"ALEXANDRU IOAN CUZA" UNIVERSITY OF IAŞI
FACULTY OF BIOLOGY
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Summary of the doctoral thesis

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RESEARCH ON THE BIOLOGY OF SOME TAXA FROM THE ARACEAE FAMILY CULTIVATED UNDER EXPERIMENTAL CONDITIONS ON MEDIA WITH SYNTHETIC NANOMATERIALS ADDITION

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List of abbreviations

ABA – abscisic acid
DNA – deoxyribonucleic acid
RNA – ribonucleic acid
β-ACT – beta-actin (used as a reference gene)
CNP – carbon nanoparticles
CNT – carbon nanotubes
-COOH – carboxyl functional group
CPD – critical point drying
DAD1 – Defender Against Apoptotic Death (gene involved in apoptosis regulation)
DPPH – 2,2-diphenyl-1-picrylhydrazyl
MWCNT – multi-walled carbon nanotubes
MWCNT-COOH – carboxylated multi-walled carbon nanotubes
NBT – nitro blue tetrazolium
NCED9 – 9-cis-epoxycarotenoid dioxygenase (gene associated with hormonal metabolism)
NMC – carbon nanomaterials
NP – nanoparticles
-OH – hydroxyl functional group
PCR – polymerase chain reaction
POD – peroxidase
PSII – photosystem II quantum efficiency
RBSC – RuBisCO (marker gene for photosynthetic activity)
ROS – reactive oxygen species
RT-qPCR – real-time quantitative PCR
SEM – scanning electron microscope
SOD – superoxide dismutase
SWCNT – single-walled carbon nanotubes

Introduction

Nanotechnology, a new field of science that develops advanced research for the production of engineered nanomaterials such as carbon nanotubes, could open up new applications in biotechnology, agriculture and industry (Fang et al., 2017; Patel et al., 2020; Mathew and Victório, 2022). The remarkable development in recent years of the field of nanotechnologies has allowed the design of specific applications of carbon-based nanomaterials in the agricultural, industrial, biomedical and environmental sectors.

Due to their distinct physico-chemical properties, carbon nanotubes (CNTs) have been widely used in laboratory experiments as a growth promoter, their use constituting a possible advantage for increasing the biomass of agricultural products; research in this regard suggests that CNTs help increase plants' ability to absorb water and essential nutrients, thus improving their growth. At the same time, CNTs have been tested for their use in genetic engineering for the administration of genes, proteins or drugs, some research revealing, however, even mixed effects of CNT exposure on plants, such as the induction of oxidative stress by the generation of reactive oxygen species (ROS) (Tan et al., 2009; Jordan et al., 2020).

Remarkable for their unique characteristics such as size, structure and topology, CNTs are today one of the most studied and exploited types of engineered nanomaterials, characterised by specific electrical, mechanical, optical and structural properties. Their use includes numerous application areas, including biomedicine, nanoelectronics, bioengineering and mechanical engineering. The use of CNTs currently continues to expand in medicine and agriculture to improve the quality of life, it being considered today that carbon nanotubes can also be used in solving environmental problems such as air, water and soil pollution, areas where already established remediation technologies are limited (Mittler, 2002; Ren et al., 2021). In the context of the above, research on the interaction of CNTs with plant systems is, according to the information currently presented in the specialised literature, at an early stage, suggesting the need for additional investigations to understand the mechanisms that determine the appearance of more or less toxic effects of these particles on plants, with unfavourable/favourable repercussions on their growth and development.

Plants, organisms with the largest share among primary producers, have developed, throughout evolution, structures that have allowed them to adapt to aquatic and terrestrial environments, thus managing to occupy the entire surface of the planet.

They represent the food source for numerous animal species, an important source of fibres and oils obtained through various industrial processes, as well as the raw material for the extraction of numerous substances with an important role in the pharmaceutical industry.

Due to the anthropogenic impact on the natural environment at present, terrestrial and aquatic ecosystems are, unfortunately, deeply affected by various pollutants resulting from industrial processes, agricultural activities, naval, air and terrestrial traffic systems, the pollutants thus generated having unfavourable effects on living organisms at several levels. Among these pollutants, a new category of possible environmental pollutants is considered to be carbon nanotubes, particles with applications in various fields, whose industrial use can inevitably lead to widespread dispersion in nature (Jackson et al., 2013).

In the effort to understand the mechanisms of action of various pollutants on plants, as a first stage of investigation, an evaluation of the morphological and anatomical changes suffered by them can be carried out, an activity that does not require a large consumption of resources and can, at the same time, indicate the state and degree of affection of the investigated organisms due to pollution.

Forced to adapt to unfavourable environmental conditions, many plant organisms today manage to survive in nature in adverse conditions. It is thus known that they manifest, as a result of the action of certain pollutants, anatomical and functional changes, a reality also observed in aquatic species.

An example of a group that has been the subject of many aquatic toxicity studies is the subfamily *Lemnoideae*, a family that includes plants widespread in many aquatic ecosystems globally, which can assimilate certain pollutants (Nasu and Kugimoto, 1981; Bokhari et al., 2016; Lanthemann and van Moorsel, 2022).

In numerous cases, aquatic environments are polluted with various categories of pollutants: heavy metals, pesticides, chemical fertilisers, textile dyes, etc., among these categories of pollutants there is the possibility of including carbon-based nanomaterials, whose toxicity has been tested on microorganisms, protozoa and algae, but not on aquatic higher plants, which is why this paper aims to understand some of the effects of this category of particles that may accidentally appear in aquatic environments on specimens of *Lemna minor* L. and *Lemna minuta* Kunth cultivated under experimental laboratory conditions.

Lemna species, in particular *Lemna minor*, constitute the preferred plant material in studies aimed at wastewater remediation, in physiology, ecology studies, as well as in foreshadowing aquatic biotechnologies, often being considered standardised ecotoxicity indicator organisms. The advantages of these species are numerous, which gives them uniqueness in aquatic ecology studies: they multiply vegetatively, have a high biomass production, have demonstrated an extreme ability to absorb and metabolise large quantities of toxic organic compounds (phytotransformation) and to immobilise heavy metals by precipitation, reduction or absorption through the roots (rhizofiltration).

In most of the specialised works that we had the opportunity to consult and which had as a relatively common subject the possible response reactions of *Lemna* species to interaction with microplastics, we find almost invariably the same conclusions: the growth of colonies upon contact with microplastics was not significantly affected; generally, root length was affected; the bioadhesion of microplastics can be affected by aquatic microorganisms that can form a biofilm on them, which attenuates their effects on higher plant organisms (*Lemna* specimens).

A limited number of studies show that *Lemna* sp. does not process and metabolise pollutants, being in fact collector species; therefore, it means that when the plants die, they will release contaminants back into the aquatic environment. Therefore, to be effective in wastewater treatment, *Lemna* must be removed and properly disposed of from these environments after their purification. Synthetic carbon-based nanomaterials (CNTs) are currently being studied as hopes that can contribute to solving environmental problems in areas where other technologies have already demonstrated their limits. Here, the specialised literature and existing results are still in early stages as answers, especially since these nanomaterials have proven to be both saviours and new sources of pollution, which complicates the situation.

In this context of existing knowledge, the present thesis aims to bring new, unprecedented information, some of which cannot, for now, be compared with similar information included in other studies. And here we refer to our experimental studies that aimed at the interaction between individuals of *Lemna minor* L. and *Lemna minuta* Kunth. with various categories of nanomaterials by investigating the possible response reactions of their foliar surfaces, morphology, structure and physiological, biochemical and genetic mechanisms.

Starting from the certainty that both entities (one living, the other non-living) are involved through particular mechanisms in reducing aquatic toxicity, the experimental models used aimed at the reactions of *Lemna* individuals when they are together, in the same aquatic space, with nanomaterials chosen for study. And we are not talking about *Lemna* colonies at different stages of development, which interacted at some point with nanomaterials, but we are talking about *Lemna* individuals that developed colonies in the presence of nanomaterials and therefore interacted with them from the one or two-individual phase, this aspect particularising the research thus carried out and giving it uniqueness.

A series of questions that we tried to answer through the experiments thus carried out are: would the two entities work together? does the living entity react to the presence of the non-living entity? whom should we prefer in aquatic bioremediation processes? can cuticular and morpho-anatomical foliar responses, along with physiological and biochemical reactions, be bioindicator tests?

Scope and objectives

Scope:

Evaluation of the effects of synthetic carbon-based nanomaterials on the biology of *Lemna minor* L. and *Lemna minuta* Kunth species, cultivated under experimental laboratory conditions.

Objectives

O1 - Analysis of the possible effects of synthetic carbon-based nanomaterials on the growth processes of *Lemna minor* L. and *Lemna minuta* Kunth specimens grown under experimental conditions.

O2 - Research into the morphological and anatomical changes induced in *Lemna minor* L. specimens by the presence of synthetic carbon-based nanomaterials in the culture medium.

O3 - Research into the morphological reactions of *Lemna minuta* Kunth specimens as an effect of their interaction with synthetic carbon-based nanomaterials in the culture medium.

O4 - Research into the influence of synthetic carbon-based nanomaterials on the functioning of the photosynthetic apparatus of *Lemna minor* L. and *Lemna minuta* Kunth specimens grown under experimental conditions.

O5 - Research into the influence of synthetic carbon-based nanomaterials on the biosynthesis and functioning of certain compounds of secondary metabolism involved in the protection of *Lemna minor* L. and *Lemna minuta* Kunth specimens against abiotic stress induced by the presence of these particles in the culture medium.

O6 - Quantification of the expression of genes involved in essential physiological and cellular processes in individuals belonging to the species *Lemna minor* L. grown in the presence of synthetic carbon-based nanomaterials under experimental conditions.

Part I - The current state of knowledge

Chapter I – Carbon-based nanomaterials

I.1 Nanotechnology

Nanotechnology involves manipulating materials at a scale of 1 to 100 nanometres (nm) to create functional materials, sensors, and devices. At such a scale, carbon-based nanomaterials (CNM = carbon nanomaterials) possess novel physicochemical properties, a large contact surface relative to their size, increased chemical reactivity, etc. These properties vary depending on the degree of agglomeration, shape, surface structure, and size of the nanomaterials (Hussain, 2020).

The special properties of CNM are due to their stable molecular architecture and their ability to disperse in the environment in which they are introduced. Different applications in nanopharmacology and nanomedicine have been developed by exploring CNM as intelligent drug delivery systems in the human body (Chandra Kanth et al., 2020). CNM can be conveniently loaded with an adequate dose of drugs or other active substances for delivery to specific target sites within the cell (He et al., 2013). Although the same principles are applicable to plant systems, nanotechnology applications in plant science and sustainable crop production (especially in the agricultural sector) have not attracted much interest within the scientific community. Thus, the effects of CNM on plant growth and development are rather poorly investigated and less understood compared to studies conducted on animals (van der Zande et al., 2011; Hassan et al., 2020). However, research reports on the effects of CNM on different plant species have recently begun to emerge. Despite these efforts, the effects of CNM on morphological, anatomical, physiological, biochemical, and molecular processes and their mechanisms in different plant systems are not yet fully understood and require detailed investigation.

I.1.1 Carbon-based nanomaterials and the discovery of their special properties and applications.

1. Multiwalled carbon nanotubes (MWCNT)

Multi-walled carbon nanotubes are composed of several rolled graphene sheets, forming concentric cylinders. MWCNTs consist of many hollow concentric cylinders, with a spacing between them of 0.34 to 0.39 nm (Ajayan and Ebbesen, 1997). The diameter of the inner wall is independent of the number of walls (from 0.4 nm to several nm), while the diameter of the outer layer varies from 2 to 30 nm. MWCNTs, depending on the position of the carbon atoms, are classified into different models, such as the parchment model and the Russian doll model (Lehman et al., 2011; Eatemadi et al., 2014; European Commission. Joint Research Centre. Institute for Health and Consumer Protection., 2014).

2. Fullerenes and fullerene soot

Fullerenes (also known as Buckyballs) were discovered in 1985 (Kroto et al., 1985) and are modified allotropic carbon atoms, existing as C60, C70, C80, etc. (Ugarte, 1992; Wang et al., 2001). They are soluble in organic liquids (e.g., toluene) and each type imparts a special colour to the solution (e.g., C60 solution is violet, and C70 solution is reddish-brown) (Ramsden, 2011). Fullerenes have wide applicability due to their special structure and characteristics, such as versatility, electron affinity, high electrical conductivity, and high resistance (Yadav and Kumar, 2008; Schwerdtfeger et al., 2015). The aforementioned characteristics make them suitable for various medical, electrical, and industrial applications (Bakry et al., 2007). Fullerene soot, also known as carbon soot, is a complex carbonaceous material, a by-product of fullerene synthesis processes, especially C_{60} and C_{70}, using methods such as electric arc discharge, laser ablation, or controlled combustion (Krätschmer et al., 1990; Keypour et al., 2013). This substance generally appears as a fine, black powder, composed of a heterogeneous mixture of nanostructured carbon forms, including amorphous carbon, graphitic fragments, and a variable content of fullerene molecules.

I.1.2 Effects of carbon-based nanomaterials on plants

The exposure of plants to carbon nanomaterials and their interactions with them cause several physiological changes, depending on the properties of the CNM, including type, size, shape, chemical composition, reactivity, and dosage. The outcome (null, beneficial, adverse) of CNM-plant interactions varies from plant to plant, always depending on the concentration of the nanomaterial used.

I.1.2.1 The impact of carbon nanoparticles (CNP) on plants

Carbon nanoparticles (CNPs) possess excellent electrical, thermal conductivity, and mechanical properties. CNPs are composed of pure carbon, therefore exhibiting high stability, low toxicity, and good conductivity (Yan et al., 2017).

These nanoparticles are used in many biomedical applications, such as drug delivery at the cellular level, gene transfer, and bio-imaging (He et al., 2013).

According to research conducted by Khodakovskaya et al. (2012), the application of CNPs (variable concentrations 0-125 mg/pot) to Nicotiana tabacum L. plants led to an increased growth rate at different stages, compared to the growth rate induced by the application of conventional fertilisers. These authors also reported that the application of CNPs to Nicotiana tabacum L. plants increased the nitrogen and potassium content in their organs.

In turn, Kumar et al. (2018) investigated the effects of CNPs on photomorphogenesis and flowering time in Arabidopsis thaliana L. It was thus observed that CNPs (in a concentration of 0-500 mg/l) were taken up by the plant, accumulated in leaf tissues, and induced earlier flowering by modifying both phytochrome b (PhyB) and photoperiod-dependent pathways, and other research has shown that CNPs (of unspecified size) promoted hypocotyl elongation under red light or red and blue light (Kumar et al., 2018). Exploring the importance of CNPs in crop plants grown under in vitro or in vivo conditions could develop an innovative non-transgenic strategy for improving economically important crops (Kumar et al., 2018), noting that the application of CNPs increased plant growth and promoted the absorption and accumulation of micro-elements, thus enhancing fertiliser efficiency and improving plant quality.

Recently, several types of NMC (fullerenes, fullerols, CNPs, and CNTs) have gained importance due to their potential use in regulating plant growth. In this regard, research reports have mostly demonstrated a beneficial, but also adverse, influence on plants, depending on the NMC types, the applied concentration, the exposure time, as well as the plant species on which they are applied and their growing conditions.

In the case of experiments carried out on Phaseolus mungo L. specimens, it was found that black bean seeds germinate normally at concentrations of 10, 20, and 40 mg/l MWCNT in the medium, results that indicated the non-hazardous nature of CNTs (Ghodake et al., 2010). At the same time, it was shown that water-soluble CNTs support the growth of Cicer arietinum L. (chickpea) plants by improving their ability to absorb water (Tripathi et al., 2017). Carbon nanotubes also induced, at a concentration of 40 mg/l, beneficial effects on seed germination and plant growth of Lycopersicum esculentum M. (var. Arka Vikas) compared to the control group (Verma et al., 2019). In addition, the germination of L. esculentum M. seeds in MS medium (containing functionalised MWCNTs) and the plants grown in it were shown to assimilate MWCNTs and distribute them in roots, leaves, and fruits (Alimohammadi et al., 2011). A predominantly stimulating effect of MWCNTs on seed germination and/or root elongation of seedlings was also observed in Triticum aestivum L. (Wang et al., 2012).

The activity of genes responsible for regulating water channels [aquaporin (LeAqp1)] was triggered in tomato plants by CNTs activated with several groups [such as COOH- and polyethylene glycol (PEG)], compared to untreated plants (Villagarcia et al., 2012), it being well known that water helps channel proteins for plant development and is directly involved in the process of seed germination and water absorption by the resulting individuals through roots (Maurel, 2007).

Khodakovskaya et al. (2013) demonstrated that the expression of the LeAqp2 gene (aquaporin) can be significantly activated in tomato roots by exposure to CNTs, compared to the control group (without CNTs). Also, the generation of reactive oxygen species (ROS) evaluated by the dichlorodihydro-fluorescein diacetate (DCFH-DA) test, the detection of hydrogen peroxide by staining with diamino-benzidine (DAB) and nitroblue tetrazolium (NBT) confirmed the presence of ROS generated in the leaves of red spinach (Amaranthus tricolor L.) grown with MWCNTs (Begum et al., 2011).

In their research on rice (Oryza sativa L.), Jiang et al. (2014) found that CNTs applied at concentrations from 0 to 100 mg/l increased the stem and root length of seedlings. The general stimulation of plant growth in the presence of CNTs was attributed to increased nutrient uptake through improved water delivery by MWCNTs. Continuing the experiments with higher concentrations, it was observed that when the experimentally applied CNT concentration increased to 150 mg/l, the root length and stem length decreased, compared to the previously experimented concentration of 100 mg/l. Thus, the obtained results demonstrated that CNTs could promote seed germination of O. sativa L. and root growth at lower concentrations, but could have phytotoxic effects at high concentrations.

Miralles et al. (2012) conducted research on the germination of Triticum aestivum L. caryopses and Medicago sativa L. seeds, to investigate the effects of MWCNTs and their impurities on the germination process. The nanomaterial was activated with Fe3O4 nanoparticles, and the adsorption of CNTs on the root surfaces was detected, which led to a substantial elongation of alfalfa and wheat roots.

In contrast, Khodakovskaya et al. (2013) reported an enhancement of tomato plant growth through the use of CNTs. The mentioned researchers established, using analytical methods, that CNTs (in concentrations of 10-40 mg/l) managed to penetrate the seed coat and facilitate water entry into them, concluding that seed germination and seedling growth of tomatoes were affected by the application of the respective compounds.

It was thus demonstrated that low doses of different types of CNTs penetrate the seed coat and activate their germination and seedling growth (Lahiani et al., 2013).

Using PCR analysis, immunostaining, and electron microscopy, Yan et al. (2013) confirmed that the penetration and accumulation of SWCNTs in the roots of Zea mays L. plants could change the expression of genes controlling root and absorbing hair growth.

In turn, Serag et al. (2013) studied the effect of CNTs at the molecular level. These authors showed that the length and diameter of SWCNTs are limiting factors for their penetration into plant cell walls. Smaller nanotubes can diffuse and penetrate cells, but if they are too large, they can remain outside the cell and are immobilised, even if they have managed to penetrate the cell wall. It was also observed that CNTs can facilitate nutrient absorption and their transport into plant tissues. However, nutrients containing sodium could be prevented by CNTs from entering plant cells (Taha et al., 2016).

In wheat plants (Triticum aestivum L.), the use of MWCNTs resulted in an increase in xylem size, epidermal cells, stomatal density, number of caryopses, biomass, and water absorption (Joshi et al., 2018).

Investigations into the effects of carbon nanotubes on rice cell cultures revealed an increase in reactive oxygen species, associated with reduced cell viability (Tan et al., 2009). The ability of simple nanotubes to penetrate the cell wall and cell membrane of intact plant cells was examined by Liu et al. (2009).

Using confocal microscopy, these authors obtained evidence of cellular uptake of both SWCNT-DNA conjugates and SWCNT-fluorescein isothiocyanate, these studies demonstrating that SWCNTs acted as cellular nanocarriers. Furthermore, based on these experiments, the cited authors hypothesised that single-walled carbon nanotubes could deliver different cargoes to different organelles of the plant cell (Liu et al., 2009).

I.2 Multiwalled carbon nanotubes – uses and biological effects

In recent decades, nanomaterials have attracted increasing attention from researchers, with numerous international research projects being proposed, aiming both to evaluate their potential for technological innovation and to understand their possible adverse effects (Chen et al., 2018). Today, it is considered of special interest to identify whether the nano form of a compound induces adverse effects (different effects or potencies) compared to its non-nano forms.

For the purpose of nanosystem research, it is desirable to know the availability of nanomaterials from a single batch to enhance the comparability of results between different laboratories and research projects and to overcome the question of whether a nanomaterial tested in one project is the same or similar to a nanomaterial tested in other projects.

Addressing this need and supporting the testing programme for a representative set of manufactured nanomaterials by the Organisation for Economic Co-operation and Development (OECD), the European Commission's Joint Research Centre (JRC) has established a repository of representative test materials (RTM), which hosts different types of nanomaterials, the role of representative test materials being described in a recently edited publication (Roebben et al., 2015).

Carbon nanotubes are cylinders of carbon atoms that appear as rolled tubes of graphite formed in large bundles of single or multiple sheets of graphene, to give single-walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNT), respectively. Practically, we are talking about a honeycomb-like network rolled upon itself, with lengths varying from a few hundred nanometres to several micrometres and diameters of a few nanometres (SWCNT), up to tens of nanometres (MWCNT) (Herrero-Latorre et al., 2015). These structures constitute a large group of tube-type carbon-based nanomaterials, which not only differ in terms of length and the number of layers they are made of, but also vary in terms of included impurities, content, and surface modification.

Carbon nanotubes can be found in finished products such as conductive polymers and composites (automotive and electronics), aerospace structural components, sports equipment, and sensors. Such cylindrical graphitic polymeric structures have novel or improved properties, making them potentially useful in a wide range of applications in electronics, optics, and other fields of materials science. Carbon nanotubes are distinguished by a remarkable set of physical properties, including high electrical and thermal conductivity, as well as exceptional mechanical strength, stiffness, and specific hardness (OECD, 2009).

Furthermore, nanotubes are pure carbon polymers and can be manipulated using the rich chemistry of carbon, which offers the possibility to modify their structure and optimise their solubility and dispersion, allowing innovative applications in material production, electronics, chemical processing, and energy management, to name just a few such application directions. In conclusion, three properties of these nanomaterials are of interest to industry: electrical conductivity, mechanical strength (up to 15-20 times greater than steel), and thermal conductivity (more than five times greater than copper). A combination of these impressive properties allows for the development of a new variety of useful and beneficial applications of this type of compounds.

A group of nanomaterials tested by the OECD Working Party on Manufactured Nanomaterials (OECD WPMN) is represented by multi-walled carbon nanotubes (MWCNT), which consist of several layers of graphite superimposed and rolled into themselves to form a tubular shape, widely used as structural composites and also in energy applications such as the manufacture of lithium-ion batteries (Fig. I.2, Photo I.1).

Multi-walled carbon nanotubes (MWCNT) are used in various finished products, including conductive polymers and composite materials for the automotive and electronics industries, aerospace structural components, sports equipment, and sensors. Due to their cylindrical configuration and polymeric structure, these materials exhibit novel or significantly improved properties, which give them extensive application potential in fields such as electronics, optoelectronics, and advanced materials science (Myojo and Ono-Ogasawara, 2018).

I.2.1 Functionalised multi-walled carbon nanotubes

The functionalisation of multi-walled nanotubes is carried out in two stages.

In the first stage, MWCNTs are treated with hydrochloric acid to remove surface impurities. For this purpose, 2 g of dry multi-walled nanotubes (kept in an oven at 350°C for 4 hours) are added to a 500 ml round-bottom flask containing 400 ml of hydrochloric acid (6M). The reaction mixture is kept at 120°C, under strong magnetic stirring, for 2 hours. Then, the purified MWCNTs are centrifuged and washed several times with distilled water until a neutral pH of the washing water is obtained. Finally, the obtained product is dried at 90°C for 24 hours and stored in an oven, under vacuum, for later application.

In the second stage, 1 g of purified MWCNTs is added to a 500 ml round-bottom flask containing 200 ml of concentrated nitric acid and a concentrated mixture of sulphuric acid (1:3 v/v) and dispersed, using ultrasound, for one hour. Then the temperature of the reaction mixture is increased to 75°C with reflux under magnetic stirring for 12 hours. Once the reaction is complete, the solution is filtered, and the obtained precipitate is washed several times with distilled water. Finally, the finished product is dried in a vacuum oven at 80°C for 24 hours (Gohari et al., 2020).

I.2.2 Biological effects

Knowledge regarding the toxicological properties of carbon nanomaterials in general, and nanotubes in particular, is evolving as research in the field intensifies. Information on nanotube toxicity appeared in the global literature from the early 2000s, and research in this area is ongoing (Gao et al., 2015). Carbon nanotubes are considered rather toxic due to their mutagenic properties, as a result of increased reactive oxygen species formation and the ability to induce apoptosis (Møller et al., 2015).

Regarding the impact on plants, experimental data from the specialised literature indicate that the use of synthetic multi-walled carbon nanotubes (MWCNT) led to an increase in seed germination rate and a stimulation of seedling development in Lupinus elegans and Eysenhardtia polystachya, plants used in ecological restoration programmes for forest ecosystems affected by fires in the Mexico region. The research provides evidence supporting the possible ecological functions of MWCNT in nature (Lara-Romero et al., 2017).

Furthermore, nanotubes have been described as plant growth promoters, favouring seed germination and an increase in the fresh mass of tomato plants (M.V. Khodakovskaya et al., 2013; L. Yang et al., 2017). Nanotechnology tools have recently developed nanotubes for potential applications in agriculture, including crop protection, pollution control, waste management, pesticide detection, nanosensitisation, and nanofertilisers (Gogos et al., 2012; De La Torre-Roche et al., 2013; M.V. Khodakovskaya et al., 2013).

In parallel with the diversification of beneficial CNT applications, research has also been initiated on the possible negative effects of nanoparticles on edible plants (Miralles et al., 2012a), and thus, at present, the idea has emerged that the known effects of MWCNT on plants, as well as the responses of natural and agricultural ecosystems to their presence, whether intentionally introduced or accidentally released into these ecosystems, are still limited (J. Yang et al., 2017).

In another context, the effects of nanomaterials, such as carbon nanotubes, on plant growth and development have been documented, and it has been suggested that their effects are due to factors such as the type and concentration of applied nanoparticles, the plant species tested, and the experimental conditions used, respectively the method of nanoparticle absorption (Tiwari et al., 2014), with reported studies supporting that some nanomaterials exhibit toxic effects on several plant models (Miralles, Church, and Harris, 2012).

Today it is known that nanotubes produce phytotoxicity in Arabidopsis thaliana L. and Oryza sativa L. species, leading to the death of approximately 25% of experimental protoplast cultures (cells without cell walls) within approximately 6 hours (Shen et al., 2010). In the same context, other researchers reported that red spinach plants (Amaranthus tricolor L.) inhibited their growth after two weeks of hydroponic culture in the presence of MWCNT and exhibited a phenomenon of cell death (Begum and Fugetsu, 2012), with nanotubes causing negative effects on the morphology of leaves and roots of plants belonging to this species, under conditions where the values of nanoparticle toxicity biomarkers, reactive oxygen species (ROS) and cellular lesions in experimental plants, were significantly increased during the two weeks of cultivation in the presence of MWCNT. These harmful effects were successfully reversed when MWCNT were supplemented with vitamin C, as a cellular protective factor against reactive oxygen species, suggesting a role for ROS in nanotube-induced toxicity. It was thus deduced that the plausible mechanism of MWCNT-induced toxicity was oxidative stress at the cellular level (Begum and Fugetsu, 2012), with cell membrane damage and the phenomenon of apoptosis in Amaranthus dubius specimens being attributed to the penetration of MWCNT into plant cells (Begum et al., 2012a).

The specialised literature has described several molecular mechanisms that would underlie the manifestation of their biological effects of nanotubes. For example, genomic analyses in Lycopersicon esculentum L. individuals showed that exposure to MWCNT modified the gene expression of test plants, with over-activation of stress-related genes (Lahiani et al., 2016, 2015), while in Nicotiana tabacum L. individuals it led to total changes in gene expression, with over-activation of genes related to cell wall assembly/cell growth, regulation of cell cycle progression, and aquaporin production (Miralles et al., 2012a; Lahiani et al., 2015; Mukherjee et al., 2016; J. Yang et al., 2017). Thus, it has been suggested that the size, composition, and specific surface characteristics of engineered nanomaterials can play an important role in their toxicity (Hong et al., 2013; Mukherjee et al., 2016).

Currently, reports on the phytotoxicity of multi-layered carbon nanotubes are somewhat contradictory. Thus, some authors state that nanotubes induced positive effects on protein production in tomato plants, on the photosynthetic activity of spinach, as well as on the growth of wheat and corn roots.

In contrast, other authors observed an inhibition in the growth of test plant roots, the induction of reactive oxygen species increase, and the appearance of chromosomal aberrations upon exposure of experimental plants to carbon nanotubes. Today it is considered that many factors can affect plant growth in the presence of MWCNT, including the physicochemical properties of nanotubes, the plant species tested, and the experimental working conditions (exposure time, substrate, growth conditions) (Zhao et al., 2017).

The study of functionalised carbon nanotubes (Fig. I.4) is of great importance from the point of view of their dispersion, their compatibility with composite materials, interfacial strength, and compatibility with biological matter.

For example, polymer materials enriched with functionalised multi-layered nanotubes exhibit better interfacial bonding strength and a smoother surface, and the amount of material required for polymer production could be greatly reduced if functionalised nanotubes are used. Thus, functionalised carbon nanotubes may be more suitable for revealing the special properties of carbon nanotubes.

In the case of other plant species (kohlrabi - Brassica oleracea var. Italica), nanomaterials applied in low concentrations had a positive effect on the growth process. This experimentally obtained information could open new perspectives for the process of managing agricultural and horticultural crops, especially when plants are grown under stress conditions (Martínez-Ballesta et al., 2016).

Reports from other researchers have shown that a concentration of 50 mg/l MWCNT could have positive effects (increase in the number of absorbent hairs, intensification of photosynthesis), as well as phytotoxic effects (root shortening) when applied as specific treatments to Arabidopsis thaliana L. crops (Fan et al., 2018). At the same time, the existence of MWCNT in several plant organs of plants from all cultures subjected to contact with these nanoparticles studied by Lahiani et al. (2018) highlighted the importance of assessing the risks of nanocontaminated plants appearing in the food chain.

Other results showed that MWCNT played a negative role in the germination of Cucurbita pepo L. seeds and delayed seedling growth (Hatami, 2017), and in curly kale (Brassica oleracea L.) Deng et al. (2017) found that in a hydroponic environment, plant biomass production was dependent on the concentration of carbamazepine, used in this study as a co-contaminant, while exposure of Brassica oleracea L. specimens to MWCNT-COOH (concentration of 50 mg/l) substantially improved their growth (Deng et al., 2017).

To study the effects of MWCNT absorption in lettuce plants, Das et al. (2018) used both simple multi-walled nanotubes and carboxyl-functionalised multi-walled nanotubes, at a concentration of 20 mg/l. The mentioned authors developed and used Raman spectroscopy to detect the presence of MWCNT and found that both types of MWCNT were present in the tissues of leaves, stems, and roots of treated lettuce plants, confirming their absorption and translocation into the plant.

To aid the growth of Oryza sativa L. seedlings, Zhang et al. (2017) hypothesised a possible link between plant hormones and ROS, under CNT treatment.

At the same time, exposure to SWCNT and MWCNT led to a harmful effect on experimental Arabidopsis plants, producing chromatin condensation, cellular aggregation, decreased dry cell weight, accumulation of hydrogen peroxide, affecting cell viability, superoxide dismutase activity, and chlorophyll content values (Zaytseva and Neumann, 2016). This study demonstrated that nanoparticle-induced phytotoxicity was due to the appearance of oxidative stress (Smirnova et al., 2012).

Most of the studies presented previously found, when using lower concentrations of nanotubes, an improvement in seed germination, acceleration of vegetative growth, and improvement in fruit/seed formation yield, and when applying larger quantities of nanotubes, a decrease in these parameters, highlighting the importance of choosing a correct treatment dose when conducting experiments.

I.3 Fullerenes and fullerene soot – uses and biological effects

Fullerenes are an allotrope of carbon, in which carbon atoms are linked by single and double bonds, to form a closed or partially closed cage-like structure. They can be of various shapes and sizes, including hollow spheres, ellipsoids or tubes (Fig. I.5).

Fullerene production generally begins with the production of fullerene-rich soot. The production method involves generating a strong electric current between two graphite electrodes, in an inert atmosphere. The resulting electric arc vaporises the carbon into a plasma which then cools into the soot residue.

Fullerenes exist in two major families: closed buckyballs and open cylindrical carbon nanotubes. Between these two classes, polyhedral structures can also exist (Fig. I.6) (Schwerdtfeger et al., 2015; Zaytseva and Neumann, 2016; Malhotra and Ali, 2018).

Fullerenes have unique physical and chemical properties. For example, their behaviour and structure depend on temperature; they are stable, but not totally unreactive, and in chemical reactions, they can act as an electrophile when doped or crystallised with alkali or alkaline-earth metals, exhibiting superconductivity properties.

These substances have various uses, particularly in the medical field: they are used as light-activated antimicrobial agents and are also integrated into numerous biomedical applications, including the development of contrast agents for high-performance magnetic resonance imaging, contrast agents for X-ray imaging, photodynamic therapy, as well as for drug and gene delivery (Zaytseva and Neumann, 2016).

Fullerene soot is a complex carbonaceous material resulting as a by-product during the synthesis of fullerenes, especially C_{60} and C_{70}, by methods such as arc discharge, laser ablation or controlled combustion processes (Krätschmer et al., 1990; Keypour et al., 2013). This material comes in the form of a fine, black powder, composed of a heterogeneous mixture of nanocarbon structures, including amorphous carbon, graphitic fragments and a variable content of fullerene molecules.

The most commonly used method for obtaining soot is electric arc discharge, in which graphite electrodes are vaporised in an inert atmosphere, usually helium. In this high-energy process, fullerenes condense along with other forms of carbon, thus leading to the formation of smoke (Iijima and Ichihashi, 1993). The fullerene composition of crude smoke typically varies between 5–15% by weight, depending on the operational parameters of the synthesis (Xihuang et al., 1994).

Chemically, fullerene soot is an extremely heterogeneous material, containing various carbon allotropes, such as: simple and oxidised fullerenes (e.g. C_{60}, C_{70}, C_{60}-O), multi-layered "carbon onion" structures, graphene-like fragments, polycyclic aromatic hydrocarbons and residual amorphous carbon. This structural heterogeneity gives fullerene soot a high specific surface area and special physicochemical properties, such as hydrophobicity, redox activity and catalytic potential (Sano et al., 2001).

Although fullerene soot does not contain fullerenes in purified form, it can be used directly in applications that do not require high material purity, such as environmental remediation, catalysis or as a precursor for the synthesis of other carbon-based nanostructures (Ali-Boucetta et al., 2013).

I.3.2 Biological effects

Testing the effects of fullerenes on plants is a relatively new field of research, which involves studying the impact of these compounds and other carbon nanomaterials on plant development, with the aim of designing new agricultural and environmental protection applications.

Various studies have been carried out to understand the impact of different carbon nanomaterials, including fullerenes, on plant development. For example, some studies have tested the antifungal activity of fullerenes and other carbon nanomaterials against phytopathogenic fungi, demonstrating the strongest antifungal activity for single-walled carbon nanotubes (SWCNTs) and the largely ineffective nature of fullerenes and activated carbon (Zaytseva and Neumann, 2016).

However, fullerene soot raises concerns regarding its ecotoxicological and health impact, due to the nanometric size of the particles, their persistence in the environment, and surface reactivity. Existing research indicates the potential of this material to interact with biological systems, inducing oxidative stress and cytotoxic effects, which can vary depending on the chemical composition and degree of surface functionalisation (Sayes et al., 2004; Lyon et al., 2006).

Chapter II – Lemnoideae subfamily

II.1 Araceae family

The *Araceae* family, a large and diverse group of flowering plants, well-known for the unique structure of their inflorescences (typically a spadix surrounded by a spathe), includes a variety of plants such as *Philodendron, Anthurium, Spathiphyllum, Colocasia*, and *Lemna* (duckweed).

II.2 Lemnoideae subfamily

II.2.1 Description

The *Lemnoideae* subfamily is a subfamily of flowering plants commonly known as duckweed. These plants are often found floating on or just below the surface of stagnant or slow-moving water bodies, especially in freshwater and marshy areas. In earlier classifications, mainly dating from before the end of the 20th century, these plants were considered to form a separate family, called Lemnaceae (McIlraith et al., 1989; THE ANGIOSPERM PHYLOGENY GROUP*, 2003).

The plants exhibit a simple anatomical structure, lacking distinct stems or leaves. Each plant in this subfamily consists of a small structure called a "thallus" or "frond", which often comprises only a few layers of cells and is equipped with air sacs (aerenchyma) that allow buoyancy on or just below the water surface. Depending on the species, individual plants may lack roots or have one or more simple roots (Blodgett, 1915; Bowker et al., 1980).

Favourable environments for development:

A variety of environmental factors such as water temperature, pH, and nutrient concentration have a significant influence on the growth and survival of duckweed. Other environmental factors that influence the growth rate of duckweed colonies include the presence of toxins in the water, overcrowding due to excessive colony growth, and competition with other plants for light and nutrients. However, duckweed's growth rate is favoured by organic pollutants, as well as inorganic nutrients (Guha, 1997).

The optimal temperature for maximum growth of most individual groups is between 17.5 and 30°C (Culley et al., 1981; Gaigher et al., 1986). Although some species can tolerate temperatures close to freezing, their growth rate decreases at temperatures below 17°C (Culley et al., 1981), and most species risk dying if the water temperature rises above 35°C. The effect of temperature on growth is also amplified by light intensity, meaning that as its intensity increases, so does the plant growth rate.

Lemnaceae have a high tolerance for environmental pH. They can survive well at a pH of 5 to 9, although some authors set pH limits between 3 and 10. Generally, duckweed grows best at a pH of 6.5 - 7, with a doubling of its biomass in 2 to 4 days being observed at a pH level between 7 and 8 units (Culley et al., 1981)..

II.2.3 Duckweed distribution

Nutrient availability plays a crucial role in determining plant distribution in wetland environments, with particular importance for aquatic plant species such as duckweed. These plants are typically found in eutrophic or nutrient-rich environments. They actively spread through various mechanisms, being transported by water currents or by attaching to the bodies of aquatic birds or mammals. However, in areas characterised by strong water currents or frequent flooding, their expansion is limited because the plants are often carried downstream. There are certain locations with cyclical growth patterns influenced by weather conditions, where duckweed thrives during periods of low water levels but is affected and eliminated during periods of heavy rainfall.

In addition to their ecological importance, duckweeds represent an essential source of protein-rich food for aquatic birds and provide refuge for the fry of many fish species. These species are used as habitat by various pond organisms, such as frogs and fish, offering shade and reducing the growth of photoautotrophic algae caused by light.

The geographical distribution of duckweeds reflects their adaptability to a wide range of climates, humidity conditions, and ecological niches. Below, we present their global distribution, according to "GBIF", with an emphasis on regions, species, and preferences for environmental conditions (Fig. II.1). (Les et al., 2002; Sree et al., 2016; Lee et al., 2020; “GBIF”):

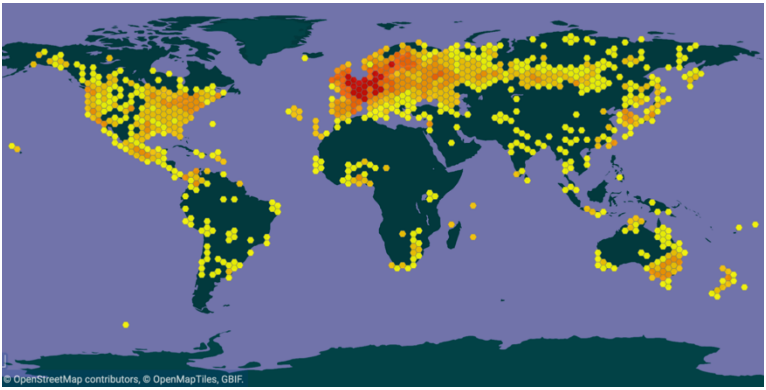

Fig. II.1 – The global distribution of the subfamily Lemnoideae. (GBIF - Global Biodiversity Information Facility, https://www.gbif.org – 25.01.2024)

Duckweeds also have several characteristics that make them uniquely useful for toxicity tests:

• their vegetative reproduction and genetically homogeneous populations allow clonal colonies to be used for all experiments, thus eliminating genetic variations (Bishop and Perry, 1981; Hillman, 1961);

• individuals can be disinfected and grown in a liquid medium or on agar (Hillman, 1961);

• *Lemna*culture can grow in the laboratory indefinitely, and controlled conditions of temperature, light and nutrition are much easier to maintain than for other angiosperms (Hillman, 1961; Wang, 1987);

• *Lemna*specimens have a large surface area to volume ratio, and the cuticle on the lower surface of the frond is absent or reduced, thus facilitating the absorption of the substances being tested (Bishop and Perry, 1981);

• plants are excellent accumulators of a large number of metallic elements, a characteristic that makes them good candidates for use in water quality monitoring and in laboratory tests for toxicity and absorption studies (Jenner and Janssen-Mommen, 1993);

• duckweed specimens are particularly sensitive to surface-active substances, hydrophobic compounds and similar substances, which concentrate at the air-water interface (Taraldsen and Norberg-King, 1990; A.S.T.M., 1991);

• unlike toxicity tests used for algae, test solutions for *Lemna*can be renewed, a process useful for replenishing them with substances that are rapidly lost from solutions due to volatilisation, photodegradation, precipitation or biodegradation. (Taraldsen and Norberg-King, 1990).

II.2.5 Duckweed usage for water depollution

The subfamily Lemnoideae includes small, free-floating aquatic plants, commonly known as duckweed, which have demonstrated considerable potential for use in water depollution, particularly in wastewater treatment activities and the removal of pollutants from aquatic environments. Various species of Lemnoideae such as *Lemna minor*, *Lemna minuta*, and *Lemna*trisulca have been studied for their abilities to absorb and accumulate nutrients and contaminants from aquatic environments, properties that have allowed researchers to consider them efficient agents for phytoremediation (Ansari et al., 2020; Ceschin et al., 2020).

1. Removal of excess nutrients:

Duckweed is highly effective in absorbing and assimilating nitrogen and phosphorus from water, common pollutants in agricultural runoff, urban wastewater, and effluent from treatment plants. High concentrations of these nutrients in water bodies can lead to their eutrophication, a process that results in excessive algal growth and depletion of oxygen levels, negatively affecting aquatic life.

By rapidly taking up these nutrients and incorporating them into their biomass, duckweed plants help mitigate the eutrophication of these waters with varying degrees of pollution (Sudiarto et al., 2019; Devlamynck et al., 2020).

In this regard, demonstrating a strong capacity to reduce ammonium (NH_4^+) and nitrate (NO_3^-) ion concentrations in polluted water, several duckweed species are considered particularly useful in treating agricultural runoff and industrial effluents with high levels of nitrogen compounds (Devlamynck et al., 2020; Sudiarto et al., 2019).

2. Removal of heavy metals:

Duckweed can accumulate heavy metals from contaminated waters in their bodies, such as cadmium, lead, chromium, mercury, arsenic, and copper, through bioaccumulation processes, where metal ions are either adsorbed onto the surface of plant organs or incorporated by absorption into their tissues. In this regard, studies have shown that species such as *Lemna minor* and *Lemna*gibba are particularly effective in removing heavy metals from the water bodies in which they grow, often tolerating and accumulating these toxic substances in their tissues (Bokhari et al., 2016; Pang et al., 2023).

By developing specific detoxification mechanisms, some duckweed species have managed to tolerate and store heavy metals in their own structures, reducing their availability in the water column and preventing them from entering the food chain (Devlamynck et al., 2020; Liu et al., 2021).

3. Degradation of organic pollutants:

Duckweed is effective in removing various organic pollutants from water, including pesticides, herbicides, pharmaceuticals, and other synthetic organic chemicals, which they can degrade either through rhizodegradation, where microbial communities associated with plant roots help degrade organic contaminants, or through phytodegradation, where the metabolic processes of these plants break down pollutants (Lobiuc et al., 2018; Ekperusi et al., 2019; Prakash et al., 2021).

4. Reduction of pathogens:

In wastewater treatment systems, duckweed plants have been found to contribute to the reduction of pathogenic bacteria and other harmful microorganisms, with dense duckweed mats creating a physical barrier that limits light penetration and thus inhibits pathogen growth. In addition, the high oxygen production during photosynthesis and its subsequent release from the plant body into the water can enhance aerobic microbial activity in this environment, a phenomenon that helps degrade organic matter and reduce pathogen levels (Ishizawa et al., 2019, 2020).

5. Biomass production for secondary uses:

Biofuel production: biomass generated from duckweed growth in nutrient-rich waters can be harvested and used as feedstock for biofuel production, including bioethanol and biogas. The high starch content of duckweed makes it an efficient source for ethanol production, while anaerobic digestion of duckweed biomass can produce methane-rich biogas (Sudiarto et al., 2019).

Animal feed and fertilisers: harvested duckweed biomass can also be used as a protein-rich feed supplement for animals and fish in aquaculture. In addition, dried duckweed can serve as an organic fertiliser, providing a sustainable way to recycle nutrients absorbed during the water depollution process (Hasan and Chakrabarti, 2010; Pagliuso et al., 2022; Demann et al., 2022).

II.3 Test species

II.3.1 *Lemna minor* L.

Lemna minor L. (common duckweed) is a small aquatic species that forms extensive populations on the surface of stagnant waters (lakes, marshes), but can also be found in slow-flowing riverbeds. (Foto II.1).

Foto II.1 – *Lemna minor* L. (original)

The plant has ovate or lanceolate fronds, with a single filiform root on the lower surface and two posterior lateral furrows from which new fronds bud, bilocular anthers, and a uniovulate ovary. The fronds have two flat surfaces, the upper one being ± keeled (Landolt, 1998; Bog et al., 2020).

A duckweed plant consists of a single frond with one or more roots. Most duckweed species multiply mainly by vegetative reproduction, through the formation of daughter fronds from two "pockets" located on each side of the narrow end of the frond. During the initial growth phase, newly formed fronds remain attached to the mother plant and, therefore, the plants appear to be composed of several fronds.

Species of the genus Spirodela have the largest fronds, measuring 20 mm in diameter, while Wolffia species measure 2 mm or less in diameter. Species of the genus *Lemna*are intermediate in size, approximately 6-8 mm. During its lifespan, which lasts from 10 days to several weeks, an individual can produce approximately 20 daughter fronds. The daughter fronds will separate from the mother plant and, in turn, produce other new fronds (Photo II.2) (Blodgett, 1915; Acosta et al., 2021).

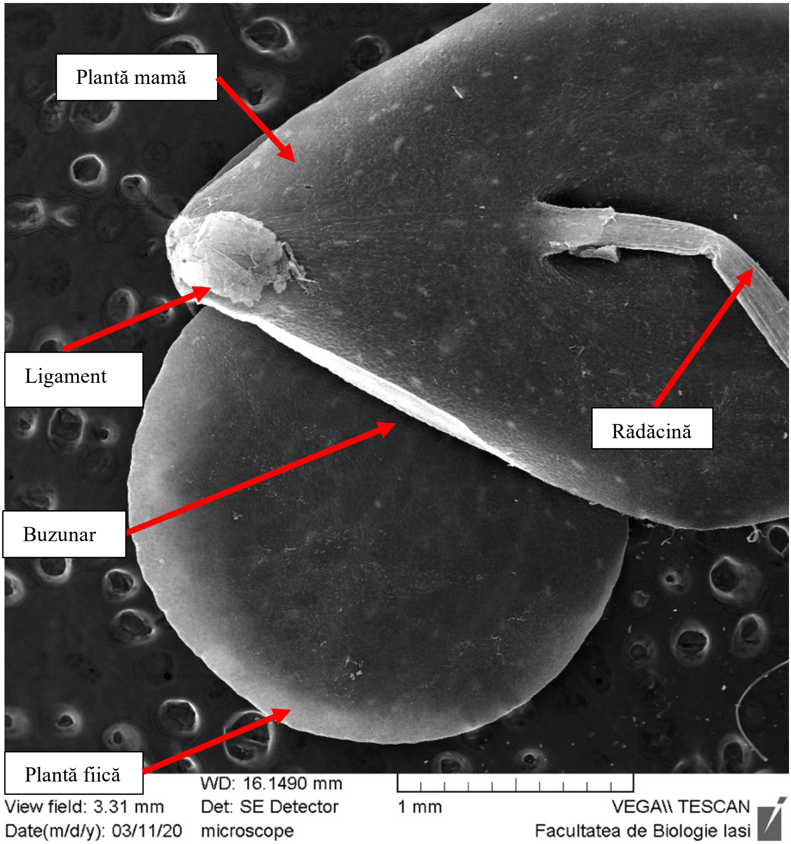

Foto II.2 - *Lemna minor* L. – lower face – photomicrograph SEM (original)

As it is a floating species, duckweed stomata are found on the upper epidermis (Photo II.3). The frond is flat, green, light green, and has a central vein. At the base of the frond, on the underside, is the root, with lengths between 0.2 and 10 cm. At the root level, there is a structure similar to the root cap of terrestrial plants, with a protective role (Photo II.4).

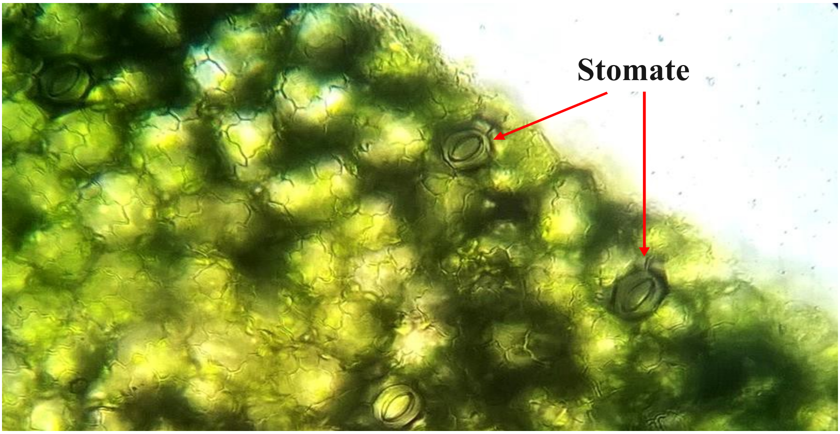

Foto II.3 – *Lemna minor* L. - Upper epidermis with stomata – optical microscope - 10x (original)

Foto II.4 – *Lemna minor* L.- Root – optical microscope 10x (original)

Observed through transparency against a light source, the frond presents 3 main conducting vessels, located as follows: two on the edge of the frond and one centrally located. All these conducting vessels start from the base of the plant, from the root attachment point. In the frond's structure, air spaces can also be identified, represented by large cells with slightly thickened walls (Photo II.5).

The duckweed frond is characterised by an envelope or pocket on each side of the base. Within each of these pockets, a frond of the next vegetative generation will appear, which does not develop at the same time. When flowers form, each grows in the position where the frond normally grows, although a young frond may develop later in the same pocket (Tippery et al., 2021).

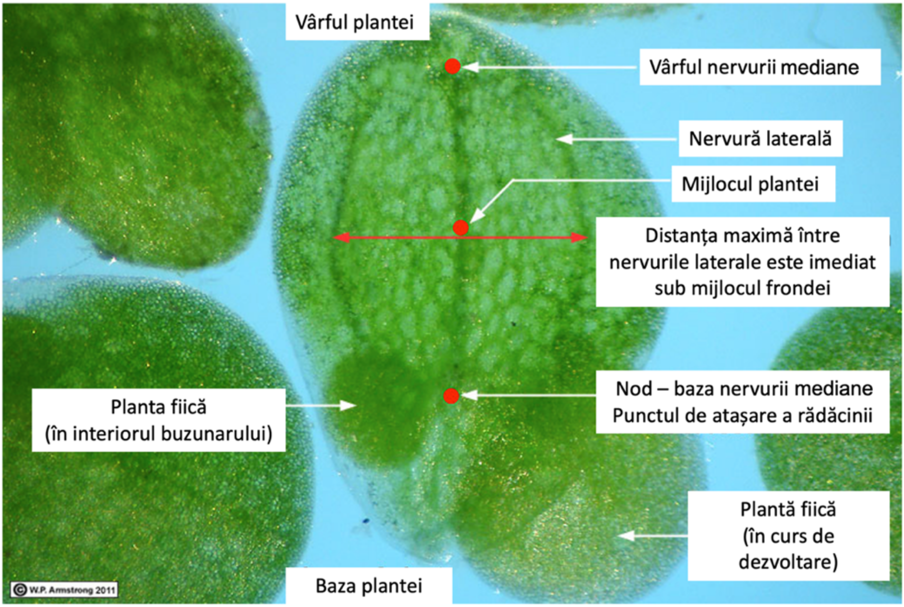

Foto II.5 – *Lemna minor* L. – Morphology (d. Armstrong, 2011)

Behind the growing frond or flower, there is a bud. Normally, the rudiments of this bud appear very early in the development of the frond, in the axil where it is located. At almost the same time as the root develops, a slight bump or cluster of cells forms on the dorsal surface of the bud. By the time the daughter frond is quite large and robust, the pocket in which it is located is enclosed by a thin membrane, which will rupture when the tissues of the daughter frond press against it (Baek et al., 2021).

The connection of the daughter plant to the mother plant is maintained by a ligament, a bundle of conducting vessels, to allow the root development of the daughter plant to be completed, as the cells forming the root could not multiply due to the limited space in the pocket. The ligament between the two plants can be retained even after development is complete (Photo II.6).

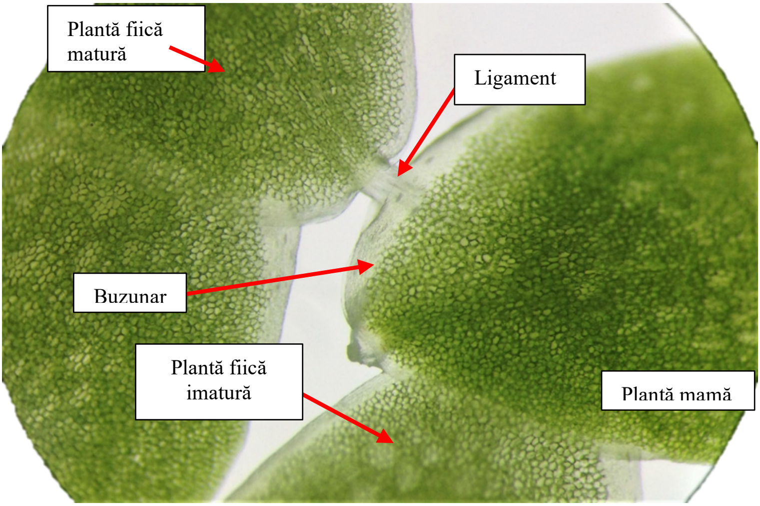

Foto II.6 – *Lemna minor* L. – individuals attached by means of a ligament; superior frontal view – stereomicroscope photo - 4x (original)

II.3.2 *Lemna minuta* Kunth

Lemna minuta Kunth, commonly known as least duckweed or dwarf duckweed, is an aquatic plant species native to the Americas. Having been introduced to various parts of Europe, Asia, and Australia, it is often considered invasive due to its rapid growth and ability to outcompete native aquatic vegetation (Hussner et al., 2017; Ceschin et al., 2018).

Lemna minuta Kunth is characterised by its small size and simple structure, typical of duckweed. The plant consists of a free-

floating, flattened frond, resembling a leaf, which performs both leaf and stem functions (Photo II.9).
The frond is very small (approximately 1.5 - 4 mm long and 1 - 2 mm wide), oval to elliptical in shape, with a rounded tip and a slightly conical base. It is light green in colour, with several air pockets or aerenchyma, which aid in buoyancy; its upper surface is usually smooth, but can sometimes present papillae (Ceschin et al., 2016).

Foto II.9 – *Lemna minuta* Kunth (original)

Each frond typically produces a single, slender, unbranched root on its underside, resembling a thread 5 to 15 mm long (Photo II.10), primarily serving as a stabilising organ, but which can also aid in nutrient absorption from the water column (Landolt, 1998; Ceschin et al., 2016).

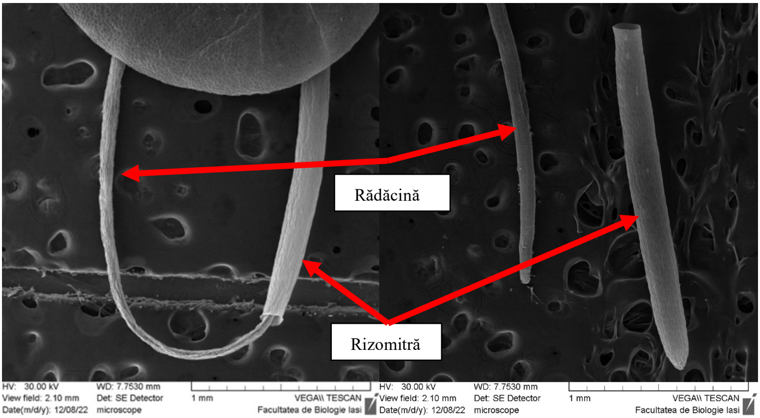

Foto II.10 – *Lemna minuta* Kunth – Micromorphological aspect of the root – microphotography SEM (original)

Lemna minuta usually reproduces asexually through vegetative budding. New daughter fronds form at the base of the mother frond, which eventually separate and become independent. Although sexual reproduction is rare in this species, the plant produces very small flowers, which are largely unnoticed as they are embedded in the frond structure and consist of a single stamen and a pistil.

Lemna minuta is known for its rapid vegetative reproduction. The plant produces new individuals at a high rate under favourable conditions, leading to exponential growth.

In nutrient-rich environments, such as ponds, slow-moving streams, or ditches with high levels of nitrogen and phosphorus, *Lemna minuta* can rapidly form dense colonies that cover the water's surface (Landolt, 1998; Ceschin et al., 2018; Chen et al., 2022).

Part II – Personal contributions

Chapter III – Matherials and methods

III.1 Test species

The plant material used in the research for this thesis consists of plant specimens belonging to two species from the subfamily Lemnoideae: *Lemna minor* L. and *Lemna minuta* Kunth.

Individuals of *Lemna minor* L. were collected on 26.08.2018 from the municipality of Iași (Nicolina River, at its confluence with the Bahlui River, GPS coordinates for the location: 47.157632 lat. N, 27.569232 long. E). Although the species typically grows on the surface of still waters, the abundant vegetation and high nutrient content in these rivers allowed for the formation of duckweed colonies that are carried by currents whenever the water level rises.

Several specimens belonging to this species have been deposited in the Herbarium of the Faculty of Biology at "Alexandru Ioan Cuza" University in Iași, receiving the registration number 207024.

Foto III.1 - *Lemna minor* L. collection site - 26.08.2018
(d. Google Maps, 2023)

Specimens of *Lemna minuta* Kunth were collected on 01.07.2022 from Staw Południowy pond, Huby Moraskie area, located on the outskirts of Poznań, Poland. The GPS coordinates of the location are: 52.466094 lat. N, 16.933088 long. E.

Several specimens belonging to this species have been deposited at the Herbarium of the Faculty of Biology of "Alexandru Ioan Cuza" University in Iași, receiving the following registration number: *Lemna minuta* Kunth – 207026.

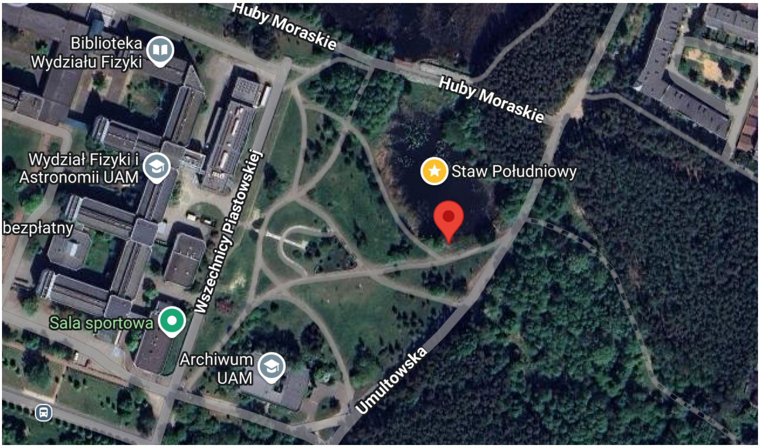

Foto III.2 - *Lemna minuta* Kunth collection site - 01.07.2022 (d. Google Maps, 2023)

III.2 Nanomaterials

In this thesis, three types of synthetic carbon-based nanomaterials were used:

• multi-walled carbon nanotubes with an outer diameter of 8 nm, purity > 96%, Manufacturer Nanografi, Ankara, Turkey – Product Code: NG01MW0101;

• carboxylated multi-walled carbon nanotubes with an outer diameter between 8 and 18 nm, purity > 96%, Manufacturer Nanografi, Ankara, Turkey – Product Code: NG01MW0303;

• fullerene soot – a combination of C60, C70 fullerenes and carbon black, which can be used in a variety of applications, such as water purification, hydrogen storage, supercapacitor production and in nanoelectronics – Manufacturer Sigma-Aldrich – Product Code: 572497-5G,

III.3 Experimental protocols

III.3.1 Experimental design

To ensure the smooth running of the testing process, we followed the provisions of testing guideline no. 221 of the Organisation for Economic Co-operation and Development (OECD), which provides detailed procedures for cultivating species of the genus *Lemna* L., particularly *Lemna gibba* L. and *Lemna minor* L.

Below, we present the main stages of the procedure for cultivating duckweed test species:

• Creating and maintaining a stock culture of *Lemna* under optimal conditions for healthy, exponential growth, an activity that involved its regular transfer to fresh medium to prevent nutrient depletion and waste accumulation. The cultures thus obtained were kept under controlled conditions, with a temperature of 24 ± 2°C and illumination for 17 hours a day, at an intensity of approximately 115 - 118 µmol/s/m2.

• Creating secondary stock cultures, starting from a single individual for each tested species, to avoid phenotypic variability of *Lemna*individuals.

• Selecting from these secondary cultures the individuals necessary for conducting the tests: plants were cultivated during the experiments in trays with 10 wells, each with a volume of 10 ml, covered on the outside with black paint to prevent the development of contaminating algae.

• Maintaining the cultures in a thermostat at a constant temperature of 25 °C and illumination for 17 hours a day, and monitoring the growth of individuals at intervals of 2-3 days.

*To ensure adequate growth and reproducibility during the testing period, each well received a defined number of fronds (1 individual = 3 fronds) (Photo III.3)

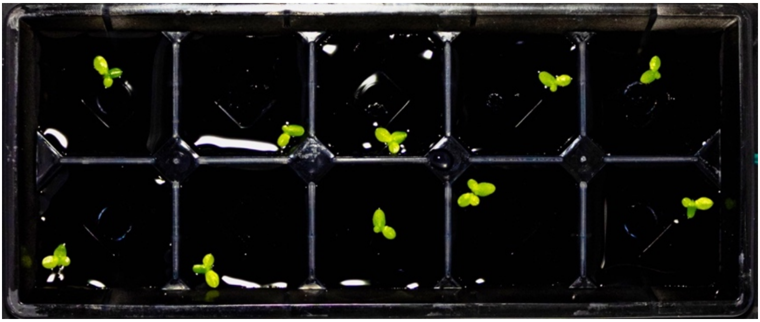

Foto III.3 – *Lemna* cultivation tray (original)

The experimental design, which aimed at the response of test plants to the presence of synthetic carbon-based nanomaterials experimentally included in the culture medium, was particularised for the two species of the genus *Lemna* L. as follows:

- in the case of the species *Lemna minor* L., recognised as a model species in aquatic ecotoxicology research, the research included morpho-anatomical, physiological, biochemical, and molecular biology determinations (subchapter IV.1 of chapter IV of the Personal Part);
- in the case of the species *Lemna minuta* Kunth, widely distributed but little studied in terms of complex morpho-functional reactivity to changes in living conditions, the research

included morphological, biochemical, and physiological determinations (subchapter IV.2 of chapter IV of the Personal Part).

Capitol IV – Results and discussion

IV.1 Interactions of plants belonging to the species Lemna minor L. with synthetic carbon-based nanomaterials

IV.1.1 Morphological and micromorphological reactions

To observe and interpret any morphological changes induced by nanomaterial treatments applied to duckweed (*Lemna minor*) plants, the plant material was analysed by transparency under a stereomicroscope in fresh microscopic preparations.

IV.1.5 Physiological reactions

IV.1.5.1 Chlorophyll fluorescence

At the initial stage of the experiment, ΦPSII parameter values were relatively high and uniform across all applied treatments, suggesting that the initial exposure of the test plants to the tested nanomaterials did not exert an immediate effect on their photosystem II efficiency. On the last day of the experiment, a clear trend of reduction in ΦPSII values is observed, especially in the variants treated with nanotubes. These changes indicate a possible cumulative toxicity of the nanomaterials, progressively manifested over time, on the photosynthetic apparatus.

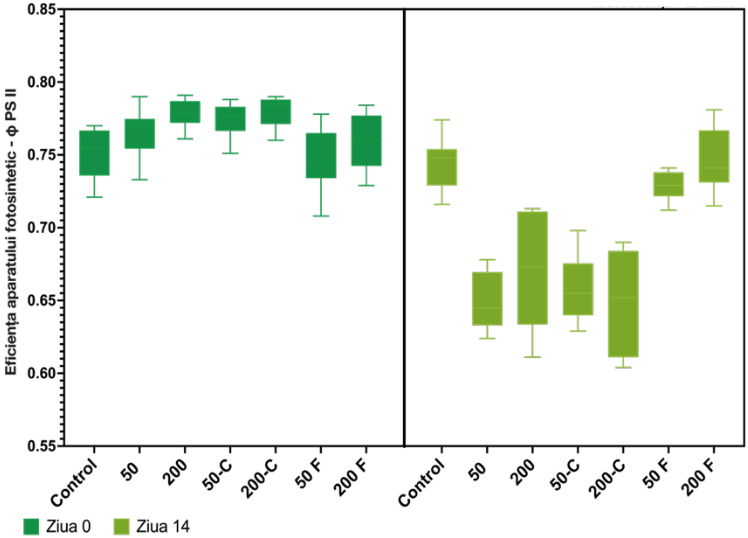

Fig. IV.5 – Evolution of chlorophyll fluorescence in *Lemna minor* L. individuals from day 0 to day 14 of the experiment. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot.

IV.1.5.2 The content of assimilatory pigments

For all categories of photoassimilatory pigments analysed (chlorophyll a, chlorophyll b, and carotenoid pigments), treatment with multi-walled carbon nanotubes led to lower quantitative levels compared to the control variant, indicating a phytotoxic effect dependent on the dose (Fig. IV.7, Annex 5). These results suggest that exposure to MWCNTs accentuates oxidative and photoinhibitory stress, an aspect previously documented in aquatic plants exposed to nanomaterials (Meften et al., 2023).

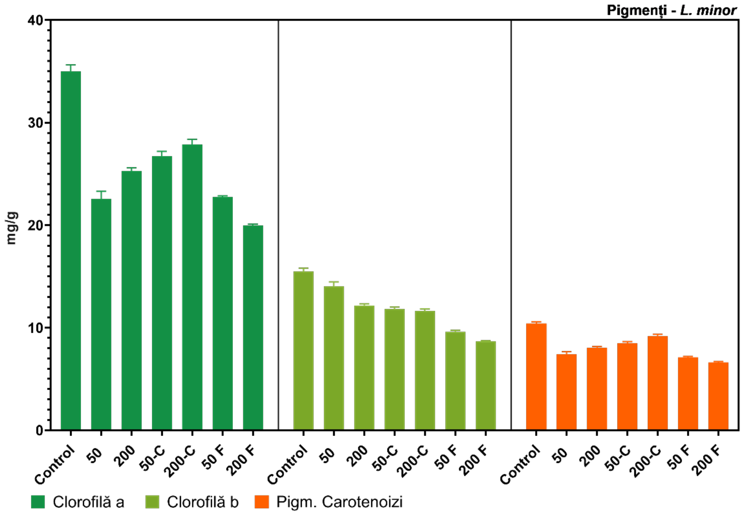

Fig. IV.7 – The content of assimilating pigments in *Lemna minor* L. plants on day 14 of the experiment. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot.

IV.1.5.3 Flavonoid content

Treatment with multi-walled carbon nanotubes functionalised with carboxyl groups (MWCNT-COOH) led to a significant increase in flavonoid content, as a clear dose-dependent response of the tested plant metabolism, with the highest accumulation of these compounds observed at a concentration of 200 mg/l MWCNT-COOH, indicating an intense activation of antioxidant pathways, most likely in response to amplified oxidative stress (Fig. IV.11, Annex 6). This effect can be attributed to the increased chemical reactivity and bioavailability of the functionalised nanotubes, which are favoured in their penetration into plant tissues and interaction with plant cells (Chen et al., 2018; Jiang et al., 2020).

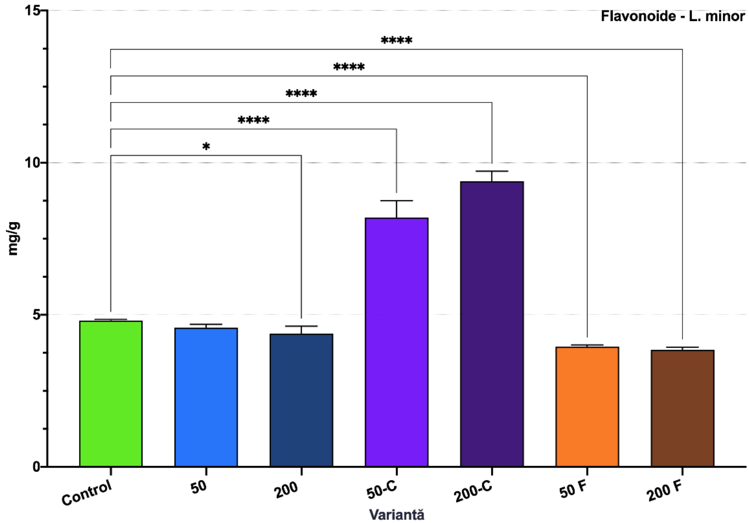

Fig. IV.11 – Flavonoid content of *Lemna minor* L. plants grown under experimental conditions. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with * (* - $p = 0.0150$; **** - $p < 0.0001$)

IV.1.5.4 Polyphenol content

Exposure of duckweed to MWCNTs led to a moderate increase in polyphenols at a concentration of 50 mg/l, compared to the control group. At 200 mg/l, polyphenol accumulation was slightly higher than at 50 mg/l, suggesting a dose-dependent response to oxidative stress. This trend correlates with specialist literature indicating the accumulation of phenolic compounds as an adaptive protective mechanism against ROS generated in the presence of nanomaterials (González-García et al., 2019).

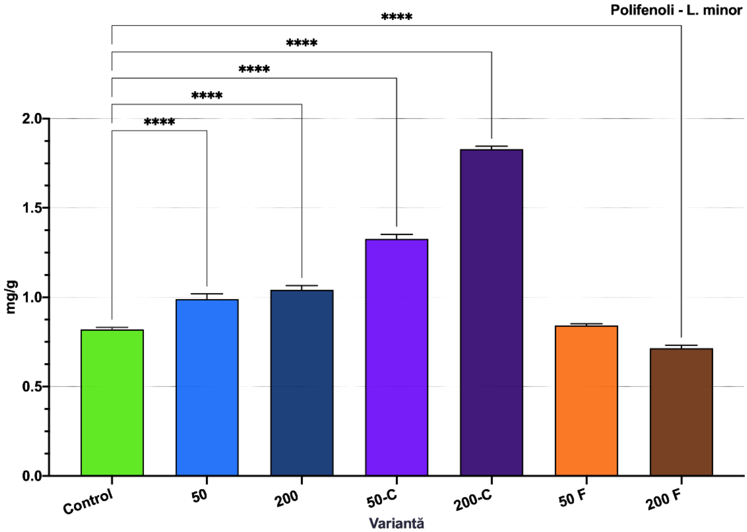

Fig. IV.12 – Polyphenol content of *Lemna minor* L. plants grown under experimental conditions. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with * (**** - $p < 0.0001$)

IV.1.6 Biochemical reactions

IV.1.6.1 Peroxidase activity (POD)

Following exposure to multi-walled carbon nanotubes (MWCNT) and carboxyl-functionalised nanotubes (MWCNT-COOH), *Lemna minor* plants showed a significant reduction in POD activity, especially at a concentration of 200 mg/l. The most pronounced reduction was recorded in the case of MWCNT-COOH treatment. This enzymatic inhibition could be attributed to the oxidative damage of the protein-enzymes directly involved or to an interference in the regulatory pathways of gene expression associated with antioxidant defence (Ma et al., 2010; Siddiqui et al., 2015). On the other hand, the decrease in POD activity may reflect an excessive accumulation of ROS, which exceeds the plant's enzymatic detoxification capacity, leading to redox imbalances and extensive cellular damage (Dietz și Herth, 2011).

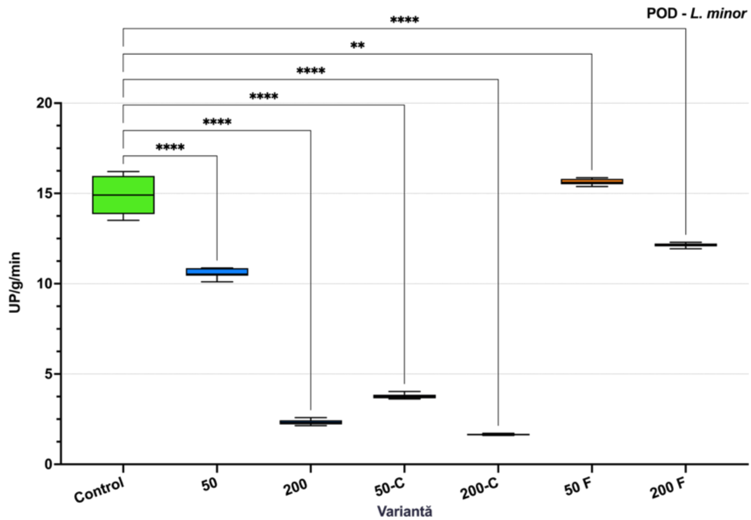

Fig. IV.13 – Peroxidase activity of *Lemna minor* L. specimens grown under experimental conditions. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with * (** - $p = 0.0026$; **** - $p < 0.0001$)

IV.1.6.2 Superoxide dismutase activity (SOD)

Following treatments with MWCNT, MWCNT-COOH, and fullerene soot, duckweed showed a significant decrease in SOD activity, with the lowest levels observed at a concentration of 200 mg/l for all analysed nanomaterials. This inhibition indicates a major disruption of the redox balance in the tested plants, possibly either through the exhaustion of SOD's antioxidant capacity due to excessive

ROS accumulation, or through direct inactivation of the enzyme at the cellular level due to oxidative stress. (Mittler, 2002; Gill and Tuteja, 2010) (Fig. IV.14, Anexa 9).

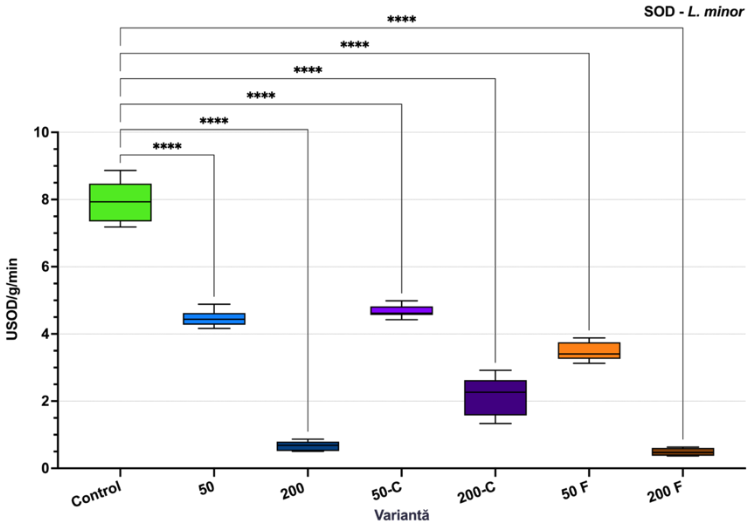

Fig. IV.14 – Superoxide dismutase activity of *Lemna minor* L. specimens grown under experimental conditions. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with * (**** - $p < 0.0001$)

IV.1.6.3 Soluble protein content

The exposure of *Lemna minor* cultures to different concentrations of multi-walled carbon nanotubes (MWCNT), carboxyl-functionalised MWCNT (MWCNT-COOH), and fullerene soot led to significant variations in total protein content compared to the control group. These results are consistent with data from the specialist literature, which describe oxidative stress and disruption of protein metabolism in response to nanomaterial exposure (Mittler, 2002; Zhang et al., 2017) (Fig. IV.15, Anexa 10).

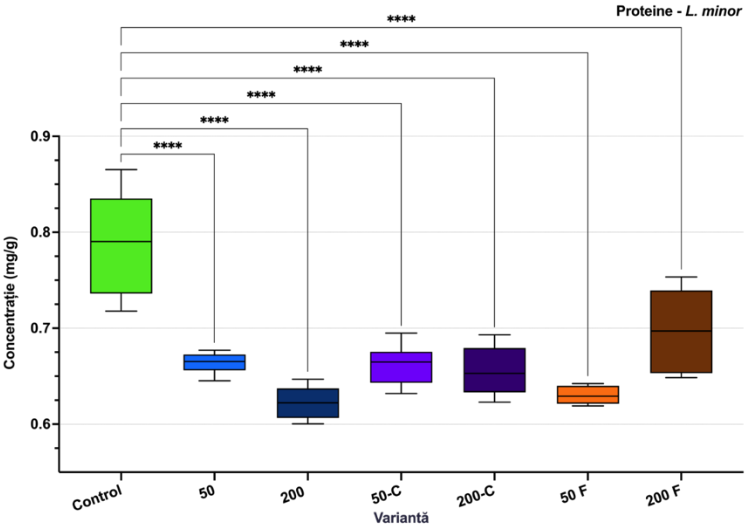

Fig. IV.15 – Protein content of *Lemna minor* L. specimens cultivated under experimental conditions. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with * (**** - $p < 0.0001$)

IV.1.6.4 Total antioxidant activity

Compared to the control variant, all treatments with nanomaterials generated significant changes in the DPPH radical scavenging activity of the tested plants, confirming that the presence of nanomaterials in the culture medium caused a systemic oxidative response in them, where the most evident increase in total antioxidant activity was observed in the treatment with MWCNT-COOH at a concentration of 200 mg/l, followed by the treatment with MWCNT at a concentration of 200 mg/l (Fig. IV.16, Annex 11).

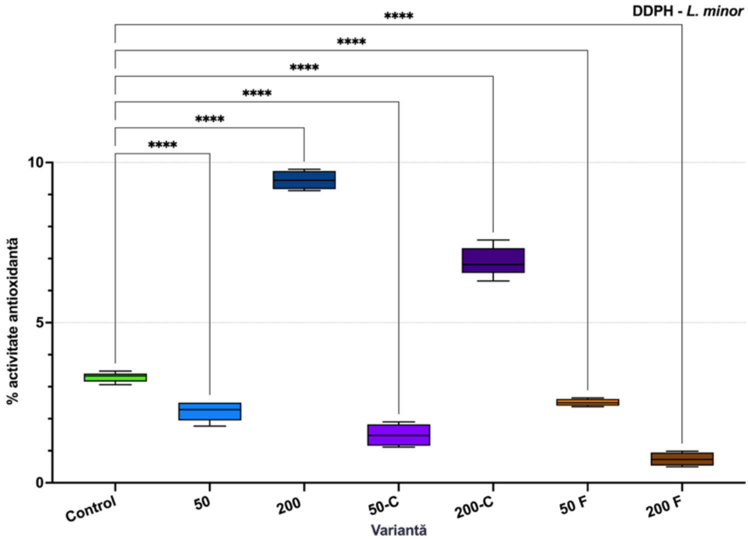

Fig. IV.16 – The total antioxidant activity of *Lemna minor* L. specimens grown under experimental conditions (Test DPPH). Variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with * (**** - p < 0.0001)

IV.1.7 Gene expression evaluation

IV.1.7.4 Marker gene expression for photosynthetic activity: RBSC

In all treatment variants, RBSC expression was significantly reduced compared to the control, indicating a decrease in the photosynthetic process evaluated at the transcriptional level. A significant decrease was recorded in the MWCNT and MWCNT-COOH treatments, highlighting the influence of the nanomaterial type on oxidative stress and on the suppression of gene expression in the tested plants. (Fig. IV.17, Annex 12) (Gill and Tuteja, 2010).

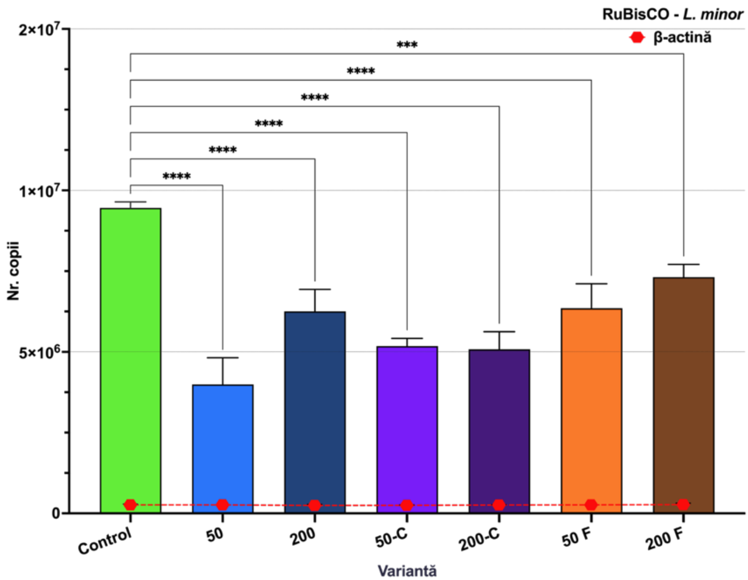

Fig. IV.17 – RBSC gene expression in *Lemna minor* L. specimens grown under experimental conditions. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with * (*** - p = 0.0003; **** - p < 0.0001)

IV.1.7.5 Gene expression associated with hormonal metabolism: NCED

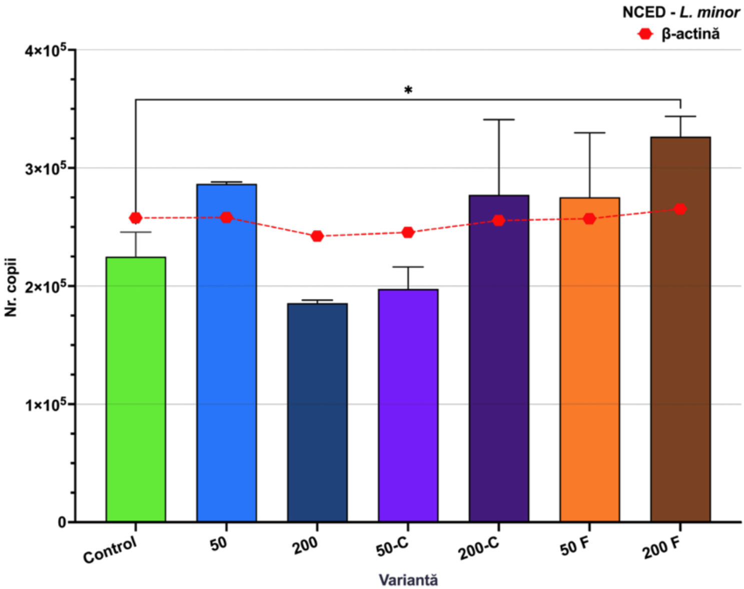

Fig. IV.18 – NCED9 gene expression in *Lemna minor* L. specimens grown under experimental conditions. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with * (* - p = 0.0121)

In variants treated with MWCNT and MWCNT-COOH, NCED gene expression was increased at both tested concentrations (50 and 200 mg/l), with a more pronounced induction level at the 200 mg/l dose. This pattern suggests a dose-dependent activation of the ABA biosynthetic pathway, reflecting the severity of oxidative stress induced by these nanomaterials. (El-Saadony et al., 2022).

IV.1.7.6 Gene expression involved in oxidative stress response: POD (peroxidaza)

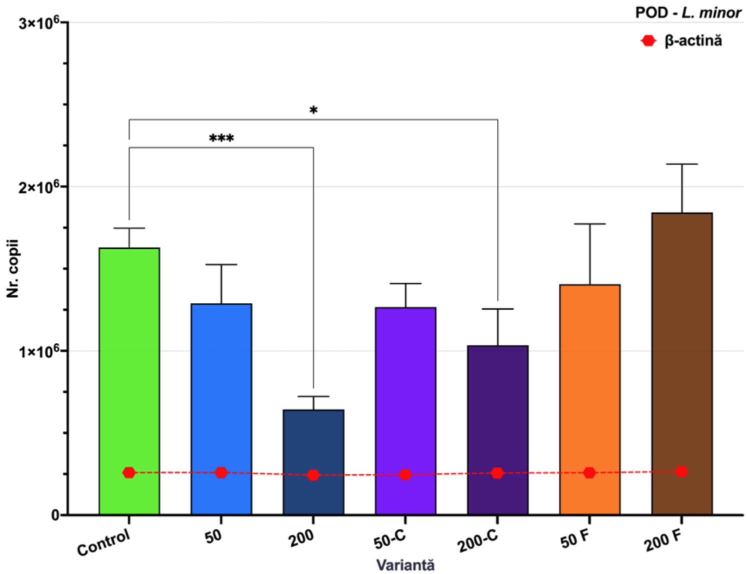

Fig. IV.19 – Gene expression of POD in *Lemna minor* L. specimens grown under experimental conditions. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with * (* - p = 0.0232; *** - p = 0.0002)

The results obtained indicate that fullerene soot, especially at high concentrations, stimulates POD transcription more efficiently than the other types of nanomaterials tested, possibly due to the continuous generation of ROS or the modification of intracellular redox signalling (Wang et al., 2024).

IV.1.7.7 Gene expression involved in oxidative stress response: SOD (superoxide-dismutase)

The relative expression of the SOD gene was increased in all treatment variants with carbon-based nanomaterials, compared to the control group, which indicates a systemic oxidative response in *Lemna minor* plants (Fig. IV.20, Annex 15).

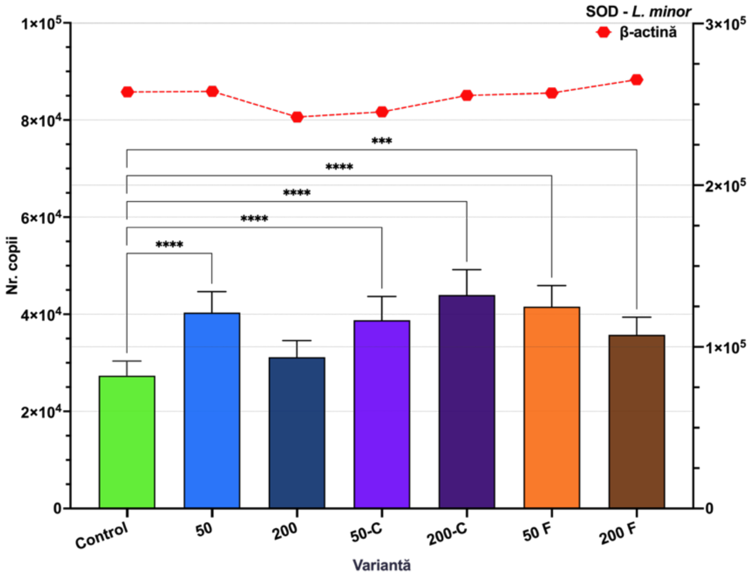

Fig. IV.20 – Gene expression of *Lemna minor* L. specimens grown under experimental conditions. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with * (*** - p = 0.0007; **** - p < 0.0001)

MWCNT at a concentration of 200 mg/l led to an increase in the expression of this gene, though slightly lower than that observed for the 50 mg/l treatment, which may reflect either negative feedback regulation or an overload of the antioxidant system under conditions of more severe stress (Cheng et al., 2016).

MWCNT-COOH at concentrations of 50 and 200 mg/l maintained high levels of SOD gene expression, supporting the hypothesis that the carboxylic functionalisation of nanotubes amplifies their interaction with plant tissues and promotes continuous ROS production in the tested plant cells.

Fullerene soot, at both 50 mg/l and 200 mg/l concentrations, led to a moderate increase in SOD gene expression, with a more pronounced induction at the higher concentration, suggesting a dose-dependent oxidative signalling (Yang et al., 2021).

IV.1.7.8 Gene expression associated with senescence and apoptosis: DAD

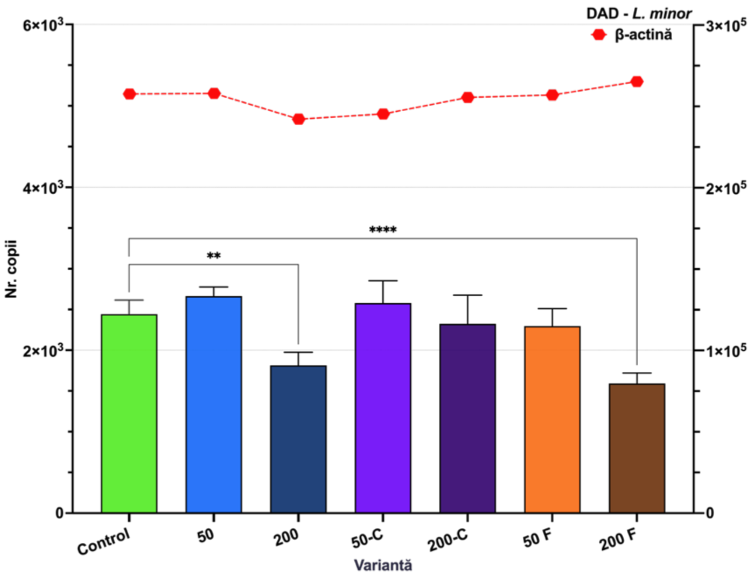

Fig. IV.21 – Expression of the DAD1 gene in *Lemna minor* L. specimens grown under experimental conditions. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with * (** - p= 0.0017; **** - p <0.0001)

In the case of MWCNT treatment, DAD gene expression was slightly increased at a concentration of 50 mg/l, indicating an adaptive response of the plants to a moderate level of oxidative stress. However, at a concentration of 200 mg/l, the expression of this gene significantly decreased, suggesting that the intensification of stress exceeded the capacity of the plants' defence system, leading to the possible activation of cell death pathways.

IV.2 Interactions of plants belonging to the species Lemna minuta Kunth with synthetic carbon-based nanomaterials

IV.2.1 Development of *Lemna minuta* Kunth cultures under experimental cultivation conditions

IV.2.1.1 Morphological reactions

In the case of treatment with multi-walled carbon nanotubes, a decline in the number of leaves/fronds belonging to the 50 mg/l concentration was observed. This experimental model indicates an inhibitory effect of the tested nanotubes, dependent on the applied dose, with the practical results obtained being consistent with previous reports regarding the effect of applying multi-walled carbon nanotubes (MWCNT), which affect the integrity of the surface of vegetative and reproductive organs of plants and suppress their growth (Begum et al., 2012b).

An even more pronounced reduction in the number of leaves/fronds of *Lemna minuta* plants was evidenced in the case of applying both concentrations of carboxylated nanotubes, a phenomenon suggesting that the carboxylation process enhances the toxicity of these nanotubes, probably as a result of their increased solubility and bioavailability, as stated in the specialised literature (Zhang et al., 2016).

Treatment with fullerene soot induced a different response: the number of newly formed leaves/fronds of *Lemna minuta* plants increased as a result of applying the 50 mg/l concentration, but slightly decreased in the case of applying the 200 mg/l concentration. This biphasic response trend may reflect a hormetic effect, where low doses of synthetic carbon-based nanomaterials favour the growth process of test plants, while higher doses trigger oxidative stress, a phenomenon reported in the specialised literature in connection with the effect of fullerene nanotubes experimentally applied to test plants (Gogos et al., 2012) (Fig. IV.22, Annex 17, Annex 31).

IV.2.2 Physiological reactions

IV.2.2.1 Chlorophyll fluorescence

In the case of testing multi-walled carbon nanotubes (MWCNT), a clear reduction in ΦPSII parameter values was obtained (dependent on the concentration of the applied treatment), with practical results indicating a progressive impairment of the photosynthetic efficiency of the photoassimilatory apparatus of *Lemna minuta* plants with increasing exposure to these nanomaterials. This finding aligns with results reported in the specialised literature, which show that MWCNTs can disrupt chloroplast structure, reduce chlorophyll content levels, and induce ROS formation, ultimately leading to photoinhibition phenomena in test plants (Meften et al., 2023).

The decrease in photosynthetic efficiency of the photoassimilatory apparatus of *Lemna minuta* plants was even more pronounced when cultivated in the presence of carboxylated nanotubes. Thus, at a concentration of 200 mg/l, ΦPSII reached the lowest value in the entire experimental series, suggesting that the functionalisation process enhances the toxicity of the nanotubes, probably due to their improved dispersion in the environment and more intense absorption at the epidermal cell level. (Wang et al., 2014).

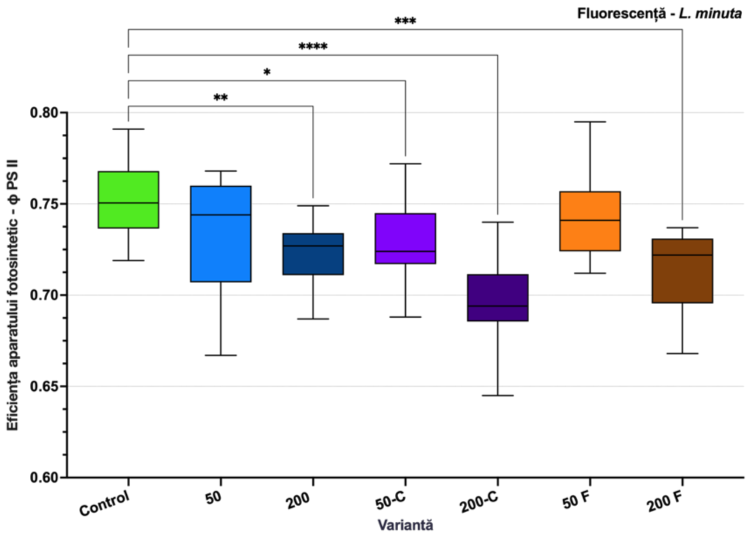

Fig. IV.26 – Photosynthetic efficiency of *Lemna minuta* Kunth plants on day 14 of the experiment. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with * (* - p = 0.0348; ** - p = 0.0064; *** - p = 0.0001; **** - p < 0.0001)

IV.2.2.2 The content of assimilatory pigments

Both tested multi-walled carbon nanotube treatments caused a slight decrease in the content of photoassimilatory pigments (chlorophyll a, chlorophyll b, carotenoid pigments) in *Lemna minuta* plants. The values thus obtained indicate the induction of a dose-dependent inhibitory effect. This practically recorded trend probably reflects the progressive disruption of pigment biosynthesis or the degradation of chloroplast structure in the photoassimilatory tissue of the test plants, processes potentially triggered by oxidative stress induced by simple nanotubes (MWCNT) present in their culture medium (Wellburn et al., 1972).

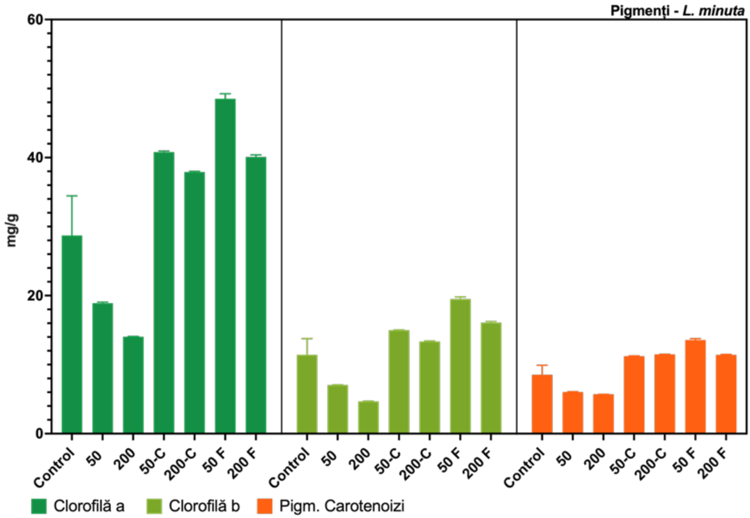

Fig. IV.27 – Comparison of assimilatory pigment content in *Lemna minuta* Kunth plants on day 14 of the experiment. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot.

IV.2.2.3 Flavonoid content

In the treatment variant with multi-walled carbon nanotubes (MWCNT), a modest increase in flavonoid content was observed as their concentration increased (from 50 mg/l to 200 mg/l). The results indicate an activation of the biosynthesis response of these compounds, which have an antioxidant role in plant tissues, dependent on the applied dose of the respective nanotubes. Both variants showed flavonoid levels below the control variant. The recorded response reflects a possible high level of oxidative stress manifested in the tissues of *Lemna minor* plants, with flavonoids being known for their role in mitigating the action of reactive oxygen species (ROS) that appear in plant organisms under conditions of abiotic stress induced by the presence of various types of chemical compounds, including synthetic nanomaterials, in their environment (Ma et al., 2010; Jiang et al., 2019).

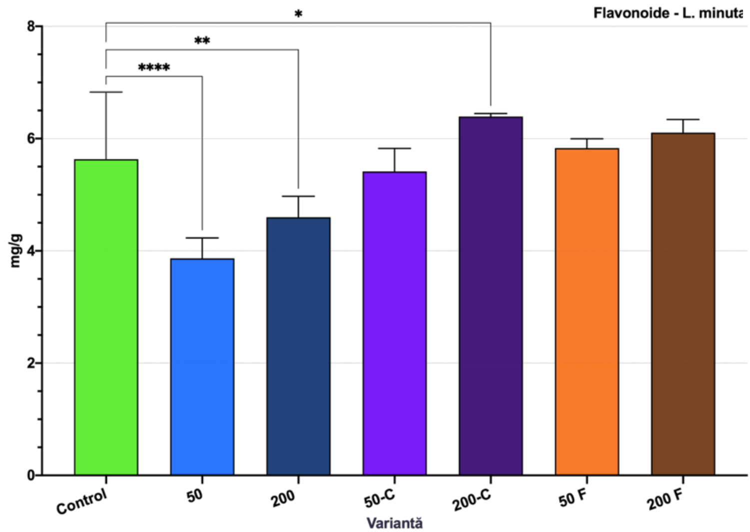

Fig. IV.31 – Flavonoid content of *Lemna minuta* Kunth plants grown under experimental conditions. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with * (* - $p = 0.0354$; ** - $p = 0.0017$; **** - $p < 0.0001$)

IV.2.2.4 Polyphenol content

In the case of treatments with carboxylated multi-walled carbon nanotubes (MWCNT-COOH), an increase in the total level of polyphenols synthesised by the test plants was observed. This type of physiological reaction of *Lemna minuta* plants implies an intense stimulation of their antioxidant defence system, a reaction probably determined by the improved dispersibility of the respective nanotubes in the aquatic environment and the increased cellular absorption of functionalised nanotubes (MWCNT-COOH) at the level of plant tissues, characteristics that enhance their functional impact with the support plants (Aryal et al., 2019).

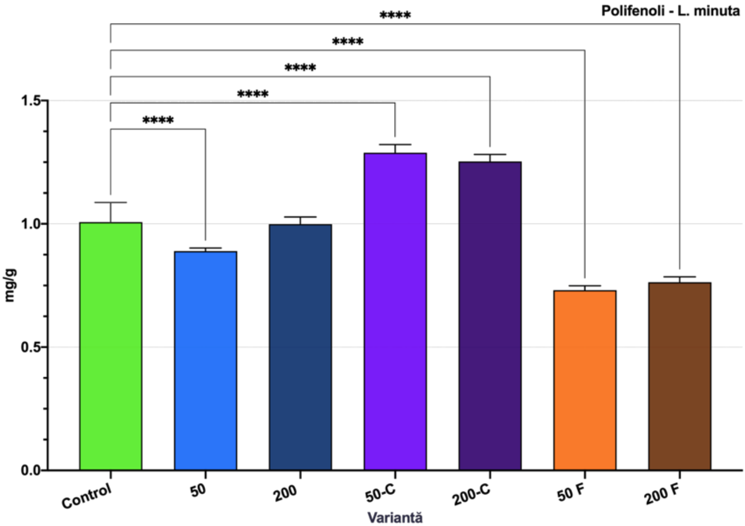

Fig. IV.32 – Polyphenol content of *Lemna minuta* Kunth plants grown under experimental conditions. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with * (**** - $p < 0.0001$)

IV.2.3 Biochemical reactions

IV.2.3.1 Peroxidase activity (POD)

Peroxidase (POD) activity showed a notable increase at a concentration of 50 mg/l for all nanomaterials experimentally applied in the culture medium of the test plants, inducing the activation of their antioxidant capacity, in response to the oxidative stress created. In the case of high concentrations of experimentally used nanomaterials (200 mg/l), POD activity decreased, even falling below the value recorded for the control variant, which suggests that higher concentrations of these synthetic chemical compounds can overwhelm the enzymatic defence system of plants, leading to cellular oxidative damage installed as a result of the inhibition of the component enzyme activity (Ma et al., 2010)(Fig. IV.33, Anexa 24).

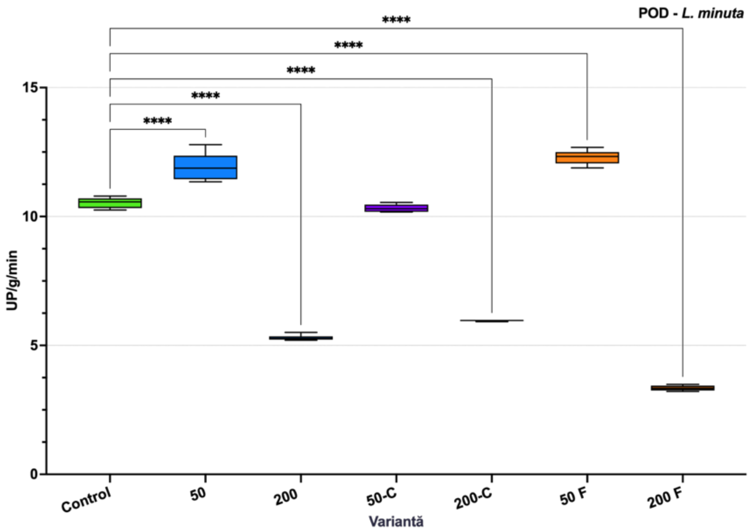

Fig. IV.33 – Peroxidase activity in *Lemna minuta* Kunth specimens grown under experimental conditions. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with * (**** - $p < 0.0001$)

IV.2.3.2 Superoxide dismutase activity (SOD)

In the case of *Lemna minuta* cultures interacting with experimentally applied carbon-based nanomaterials, SOD activity was consistently and strongly reduced by the presence of these chemical compounds in their living environment, with the most evident inhibition observed at high concentrations of nanomaterials (200 mg/l). This type of biochemical response reflects an inhibitory effect on SOD activity dependent on the experimental treatment dose, an effect possibly linked to an initial oxidative "burst" at lower concentrations of the tested nanomaterials, followed by enzyme inactivation or the appearance of severe cellular oxidative lesions in plant tissues, as a result of increasing the application dose of the respective nanomaterials (Ma et al., 2010)(Fig. IV.34, Annex 25).

The biochemical aspects discussed suggest a possible pathway for disturbing the primary antioxidant defence of plants and increasing their susceptibility to environmental oxidative stress, with the results validating SOD as a reliable biomarker of oxidative disruption and enzymatic impairment in aquatic plants exposed to the presence of nanomaterials in their living environment.

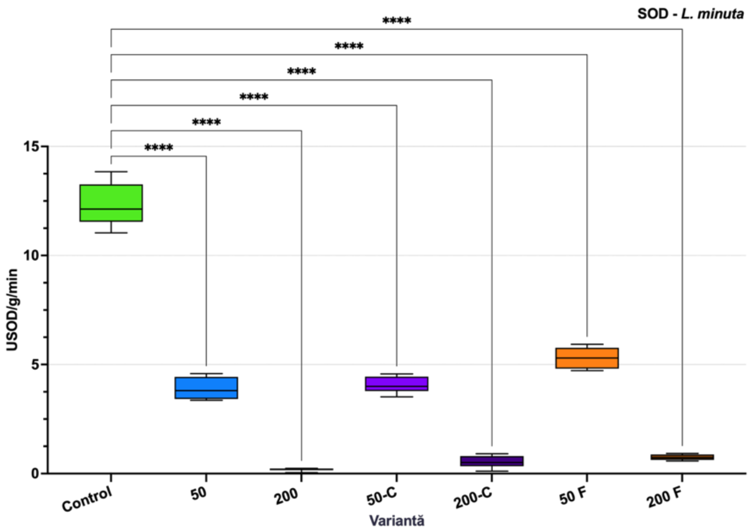

Fig. IV.34 – Superoxide dismutase activity in *Lemna minuta* Kunth specimens grown under experimental conditions. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l

Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with * (**** - $p < 0.0001$)

IV.2.3.3 Soluble protein content

The variation in protein content, which was higher in almost all test variants compared to the control variant, suggests that most synthetic carbon-based nanomaterials cause metabolic activation associated with defence processes and cellular adaptation. The only inhibitory effect, observed at a concentration of 50 mg/l multi-walled carbon nanotubes (MWCNT), indicates potential metabolic suppression or a delayed response of plants belonging to that experimental variant to the chemical stress induced by this lower concentration of MWCNT. In contrast, functionalised nanomaterials (MWCNT-COOH) and fullerene soot led to an increase in protein content, indicating their ability to stimulate detoxification mechanisms and cellular repair. These results support total protein content as a reliable indicator of metabolic changes and oxidative stress responses induced by nanomaterials in aquatic plants. (Ma et al., 2010; Zhang et al., 2017)(Fig. IV.35, Annex 26).

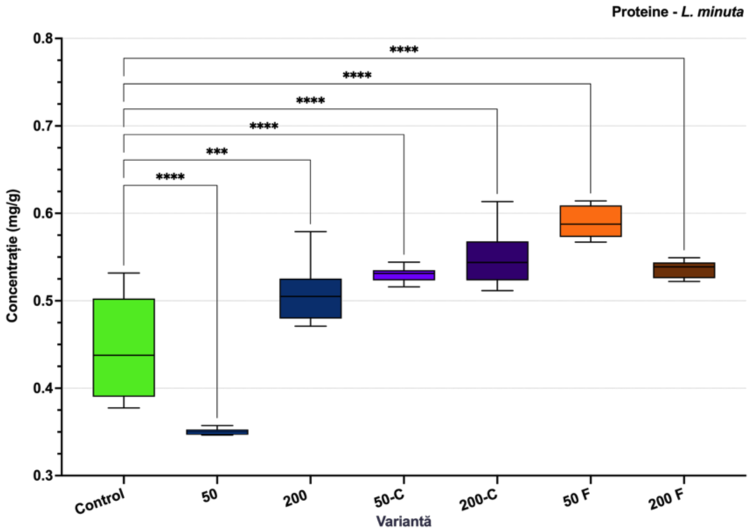

Fig. IV.35 – Protein content of *Lemna minuta* Kunth specimens grown under experimental conditions. Experimental variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with * (*** - $p = 0.0003$; **** - $p < 0.0001$)

IV.2.3.4 Total antioxidant activity

The antioxidant response of *Lemna minuta* cultures, assessed using the DPPH test, demonstrates the distinct effects of carbon-based nanomaterials depending on their composition. Thus, multi-walled carbon nanotubes and fullerene soot reduced the antioxidant capacity of the test plants, probably due to oxidative stress and damage to their endogenous defence systems. In contrast, carboxylated multi-walled carbon nanotubes significantly improved the antioxidant activity of *Lemna minor* individuals, indicating a possible activation in their tissues of pathways for neutralising reactive oxygen species, determined by improved nanotube absorption and intracellular signalling. These findings highlight the critical role of functionalisation and concentration of synthetic carbon-based nanomaterials in modulating plant physiological responses and confirm that DPPH-based tests serve as reliable indicators of adaptation or suppression of oxidative stress in aquatic plants. (Ma et al., 2010; Begum and Fugetsu, 2012; Tripathi et al., 2017)(Fig. IV.36, Annex 27).

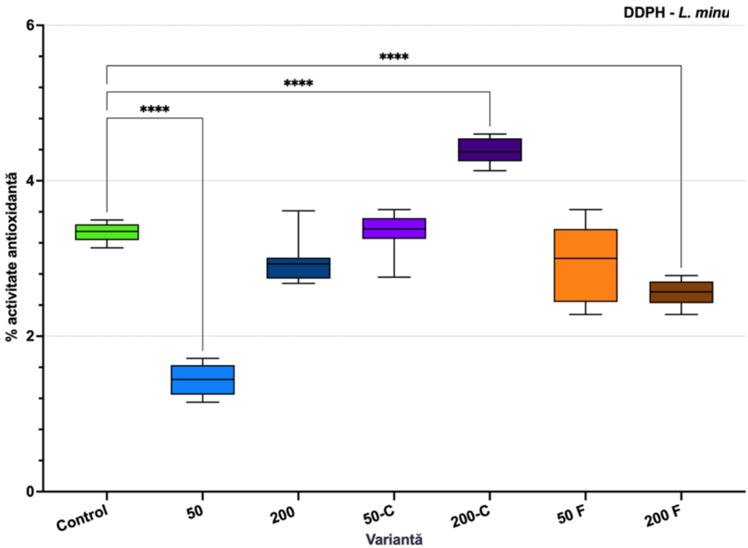

Fig. IV.36 – Total antioxidant activity of *Lemna minuta* Kunth specimens grown under experimental conditions (Test DPPH). Variants: Control, 50 - 50 mg/l MWCNT, 200 – 200 mg/l MWCNT, 50-C – 50 mg/l MWCNT-COOH, 200-C - 200 mg/l MWCNT-COOH, 50 F – 50 mg/l Fullerene soot, 200 F – 200 mg/l Fullerene soot. Statistically significant differences are marked with * (**** - $p < 0.0001$)

Final considerations

The present work aimed to provide, through the obtained results, a comparative evaluation of the morpho-physiological and biochemical impact experienced by representatives of two related aquatic plant species - *Lemna minor* L. and *Lemna minuta* Kunth - as a result of their interaction in the culture medium with synthetic carbon-based nanomaterials (MWCNT, MWCNT-COOH and fullerene soot).

• While the species *Lemna minor* L. is considered a model species, recognised in ecotoxicological research, the inclusion in the group of typical plants for such research of the widely distributed but little-studied species *Lemna minuta* Kunth, in terms of its complex morpho-functional and biochemical reactivity to changes in living conditions, highlights its potential as a sensitive bioindicator of freshwater quality and supports a new direction of research in aquatic ecology.

• The integrative analysis of morphological and micromorphological changes (colony development, leaf surface characteristics, stomatal structure), physiological and biochemical changes (chlorophyll fluorescence, content of photoassimilatory pigments, content of secondary metabolites, antioxidant enzymatic activity), as well as the expression of genes involved in the stress response of individuals belonging to the species *Lemna minor* L. provides convincing evidence that the type and concentration of synthetic carbon-based nanomaterials significantly influence the coordination and extent of their responses to the abiotic stress conditions of the living environment. These results consolidate the relevance of the species as a model organism in nanotoxicity studies and support the need for multi- and interdisciplinary research approaches in assessing the ecotoxicological impact of synthetic carbon-based nanomaterials on aquatic ecosystems.

• The morpho-physiological and biochemical changes observed in plants belonging to the species *Lemna minuta* L., as a response to their interaction with synthetic carbon-based nanomaterials, covering aspects related to colony development, stomatal structure, content and composition of photoassimilatory pigments, enzymatic activity and the concentration of some metabolic compounds with an antioxidant role, demonstrate that this species is a sensitive bioindicator of the degree of pollution of the aquatic environment. Its capacity to react to oxidative stress through metabolic disturbances and specific morphological changes, experimentally highlighted, underlines its value in the practical assessment of ecotoxicological risks represented by the contamination of freshwater ecosystems with synthetic nanomaterials.

• In the case of *Lemna minor* L., simple multi-walled carbon nanotubes (MWCNT) significantly reduced the efficiency of photosynthetic system II (ΦPSII) and the content of photoassimilatory pigments, especially at concentrations of 200 mg/l, inducing toxicity and chloroplast damage, followed by reductions in the content of chlorophylls a, b and carotenoid pigments. At the same time, antioxidant enzymes reacted asymmetrically, POD showing an increase in activity in some treatments, while SOD activity, generally inhibited, was evidently suppressed especially in the presence of carboxyl-functionalised nanotubes (MWCNT-COOH). The observed enzymatic asymmetry suggests the preferential activation of peroxidase-mediated metabolic detoxification, indicating the establishment of a redox imbalance in plant cells, as an effect of the presence of disturbing chemical factors represented by the tested synthetic nanomaterials.

• Synthetic carbon-based nanomaterials exert specific effects depending on their chemical composition and application concentration on *Lemna minuta* Kunth cultures, altering both their morphology and biochemical homeostasis. Among the tested variants, carboxyl-functionalised nanotubes (MWCNT-COOH) stand out, which showed the highest phytotoxicity, significantly affecting the growth, antioxidant capacity and photosynthetic performance of the test plants, probably due to the increased reactivity and bioavailability of their interaction surface with the support plants. In contrast, fullerene soot produced milder responses in the body of the test plants, including stimulative effects at lower application concentrations, indicating a hormetic response of plant organisms in interrelation with these compounds in the aquatic environment. These findings underline the essential role of surface chemistry and the concentration of synthetic carbon-based nanotubes in determining and quantifying their phytotoxic effects.

• *Lemna minuta* Kunth plants showed stronger responses than those belonging to the species *Lemna minor* L., manifested by visible changes in the variation of several morpho-physiological and biochemical parameters, including leaf/frond growth, photoassimilatory pigment content and accumulation of secondary metabolites with a detoxifying role in the cellular environment, reactions suggesting that the species *Lemna minuta* Kunth is a more functionally responsive bioindicator to qualitative changes in the living environment, being suitable for testing the acute nanotoxicity of freshwater aquatic ecosystems.

• Presenting both common response patterns to the two *Lemna* species under consideration, as well as specific to each of them, and demonstrating the increased sensitivity of *Lemna minuta* Kunth individuals to the presence of synthetic carbon-based nanomaterials, the obtained results complete their scientific basis for use as model systems in environmental risk assessment and support the interdisciplinary integration of nanotoxicology research for future models of monitoring and regulation of aquatic environment quality.

• Recognised as valuable model organisms in ecotoxicological research due to their rapid growth, clonal propagation and high sensitivity to pollutants, species of the genus Lemna, especially *Lemna minor* L. and *Lemna minuta* Kunth, exhibit certain traits associated with a strong invasive potential in natural aquatic ecosystems, such as the ability to reproduce vegetatively at high rates, the ability to form dense surface mats and to tolerate diverse environmental conditions, traits that allow them to compete with native macrophytes by limiting light availability and disrupting local trophic structures; in this regard, the specialised literature from several European countries presents the species *Lemna minuta* Kunth as an invasive alien taxon, its proliferation being closely linked to anthropogenic disturbances and nutrient-enriched waters.

• In the presented context, the use of *Lemna* spp. individuals in experimental cultures must be carried out under strictly controlled conditions, which avoid the risk of their accidental release into natural environments. The implementation of correct and safe measures for the use and valorisation of plant materials involved in decontamination and depollution activities of affected aquatic environments - which comply with current national and international regulations regarding their safe disposal - are essential to prevent the ecological impact associated with the escape from specially organised perimeters for the cultivation and experimental/exploitation use of these possible fast-growing aquatic plant invaders.

The design of the present multi-level experimental design, comprising aspects of growth, morphological and micromorphological characteristics of the leaf surface, functional, biochemical aspects and, last but not least, elements of gene expression involved in stress response, can constitute a model of integrative and comparative applied research in lemnaceae nanotoxicology. The emphasis placed especially on multi-walled carbon nanotubes, whose behaviour in the natural environment remains insufficiently characterised, represents our effort to cover, with statistically supported results, the existing gaps in understanding how the type of nanomaterials influences the phytotoxicity of aquatic environments populated with taxa of the genus *Lemna* L..

Prospects for research follow-up

1. Investigations into the absorption and transport of nanomaterials:

To unravel how MWCNTs and fullerene soot interact with aquatic macrophytes such as *Lemna minor* L., *Lemna minuta* Kunth, and *Lemna trisulca* L., future work will utilise advanced imaging modalities such as TEM, SEM-EDX, micro-CT, and confocal microscopy alongside Raman spectroscopy. These tools can help clarify how structural features such as root morphology and leaf architecture influence the uptake and distribution of nanomaterials at the cellular and subcellular level.

2. Species-specific stress response analysis:

Investigating transcriptomic and proteomic changes in response to nanomaterials will be essential for understanding how these species manage oxidative stress. Targeting genes and proteins related to antioxidant systems (e.g., SOD, POD, CAT), hormonal pathways, and stress metabolism could reveal key differences in the sensitivity and protective mechanisms of the species *Lemna minor* L., *Lemna minuta* Kunth, and *Lemna trisulca* L..

3. Community and ecosystem level perspectives:

Incorporating the species *Lemna minor* L., *Lemna minuta* Kunth and *Lemna trisulca* L. into multi-species aquatic systems (e.g. in a microcosm) would provide a clearer picture of how nanomaterials propagate through plant and algal communities and aquatic food webs.

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