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Original Article

Enhancing rooting tobacco (*Nicotiana tabacum*) plant by loaded indole-3-butyric acid in alginate/chitosan nanocapsule

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Abstract

Recently, nanocarriers have been utilized for encapsulating and sustained release of agrochemicals specifically auxins. Due to their potential applications such as increased bioavailability and improved crop yield and nutritional quality. Herein, the efficacy of alginate/chitosan nanocapsules as a nanocarrier for the hormone indole-3-butyric acid (IBA) loading and its effect on rooting tobacco plants has been carried out in the present study. The average particle size of IBA-alginate/chitosan nanocapsules was measured by Dynamic light scattering analysis at 321 nm. Scanning electron microscope studies revealed the spherical shape of nanoparticles with an average size of 97 nm. The average particle size of IBA-alginate/chitosan nanocapsules was measured by Dynamic light scattering analysis at 321 nm. The characteristic peaks of IBA on alginate/chitosan nanocapsules were identified by Fourier transform infrared spectroscopic analysis. Also, high efficiency (35%) of IBA hormone loading was observed. The findings indicated that the concentration of 3 mgL^{-1} of IBA-alginate/chitosan nanocapsules has the highest efficiency in increasing the rooting in tobacco (Nicotiana tabacum) plants compared to other treatments. According to our results, we can introduce alginate/chitosan nanocapsules as an efficient nanocarrier in IBA hormone transfer applications and their use in agriculture.

Keywords: Alginate, Agricultural nanotechnology, Chitosan, Indole-3-butyric acid, Nanocarrier, Rooting.

1. Introduction

Poor rooting is one of the major problems of horticultural plants, and the economic consequences are extensive, with losses of 10-25% of nursery crops and 5% of ornamental crops annually worldwide. Administering auxin hormones to stimulate rooting has provided a method to achieve early rooting. Although this method often fails, other methods of rooting with nanotechnology do not, and it strengthens rooting in species or varieties in which the propagation of shoot cuttings is difficult. In addition to being economical, this technology is a simple and safe management strategy [1].

The significance of nanotechnology in agriculture has been increasingly recognized, with recent research endeavors concentrating on creating innovative techniques to enhance the physicochemical characteristics, bioaccessibility, and efficacy of agrochemicals [2]. Using nanocarriers to encapsulate said chemicals has demonstrated enhanced efficacy and improved environmental safety, as evidenced by previous research [3]. Several nanocarriers were synthesized and utilized to examine their impact on plant tissue culture concerning fungal and bacterial contamination [4, 5], shoot proliferation [6], somatic embryogenesis and regeneration [7, 8], callus formation [9], root formation [1, 10] and leaf activity [3]. Herbicides were encapsulated within nanocarriers to achieve efficient weed control and mitigate toxic effects, as evidenced by previous research [11]. It has been suggested that encapsulated biodegradable nano-formulations may offer improved penetration through micro-cuttings and enable the regulated discharge of active agents upon reaching the intended plants, as evidenced by previous studies [12-14]. On the other hand, controlled release systems are gaining popularity as effective methods for delivering agricultural chemicals. One significant benefit of these systems is their ability to maintain the optimal dosage of the active ingredient over a specific duration in the environment [15]. This characteristic enhances the efficacy of the active agents while minimizing the quantity of chemicals introduced into the soil, thereby reducing the potential for environmental harm [16].

Indole-3-butyric acid (IBA) is a phytohormone that

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functions as a plant growth regulator, facilitating the processes of germination and root development in diverse plant species. The instability and limited utilization of IBA necessitate the development of an eco-friendly formulation that can effectively minimize its loss and degradation, as highlighted in previous research [17]. The most effective approach to fulfill this requirement is by utilizing controlled release systems, which is one of these methods It is the encapsulation of plant hormones. Therefore, it is very important to develop simple encapsulation methods using low-cost and environmentally friendly materials to produce plant hormone formulations with multiple functions, such as controlled release. Biopolymers from natural sources have great potential as encapsulating materials to protect plant hormones to prolong their activity. Cellulose, starch, chitosan, alginate, and lignin have been used in this field because they have the characteristics of biodegradability, easy access, low cost, and non-toxicity. However, chitosan and alginate biopolymers are among the best and most efficient materials for transferring and protecting agricultural chemicals [18-20].

It is possible to generate hydrogel by the use of the ionotropic gelation method through two different approaches, which vary in the cross-linking ion source. In one approach, the cross-linker ion is added externally, while in the other one, the cross-linker ion is integrated in the polymer solution inactively. External cross-linking results in films that are thinner and have smoother surfaces, and higher matrix strength, permeability and stiffness compared to films with internal cross-linking. Externally cross-linked micropellets exhibit slower drug release rates and greater drug encapsulation efficiency. Researchers have explored various synthetic and natural polymeric systems for controlled drug release, with particular attention given to hydrophilic polyionic carbohydrates, like chitosan and alginate in recent years [21, 22]. The use of aqueous solvents in the preparation of these materials for bead formation helps minimize environmental issues associated with organic solvents. Researchers have used different natural polymers and their modified forms in hydrogel systems for diverse pharmaceutical uses. Sodium alginate and chitosan, in comparison with other natural polymers, exhibit consistent viscosity and result in a more uniform gel structure with greater cross-linking and higher loading capacity for entrapped substances. In our study, nanocapsules composed of alginate and chitosan were synthesized using the ionic gelation method, which involves the cross-linking initiation through the ionic polymer interaction with oppositely charged ions. The 3D configuration and the availability of additional functional groups can affect the capacity of anions or cations to associate with cationic or anionic functionality [23].

Despite the practical solutions offered by advances in plant tissue culture techniques for rooting problems in woody plants, challenging genotypes have yet to yield satisfactory results. The rooting phase in micropropagation, a tissue culture methodology, is of utmost importance and can have significant financial consequences if unsuccessful. To mitigate these limitations, the integration of nanoparticle technology with micropropagation has been proposed [24]. Therefore, in this research, the main goal is to prepare gelatin nanocapsules loaded with IBA hormone by alginate-chitosan **(**AG/CS) nanocapsules and to investigate its effects in increasing rooting of tobacco (*Nicotiana tabacum*) plant.

2. Materials and methods

2.1. Materials

The chemical substances were procured from Fluka and Sigma-Aldrich, corporations and employed in their as-received state without undergoing additional purification procedures. The plant growth hormone indole-3-butyric acid (IBA, $C_{12}H_{13}NO_2$) used in rooting experiments was obtained and sodium alginate from Sigma-Aldrich. Commercial chitosan (average molecular weight, ≥ 75% to 85% degree of acetylation), calcium chloride ($CaCl_2$), and sodium hydroxide (NaOH) were purchased from Fluka.

2.2. Preparation of AG/CS nanocapsules and IBA auxin loading in nanocapsules

Chitosan alginate nanocapsules were prepared by the ionic gelation method. First, 10mL of 0.06% sodium alginate solution was sonicated for 3 min with 3mg L^{-1} IBA hormones. We put this solution in a container filled with water and ice during sonication. Then we added 2 mL calcium chloride 0.067% drop by drop to the previous solution and they were placed on the stirrer for 30 min. Then 1.5 mL of 0.05% chitosan solution was added drop by drop to the solution and it was placed on the stirrer for another 10 min then we adjust the pH to 4.7 and finally sonicated the solution in the water and ice container for 4 min.

2.3. Characterization of the AG/CS nanocapsules

The average size of AG/CS nanocapsules loaded with IBA was measured utilizing a zeta sizer instrument (Malvern Zetasizer Nano S90). The surface charge of IBA-AG/CS nanocapsules was assessed using Zeta potential. Detection of hormone loading efficiency (HLE) and encapsulation efficiency (EE) were analyzed by UV-visible spectrophotometer (Genesys UV spectrophotometer, USA). The morphology of the IBA-AG/CS nanocapsules was subjected to scanning electron microscopy (SEM, FEI Quanta 450). For SEM analysis, aluminum stabs were utilized to mount lyophilized nanoparticles with double-adhesive carbon tape. Additionally, the presence of IBA in the loaded AG/CS nanocapsules was investigated. The sample powder was subjected to FTIR spectra analysis from 400 to 4000 cm^{-1}. The FTIR (Shimadzu Prestige-21) was used to confirm our samples such as IBA, AG/CS nanocapsules, and IBA-loaded AG/CS nanocapsules, and distinctive peaks were observed in the samples.

2.4. Determination of encapsulation, loading efficiencies, and controlled release of IBA hormone in PBS medium

In order to assess the encapsulation efficiency of IBA hormone in alginate-chitosan nanocapsules, it was essential to establish the standard line equation for the hormone. This involved preparing various concentrations of IBA hormone and conducting UV spectroscopy in the range of 200 nm to 800 nm. Following the determination of the absorbance at each concentration, a curve standard was constructed. To address any issues with adsorption at concentrations that affected the curve standard, the T-test was utilized to remove those data points. Next, the standard line equation was computed. Subsequently, UV spectroscopy was employed in the range of 200 to 400 nm to measure the absorbance of IBA-loaded nanocapsules at

221 nm. The suspension containing the loaded nanocapsules underwent centrifugation at 20,000 rpm for 15 minutes at 4 °C. The resulting supernatant was subsequently diluted twice. Subsequently, the samples were subjected to spectroscopic examination. By utilizing the standard line equation of IBA hormone and measuring the absorption at 221 nm of the nanocapsules loaded IBA hormone, the percentage of hormone loading efficiency (%HLE) and encapsulation efficiency (%EE) were calculated using Equations (1) and (2).

Equations (1):

HLE (%) = [total amount of hormone − amount of hormone in supernatant / total amount of nanoparticle recovered] × 100 (1)

Equations (2):

EE (%) = [total amount of hormone − amount of hormone in supernatant / total amount of hormone] × 100 (2)

The present study employed the dialysis bag technique to assess the regulated discharge of indole-3-butyric acid (IBA) from AG/CS nanocapsules that were loaded with IBA in a phosphate-buffered saline (PBS) environment. The cellulose membrane from Sigma-Aldrich utilized in the experiment possessed a cut-off value of 12KD. Initially, 4 mL of AG/CS nanocapsules loaded with IBA were extracted and introduced into dialysis bag. Subsequently, the dialysis bag was introduced into a vessel that contained 200 mL of phosphate-buffered saline (PBS) solution with a pH of 5.8. The study employed a time interval of 0 to 68 hours for sampling. Specifically, 14 distinct time points were selected for the sampling procedure: 0, 1, 2, 3, 4, 5, 12, 20, 28, 36, 44, 52, 60, and 68 hours. Following each sampling event, an equivalent volume of PBS medium was introduced into the container housing the dialysis bag. The specimens were analyzed using UV spectroscopy within the λmax of IBA. Ultimately, a graphical representation of the IBA release percentage from IBA- AG/CS nanocapsules was produced.

2.5. Plant materials and growth conditions

The present study employed plant specimens of tobacco (*Nicotiana tabacum*). The plant tissue culture experiments were carried out in a culture room with controlled conditions, where the temperature was maintained at 25°C and uniform light of 1000 Lux was provided by fluorescent tubes, following a light/dark cycle of 16/8 hours. The Murashige and Skoog (MS) medium, specific to plant tissue culture, was prepared using the standard procedure and subsequently utilized for explant culture, as described in reference [25]. A medium was prepared using the MS formulation, which consisted of 3% sucrose and 0.8% agar and was adjusted to a pH of 5.8. The media and apparatus underwent sterilization through autoclaving at 121 °C for 15 minutes.

2.6. Applications of IBA-loaded AG/CS nanocapsules for *in-vitro* rooting of tobacco

About 2 cm of tobacco plant stem along with 2 lateral buds were separated and cultured in environments containing different groups of IBA-loaded AG/CS nanocapsules. The experimental groups were; (1) the control group (no hormone treatment), (2) IBA treatment (1, 2, 3 mg L^{-1} in solution), and (3) IBA-loaded AG/CS nanocapsules (1, 2, 3 mg L^{-1} in solution). Stems were planted in 90 mm × 70 mm glasses containing 50 mL of culture medium and kept in the culture room. Stems after 4 weeks were examined using the following parameters; Average root length, longest root length, fresh weight, dry weight, and day to rooting. The experiments were done with a randomized plot design with 3 replications. The number of stems in each replication was 3.

2.7. Statistical analysis

The data obtained from the present research has been analyzed by one-way ANOVA statistical method in SPSS-v26 software.

3. Results

3.1. Characterization of the IBA-loaded AG/CS nanocapsules

The IBA-loaded AG/CS nanocapsules were subjected to a comprehensive analysis using various techniques, including scanning electron microscopy (SEM), Fourier transforms infrared spectroscopy (FT-IR), UV-visible spectrophotometer, and the determination of zeta potential, polydispersity index (PDI), and mean particle size.

3.1.1. Encapsulation efficiency and release in PBS

The percentage of IBA loading in the Nano formulation was 35% and the encapsulation efficiency was 83%. The calculations of these two parameters were obtained based on the standard line equation of the IBA hormone (Fig 1).

The investigation of the release of indole-3-butyric acid (IBA) from alginate-chitosan nanocapsules loaded with IBA was conducted through a dialysis bag assay and ultraviolet-visible (UV) spectroscopy. The maximum quantity of release, amounting to 19%, was observed within the initial 28-hour period, followed by a period of 40 hours, during which the release stabilized consistently (Fig 2).

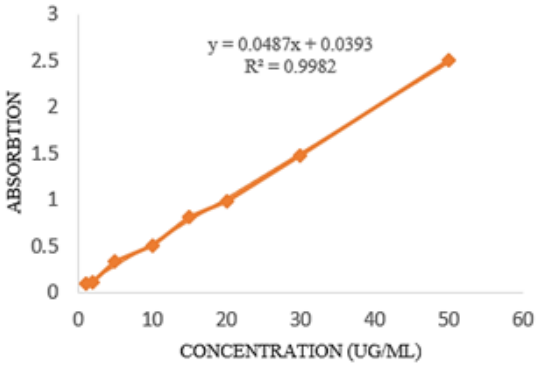

Fig. 1. IBA standard line equation in 221nm UV spectrum.

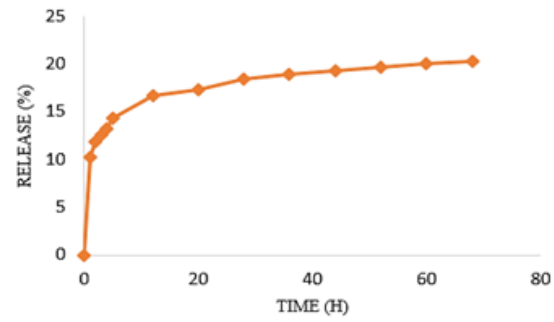

Fig. 2. Release diagram of IBA from IBA-loaded AG/CS nanocapsules.

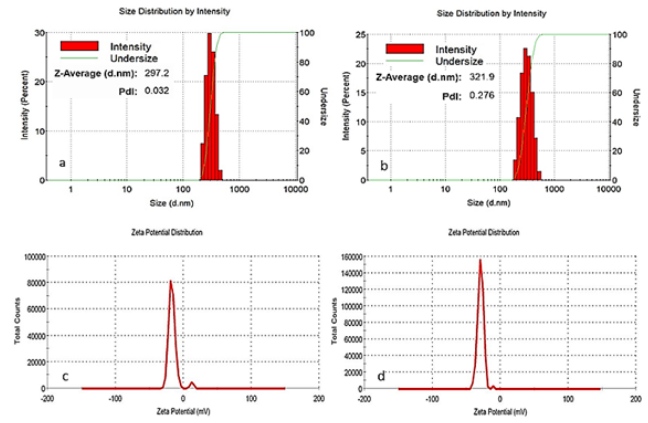

Fig. 3. Particle size distribution of a) AG/CS nanocapsules and b) IBA-loaded AG/CS nanocapsules and zeta potential (mV) values for c) AG/CS nanocapsules and d) IBA-loaded AG/CS nanocapsules.

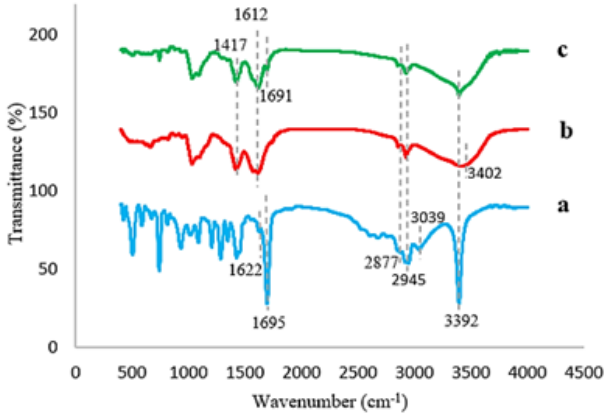

Fig. 4. FT-IR Spectra of a) IBA, b) AG/CS nanocapsules, and c) IBA-loaded AG/CS nanocapsules.

3.1.2. Determining the size and surface charge

The IBA-loaded AG/CS nanocapsules were characterized using zeta-sizer and zeta-potential techniques. The mean diameter of AG/CS nanocapsules was determined to be 297 nm. Following hormone loading, the synthesized IBA-loaded AG/CS nanocapsules showed mean diameter of 321 nm. The zeta potential value for AG/CS nanocapsules was obtained at -15.9 mV, and after the loading of the hormone, it increased to -28.9 mV, which indicates good stability of the formulated IBA-loaded AG/CS nanocapsules (Fig 3).

3.1.3. Fourier transform infrared (FT-IR) spectra

Fig 4 displays the FT-IR spectra of IBA, AG/CS nanocapsules, and IBA-AG/CS nanocapsules. The spectrum obtained for IBA, as depicted in Figure 4a, displays distinct peaks that correspond to various molecular vibrations. Specifically, a band located at 3392 cm^{-1} corresponds to the N-H stretching vibration. The observed peaks at 2945, 2877, and 1622 cm^{-1} represent the stretching and bending vibrations of -CH_2 groups, respectively. The aromatic C-H stretching vibration signal at 3039 cm^{-1}, and vibrations at 1695 cm^{-1} reflect the presence of the carboxylate ion band (COO^-)[26, 27]. In Fig 4b the peaks observed at 1417 cm^{-1} and 1612 cm^{-1} are attributed to symmetric and asymmetric stretching vibrations of chitosan and alginate carbonyl (C=O) bonds, respectively. Due to the hydrogen bonding among the NH_2 of chitosan and COOH of alginate, the expansive band is observed at 3402 cm^{-1}. The band at 1031 cm^{-1} is associated with the stretching vibration band of the C-O [28]. In Fig 4c which belonged to IBA-loaded AG/CS nanocapsules, it shows a band at about 3392 cm^{-1} attributed to stretching vibration of N-H groups of IBA, and the signal at about 1691 cm^{-1} attributed to carbonyl bond (C=O). The strong peaks at 2852 and 2924 cm^{-1} belonged to C-H stretching vibrations, which indicated that IBA was successfully loaded into the AG/CS nanocapsules.

3.1.4. Electron microscopy analysis

Morphological characterization of IBA-loaded AG/CS nanocapsules was determined using field-emission scanning electron microscopy (SEM, FEI quanta 450). The SEM image of IBA-loaded AG/CS nanocapsules (Fig 5) shows a separate and spherical morphology with an average size of ~97 nm.

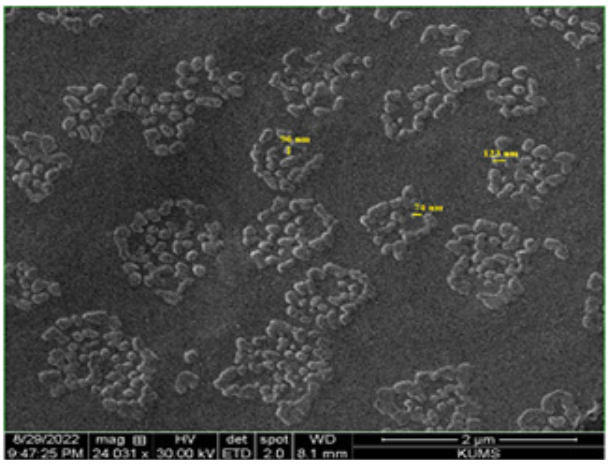

Fig. 5. SEM image of IBA-loaded AG/CS nanocapsules.

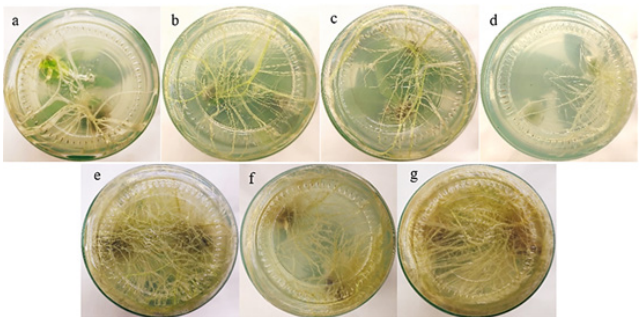

Fig. 6. Effects of free IBA, and IBA-AG/CS nanocapsules on in vitro rooting of tobacco plant stem; Control 0.0 mg L^{-1} IBA (a), 1.0 mg L^{-1} IBA (b), 2.0 mgL^{-1} IBA (c), 3.0 mgL^{-1} IBA (d), IBA-AG/CS nanocapsules (1.0 mg L^{-1} IBA) (e), IBA-AG/CS nanocapsules (2.0 mgL^{-1} IBA) (f), and IBA-AG/CS nanocapsules (3.0 mgL^{-1} IBA) (g).

3.1.5. The effect of IBA-stabilized AG/CS nanocapsules on in vitro treated stem Nicotiana tabacum

The results and figures of the effects of IBA and IBA-AG/CS nanocapsules on the characteristics of average root length, longest root length, root fresh weight, root dry weight, and days to rooting are presented in Table 1 and Figure 6. In terms of the root length attribute, the highest value of 9.83 cm belongs to the IBA-AG/CS nanocapsules treatment with a concentration of 3 mgL^{-1}, and the lowest value of the root length belongs to the control group, 5.60 cm. The length of the longest root belongs to the IBA-AG/CS nanocapsules treatment with a concentration of 3 mgL^{-}

Table 1. The effect of IBA-AG/CS nanocapsules on the rooting of tobacco plants *in vitro.*

Treatment	Auxin concentration (mg L^{-1})	Root length (cm)	Longest root length(cm)	Fresh weight (g)	Dry weight (g)	Day to rooting (day)
Control	0.0	5.60 e	6.10 c	0.04 e	0.03 cd	9.33 b
IBA	1.0	7.50 c	7.80 b	0.40 d	0.02 cd	12.33 a
	2.0	7.66 c	8.00 b	0.53 c	0.03 c	9.33 b
	3.0	6.40 d	6.70 c	0.33 d	0.02 d	12.66 a
IBA-AG/CS nanocapsules	1.0	8.90 b	8.56 b	0.67 b	0.04 b	5.66 d
	2.0	6.50 d	6.80 c	0.53 c	0.03 cd	7.33 c
	3.0	9.83 a	10.57 a	0.83 a	0.06 a	4.00 e

Different letters within each column indicate statistical differences at $P < 0.05$.

^{1} and a value of 10.57 cm. Also, in the same treatment and the same concentration, in terms of the properties of root fresh weight and root dry weight, they have the highest values of 0.83 g and 0.06 g, respectively, which has a statistically significant difference with other treatments. Also, we observed the fastest rooting time in different treatments in the IBA-AG/CS nanocapsules treatment with a concentration of 3 mgL^{-1} with a rate of 4 days, which has a statistically significant difference with other treatments.

4. Discussion

In this study, AG/CS nanocapsules loaded with plant growth hormones IBA were prepared for use in agricultural applications. IBA-AG/CS nanocapsules were characterized, focusing on their morphology, chemical composition, hydrodynamic diameter, and colloidal properties. FT-IR analysis showed the successful loading of hormones. SEM images showed separate, spherical nanocapsules in the size range of 97 nm. It seems that the amino group of IBA has been effective in the shape of nanocapsules in interaction with alginate and chitosan polymers. On the other hand, due to water retention and high hygroscopic properties of chitosan and hydrogen bonding, it can be expected that chitosan mechanical properties have changed and it has been effective in the shape of nanocapsules. A narrow hydrodynamic diameter distribution was observed with an increase in the hydrodynamic diameter after hormone loading. The obtained polydispersity index (PDI) of 0.276, which is less than 0.5, suggests that the suspension is colloidal and exhibits a uniform size distribution. Furthermore, after administering hormones, there was a discernible discrepancy in the hydrodynamic diameter. The observed discrepancy in particle size determination could be attributed to particle agglomeration during sample measurement preparation. This phenomenon has been frequently documented in the context of nanoparticles dispersed in water [29]. The presence of a negative zeta potential suggests the existence of free-COO groups on the surface, which may be attributed to higher concentrations of alginate compared to chitosan. This is because alginate contains carboxyl groups that contribute negative charges. These carboxylic acid groups in alginate can interact electrostatically with positively charged molecules, leading to gel formation. This characteristic of alginate, combined with chitosan, is a notable advantage [30].

In a recent study conducted by Korpayev et al. It was demonstrated that the *in vitro* adventitious rooting of micro cuttings of Malling Merton 106 (MM 106) was notably increased in the presence of chitosan and silver nanoparticles loaded with IAA or IBA (ranging from 62.5% to 91.7%) as compared to the application of free IAA or IBA (ranging from 33.3% to 50.0%) [24]. Karakeçili et al. conducted research indicating an increase in in-vitro rooting efficiency by two times for the difficult-to-root wild pear (*Pyrus elaeagrifolia Pallas*) with IBA-loaded ZnO or IAA NPs. In this particular genotype, the largest percent of rooting was observed when treated with IBA-loaded zinc oxide NPs (IBA-nZnO) and IAA-loaded zinc oxide nanoparticles (IAA-nZnO) at a concentration of 400 mg/L. The rooting percentage for IBA-nZnO was recorded at 50.0%, while IAA-nZnO exhibited a rooting percentage of 41.7% [13]. Reed et al. conducted a similar study on different Pyrus genotypes, focusing on auxins' impacts on the rooting functioning of challenging-to-root genotypes. It was found that when auxins were applied conventionally, the maximum rooting percentage achieved was 31.8% [31].

According to our results, IBA-loaded AG/CS nanocapsules increase the growth of tobacco plant roots. It has a good potential application as a controlled-release formulation and environmentally friendly plant growth regulator.

5. Conclusion

From the perspective of nanobiotechnology, IBA-AG/CS nanocapsules are a suitable carrier for the transfer of IBA hormones. Its physicochemical properties were confirmed through different analyses. which causes rapid root growth in plants. Also, this nanoparticle has a loading capacity of 35% of the IBA hormone. Among the different concentrations of this nanoparticle, the concentration of 3 mgL^{-1} of IBA-AG/CS nanocapsules has more effective and promising results than other treatments in increasing root growth.

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Declaration of competing interest

The authors hereby assert that they do not possess any known financial interests or personal relationships that may have been perceived as conflicting and could have potentially exerted an influence on the research presented in this paper.

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