Secondary Metabolites Evaluation On *Medicago Sativa L.* Plants Treated By (Fe, Ag, Cu)-TiO₂ Nanostructured Materials Towards Sustainable Agriculture.

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Abstract

The present study analyzed *Medicago sativa L.* crops irrigated by TiO_2 in the anatase phase and TiO_2 doped with Ag, Fe, Cu ions at 0.1%w synthesized by the sol-gel method (SG) and the sol-gel coupled with microwaves (Mw-SG). The materials were added to the irrigation water at different concentrations (50, 100, and 500 ppm). Affection levels were observed by measuring stem morphology, chlorophyll content, secondary metabolite content (total phenolic, flavonoids), and antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl radical assay. The results revealed a reduction in stem and leave size at different concentrations and chlorophyll content, showing no correspondence with the applied dose. Nanostructured materials in the soil generated nitrogen, boron, and potassium deficiencies observed in leaves. No linear effect related to the increase in total gallic acid, rutin content, and antioxidant activity dependent on concentration was determined. The stress factor depended on the dopant type, generating different stress levels at the three organs investigated (leaves, roots, stem). The metabolite augmentation was mainly obtained at 100 and 500 ppm for both synthesis methods.

Highlights

- The toxicological effects observed differ due to the physicochemical properties.
- Morphological and biochemical changes due to the nanomaterial's exposure.
- Increased secondary metabolite content in plants treated with nanomaterials.
- Alteration of chlorophyll content after nanomaterial's exposure.

Introduction

Nanostructured nanomaterials find many applications in agriculture. They can serve as fertilizers to promote better crop development due to their high selectivity and availability (Zulfiqar et al., 2019). There is also the possibility of using nanomaterials as pesticidal agents for protection against microorganisms or other detrimental species (Fraceto et al., 2020), or as well as the protection against biotic and abiotic stresses for the development of the plants and the improvement in the production of secondary metabolites, which allows generating added value products (Khan et al., 2021).

Nowadays, the excessive use of nanomaterials is worrying without considering the interactions that they may have with multiple organisms (Millán-Chiu et al., 2020), generating toxic reactions that can compromise the development of plants, microorganisms, and animals. An area such as Nanotoxicology is developed to understand the toxicological mechanisms of various nanomaterials (Ananthi et al., 2020; Zia-ur-Rehman et al., 2018). Which are different from their bulk counterparts, the study and understanding of these mechanisms will make it possible to better use of the nanomaterials, preventing these new products from generating conflicts with various organisms.

Titanium dioxide (TiO_2) is one of the most produced nanomaterials worldwide (Piccinno et al., 2012). Its applications vary from solar cells to the photocatalytic removal of persistent pollutants (Haider et al.,

2019). The increase in its production means a greater risk for introducing nanomaterials (NMs) in the environment without considering the expected applications for agriculture (Klaine et al., 2008). An increasing presence in the environment has meant increased studies related to the toxicological effects that TiO₂-NMs can have when interacting with living organisms (Ananthi et al., 2020). The interaction mechanisms vary and depend on the nanomaterial characteristics compared to their bulk, making it a complex study (Zia-ur-Rehman et al., 2018). The distribution of sizes, morphology, dose, concentration, and the crystalline phase determines the behavior of the TiO₂-NMs when they encounter living organisms (Millán-Chiu et al., 2020; Zia-ur-Rehman et al., 2018). With living organisms, modifying the synthesis of a material to obtain new physiochemical characteristics such as a smaller size, higher crystalline quality, or different morphology will differ in the effects when they are exposed, generating a new toxicological profile (Hossain et al., 2020).

Most of those characteristics are conferred by the synthesis methodology, such as size, which defines the TiO₂-NM's ability to enter into several types of tissue (Tan, Peralta-Videa, et al., 2018). The modification by coating or doping elements in metal oxides can modify the NM/cell interaction pathway (Medina-Velo et al., 2017). At the same time, the introduction route of the TiO₂-NMs to the plant system plays an essential role in the toxicological mechanisms that may arise due to the variety of tissues or organs with which they can interact (roots, leaves, stem, etc.) (R. Singh et al., 2021).

Based on previous reports, the interaction between plant/TiO₂-NM can positively or negatively affect the plant product's quality, such as a higher or lower production rate, depending on the plant species (R. Singh et al., 2021). However, one area with potential applications is the stress induction by TiO₂-NMs. The stress in a plant generates secondary metabolites, which act as defense molecules to mitigate the effects of such stress (Modarresi et al., 2020; Wu et al., 2017). For humans, these molecules derived from secondary metabolism vary from nutrients with antioxidant capacities or products such as medicines. TiO₂-NMs could be efficient products for developing crops with higher nutrient content and increased valuable content compound (Chutipaijit & Sutjaritvorakul, 2020; Hatami et al., 2019; Shabbir et al., 2019). It is known that the capacity for an NM to generate stress in a plant will depend entirely on its physical and chemical characteristics and its introduction path (Ananthi et al., 2020; Su et al., 2019).

Alfalfa crops are one of the most significant fodder crops for cattle feeding. The effects on the alfalfa plant by NMs have been studied (Hong et al., 2015; Ramírez-Valdespino et al., 2020; Stegemeier et al., 2015). Although to our concern, there is no study relating the exposure effects with such an essential photocatalytic nanomaterial like TiO₂. This research aims to highlight the effects produced in the morphologic characteristics of the plant and the production of secondary metabolites when it interacts with the pristine TiO₂ as well as with the doped-TiO₂ (F. Huang et al., 2016).

Materials And Methods

2.1. Titanium dioxide and doped TiO₂ materials synthesis and characterization

Titanium isopropoxide (Sigma Aldrich 97%) was dissolved in isopropanol (J.T. Baker 99%). The solution was stirred for 20 minutes under a nitrogen atmosphere to prevent the oxidation of the titanium precursor. The hydrolysis process was then performed by adding water into the precursor/solvent solution, and this new solution was then stirred for 1 hour. For the Ag modified TiO₂, the precursor AgNO₃ (J.T. Baker) was used, for the Fe-TiO₂, the precursor was FeSO₄•7H₂O (J.T. Baker), and for the Cu-TiO₂, the precursor was CuSO₄•5H₂O (J.T. Baker), these compounds were added by dissolving them into the water used for the hydrolysis in a 0.1 wt%, the obtained product was dried at room temperature and then calcined at 450°C for 3 hours to promote the anatase crystal phase. For this synthesis, the materials were identified as sol-gel (SG) materials (Esquivel et al., 2013). The TiO₂ samples synthesized by the microwave-assisted sol-gel method was prepared by the sol obtained after the hydrolysis process. It was transferred into a Teflon vessel and placed on a microwave reaction system (Flexiwave Milestone). The process was carried out at a temperature of 180°C for 30 minutes. Once the product is obtained, it was filtered, dried, and calcined at 450 ° C for 3 hours. For this synthesis, the materials were identified as microwave-assisted sol-gel (Mw-SG) materials (Hernández et al., 2020).

Morphology and elemental analysis were carried out using a JEOL JSM-6060 LV scanning electron microscope (SEM) operating at a voltage of 15 keV. Elemental analysis was performed by Energy Dispersive X-ray Spectroscopy (EDS Oxford Inca X-Sight coupled to a MT 1000, Hitachi). The crystallinity was determined by X-ray Diffraction analysis (XRD) using a Bruker D8 advanced diffractometer equipped with a Cu seal tube to generate Cu K α radiation ($\lambda = 1.5406 \text{ \AA}$) with angles of $10 < 2\theta < 80^\circ$ in a pitch of 0.01° , Raman analysis was made using a LabRam HR Horiba Scientific with a NdYGa ($\lambda = 532 \text{ nm}$).

2.2. Plant harvest and growth parameters

Alfalfa seeds (*Medicago sativa* L.) were purchased from a local distributor brand Hortaflor, Mexico. They were placed in seedbeds using peat moss substrate (Jiffy) with a pH of 5.8, an electrical conductivity of 0.4 mS/cm, moisture fraction of 15%, and particle size of < 10 mm inside a plasticized greenhouse of 68 X 49 X 156 cm of length, wide and height, respectively. For the experiment, three replicates with a population of 6 crops were maintained during development. Sprouts were kept in seedbeds for 15 days before being transferred to plastic containers of 500 mL. Crops were treated by direct soil irrigation with 5 mL solutions of 50, 100, and 500 ppm of TiO₂ and M-TiO₂ (M = Ag, Cu, Fe) with no nutritive solution, during the seedbed development and 50 mL at the bigger container every three days, after completing 80 days of treatment.

Half of the plants were randomly selected for morphological analysis. After harvest, the samples were divided into leaves, stems, and roots immersed in liquid nitrogen to prevent any chemical structural change for future tests. Then the samples were milled and kept under refrigeration at 4°C for further metabolomics quantifications assays. The greenhouse temperature was recorded using a hygrometer

(YASSUN) and obtaining temperature and humidity values at midday. Climatic data were taken from the geo-electromagnetic center of the National Autonomous University of Mexico, Juriquilla campus (longitude: 100°26'48.81" west, latitude:20°42'14.87" north) (Levresse et al., s/f) at noon each day.

2.3. Secondary metabolite quantification

The extracts' total phenolic and flavonoid contents were determined according to the Folin-Ciocalteu spectrophotometric method (Bobo-García et al., 2015) modified for 96-well microplate. Total phenol content results were expressed as equivalent mg of gallic acid per gram of fresh sample, and rutin hydrate (flavonoid) is expressed as equivalent mg of rutin per gram of fresh sample and was determined by the 2-aminoethyl-diphenyl borate reagent method (Garcia-Mier et al., 2021).

2.3.1. Antioxidant activity

The extracts' antioxidant activity was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical method (Parit et al., 2018), and the results were expressed as the percentage of DPPH discoloration (% radical inhibition) named as well as percentage inhibition (IHB), which was calculated with Eq. 1

$$IHB\% = \left(\frac{A_{DPPH} - A_S}{A_{DPPH}} \right) * 100 \quad (1)$$

Where (A_S) is the absorbance of the solution containing the sample, and (A_{DPPH}) is the DPPH solution's absorbance. All the spectrophotometric measurements were obtained in a Thermo Scientific Multiskan Go spectrophotometer.

2.3.2. Chlorophyll content

The chlorophyll content was quantified using a SPAD 502 Plus Chlorophyll Meter from Minolta Co. Ltd. SPAD values were determined for plants in each treatment group, taking three readings per plant for an average amount per plant (Doğaroğlu et al., 2021; Monostori et al., 2016).

2.4. Data analysis

Statistical analysis was performed using Minitab 18.1 (Minitab 2017). Significant statistical difference was determined by ANOVA test using a Tukey pairwise comparison (Li et al., 2021). A Games-Howell pairwise comparison was made for data not showing equal variances, and the significance value was set to $p < 0.05$.

Results

3.1. Physicochemical Characterization of TiO₂ materials

Figure 1 shows a structural comparison between the materials synthesized by SG and Mw-SG. Figure 1(a) undoped TiO₂, (b) Fe-doped, (c) Cu-doped, and (d) Ag-doped. The synthesized materials by Mw-SG

show aggregates with lesser size than the pure sol-gel method. The internal heat generated through this process helps to form NMs with high crystallinity, small and uniform size (Hernández et al., 2020; Kadam & Park, 2018).

EDS shows the elemental composition of the M-TiO₂, M = Ag, Fe, Cu NPs, synthesized by the sol-gel method, where the presence of the Ti and O elements can be seen in Fig. 2(a) for the undoped TiO₂. In Fig. 2(b-d), the elemental mapping for the doped materials is observed, where the presence of the elements Cu (Fig. 2(b)), Fe (Fig. 2(c)), and Ag (Fig. 2(d)) are observed. Identical results were obtained for the Mw-SG synthesis method (results not shown).

X-ray diffraction patterns for the SG and Mw-SG synthesized materials are shown in Figs. 3(a) and (b). Where the signals detected at 2θ angles of 25.1 °, 37.7°, 47.8 °, 53.6 °, 54.8 °, 62.5 °, 68.7 °, 70.1 °, and 75.1 °, indicates the presence of the anatase crystalline phase with no presence of the rutile or the brookite crystal phases (Esquivel et al., 2013), and a preferential growth in the (101) Bragg diffraction. There are no diffraction signals related to the dopants due to their low concentration (Esquivel et al., 2013).

The crystallite sizes for both synthesis methods were calculated using the Scherrer equation showed in Eq. 2, where (D) is the diameter of de crystallite, (λ) is the wavelength of the X-rays, (k) is the Scherrer constant with a value of 0.9 for spherical nanoparticles, (β) is de full width at half maximum obtained from the diffraction signals in the XRD pattern and (Θ) the peak position.

$$D = \frac{k\lambda}{\beta \cos\theta};$$

Crystallite size was also determined using the Williamson-Hall equation (Eq. 3), which considers the structural stress of the crystallite. The equation represents a straight line where (ε) is the slope that provides the strain of the crystallite.

$$\beta \cos\theta = \epsilon(4\sin\theta) + \frac{k\lambda}{D} \quad (3)$$

The crystallite size for both synthesis methods is compiled in Table 1. The sol-gel synthesis method gives an average crystallite size of 9 nm, being the undoped TiO₂ the smallest size calculated by the Sherrer equation (Eq. 2). The Williamson-Hall method shows an average size of 17 nm. Since this equation considers the stress of the crystal lattice, either compression or relaxation, the calculated size is bigger than the Scherrer method, which refers to a crystallite with stress in the materials' lattice. The crystallinity degree has values greater than 90%.

Table 1 Crystallite size by Sherrer and Williamson-Hall method and degree of crystallinity for the SG and Mw-SG synthesized materials.

Material	Sherrer		Williamson-Hall		Crystallinity	
	(nm)		(nm)		(%)	
	SG	Mw-SG	SG	Mw-SG	SG	Mw-SG
TiO ₂	8.65	8.04	19.80	12.49	93.94	93.69
Ag-TiO ₂	9.62	7.42	14.00	12.27	93.15	92.90
Fe-TiO ₂	9.28	6.81	18.73	11.45	92.53	93.20
Cu-TiO ₂	8.75	7.69	16.31	12.27	92.72	91.09

The Scherrer equation obtained an average of 7.4 nm crystallite size for the Mw-SG synthesized materials, showing that microwave coupling helps to obtain particles with smaller crystallite sizes (Hernandez et al., 2020). The Williamson-Hall equation shows an average crystallite size of 12.2 nm, and the crystallinity also had values greater than 90%, considering the structural stress present in the crystallite.

The crystal phase was also confirmed with Raman spectroscopy, where the spectra of the materials synthesized by the sol-gel method are shown in Fig. 4(a). Mw-SG (Fig. 4(b)) and SG synthesized materials shows four signals at 142.7 (E_g), 396.8 (B_{1g}), 517 (B_{1g}/A_{1g}), and 637.7 (E_g) cm^{-1} , which are indicative of the presence of the anatase crystalline phase (Esquivel et al., 2013), without the presence of the brookite and rutile phases of the TiO₂. No band shifting or new signals are observed due to the low concentration of dopants (Esquivel et al., 2013).

3.2. Germination and morphological data

The germination of the alfalfa seeds was not affected by the direct irrigation of the TiO₂ materials (with or without doping) at three concentrations for both synthesis methods. For all concentrations except 500 ppm to the doped materials (Ag, Fe-TiO₂), the seeds germinated on the 5th day; for this concentration, the seeds germinated on the 6th day. The real leaf was observed for the control on the 11th day, while for the rest of the treatments, the real leaf was observed on the 12th day. For Mw-SG NPs, seed germination and real leaf were also not affected, where for all treatments, germination occurred on the 5th while real leaf appeared on the 11th day.

Nutrient deficiency in the alfalfa crops was observed in leaves based on texture and colors, as shown in Fig. 5. Which corresponds to control plants with non-visual nutrient deficiency, and M-TiO₂ plants treated plants showing boron deficiency (Fig. 5(b)), nitrogen deficiency (Fig. 5 (c)), and potassium deficiency (Fig. 5(d)). The boron deficiency was observed only in alfalfa treated with Ag-doped at concentrations of 50

and 100 ppm. At the same time, the control did not show apparent effects of nutrient deficiency or another type of adversity (Undersander et al., 2011).

To our best of knowledge, no studies visualize the effect of TiO₂ NMs in alfalfa. However, some studies have determined a reduced nutritional quality and micronutrient content for metal base NMs in other species (Bellani et al., 2020; Hu et al., 2020; Missaoui et al., 2021; Rui et al., 2018). Micronutrient deficiency may be subjected to multiple factors like climate or microbiota present in the soil and the interaction between root/nanoparticles.

Some studies are finding multiple cases of root cells damage by nanoparticle and reactive oxygen species (ROS) interactions, which can cause root blockage or cellular damage, resulting in micronutrient uptake imbalance (Hu et al., 2020; Rui et al., 2018).

The stem length of the alfalfa crops was measured in the central and secondary stem. Figure 6(a) contains the measurements obtained from the length (cm) of the central stem, showing a reduction in size for the four types of SG synthesized NMs at the three concentrations. At 50 ppm, SG-NMs caused a 21.8% reduction, 100 ppm (31.9%), followed by a cease in size reduction for 500 ppm with only a 22.2%; undoped NPs treatments showed statistical significance compared to control, for Ag-doped 50 ppm caused a 42.9% reduction, 27.78% at 100ppm and 11.18% at 500 ppm. Meanwhile, Fe-doped showed a reduction of almost 25% for all doses with a statistical significance. Finally, Cu-doped showed the lowest effect among the NMs with only a reduction of 9% at 50 and 100 ppm, increasing at 500 ppm (17%), where the highest concentration caused a significant reduction in stem height.

The measurements obtained from the secondary stem (Fig. 6(b)) also show a size reduction. For undoped TiO₂, a 41.16% reduction was obtained at 50 ppm, 55.0% at 100 ppm, and 30 % at 30.3% at 500 ppm, where the three concentrations reached statistical significance compared to control. Ag-dope material showed a reduction as concentration was increased, although the three concentrations were statistically similar, obtaining a reduction of 42.9% (50 ppm), 21.6% (100 ppm), and 18.3% (500 ppm). Fe-doped material showed a high reduction in the size while the dose is augmenting, showing statistical significance among the three concentrations, causing a 37% (50 ppm), 44.3% (100 ppm), and 50.0 % reduction at 500 ppm. Finally, the Cu-doped material also caused a reduction of almost 30% for all three concentrations. Although 50 and 100 ppm caused a reduction, these two treatments showed no statistical difference compared to the control.

In the case of the leaf length (Fig. 6(c)) measured in the central stem for the undoped TiO₂, it generates a reduction of about 5% for the three concentrations, while Fe-doped had its maximum reduction at 100 ppm (25.5%). Nevertheless, at 500 ppm, there is an inhibition effect (17.2%). Ag-doped has the same effect with the maximum at 100 ppm (11.9%) and inhibition at 500 ppm. The Cu-doped had no reduction effect at 50 and 100 ppm, while 500 ppm caused a reduction of 28.8%.

Central stem length measurement for alfalfa treated with NMs synthesized by the Mw-SG method Fig. 7(a), it reveals a reduction in the stem length at different materials concentrations. For the undoped TiO₂

caused a reduction of 21.9% (50 ppm), 12.2% (100 ppm), and 9.1% (500 ppm). Fe-TiO₂ shows a reduction of 16.7% (50 ppm), 29.8% (100 ppm) and 25.3% (500 ppm). For Ag-TiO₂ results showed 9.9% (50 ppm), 8% (100 ppm) and 28.6% (500 ppm). Finally, Cu-TiO₂ generated 9.2% (50 ppm), 30.6% (100 ppm) and 16.9% (500 ppm) reduction in the stem length.

For the secondary stem (Fig. 7(b)), there was a reduction of 20.9% (50 ppm), 13% (100 ppm,) and 2.8% (500 ppm) for the undoped material. Fe-TiO₂ caused a reduction of 18.8% (50 ppm), 26.9% (100 ppm), and 15.5% (500 ppm). For Ag-TiO₂, results showed 21.5% (50 ppm), 10.7% (100 ppm,) and 23.6% (500 ppm). Finally, Cu-TiO₂ generated 11.4% (50 ppm), 20.7% (100 ppm), and 11.8% (500 ppm) reduction in the secondary stem length.

The leaf length in the central stem (Fig. 7(c)) after an 80-day exposure with the Mw-SG synthesized materials also shows a reduction of 22% (50 ppm), 17.6% (100 ppm), and 11.6% (500 ppm) for undoped TiO₂. Fe-TiO₂ caused a reduction of 11.9% (50 ppm), 17% (100 ppm) and 19.5% (500 ppm). The Ag-TiO₂ showed a 22.9% (50 ppm), 31.3% (100 ppm) and 22.9% (500 ppm). Finally, Cu-TiO₂ generated 10.3% (50 ppm), 33.1% (100 ppm) and 25.2% (500 ppm) reduction in leaf length. The leaf width in the central stem also showed a reduction of 21.5% (50 ppm), 22.5% (100 ppm), and 14.3% (500 ppm) for the undoped material. Fe-TiO₂ caused a reduction of 8.9% (50 ppm), 13.9% (100 ppm), and 14.1% (500 ppm). For Ag-TiO₂, results showed 25.1% (50 ppm), 27.8% (100 ppm), and 12.9% (500 ppm) of reduction in leaf length. Finally, Cu-TiO₂ generated 15.2% (50 ppm), 25.7% (100 ppm), and 26% (500 ppm) reduction in leaf length. Both synthesis methods cause a reduction of leaf dimensions and stem length. The effect observed differ from both methods, possibly due to the different physicochemical characteristics of both types of NMs. For both measurements (central and secondary steam) in both synthesis methods, the Tukey pairwise comparison test reveals significant morphological reductions caused by the treatments at different concentrations compared to control and among each treatment. This shows that NMs have different properties that cause different degrees of stress that reflect on the morphological of stem and leaf.

3.3. Secondary metabolite and chlorophyll content

The UV-visible method of secondary metabolite quantification compares the rutin, gallic acid, and inhibition percentage between the NMs used in the treatments at each concentration. After completing 80 days of treatment, the alfa plants were divided into groups containing only leaves, shoots, and roots for a sectional analysis of the metabolite content.

The effect on crop growth was observed in the secondary metabolites content in leaves (Table 2). A statistical analysis using a Tukey assay for comparing data pairs was done to identify significantly statistical data. The assay compared the data obtained for gallic acid and rutin with the three doses and the four types of NMs synthesized by the SG method. The results showed no significant augmentation of the equivalent grams of gallic acid than control except for the plants treated with the undoped TiO₂ at 100 ppm with an augmentation of 70.85%. The rutin content also had no significant affectation due to

the materials, even though the equivalents grams of rutin appear to be higher than the control plant. All treatments showed no significant effect on this secondary metabolite content in leaves at all concentrations (50, 100, 500 ppm).

Table 2. Secondary metabolites and inhibition % in plant leaves by the materials obtained by the SG synthesis method.

Leaves									
	Gallic acid (mg/g)	SD (±)	%	Rutin (mg/g)	SD (±)	%	IHB %	SD (±)	%
50 ppm									
Control	49.21 ^B	6.82	-	305.09 ^A	30.61	-	30.69 ^B	4.08	-
TiO ₂	58.21 ^B	6.26	18.28	313.27 ^A	90.92	2.68	43.47 ^{AB}	1.54	41.64
Ag-TiO ₂	64.75 ^{AB}	6.31	31.57	426.8 ^A	53.7	39.89	43.88 ^{AB}	2.69	42.97
Fe-TiO ₂	62.00 ^B	6.56	25.99	424.2 ^A	49.75	39.04	39.86 ^{AB}	4.27	29.87
Cu-TiO ₂	59.18 ^B	7.59	20.26	327.5 ^A	26.58	7.35	38.19 ^{AB}	1.97	24.43
100 ppm									
Control	49.21 ^B	6.82	-	305.09 ^A	30.61	-	30.69 ^B	4.08	-
TiO ₂	84.08 ^A	5.80	70.85	355.37 ^A	105.50	16.47	46.28 ^A	11.44	50.79
Ag-TiO ₂	59.36 ^B	5.50	20.62	512.8 ^A	115.55	68.09	40.47 ^{AB}	4.34	31.86
Fe-TiO ₂	49.99 ^B	1.87	1.58	384.5 ^A	16.06	26.02	35.32 ^{AB}	0.54	15.08
Cu-TiO ₂	63.20 ^B	9.24	28.42	397.4 ^A	188.11	30.26	37.93 ^{AB}	1.70	23.59
500 ppm									
Control	49.21 ^B	6.82	-	305.09 ^A	30.61	-	30.69 ^B	4.08	-
TiO ₂	67.03 ^{AB}	11.27	36.21	345.83 ^A	78.13	13.35	40.80 ^{AB}	6.19	32.94
Ag-TiO ₂	52.26 ^B	4.49	6.19	330.0 ^A	69.22	8.18	41.06 ^{AB}	6.27	33.79
Fe-TiO ₂	59.02 ^B	1.16	19.93	393.5 ^A	55.83	28.99	39.10 ^{AB}	1.52	27.40

Means that do not share a letter indicate a significant difference ($p < 0.05$) between treatments with different TiO₂ for each concentration. Different letters per row indicate a statistical difference in comparison of means (Tukey).

Leaves									
Cu-TiO ₂	53.62 ^B	8.24	8.96	404.5 ^A	32.92	32.58	34.98 ^{AB}	4.58	13.97
Means that do not share a letter indicate a significant difference ($p < 0.05$) between treatments with different TiO ₂ for each concentration. Different letters per row indicate a statistical difference in comparison of means (Tukey).									

Metabolite analysis at the stem of the alfalfa treated with SG-Titania materials is shown in Table 3. It was possible to observe statistical significance towards the metabolite content compared to the control. However, these results show no concentration-dependent manner rewarding the NMs' application and the administrated concentration. The gallic acid content was significantly higher for the undoped TiO₂ at 500 (96.29%) and 100 ppm (92.95%). In comparison, Ag and Cu-TiO₂ doped showed gallic acid accumulation at 100 ppm, increasing 72.12% and 69.21%, respectively. The rest of the treatment's concentration, including the complete treatment Fe-doped, showed no significant change compared to control. Even though the treatments show some enhanced production of gallic acid, none of the treatments shows a significant augmentation or reduction of stem rutin content.

Table 3. Secondary metabolites and inhibition % in the stem of plants by the materials obtained by the SG synthesis method.

Stem									
	Gallic acid (mg/g)	SD (±)	%	Rutin (mg/g)	SD (±)	%	IHB %	SD (±)	%
50 ppm									
Control	25.40 ^D	6.73	-	119.06 ^A	19.5	-	25.65 ^D	2.19	
TiO ₂	35.13 ^{ABCD}	2.46	38.30	128.35 ^A	11.91	7.80	39.28 ^{ABC}	2.02	53.31
Ag-TiO ₂	29.9 ^{CD}	2.78	17.78	125.58 ^A	12.23	5.28	41.15 ^{AB}	4.10	60.61
Fe-TiO ₂	31.42 ^{BCD}	3.37	23.70	160.25 ^A	22.78	34.59	29.65 ^{ABCD}	1.04	15.72
Cu-TiO ₂	35.90 ^{ABCD}	6.40	41.33	139.97 ^A	22.12	17.56	33.68 ^{ABCD}	4.17	31.30
100 ppm									
Control	25.40 ^D	6.73	-	119.06 ^A	19.5	-	25.65 ^D	2.19	
TiO ₂	49.01 ^{AB}	2.83	92.95	174.9 ^A	48.0	46.20	43.76 ^A	1.26	70.60
Ag-TiO ₂	43.72 ^{ABC}	7.87	72.12	165.87 ^A	10.14	39.31	32.07 ^{ABCD}	2.67	25.02
Fe-TiO ₂	34.77 ^{ABCD}	6.24	36.88	142.6 ^A	31.59	19.77	28.01 ^{CD}	1.50	9.2
Cu-TiO ₂	42.98 ^{ABC}	5.72	69.21	134.96 ^A	10.11	13.35	30.15 ^{ABCD}	5.69	17.54
500 ppm									
Control	25.40 ^D	6.73	-	119.06 ^A	19.5	-	25.65 ^D	2.19	
TiO ₂	49.86 ^A	9.08	96.29	146.46 ^A	13.67	23.01	40.41 ^{ABC}	9.60	57.72
Ag-TiO ₂	37.72 ^{ABCD}	3.95	48.50	143.3 ^A	37.4	20.35	32.50 ^{ABCD}	6.51	26.85
Fe-TiO ₂	37.59 ^{ABCD}	5.72	47.99	165.44 ^A	27.8	38.95	32.21 ^{ABCD}	2.59	25.57

Means that do not share a letter indicate a significant difference ($p < 0.05$) between treatments with different TiO₂ for each concentration. Different letters per row indicate a statistical difference in comparison of means (Tukey).

Stem									
Cu-TiO ₂	38.24 ^{ABCD}	3.42	50.55	167.60 ^A	28.40	40.76	28.33 ^{BCD}	2.42	10.44
Means that do not share a letter indicate a significant difference ($p < 0.05$) between treatments with different TiO ₂ for each concentration. Different letters per row indicate a statistical difference in comparison of means (Tukey).									

Since the NMs were added to the water used for irrigation of the plants, the roots were the primary organ exposed to the NMs, so the highest stress effect related to the production of secondary metabolites should be seen in this organ.

The data showed in Table 4 correspond to the analysis of the roots, where it can observe at first instance by the Tukey pairwise comparison that the SG synthesized undoped TiO₂ at 50 (70.91%) and 100 ppm (61.01%) showed the significative higher effect on the gallic acid production as well for the Cu-doped material at 500 ppm (60.03%) and Ag-doped material at 100 ppm (58.22 %).

Table 4 Secondary metabolites and inhibition % in plants' roots by the materials obtained by the SG synthesis method.

Root									
	Gallic acid (mg/g)	SD (±)	%	Rutin (mg/g)	SD (±)	%	IHB %	SD (±)	%
50 ppm									
Control	21.52 ^B	4.10	-	11.28 ^C	0.94	-	12.63 ^C	0.92	-
TiO ₂	36.78 ^A	2.22	70.91	16.06 ^{BC}	0.82	42.37	19.85 ^{ABC}	3.33	57.16
Ag-TiO ₂	26.39 ^{AB}	4.70	22.63	31.36 ^A	2.04	178.01	17.41 ^{ABC}	3.19	37.84
Fe-TiO ₂	25.94 ^{AB}	1.58	20.53	20.16 ^B	0.85	78.74	23.97 ^{AB}	1.58	89.78
Cu-TiO ₂	24.91 ^{AB}	1.29	15.75	15.42 ^{BC}	3.17	36.70	17.75 ^{ABC}	5.32	40.53
100 ppm									
Control	21.52 ^B	4.10	-	11.28 ^C	0.94	-	12.63 ^C	0.92	-
TiO ₂	34.65 ^A	8.12	61.01	21.04 ^B	0.52	86.52	22.60 ^{AB}	0.45	78.93
Ag-TiO ₂	34.05 ^A	6.37	58.22	21.02 ^B	4.25	86.40	21.13 ^{AB}	2.36	67.30
Fe-TiO ₂	27.43 ^{AB}	1.69	27.46	21.59 ^B	2.24	91.40	25.78 ^A	1.37	104.11
Cu-TiO ₂	33.21 ^{AB}	3.72	54.32	18.04 ^{BC}	4.64	59.91	15.73 ^{BC}	2.56	24.54
500 ppm									
Control	21.52 ^B	4.10	-	11.28 ^C	0.94	-	12.63 ^C	0.92	-
TiO ₂	32.47 ^{AB}	2.73	50.88	21.09 ^B	3.39	86.86	20.84 ^{ABC}	2.14	65.00
Ag-TiO ₂	24.65 ^{AB}	5.91	14.54	11.66 ^C	2.23	3.36	21.20 ^{AB}	4.04	67.85
Fe-TiO ₂	25.14 ^{AB}	3.14	16.82	19.78 ^B	2.17	75.35	24.54 ^A	2.99	94.29

Means that do not share a letter indicate a significant difference ($p < 0.05$) between treatments with different TiO₂ for each concentration. Different letters per row indicate a statistical difference in comparison of means (Tukey).

Root									
Cu-TiO ₂	34.44 ^A	1.66	60.03	19.90 ^B	2.19	76.41	21.38 ^{AB}	2.53	69.27
Means that do not share a letter indicate a significant difference ($p < 0.05$) between treatments with different TiO ₂ for each concentration. Different letters per row indicate a statistical difference in comparison of means (Tukey).									

The effect on the rutin production was much clearer in the roots than the other plants' sections, where more treatments resulted in a significant increase of the metabolite content in roots compared to the control.

Ag-doped material caused an increase of rutin at 50 (39.61 %) and 100 ppm (86.40 %) as well the Fe-doped material at 50 ppm (78.74%), 100 ppm (91.46 %), and 500 ppm (74.22 %). The undoped TiO₂ only increase the rutin content at 100 ppm (86.52%) and 500 ppm (86.86), and lastly, Cu-doped material only showed a significant effect at 500 ppm (76.41%). These results show that the undoped and doped TiO₂ NMs at specific concentrations treatments can induce the production of rutin in the roots causes by possible stress.

Plant irrigated with the materials obtained by the Mw-SG method, after 80 days of exposure, the analysis to leaves, stem, and roots were made for metabolite quantification. In Table 5 it is presented the quantification of gallic acid, rutin, and inhibition % in leaves. Comparing the treatments with the control using a Tukey assay shows that the Mw-SG materials cause an augmentation of gallic acid content compared to the SG materials.

Table 5. Secondary metabolites and inhibition % in plant leaves by the materials obtained by the Mw-SG synthesis method.

Leaves									
	Gallic acid (mg/g)	SD (±)	%	Rutin (mg/g)	SD (±)	%	IBH %	SD (±)	%
50 ppm									
Control	45.72 ^E	4.01	-	396.54 ^C	57.90	-	33.93 ^E	1.78	-
TiO ₂	60.71 ^{ABCD}	7.34	32.78	540.43 ^{ABC}	113.01	36.28	55.88 ^A	1.80	64.69
Ag-TiO ₂	64.08 ^{ABCD}	9.87	40.16	400.75 ^C	64.27	1.06	49.05 ^{BCD}	4.24	44.55
Fe-TiO ₂	59.83 ^{ABCD}	3.45	30.86	529.41 ^{ABC}	59.66	33.50	44.55 ^D	1.63	31.29
Cu-TiO ₂	54.96 ^{DE}	13.89	20.20	540.57 ^{ABC}	89.75	36.32	55.91 ^A	3.43	64.77
100 ppm									
Control	45.72 ^E	4.01	-	396.54 ^C	57.90	-	33.93 ^E	1.78	-
TiO ₂	67.55 ^{ABCD}	5.11	47.74	401.23 ^{B^C}	24.29	21.35	47.49 ^{CD}	2.96	40.84
Ag-TiO ₂	58.83 ^{BCDE}	7.19	28.68	504.95 ^{BC}	93.32	27.33	54.14 ^{AB}	4.31	59.56
Fe-TiO ₂	65.81 ^{ABCD}	4.58	43.95	574.86 ^{AB}	82.32	44.96	43.78 ^D	2.87	29.03
Cu-TiO ₂	72.76 ^{AB}	5.93	59.14	534.76 ^{ABC}	58.56	34.85	46.74 ^D	5.51	37.75
500 ppm									
Control	45.72 ^E	4.01	-	396.54 ^C	57.90	-	33.93 ^E	1.78	-
TiO ₂	69.45 ^{ABC}	3.10	51.90	408.19 ^C	42.08	2.93	46.67 ^D	0.91	37.54
Ag-TiO ₂	58.31 ^{CDE}	4.88	27.53	442.14 ^{BC}	24.20	11.49	47.65 ^{CD}	2.19	40.42

Means that do not share a letter indicate a significant difference ($p < 0.05$) between treatments with different TiO₂ for each concentration. Different letters per row indicate a statistical difference in comparison of means (Tukey).

Leaves									
Fe-TiO ₂	73.85 ^A	8.84	61.53	688.59 ^A	185.01	73.65	46.55 ^D	3.56	37.20
Cu-TiO ₂	65.67 ^{ABCD}	5.88	43.63	504.01 ^{BC}	42.06	27.10	53.19 ^{ABC}	2.64	56.76
Means that do not share a letter indicate a significant difference ($p < 0.05$) between treatments with different TiO ₂ for each concentration. Different letters per row indicate a statistical difference in comparison of means (Tukey).									

Where none of the treatments caused a significant augmentation of gallic acid content when treated with Mw-SG materials, only the Ag-doped material at 100 and 500 ppm and Cu-doped material at 50 ppm were not significant compared to the control test.

Results related to the gallic acid content in the stem appeared to be no different in variance, so the Tukey assay for pairwise comparison could not be done. For the gallic acid content, a Game-Howell pairwise comparison shows that the treatments significantly different compared to the control are the Fe-doped material at 100 (67.53%) and 500 ppm (71.48%), and the undoped TiO₂ at 100 ppm (86.84%). The Cu-doped material at 500 ppm (125.04%), as seen in Table 6, results obtained to quantify rutin content had a significant difference in variance so that the Tukey assay could be performed. In this case, the treatments statistically different were the Fe-doped material at 100 ppm (73.20%), 500 ppm (33.46%), the Ag-doped material at 100 (33.83%) and 500 ppm (30.94%), and the Cu-doped material at 500 ppm (32.44%), the inhibition percentage augmentation caused by the NMs interaction appeared to be all significant compared to the control.

Table 6. Secondary metabolites and inhibition % in the stem of plants by the materials obtained by the Mw-SG synthesis method.

Stem									
	Gallic acid (mg/g)	SD (±)	%	Rutin (mg/g)	SD (±)	%	IBH %	SD (±)	%
50 ppm									
Control	20.48 ^C	2.38	-	132.03 ^D	10.09	-	23.24 ^F	2.58	-
TiO ₂	28.27 ^{ABC}	1.92	38.05	146.83 ^{BCD}	8.30	11.21	36.94 ^D	1.39	58.95
Ag-TiO ₂	24.57 ^{BC}	2.57	19.97	141.14 ^{BCD}	12.71	6.89	42.09 ^{BC}	1.46	81.11
Fe-TiO ₂	30.57 ^{ABC}	9.08	49.26	165.93 ^{BCD}	10.54	25.67	42.80 ^{AB}	1.53	84.16
Cu-TiO ₂	32.46 ^{ABC}	5.85	58.49	142.64 ^{BCD}	34.04	8.04	42.75 ^{AB}	4.54	83.95
100 ppm									
Control	20.48 ^C	2.38	-	132.03 ^D	10.09	-	23.24 ^F	2.58	-
TiO ₂	38.26 ^{AB}	7.02	86.84	162.61 ^{BCD}	9.59	23.16	37.36 ^{CD}	1.29	60.76
Ag-TiO ₂	28.09 ^{ABC}	3.14	37.16	176.70 ^{BC}	19.17	33.83	44.35 ^{AB}	4.08	90.83
Fe-TiO ₂	34.31 ^{AB}	6.51	67.53	227.5 ^A	40.93	72.30	41.83 ^{BC}	2.20	79.99
Cu-TiO ₂	28.68 ^{ABC}	7.47	40.03	164.16 ^{BCD}	19.06	24.33	31.96 ^E	2.43	37.55
500 ppm									
Control	20.48 ^C	2.38	-	132.03 ^D	10.09	-	23.24 ^F	2.58	-
TiO ₂	26.99 ^{BC}	1.71	31.78	136.10 ^{CD}	11.50	3.08	34.05 ^{DE}	1.36	46.51
Ag-TiO ₂	30.01 ^{ABC}	3.98	46.53	172.88 ^{BC}	10.87	30.94	42.17 ^{BC}	1.41	81.45
Fe-TiO ₂	35.12 ^{AB}	3.16	71.48	176.21 ^B	18.55	33.46	42.07 ^{BC}	2.65	81.04

Means that do not share a letter indicate a significant difference ($p < 0.05$) between treatments with different TiO₂ for each concentration. Different letters per row indicate a statistical difference in comparison of means (Tukey).

Stem									
Cu-TiO ₂	46.09 ^A	8.34	125.04	174.86 ^{BC}	16.76	32.44	47.25 ^A	2.11	103.31
Means that do not share a letter indicate a significant difference ($p < 0.05$) between treatments with different TiO ₂ for each concentration. Different letters per row indicate a statistical difference in comparison of means (Tukey).									

A Games-Howell pairwise comparison was made to a lack of variance between the data for the gallic acid content in roots. The data expressed in Table 7 shows that the root's gallic acid content is significantly higher for the Cu-doped materials at 100 (70.42%) and 500 ppm (73.97%). In contrast, the rest of the treatments at the different comparisons were statistically equal to the control. The Cu-doped materials at 500 ppm (41.99%) resulted in statistical significance compared to the control in the rutin content. Finally, the inhibition percentage calculated in roots is significantly higher for the three doses of undoped NMs, copper, and iron-doped titania. Only silver-doped titania at a concentration of 50 ppm was statistically similar to the control.

Table 7. Secondary metabolites and inhibition % in plants' roots by the materials obtained by the Mw-SG synthesis method.

Root									
	Gallic acid (mg/g)	SD (±)	%	Rutin (mg/g)	SD (±)	%	IBH %	SD (±)	%
50 ppm									
Control	21.71 ^{ABC}	7.76	-	19.55 ^B	1.93	-	20.40 ^E	1.71	-
TiO ₂	29.92 ^{ABC}	5.02	37.81	23.84 ^{AB}	2.74	21.94	45.47 ^A	6.22	122.89
Ag-TiO ₂	22.28 ^{BC}	1.14	2.62	24.69 ^{AB}	3.91	26.29	26.20 ^{DE}	2.72	28.43
Fe-TiO ₂	18.09 ^C	3.87	6.33	21.44 ^{AB}	3.43	9.66	33.80 ^{CD}	1.36	65.68
Cu-TiO ₂	29.08 ^{ABC}	3.54	33.94	24.01 ^{AB}	2.85	22.81	38.80 ^{ABC}	1.68	90.19
100 ppm									
Control	21.71 ^{ABC}	7.76	-	19.55 ^B	1.93	-	20.40 ^E	1.71	-
TiO ₂	21.80 ^{BC}	5.39	0.41	24.20 ^{AB}	4.15	23.78	44.06 ^A	3.86	115.98
Ag-TiO ₂	27.28 ^{ABC}	4.90	25.65	25.83 ^{AB}	4.16	32.12	39.14 ^{ABC}	2.40	91.86
Fe-TiO ₂	22.32 ^{BC}	6.18	2.80	26.09 ^{AB}	2.11	33.45	45.12 ^A	1.98	121.13
Cu-TiO ₂	37.00 ^{AB}	8.77	70.42	24.4 ^{AB}	4.82	24.80	35.48 ^{BC}	7.06	73.92
500 ppm									
Control	21.71 ^{ABC}	7.76	-	19.55 ^B	1.93	-	20.40 ^E	1.71	-
TiO ₂	25.82 ^{ABC}	2.08	18.93	25.48 ^{AB}	4.38	30.33	42.52 ^{AB}	5.22	108.43
Ag-TiO ₂	25.64 ^{ABC}	4.60	18.10	21.27 ^{AB}	1.49	8.79	38.17 ^{ABC}	3.79	87.10
Fe-TiO ₂	29.94 ^{ABC}	6.61	37.89	22.33 ^{AB}	1.59	14.21	37.50 ^{ABC}	5.50	83.82

Means that do not share a letter indicate a significant difference ($p < 0.05$) between treatments with different TiO₂ for each concentration. Different letters per row indicate a statistical difference in comparison of means (Tukey).

Root									
Cu-TiO ₂	37.77 ^A	6.44	73.97	27.76 ^A	3.07	41.99	37.66 ^{ABC}	4.32	84.60
Means that do not share a letter indicate a significant difference ($p < 0.05$) between treatments with different TiO ₂ for each concentration. Different letters per row indicate a statistical difference in comparison of means (Tukey).									

The measurement of chlorophyll by the Konica Minolta SPAD 502 Plus chlorophyll meter revealed a reduction in chlorophyll content in leaves for the plants treated with the sol-gel synthesis method shown in Fig. 8(a). The undoped TiO₂ caused significant chlorophyll reduction at 50 ppm (32.5%), 100 ppm (40%), and 500 ppm (11.6%), where treatments at 50 and 100 ppm were statistically significant compared to the control. A similar effect is shown with Fe-doped material where 50, 100, and 500 ppm caused a reduction of 16.9, 27, and 6.9% of chlorophyll content, respectively. Although Fe-doped material at 100 ppm showed only a significant difference, Ag-doped material caused a reduction of almost 10% for the three concentrations showing no statistical variation upon this treatment compared to the control. Cu-doped material caused less chlorophyll reduction in the 50 and 500 ppm doses, and 100 ppm presents no reduction effect.

However, all three treatments showed no significant difference compared to the control. The chlorophyll content shows interesting results in plants treated with the materials obtained by the Mw-SG synthesis method (Fig. 8(b)). The lowest dose in undoped TiO₂, as shown in Fig. 8(b), causes an increase of 7% compared to control. In contrast, the content decreases to about 14% and 25% at higher doses for 100 and 500 ppm, respectively. The Fe-TiO₂ shows a significant reduction at 50 ppm (31.5%), and at higher doses of 100 and 500 ppm, there is a reduction of 15%. For the Ag-doped material, there is also an increase of chlorophyll at 50 ppm of about 13%, and for the 100 and 500 ppm treatments, it only increases 15%. Finally, Cu-TiO₂ material caused a reduction of 7.6% (50 ppm), 24.8% (100 ppm), and 30.1% (500 ppm).

3.4. Antioxidant activity

DPPH radical inhibition was determined for each treatment in the three organs studied. As shown in Tables 2–7, leave analysis shows an overall augmentation of radical IHB for plants interacting with NMs compared to control. No apparent effect towards IHB% augmentation with NMs concentrations was determined for all treatments. At 50 ppm, the highest radical inhibition was obtained with Ag-doped and undoped NMs. At 100 ppm, the highest effect was determined for undoped NPs when for the rest of the treatments a reduction in %IHB was observed, at the highest concentration Ag-doped and undoped NMs where undoped TiO₂ showed a final reduction of IHB%. For the stem, the highest reduction at 50 ppm was obtained Ag-doped followed by undoped TiO₂, at 100 ppm highest antioxidant activity was obtained with undoped NMs, at the highest concentration, the same result was obtained where highest undoped TiO₂ gave the highest radical inhibition. In roots, the NMs effect on the inhibition percentage augmentation

compared to control was expressed in much more concentrations than in the leaves and stem of the alfalfa. Roots exposed to Fe-doped material at the three doses showed a higher inhibition percentage than control. The undoped TiO₂ only showed apparent effect at 100 ppm (78.93 %), while Cu-doped material at 500 ppm (69.27 %). The Ag-doped NPs caused a significant effect at 100 (67.30 %) and 500 ppm (67.85%).

For the Mw-SG treated plant IHB% was also augmented. However, no relation between an augmenting NPs concentration and an increase in radical inhibition was observed. Radical imbibition analysis at leaves (Table 5) showed that the highest IHB% at 50 ppm was obtained with undoped and Cu doped NPs. At the same time, Fe and Ag-doped caused an augmentation between 30 and 40%. At 100 ppm, Ag-doped caused the highest radical inhibition.

In contrast, the lowest was caused by Fe-doped (29.0%). At the highest concentration, Cu-doped NPs showed the highest radical inhibition. Overall, all treatments compared to control are statistically significant. For radical inhibition in the stem, all treatments also showed statistical significance when compared to control, where at 50 ppm, the Ag, Fe, and Cu-doped NPs had the highest augmentation reaching between 80–84% when compared to control, Ag-doped NPs maintained the highest IHB% augmentation at 100 ppm. In contrast, some treatments reduced its effect at different degrees, Cu-doped the lowest imbibition, at 500 ppm, Cu-doped increased its radical inhibition, reaching 103% augmentation being the highest. For roots treated with NMs at 50 ppm, undoped TiO₂ caused a 122% augmentation. In contrast, the lowest (28.43%) was caused by Ag-doped at 100 ppm. Fe-doped NMs increased their radical inhibition reaching a 121.1% augmentation with undoped TiO₂. Finally, at 500 ppm, all NMs caused an augmentation between 80 and 110%.

Discussion

NMs can cause multiple effects, which can generate a reduction of the morphological parameters. Studies of TiO₂ interaction with plants have mixed effects where the presence of TiO₂ enhances some species growth of multiple organs like roots or stems while other cases show detrimental effects in the growth of these organs (Bellani et al., 2020; Gordillo-Delgado et al., 2020; Tan, Du, et al., 2018; Tan et al., 2017).

SEM images reveal the differences in morphology obtained for both synthesis methods; the SG method generates large granulates with smaller aggregates growing on the surface and heterogeneous sizes. On the other hand, adding microwaves to the synthesis process gave particles with smaller and more uniform aggregates. Both synthesis methods gave an amorphous geometry for the materials (Hernandez et al., 2020). By using the Scherrer equation, it can be observed that the materials obtained from the Mw-SG method have a smaller crystallite size compared to the SG method. When considering structural stress by the Williamson-Hall equation, it can be observed that crystallite size for both methods appears to be bigger. However, Mw-SG materials maintain a smaller crystallite size than the SG method between these two methods. Further evaluation through Transmission electronic microscopy (TEM) needs to be realized to obtain the materials' accurate sizes. It may help to relate the observed effects in the three

organs analyzed, relating NMs size with their availability for being translocated through organs due to size differences, where smaller NMs will tend to move easily through plant tissues (Wojcieszek et al., 2019).

Crystallinity plays a crucial role in the interactions between plants and NMs; however, both synthesis methods give NMs with high crystal quality, as seen in the XRD diffractograms and Raman spectra. Further analysis like Rietveld could permit us to understand dopant position in anatase structure and phase composition (Hernández et al., 2020), helping to predict the availability of the metal ions to plant nutrition or the interaction with other molecules present in the soil root exudates.

It is found that NMs can block root hair, preventing the plant from absorbing nutrients from the soil (Rui et al., 2018). Root hair damage can also occur by mechanical or reactive oxygen species (ROS) interactions, which further reduces the ability to absorb nutrients, decaying plant growth, and altering multiple morphological traits (Kole et al., 2016; Tang et al., 2016; Tripathi et al., 2017). Although NMs did not affect seed germination, a more strict assay should be performed to evaluate the interaction and determine the consequences in seedling development.

The effect observed in the chlorophyll content could be related to the plant's growth stage as determined by (Phothi & Theerakarunwong, 2020). Chlorophyll content in alfalfa leaves appeared to have its highest reduction at 100 ppm for undoped NMs. At the same time, other doped NMs treatments also showed a reduction, although it was less, compared to undoped NMs. However, some results are not significantly different from control, like those observed in Mw-SG NMs, where the lowest concentration of undoped and Ag-doped appeared to increase chlorophyll. These results are similar to those obtained after treating wheat plants with ZnO and Ni:ZnO doped NMs, where undoped NMs caused a higher reduction than doped NMs (Doğaroğlu et al., 2021). A reduced concentration of chlorophyll caused by the presence of the NMs can lower the photosynthetic rate, which slows down growth (Szymańska et al., 2016). With the TiO_2 , the effect on the photosynthetic rate varies from the species investigated, in which some treatments significantly reduce the chlorophyll content. At the same time, others appear to increase it, although the data appear to be statistically the same as control, results indicating reduction or augmentation of photosynthetic pigments in plants treated with TiO_2 have also been determined (Daghan, 2018; Hu et al., 2020; Rafique et al., 2018; Raliya et al., 2015; Rizwan et al., 2019; Satti et al., 2021)

The possibility of TiO_2 translocated into higher organs could mean different adversities caused by the interaction of NMs with molecules and biochemical processes present in different organs.

Different affectations in each section depended on NMs' capability to move through plant tissues as observed with the metabolic assays. NMs with structural differences appeared to have different degrees of metabolic stress at each organ measured (Silva et al., 2017).

Another critical factor for the stress-inducing effect is the concentration of NMs that interact with the plant tissue. After treatment with three different concentrations, the results show no linear effect in increasing the metabolite content proportional to NMs' exposure concentration for both types of

synthesis. Even though, to our knowledge, there are no studies of the interaction of TiO₂ with alfalfa. Some studies of multiple metal oxide nanoparticles also showed no proportional relation of the stress factor with the exposure concentration of NMs. This behavior could be related to no linear response relationships like hormesis (Agathokleous et al., 2019).

Secondary metabolite analysis revealed different secondary metabolite and radical inhibition modification degrees at leaves, stems, and roots. Comparing the two synthesis methods, the results demonstrate that gallic acid content is mainly augmented in leaves treated with Mw-SG NMs. For stems, the same quantity of treatments significantly alters the gallic acid content. However, both differ in concentration and type of NMs that induce the observed effect. Compared with leaves, an opposite effect in gallic acid augmentation in roots is observed where SG NMs had more effective treatments than Mw-SG. Rutin content in leaves and stem were not statistically significant for SG treatment compared with Mw-SG, where some concentration significantly increased the rutin content. The opposite effect was observed in rutin root content, where a higher number of treatments increases its content in SG-treated plants compared with Mw-SG. An overall analysis reveals that radical inhibition was mainly augmented in the three organs of plants treated with Mw-SG NMs.

Increased secondary metabolite content could be mainly due to reactive oxygen species (ROS), one of the principal mechanisms described for NMs plant interaction and stress induction (Marslin et al., 2017). The excess of these oxidating molecules affects the vegetable cell disrupting the membrane and unbalancing the plant cellular development affecting molecules like proteins, DNA, and lipids (Czarnocka & Karpiński, 2018), including micronutrient uptake, which can be related to the deficiencies observed in leaves, quantification of micronutrient content in the plant is needed to assure an imbalance caused by the NMs treatment. For mitigating the effect caused by the ROS, the plant synthesizes metabolites that can scavenge the molecules reducing the stress factor caused by the cellular damage, increasing its radical inhibition capability as observed in results.

Where the treatments showed an augmentation in radical inhibition, which is related to an increase in the production of antioxidant compounds which can be found among the phenols and flavonoids classification (Ahmed et al., 2017; H. Huang et al., 2019), although some treatments appear to increase IHB% while having no significant augmentation of specific metabolites the effect could be due to other antioxidant structures from secondary metabolism that are not measured with the techniques used, such as saponins (Rafińska et al., 2017).

No significant increase or reduction of the metabolite content present in plants is related to multiple effects. Nanoparticle characteristics play a crucial role in defining toxicity and stress-inducing factor (Zia-ur-Rehman et al., 2018). However, plant development and biologic characteristics also define the effects observed in the secondary metabolite content, plant tissue characteristics, development stage, and exposure time to NMs to define the stress-induced factor (Lv et al., 2019). NMs can affect germination, floration, or growing phases of the plant in different ways. No stress or toxic effects observed at specific exposure to NMs imply that the plant can subsist to different NMs concentrations and different exposure

times. Plants with long-term exposure to the NMs can adapt to the stress effects generated by the same NMs (ASLI & NEUMANN, 2009; Marslin et al., 2017). As shown in this research, several concentrations did not significantly affect the metabolite content modification. However, it does not imply that alfalfa crops can resist the effect at higher exposure times or NPs with different morphological characteristics. Lower and higher concentrations and the comparisons of metabolite content in different development stages can bring down more insight into NMs plant interaction and secondary metabolism modification.

Conclusions

It can be showed that alfalfa treated with TiO_2 NMs caused a stress effect on alfalfa crops which resulted in lower growth rate and a higher metabolic content as well as some affectations in chlorophyll content and micronutrient uptake, the results suggest that the degree of stress depends upon on concentrations and doping of TiO_2 NMs, the fact that NMs generated high concentrations of phenols and flavonoids could significate in an effective mechanism of generating alfalfa crops with higher nutritional content than traditional crops but further assays for determine NMs uptake by roots and translocation are suggested to differentiate NMs size with stress factors as well as safety concerns as well of investigating a higher spectrum of concentrations for achieving an ideal point for secondary metabolite production improvement including a deeper understatement of the toxicological model involved in the NPs-alfalfa interaction, associating it with hormesis curves comprehending the stress generated at low and higher doses which may help in the understanding of other toxicological behavior of photocatalytic materials like TiO_2 .

The NMs of both synthesis methods reduce the chlorophyll content and stem size of treated plants. The presence of dopants was a factor to generate differences in the observed effects. The morphological changes generate differences in the metabolite content. The increase of specific metabolites and antioxidant capacity in leaves, stems, and roots was marked by the physicochemical characteristics of both synthesis methods and the presence of dopants.

Specific and complete structural and chemical characterization of NMs is essential for relating the effects observed when interacting with living beings. It is essential to observe that synthesizing methods for obtaining NMs with different structural and chemical characteristics will help understand nanomaterials' toxic effects. Also, more insight toward exposure time, concentration, and dopant content could help to increase secondary metabolite production. Furthermore, without compromising plant health and NMs overuse, based on the results obtained, lower concentrations of titania could help as well to increase chlorophyll content in leaves increasing its photosynthetic activity.

Declarations

6.1 Ethical Approval

Not Applicable

6.2 Consent to Participate

All authors were agreed to participate in this research.

6.3 Consent to Publish

All authors have read and agreed to the published version of the manuscript.

6.4 Competing Interests

Not Applicable

6.5 Availability of data and materials

If it is needed can be requested to the corresponding author (Karen Esquivel).

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6.7 Author Contributions

Conceptualization: Karen Esquivel; Methodology: Karen Esquivel, Luis Páramo, Ana Angélica Feregrino; Formal analysis and investigation: Karen Esquivel, Luis Páramo, Ana Angélica Feregrino, Ramón Guevara, Marina Vega, Luis Escobar-Alarcón; Writing - original draft preparation: Karen Esquivel, Luis Páramo; Writing - review and editing: Karen Esquivel, Ana Angélica Feregrino, Ramón Guevara, Marina Vega, Luis Escobar-Alarcón; Funding acquisition: Karen Esquivel, Ana Angélica Feregrino; Resources: Karen Esquivel, Ana Angélica Feregrino, Ramón Guevara, Marina Vega, Luis Escobar-Alarcón; Supervision: Karen Esquivel, Ana Angélica Feregrino. All authors have read and agreed to the published version of the manuscript.

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Figures

Figure 1

SEM images (5000X) of the SG and Mw-SG synthesized materials, (a) TiO₂, (b) Fe-TiO₂, (c) Cu-TiO₂, (d) Ag-TiO₂.

Figure 2

Elemental analysis of the (a) SG-TiO₂ and elemental mappings of (b) Cu, (c) Fe, and (d) Ag-doped TiO₂.

Figure 3

X-ray diffraction patterns of (a) SG synthesized and (b) Mw-SG synthesized materials.

Figure 4

Raman spectra of (a) SG synthesized and (b) Mw-SG synthesized materials.

Figure 5

(a) Healthy leaves, (b) boron-deficient leaves, (c) nitrogen-deficient leaves, and (d) potassium-deficient leaves.

Figure 6

(a) Central stem length and (b) secondary stem length (c) leaf length of plants treated with SG synthesized NMs for 80 days.

Figure 7

(a) Central stem length and (b) secondary stem length (c) leaf length of plants treated with Mw-SG synthesized NMs for 80 days.

Figure 8

Chlorophyll concentration measured in SPAD units for alfalfa treated with (a)SG and (b) Mw-SG synthesized materials.

Supplementary Files

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