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Graphene oxide nanoparticles and graphite microparticles on seeds germination and growth of *Solanum lycopersicum* seedlings◊

Nanopartículas de óxido de grafeno y micropartículas de grafito en la germinación y crecimiento de semillas de plántulas de *Solanum lycopersicum*

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ABSTRACT: Nanotechnology (NT) can modernize agriculture with new tools that allow better nourished and protected crops. Graphene oxide (GO) is a new kind of carbon-based nanomaterial with unique structural and physicochemical properties, which is very useful for many agricultural applications. GO, the two-dimensional carbon nanoparticles, have attracted increasing attention in the last few years because these contain large amounts of functional oxygen groups; therefore, they could be used as a fertilizer carrier to slow the release rate and improve the nutrients use efficiency, which makes this material suitable for developing new slow-release fertilizers. In this study, the application of GO nanoparticles (NPs) and graphite microparticles were compared as potential promoters of tomato seed germination and seedlings growth. Concentrations of 0, 50, 100, 200, and 500 mg L^{-1} were applied, using distilled water and micro-size graphite as controls. GO treatments improved root growth dose-dependently by increasing the seed vigor and showing significant differences ($P \leq 0.05$) between treatments applied, increasing antioxidant enzymes activities. When using the dose of 200 mg L^{-1} GONPs, the radicle length was stimulated (31%) compared to the control seedlings. The graphite NPs performed better than the control in all variables; however, they were surpassed by the treatments with GONPs.
KEYWORDS: agronanotechnology, nanocarbon, nanofertilizers, tomato.

RESUMEN: La nanotecnología (NT) puede modernizar la agricultura con nuevas herramientas que permitan cultivos mejor nutridos y protegidos. El óxido de grafeno (GO) es un nuevo tipo de nanomaterial basado en carbono con propiedades estructurales y fisicoquímicas únicas, muy útil para muchas aplicaciones agrícolas. El GO son nanopartículas de carbono bidimensionales, que

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han atraído una atención cada vez mayor en los últimos años porque contienen grandes cantidades de grupos funcionales de oxígeno, por lo tanto, podrían usarse como portadores de fertilizantes para reducir la velocidad de liberación y mejorar la eficiencia de utilización de nutrientes, lo cual hace que este material sea adecuado para el desarrollo de nuevos nanofertilizantes de liberación lenta. En el presente estudio, se evaluó la aplicación de nanopartículas de GO (GONPs) y micropartículas de grafito (MG), como potenciales promotores de la germinación de semillas y el crecimiento de plántulas de tomate. Se aplicaron concentraciones de 0, 50, 100, 200 y 500 mg L^{-1}, utilizando como controles agua destilada y micrografito. Los tratamientos GO mejoraron el crecimiento de las raíces de manera dependiente de la dosis, al aumentar el vigor de las semillas, revelando diferencias significativas ($P \leq 0.05$) entre los tratamientos aplicados, lo anterior fue acompañado por un incremento en la actividad de las enzimas antioxidantes. Al utilizar la dosis de 200 mg L^{-1} de GONPs se estimuló la longitud de la radícula (31%) con respecto a las plántulas testigo. Las MG se desempeñaron mejor que el control en todas las variables; sin embargo, fueron superadas por los tratamientos con GONPs.
PALABRAS CLAVE: agronanotecnología, nanocarbón, nanofertilizantes, tomate.

Introduction

Nanotechnology (NT) studies using nanomaterials (NMs) of nanometric size (10^{-9}) have great potential for modern production systems, including agriculture, as is introducing new tools to improve existing crop management techniques through the incorporation of nanopesticides, nanofertilizers, nanoherbicides, and plant growth regulators; which promise to be more environmentally friendly and less toxic to humans and animals (Shing *et al*., 2023). Using NMs in agriculture also includes nanosensors to monitor biotic and abiotic stresses affecting plant nutrition, crop quality, water, and nutrient availability (Johnson *et al*., 2021; Tshabalala *et al*., 2022). It is essential to point out that carbon NMs include different forms of low-dimensional carbon, such as single-walled and multi-walled carbon nanotubes, fullerenes, graphene, GO, and carbon dots (Gazzi *et al*., 2020), which have proven to stimulate and promote beneficial plant responses on alfalfa crop (Chen *et al*., 2022). The report by Kalwani *et al*. (2022) shows that the application of NT in the form of nanofertilizer provides an innovative, efficient, and eco-friendly alternative to synthetic fertilizers, because not only supports plant growth but also conserve the diversity of the beneficial microbiome.

Due to its excellent material properties, such as its large surface area and superb mechanical and thermal characteristics, GO is likely to be applied in various biomedical and agricultural fields (Andelkovic *et al*., 2018). These applications may lead to GO entrance into terrestrial ecosystems; however, there are few scientific reports regarding the impact of GO on plants upon such entrances (Lee *et al*., 2021). The superiority of modified GO for enhancing the growth, yield, and antioxidant potential of pearl millet plants (*Pennisetum glaucum* L.) under salt stress, has been reported by Mahmoud and Abdelhameed (2021). Similarly, the GO treatments applied by Guo *et al*. (2021) considerably improved tomato growth in a dose-dependent manner by increasing the cortical cells number, cross-sectional area, diameter, and vascular-column area. In addition, these authors argue that GO promoted morphological root system development and increased biomass accumulation.

López-Vargas *et al*. (2020) reported that tomato seeds primed with graphene increased chlorophyll content, vitamin C, β-carotene, phenols, flavonoids, and H_2O_2. Therefore, they suggest that this nanomaterial can induce biostimulation and provide an easy way to apply carbon NMs to plants. According to Park *et al*. (2020), GO positively affected plant growth by increasing root length, leaf area, leaf number, and flower bud formation. In addition, GO affected the watermelon ripeness, increasing the perimeter and fruit sugar content. Hence, they consider that GO may be used to accelerate both plant growth and the fruit ripening process. Lira-Saldivar *et al*. (2018) pointed out that carbon-based materials like GONPs represent potential NMs for developing modern sustainable agrochemicals for their use as nanopesticides and nanofertilizers of low environmental impact, and Kalwani *et al*. (2022) indicate that using nanofertilizers provides an innovative, efficient, and eco-friendly alternative to synthetic fertilizers, because permit a slow and sustained release of nutrients, supporting plant growth and preserves the biodiversity of beneficial microbiome in the soil.

Different physiological and biochemical responses of *Vigna radiata* and *S. lycopersicum* seeds induced by graphene quantum dots (GQDs) were studied by Feng *et al*. (2019). Results showed that both seeds exposed to GQDs could germinate easily. However, the growth of seedlings was harmfully affected by the GQDs, being *V. radiata* plants more sensitive than tomato seedlings. In hydroponic experiments, the appropriate concentration of GQDs enhanced the accumulation of chlorophyll in seedlings of *V. radiata* (250–1250 mg L^{-1}) and tomato (250–500 mg L^{-1}), after exposure for two weeks. He *et al*. (2018) describe that GO may act as a water transporter and promote plant germination in soil. In this sense, Zhang *et al*. (2021) described that GO membranes with stable porous structures have the potential for ultrahigh permeability and ultrafast water transport. It has been reported that wild-type (WT) tomato germplasm 'New Yorker' and corresponding transgenic plants (Prd29A: LeNCED1), were evaluated by Jiao *et al*. (2016) with suspensions of GO. The seminal root length of the WT tomato was longer than that of the control samples, when the seedlings were exposed to 20 mg L^{-1} GO during 15 days. By contrast, the same treatment resulted in shorter seminal roots length in transgenic plants compared with the control samples. In addition, GO treatments led to lower enzymes activities of superoxide dismutase, peroxidase, and malondialdehyde content in the WT tomato and transgenic plants.

Germination and vigor are the two most important attributes associated with the physiological quality of seeds in the growth and development of plant stands, crucial to achieving an adequate establishment and plant performance under protected agriculture conditions and in open field environments (Wijewardana *et al*., 2019). Our study hypothesized that GONPs would improve seed germination and growth of *S. lycopersicum* seedlings. So, our objective was to determine the responses of tomato seeds primed with several concentrations of GONPs and graphite microparticles to determine the changes in seeds vigor, percent germination, growth, and antioxidant enzymatic activation of tomato seedlings.

Materials and methods

Graphene oxide nanoparticles

HD PlasTM graphene from Cheap Tubes Inc., was used, with an average flake length of 1-3 µm, surface area > 700 m^2/g, purity of approximately 99% by weight, and density of 2.2 g/cm^3. Concentrated stock suspensions of 500 mg L^{-1} were prepared, and later different dilutions were made to obtain the suspensions of each treatment. Before application, they were dispersed in water, using a Branson 2510 ultrasonic cleaner; the suspensions were sonicated for 30 min (two times of 15 min). The graphite microparticles used as the control were evaluated and compared to GONPs as if they were a double control since the absolute control was pure deionized water. This was done to elucidate whether the effect derived from the treatments was due to the product itself or the size of the particles applied to tomato seeds.

Treatments applied

In order to determine the effect of the GONPs treatments on tomato seeds Floradade variety, *in vitro* bioassays were performed, which were carried out according to the standards of the International Seed Testing Association (ISTA, 2004), to evaluate the physiological quality of seeds by its germination capacity and vigor. Treatments consisted of GONPs suspensions at concentrations of 0, 50, 100, 200, and 500 mg L^{-1}. Regarding graphite microparticles assessed, stock suspensions were prepared in similar concentrations as the GONPs. The bioassays, with nano and micro carbon-based particles treatments, were established using a completely randomized experimental design with nine treatments and four replicates, with 25 seeds per replicate. Treatments were applied once by priming the seeds in 25-30 mL of the GONPs and microsized graphene solutions for 18 hours. The seeds were placed on filter paper in Petri dishes and kept in a bioclimatic chamber (EICS 351 HR, Equitec). When the priming period was completed, four replicates of 25 seeds were sown on anchor paper, which was moistened with distilled water, later the seeds were placed in a row horizontally on the paper, taking care that the embryo of the seed were situated downwards; then another anchor paper of the same size was moistened to cover the seeds, and it was rolled into a *taco* shape. Subsequently, the plugs were randomly settled in a polyethylene bag that was placed inside a basket, which was kept in a Lab-line Instruments germinating chamber at a constant temperature of 25 °C ± 2 °C and 80% relative humidity.

Assessed variables

The computations of the analyzed variables were made on the sixth and 14th day after sowing. On the sixth day, the number of normal seedlings was evaluated (seedlings with roots and plumule, each one with 2 centimeters of development), indicating the seeds' vigor. In addition to counting the total number of germinated seeds, a second count was made on seedlings on the 14th day. This allowed us to determine the germination percentage, measuring the normal, abnormal, and ungerminated seedlings. Plumule and radicle length were also determined in all normal seedlings.

Vigor index

The first count of normal seedlings was carried out on the sixth day after sowing and was expressed as a percentage. This variable is an indicator of the vigor that the seed possesses to germinate in less time and that the plants can be properly established under field conditions. Normal seedlings are those that show the potential to continue developing with satisfactory characteristics when grown under favorable conditions of humidity, temperature, and light (FAO, 2019). The equation used to calculate this variable is the one indicated by Peretti (1994):

$$Vigor\ index = \frac{Normal\ seedlings\ (First\ counting)}{Total\ seeds\ sown} * 100$$

Germination percentage

At the end of the bioassay, a count of normal seedlings was performed and the information obtained was expressed as GP (%). The equation used to calculate this variable was the following:

$$Germination\ percentage = \frac{Normal\ seedlings\ (Second\ count)}{Total\ seeds\ sown} * 100$$

Mean plumule length (MPL) and radicle length (MRL)

Regarding the effect of applied treatments on seedlings' growth, all normal seedlings were characterized, which were those that did not present any abnormality trait; this variable was expressed in cm.

Statistical analysis

The data obtained was analyzed in the statistical program INFOSTAT (Balzarini *et al*., 2001), using the one-way or wholly randomized analysis of variance and the Tukey multiple range test ($P \leq 0.05$) to determine its statistical significance.

Antioxidant enzyme assays

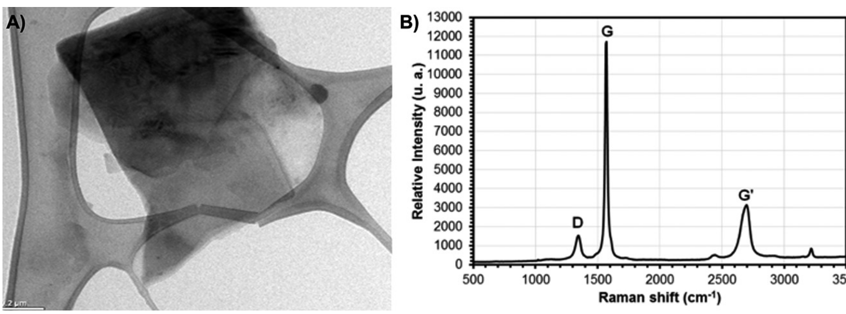
According to Elevarthi and Martin (2010), protein was extracted for assessing the enzymatic activities. Frozen plantlets (200 mg) were ground with liquid nitrogen to a fine powder in a prechilled mortar with 5% (w/w) polyvinylpolypyrrolidone. Extraction buffer (0.1 M potassium phosphate pH 7.8, 0.1 mM EDTA) in a 1:2.5 ratio (v/w) was added and mixed to obtain a homogeneous slurry. After centrifugation at 10,000 g for 15 min at 4 ºC, the supernatant was collected. The eluted samples were used for enzyme assays. Protein preparations were stored at –20 ºC until use. Protein concentration was determined using the Bradford Reagent (Sigma, USA) and bovine serum albumin as standard. Peroxidase activity (POX) was carried out spectrophotometrically using guaiacol, the formation of the oxidized product (tetraguaiacol) was measured at 470 nm using the extinction coefficient of 26.6 $mM^{-1} cm^{-1}$ (Putter, 1974). Ascorbate peroxidase activity (APX) was determined by measuring the decrease in absorbance of ascorbate at 290 nm (ε 2.8 $mM^{-1} cm^{-1}$). One unit of enzyme activity was defined with the oxidization of 1 μmol AsA at 25 ºC in 1 min (Nakano and Asada, 1987). Catalase activity (CAT) was measured by following the decline in absorbance at 240 (ε 36 M^{-1} cm^{-1}) as H_2O_2 was catabolized, according to Aebi (1984).

Results and discussion

Graphene oxide nanoparticles

To reduce the number of graphene layers, treatment was performed using ultrasound in the absence of solvent (González-Morones *et al*., 2011). The GONPs were characterized microscopically and by *Raman spectroscopy* (figure 1). Figure 1A shows the graphene treated with ultrasound, where it can be seen that the particle size ranges between 1.0 and 5.0 micron. Likewise, it can be seen that graphene is composed by a smaller number of stacked layers and has well-defined edges. Figure 1B shows the Raman spectrum of graphene exfoliated by ultrasound, where it is possible to detect the three main bands for graphene. The D band at 1336 and 1342 cm^{-1} was associated with sp3 hybridization caused by defects in the graphene layer. The G band at 1559 and 1566 cm^{-1} is related to all forms of sp2 carbon, and finally is presented the 2D band at 2692 and 2676 cm^{-1} (Sole *et al*., 2014).

Figure 1. Micrograph of a graphene oxide slide obtained by transmission electron microscope (TEM).



Source: Author’s elaboration.

Seed germination and vigor

Results indicate that seeds primed with GONPs and graphite MPs improved their vigor, by reporting higher values on this variable than control treat- ments. The dose of 50 mg L^{-1} graphite MPs reported the best vigor average (95.0 ± 1.0%), superior to the treatment with 50 mg L^{-1} of GONPs, which reached a vigor value of 88.0 ± 1.63% (table 1). These results show differential effects on the physiological quality of the seeds due to the size of particles applied (GONPs and graphite MPs). Regarding the effect of graphite MPs, the data shows high germination (%) at concentrations of 50, 100, and 200 mg L^{-1}, being these values higher compared to the control treatment (distilled water). However, the germination percentage decreased compared to the control treatment (distilled water) at higher concentrations of GONPs.

Table 1. Comparison on the effect of graphene oxide (GO) nanoparticles and graphite microparticles (GMPs), on vigor and germination of tomato seeds cv. Floradade.

Treatments (mg L^{-1})	Vigor (%)		Germination (%)	
	GONPs	GMPs	GONPs	GMPs
Control	80.0 ± (2.31)b		93.0 ± (1.91)a	
50	85.0 ± (4.12)ab	95.0 ± (1.0)a	92.0 ± (2.83)a	97.0 ± (1.0)a
100	88.0 ± (1.63)ab	89.0 ± (1.91)ab	98.0 ± (2.0)a	95.0 ± (1.91)a
200	85.0 ± (4.12)ab	90.0 ± (3.83)ab	95.0 ± (1.91)a	95.0 ± (2.52)a
500	84.0 ± (4.32)ab	88.0 ± (2.31)ab	93.0 ± (3.42)a	91.0 ± (1.0)a
p ≤ F	0.1105		0.3943	

Note: Different letters represent significant statistical differences * Significant differences, (Tukey P ≤ 0.05).
Source: Author's elaboration.

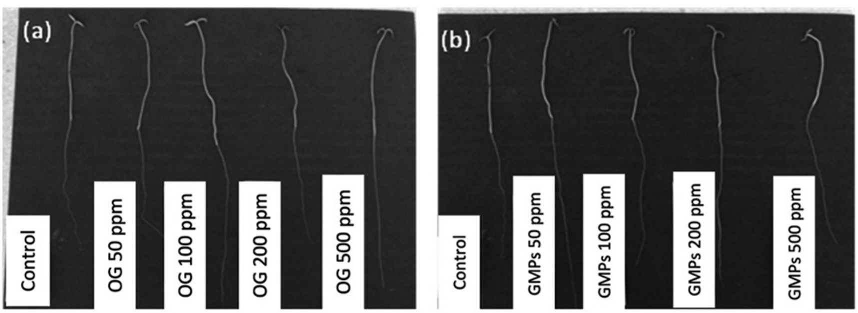
The results of the present study agree with those of Lee *et al*. (2021), who analyzed the effects of GO concentrations (0, 0.2, 0.4, 0.8, and 1.6 mg mL^{-1}) on germination and growth of various plants (lettuce, radish, perennial ryegrass, alfalfa, and cucumber). These authors also state that the germination rate decreased with GO concentration for lettuce plants. However, no significant effects were observed on the germination rate of other species. Similarly, the growth of lettuce, alfalfa, and radish seedlings decreased by GO treatment. Such outcomes suggest that germination and early growth of seedlings are adversely affected in a species-specific manner under high concentrations of GO particles.

On the other hand, GO exhibited positive effects on the growth of *Aloe vera* plants. Zhang *et al*. (2021), demonstrated that GO with a dose of 50 mg L^{-1}, enhance the photosynthetic capacity, increases yield and morphological characteristics of root and leaf, and improves the nutrient contents of leaves (protein and amino acids), without reducing the content of aloin, the main bioactive compound. Although the electrolyte leakage and malondialdehyde content were increased at high concentrations, GO treatments did not increase the root antioxidant enzyme activity or decrease the root vigor.

Plumule and radicle length

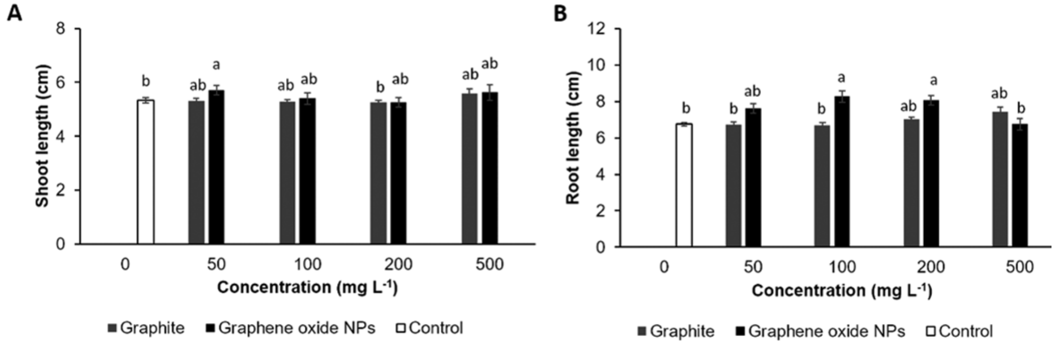
Figure 2 shows the morphological changes induced by the materials assayed. The size of tomato seedlings was altered by treatments of carbon-based materials since the length of plumule and radicle revealed statistically significant differences ($P \leq 0.05$) due to the treatments with GONPs (figure 3). The 50 mg L^{-1} doses induced a more significant plumule length (6.58 ± 0.15 cm), increasing this value by 11% compared to the control treatment (5.93 ± 0.22 cm). Highly significant differences ($P \leq 0.01$) were revealed for radicle length when the seeds were primed with GONPs. The 200 mg L^{-1} dose reported the maximum value (9.32 ± 0.36 cm) reached.

Figure 2. Effect of graphene oxide nanoparticles (a) and graphite microparticles (b) on tomato seedlings.



Source: Author's elaboration.

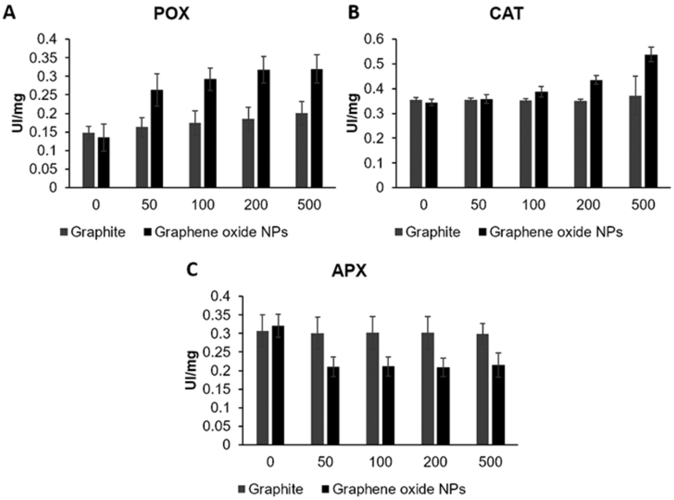
Figure 3. Comparison of the effect of graphene oxide nanoparticles and graphite microparticles on the length of plumule (A) and radicle of tomato seedlings (B) cv. Floradade.



Source: Author's elaboration.

This shows that GONPs positively influenced the attributes related to seedlings' growth (figure 4); since, at this concentration, the radicle length increased by 31% to the control (7.11 ± 0.30 cm). It was also observed that when GONPs increased to 500 mg L^{-1}, this variable's values were harmed, indicating this a possible effect of toxicity in plants (Park *et al*., 2020).

Figure 4. Effect of graphene oxide nanoparticles on the antioxidants enzymes of tomato seedlings: A) peroxidase activities (POD); B) catalase activities (CAT), and C) ascorbate peroxidase activities (APX). The bars correspond to the standard error.



Source: Author's elaboration.

Those mentioned above could be related to the adverse effect GONPs could cause on cell structure and function at relatively high concentrations. This outcome agrees with Zhang *et al*. (2016), who found that concentrations of graphene in the range of 250-1500 mg L^{-1} inhibited the growth of *Triticum aestivum* seedlings due to the oxidative stress caused by GONPs. Shen *et al*. (2018) found that GO affected the hormonal level since ABA and IAA concentrations caused a negative impact on the roots growth of *Oryza sativa* plants. However, these results depended on plant genotype and complex regulation in the hormone content level. The present study exposed the role of GONPs in tomato seedlings, on growth promotion by stimulating seeds germination, vigor and seedlings growth, which could provide some basis for GONPs application as a potential nanofertilizer in some agricrops of high economic importance such as *S. Lycopersicon*.

Antioxidant enzyme assays

We evaluated the antioxidant enzyme activity in 14 days-old seedlings after GO treatment. The results showed that at the tested graphene concentration range of 50 - 500 mg L^{-1}, the activity of POD and CAT increased dose-dependent (figure 4A, B). However, APX activity decreased with respect to control plants (figure 4C). These results are similar to the effect of graphene nanomaterials on other higher plants (Ren *et al*., 2020; González-García *et al*., 2019). Compared with the control, POD, and CAT activities at 500 mg L^{-1} were increased by 59% and 44%, respectively.

GO could stimulate the generation of reactive oxygen species in plants at high concentrations. As a result, maintaining cellular homeostasis on plants activates a complex system to control the stresses, which requires several physiological and biochemical mechanisms, including enzymatic and non-enzymatic compounds to develop stress tolerance. On the other hand, some reports suggest that the activation of APX is under catalase deactivation (Rajput *et al*., 2021). Our result showed a 27 % inhibition of APX activity at 500 mg L^{-1}, corresponding with a more significant CAT activity. These oxidative and biochemical disturbances are among the significant causes of successful germination.

The results obtained in the present work indicate that differential effects were generated between the application of OGNPs and MPs of graphite oxide with respect to the seeds of physiological quality. This behavior described above could be because the suspensions of OGNPs decreased the seeds development phases, inhibiting germination and preventing cell division and elongation, thus causing a negative effect on the vigor percent due to the possible toxic effect of these suspensions. In previous studies, the NPs derived from coal at high concentrations (500 mg L^{-1}) suggest that the electrical conductivity (EC) of a suspension increases when plant tissues are immersed in NMs of carbon, so as the EC increases, the freedom of ions movement and molecules decreases, causing negative effects on seedlings development (Xie *et al*., 2005).

The above-mentioned suggests that the OG penetrated the interior of seeds, allowing the absorption of water at low concentrations and promoting root growth (less than 200 mg L^{-1}), which agrees with Zhang *et al.* (2015), who made TEM micrograph observations of the peel of tomato seeds treated with graphene, having observed OG sheets inside the peel. It is interesting to note that the seeds showed a significantly higher level of moisture compared to the seeds of the control treatment, endorsing the hypothesis that OG is capable of penetrating the seed coats and modifying certain physiological aspects. In a similar way (Wu *et al*., 2023) reported that the effectiveness of graphite-derived NMs in phytonanotechnology was reflected by the promotion of seed priming and germination, root and seedling growth, biomass accumulation, and the improvement of final yield.

Conclusion

Graphene oxide NPs, derived from graphene, contains various functional groups with unique physical properties that can translate into potential applications in agriculture, such as nanofertilizer and promoter of tomato plants growth. In the present work, this GO accelerated seed germination and seedling growth through increasing ROS at concentrations as low as 200 mg L^{-1} obtaining rapid seed germination and higher germination rates, which was attributed to better water uptake. Excess ROS resulted in oxidative stress, the most important mechanism in growth-limiting effects on plants. Therefore, it is feasible to consider that this carbon-based nanomaterial at the right concentration could serve as a promising non-toxic chemical to increase seeds germination and vigor, as well as growth and possibly the yield of *S. lycopersicum* plants.

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