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Green Synthesized Chitosan Nanoparticles for Strawberry Micropropagation Protocol

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ABSTRACT
The study was conducted to confirm the possibility of obtaining Nano chitosan using green-synthesis technology using aqueous extract of palm fronds and to test adding nano chitosan produced at concentrations of (1.5, 1, 0.5, 0 mg L-1) to the nutrient medium to improve the propagation of strawberry (Fragaria X ananassa) Strawberry Ruby Gem variety. The results of the study showed the possibility of producing nano chitosan particles using green date palm (Zahdi variety) frond extract. The scanning electron microscope (SEM) results showed the formation of spherical shapes with nano-diameters ranging in size from 49.38 to 35.36 and 26.54 nm at an average of 37.08. This was consistent with the results of the UV-Vis spectroscopy, which showed the appearance of the highest peak of chitosan nanoparticles at a wavelength of 226 nm. The X-ray diffraction study of the produced nano-chitosan showed the absence of clear peaks, confirming that the nano-chitosan is of an amorphous nature. The strawberry micropropagation program was evaluated by adding different concentrations of manufactured nano-chitosan to the strawberry propagation medium at its different stages (sprouting, multiplication and rooting). It was found that the produced nano-chitosan showed a significant effect in reducing the percentage of contamination in the plants and increasing the response rate and the number and length of the branches formed when added at the branch emergence stage. At an advanced stage, adding chitosan to the culture medium led to improving the characteristics of Vegetative growth in number of branches, lengths, number of leaves, fresh and dry weight were significantly different from those obtained from the standard medium. It was also observed that the manufactured nano chitosan recorded the highest rooting percentage and the highest rate of number and length of roots when it was added to the rooting medium.

Keywords: Strawberry, Micropropagation, Nanoparticle Chitosan. Green synthesis.

INTRODUCTION

Duch Fragaria X ananassa (strawberry) belongs to the Rosales order and the Rosaceae plant family. Strawberries are propagated in several ways, mostly by dividing the crown, by runners, and or by tissue culture method. The latter considered to be the most beneficial as one of its advantages is the production of large numbers from a small plant part (apical meristem) in a short time without being restricted by the propagation date (Dutta and Sen, 2019).

In the field of plant tissue culture, nanoparticles are of particular interest as they can be added to the culture media as more soluble nutrients and from diffusible sources to increase the growth of ex vivo transplanted parts. The smaller size and higher surface area of nanoparticles can affect the solubility, diffusion and availability compared to conventional nutrients (Singh and Ahamad, 2022). Therefore, nanoparticles have been employed in plant tissue culture applications such as in vitro seed germination, improved plant growth, elimination of microbial contaminants in plant parts, callus induction, plant organ formation, somatic embryogenesis, plant micropropagation, genetic transformation and secondary metabolite production (Feizi, 2023).

Despite the diversity of physical and chemical methods for manufacturing nanoparticles, some of them are described as unsafe from a medical and environmental point of view. Therefore, the use of biological sources in nanotechnology has become very important because they are simple, with an increasing success rate, and their diversity (bacteria, fungi, yeasts, plant extracts, algae and viruses). However, the use of plants has caught the attention of researchers as a fast, low-cost and environmentally friendly means with very few steps in the process of greening synthesis.

Abogarr et al. (2022) tested the efficiency of nanoparticles (chitosan, zinc, copper and silver nitrate) in overcoming the problem of contamination in tissue cultures of date palm Phoenix dactylifera L. where different

concentrations of nanoparticles were used (5 and 15 mg. L-1 chitosan, 4 and 7 mg L-1 silver and zinc nitrate, 0.5 and 1 mg L-1 copper) where no contamination was recorded when using nano chitosan at both concentrations. Rohim et al. (2022) indicated the effect of adding nano chitosan and silver to the nutrient medium on sterilizing immature flowers of male date palm seedlings, where the concentrations (150 and 200 mg L-1 nano chitosan) recorded the highest percentage of plant survival (72, 76%) respectively with the lowest percentage of contamination not exceeding (28, 24%) respectively. It was also found that treating the culture medium with nanoparticles (chitosan, silver and selenium) showed high efficiency in sterilization and multiplication of green branches of olive Olea europaea, effectively inhibiting pathogens, especially nano silver, which excelled in the multiplication stage with higher values for the length and number of branches, the number of leaves and the multiplication rate compared to chitosan and selenium nanoparticles (Darwesh et al. (2023).
Based on the above and due to the increasing importance of strawberry crop in Iraq day after day and the lack of studies on the effect of nano chitosan in the field of tissue propagation of plants, including strawberries in Iraq, the study aimed to produce nano chitosan using the green synthesis method from palm frond extract, and to test the nano product in the culture medium for tissue propagation of strawberries in the stages of initiation, multiplication and rooting.

MATERIALS AND METHODS

The study was carried out in the Palm Propagation Laboratory, Plant Tissue Culture, Department of Horticulture and Landscape Engineering - College of Agriculture, University of Kufa during the period from November 2022 to November 2024. As for preparation of nano chitosan using green synthesis, plant samples of date palm leaves (Zahdi cultivar) were prepared and washed to remove dust residues, then cut into small pieces. To prepare the aqueous extract, the modified method of Soro et al. (2009) was followed. 2 g of chopped palm fronds were added to 20 ml of distilled water and stirred by magnetic stirrer at 80 0 C for 30 minutes. Then left at room temperature for 24 hours, after which the mixture was filtered using filter paper (Whatman No. 1) and the filtrate was used in the biosynthesis of nano chitosan.

Green synthesis of nano chitosan

The biosynthesis of nano chitosan was carried out according to Rasaee et al. (2016). 2% chitosan was mixed with 0.5% acetic acid, then the pH was adjusted to 5 using sodium hydroxide, placed under a magnetic thermomixer for 24 hours and the volume was completed to 200 ml of distilled water. Then 10 ml of palm leaf extract was added to 40 ml of nano chitosan solution to form the reaction mixture under a magnetic mixer at 60°C and 110 rpm. Then the resulting particles were separated from the solution by drying.

Detection and Characterization of Nanochitus Nano

The resulting particles were characterized using Ultraviolet-visible spectroscopy (UV-vis), then using X-ray diffraction (XRD) and Fourier Transform Infraredspectroscopy (FTIR). The product was also characterized by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM).

Preparation of the culture medium

The ready-made culture medium (Murashige and Skoog Medium MS) produced by the Indian company Himedia was used. 4.43 g of the ready-made culture medium was dissolved according to the manufacturer's recommendations in 600 ml of distilled water free of ions with the addition of growth regulators in different concentrations, prepared in advance as stock solutions (stored at 4 °C) with the addition of sucrose 30 g. L^{-1}, then the volume was completed to 1 liter.

The pH of the medium was adjusted to 5.7 ± 0.1 with a 1-norm solution of hydrochloric acid (HCl) or a 1-norm solution of sodium hydroxide (NaOH), and 7 g of agar (type Agar-Agar) was added to it. The components of the medium were mixed and the agar was dissolved using a hot plate magnetic stirrer. The culture medium was then distributed into bottles that had been previously sterilized with a sterilizer at a rate of 40 ml for each vial. The vials were covered with plastic caps and the medium was sterilized with an autoclave for 20 minutes at a temperature of 121°C and a pressure of (1.04) kg. cm2. After the sterilization was completed, it was stored in a clean place until use.

Effect of Nano-**chitosan in the development stage**

The growing tip of strawberry variety Ruby Jim was used in the development stage of plants. The growing tip of strawberries was taken, the root and vegetative system were removed, and the growing tip was separated to a length of 2 cm. It was cleaned with running water and then treated with 0.5% of the pesticide botanol for 15 minutes under a magnetic mixer. Then it was sterilized with sodium hypochlorite solution at a concentration of 3.0% (volume/volume) for 10 minutes, after which the plant parts were washed with sterile distilled water three times each time for five minutes. The growing tip was transferred to the culture tubes by a sterile needle, and planted in MS medium containing 0.5 mg L^{-1} benzyl adenine in the presence of 0.1 mg L^{-1} IBA at a rate of 10 ml. Laboratory bottle with chitosan at concentrations (1.5, 1, 0.5, 0 mg L-1) 10 replicates for each concentration. The cultures were incubated at a temperature of 25 °C and a light intensity of 1000 lux and a daily light period of 16 hours of light and eight hours of darkness in the growth room for 4 weeks. After the end of the incubation

period, data were collected that included the percentage of contamination, the response rate (%), the number of branches formed, and the average length of the branches.

Effect of Nano-**chitosan on branches multiplication stage**

An experiment was conducted to show the effect of adding chitosan at concentrations (1.5, 1, 0.5, 0 mg L-1) to the multiplication medium (MS medium containing 1 mg L^{-1} benzo adenine in the presence of 0.1 mg L^{-1} IBA) with twenty replicates for each treatment and they were incubated in the growth chamber for 6 weeks under a light intensity of 1000 lux and 16 hours of light and eight hours of darkness, after which the average number of branches formed, the average length of branches, the average number of leaves, and the average fresh and dry weight of the vegetative group (g) were calculated.

Effect of Nano-**chitosan on branch rooting stage**

The branches resulting from the vegetative multiplication stage (from the best treatment) were transferred to MS medium to show the effect of adding four concentrations of traditional chitosan to the MS medium for rooting (1.5, 1, 0.5, 0 mg L^{-1}) and the plants were incubated in the growth chamber for 6 weeks where measurements included Rooting percentage, number of Roots and Root length (cm).

Experimental design and statistical analysis

All experiments were carried out in the stages of initiation, multiplication and rooting using simple experiments (CRD) with one factor (chitosan concentration) with three replicates for each treatment and one plant part for each replicate in the initiation and multiplication stages and rooting stage (Compton, 2018). The data were analyzed using 12th (VSN International GenStat 2009) oparated by Windows computer operating system, where Analysis of Variance was performed.

RESULTS AND DISCUSSION

Detection and Characterization of green-synthesis Chitosan Nanoparticles

The UV-visible spectroscopy results (Fig. 1) indicated the appearance of the highest peak of chitosan nanoparticles at the wavelength of 226nm. This result is consistent with what was found by Agarwal et al. (2018). The results of the study (X-Ray diffraction analysis of nano-chitosan showed the absence of clear peaks, which indicates the absence of long-range order in the structure, which means that nano-chitosan is of an amorphous nature. This characteristic increases the absorption of metal ions, and can be attributed to the bio-preparation process that leads to a change in the crystalline structure of the original chitosan. This result is consistent with (Qi et al., 2004; Vaezifar et al., 2013; Safari et al., 2015; Rasaee et al., 2016; Ali et al., 2018; Sathiyabama et al., 2024).

Fourier Transform Infrared Spectroscopy (FTIR)

In its results shown in Figure (3), it showed the presence of values in the chart in the form of peaks starting from the frequency 3419.79 cm-1 and ending at 410.84 cm-1 (Table 1).

Table 1. Values of frequencies affecting the active groups present in the nano chitosan sample

Peak No.	cm^{-1}	Chemical substance/active part-root
1	3419.79	(0-H) Phenols, carbohydrates, proteins
2	2918.30	C-H) and (C-H2) aliphatic group
3	2850.79	CH and CH2 aliphatic group
4	1624.06	C=C extension
5	1404.18	C=O inorganic carbons
6	1261.45	C–O–C polysaccharides
7	1224.80	C-C C(CH3)3 Alkane
8	1101.35	Aromatic compounds
9	1066.64	C–O–C polysaccharides
10	1022.27	C–O–C polysaccharides
11	929.69	C-C Alkane
12	792.74	C-H Benzenoid
13	721.38	C-H Benzenoid
14	613.36	Vinyl hydrocarbon compounds C-H
15	514.99	Aliphatic iodo compounds, C-I strehch
16	474.49	prim aliphatic amides C-C=O
17	451.34	prim aliphatic amides C-C=O
18	410.84	C-OH Phenol

The results of the precise characterization of the resulting chitosan particles based on (SEM) Scanning Electron Microscopy Figure (4) indicate the formation of spherical chitosan nanoparticles with nano diameters ranging in size from (49.38, 35.36, 26.54) nm and an average of 37.08, which is consistent with similar results in previous studies (Gamal et al. (2022). Similar results were obtained (TEM) Transmission Electron Microscopy Figure (5) for the study of the size and shape of chitosan nanoparticles, where spherical particles were observed. This result is consistent with the shape of the particles obtained by Thamilarasan et al. (2018). The studied particles recorded an average particle diameter ranging from 22.79nm to - 23.24nm. These results are consistent with the average diameter of chitosan nanoparticles indicated in the study of Hasaneen et al. (2014).

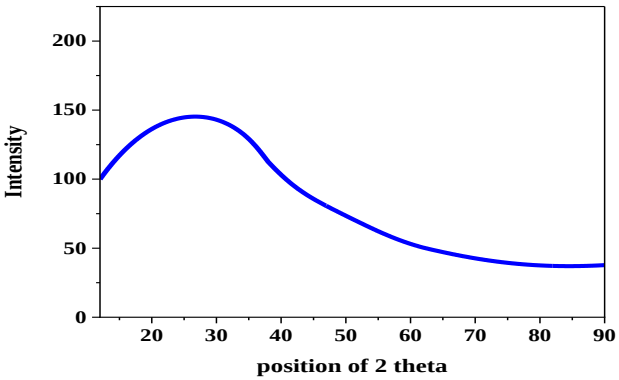

Figure 1. UV-Vis spectroscopy results of chitosan nanoparticles

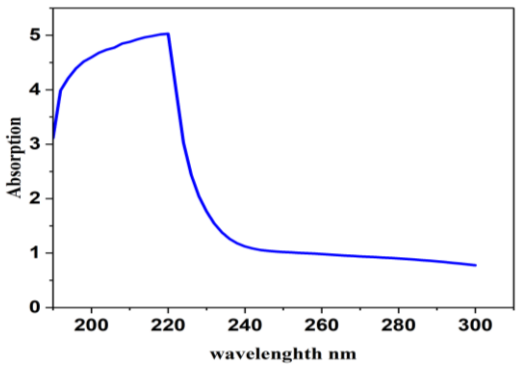

Figure 2. X-ray diagram of the manufactured nanochitosan

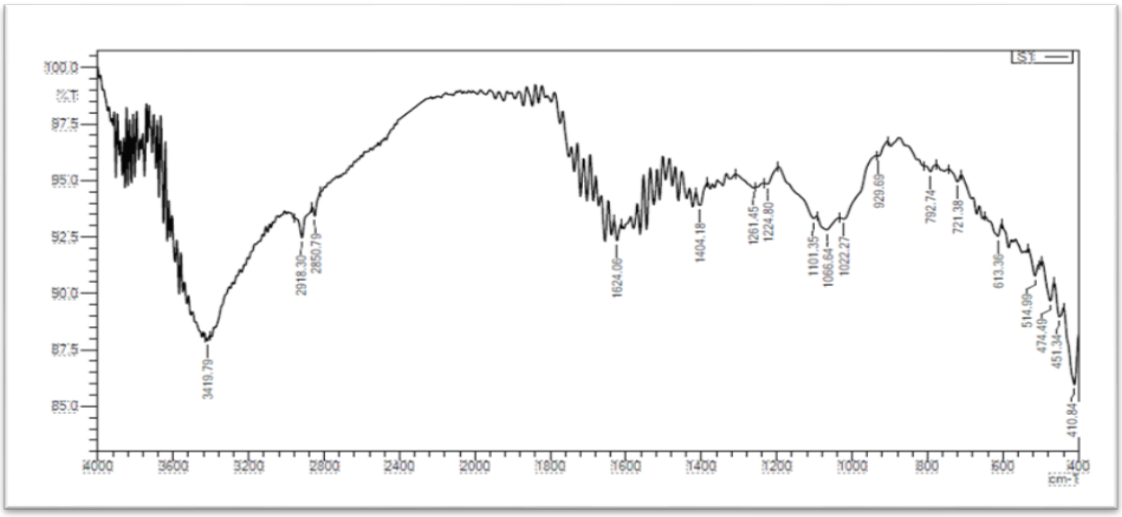

Figure 3. FTIR results of chitosan

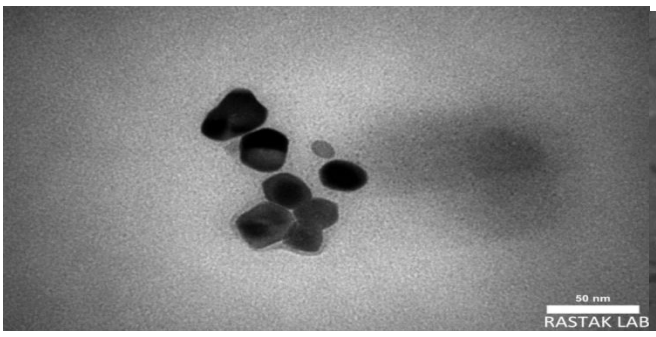

Figure 4. Chitosan nanoparticles under (scanning electron microscope (SEM

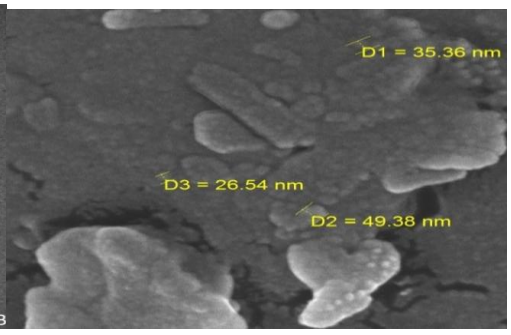

Figure 5. Chitosan nanoparticles under the transmission electron microscope

Effect of green synthesis nano-chitosan in the initiation stage of vegetative branch

The results shown in Figure (6) showed significant differences in the percentage of contamination, the percentage of response, and the number and length of branches formed as a result of adding nano-chitosan at different concentrations to the nutrient medium for plant growth.

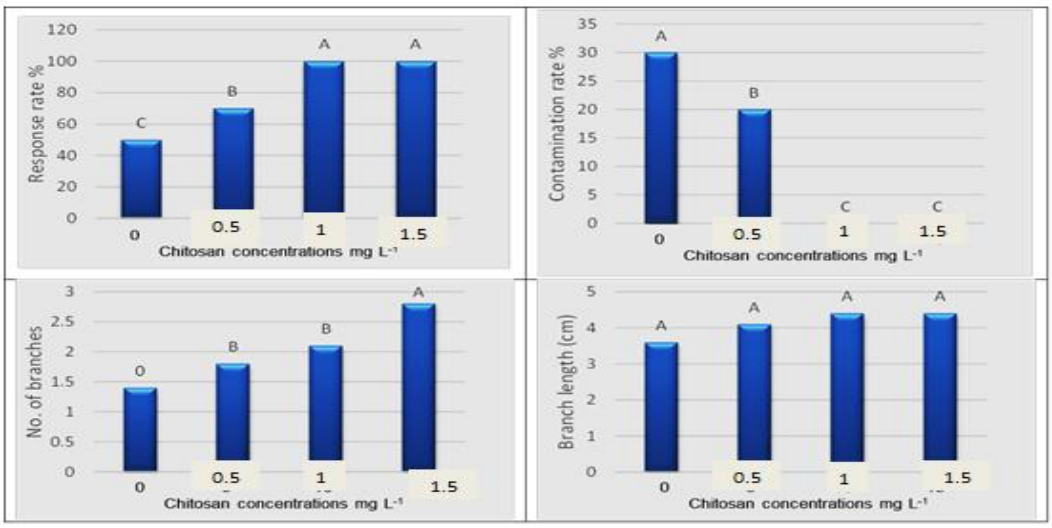

Figure 6. Effect of culture medium treated with chitosan on plantlets contamination %, growth response (%), and the number and length of branches of strawberry plantlets after 4 weeks

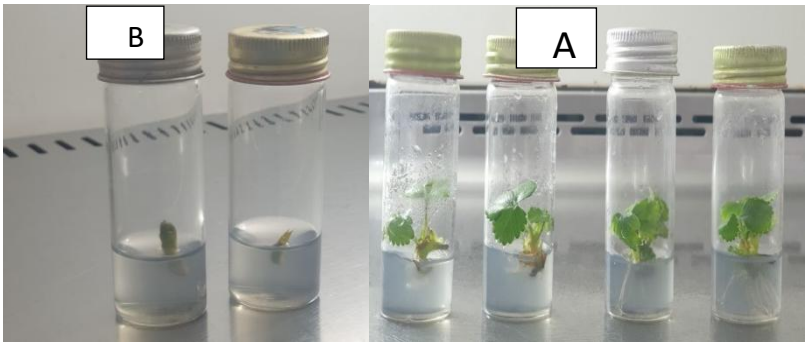

Figure 7. Effect of nanotechnology on plants development. young plantlets grown on the medium treated with Chitosan A: at first day of cultivation, B: after four weeks**.**

As for the effect of chitosan on growth characteristics in the multiplication stage, the results (Table 2) indicate that the number of branches, number of leaves, fresh weight and dry weight recorded a significant increase in the medium prepared with chitosan compared to the standard medium. the highest average of number of branches 11.66 and branch length of 5.33 cm were recorded in the nutrient medium prepared with 1.5 mg L^{-1} chitosan. The highest values in the number of fresh weight of 7.50 g and dry weight 0.40g and number of leaves 5.66 recorded in the medium treated with 0.5 mg L^{-1} chitosan.

Table 2. Effect of culture medium treated with chitosan on strawberry plantlets growth indicators at multiplication stage after 4 weeks

Chitosan mg L^{-1}	**N0. of branches Branch plantlet^{-1}**	**Branch length cm**	**No. of leaves Leaf branch^{-1}**	**Fresh weight (g)**	**Dry weight (g)**
0	3c	3.00bc	4.00b	0.73c	0.14b
0.5	11.33ab	2.00c	5.66a	7.50a	0.40a
1	6.3bc	4.00b	4.00b	2.03b	0.23ab
1.5	11.66a	5.33a	3.00c	2.56b	0.13b

Values are means of 10 replications, where means followed by the same letter(s) within in a column do not differ according to Duncan's multiple range test ($P \leq 0.05$)

Figure 8. Effect of Nano-chitosan (1.5 mg L^{-1}) on the vegetative growth characteristics of strawberry plantlets in multiplication stage 6 weeks after treatment

Rooting stage

The results of Table (2) show significant differences in the rooting percentage resulting from the use of different concentrations of chitosan. The medium prepared with a concentration of 1.5 mg L^{-1} of chitosan outperformed by recording the highest rooting percentage of 80%, number of roots of 6.33 root $plantlet^{-1}$ and root length 3.73 cm compared to the lowest value of rooting rate 40% in the 1 mg L^{-1} and lowest root length 1.8 cm in the medium treated with 0.5 mg L^{-1}.

Table 2. Effect of culture medium treated with chitosan on rooting indicators of strawberry Ruby Gem plantlets at rooting stage after 6 weeks

Chitosan mg L^{-1}	**Rooting rate (%)**	**No. of roots (root $plantlet^{-1}$)**	**Root length (cm)**	**No. of days to 1st visible rooting**
IBA (1 mg L^{-1})	50	3.33b	3.00ab	12
0.5	70	3.6ab	1.83c	17
1	40	3.33ab	2.16bc	20
1.5	80	6.33a	3.7a	17

Values are means of 10 replications, where means followed by the same letter(s) within in a column do not differ according to Duncan's multiple range test ($P \leq 0.05$)

Figure 9. Effect of chitosan 1.5 mg L^{-1} on rooting of strawberry plantlets at rooting stage after 6 weeks

The nutritional medium is one of the most important factors that affect the growth of plant parts grown outside the living body, due to its importance in providing the necessary nutrients for growth. The cultivated part reveals

that the cultivated cells and tissues live in a state of decay depending on what the nutritional medium provides them (Vinay and Afroze, 2015). Therefore, serious attention to the components of the nutritional medium and its physical condition is very important with a high degree of accuracy, as the requirements of the cultivated part of each element of the medium must be calculated with great care. This is due to the importance of the medium for the growth of the plant part at a specific stage, and its subsequent effect on the following stages (George et al., 2008). The results show significant differences in the vegetative and root characteristics of strawberry plants due to the addition of nano-chitosan compared to the control treatment. This may be due to the chemical and physical properties of nanoparticles that reflect increased ionic exchange and increased chemical reactions, as well as the small size of nanoparticles, which increases the efficiency of their entry and spread into the tissues of the cultivated plant part, compared to traditional material particles present in large quantities of micron or millimeter size (Tanou et al., 2017). Thus, giving positive results that are reflected in improving growth in crops (Khan et al., 2017).

The positive effects of nanochitosan on the response rate, pollution reduction, and branch number and length in the emergence stage are similar in their general line to the same indicators when using regular chitosan. This is due to the importance of regular chitosan in the emergence stage, which is consistent with the results of the current study in terms of increasing the response rate, reducing pollution, and increasing the number and growth of branches in harmony with the study of Rohim et al. (2020) in bananas and Abogarra et al. (2022) and Rohim et al. (2022) in palm trees. As for the increase in the number of branches and fresh and dry weight in the branch multiplication stage in general, it is due to the fact that chitosan enhances the entry of nutrients into plant tissues in the planted branches (Salachna and Zawadzińska, 2014). It also stimulates the plant to produce and regulate other plant hormones such as cytokinins (Jogaiag et al., 2020). Cytokinins are known for their effective role in eliminating the apical dominance of buds, increasing branching, and stimulating branch growth. Therefore, their effect has been shown in the growth of plants, increasing the number of branches and their lengths, and increasing dry weight, provided that nano particles are added at appropriate low concentrations at precise rate to regular nutrient medium.

Chitosan also provides organic nitrogen in the nutrient medium as it contains a high percentage of nitrogen up to 6.8%, which helps cells grow and divide. This is due to the importance of containing the amine group (NH2), which is the backbone of nitrogen, which is more readily absorbed than inorganic nitrogen (Sun and Li, 2011). This was confirmed by Zhao et al. (2011) that the amine group in the chitosan compound gives chitosan many special advantages that make it easily applicable in agricultural fields. This is also reflected in the vegetative indicators of crops, as the results are consistent with the experiment of the effect of nano-chitosan on branch multiplication Darwesh et al. (2023) in olives. The positive effect of nano-chitosan added to the nutrient medium for rooting of scallion plants outside the body was also observed in the rooting percentage, and the number and length of the roots formed. This may be due in general to the effect of chitosan in maintaining the nature of the medium or the physiological effect of auxins in the formation of adventitious roots on the branches in addition to its effect in encouraging cell divisions and elongation, which increases the formation and growth of adventitious roots (Davies, 2010). The increase in the rate of the number of roots formed on the branches is due to the effect of chitosan on the level of auxins (Uthairatanakij et al. 2007), as the role of the latter has been proven in increasing the number of meristematic regions at the base of the branch, and then increasing the number of roots formed from it by the process of losing differentiation of specialized tissues and converting them into meristematic cells (De-differentiation). These in turn divide to form the root initial, which continues to grow and develop into the root primordium that grows out of the stem tissues forming the adventitious root (Hartmann et al., 2014).

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