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SUPERIORITY OF NANO-SILVER NITRATE AND NANO-CHITOSAN IN CONTROLLING BACTERIAL CONTAMINATION AND PROMOTING GROWTH OF *In vitro* DATE PALM CULTURES

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ABSTRACT

Date palm (*Phoenix dactylifera* L.) a perennial monocotyledonous tree it's have an ecological importance and economical role in several countries of Middle East and North Africa, date palm can't be propagate by seeds, meanwhile the offshoots produced low numbers of offshoots for the given period, so tissue culture technique become a great important for produced the huge demand plantlets, the in vitro technique facing a problem as the contamination by bacterial and fungus that lead to loss the cultures, for eliminate this problem many treatments were used as chitosan 5 and 15 ml/l, $AgNO_3$ 4.0 and 7.0 ml/l, Zn 4.0 and 7.0 mg/l and cu 0.5 and 1.0 mg/l. as the main forms or as a nanoparticles NPs in addition to streptomycin 40 and 50 mg/l and $AgNO_3$ + Sodium thiosulfate 0.5 and 1.0 ml/l were added to rooting stage medium for 3 subcultures for each, then these plantlets were acclimatized under greenhouse condition. Collected data were subjected to analysis of variance (ANOVA) and the means were compared following t-test using LSD values at 5%. The percentage of cultures contamination was to be found zero % with the treatments chitosan, silver nitrate $AgNO_3$, in addition to the combination of Chitosan + $AgNO_3$, chitosan + Zn and finally chitosan + Cu at all tested levels produced, while the treatments Zn and Cu at two tested levels produced 11% contamination, the observation of the vegetative growth and leaves contents of indoles and amino acids mg/g f.w. were found highest values under chitosan and $AgNO_3$ and the combination in

between as the main forms or as NPs treatments, as well as, the acclimatization stage presented the great survival percentage % under Chitosan and $AgNO_3$ and combination in between as the main form or as NPs. The present study have been suggest that NPs chitosan, $AgNO_3$ and the combination in between might be a promising method for reduced or eliminate the in vitro microbes, reduced the harmful effects on the valuable date palm plantlets and increasing growth parameters.

Keywords: Chitosan; silver nitrate; bacteria; contamination; date palm; *In vitro*.

INTRODUCTION

Estimations of global population in 2050 is 9 billion, which corresponds to increasing demand for food, in addition to the climate changes which negatively impacts the food production as well as the confrontation of continuously decreasing water and cultivated land availability, all together increases the world hunger [1,2]. Date palm (*Phoenix dactylifera* L.) is a perennial monocotyledonous tree and its fruits are the major source of food and of high economic as well as ecological importance in several countries in the Middle East and North Africa [3]. Many limitations of the traditional seed and offshoot propagation methods have been arisen. Therefore, date palm tissue culture has become an important tool to produce huge numbers of date palm plantlets that are genetically uniform, which are described as true-to-type, in a short time. Along the stages of *in vitro* date palm technique, stages as initiation of callus or sub-culturing, it faces many constraints and challenges such as tissue browning, vitrification and, the worst, fungal and bacterial contamination, which lead to the loss of huge numbers of explants and therefore reduces the desired plantlets that should eventually suffice the producers demand [4,5]. The origin of contamination of plant tissue cultures is potentially from one of two major sources, either through carry over of microorganisms on the surface or in the tissues of explants due to inefficient methods for sterilizing explants taken from *in vivo* plants, or through unsanitary procedures in the laboratory, e.g., aseptic handling of plant material; or the faulty sterilization of culture vessels, working tools and growth media [6-10]. To control such contaminations, it is required that all glassware, media, and tools should be sterilized, and good microbiological procedures should be maintained including a viable technique of explant surface sterilization and supplementing the media with antimicrobial agents, preferably, biomaterial based and biodegradable [11,12].

In the last three decades, various antibiotics have been developed to eliminate bacterial contamination during *in vitro* propagation, yet their continuous use has led to the emergence of antibiotic-resistant bacterial strains and most of them have shown inhibitory effects in the plants. Antibiotics are commonly used incorporated in the medium to eliminate unwanted contaminants from plant systems. Streptomycin and kanamycin were used effectively for controlling bacterial contaminants as media component of plant tissue cultures [13,14].

Chitosan is a safe broad-spectrum antimicrobial agent *naturally* found in the shells of crustaceans, such as crab, shrimp, squid pen and crawfish [15]. It can be produced commercially by the de-acetylation of chitin, which adds the inexpensiveness to its multiple advantages [16,17]. In addition, it stimulates plant growth and improves crops quality. Chitosan-supplemented MS medium increased green shoots regenerated of the yellow compact calluses oil palm from zygotic embryos [18]. In 1700, silver nitrate $AgNO_3$ was used for the treatment of microbial human diseases [19]. Toxicity of silver ion (Ag^+) and its compounds towards microbes is characterized by its stronger antibacterial activity, broad antibacterial spectrum and higher stability [20]. Silver nanoparticles (NPs) are toxic to bacteria at low concentrations, and are currently used in numerous life applications to inhibit microbial growth [21]. When incorporated in modified MS medium of culturing tobacco plants, Ag (NPs) had a good potential for removing of the bacterial contaminants in tobacco plant tissue culture procedures [22]. Silver/chitosan nano- formulation has antifungal activity against seed borne plant

fungal pathogen throughout inhibiting mycelium growth [23]. Copper (Cu) and zinc (Zn), among various inorganic antimicrobial materials, have been developed to be used in different life applications. Nanoparticles of copper (Cu NPs) with non-nano copper such as copper oxychloride (Cu_2 $(OH)_3$ Cl) at 50 mg l^{-1} recorded 76% growth inhibition of the oomycete *Phytophthora cinnamomi in vitro*, effective antimicrobials [24]. However, phytotoxicity of Cu and Zn was extensively investigated. It has been reported that phytotoxicity threshold for Zn concentrations in plant tissues are in the range of 200–500 mg kg^{-1}, moreover, zinc toxicity seen in the nucleus of the root tip [25], meanwhile, CuO NPs at 1,000 mg L^{-1} caused a decline in photosynthetic rate, transpiration rate, stomatal conductance, maximal quantum yield of photochemistry, and photosynthetic pigment contents, with a complete loss of PSII photochemical quenching [26].

The aim of this study is to (1) identify bacterial contaminants of date palm *in vitro* cultures (i.e. the rooting stage); (2) evaluate the efficacy of biological-based and inorganic antibacterial agents in their ordinary forms as well as their nano formulations for preventing/reducing any bacterial contaminants and the regenerated explant growth characteristics and (3) investigate their effect on the plantlet survival percentage during the greenhouse acclimatization stage.

MATERIALS AND METHODS

The experiment was conducted during 2019 to 2022.

Plant Material and Sample Collection

In this research, we used date palm (*Phoenix dactylifera* L.) cv. Batamoda from Central Laboratory for Research and Development of Date Palm, Agricultural Research Center (A.R.C.), Giza, Egypt. Explants, not showing visible symptoms or signs of bacterial contamination, were used as explants for *in vitro* cultures. Samples of contaminated cultures were obtained from date palm tissue cultures at the same laboratory. Contamination was assessed visually by examining plants and growth media under fluorescent light. Most detected contaminations were only marginally visible as a tiny faint halo around the base or roots of the *in vitro* plant. Ten contaminated cultures were collected out of 200-two month's old *in vitro* cultures.

Isolation of Bacterial Contaminants from *in vitro* Cultures

Collected samples were used for isolating bacterial contaminants. Microbial cultures were initiated from all plants by placing 1–2 mm of stem segments (cut at the base of the plant) and 5 mm of root tissue in tubes (16 mm × 150 mm) with 3 ml of modified Nutrient Broth (NB) (Atlas, 2004). Each tube was incubated at room temperature (RT) for 3 days.

Morphological, Nutritional and Biochemical Characterization of Isolated Bacteria

A primary identification of the bacterial isolates was conducted on the bases of their morphological, nutritional, and physiological characteristics according to the schemes suggested by Garrity et al. [27]. Bacteria were grown on Nutrient Agar (NA), incubated at 28°C for 3 days. Bacteria growing on the NA plates were examined based on the following morphological characteristics of the bacterial colonies: margin, surface structure, form, elevation, texture, color and diameter [28]. According to Bergey's Manual [27], physiological tests of isolated bacteria were carried out; i.e. growth at different temperatures, growth on different concentration of NaCl, citrate utilization, lactose utilization, sucrose utilization, D-glucose utilization, glycerol utilization. Also, the Gram reaction was performed.

Preparation of Antibacterial Agents

Sodium thiosulfate

For the preparation of 200 ml of 1000 ppm STS solution, two sock solutions were prepared. 204 mg of silver nitrate ($AgNO_3$) was dissolved in 100 ml of double distilled water. A weigh of 2,382 mg of sodium thiosulfate ($Na_2S_2O_3.5H_2O$) was dissolved in 100 ml of double distilled water. A 100 ml of each solution was combined by slowly pouring solution No. 1 into solution No. 2. The 200 ml final solution should be clear (non-

precipitated). If you combine the two solutions in the opposite direction you will get black precipitate. This solution can be stored for up to 3 months in brown bottle in dark.

Silver nanoparticles

100 ml of $AgNO_3$ solution was prepared in a 250 ml Erlenmeyer flask with a magnetic pill. 20 ml of sodium citrate in a burette. The flask was placed on a heating/stirring plate, heated to 100°C (boiling) and stirred vigorously (400 rpm). A drop wise the sodium citrate solution was added drop wise until a light-yellow color was observed and remained stable. The solution was then cooled down to room temperature. And used immediately or stored in a sealed container, preferably opaque and with as little air as possible [29,30].

Chitosan nanoparticles

Chitosan nanoparticles were synthesized via the ionotropic gelation of chitosan with TPP (tripolyphosphate) anions. Chitosan (0.2%) was dissolved in ascorbic acid solution (1%) and stirred (1000 rpm, 1 hour) at room temperature. Stock solution of TPP will be prepared by using 0.03 g of TPP in 11 ml of water. Chitosan nanoparticles were spontaneously fabricated with adding 1 ml of TPP stock solution drop wise to chitosan solution under magnetic stirring (1000 rpm, 1 hour) at room temperature. Then chitosan nanoparticles were sonicated for an hour to make sure that they are in small size [31].

Zinc nanoparticles

Zinc nitrate and KOH as precursors, the aqueous solutions of zinc nitrate ($Zn(NO_3)_2.6H_2O$) (0.2 M) and KOH (0.4 M) were prepared in deionized water. The KOH solution was slowly added into zinc nitrate solution at room temperature under vigorous magnetic stirring, which resulted in the formation of a white suspension. The later white product was centrifuged at 5000 rpm for 20 min and washed three times with distilled water then washed with absolute alcohol at last. The obtained product was calcined at 500°C in air atmosphere for 3 hours [32].

Copper Nanoparticles

2.49 g of copper sulfate pentahydrate ($CuSO_4.5H_2O$) was dissolved into 200 ml of deionized water, and temperature was raised to 85°C. 10.46 g of CTAB together with 19.37 g of L-ascorbic acid were dissolved into 800 ml of deionized water; then NaOH solution was used to adjust the pH at 6.8 and temperature was raised to 85°C under magnetic stirring. During stirring of the previous solution of CTAB and L-ascorbic acid, copper sulfate pentahydrate solution was introduced drop wise into the mixture, the temperature was kept at 85°C. At the beginning, the reaction mixture was colorless, then it turned into yellow then it darkened gradually until reddish brown color was developed [33].

Antibacterial Treatments

Different antibacterial agents (Table 1) were added to establishment medium ¾ MS medium [34] + 0.1 mg l^{-1} NAA + 0.05 mg l^{-1} BA + 2 mg l^{-1} PBZ + 0.4 mg l^{-1} thiamine-HCL + 30 g/l sucrose + 6 g/l agar for shoot stage and then explants were placed on it.

To ensure the effectiveness of the antibacterial agents and antibiotics used in the shooting and rooting stages, three consequent subcultures were carried out for all treatments. In every subculture, reoccurrence of bacterial contamination was monitored, and the presence of clean plantlets was assessed. The plantlet is best described as about 8-10 cm in length, 2-3 leaves and 1-2 roots. The plantlets were incubated for 3 weeks/subculture.

The experiment was conducted as a completely randomized design in a factorial arrangement with three replications.

Data Collecting

After the three subcultures of plantlet growth, plantlets not showing visible symptoms or signs of bacterial contamination were re-subcultured into rooting stage for three subcultures in a medium contains ¾ MS + 2 mg l^{-1} PBZ + 2 mg l^{-1} IBA.

After each culture stage the following assessments were recorded; bacterial contamination percentage (%), plantlet height (cm), leaves number/plantlet, root length (cm), roots number/plantlet, leaves fresh and dry weight (g) and roots fresh and dry weight (g) at the end of rooting stage.

Table 1. The evaluated antibacterial agents added in the culture medium and their concentrations

Antibacterial agents	Concn. (mg l^{-1})	Nanomaterial antibacterial agents	Concn. (mg l^{-1})
Chitosan	5	Chitosan (NPs)	5
	15		15
$AgNO_3$	4	$AgNO_3$ (NPs)	4
	7		7
Zn	4	Zn (NPs)	4
	7		7
Cu	0.5	Cu (NPs)	0.5
	1.0		1.0
$AgNO_3$ + Sodium thiosulfate	0.5	Chitosan + $AgNO_3$ (NPs)	15+7
	1.0		
Streptomycin*	40	Chitosan + Zn (NPs)	15+7
	50		

* $C_{21}H_{39}N_7O_{12}$, Molecular weight 581.6 g/mol

Acclimatization Stage

The plantlets which were driven from tissue culture rooting stage at different treatments were described as (10-15 cm for plant height, 2-3 roots/plantlet, 3-4 cm for root length and 1-3 cm for root thickness), the plantlets were washed with tap water to remove the adhering culturing medium and dipped in fungicide copper oxi chloride solution (0.5%) for 10 minutes, to protect them from fungal attacks, then transplanted into plastic pots (8 cm diameter and 12 cm length) containing a sterile soil mixture of peat moss and perlite (2 : 1), and grown in a greenhouse under plastic tunnels at 29°C and 100% relative humidity for one month and the plastic tunnel was opened daily for 5 min. After this period, the plastic tunnel was opened daily for 15 min to help the growth of new leaves or new roots. After 3 months of acclimatization stage the following data were recorded: plant survival percentage (%), plant height (cm) and leaves number/plant [35].

Biochemical Assessments

At the end of rooting stage, plantlet leaves were used for the assessment of total indoles (mg/g FW) as described by Salim et al. [36]; Larsen et al. [37] and total amino acids contents (mg/g DW) using ninhydrin reagent according to Rosen [38].

Statistical Analysis

For statistical analysis, collected data were subjected to analysis of variance (ANOVA) and the means were compared following *t*-test using LSD values at 5% level [39].

RESULTS

Identification of Isolated Bacterial Contaminants

Microscopic examination, gram staining (Fig. 1) and biochemical tests were used for identification according to Collins et al. [28]. Two species could be identified based on this test; a Gram-positive isolate identified as *Bacillus subtilis*, which belongs to family *Bacillaceae*, and a Gram-negative isolate was identified as *Serratia marcescens*, which belongs to family *Enterobacteriaceae*.

Effect of Different Antibacterial Agents as the Ordinary Forms on the Contamination of the *in vitro* Date Palm Plantlets (rooting stage)

Supplementing the MS medium with antibacterial agents (Table 1), then carrying out 4 subcultures resulted in the following:

Contamination Percentage

As shown in Fig. 2, significant differences were observed among the tested antibacterial agents on *in vitro* shooting stage. Under control, as well as the treatment with streptomycin at 40 mg l^{-1},55.6% of shoots were visibly contaminated with bacteria meanwhile streptomycin (50 mg l^{-1}) and $AgNO_3$ + sodium thiosulfate at 0.5 ml/l resulted in a contamination reduction to 44.4%. A greater reduction has occurred (to 33.3%) with each of AgNO3 + sodium thiosulfate (1.0 ml/l), Zn (4.0 mg l^{-1}) and Cu (0.5 and 1.0 mg l^{-1}) , while 22.2 %

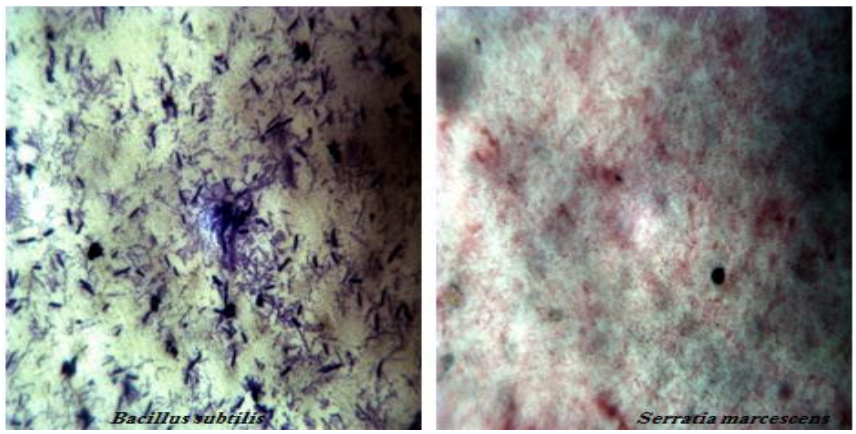

Fig. 1. *Bacillus subtilis* (left) and *Serratia marcescens* (right)

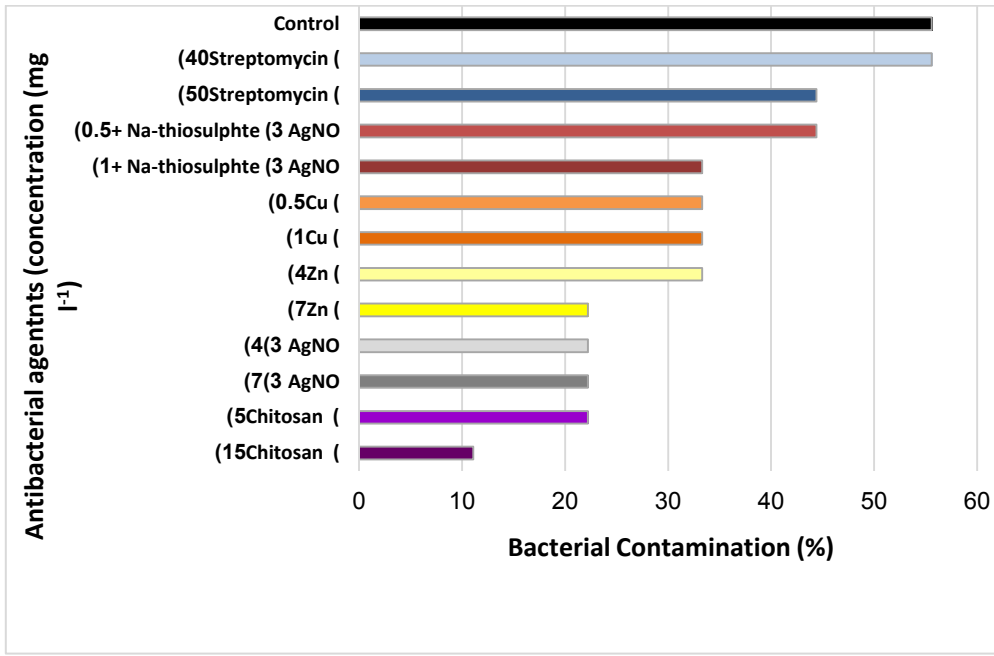

Fig. 2. Effect of different antibacterial agents, at their ordinary form, on bacterial contamination percentage of date palm *in vitro*-shooting stage cultures

take place from chitosan (5 mg l^{-1}), $AgNO_3$ (7 mg l^{-1}) and Zn (7 mg l^{-1}), finally the highest reduction of bacterial contamination (11%) was obtained by the chitosan (15 mg l^{-1}) treatment.

Plantlet Growth Parameters

Data and photographs presented in Table 2 and Fig. 3 show the effect of supplementing the culture media with different antibacterial agents on certain plantlet growth parameters (i.e., plant height (cm), leaves number/ plantlet, root length (cm), and roots number/ plantlet). As compared to control (culturing medium was free of any antibacterial agent), the highest significant difference of plantlet height was recorded with the treatments of 15 and 5 mg l^{-1} chitosan and 7 mg l^{-1}

$AgNO_3$ (23.0, 21.8 and 21.7 cm, respectively). On the contrary, the Cu treatment at 0.5 and 1 mg l^{-1} affected the plant height negatively as it resulted in the highest negative effect on the plantlets height i.e., 14.5 and 13.0 cm, respectively. As for leaves number/ plantlet, chitosan treatments resulted in the highest significant increases while, Cu and Zn treatments caused significant reductions compared to control. No significant induction of the root length was recorded by any of the antibacterial agents; however, means were significantly decreased by Cu treatments followed by Zn treatments. Roots number/ plantlet was slightly increased over the control mean by both chitosan and $AgNO_3$, yet non-significantly. On the contrary, Cu and Zn treatments resulted in significant reductions, compared to control.

Table 2. Effect of different antibacterial agents at their ordinary form on the growth characteristics of *in vitro* date palm cultures at the rooting stage

Antibacterial agent	Conc. (mg l^{-1})	Means of growth parameters of plantlets			
		Plant height (cm)	Leaves number/ plantlet	Roots length (cm)	Roots number/ plantlet
Chitosan	5.0	21.8 *b*	3.0 *ab*	4.0 *ab*	3.0 *ab*
	15.0	23.0 *a*	3.3 *a*	4.5 *a*	3.3 *a*
$AgNO_3$	4.0	20.0 *de*	2.9 *abc*	3.3 *abc*	2.7 *abc*
	7.0	21.7 *bc*	3.0 *ab*	3.6 *abc*	3.3 *a*
Zn	4.0	19.3 *e*	2.0 *abc*	3.0 *bc*	2.0 *abcd*
	7.0	17.0 *g*	1.5 *bc*	2.7 *cd*	1.0 *cd*
Cu	0.5	14.5 *h*	1.7 *abc*	1.5 *de*	1.3 *cd*
	1.0	13.0 *i*	1.3 *c*	1.3 *e*	1.0 *d*
$AgNO_3$ + Sodium thiosulfate	0.5	19.5 *e*	2.3 *abc*	3.3 *abc*	2.3 *abcd*
	1.0	20.8 *cd*	2.6 *abc*	3.6 *ab*	2.3 *abcd*
Streptomycin	40.0	18.2 *f*	2.3 *abc*	3.2 *bc*	2.3 *abcd*
	50.0	19.5 *e*	2.3 *abc*	3.5 *abc*	2.0 *abcd*
Control		19.5 *dc*	2.1 *abc*	3.5 *abc*	2.7 *abc*
LSD (0.05)		0.9	1.6	1.2	1.5

Fig. 3. Date palm plantlets from *in vitro* cultures supplemented with different antibacterial agents at their ordinary forms. Growth parameters, i.e., plant height (cm), leaves number/ plantlet, root length (cm), and roots number/ plantlet were recorded. Control = no antibacterial agent was added

Plantlet Leaves Content of Indoles and Amino Acids

The assessment of indole level in date palm plantlets subjected to different antibacterial agents showed that in comparison to control (1.7 mg g^{-1} FW), the indoles significantly increased with both treatments of chitosan (2.7, 3.6 mg g^{-1} FW) and both treatments of $AgNO_3$ (2.9, 3.1 mg g^{-1} FW), wherein the only non-significant decrease (1.5 mg g^{-1} FW) was assessed with the treatment of 1 mg l^{-1} Cu. All other treatments slightly increased the indoles to a non-significant level (Fig. 4).

As for aminoacids content, all concentrations tested of AgNO3 significantly highly increased the content of aminoacids (3.5, 3.6 mg g^{-1} FW) followed by $AgNO_3$ + sodium thiosulphate at 0.5 mg l^{-1} and chitosan at 15 mg l^{-1} (3.3 mg g^{-1} FW). The decline of aminoacids levels in the treatment of $AgNO_3$ + sodium thiosulphate at the higher concentration (1 mg l^{-1}) to 2.4 mg g^{-1} FW was noteworthy. Cu treatment at both concentrations caused a significant reduction in aminoacids level (0.7 and 0.8 mg g^{-1} FW) compared to control (Fig. 4).

Effect of Different Antibacterial Agents as Nanomaterials on the Contamination of the *in vitro* Date Palm Plantlets (rooting stage)

The aim of this experiment was to evaluate nanoparticles (NPs) of chitosan, $AgNO_3$, Zn and Cu and their combinations as antibacterial agents when supplemented in the *in vitro* culture medium of date palm. Bacterial contamination percentage and growth parameters were recorded after 4 subsequent subcultures after treatments. At the end of rooting stage, biochemical assessments were carried out.

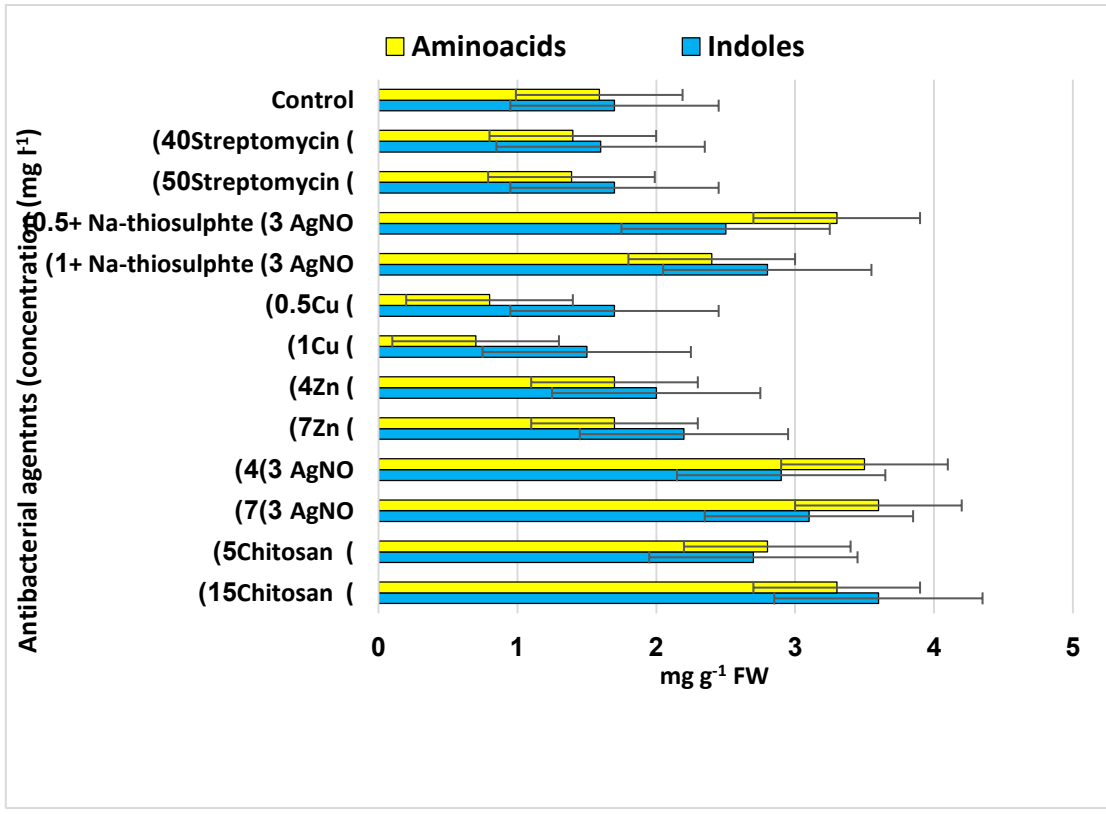

Fig. 4. Effect of different antibacterial agents, at their ordinary form, supplemented into date palm *in vitro* cultures on driven plantlet content of indoles and total aminoacids

Contamination Percentage

Perfect inhibition of bacterial contamination in *in vitro* cultures were recorded with the treatments of chitosan NPs as well as chitosan NPs combinations with $AgNO_3$, Cu or Zn. On the other hand, Cu NPs and Zn NPs equally affected the bacterial contamination as they caused a significant reduction to 11% at all concentrations tested (Fig. 5).

Plantlet Growth Parameters

In this experiment, the shoots were transferred to fresh MS medium enriched with rooting growth regulators and supplemented with nano-antibacterial agents (Table 1). As listed and shown in Table 3 and Fig. 6, the treatment of chitosan NPs at 15.0 mg l^{-1} + $AgNO_3$ NPs at 7.0 mg l^{-1} developed the best growth of the plantlets for all the assessed parameters. On the contrary, Cu NPs, Zn NPs or their combinations with chitosan resulted in a remarkable decrease in the plantlet growth for all the assessed parameters. As for the plant plantlet height, the chitosan NPs at 15.0 mg l^{-1} + $AgNO_3$ NPs at 7.0 mg l^{-1} treatment highly significantly promoted the tall of the plantlets to 26.7 cm, followed by chitosan NPs (15.0 mg l^{-1}); 25.7 cm compared to the control 20.0 cm. Meanwhile, the shortest plantlets (12.3 cm) were

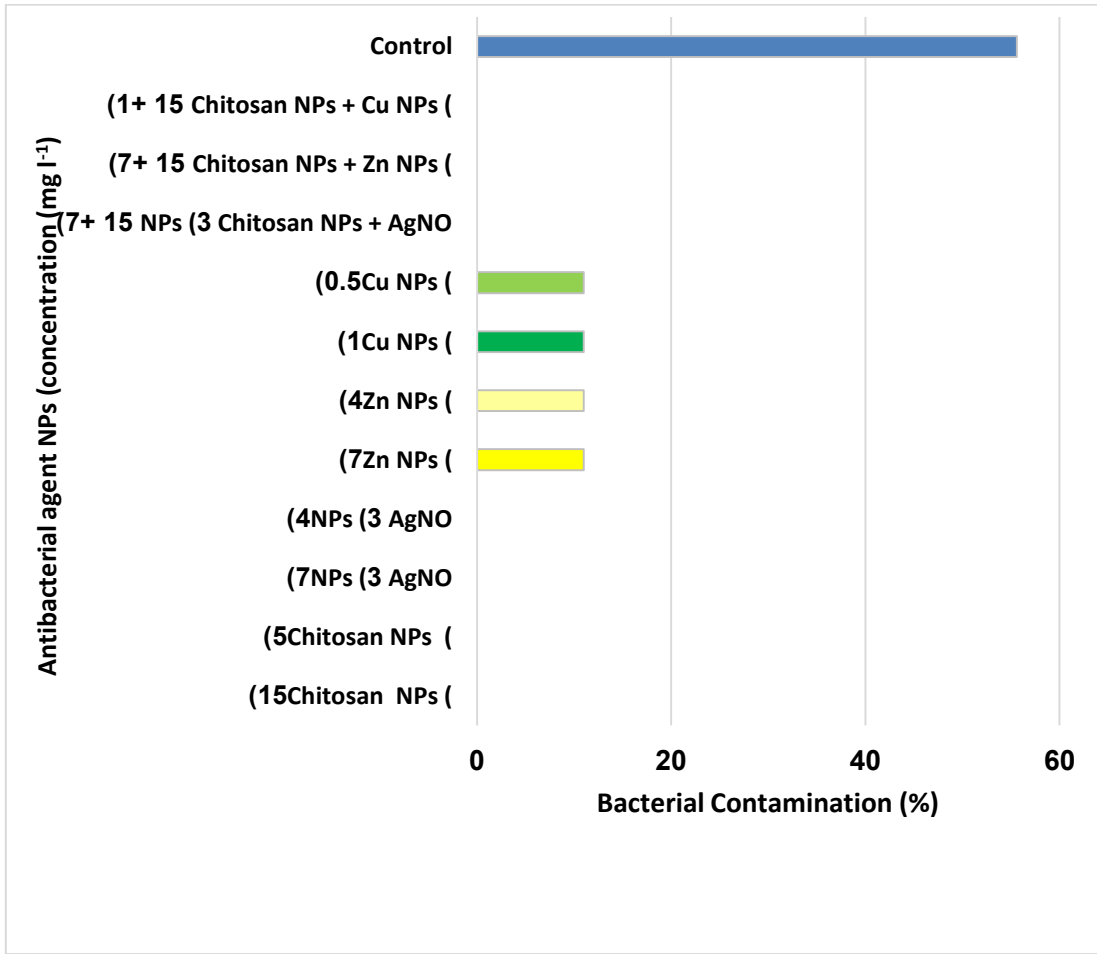

Fig. 5. Effect of different antibacterial agents on bacterial contamination percentage of date palm *in vitro*-shooting stage cultures

obtained with the medium contained Cu NPs at 1 mg l^{-1}. As for the leaves number/ plantlet, the only highly significant increase over control was recorded with the treatment of chitosan NPs + $AgNO_3$ NPs. Other observed increases, yet non-significant, were recorded with other treatments, i.e. chitosan NPs (15 mg l^{-1}). On the other hand, Cu NPs resulted in the lowest number of leaves amongst all treatments. As for the root length measurements, most of the nano-antibacterial agents induced the growth of root to differ significantly from control, to be led by the treatment of chitosan NPs + $AgNO_3$ NPs. The lowest non-significant increases in root length were achieved by Cu then Zn treatments. Finally, it is obvious that the root number/plantlet measurements followed the same general trend of the previously presented parameters; since the highest significant difference over control was achieved by the treatment of chitosan NPs + $AgNO_3$ NPs followed by chitosan NPs. Cu NPs and Zn NPs (7mg l^{-1}) caused significant reductions to the root length of plantlets compared to control.

Table 3. Effect of different nanoparticle formulations of antibacterial agents on the growth characteristics of *in vitro* date palm cultures at the rooting stage

Nano-antibacterial Agent	Conc. (mg l^{-1})	Means of growth parameters of plantlets			
		Plant height (cm)	Leaves number/ plantlet	Roots length (cm)	Roots number/ plantlet
Chitosan NPs	5	24.5 *b*	3.0 *ab*	4.3 *abc*	3.3 *ab*
	15	25.7 *b*	3.3 *ab*	4.8 *ab*	3.7 *ab*
$AgNO_3$ NPs	4	22.0 *c*	2.7 *ab*	3.6 *bcde*	3.0 *abc*
	7	24.6 *b*	3.0 *ab*	3.9 *abcd*	3.3 *ab*
Zn NPs	4	16.7 *e*	2.3 *b*	2.7 *defg*	2.0 *bcd*
	7	11.3 *g*	2.0 *bc*	2.1 *efgh*	1.2 *d*
Cu NPs	0.5	14.0 *f*	1.8 *bc*	1.8 *fgh*	1.3 *cd*
	1	11.3 *g*	2.0 *bc*	2.1 *efgh*	1.2 *d*
Chitosan NPs + $AgNO_3$ NPs	15+7	26.7 *a*	4.3 *a*	5.3 *a*	3.8 *a*
Chitosan NPs + Zn NPs	15+7	17.2 *e*	2.9 *ab*	3.3 *bcdef*	2.7 *abcd*
Chitosan NPs + Cu NPs	15+1	16.3 *e*	2.7 *ab*	3.0 *cdefg*	2.5 *abcd*
Control		20.0 *d*	2.1 *bc*	1.5 *gh*	2.3 *abcd*
LSD (0.05)		1.7	1.6	1.5	0.9

Fig. 6. Date palm plantlets from *in vitro* cultures supplemented with different nano-antibacterial agents. Growth parameters, i.e., plant height (cm), leaves number/ plantlet, root length (cm), and roots number/ plantlet were recorded. Control = no antibacterial agent was added

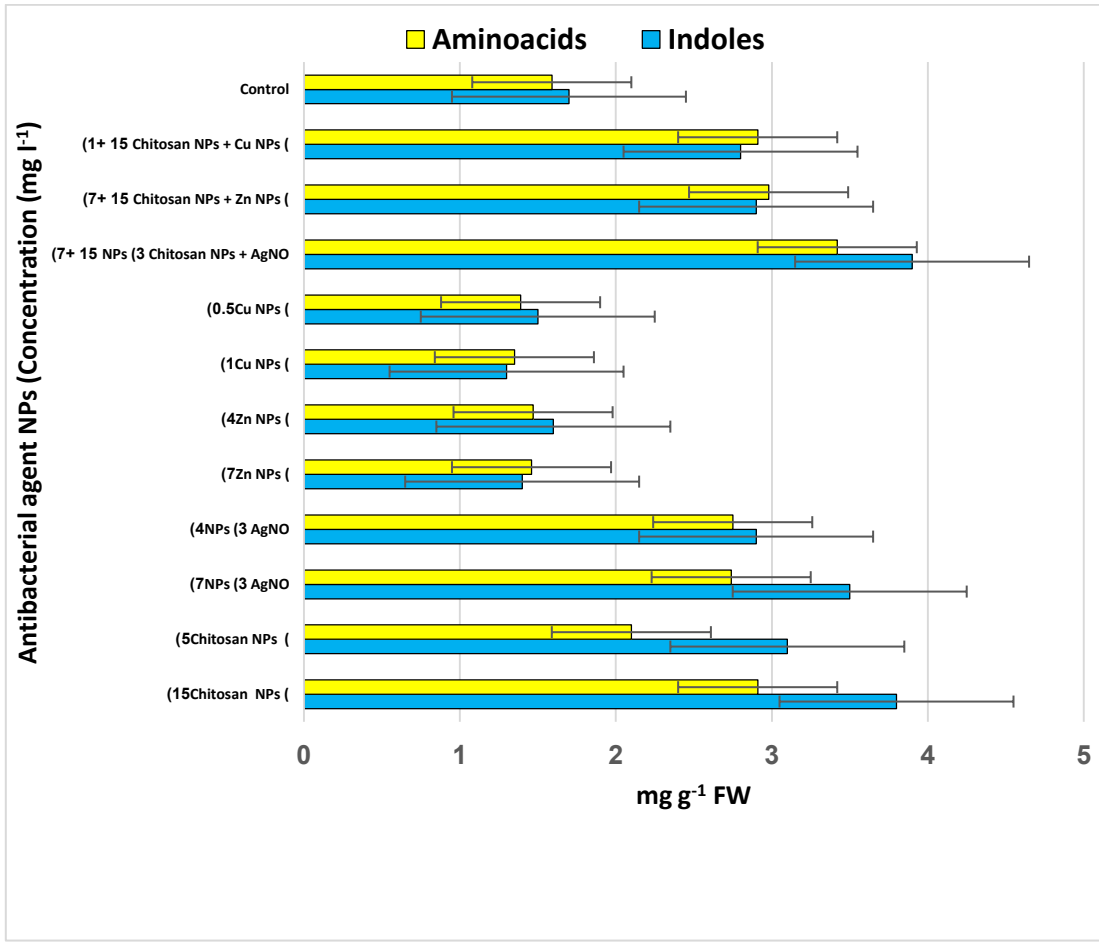

Fig. 7. Effect of different nano-antibacterial agents supplemented into date palm *in vitro* cultures on driven plantlet's content of indoles and total aminoacids

Plantlet Leaf Content of Indoles and Amino Acids

As shown in Fig. 7, except of the Zn NPs and the Cu NPs, which resulted in valued less than the control content, all other tested nano-antibacterial agents considerably changed the amount of indoles and aminoacids detected. For the indoles, chitosan (15 mg l^{-1}) and chitosan + $AgNO_3$ gave the highest significant increase; 3.9 and 3.8 mg g^{-1} FW, respectively, which considered the best levels of indoles in the entire study. As for the aminoacids, a remarkable highly significant increase occurred with the chitosan + $AgNO_3$ treatment (3.42 mg g^{-1} FW), followed by chitosan, chitosan combinations with Zn and Cu and $AgNO_3$.

Plantlet Survival Rate (%) after Acclimatization Phase

This experiment was to evaluate the effect of all antibacterial agents tested in this study on the survival rate of plantlets driven from tissue culture rooting stage, at the acclimatization phase in the greenhouse. The percentage was calculated after 3 months of the transplantation. Results presented in Fig. 8 show that most of the treatments of this study had a positive effect on the plantlet survival rate. The highest survival rate of plantlets was achieved by plantlets recovered from the chitosan NPs + $AgNO_3$ NPs treatment (77.8%) followed equally by chitosan NPs, $AgNO_3$ NPs and chitosan (72.2%). $AgNO_3$ and $AgNO_3$ + sodium thiosulphate resulted in a slight increase in the

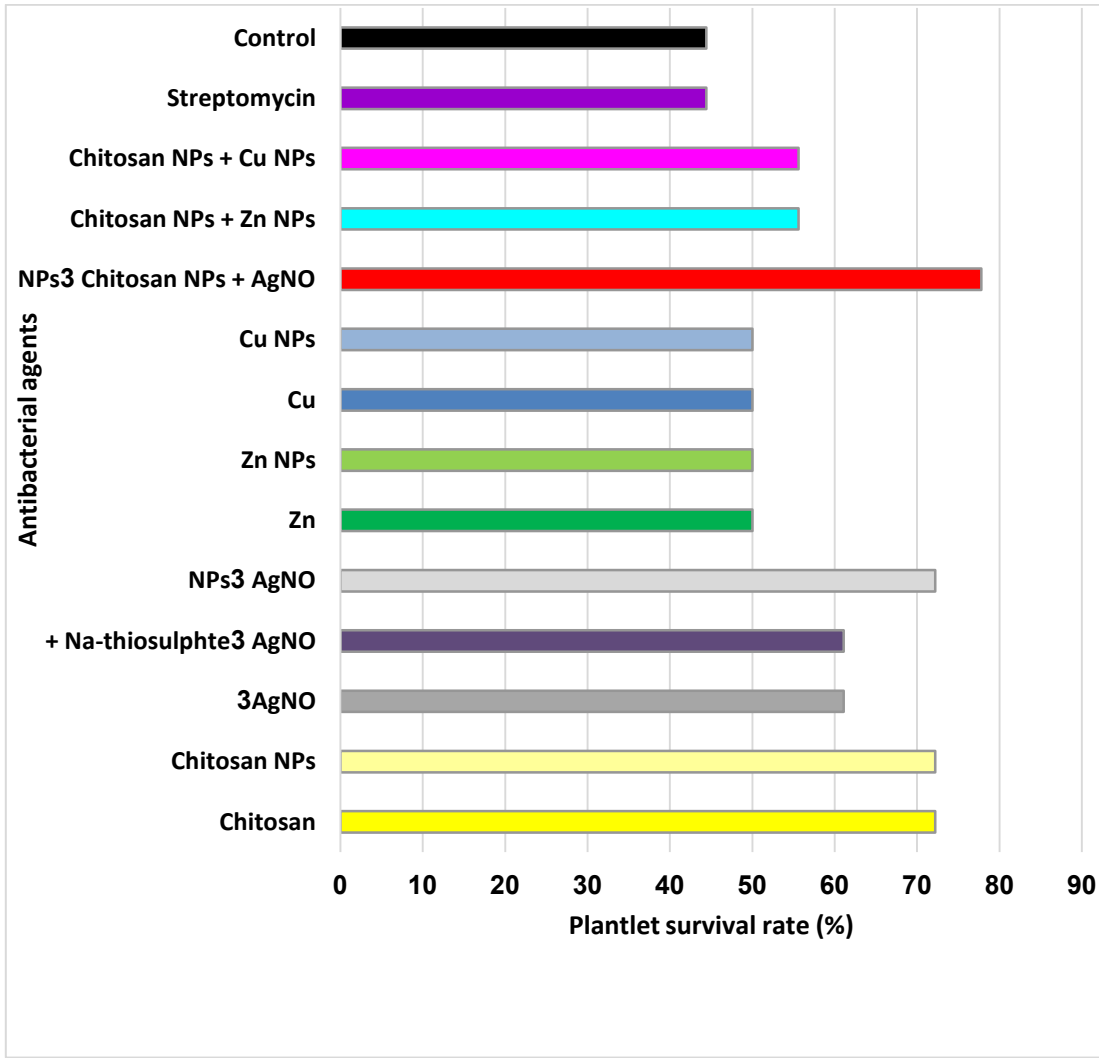

Fig. 8. Effect of antibacterial agents incorporated in date palm in vitro cultures on the recovered plantlet survival rate (%) after 3 months of greenhouse acclimatization

survival rate (61.1%). In the next place, plantlets recovered from chitosan NPs + Zn NPs and chitosan NPs + Cu NPs treatments (55.6%). At the end, the plants recovered from Zn NPs, Zn, Cu NPs and Cu treatments (50%). Streptomycin had no effect on the survival rate as it was equal to the control (44.4%).

DISCUSSION

The present study aimed to investigate the potential of certain antibacterial agents, i.e. chitosan, $AgNO_3$, $AgNO_3$ + sodium thiosulphate, zinc and copper for their potential to eliminate bacterial contamination in *in vitro* date palm cultures in comparison to regularly used antibiotic; i.e. streptomycin and bactericide-free cultures. In addition, the study aimed to investigate the advantages of using the nanoparticles of the aforementioned agents and some combinations of them for the same purpose and finally, to investigate their potential to improve the growth of retrieved plantlets and enhance their survival rate after acclimatization phase.

We could isolate and identify two predominant bacterial isolates from date palm *in vitro* cultures,

Bacillus subtilis and *Serratia marcescens*. *Bacilli* species have been repeatedly isolated from bacterial contaminations of plant tissue cultures; i.e. *Bacillus subtilis and B. cereus* [10], *B. cereus* and *B. subterraneus* [40]. On the other hand, specifically, in Iraqi date palm tissue culture lab, the following bacterial contaminants could be isolated from the cultures; *Bacillus* spp., *Staphylococcus* spp. and *Proteus* spp. [41].

Tested antibacterial agents were added to the *in vitro* date palm micropropagation medium. Chitosan and $AgNO_3$ were the most effective in the inhibition of the bacterial contaminants in the treated cultures. These data support earlier investigations made in this field, which reported that chitosan inhibited the *in vitro* growth of *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecium*, *Lactobacillus rahmnosus*, *Pseudomonas lachrymans* [42,43].

Chitosan has been reported to be one of the efficient natural antibacterial agents. The ability of chitosan to retard its growth was formerly confirmed by López-Mata et al. [44]. The inhibitory activity of chitosan against bacteria depends on type of bacteria (Gram-positive and Gram-negative), the concentration and molecular weight of chitosan, exposure time, type of solvent if there is any, and abiotic factors [45]. The inhibitory effect of chitosan was investigated and attributed to reducing bacterial respiration (López-Mata et al. 2013), the electrostatic bond built between the negatively charged bacterial cell wall, or the residues on its surface and the positively charged - cationic - chitosan [46], its ability to bind to and weaken the barrier function of the outer membrane of Gram-negative bacteria [47,48], its ability to interact with the bacterial cell membrane, in which it increases membrane permeability that leads to the loss of intracellular substances or allowing foreign substances to enter the cell, eventually leads to killing the cell [47].

On the other hand, the current results showed that chitosan stimulated better growth of the date pam plantlets either in its ordinary form or in its nanoparticle form. This is consistent with [49] who found that chitosan improved development, and stimulated related parameters (i.e. shoot length, shoot and root dry weight) of treated grapevine plantlets. Similarly, in many former studies, chitosan, either supplemented in culture medium or foliar-sprayed, stimulated plant regeneration and growth parameters of plantlets in *in vitro* cultures and acclimatization stage of various plant species [16,50-56]. This positive effect may be due to the effect of chitosan in improving photosynthetic systems, which is closely related to the growth the growth and biomass production and increasing the antioxidant capacity of the plant [49,57,58].

Our results showed that $AgNO_3$ (4 and 7 mg l^{-1}) was as effective as chitosan in inhibiting bacterial growth in *in vitro* cultures of date palm. This is in agreement with [59] who obtained a minimum contamination percentage in the date palm cv. Barhee MS medium treated with silver nanoparticles at 4 mg l^{-1} and chitosan nanoparticles at 4 mg l^{-1}, in addition to the highest survival percentage of the plantlets acclimatized in the greenhouse. Parzymies [60] found that a treatment of Ag (NPs) at 5 mg l^{-1} to *Aldrovanda vesiculosa* (waterwheel plant) tissue cultures reduced the bacterial contaminations. Habiba et al. [61] recommended the use of 2.5 and 5 mg l^{-1} $AgNO_3$ to inhibit the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacteria, respectively. $AgNO_3$ (NPs) controlled the microbial infection from explants and a positive role of Ag (NPs) was demonstrated for callus induction, organogenesis, somatic embryogenesis [62].

In addition to its antibacterial effect, silver nitrate ($AgNO_3$) at its ordinary molecular form as well as its nanoparticle form has profound effects on the date palm plantlet development and growth (i.e. plant height, leaves number/plantlet, root length and roots number/plantlet). Those results are consistent with many former investigations that used $AgNO_3$ at concentrations ranges from 2 to 10 mg l^{-1}, which are relatively close to this study, in micropropagation cultures of various plants such as date palm cv. Sakkoty [63], date palm cv. Medjool [64], potato [65,66], banana [67], olives [68,69], lemon [70], wheat [71], *Hibiscus sabdariffa* [72] and *Prosopis cineraria* (L.) Druce; a medicinal tree [73]. There is an evidence that silver ion (Ag^+) is able to or due to its characteristic electrostatic attraction and affinity to

sulphur proteins, it can adhere to the cell wall and cytoplasmic membrane and changes the latter, consequently, cell death [74,75].

In the present study, both chitosan and $AgNO_3$ antibacterial and growth stimulator positive effects was more evident in their nanoparticle form. The experimentally proven fact is, there has been an increasing success of using nanoparticle materials (NPs), over the antibiotics and traditional bactericides, as antibacterial agents. Although the detailed antibacterial mechanisms of NPs are poorly understood, but the currently accepted mechanisms include oxidative stress induction through the generation of reactive oxygen species, leading to cell death [76], metal ion release that interfere with the cell granules leading to the formation of condensed particles [77], or non-oxidative mechanism, i.e. the cell membrane damage leading to cell death [78]. In general, it has been proposed that nanoparticle size, core composition, shape, surface properties, purity, stability, and method of manufacturing are responsible for nanoparticles reactivity with biomolecules [79,80].

Although earlier studies demonstrated the antimicrobial role for Zn and Cu [81], unexpectedly, the results of this study showed that Zn and Cu in their ordinary form were impaired compared to the rest of tested agents. Moreover, they negatively affected the plantlet growth. Noteworthy, supplementing the culturing medium with Zn or Cu in their ordinary forms was less harmful to the plantlet than their nano-formulation, as the growth parameters of the plantlets showed better measurements in the first case than the latter. The negative effect of copper and zinc on plant growth could be attributed to a decrease in photosynthetic rate and limitations in photosynthesis [82]. Nanoparticles of copper oxide (CuO NPs) produced disruption of mitochondria, dilation of chloroplast membrane, distortion of stroma and grana of the chloroplasts, and alteration of photosynthetic pigments [83].

Treatment of streptomycin at the recommended concentration had a little or no detrimental effect on the growth parameters of the date palm plantlets. Although this represents the common status in this field, reports varied about the effect of antibiotics on plantlet growth. Streptomycin is considered as the best tested antibiotic to reduce contamination and to enhance the growth of potato plant tissue [84] and its application at lower concentrations (10 and 20 mg l^{-1}) improved the vigour of potato plantlets, whereas all the concentrations of gentamycin had negative effect on all the morphological characters [85]. Panathula et al. [86] reported that bavistin (150 mg l-1), cefotaxime (100 µM l^{-1}) and kanamycina (80 µM l^{-1}) promoted plantlet growth parameters while a higher concentration of kanamycin (100 µM l^{-1}) Kanamycin caused reduction of *Centella asiatica* L. shoot regeneration.

The combinations of chitosan with Cu or Zn completely inhibited the bacterial contaminants in the date palm *in vitro* cultures. On the other hand, those treatments significantly, increased two of the growth parameters (i.e., root length and leaves number), whereas significantly reduced the plant height. In this context, Cu-chitosan NPs increased the plant height, stem diameter, root length and roots number in maize [87].

Survival rate of transplanted date palm plantlets during acclimatization stage reacted differently to various treatments. The best survival rate was obtained by combination of chitosan NPs and $AgNO_3$ NPs followed by $AgNO_3$ NPs, $AgNO_3$ and chitosan in both forms. Chitosan has been reported to have a positive effect on plantlets during acclimatization, extensively and repeatedly for orchid plants [50,88-91] and for banana [92].

In the present study, a positive relationship was observed between the maximum effect of antibacterial agents on preventing/reducing bacterial contamination in *in vitro* cultures and their effect on the plantlet leaf content of amino acids. This is in line with [93] who found that $AgNO_3$ increased proline contents of *in vitro* strawberry and [94,95] who found that chitosan stimulated the production of amino acids.

CONCLUSION

The present study can produce broader seeing effects of different anti-bacterial treatments as the ordinary form or as NPs, on date palm in vitro cultures to eliminate the harmful effects of

bacterial that can be lead to cultures death. In general, chitosan, $AgNO_3$ as the ordinary form or as NPs at tested concentrations or either in their combination in between, Chitosan and $AgNO_3$ possess considerable unique properties that make its could be used as an inhibitor agent against in wide range of vitro microbial. Additionally, the plants growth promotion effects of these agent, likewise, the useful effects of these agent on desirable contents leaves chemical contents.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this published article.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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