concerning the effects of agitation on the interaction between different pollutants and phytoplanktonic microorganisms. This study aimed to investigate the impact of agitation on the growth and cellular response of *Tetraselmis* sp. exposed to BP3 and polyethylene nanoparticles. Results showed that agitation significantly inhibited microalgae growth, in the presence of BP3 and nanoplastic. Considering photosynthetic pigments, there was a reduction in the concentration of Chl a under agitation, regardless of contaminants. Furthermore, Chl b concentrations remained unchanged across all experimental conditions. Additionally, a noticeable increase in carotenoid levels was observed in groups with higher concentration of BP3 (with agitation) and BP3 + NP (with or without agitation). An elevation in SOD and CAT enzyme activity was observed, indicating oxidative stress. But agitation did not impact MDA levels. These findings highlight the importance of considering agitation when evaluating the interaction of pollutants with planktonic organisms in toxicological studies, enhancing the understanding of environmental stressors in natural ecosystems.

Keywords: Microalgae, Plastic Pollution, Polyethylene, Agitation, Photosynthetic Pigments, Oxidative Stress

Introduction

Microalgae are essential components of both the environment and the economy, playing a significant role in various aspects. As photosynthetic organisms, they form the basis of the aquatic food chain, facilitating the transfer of energy from lower to higher trophic levels. Moreover, microalgae contribute significantly to climate change mitigation, through to their ability to fix CO₂ and generate atmospheric oxygen through photosynthesis. Furthermore, certain microalgae species are particularly abundant in lipids, making them suitable candidates for biofuel production (Mata *et al.*, 2010; Maeda *et al.*, 2018; Sarwer *et al.*, 2022).

Several factors can impact the growth of microalgae in their natural environment. These include factors such as

light intensity, temperature, nutrient availability, pH levels, aeration and exposure to marine currents. Among them, agitation movements can play a crucial role, exerting a substantial influence on a range of processes. These processes include metabolic rates, biomass generation, as well as the intricate interactions between microalgae and environmental pollutants (Sarker and Kaparaju, 2023).

The use of plastics as a substitute for traditional materials such as glass, metals and wood has increased significantly. The increase in plastic waste in natural ecosystems has shown a significant increase because of widespread production and improper disposal practices (Geyer *et al.*, 2017).

In the environment, plastic fragments occur in different dimensions. Macroplastics (>5 mm) are subject

to a process of degradation, resulting in the formation of progressively smaller particles. The degradation process results in the formation of Microplastic (MP) particles (<5 mm) and Nanoplastics (NP) (<1 µm), which have a tendency to accumulate in the environment, particularly in aquatic ecosystems (Sommer *et al.*, 2018).

Microplastics (MPs) and Nanoparticles (NPs) possess a high surface-to-volume ratio due to their small size, making them susceptible to contamination by different pollutants. These pollutants can adhere to their hydrophobic and usually irregular surfaces, in addition to additives that are incorporated during the plastic manufacture (Sommer *et al.*, 2018).

Polyethylene (PE) is the most common plastic polymer that is used in both production and disposal, constituting approximately 60% of the worldwide plastic material consumption. In addition, PE exhibits a remarkable resistance to degradation in the natural environment, which enables it to withstand damage and persist over extended periods of time. As a consequence, polyethylene tends to accumulate significantly in the environment (Ghatge *et al.*, 2020).

In addition, chemical components also have an impact on the environment in addition to the presence of plastic particles.

Benzophenone-3 (BP3), a compound commonly found in personal care products such as cosmetics, is used to protect the human body against the damaging effects of Ultraviolet (UV) radiation by means of its capacity to absorb and disperse the UV rays. The use of BP3 started in the 1950s for the production of organic sunscreens and cosmetics filters. Furthermore, BP3 has been used on a global scale in insecticides and as an additive in plastics for UV protection and stabilization (Sanad *et al.*, 2010; Oliveira *et al.*, 2004; Sánchez-Quiles and Tovar-Sánchez, 2015).

This study was carried out due to the lack of data on the effects of substances and NPs on phytoplanktonic organisms in conditions under agitation in the cultivation medium. For this purpose, *Tetraselmis* sp. was cultured in the presence of BP3 and BP3 combined with NP (BP3 + NP), with and without agitation of the cultivation medium. The experimental condition was established considering the continuous movement of phytoplanktonic organisms in their natural environment, which is influenced by marine currents and human activities.

Materials and Methods

Materials

Nanoplastics of Polyethylene (NP) with dimensions from 200-990 nm were purchased from Cospheric LLC and Benzopheno-3 (BP3) (PHR1080) from sigma-aldrich® with 99.5% purity was used as a contaminant.

Microalgae of *Tetraselmis* sp. were collected from Guanabara Bay (Rio de Janeiro, Brazil) close to Ilha do

Fundo (22°50'40"S and 43°12'40"W). For this research, microalgae were cultivated in the Laboratório de Fitoplâncton Marinho at the instituto de Biologia at UFRJ.

Microalgae cultures were kept in Guillard's (F/2) marine water enrichment medium (salinity between 28 and 30%), being prepared with filtered natural seawater (0.22 µm pore filter) and previously autoclaved. The cultures were maintained in a germination chamber (BOD incubator) with 100 mE/m²/s of irradiance at 22°C and a photoperiod of 16 h of light and 8 h of darkness.

Methods

All methods were performed in triplicate in 6 or 12-well plates with an initial number of 3×10⁴ cells and a duration of 96 h. Different tests were performed to investigate the effects of BP3 in isolation and in combination with NP on microalgae. Since BP3 is insoluble in water but soluble in alcohol, 10 mg of BP3 was first dissolved in 10 mL of absolute ethanol. Then, the solution was diluted in F/2 medium to form a stock solution of BP3 with a concentration of 1 mg. L⁻¹. In order to assess the effects of ethanol on the growth of microalgae, a solvent control was used by adding ethanol into the medium, resulting in a final concentration of 0.01% (v/v).

The concentration of NP was determined based on preliminary assays. Test concentrations ranged from 1-300 mg. L⁻¹ and showed no significant changes. The absence of precise estimates of nanoplastic concentrations resulted in the selection of an intermediate concentration within the tested range. The concentration of 100 mg. L⁻¹ was defined for assays in which microalgae were exposed to BP3 combined with NP, the assay, in which the microalgae were exposed to ethanol, showed that ethanol did not interfere with cell growth.

All experimental conditions were performed both without and with agitation using rocking shakers Kasvi® (30 movements per min). The tested groups are shown in Table 1.

Table 1: Experimental groups, namely BP3 and BP3 + NP and their respective conditions, including both with agitation and without agitation

Teste groups	Group		Experimental
	Without shaker®	With shaker®	
Microalgae	A-0	SA-0	Control (without BP3 or NP)
	A-0.1	SA-0.1	BP3 at 0.1 mg.L ⁻¹
Microalgae	A-0.5	SA-0.5	BP3 at 0.5 mg.L ⁻¹
	exposed only to BP3	A-1	BP3 at 1 mg.L ⁻¹
Microalgae	A-2	SA-2	BP3 at 2 mg.L ⁻¹
	exposed only to NP	B-0	NP (100 mg.L ⁻¹)
Microalgae	B-0.1	SB-0.1	NP (100 mg.L ⁻¹) + BP3 (0.1 mg.L ⁻¹)
	exposed to plastic mixture (NP + BP3)	B-0.5	NP (100 mg.L ⁻¹) + BP3 (0.5 mg.L ⁻¹)
	B-1	SB-1	NP (100 mg.L ⁻¹) + BP3 (1 mg.L ⁻¹)
	B-2	SB-2	NP (100 mg.L ⁻¹) + BP3 (2 mg.L ⁻¹)

NP = Nanoparticle; S = using Shaker®

Firstly, pilot assays were carried out using only NP at different concentrations in order to find out the possible effects of NP on the growth of microalgae. The concentration of NP at 100 mg. L⁻¹ was selected as the basic concentration used for NP + BP3 solution (the plastic mixture solution). For this, 100 mg of NP was added to a volume of 1,000 mL of F/2 medium. Based on the obtained EC₅₀ value (0.67 mg. L⁻¹), solutions of BP3 at concentrations of 0.1, 0.5, 1 and 2 mg. L⁻¹ were used for the entire experiment.

All assays were performed in 12-well plates with a volume of 4 mL; the initial number of cells was 3×10⁴ cells/well. To verify the cell growth, the microalgae were fixed in a 1% acetic Lugol solution. The measurement of cell density was performed at 96 h using a Neubauer chamber under a light microscope (Leica DM750). For each condition, the average rate and the amount of the average specific growth rate were determined for each experimental condition (group).

Statistical analyses were performed on the GraphPad prism (version 7) using a nonparametric one-way ANOVA test with a Tukey post-test.

Photosynthetic Pigments

Photosynthetic pigments play a vital role in the survival of microalgae and serve as the primary internal factor that can potentially restrict photosynthesis. For the determination of photosynthetic pigments, such as Chlorophyll a (Chl a), Chlorophyll b (Chl b) and carotenoids, 10⁵ microalgae were centrifuged at 10,000 rpm for 5 min. Subsequently, the pellet was resuspended in methanol (99%) and incubated in the dark for 24 h at 45°C. After incubation, the reading of Chl a, Chl b and carotenoids was performed at 470, 652 and 665 nm on a spectrophotometer (SpectraMax M5). The content of photosynthetic pigments was determined according to Lichtenthaler (1987); Pancha *et al.* (2015).

Enzymes Assays

For oxidative stress analysis, Superoxide Dismutase (SOD), Catalase (CAT) and Malondialdehyde (MDA) were measured using a spectrophotometer spectra max M5. All assays were performed in triplicate.

The activity of total SOD, an enzyme that catalyzes the dismutation of superoxide radicals (O₂⁻) into Hydrogen Peroxide (H₂O₂) and molecular Oxygen (O₂), was measured using the SOD assay kit (19160-1kT-F; Sigma-Aldrich). For evaluating the activity of the CAT enzyme, samples were incubated with H₂O₂ in Phosphate buffer (PBS) 0.1 m at a proportion of 20 µL of sample/180 µL of H₂O₂. adapted from (Aebi, 1984; Bannister and Calabrese, 1987).

The Thiobarbituric Acid Reactive Substance (TBARS) assay was used to detect lipid oxidation. This assay measures MDA, which is one of several end products of lipid peroxidation. For this, the samples were treated with thiobarbituric acid and the absorbance was read at 450, 532 and 600 nm (Hodges *et al.*, 1999; Pancha *et al.*, 2015).

Results

Growth of *Tetraselmis* sp.

An evident decrease in the growth of microalgae was observed after their exposure to BP3, especially in groups A-0.5, A-1 and 2, where the microalgae were only exposed to BP3. Nevertheless, the growth of microalgae was significantly inhibited when exposed to BP3 while being shaken (control groups SA-0 and SB-0). A significant reduction in growth was observed when microalgae were exposed to BP3 under shaking conditions, which was more evident in the groups SA-1 and SA-2. Furthermore, when microalgae were exposed to BP3 + NP (groups SB-0.1, SB-0.5, 1 and 2), the decrease in growth was even more pronounced (Fig. 1).

Evaluation of Photosynthetic Parameters

The findings of the photosynthetic pigment analysis indicated that there was no statistically significant alteration in the concentrations of Chl a for the groups that were not in agitation. However, Chl a concentration was increased in all groups that were exposed to BP3 and to BP3 + NP while in agitation (Fig. 2A), but the concentrations of Chl b did not change much in any groups (Fig. 2B). An increase in carotenoid levels was also observed in the groups treated with the highest concentrations of BP3 and BP3+NP, with or without agitation (Fig. 2C).

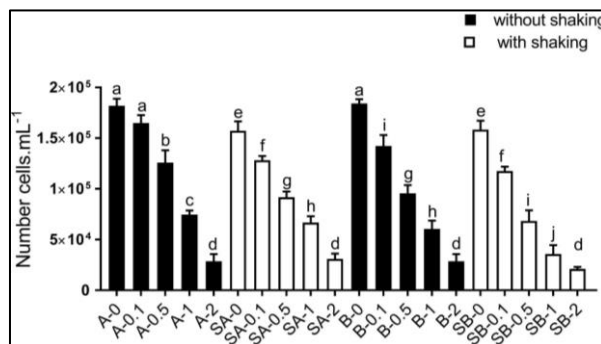
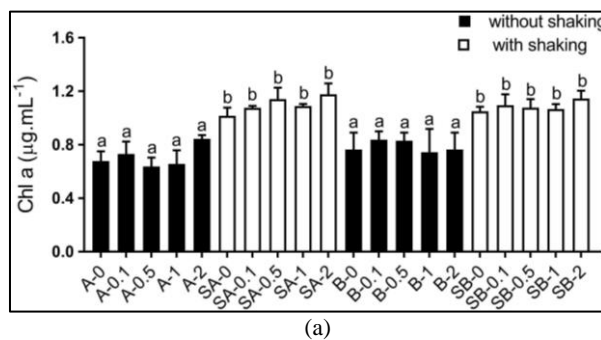
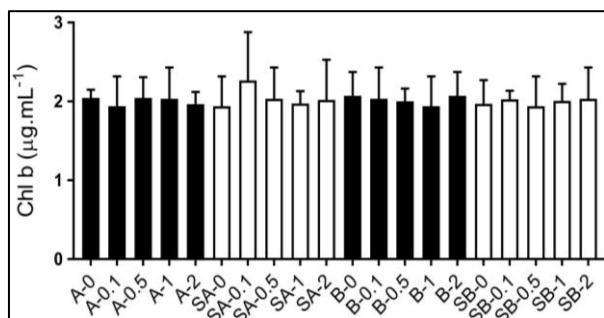


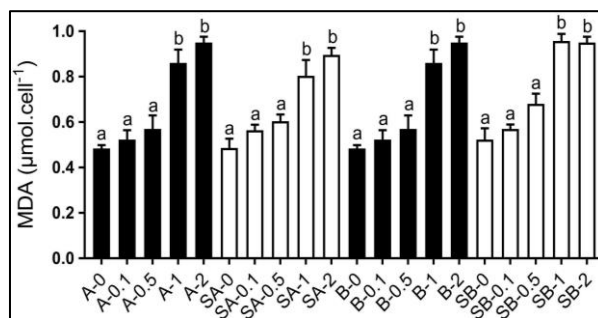
Fig. 1: Note the growth of microalgae exposed to different concentrations of BP3 alone (A and SA) and combined with NP (B and SB), without and with agitation. Different letters indicate statistically significant differences between the groups (p<0.05)



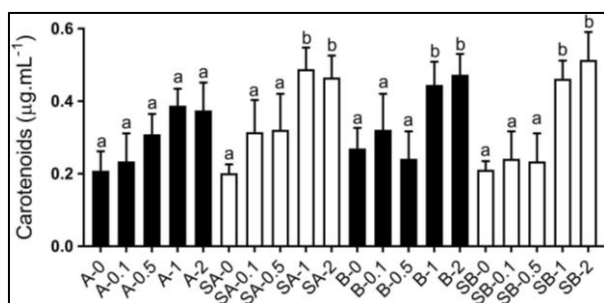
(a)



(b)

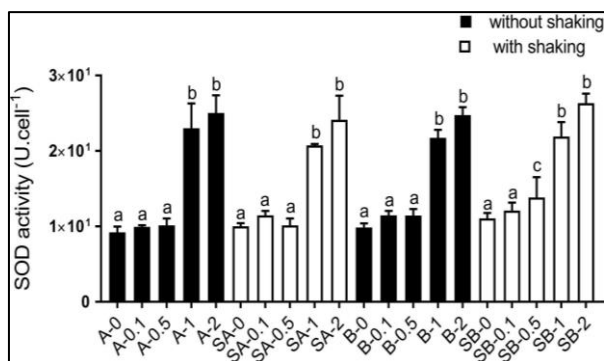


(c)

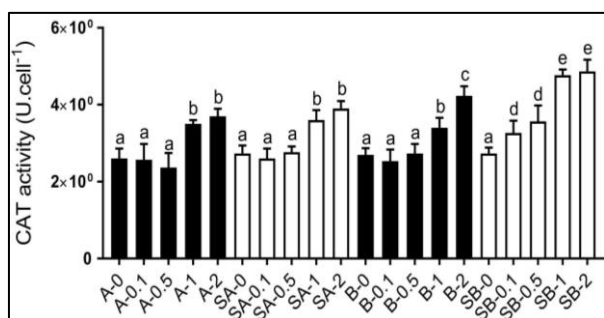


(c)

Fig. 2: Analysis of photosynthetic pigments in microalgae exposed to BP3 and to BP3 + NP with and without agitation. The letters indicate differences statistically significant among the groups ($p < 0.05$)



(a)



(b)

Fig. 3: CAT and SOD activities and MDA levels in microalgae exposed to BP3 and to the mixture (BP3 + NP) with and without agitation. Different letters indicate statistically significant differences among the groups ($p < 0.05$)

SOD, Catalase and MDA

The SOD activity was increased in all groups exposed to higher concentrations of BP3 (A-1, 2, SA-1, 2, B-1, 2, SB-1 and 2), regardless of shaking or the presence of NP. But the group SB-0.5 also showed a significant increase in SOD activity (Fig. 3A).

Concerning CAT activity, the same pattern of activity was seen in microalgae exposed to BP3 in groups without (A-1 and 2) and with (SA-1 and 2) agitation, as well as in groups exposed to BP3 + NP without agitation (B-1 and 2). In addition, CAT activity was increased even further when microalgae were exposed to SB-0.1, SB-0.5, SB-1 and SB-2 (Fig. 3B).

In relation to MDA, there was an increase in its levels in all groups exposed to higher concentrations of BP3 (A-1, 2, SA-1, 2, B-1, 2, SB-1 and 2), regardless of the presence of NP or whether the solution was at rest or undergoing agitation (Fig. 3C).

Discussion

In the aquatic environment, microalgae are exposed not only to the constant movement of the usual marine currents but also to anthropogenic influences, such as ship traffic. Thus, this movement of the aquatic environment can interfere with the development of these microorganisms.

Microalgae are used as a biological model in numerous metabolic processes. For instance, in biocompost production, microalgae are cultivated under agitation through air bubbles in the cultivation solution. This agitation process is crucial to avoiding the formation of dead zones, where densely packed algae prevent nutrient absorption from the medium. However, excessive mechanical agitation can be harmful to microalgae due to their sensitivity to shear stress, significantly restricting productivity in bioreactors (Camacho *et al.*, 2000; Sobczuk *et al.*, 2006; Tan *et al.*, 2020). Sobczuk *et al.* (2006) evaluated the effect of mechanical agitation using

a shaker (Phillips TLD W154) on microalgae *Phaeodactylum tricornutum* and *Porphyridium cruentum*. The results indicated that the maximum tolerable agitation velocity varied according to the microalgae species, being over 1.56 m/s for *P. tricornutum* and between 2.45 and 2.89 m/s for *P. cruentum*. Furthermore, the authors noticed that the increasing agitation speed resulted in a decrease in biomass concentration. Thus, the mechanical agitation itself was not the direct cause of cellular damage, but the rupture of small gas bubbles on the surface of the culture medium would be responsible for cell disruption.

In this study, to maintain the solution in constant movement during the experimentation, a continuous mechanical shaking system was employed. This procedure was employed because the experiments were conducted on cultivation plates, which allowed for more effective control over the concentrations of contaminants (BP3 and NP) as well as a reduction in the generation of contaminated wastewater at the end of the trials.

However, when *Tetraselmis* sp. were subjected exclusively to agitation, a reduction in their growth was observed. A similar response was noticed for the microalgae *P. tricornutum* and *P. cruentum* (Sobczuk *et al.*, 2006). Furthermore, the growth reduction was even more significant when the microalgae were exposed to BP3 at all concentrations (SA groups), with the most pronounced effect observed in the microalgae groups treated with the BP3 + NP mixture (SB groups). These results demonstrated that agitation can act as a stressor and suggest that the movement of the medium facilitates interaction with pollutants.

As autotrophic organisms, photosynthetic pigments are crucial for the survival of microalgae in the environment. These pigments are commonly utilized to assess the impacts of xenobiotics and microplastics on phytoplanktonic organisms (Li *et al.*, 2021).

In *Tetraselmis suecica* exposed to microplastics made of polyester, the concentration of chlorophyll varies according to the time of exposure. When these microalgae are exposed for a short period of time, growth and concentrations of Chlorophyll (Chl a and b) increase compared with long-term exposures, where chlorophyll concentrations decrease (Raju *et al.*, 2022). Mao *et al.* (2017) noticed that when *Chlamydomonas reinhardtii* was exposed to BP3, an alteration in growth and production of the photosynthetic pigments (Chl a, b and carotenoids) was observed. In *C. reinhardtii*, Chl a production is significantly inhibited, while the production of carotenoids is stimulated depending on the concentration of BP3.

In this study, when *Tetraselmis* sp. were exposed to BP3 and to BP3 + NP mixture while agitation, the level of Chl a increased in all of the experimental groups. However, the concentration of Chl b remained unchanged under all experimental conditions. Carotenoid

concentrations increased in the experimental groups with the highest concentrations of BP3 and BP3 + NP, irrespective of agitation. Thus, the tests suggest that Chl a is more susceptible to agitation, while carotenoids are more sensitive to contaminants.

The literature reports that both BP3 and NP can cause oxidative stress in microalgae, interfering with protective proteins such as antioxidant enzymes. These enzymes are responsible for regulating the production of Reactive Oxygen Species (ROS), including Superoxide (O₂⁻), Hydrogen Peroxide (H₂O₂) and Hydroxyl Radical (HO⁻). The excessive accumulation of these reactive oxygen species can damage lipids, resulting in increased Lipid Peroxidation (LPO) and an increase in MDA levels, a by-product of lipid peroxidation (Piddington *et al.*, 2001; Yang *et al.*, 2020).

The microalgae of the groups SA-1 and 2 (exposed only to BP3 and under agitation) showed a significant increase in SOD activity. This response was also detected in microalgae of the group SB-05 (microalgae exposed to NP + BP3 at a concentration of 0.5 mg. L⁻¹ with agitation), but was not observed in any other condition. CAT was also sensitive, revealing that CAT activity increased when microalgae were exposed to the mixture of BP3 + NP with agitation, independent of BP3 concentration. Thus, the mixture of BP3 with NP under agitation was able to affect the activity of SOD and CAT in microalgae, even at low concentrations. In relation to MDA, it was possible to see that when microalgae were exposed to BP3 and the BP3 + NP mixture, the agitation did not interfere with MDA levels.

The literature reports that contaminants cause changes in ROS response parameters. The inhibition of growth is noticed when microalgae are exposed to microplastics made of polystyrene and nonylphenol, an endocrine disruptor and xenoestrogen. According to Yang *et al.* (2020), microplastics with dimensions of 100-150 µm also cause increased activity of the enzymes SOD, CAT and MDA.

Song *et al.* (2021), in studying the effects of BP3 and PE + BP3 on the microcrustacean *Daphnia magna*, commented that PE combined with BP3 reveals whether it is more toxic in *D. magna* (EC₅₀ = 0.99 mg. L⁻¹) when compared with the exposure of microcrustaceans to PE fragments (EC₅₀ = 3.90 mg. L⁻¹) or BP3 (EC₅₀) alone. The authors also noticed that exposing to MP + BP3 caused a synergistic effect that increase in reactive oxygen species, total antioxidant capacity and lipid peroxidation in *D. magna*. However, no reports have been found on the impact of agitation on microalgae interactions with xenobiotic compounds.

Conclusion

The current investigation demonstrated the significant impact of medium dynamics on the cellular response of microalgae. Furthermore, the toxicity of BP3 and NP

increases when mechanical agitation is combined with the presence of BP3 and NP in the cultivation of *Tetraselmis* sp. The changes include changes in cell growth, photosynthetic pigments and the induction of oxidative stress. Thus, the agitation of the medium promotes a greater interaction between such elements and microalgae, making them more susceptible to adverse effects.

Although there are a few studies that cite the use of microalgae cultures kept in agitation, mainly through aeration, the studies do not consider constant agitation an additional stressor for these organisms. Our findings highlight the importance of considering both agitation and pollutants in toxicological studies using planktonic organisms in order to improve our understanding of environmental stressors in natural environment.

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Author’s Contributions

Rafaela Luiza Dias Da Cunha: Improving the idea presented, experimental assays (morphological and biochemical analysis and photosynthetic parameters) presented in this research and written-reviewed and edited the manuscript.

Camyla Lais Costa Leal: Experimental assays (morphological and biochemical analysis) in this research.

Lycia De Brito-Gitirana: Improving the idea presented in this research, written-reviewed and edited the manuscript.

Ethics

This study adhered to ethical principles, ensuring the integrity of the research. Neither animals nor humans were used.

Conflict of Interest

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 study.

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