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In vitro application of silver nanoparticles as explant disinfectant for date palm cultivar Barhee

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Abstract

The objective of the research study was to determine the effect of different concentrations of silver nanoparticles (1, 5, 10 and 20 mg L^{-1}) alone and in combination with commonly used disinfectants (80% sodium hypochlorite and 0.2% mercuric chloride) on *in vitro* grown explants of date palm *cv.* Barhee. Seventeen treatment combinations were made to study the survival, contamination and mortality percentage of *in vitro* grown date palm explants. The laboratory experiment was laid out on completely randomized design with three replicates in each treatment. The findings revealed that application of 5 mg L^{-1} silver nanoparticles alone and in combination with 80% sodium hypochlorite and 0.2% mercuric chloride statistically behaved alike. However, maximum survival of explants (88.89%) and zero percent mortality was observed when 5 mg L^{-1} silver nanoparticles was used alone. Higher concentrations of silver nanoparticles (10 and 20 mg L^{-1}) when combined with sodium hypochlorite and mercuric chloride had a detrimental effect and caused highest explant mortality. Application of sodium hypochlorite and mercuric chloride showed 33.33% contamination and 11.11% explant mortality. It is therefore, concluded to use 5 mg L^{-1} silver nanoparticles alone for explant sterilization of date palm *cv.* Barhee, which is non-hazardous and environment friendly.

Key word: Date palm, *Phoenix dactylifera* L., explant sterilization, disinfectants, silver nanoparticles

Introduction

Date palm (*Phoenix dactylifera* L.) is extensively grown in many parts of the world including Egypt, Iran, Arabian Peninsula, Algeria, Iraq, Pakistan and Tunisia. Egypt is the world largest date producing country *i.e.* more than 1.5M tonnes/annum. (Food and Agriculture Organization, 2013). Date palm is commonly propagated by ground offshoots, however, a female date palm produces only 10-20 offshoots in its entire life (Zaid and deWet, 1999), which is a limiting factor for the propagation of commercial cultivars. A non-conventional technique of *in vitro* culture is widely used in many species (Munir *et al.*, 2015) including date palm (Rashid and Quraishi, 1994; Al-Khalifah and Shanavaskhan, 2012; Ghazzawy *et al.*, 2017)

The disease-free germplasms derived from this technique are used for the production of pharmaceuticals and other natural products, the genetic improvement, and rapid clonal multiplication (Withers and Alderson, 1989). However, an aseptic growing environment is needed to produce plants through this non-traditional method. The most highlighted problem is the contamination that commonly arises with the introduction of explants into the culture infected with microorganisms such as bacteria or fungi (Al-Khalifah and Shanavaskhan, 2012). Plant *in vitro* media composed of various nutrient elements, which can attract many common microorganisms to flourish. The distribution and planting of the contaminated plants produced through *in vitro* culture encourage the spread of diseases at large-scale in the field. To avoid this problem, it is required that all glassware, media, and devices used in handling plant tissues, as well as explant itself should be sterilized. Therefore, it is vital to adopt best aseptic culture plan to control *in vitro* contamination and to uphold a safe laboratory procedure including a viable technique of explants sterilization (Moisander and Herrington, 2006). Currently, explants are commonly sterilized with different type of disinfectants such as sodium hypochlorite, calcium hypochlorite, mercuric chloride, hydrogen peroxide, ethyl alcohol, silver nitrate and benzalkonium chloride, which are used through different techniques (Thakur and Sood, 2006; Barampuram *et al.*, 2014; Eziashi *et al.*, 2014). The antibacterial properties of silver (Ag) salts have been observed since olden times and is currently used to control bacterial growth in various ways (Silver and Phung, 1996; Kim *et al.*, 2007). Since the past few years, silver nanoparticles (size between 1-100 nm) are widely used as an antimicrobial agent in various industries, which releases a low level of silver ions to provide safety against microorganisms (Li *et al.*, 2010; Ouay and Stellacci, 2015; Thombre *et al.*, 2016). They exist as powerful inhibitory agents due to their microscopic surface area, chemical properties, and antimicrobial capabilities (Mapara *et al.*, 2015). Araújo *et al.* (2012) reported that concentrated silver nanoparticles alone and with added sodium chloride had high antimicrobial activities. Salisu *et al.* (2014) reported that the leaf explants of *Cymbopogon citratus* treated with silver nanoparticles and cultured in MS media significantly decreased the bacterial infection after four weeks of culturing. It was observed that the date palm explants are exposed to microbial infection at all stages of *in vitro* culture (Al-Mussawi, 2010). Keeping in view the importance and role of silver nanoparticles as antimicrobial agent, a comparative research study was designed to observe the survival response of explants of date palm *cv.* Barhee under *in vitro* growth conditions. The suitability and efficacy of silver nanoparticles as disinfectant along with sodium hypochlorite and mercuric chloride was also assessed.

Materials and methods

Choice of explant and sterilization: The study was carried out in the Tissue Culture Laboratory for Date Palm Research and Development, Agriculture Research Center, Giza, Egypt during 2015-2016. Four-year-old female ground offshoots of date palms *cv.* Barhee were obtained from Ismailia Governorate, Egypt. The primary preparation of explants was done outside the laboratory by removing the roots, brown fibrous leaf sheaths and outer green mature leaves from the offshoots reducing the size to 30 cm. In the laboratory, remaining mature leaves were removed gradually from the bottom offshoot to the top, exposing the white young centered leaves. The gradual removal of white young leaves and surrounding white fibrous leaf sheath resulted in 5 cm shoot tips, which were further trimmed to 2 cm for explant use. The explants were immersed in solution supplemented with combinations of ascorbic acid and citric acid at concentrations of 100 and 150 mg L^{-1}, respectively for one hour to reduce explants browning and their subsequent death at initiation stage.

Disinfectant treatments: Shoot tips (explants) were washed three times with sterilized double-distilled water (SDDW) and immersed in for 30 minutes. Afterward, they were treated with different combinations of disinfectants (sodium hypochlorite and mercuric chloride) along with silver nanoparticles (Table 1). After washing the explants, they were dipped in 70% Ethanol for 3 minutes and rinsed three times with SDDW followed by immersing in 80% sodium hypochlorite or 0.2% mercuric chloride solutions for 20 minutes and then washed three times with SDDW. Similar procedure was adopted when different concentrations of silver nanoparticles (1, 5, 10 and 20 mg L^{-1}) was used as disinfectant, however, the explants were immersed in the respective silver nanoparticle solutions for half an hour.

Media preparation and explant culture: Murashige and Skoog (1962) basal medium containing macro and micro elements and vitamins was used throughout the study. The pH of media was adjusted to 5.7 and then 7 $g.L^{-1}$ agar was added. Twenty mL of media were dispensed into 100 mL glass jars, which were then sterilized by autoclaving under pressure of 1.5 kg cm^2 at 121ºC for 20 minutes. Thereafter, the glass jars were transferred to the culture cabinet (laminar flow hood) and left to cool in a slant position until they were used for explant culture. Explants were cultured on MS media and incubated in dark at 25±2 ºC for eight weeks in order to initiate the explant growth.

The experimental design was completely randomized with three replicates in each treatment. Data recorded of seventeen treatments were first analyzed as a whole using the aforementioned statistical design and then it was divided into four groups. Group included T_1 to T_5 treatments (T_1, 80% sodium hypochlorite+0.2% mercuric chloride; T_2, 80% sodium hypochlorite+0.2% mercuric chloride+1 mg L^{-1} silver nanoparticles; T_3, 80% sodium hypochlorite+0.2% mercuric chloride+5 mg L^{-1} silver nanoparticles; T_4, 80% sodium hypochlorite+0.2% mercuric chloride+10 mg L^{-1} silver nanoparticles; T_5, 80% sodium hypochlorite+0.2% mercuric chloride+20 mg L^{-1} silver nanoparticles). Group two comprised of T_6 to T_9 treatments (T_6, 80% sodium hypochlorite+1 mg L^{-1} silver nanoparticles; T_7, 80% sodium hypochlorite+5 mg L^{-1} silver nanoparticles; T_8, 80% sodium hypochlorite+10 mg L^{-1} silver nanoparticles; T_9, 80% sodium hypochlorite+20 mg L^{-1} silver nanoparticles). Group three contained T_{10} to T_{13} treatments (T_{10}, 0.2% mercuric chloride+1 mg L^{-1} silver nanoparticles; T_{11}, 0.2% mercuric chloride+5 mg L^{-1} silver nanoparticles; T_{12}, 0.2% mercuric chloride+10 mg L^{-1} silver nanoparticles; T_{13}, 0.2% mercuric chloride+20 mg L^{-1} silver nanoparticles) and group four had T_{14} to T_{17} treatments (T_{14}, 1 mg L^{-1} silver nanoparticles; T_{15}, 5 mg L^{-1} silver nanoparticles; T_{16}, 10 mg L^{-1} silver nanoparticles; T_{17}, 20 mg L^{-1} silver nanoparticles). The data were statistically analyzed using GenStat version 18 (VSN International Ltd., UK).

Table 1. Different combinations of disinfectants (80% sodium hypochlorite and 0.2% mercuric chloride) along with silver nanoparticles (1, 5, 10 and 20 mg L^{-1})

T_1	80% sodium hypochlorite+0.2% mercuric chloride
T_2	80% sodium hypochlorite+0.2% mercuric chloride+1 mg L^{-1} silver nanoparticles
T_3	80% sodium hypochlorite+0.2% mercuric chloride+5 mg L^{-1} silver nanoparticles
T_4	80% sodium hypochlorite+0.2% mercuric chloride+10 mg L^{-1} silver nanoparticles
T_5	80% sodium hypochlorite+0.2% mercuric chloride+20 mg L^{-1} silver nanoparticles
T_6	80% sodium hypochlorite+1 mg L^{-1} silver nanoparticles
T_7	80% sodium hypochlorite+5 mg L^{-1} silver nanoparticles
T_8	80% sodium hypochlorite+10 mg L^{-1} silver nanoparticles
T_9	80% sodium hypochlorite+20 mg L^{-1} silver nanoparticles
T_{10}	0.2% mercuric chloride+1 mg L^{-1} silver nanoparticles
T_{11}	0.2% mercuric chloride+5 mg L^{-1} silver nanoparticles
T_{12}	0.2% mercuric chloride+10 mg L^{-1} silver nanoparticles
T_{13}	0.2% mercuric chloride+20 mg L^{-1} silver nanoparticles
T_{14}	1 mg L^{-1} silver nanoparticles
T_{15}	5 mg L^{-1} silver nanoparticles
T_{16}	10 mg L^{-1} silver nanoparticles
T_{17}	20 mg L^{-1} silver nanoparticles

Results and discussion

Significant variation ($P \leq 0.05$) among different disinfectant treatments regarding the explant survival percentage of date palm *cv.* Barhee were recorded (Fig. 1). Maximum explant survival percentage (88.89 %) was observed when 5 mg L^{-1} silver nanoparticles alone (T_{15}) was used as disinfectant followed by the same concentration of silver nanoparticles in combination with 80% sodium hypochlorite and 0.2% mercuric chloride *i.e.* T_3, T_7 and T_{11} (77.78%). These four treatments were statistically at par. Similarly, T_1, T_2 T_4, T_6, T_8, T_9, T_{10}, T_{12}, T_{14}, and T_{17} also behaved alike and only 55.56% explants survived. Only 66.67% explants survived when treated with 10 mg L^{-1} silver nanoparticles alone. However, minimum explant survival was observed in T_5 and T_{13} (44.44%). Data regarding *in vitro* explant contamination percentage showed significant difference ($P \leq 0.05$) among various means of sterilizing treatments of date palm *cv.* Barhee (Fig. 2). Zero percent contamination was noted in T_4, T_5, T_9, T_{12}, T_{13}, T_{14} and T_{16}. However, maximum contamination (33.33%) was observed in T_1 (Fig. 8c), T_2 (Fig. 8a), T_6, T_{10} (Fig. 8d) and T_{17} treatments followed by T_3, T_7 T_8, and T_{11} (22.22%). Explants treated with 5 mg L^{-1} silver nanoparticles alone (T_{15}) showed 11.11% contamination (Fig. 8b). The explant mortality percentage was affected significantly ($P \leq 0.05$) by the application of different concentrations of disinfectants (Fig. 3). Zero explant mortality was recorded in T_3, T_7, T_{11}, and T_{15} treatments. However,

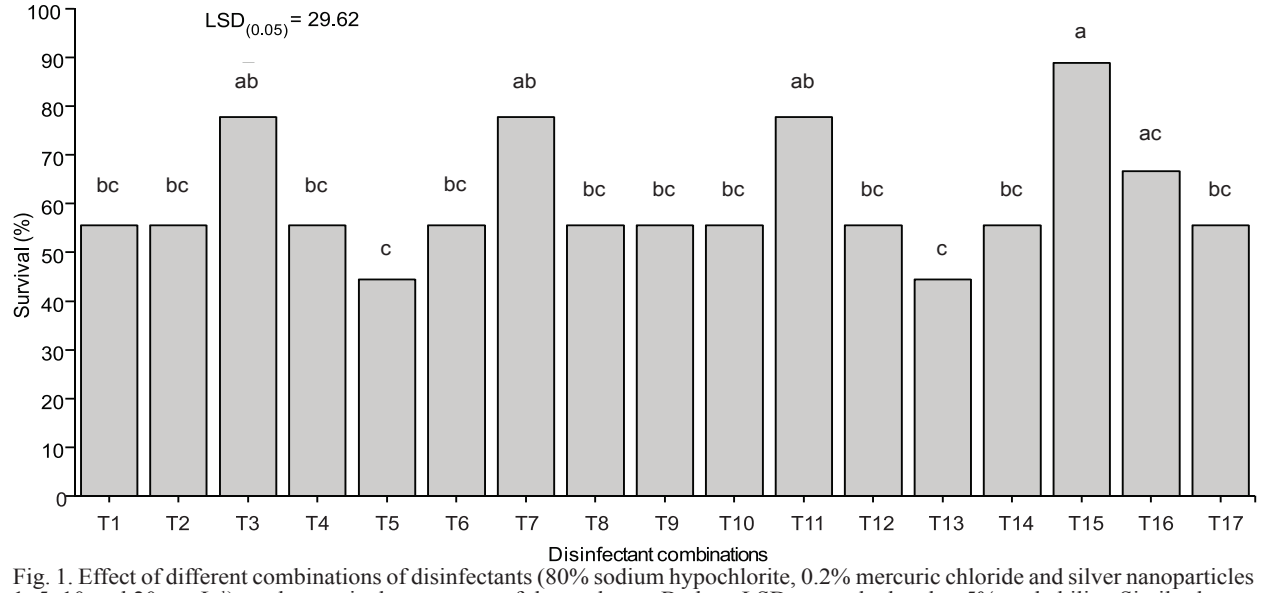

Fig. 1. Effect of different combinations of disinfectants (80% sodium hypochlorite, 0.2% mercuric chloride and silver nanoparticles 1, 5, 10 and 20 mg L^{-1}) on the survival percentage of date palm *cv.* Barhee. LSD was calculated at 5% probability. Similar letters above bar are non-significantly different.

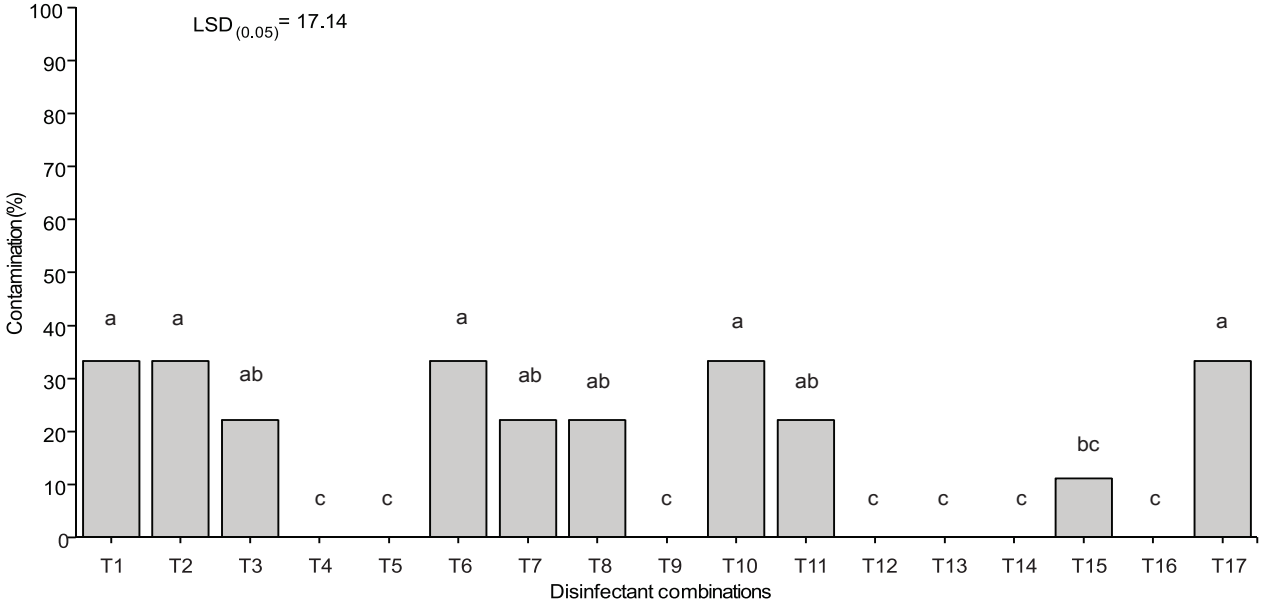

Fig. 2. Effect of different combinations of disinfectants (80% sodium hypochlorite, 0.2% mercuric chloride and silver nanoparticles 1, 5, 10 and 20 mg L^{-1}) on the contamination percentage of date palm *cv.* Barhee. LSD was calculated at 5% probability. Similar letters above bar are non-significantly different.

maximum explant death (55.56%) was observed in T_5 (Fig. 8e) and T_{13} treatment combinations followed by T_4, T_9, T_{12}, and T_{14} (44.44%), T_{16} (33.33%) and T_8 and T_{10} (22.22%) treatments. Similarly, explants in T_1, T_2, T_6, and T_{17} had 11.11% mortality.

Data recorded in present study was further divided into four groups. The analysis of group one indicated non-significant difference of means of five treatment combinations regarding explant survival (Fig. 4a), however, contamination (Fig. 4b) and mortality percentage (Fig. 4c) significantly varied among the treatments. T_3 (80% sodium hypochlorite+0.2% mercuric chloride+5 mg L^{-1} silver nanoparticles) showed higher survival percentage (77.78%) and zero mortality. Similar statistical trend was observed in group two, where explant survival (Fig. 5a) parameter was non-significant and the contamination (Fig. 5b) and mortality (Fig. 5c) were significant. However, higher explant survival (77.78%) and zero mortality were recorded in T_7 (80% sodium hypochlorite+5 mg L^{-1} silver nanoparticles). All treatment combinations in group three were statistically at par regarding explant survival (Fig. 6a) whereas significant difference was observed among treatments regarding explant contamination (Fig. 6b) and mortality (Fig. 6c). Higher explant survival (77.78%) and zero mortality was noticed in T_{11} (0.2% mercuric chloride+5 mg L^{-1} silver nanoparticles). Similarly, treatment combination in group four showed significant variation among mean regarding explant survival (Fig. 7a), contamination (Fig. 7b) and mortality (Fig. 7c) percentage. Maximum explant survival (88.89%) and zero mortality was noticed in T_{15} (5 mg L^{-1} silver nanoparticles) treatment.

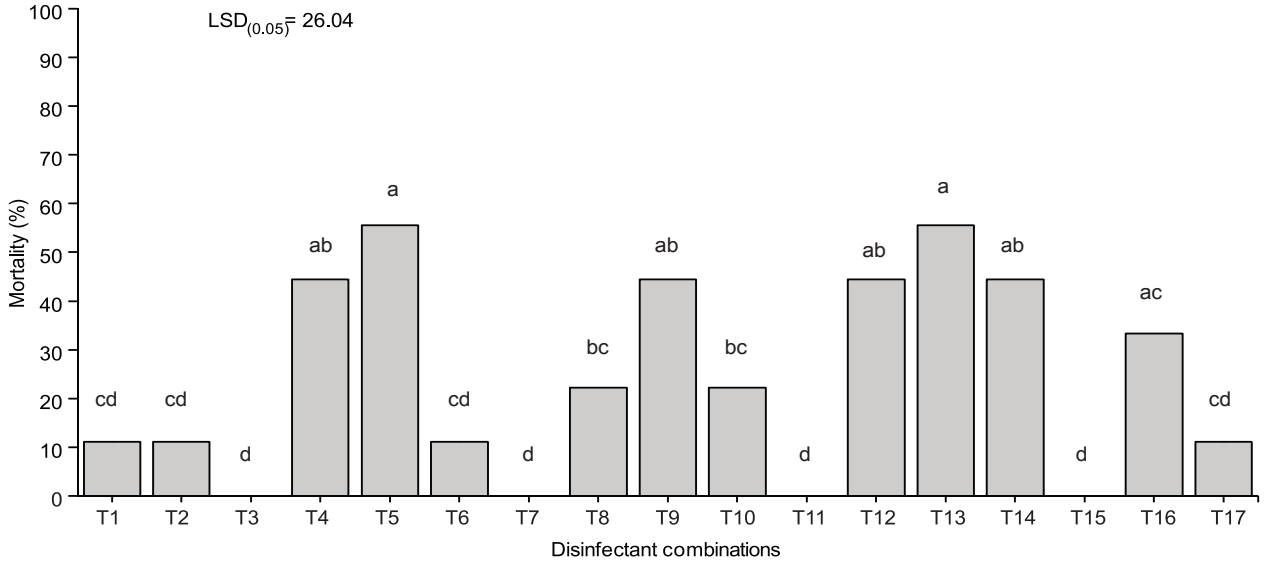

Fig. 3. Effect of different combinations of disinfectants (80% sodium hypochlorite, 0.2% mercuric chloride and silver nanoparticles 1, 5, 10 and 20 mg L^{-1}) on the mortality percentage of date palm *cv.* Barhee. LSD was calculated at 5% probability. Similar letters above bar are non-significantly different.

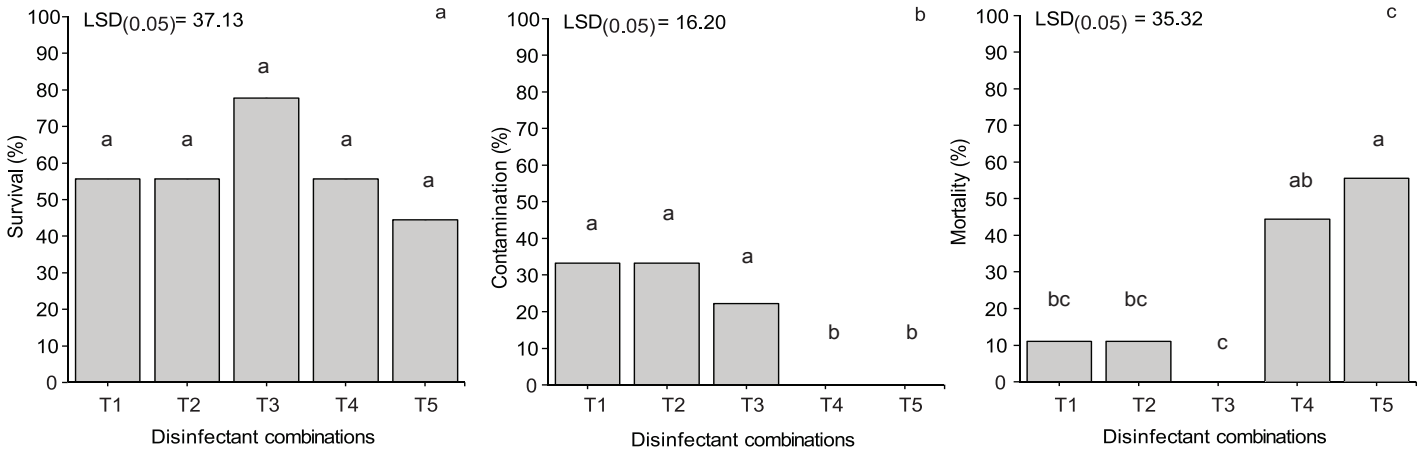

Fig. 4. Effect of different combinations of disinfectants (T_1, sodium hypochlorite + mercuric chloride; T_2, sodium hypochlorite + mercuric chloride + 1 mg L^{-1} silver nanoparticles; T_3, sodium hypochlorite + mercuric chloride + 5 mg L^{-1} silver nanoparticles; T_4, sodium hypochlorite + mercuric chloride + 10 mg L^{-1} silver nanoparticles; T_5, sodium hypochlorite + mercuric chloride + 20 mg L^{-1} silver nanoparticles) on explant (a) survival, (b) contamination and (c) mortality percentage of date palm *cv.* Barhee. LSD was calculated at 5% probability. Similar letters above bar are non-significantly different.

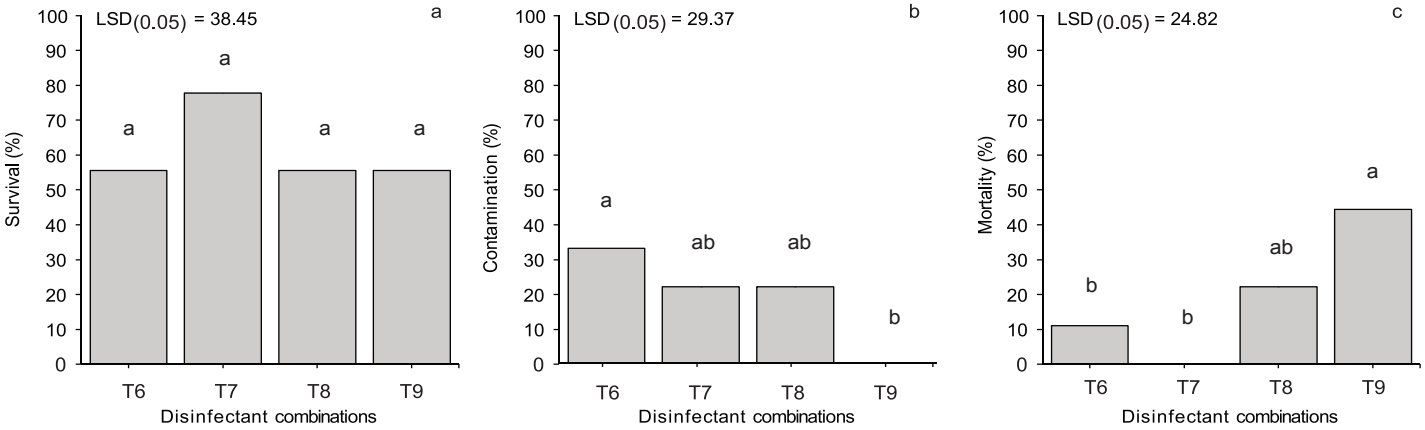

Fig 5. Effect of different combinations of disinfectants (T_6, 80% sodium hypochlorite+1 mg L^{-1} silver nanoparticles; T_7, 80% sodium hypochlorite+5 mg L^{-1} silver nanoparticles; T_8, 80% sodium hypochlorite+10 mg L^{-1} silver nanoparticles; T_9, 80% sodium hypochlorite+20 mg L^{-1} silver nanoparticles) on explant (a) survival, (b) contamination and (c) mortality percentage of date palm *cv.* Barhee. LSD was calculated at 5% probability. Similar letters above bar are non-significantly different.

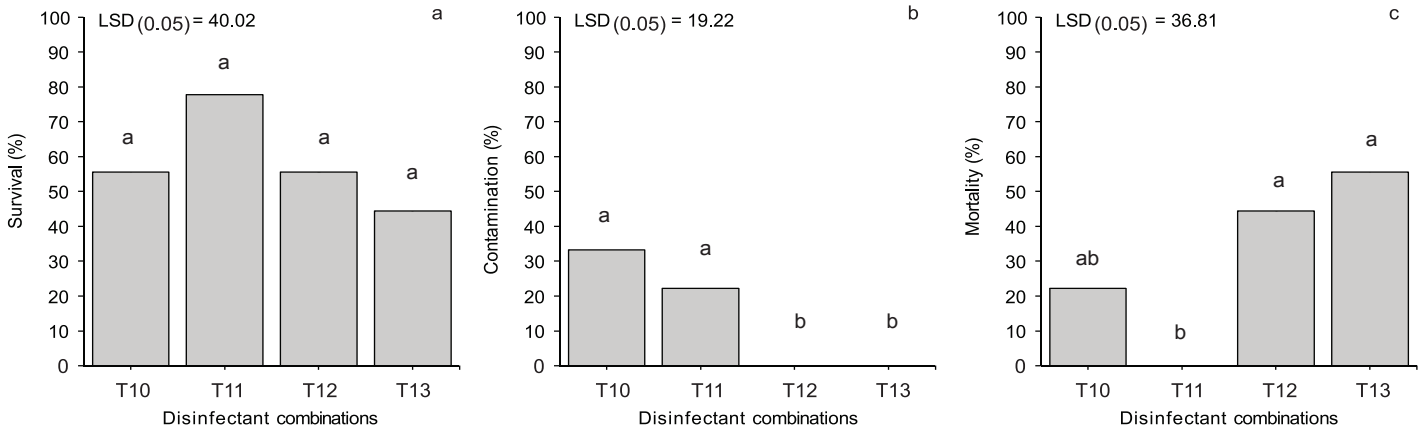

Fig 6. Effect of different combinations of disinfectants (T_{10}, 0.2% mercuric chloride+1 mg L^{-1} silver nanoparticles; T_{11}, 0.2% mercuric chloride+5 mg L^{-1} silver nanoparticles; T_{12}, 0.2% mercuric chloride+10 mg L^{-1} silver nanoparticles; T_{13}, 0.2% mercuric chloride+20 mg L^{-1} silver nanoparticles) on explant (a) survival, (b) contamination and (c) mortality percentage of date palm *cv.* Barhee. LSD was calculated at 5% probability. Similar letters above bar are non-significantly different.

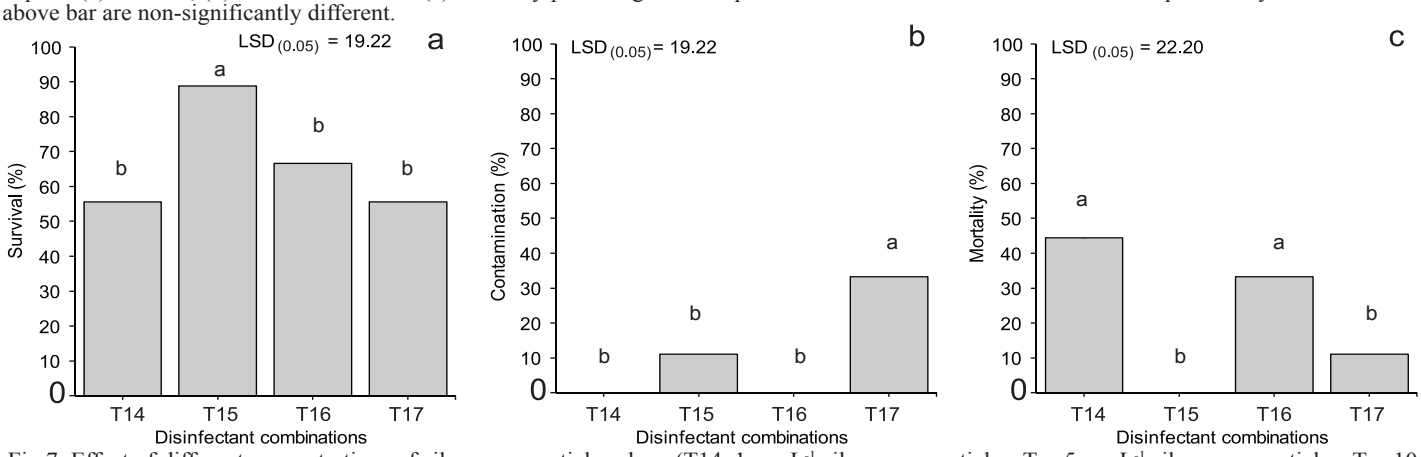

Fig 7. Effect of different concentrations of silver nanoparticles alone (T14, 1 mg L^{-1} silver nanoparticles; T_{15}, 5 mg L^{-1} silver nanoparticles; T_{16}, 10 mg L^{-1} silver nanoparticles; T_{17}, 20 mg L^{-1} silver nanoparticles) on explant (a) survival, (b) contamination and (c) mortality percentage of date palm *cv.* Barhee. LSD was calculated at 5% probability. Similar letters above bar are non-significantly different.

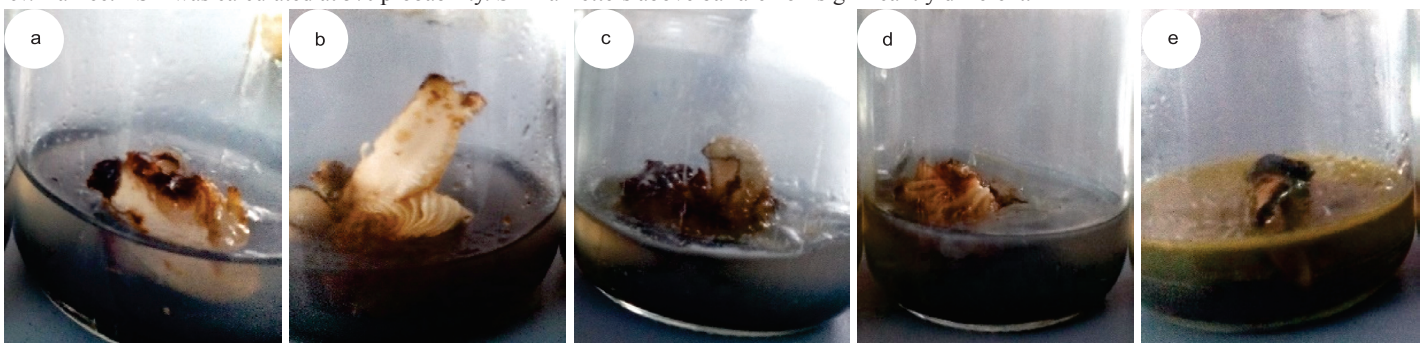

Fig. 8. Response of date palm shoot tip explants of *cv.* Barhee to various surface sterilization treatments. (a) *In vitro* contamination when explants were sterilized with sodium hypochlorite, mercuric chloride, 1 mg/L silver nanoparticles, T_2. (b) Contamination of explants sterilized with 5 mg/L silver nanoparticles alone, T_{15}. (c) Contamination of explants sterilized with sodium hypochlorite, mercuric chloride, T_1. (d) Contamination of explants sterilized with mercuric chloride, 1 mg/L silver nanoparticles, T_{10}. (e) Death of explants sterilized with sodium hypochlorite, mercuric chloride + silver nanoparticles 20 mg/L, T_5.

Microorganism such as fungi and bacteria are most commonly found on or in plant tissues. Establishing a disinfected *in vitro* culture is the most essential step for the success in micropropagation of plants (Aghaye and Yadollahi, 2012; Rostami and Shahsava, 2012). To achieve this the sterilization of explant is one of the important steps to be taken (Munir *et al.*, 2015). For this purpose, various chemicals such as alcohol derivatives, sodium hypochlorite, mercuric chloride and antibiotic solutions with advantages and disadvantages to eliminate contamination are generally used. Therefore, to control contamination in micropropagation and to reduce the impact of toxic chemicals it is necessary to consider new antimicrobial agents (Counter and Buchanan, 2004; Fakhrfeshani *et al.*, 2012). In present study, we have compared an environment friendly disinfectant, silver nanoparticles, with two chemical sterilizers, which have a good potential for removing the contaminants in plant tissue culture procedures (Safavi, 2012). The application of silver nanoparticles in tissue culture media as an alternative to antibiotics and other disinfectants to control microbial contaminations during plant morphogenesis has already been reported in few studies (Mahna

et al., 2013; Arab *et al.*, 2014; Salisu *et al.*, 2014). However, in present investigation we have used different concentrations of silver nanoparticles as an explant sterilizer, which is not studied earlier in date palm.

Our results showed the potential use of silver nanoparticles alone and in combination with chemical disinfectants such as sodium hypochlorite and mercuric chloride. Although, the use of 5 mg L^{-1} silver nanoparticles with sodium hypochlorite and mercuric chloride presented more or less statistically similar results when compared with 5 mg L^{-1} silver nanoparticles alone, however, we have recommended the use of 5 mg L^{-1} silver nanoparticles alone if preference is given to use non-hazardous disinfectant. Moreover, explants treated with 5 mg L^{-1} silver nanoparticles alone had highest rate of survival (88.89%), lowest contamination rate (11.11%), and zero mortality rate. Although, 5 mg L^{-1} silver nanoparticles when combined with sodium hypochlorite and mercuric chloride showed zero mortality rate, the contamination rate was higher (22.22%) than the same concentration of silver nanoparticles alone. Mahna *et al.* (2013) reported that the lower concentrations of silver nanoparticles functioned as an antimicrobial agent without any adverse effects on explant viability when the explants of Arabidopsis, tomato and potato were soaked in different concentrations of silver nanoparticles at various exposure times, and then transferred onto the MS medium. Similarly, Abdi *et al.* (2008) and Sarmast *et al.* (2011) used silver nanoparticles for *in vitro* decontamination of *Valeriana officinalis* and *Araucaria excelsa* explants, respectively. Salisu *et al.* (2014) reported that adding silver nanoparticles at the rate of 40 mL L^{-1} into the growth media for *Cymbopogon citratus* was fully effective to control the bacterial infection after four weeks of culturing. The difference between our findings and the previously mentioned study could be because Salisu and co-workers used the same material (silver nanoparticles) as media sterilizer while we used it for explant sterilization prior to transfer them on the media, that is why lower concentration of silver nanoparticles worked well. The other possible reason could be the difference in plant species.

The use of chemical disinfectants to sterilize explants is a common procedure in *in vitro* studies. However, in present study, apart from chemical disinfectants (sodium hypochlorite and mercuric chloride) we included different concentrations of silver nanoparticles (1, 5, 10 and 20 mg L^{-1}) to observe the survival, contamination and mortality attributes of *in vitro* raised explants. Our findings validate the suitability of chemical disinfectants along with silver nanoparticles. Although, the application of 5 mg L^{-1} silver nanoparticles alone and in combinations with sodium hypochlorite and mercuric chloride were statistically non-significant, we recommend the use of 5 mg L^{-1} silver nanoparticles alone to avoid the extra cost of chemical disinfectants. Moreover, the results of present study will be useful to improve explant sterilization protocol for the use of silver nanoparticles, which are non-hazardous and environment friendly.

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