EGYPTIAN JOURNAL OF BOTANY (EJBO)

Print ISSN: 0375-9237
Online ISSN: 2357-0350

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PUBLISHED BY
THE EGYPTIAN
BOTANICAL SOCIETY

Nano carbon application for pineapple (*Ananas comosus* L.) micropropagation, secondary metabolites production and potential scavenging activity

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Carbon nanotubes application attracts researcher's attention due to benefits occurred. The safest way to benefit from nanotechnology's advantages and stay away from most of its drawbacks is to use nanomaterials in controlled in-vitro experiments. This study describes the effect of carbon nanotubes (CNTs) on regeneration of pineapple cultures, its active ingredients and antioxidant activities. Carbon nanotubes were used at 0.0, 0.2, 0.02 and 0.002 g/l to study their effect on pineapple multiplication and rooting stages. Results showed that CNTs treated cultures showed a higher shoot multiplication rate as its concentration at 0.2 g/l gave the highest shoot number followed by 0.02 and 0.002 g/l compared with the control treatment (0.0 g/l). It is worth mentioning that all plantlet parameters, in rooting stage, showed significant higher results with CNTs compared with the control. As for the chemical composition analysis, the positive effect of CNTs was reversed by increasing the application rate. It is assumed that the low level of carbon nanotubes used has a role as an antioxidant due to their radical capacity. These results clearly encourage the application of carbon nanotubes with low concentration as a potent natural candidate for antioxidation.

Keywords: antioxidants, micropropagation, carbon nanotubes, pineapple, phenolics

ARTICLE HISTORY
Submitted: December 14, 2023
Accepted: October 01, 2024

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DOI: 10.21608/ejbo.2024.255539.2612

EDITED BY: R. Hafez

INTRODUCTION

Pineapple (*Ananas comosus* L.) is a globally important fruit crop (FAO, 2017). It is a monocot plant that belongs to the family Bromeliaceae. It can be a rich supply of vitamin A, B, and C plus minerals like calcium, phosphorous, and iron. The bioactivity of this fruit is because of excellent compounds like polyphenols and ascorbic acid (Hossain and Rahman, 2011). Because of its rapid production, contribution to job creation and trade promotion, and position as a component of a healthy diet, pineapple is one of the tropical fruits that is mainly cultivated in developing countries (FAO, 2022). Its advantageous properties include increasing collagen synthesis, promoting B, T lymphocyte development, eradicating infections and aiding in the manufacture of antibodies, participating in vasodilation, and shielding DNA from oxidative damage (Farzana et al., 2022). Also, pineapple leaf fibers and their mixed textiles may be utilized to make ornamental fabrics, allowing them to be employed in a variety of apparel and textile industries (Jalil et al., 2022).

To produce large number of pineapple plants, micropropagation is an important tool for that. Many micropropagation techniques could be used (direct or indirect) for providing numerous numbers of plants (Hassan et al., 2021a). Many types of explants can serve as plant source for its initiation; shoot tip, node, inflorescence, etc. (Taha et al., 2021). Various problems affect the production of plants with tissue culture technique; browning, contamination, low growth, slow response and more (Hassan et al., 2021b).

Nanotechnology is a promising technology that has the potential to boost agricultural output (Azeez, et al., 2021; Hassan et al., 2022; El-Khouly et al., 2024; Soliman et al., 2024). However, further studies are required to investigate its impact on the plant, as well as to assure the safety and eliminate the health concerns linked with this technique (Abou-Baker et al., 2020). Due to their appealing physiochemical characteristics, such as their small size, high surface area, and superior mechanical and thermal strength, carbon nanotubes (CNTs) are one of the most promising carbon-based nanomaterials, providing better opportunities for applications in the agriculture sector (Safdar et al., 2022). Although studying CNTs-influence on plants has been conducted but assuring if it is helpful or harmful to the plant has not been fully assessed yet. It has been confirmed in some plant species that CNTs are safe at low concentrations and are capable to enhance plant growth and encouraging nitrogen metabolism and photosynthesis (Klaine et al., 2008).

The present study focuses on the role of carbon nanotubes in plant production with micropropagation process and their effect on the

content of phenolics, flavonoids and antioxidant activities in pineapple tissues.

MATERIALS AND METHODS

This study was conducted in the Tissue Culture Technique Lab of the Central Laboratories Network, National Research Center (NRC). Analysis was conducted at the Biochemistry Department, and the Soils and Water Use Department of the Agricultural and Biological Research Institute of the NRC in Giza, Egypt.

Plant collection and explants preparation

Ananas comosus L. "Queen" shoots tips were possessed from the crown of mature fruits that were 20 to 25 cm in length. The tips comprised the apical meristem and 2-4 primordial leaves were cleaned for 30 minutes under running water. The isolated shoot tip explants were immersed for 20 minutes in 15% Clorox with one drop of Tween 20 under aseptic conditions (Taha et al., 2019).

Shoot initiation and multiplication

MS media (Murashige and Skoog, 1962) improved with 2.0 mg/l BA + 0.1 mg/l NAA + 30 g/l sucrose + 0.4 mg/l thiamine-HCl + 6 g/l agar was used for this stage. Each culture jar (325 ml) containing 50 ml of the prepared media was right away sealed with polypropylene closures before being autoclaved for 20 minutes at 121°C and 15 Ibs/in^2 (Taha, 2022). Sterilized shoot-tips were grown for one month in this media. All of the explants that had survived were moved and recultured on the same medium for multiplication. Thereafter, shoots produced *in vitro* were used in this investigation after three months of culture.

Carbon nanotubes experiment

Multiwalled carbon nanotubes (CNTs), ALDRICH®, were initially studied using a transmission electron microscope (TEM) (Figure 1). The diameters and lengths of its particles range between 110 and 170 nm and 5 and 9 µm, respectively. Before the media were autoclaved, CNTs were added at concentrations of 0.0, 0.2, 0.02 and 0.002 g/l. The multiplication and rooting stages were examined. MS medium supplemented with 2.0 mg/l BA + 0.1 mg/l NAA + 30 g/l sucrose + 0.4 mg/l thiamine-HCl + 6 g/l agar was used for multiplication stage. Otherwise, ¾ MS medium supplemented with 1.0 mg/l IBA was used for rooting stage. In the multiplication stage, leaf number, shoot length, and shoot number were recorded. In addition, some measurements for rooted plantlets (plantlet lengths, leaf counts, and stem thicknesses) were determined.

Adaptation of Pineapple Plantlets

After rooting, explants were transplanted to a greenhouse, washed in tap water to eliminate all traces of agar, soaked in a systemic fungicide at 1.0 g/l, and then cultivated in pots made of black plastic filled with a 1:1 (v/v) mixture of peat moss and vermicompost (Hassan et al., 2022). Transparent plastic bags were placed over the plantlets to keep the humidity between 80 and 90 percent. After 7 to 10 days, plastic bags were gradually removed, and after 14 days, they were completely grown. Plants were frequently fertilized and irrigated as needed using tap water.

Biochemical study

Butyl Hydroxy toluene (BHT) and, potassium ferricyanide ($K_3[Fe(CN)_6]$); DPPH· (2, 2`-diphenyl -1-picrylhydrazyl); Ferrozine: (3- (2 - pyridyl) - 5, 6- bis- (4-phenylsulfonic acid)-1, 2, 4-triazine; Folin-Ciocalteau reagents; Gallic acid; quercetin were all obtained from Sigma Chemical Co. in St. Louis, Missouri, USA. The tissues of the pineapple shoot were extracted. One hundred ml of 80% methanol was added to 10 g of dry powder, and the mixture was shaken at room temperature for 48 hours. Filter paper (Whatman No.1) was used to purify the extract. Methanolic extracts were utilized for further antioxidants and phytochemical analyses. Folin Ciocalteu reagent assay was used to assess the total phenolic (TP) content (Singleton and Rossi, 1965). The content of total flavonoids (TF) was determined using the aluminum chloride technique according to Zhishen et al. (1999). The Folin-Ciocalteu reagent assay was used to determine the total tannins (TT) of several pineapple extracts as described by Polshettiwar et al. (2007).

Antioxidant activity

To evaluate the DPPH• radical scavenging activity of pineapple extracts, the procedure reported by Chu et al. (2000) was utilized. The following equation was used to calculate the capacity to scavenge the DPPH• radical: DPPH• scavenging effect (Inhibition %) = [(the absorbance of control (Ac) – the absorbance in presence of plant extract (As))/ Ac] X 100. The results were shown as IC_{50} (the concentration of plant extract (mg/ml) that scavenge 50 percent of DPPH• radical).

Iron chelating effects were determined according to Hsu et al. (2003). The same equation was utilized to calculate the ferrous ion chelating activity (Inhibition percentage), where: A_s and A_c were the absorbance in the presence of the plant extracts, and in the control reaction, respectively. The results were presented as IC_{50} (the concentration of plant extract (µg/ml) that chelate 50 percent of ferrous ion). According to the method that was described by Kuda et al. (2005), the reducing power was determined. The records were presented as EC_{50} (the concentration of plant extract (mg/ml) that provided the reading of 0.5 absorbance at 700 nm).

Minerals analysis

Pineapple samples were cleaned and dried at 65°C in an electric oven. The percentages of water in cluster tissues were calculated as mentioned by Romero-Aranda et al. (2006). Dried samples were digested by using sulphuric and perchloric acids mixture. The concentration (%) and uptake (mg/cluster) of N, P, K, Ca, Mg and Na were recorded according to Cottenie et al. (1982). Also, some mineral percentages (Na/K, Na/Ca, Ca/(Na+K) and K/Ca) were calculated.

Statistical analysis

All samples were replicated three times in a completely randomize design. The CoStat statistical package was used to analyze the data (Anonymous, 1989).

RESULTS

Effect of CNTs treatment on pineapple multiplication stage

Data in Table 1 and Figure 2 show that all used CNTs concentrations significantly increased multiplication rate. CNTs at 0.2 g/l gave the highest shoot number followed by 0.02 and 0.002 g/l, compared with the control. In addition, 0.02 g/l gave the highest shoot length followed by 0.2 g/l and the latest result was not significant with the control. Meanwhile, leaf number showed a decrease with CNTs treatments compared with the control.

Effect of CNTs treatment on pineapple rooting stage

Data in Table 2 and Figure 3 show that all CNTs treatments showed a significant increase in parameters of rooting stage of pineapple cultures. Plantlet length, leaf number, plant thickness, root number and root length are significantly higher compared with the control. All CNTs treatments gave the highest plantlet length compared with the control. Generally, CNTs at 0.02 g/L showed the highest plantlet length, leaf number, plant thickness, root number and root length compared with other treatments. Meanwhile, the lowest parameters were achieved with the control treatment.

Data presented in Figure 4 showed that CNTs treatments led to increased fresh and dry weight compared with the control. The highest weight resulted in the addition of 0.02 g/l CNTs which increased fresh and dry weight by 43.0 and 38.1% compared with the control, respectively. Increasing/decreasing of CNTs application dose reduced both fresh and dry weight compared with the mediated dose (0.02 g/l) but still higher than the control. The water content % of pineapple cluster tissues took the opposite line of weight where plant clusters treated with 0.2 g/l CNTs had the highest value of water content while the lowest record was obtained by the control.

Total phenolic (TPC), total flavonoids (TFC), and total tannins (TTC) content

The levels of phenolic compounds in 80% methanol pineapple extract are shown in Table (3). Tissues of pineapple treated with CNTs exhibited lower content of total phenolic, total flavonoids and total tannins compared with the control. The level of 0.002 and 0.02 g/l CNTs exhibited lower content of total phenolic, total flavonoids, and total tannins compared with 0.2 g/l CNTs.

Antioxidant activities

The antioxidant activity using $DPPH^{\cdot}$, Fe^{+2} chelating expressed as IC_{50} and reduced power activity expressed as EC_{50} of pineapple 80% methanol extract were shown in Table 4. Regarding $DPPH^{\cdot}$ assay, the antioxidant capacity (IC_{50}) is ranged from 100.71 – 139.45 µg/ml. The lowest IC_{50} means the highest antioxidant capacity. As shown in Table 4 the scavenging activity of pineapple tissues treated with CNTs at 0.002 mg/l showed the highest value (100.71 µg/ml), while the lowest activity (139.45 µg/ml) was shown at 0.2 mg/l.

In the present study, Fe^{+2} chelating ability as IC_{50} in pineapple 80% methanol extract ranged from 114.44 to 177.29 µg/ml. All the treatments of nano carbon demonstrated considerable ability to chelate metal ions (Fe^{+2}). Meanwhile, untreated pineapple extract had the highest Fe^{+2} chelating capacity (114.44 µg/ml), while 0.2 mg/l CNTs treatment had the lowest ones (177.29 µg/ml).

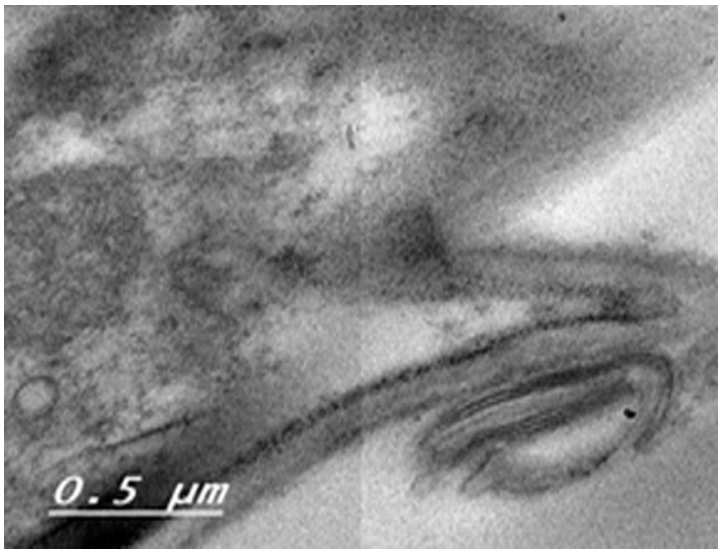

Figure 1. Transmission electron microscope image of carbon nanotubes shows its absorption in plant cell.

Table 1. Impact of carbon nanotubes (CNTs) on pineapple shoots' multiplication *in vitro*

CNTs (g/l)	Shoot No.	Shoot length (cm)	Leaf No.
0.000	17.5c	1.30b	5.94a
0.002	46.5b	1.00c	4.43b
0.020	46.5b	1.75a	4.52b
0.200	47.0a	1.37b	3.36c

Means with dissimilar letters are significantly different at *P* 0.01.

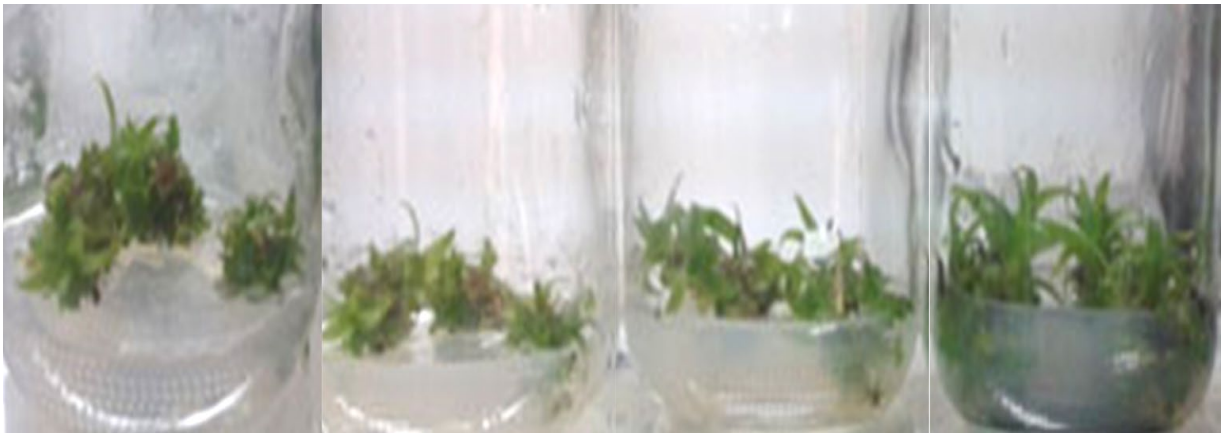

Figure 2. Multiplication of pineapple shoots with CNTs treatments from left to right: 0.0, 0.002, 0.02 and 0.2 g/L.

Table 2. Effect of carbon nanotubes (CNTs) on rooting of pineapple shoots in vitro

CNTs (g/l)	Plantlet length (cm)	Leaf number	Thickness (mm)	Root number	Root length (cm)
0.000	5.22b	8.22c	1.50c	4.33c	4.53c
0.002	12.23a	11.17b	3.00b	7.33b	5.32b
0.02	12.33a	11.50a	3.83a	7.83a	5.77a
0.2	12.13a	11.33ab	3.00b	7.17b	5.02b

Means with dissimilar letters are significantly different at *P* 0.01

Figure 3. Rooting of pineapple shoots with CNTs treatments from left to right: 0.0 and 0.02 g/L, notice the thickness of the stem with 0.02 g/L CNTs.

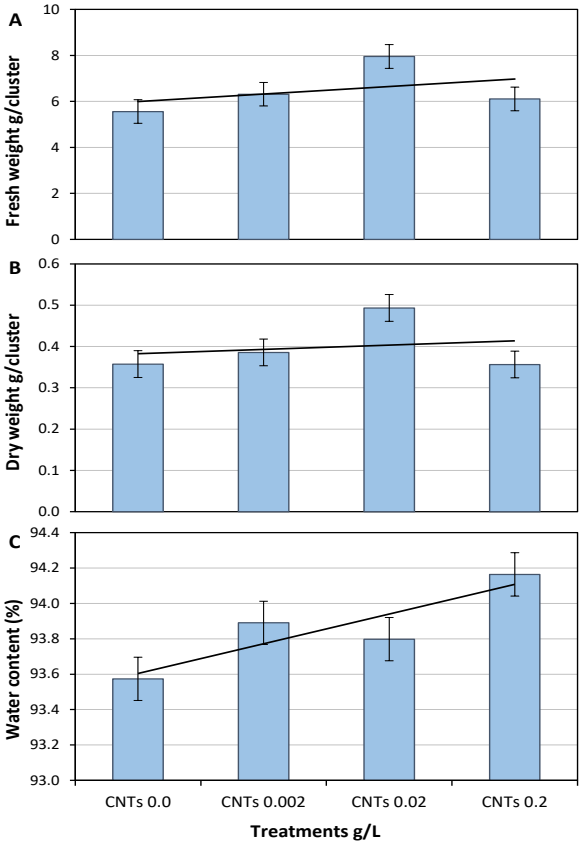

Figure 4. Effect of carbon nanotubes rates on fresh (A), dry weight (B) of pineapple (g/cluster) and water content (C) in tissues.

Table 3. Effect of carbon nanotubes (CNTs) on total phenolic (TPC), total flavonoids (TFC) and total tannins (TTC) content of pineapple

CNTs (g/l)	TPC mg GAE /g DW	TFC mg QE /g DW	TTC mg TE /g DW
0.000	20.38^{a}	14.19^{a}	5.03^{a}
0.002	17.24^{c}	11.64^{d}	4.65^{b}
0.02	17.03^{c}	12.79^{c}	3.39^{d}
0.2	18.66^{b}	13.14^{b}	3.90^{c}

The means of three replicates are significantly different at 1%, gallic acid equivalents (GAE), quercetin equivalents QE, Tannic equivalents (TE).

Table 4. Effect of carbon nanotubes (CNTs) on scavenging activities by DPPH$^{\cdot}$, Fe^{2+}-chelating and reducing power activities of pineapple

CNTs (g/l)	DPPH$^{\cdot}$ IC_{50} µg/ml	Fe $^{2+}$- chelating IC_{50} µg/ml	Reducing power EC_{50} µg/ml
0.000	112.06^{c}	114.44^{d}	111.75^{d}
0.002	100.71^{d}	129.07^{c}	155.75^{c}
0.02	128.74^{b}	163.36^{b}	188.45^{b}
0.2	139.45^{a}	177.29^{a}	278.44^{a}
Standard	29.64^{e}	23.12^{e}	11.01^{e}

The means of three replicates are significantly different at 1%.

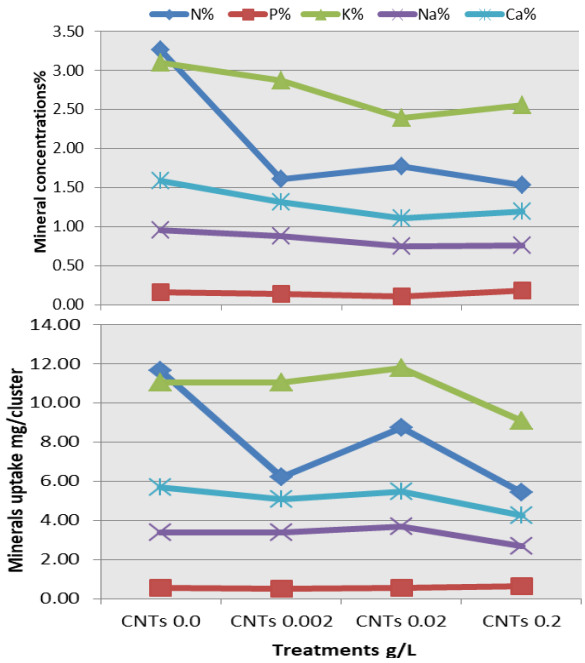

Figure 5. Minerals concentrations (%) and content (mg/cluster) of pineapple as affected by carbon nanotubes rates.

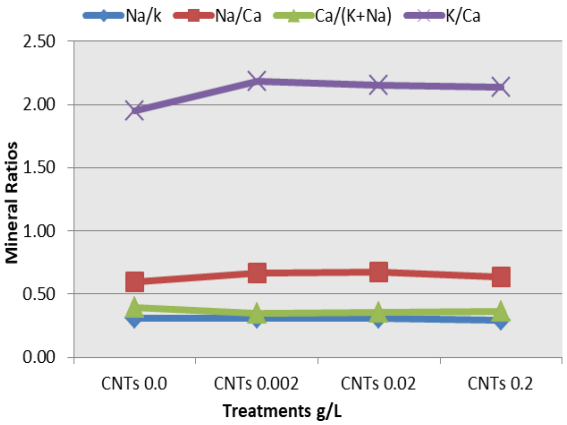

Figure 6. Some elements ratios in pineapple tissues as affected by carbon nanotubes rates.

It was mentioned that samples included a reductant showed a reduction of the ferric (Fe^{3+}) to ferrous ion (Fe^{2+}). The reducing power activity of a compound could be considered an indicator of its potential antioxidant activity. The reducing power activity as EC_{50} of pineapple extracts ranged between 111.75 and 278.44 µg/ml. Results showed that the fewer CNTs concentrations used the more antioxidant capacity occurred as CNTs at 0.2 µg/l exhibited the minimum antioxidant capacity (EC_{50} = 278.44 µg/ml) while the lower concentration exhibited 155.75 µg/ml (Table 4).

Minerals analysis

Data presented in Figure 5 showed that the highest mineral concentration values were observed in untreated cluster tissues (the control). Thus, the advantage of increasing weight by adding CNTs at 0.02 g/l was interpreted by increasing water absorption and did not induce mineral acquisition. As for mineral content, the highest value of nitrogen and calcium was shown by the control, while the highest potassium and sodium were observed in CNTs at 0.02 g/l. Data presented in Figure 6 showed that no significant difference between treatments in their effect on element ratios. Few differences were observed in K/Ca ratio that increased with increasing CNTs rate.

DISCUSSION

All used CNTs concentrations significantly increased multiplication rate. The latest values of shoot number and shoot length resulted from the control. Generally, CNTs at 0.02 g/l showed the highest plantlet length, leaf number, plant thickness, root number and root length compared with other treatments. Meanwhile, the lowest parameters were achieved with the control treatment. Similarly, Taha *et al*. (2016) found that CNTs increased the number of date palm germinated embryos, plantlet length and leaves number. In addition, Gaafar et al. (2018) assured that jojoba cultures treated with CNTs exhibited a more multiplication rate than the control. CNTs was found to be a prospective conductive substrate for cell cultivation (Chetyrkina et al., 2022).

Polyphenols, flavonoids and tannins are antioxidant constituents that provide some important biological and pharmacological roles (Rudrapal et al., 2022). The level of 0.002 and 0.02 g/l CNTs exhibited lower content of total phenolic, total flavonoids, and total tannins compared with 0.2 g/l CNTs.

In the presence of a hydrogen donor, the $DPPH^{\cdot}$ assay was based on the reduction of the stable radical $DPPH^{\cdot}$ to yellow-colored diphenylpicryl hydrazyl (Gerasimova et al., 2022). The lowest IC_{50} means the highest antioxidant capacity. In this concern, the increased level of antioxidant activity in tissues treated with nano carbon might be due to oxidative stress at low concentration of CNTs (0.002), and this increase of activity may be due to bioactive compounds that help the plant to resist that stress (Lester, 2008; Callohuanca-Pariapaza et al., 2021). Also, the presence of total phenolic compounds might contribute to antioxidant activity (Rahim et al., 2022).

All the treatments of nano carbon demonstrated considerable ability to chelate metal ions (Fe^{+2}). It was mentioned that samples included a reductant showed a reduction of the ferric (Fe^{3+}) to ferrous ion (Fe^{2+}) as mentioned by Gordon *et al*. (2012). However, Oyaizu (1986) claimed that nanoparticles at different concentrations did not show a stable trend for antioxidant activity. The reducing power activity as EC_{50} of pineapple extracts ranged between 111.75 and 278.44 µg/ml. Woo et al. (2021) assured that CNTs have a high reducing power at the early stages of exposure but a low reducing power later.

Our result conforms with the finding of Taha et al. (2016) on date palm. However, the response of plant tissue to nanomaterial application could differ between plants species as described by Begum et al. (2014) where they have shown that carbon nanotubes are toxic to plants, and the potential effects of exposure remain differ from one plant to another. Lettuce and red spinach were most sensitive to carbon nanotubes application followed by cucumber and rice. No toxic impacts were recorded for chili pepper and soybean. In close agreement with our present study, the growth of maize seedlings was enhanced by low concentrations of nano carbon but depressed by the higher concentration (Begum et al., 2014).

CONCLUSION

The controlled usage of nanomaterials in the tissue culture process before plant adaptation may be the solution to the unsafe use of some nanotechnology applications in agriculture. Carbon nanotubes enhanced the growth and production of pineapple plants grown in vitro. Due to the reverse effect of nano-carbon with increasing the application rate,

further studies on its impact on the plant and users' health are required.

AVAILABILITY OF DATA AND MATERIAL

All the data is presented in the manuscript.

COMPETING INTERESTS

The authors have no competing interests.

FUNDING

The authors affirm that they did not receive any fund or other support.

AUTHORS' CONTRIBUTIONS

Dr. Rania A. Taha performed the tissue culture experiments and wrote the manuscript. While Dr. Alaa A. Gaafar conducted the physiological analysis and applied analysis. Dr. Abou-Baker N.H. was responsible for determining the nutritional status of pineapple. Dr. Nagwa Zaied and Dr. Esam Shaaban reviewed the manuscript. All authors approved the work and final manuscript status.

LIST OF ABBREVIATIONS

CNTs: carbon nanotubes
DNA: deoxyribonucleic acid
TEM: transmission electron microscope
TP: total phenolic
GAE: gallic acid equivalents
TF: total flavonoid
QE: quercetin equivalents
TT: total tannins
TE: tannic acid equivalent
DPPH•: 2, 2-diphenyl-1-picryl hydrazyl
EDTA: ethylenediaminetetraacetic acid
N: nitrogen
P: phosphorus
K: potassium
Ca: calcium
Mg: magnesium
Na: sodium

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