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Biologically synthesized silver nanoparticles eclipse fungal and bacterial contamination in micropropagation of *Capparis decidua* (FORSK.) Edgew: A substitute to toxic substances

Jyoti Ahlawat[1], Anita Rani Sehrawat[1], Ravish Choudhary[2] & Shiv Kumar Yadav[2]

[1]Department of Botany, Maharshi Dayanand University Rohtak, Haryana-124 001, India
[2]Division of Seed Science and Technology, ICAR-Indian Agricultural Research Institute, New Delhi-110 012, India

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Microbial contamination is a serious challenge in plant tissue culture, particularly in micropropagation of threatened and rare medicinally important plants for conservation purpose. Use of antibiotics exhibit harmful effects on plants, and continuous use makes bacteria more resistant. Also, chemicals used to control such contaminations are either toxic to the explant or have limited efficiency. Though nanobiotechnology offers an effective alternate to deal with the bacterial and fungal contamination, chemical synthesis of metal nanoparticles has limitations and found to be toxic, flammable and hard to get disposed. Green synthesis of silver nanoparticles (AgNPs) employing plant extracts, being environment friendly, cost-effective, and single step, is gaining attention as better alternative method. In this study, the green synthesised silver nanoparticles were confirmed by UV-Vis spectroscopy (462.73 nm, 0.473 Abs) and Transmission Electron microscopy (TEM). The fruit extract of *Capparis decidua* served as an environmentally benign reducing agent and the phytochemicals of the extract as non-toxic agent to stabilize the AgNP (FTIR) upholding its significance as an eco-friendly approach compared to hazardous chemicals. The nano size (1.5-15 nm) makes the green synthesized AgNPs a better antimicrobial agent allowing easy diffusion into the cells. Evaluation of decontamination as well as the survival rate of the explants was monitored using the explants (shoot tip and nodal segment) immersion in three different concentrations of AgNP solution (100, 300, and 500 mg/L) and controlled by 0.1% mercuric chloride treatment demonstrating promising decrease in decontamination. However, the survival was expedient excluding immersion in 100 mg/L for 20 or 30 min. The MS media supplementation by AgNP solution (50, 100, 300 and 500 mg/L), controlled by 70% ethanol treatment divulged the superior decontamination rate at 150 mg/L of AgNPs (90.2% for bacteria and 94.4% for fungal contamination) with 80.5% survival. The increased concentration gave 100% bacterial and 98.6% fungal decontamination but a reduced survival percent (68.5%). This work potentially showed that nanosized AgNPs could serve as an appropriate antimicrobial substitute to chemicals being innocuous to the explant regeneration.

Keywords: Bare caper, Caper berry, Green synthesis, Karira, Microbial contamination

Plant tissue culture is an efficient and viable technique for maintaining biological diversity, conserving threatened and rare medicinally important plants[1]. The most important step of the entire process to get an efficient micropropagation is its successful initialization which can be tolled by microbial contamination. The explants from fields and the environmental state of laboratory is the grievous source of contamination. Various techniques, such as use of antibiotics and toxic substances as decontaminating agents in tissue culture process are being employed to contain the contaminants. Antibiotics are either microbiocidal or bacteriostatic. Most antibiotics show prohibitory effects on the plants. Continuous use of antibiotics renders bacteria more resistant to them. Some chemical moieties are being used to control these contaminations; however, these chemicals are either toxic to the explant or have limited efficiency. For instance, ethanol reduces the survival rate of explants in tissue culture by complete browning of the tissue or by bleaching it; whereas, due to high toxicity of mercuric chloride its usage demands precautionary measures[2]. The threatening challenge to medical and health lines is the lack of success towards infectious diseases due to enhanced resistivity to antibiotics. The current status demands an effective alternate to eliminate the bacterial and fungal contamination[3].

Nanobiotechnology is an emerging field casting wide applications of metal nanoparticles *viz*. silver, gold, titanium and platinum as antimicrobial agents[4]. Metallic nanoparticles were ordinarily been

*Correspondence:
Phone: +91 9811095991 (Mob.)
E-mail: j.10dec@gmail.com (JA); ravianu1110@gmail.com (RC)

synthesized by physical and chemical methods, however, the chemicals used are often toxic in the process, are flammable and are not disposed of easily in the environment. The green synthesis of silver nanoparticles employing plant extracts, being environment friendly, cost-effective, and single step, is advantageous alternative to other way of synthesis (i.e. chemical and physical methods) and can be efficiently accounted for expensive synthesis[5,6]. Few disinfecting agents were found unsuccessful in eliminating contamination in explants, rather they affected the organogenesis to a higher extent due to their phytotoxicity.

Silver nanoparticles evidenced to be most effective than nanoparticles of copper[4], zinc, titanium[7], magnesium and gold, as they display formidable antimicrobial potential against various microorganisms[8-10]. Nano-silver, at low concentration, has a good potential to eliminate contamination without affecting the plant growth factors[11]. Recently, Kalia *et al*.[12] have demonstrated the antimycotic efficacy of biogenically synthesized silver nanoparticles using *Trichoderma harzianum* hyphal or mycelial extract against plant pathogenic fungus *Fusarium moniliforme*. Though the mechanism of action of nanosilver is not completely understood, it portrays strong aspects of potential to prevent contamination due to the working environment. The extensive antimicrobial activity of nanosilver causes inactivation of bacterial transport chain *in vitro* by binding to the sulfhydryl group of enzymes involved in the metabolic activities, and also cohere to the microbial DNA to arrest the bacterial replication[13,14]. The enhanced need of silver nanoparticles as an anti-microbial agent raise an alarm to the research field to synthesize AgNP of various characteristics, such as shapes, sizes, chemical composition and manageable polydispersity that decides their effectivity of penetrance and expressivity against microorganisms[15]. The chemical method of AgNP synthesis is the most common process till now, however, it involves hazardous chemicals in the procedure. The first report of effective control of bacterial contamination was by Abdi *et al.*[16] in *Valeriana officinalis*, where the nodal explants were sterilised using 70% ethanol, 10% clorox (containing 5.25% sodium hypochlorite) followed by 100 mg/L AgNP, resulting 89% decontamination without affecting the growth parameters (shoot multiplication and rooting).

The present study provides an alternative green method to synthesize AgNPs using fruit extract of a common herb used in folk medicine, called Bare caper or Karira, *Capparis decidua* (FORSK.), as a reducing agent. In addition, the present work also evaluated the potential of green synthesized silver nanoparticles to eradicate fungal and bacterial contaminants in the explants of *C. decidua*.

Material and Methods

Chemicals and Plant material

The fruits/seeds were washed under running tap water followed by surface sterilising with water supplemented with 0.01%Tween-20 for 30 min. After complete removal of detergent by washing thoroughly under tap water, the explants were submerged in a solution of 0.05% bavistin to remove fungal contamination from the fields for green synthesis of silver nanoparticles. For tissue culture, explants i.e., shoot tips and nodal segments were separated and cut (5-10 cm), and were surface sterilized by washing with the 0.01% Tween-20 in water. A final wash for one hour was given under running tap water. Rest of the procedures were carried out in a laminar flow. The explants were cut into 1.5-2 cm pieces before inoculating in MS media.

Green synthesis of silver nanoparticles

Fruits of *Capparis decidua* were collected from (Bani) fields of Kamal Farms, Manesar, Gurugram, Haryana (India) in the month of Feb-March 2017. About 50 g of fruits were thoroughly sterilized, washed in distilled water, dried at room temperature (25°C) and then dried in an oven set at 40°C and were crushed in 250 mL of sterile distilled water. The mixture was then heated on a water bath to 70-80°C for 10 min and were centrifuged at 20000 rpm for 30 min upon cooling. The supernatant was then used for silver nanoparticle synthesis and the remaining aqueous extract was stored at 4°C for further use. On treatment of 200 mL of aqueous fruit extract with 800 mL of 1.0 mM of silver nitrate (HI MEDIA, India), the stable silver nanoparticles were formed when the reaction mixture was kept at room temperature for 24 h. The reaction was monitored as change in colour over time. Once the pinkish brown colour stabilized (Fig. 1), the AgNP solution was subjected to the characterization via TEM, UV-Vis spectroscopy and FTIR.

Preparation of explants and different concentrations of AgNP solution

In first experiment, these pre-sterilized explants were soaked in 3 different concentrations *viz.*, 100, 300

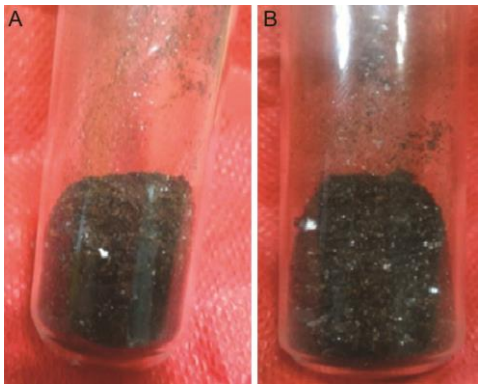

Fig. 1 — Green synthesized silver nanoparticles (AgNPs) via fruit extract of *Capparis decidua* in dried form

and 500 mg/L of AgNP solution for three different immersion times (i.e., 20, 30 and 60 min). The experiment was controlled by 0.1% mercuric chloride treatment. Simultaneously, in another experiment, the explants were treated with 70% ethanol for 2 min as a control treatment, followed by washing with distilled water to remove traces of ethanol. The explants were then cultured on MS1 (BAP 5 mg/L, NAA 0.1 mg/L, adenine sulphate 10 mg/L, citric acid 25 mg/L) and MS2 media (BAP 5 mg/L, NAA 0.1 mg/L, adenine sulphate 10 mg/L) at pH 5.8, supplemented with 50, 100, 150 and 200 mg/L of AgNPs[16]. Fungal and bacterial contamination and *C. decidua* viability percent were scored after one week.

Experimental design and statistical analyses

Soaking experiments were conducted in three replications and each experiment was conducted thrice. The second (media supplementation) experiment was replicated in 24 tubes and was also conducted thrice. The data was tested for difference in the significance level using ANOVA. Further, the significance among the means was calculated by HSD Tukey test. P value less than 0.05 was considered as significant.

Results and Discussion

Biological synthesis of silver nanoparticles and its characterization

Diverse plant materials, due to presence of phytochemicals, secondary metabolites, etc have potential of synthesizing silver nanoparticles (AgNP), acting as a better alternative to the traditional chemical methods[17]. The biologically synthesized silver nanoparticles using fruit extract of *Capparis decidua* acting as a good reducing agent were significantly identified and characterized using UV-Vis spectro scopy and Transmission Electron Spectroscopy. The UV-Vis spectroscopy (SHIMADZU UV-Vis spectro-

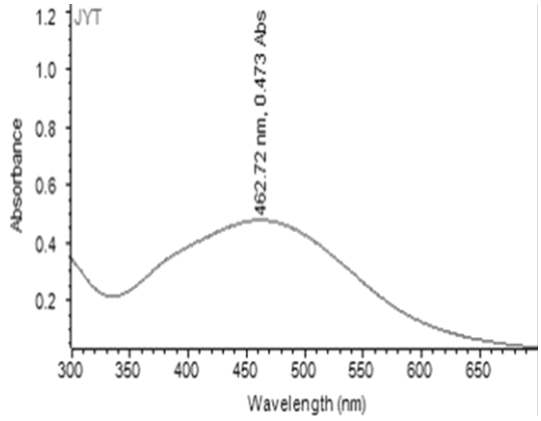

Fig. 2 — Fruit mediated AgNPs showing 0.473 absorbance at 462.72 nm wavelength in UV-Vis spectroscopy

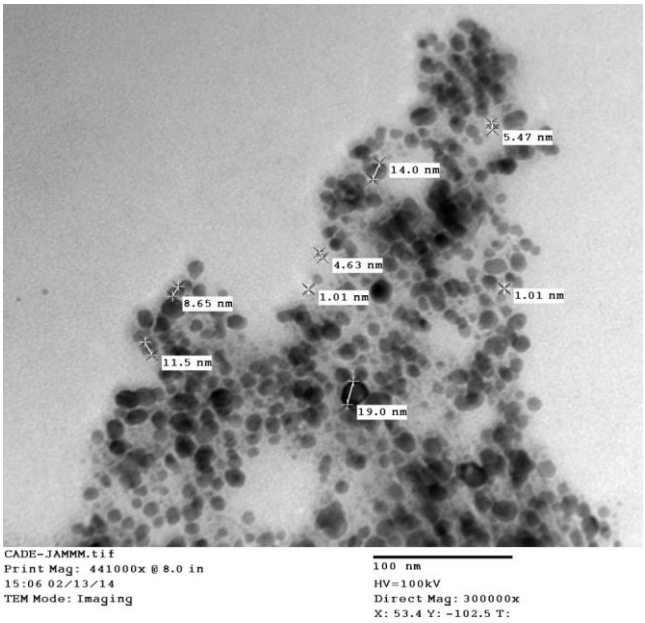

Fig. 3 — TEM images of AgNPs synthesized using fruit extract of *Capparis decidua*

photometer 2450) showed the maximum absorbance at 460 nm (Fig. 2). The stability of nanoparticles after two months depicts almost no shift in the absorption intensity and the absorption maxima which indicated that the particle size remained the same. The broadened peak specified the polydispersity of particles. Observation of identical results found earlier in case of silver nanoparticles synthesis using the leaf extract of *C. decidua*[18]. The peak of absorbance was at 462.72 nm, 0.473 Abs.

The morphology of the particles was predominately of circular, triangular, rectangular and oval shape (with size range of 1.5-15 nm as per TEM (Fig. 3).

The FTIR analysis, with the characteristic peaks and their functional groups, showed the potential biomolecules that capped and efficiently stabilised the silver nanoparticles of *C. decidua* synthesized using the fruit extract (Fig. 4). The broadened peaks specified the polydispersity of the nanoparticles. The capping was confirmed by the existence of bands. The absorbance band peaks were observed at 3282, 1637, 585,553, 531 and 499 cm^{-1} in the region of 470-4000 cm^{-1}. The absorbance peaks were analyzed based on the table adopted from Pongpiachan[19]. The peak observed at 3282 represents N-H stretch (amines and amides), The peak at 1637 i.e. between the range of 1614-1640 cm^{-1} is C=C bond in alkene rings and C=C stretch of aromatic rings 585, 553 and 531 cm^{-1} lies in the range of 690-515 cm^{-1} is C-Br stretch, which is characteristic of alkyl halides, whereas 499-470 cm^{-1} range may also be assigned to the first overtone mode of the methyl torsion vibration. Stabilization of the nanoparticle depends upon the functional groups like amines, amides, alkynes, alkenes, bromoalkanes, etc. as identified by FTIR analysis of fruit extract and Fruit extract synthesized AgNPs. The green synthesis of silver nanoparticles from *C. decidua* supported that the fruit extract can act as a good template for the biological synthesis of AgNPs by playing a dual role i.e., a capping as well as a reducing agents due to the presence of several plant phytochemicals, such as fatty acids, oxygenated heterocyclic constituents, sterols, alkaloids, flavones, and an isothiocyanate glucoside[20] in different parts of the *C. decidua* plant which serves as reducing agent. Similar results have been reported in berry extract of *Solanum xanthocarpum*[21].

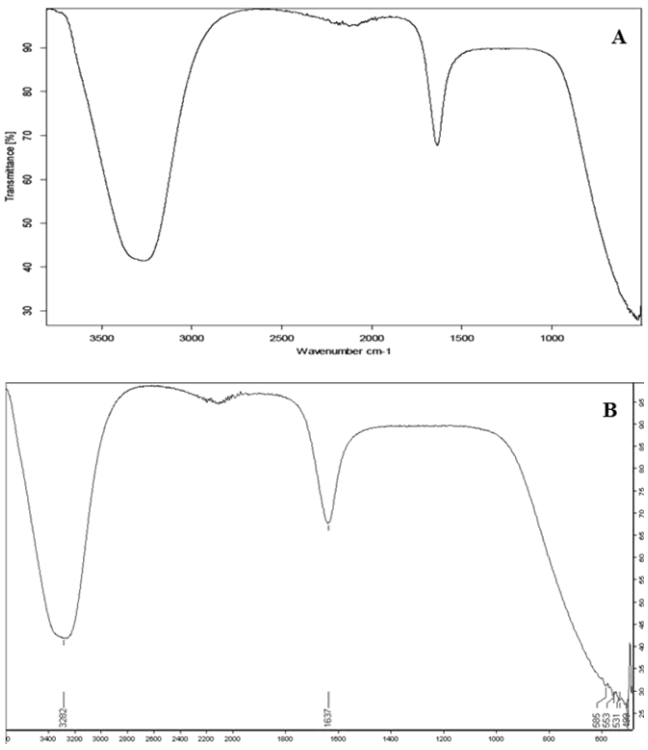

Fig. 4 — FTIR spectra of (A) fruit extract; and (B) Fruit extract synthesized AgNPs of *Capparis decidua*

Green synthesized AgNPs as decontaminating agents in plant tissue culture

Interaction between various conc. of AgNPs and explants (*Soaking method*)

Soaking of the explants in the green synthesized and dried AgNP were used in solution form to sanitize the explants completely (ANOVA; F= 16; P <0.001) and also effects the survival rate (ANOVA; F= 23.33; P <0.001). The experiment showed that 100 mg/L of AgNP sterilized the explants completely within 20 min of soaking with 95.8% survival rate which was significantly higher than the corresponding control treatment (79.16%) (HSD Tukey; P <0.05). After 30 min of soaking in 100 mg/L AgNPs, the survival was same as that of the control, however, it decreased to 62.5% after 60 min of soaking (HSD Tukey; P <0.05). Soaking of the explants in AgNPs at the concentrations ascended to 100 mg/L concentration led to the substantial decrease in the survival rate (HSD Tukey; P <0.01); although the decontamination remained 1absolute (Fig. 5A-C and Fig. 6) The results indicate that the AgNP of *C. decidua* acts as a decontaminating agent while innocuous to the plant itself. The obtained results favour the pre-treatment of the explants with 5% Teepol for 10 min and subsequent washing before surface sterilisation. The pretreatment was found effective even in the explants brought directly from the fields and which are supposed to have high as well as diverse level of pathogenic contamination.

Effect of various conc. of AgNP on bacterial, fungal contamination and regeneration of explants (*MS Media supplementation method*)

The results of second experiment, specified that media supplementation of various concentrations (50, 100, 150 and 200 mg/L) of AgNP had a remarkable outcome on bacterial (ANOVA; F= 7.72; P <0.005) and fungal decontamination (ANOVA; F=9.21; P <0.005) as well as on the survival rate of explants (ANOVA; F=14.28; P <0.0005). 150 mg/L of AgNP resulted in the highest rate of survival (80.5%) (HSD Tukey; P <0.01) with bacterial (90.2%) and fungal (94.4%) decontamination, which was again significantly

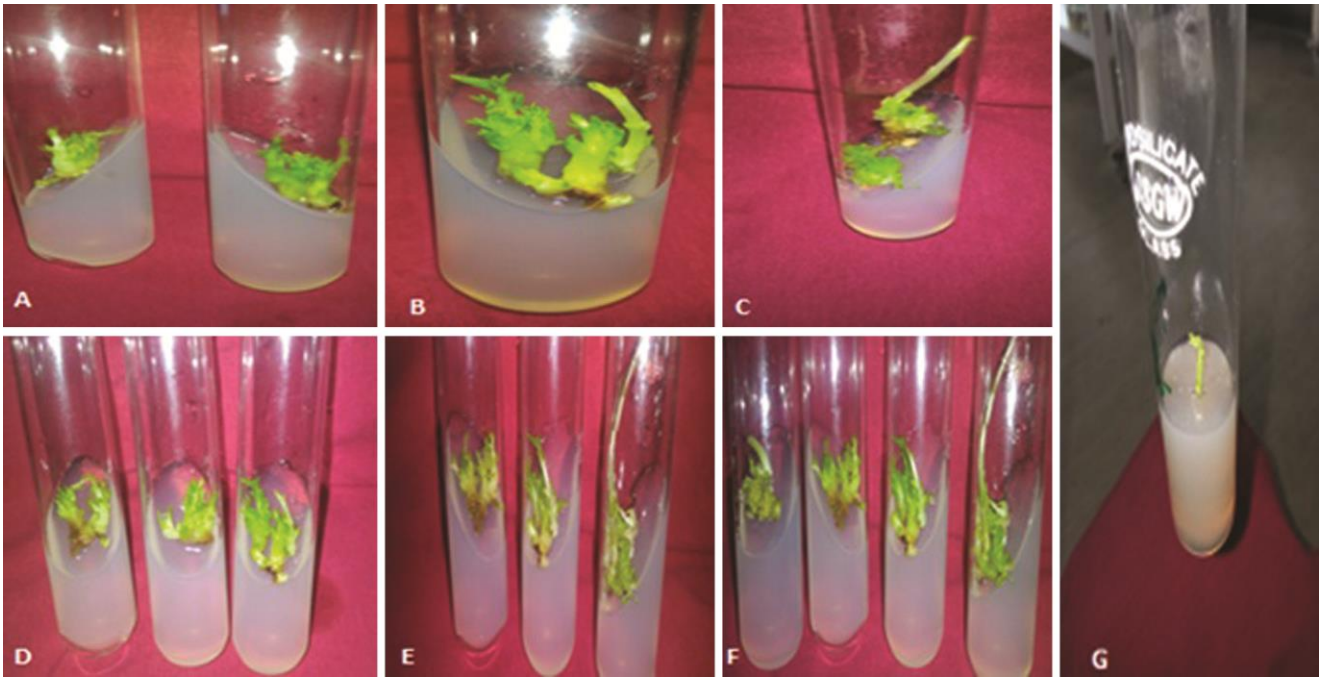

Fig. 5 — Antimicrobial potential of green synthesized silver nanoparticles in tissue culture media (A-F). [(A-C) 100 mg/L AgNP in soaking media decontaminates the culture 100%; (D-F) Stages of development at 150 mg/L of AgNP supplementation in tissue culture media yields a healthy, decontaminated culture with no phenol exudation and (G) Control showing phenolic exudation]

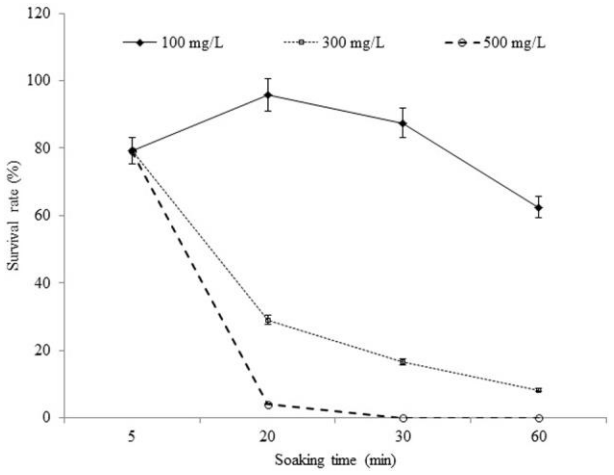

Fig. 6 — Analysis of survival rate of explants when soaked in various concentrations of AgNPs at different time intervals

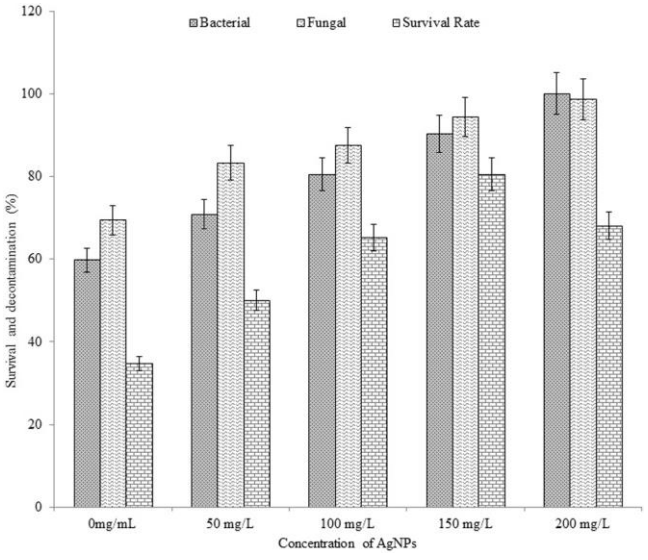

Fig. 7 — Survival rate of explants, fungal and bacterial decontamination when various concentrations of AgNPs were supplemented in the Media

higher than the control (HSD Tukey; P <0.05 and <0.01, respectively). The nano dimensions i.e 1.5-15 nm of AgNP synthesized from the fruit extract of *C. decidua*, seems to be an important contributory factor in clearing the fungal contamination[11,17]. At higher concentration of 200 mg/L AgNP in tissue culture media, bacterial and fungal decontamination reached to maximum that is 100% and 98.6, respectively however, the survival rate was decreased to 68.05% (Fig. 5D-F and Fig. 7).

Whilst, the ascending concentrations of AgNPs remarkably decreased bacterial contamination in soaking and media supplementation experiments, increased concentrations of AgNPs had a harmful effect on the survival rate of the explants. A noteworthy observation about the immersion of explants in the AgNP solution

is that it affects more adversely than that of the media supplementation as concluded from the results obtained on survival percentage and regeneration of plantlets. The statistical analysis has specified that the perfect treatment for decontamination and maximum survival rate of explants were 100 mg/L in the soaking treatment and 150 mg/L in media supplementation experiment. The results here demonstrated a remarkable difference with other treatments as they achieved highest survival rate and an optimum level of decontamination.

Additionally, it was noted that MS2 (deprived of citric acid) yield similar results as of MS1 with negligible phenol exudation leading to a conclusion that AgNP can be served as a replacement to citric acid in successfully eradicating phenolic exudation (Fig. 5G). Green synthesized silver nanoparticles could be used as an antimicrobial agent showing no harmful effects on the plant growth parameters[12]. In the present study, we observed the nano size of biosynthesized AgNPs (1.5-15.0 nm) from the *C. decidua* fruit extract to play a remarkable role in decontaminating the explants as smaller the size higher the antimicrobial capacity. Similarly, Syu *et al*.[22] demonstrated the AgNPs size and shape impacts on the plant growth and gene expression in *Arabidopsis*. Mahna *et al*.[23] reported the potent efficiency of silver nanoparticles on surface sterilisation of tomato cotyledons, arabidopsis seeds and potato leaves, 100 mg/L completely (100%) decontaminated the cotyledons and seeds leaving no harmful effect on the viability of the explants. However, the survival rate of cotyledons and seed germination was reduced when exposed/treated to the increased concentrations of silver nanoparticles.

High concentration of AgNPs 300-500 mg/L was found to be cytotoxic in our work and likewise, Abdi *et al*.[16] indicated that 100 mg/L NS had a good potential of removing the bacterial contaminants in plant tissue culture procedures. Sarmast *et al*.[24] also reported surface sterilization by nanosilver using concentrations of 200 mg/L for reducing decontamination range from 61.5 to 11.3% and concentrations of 400 mg/L nanosilver attained the reducing range on decontamination from 81.25 to 18.75%. They reported that nanosilver played no harmful role in the growth and development of the plant. Some other nanoparticles like Cu, Ce, Feand Zn when tested at optimum concentrations have also shown improved growth parameters and checked fungal infection in plants[12,25]. Our work reports safe concentration levels *viz*. 100 and 150 mg/L in soaking and media supplementation experiments and reduced the *in vitro* contamination to a significant level. Soaking explants in nanosilver (NS) solution was reported to eliminate microorganisms in tissue culture of *Valeriana officinalis* L[16]. Our experiment results concurrent with earlier report that silver nanoparticles has significant effect on bacterial decontamination[26]. These results were supported by Safavi *et al*.[13] who demonstrated that surface sterilisation of explants when followed by AgNPs treatments in culture media displayed a noteworthy effect on decontaminating bacterial and fungal infections. Concentrations above 200 ppm NS in soaking and media supplementation experiment starts lowering the regeneration and viability of buds. Findings of the present study are in agreement with Arab *et al*.[27], those of that reported using high concentration of AgNPs is highly toxic to regeneration of plants rather up to 150 mg/L, of AgNP as a disinfecting agent in plants tissue culture media is recommended. Our results too reveal that AgNPs can be an efficient tool for removing contaminants from plant tissues, only if the right dose and exposure time are used. Mahna *et al*.[23] and Jain *et al*.[28] also described a cost effective and eco-friendly technique for green synthesis of silver nanoparticles from 1.0 mM $AgNO_3$ solution using papaya fruit extract as reducing agent. The use of green synthesized nanoparticles and assay of its antimicrobial[29] and viral[30] activities in various fields is receiving attention from many researchers because of their eco-friendly nature[31-34]. The potential of green synthesized silver nanoparticles using fruit extract of *Capparis decidua* in eradicating the fungal and bacterial contaminants could be attributed to its richness in plant phytochemicals, such as sterols, fatty acids, flavones, oxygenated heterocyclic constituents, alkaloids and an isothiocyanate glucoside[30] in different parts of the plant which serves as reducing agent in reducing Ag+ to silver nanoparticle.

Conclusion

In the present study, we experimented permissible concentrations of silver nanoparticles (AgNPs) from fruit extract of *Capparis decidua* in soaking and media supplementation for decontamination and survival rate of *C. decidua* explants in tissue culture. The green synthesized AgNPs (1.0-15 nm) did not have any toxic effects. Findings of this study suggest a better alternative method of simple, rapid and eco-friendly

synthesis of AgNPs from *C. decidua*. The soaking experiment has higher rate of decontamination which may be indicative of higher degree of diffusion due to higher concentration differences and more exposed surface area. The findings recommend that lower concentrations of AgNP (1.0-15 nm), being innocuous to plant growth factors and leading negligible phenolic exudation in the tissue culture, may be considered as a replacement of antibiotics and other chemicals as a decontaminating agent in tissue culture process.

Conflicts of interest

The authors declare no conflict of interests.

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