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IN VITRO CULTURE OF *ARGANIA SPINOSA* AND INFLUENCE OF NANOPARTICLES ON GROWTH IMPROVEMENT

Ghada Abd El-Moneim Hegazi[1*], Walaa Mohamed Ibrahim[1], Mohamed Hamed Hendawy[1] and Hanaa Mohamed Salem[2]

[1]Department of Genetic Resources, Desert Research Center, El-Matareya, Cairo, Egypt

[2]Department of Botany and Microbiology, Faculty of Science, Al-Azhar University (Girls Branch), Cairo, Egypt

[*]Corresponding author

E-mail: ghada.hegazi1211@gamil.com

Abstract

Argan (*Argania spinosa*) is a drought tolerant multipurpose tree, endemic to Morocco and has high economic and ecological importance, particularly for its valuable oil. The plant is endangered; therefore, the *in vitro* culture of this plant could conserve its natural populations. The aim of this research was the *in vitro* culture of argan and study the influence of titanium and silicon dioxide nanoparticles (TiO_2 and SiO_2 NPs) on plant growth improvement. Argan seeds were germinated *in vitro* in liquid Murashige and Skoog (MS) medium supplemented with 0.537 µM β–naphthalene acetic acid (NAA), 1.445 µM gibberellic acid (GA_3) and 4.44 µM benzyl adenine (BA) with 80% germination percentage. Shoot tips and stem node segments from seedlings were *in vitro* initiated on MS medium supplemented with 0.289 µM GA_3, 2.325 µM kinetin (Kin) and 8.88 µM BA. Axillary shoots were multiplied on MS medium supplemented with 2.27 µM thidiazorun (TDZ) and 0.537 µM NAA giving 5.1 axillary shoots/explant. Elongated shoots were rooted on 9.8 µM indole butyric acid (IBA) and 5.37 µM NAA, with 30% root induction percentage. Nanoparticles of TiO_2 and SiO_2 were added in the best multiplication and rooting media. The application of TiO_2 NPs at 10 ppm enhanced the multiplication of axillary shoots and gave the highest mean number of axillary shoots of 6.2 axillary shoots/explant. However, the rooting induction percentage was increased up to 40% when shoots where cultured on the rooting medium supplemented with 5 ppm SiO_2 NPs. Well rooted plantlets where transferred to greenhouse for acclimatization and the percentage of survived transplants reached 60%. The addition of nanoparticles to the nutrient medium was promising towards plant improvement.

Keywords: Argan, microproduction, TiO_2 NPs, SiO_2 NPs

Introduction

Moroccan *Argania spinosa* (L.) Skeels, belongs to family Sapotaceae and is known as argan. It is a slowgrowing tree, reaching 20 m in height and has a great importance in agroforestry systems. The plant is a multipurpose, oil-yielding tree that has a great ecological and socio-economic value. Due to its deep roots, argan tree can stand with drought and other stress conditions and can live for longer than 200 years (Villareal *et al*., 2013).

Plant leaves are used for feeding cattle (Benaouf *et al*., 2014). Argan oil has an effective role in disease prevention and has antioxidant potential, chemopreventive, hypocholesterolemic, hypolipidemic, antihypertensive and anti-inflammatory properties (Drissi *et al*., 2004 and Charrouf and Guillaume, 2010). Argan oil is traditionally found to be effective as a choleretic and hepatoprotective agent and for atherosclerosis, hypercholesterolemia and also used in rheumatology. Argan oil has a higher tocopherol and polyphenol content and antioxidant capacity than other edible vegetable oils (Marfil *et al*., 2011). It is used for cosmetics as a skin lotion to cure all kinds of skin pimples, juvenile acne and chicken pox pustules (Kechebar *et al*., 2013). It is also known to reduce dry skin diseases and slow down the rate of senescence as well as to resist the hair loss (Charrouf and Guillaume, 2002).

Due to the decline of this valuable oil crop as a result of human activities, climate change and ecological imbalances, in addition to the low seed germination and the genetic diversity generated by seeds, argan tree is endangered (Charrouf and Guillaume, 2010). Therefore, it is necessary to develop *ex situ* conservation methods to overcome the loss of the genetic resources of this valuable plant. In vitro culture is an important tool for conservation and management of plant genetic resources (Paunescu, 2009). In vitro propagation of argan are facing many difficulties; multiplication rate of axillary shoots was low with limited elongation through repeated subcultures, with hyperhydricity and high rate of apical and leaf necrosis. In addition, poor adventitious roots are formed (Nouaim *et al*., 2002 and Martinez-Gómez *et al*., 2010). The first positive results of *in vitro* propagation of argan were achieved in 2002, but multiplication showed large variations between clones and rooting percentage was high in some clones and absent in others (Nouaim *et al*., 2002). Obviously, the high genetic variability of this tree is an obstacle for using the same treatments with various clones. Lamaoui *et al*. (2019) micropropagated 30 genotypes of argan and they reported well shoot regeneration efficiency, but the rooting rates were still not sufficient.

Nanoparticles have unique physicochemical properties that differ from their macroscopic or ionic counterparts. These properties result from their small size, high surface area, shape, chemical composition and reactivity. Therefore, they have been widely used in plant biotechnology and agriculture to enhance seed germination, plant growth and yield, facilitate site target delivery of various nutrients needed for better growth and high productivity of plants and improve plant resistance to abiotic and biotic stresses with positive environmental impact (Singh *et al*., 2015; Gowayed *et al*., 2017 and Timoteo *et al*., 2019). Also, nanoparticles such as TiO_2 and SiO_2 NPs had positive effects on plant growth enhancement, biomass, crop production, root and shoot length, chlorophyll and protein content (Ullah and Ullah, 2019). In plant *in vitro* culture, there are promising studies of the utilization of nanotechnology. Many researchers studied the beneficial effects of NPs on the growth of micropropagated plants; such as TiO_2 NPs with *Plantago psyllium* (Farahani *et al*., 2012) and *Petroselinum crispum* (Dehkourdi and Mosavi, 2013), and SiO_2 NPs with *Larix olgensis* (Lin *et al*., 2004) and banana (El-Boray *et al*., 2017).

The aim of this study was finding a successful protocol for the *in vitro* culture of argan as an endangered species for

conservation and introducing this valuable plant to Egypt. Also, the study aimed to evaluate the influence of TiO_2 and SiO_2 NPs on the multiplication and rooting of shoots for growth enhancement.

Materials and Methods

In vitro culture of argan was conducted in Tissue Culture Unit, Department of Genetic Resources, Desert Research Center, Cairo, Egypt.

1. Plant material and explant preparation

Seeds of argan were purchased from Progress Company, Cairo, Egypt. The hard seed coats were removed and the internal nuts (kernels) were disinfected under complete aseptic conditions in the laminar air flow hood (Holten LaminAir HVR 2448, USA), by immersing in sterile distilled water with two drops of Dettol (cloroxylenol 4.8%) for 10 min, then rinsing three times with sterile distilled water. Decoated seeds were given a quick rinse (10 s) in 95% ethyl alcohol, then rinsed again three times with sterile distilled water. After that, they were soaked in 15% commercial bleach [Clorox containing 5.25% sodium hypochlorite (NaOCl)] for 15 min, before rinsing five times with sterile distilled water to remove all traces of the disinfectants.

Decoated seeds were cultured for germination on liquid Murashige and Skoog (Murashige and Skoog, 1962) medium (Duchefa, Haarlem, the Netherlands) supplemented with 3% (w/v) sucrose, 0.01% (w/v) meso-inositol (Fluka AG, Switzerland), 0.537 µM β–naphthalene acetic acid (NAA) and 1.445 µM GA_3 (Sigma Cell Culture, min. 90%, St. Louis, USA) in combination with 4.44, 8.88, 13.32 and 17.76 µM 6- benzyladenine (BA). MS medium without plant growth regulators (PGRs) served as control. The pH of the nutrient media was adjusted to 5.7±0.1, then media were dispensed by pouring 50 ml volumes into 300 ml jars containing a piece of cotton and covered with polypropylene caps before autoclaving at a pressure of 1.06 kg/cm and 121°C for 15 min (Harvey Sterilemax autoclave, Thermo Scientific, USA). Thermolabile GA_3 was filter-sterilized by using 0.25 µm diameter filter, then added to the autoclaved media after cooling. Cultures were incubated at 16-hour photoperiod with light intensity of 2500-3000 lux, provided by cool white light fluorescent tubes (F140t9d/38, Toshiba) at 25±2°C and 60-65% relative humidity.

On the other hand, shoot tips and stem node segments were excised from seedlings germinated in the greenhouse and washed under tap water for one hour, then disinfected by a quick immersion in Dettol (10 min) and 95% ethyl alcohol (10 s), then by commercial bleach at concentration of 10% for 15 min for shoot tips and 25% for 20 min for stem node segments. Traces of disinfectants were removed by rinsing explants with sterile distilled water for five times.

Germination percentage (%), mean number and length (cm) of shoots/explant were recorded after eight weeks of seed culture. The produced seedlings were cut into shoot tips and stem node segments to be used as explants for the *in vitro* culture.

2. In vitro culture establishment

Shoot tips and stem node segments from germinated seedlings were cultured on solid MS medium supplemented with 3% (w/v) sucrose, 0.01 (w/v) meso-inositol, 2.325 µM kinetin (Kin; Sigma Cell Culture, min. 90%, St. Louis, USA), 0.289 µM GA_3 in combination with 4.44, 8.88, 13.32 and 17.76 µM BA. MS medium without PGRs served as control. The pH of the media was adjusted to 5.7±0.1 and gelled with 0.3% (w/v) phytagel (Duchefa, Haarlem, the Netherlands), then dispensed and autoclaved. Cultures were maintained at the same conditions as mentioned and the percentage of growth initiation (%), mean number and length (cm) of axillary shoots/explant were recorded after eight weeks of culture.

3. Multiple shoots formation

Axillary shoots raised from the establishment stage were multiplied on MS medium supplemented with 3% (w/v) sucrose, 0.01% (w/v) meso-inositol and 4.44, 8.88, 13.32, 17.76 and 22.2 µM BA in combination with 0.537 µM NAA. In another experiment, axillary shoots were cultured on MS medium supplemented with 2.27, 3.405, 4.54 and 6.81 µM thidiazorun (TDZ; Sigma Cell Culture, min. 90%, St. Louis, USA) in combination with either 0.537 µM NAA or 0.289 µM GA_3 or both. MS medium without cytokinins served as control. The pH of the media was adjusted to 5.7±0.1 and gelled with 0.3% (w/v) phytagel, then dispensed and autoclaved. Percentage of explants forming growth (%), mean number and length (cm) of axillary shoots/explant were recorded after eight weeks of culture. Subculturing of axillary shoots on the best medium for multiplication was done every eight weeks.

4. Rooting of axillary shoots

Elongated axillary shoots of argan with 3-4 nodes were excised and cultured on root induction medium of solid half strength MS (½ MS) medium supplemented with 1.5% (w/v) sucrose, 0.01% (w/v) meso-inositol and indole butyric acid (IBA; Sigma Cell Culture, min. 90%, St. Louis, USA) at 4.9, 9.8, 14.7, 19.6 and 24.5 µM or NAA at 5.37, 10.74, 16.11, 21.48 and 26.85 µM, individually or the same concentrations of IBA in combination with 5.37 µM NAA. Half strength MS medium without auxins served as control. The pH was adjusted, the media were gelled and autoclaved. Cultures were incubated as mentioned in the previous stages. Percentage of root induction (%), mean number and length (cm) of roots/explant were recorded after eight weeks of cultures.

5. Nanoparticles application

Nanoparticles of TiO_2 and SiO_2; 21 and 5-15 nm in size (Sigma-Aldrich, Germany), respectively, were tested for their effect on the growth performance of argan cultures through multiplication and rooting stages. Nanoparticles were added at 5, 10 and 20 ppm to the best multiplication and rooting media. MS medium without NPs served as control. Growth parameters were recorded as mentioned previously for each stage after eight weeks of nanoparticles application.

6. Acclimatization of plantlets

Rooted plantlets with well-developed root system were washed from the medium residues and transplanted into pots containing a sterilized mixture of sand, peat moss and vermiculite (1:1:1 v/v/v). Pots were placed in the greenhouse covered with transparent polyethylene bags to maintain a high level of relative humidity around shoots. Irrigation took place once a week with ½ MS medium. Relative humidity was reduced by gradual removal of the covers within two months. The percentage of survived transplants (%) was recorded.

7. Experimental design and Statistical analysis

Experiments were subjected to completely randomized design. Each treatment in all experiments consisted of at least 10 replicates. Variance analysis ANOVA was done using Costat software program for statistical analysis. The differences among means of all treatments were tested for their significance at 5% level using Duncan (1955) multiple range test as modified by Snedecor and Cochran (1990).

Results and Discussion

1. In vitro seed germination

Argan seeds germination took place when they were cultured on MS medium supplemented with 0.537 µM NAA and 1.445 µM GA_3 in combination with different concentrations of BA, as represented in Table (1). After eight weeks of seed culture, BA at 4.44 and 8.88 µM showed the highest percentage of seed germination (80%) with 1.4 and 1.8 mean number of axillary shoots/explant and mean length of axillary shoots of 5.5 and 4.5 cm, respectively (Fig. 1). However, by increasing the concentration of BA, the seed germination percentage decreased gradually to reach 30% using 17.76 µM BA, also the mean number and length significantly decreased. The control treatment without BA recorded 50% of germination. Although, Marfil *et al*. (2011) got a high germination percentage for argan seeds when cultured on MS medium supplemented only with GA_3 at 0.145 µM. Also, Justamante *et al*. (2017) obtained the highest germination percentage of argan seeds on MS medium supplemented with 2.89 µM GA_3.

Table 1: Influence of MS medium supplemented with 0.537 µM NAA and 1.445 µM GA_3 in combination with different concentrations of BA on the germination of argan seeds.

BA conc. (µM)	Germination (%)	No. of shoots/explant	Length of shoots/explant (cm)
0.00	50^{ab}	1.2^{c}	2.0^{d}
4.44	80^{a}	1.4^{b}	5.5^{a}
8.88	80^{a}	1.8^{a}	4.5^{b}
13.32	60^{ab}	1.2^{c}	3.5^{c}
17.76	30^{b}	1.0^{c}	1.8^{e}

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$.

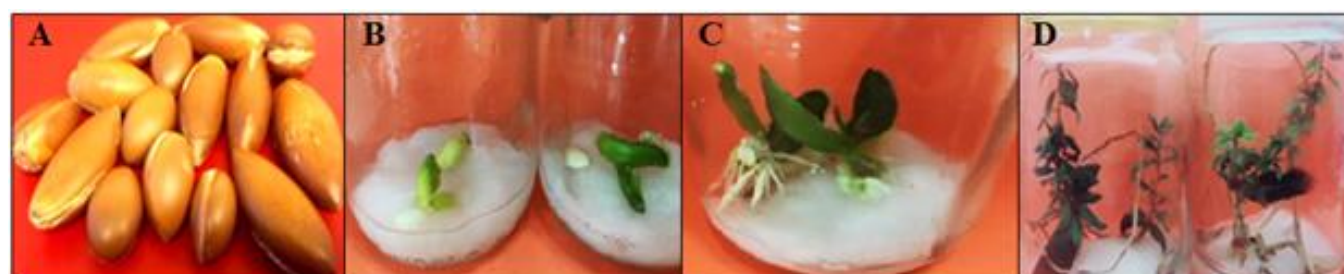

Fig. 1: Argan seeds germination. **(A)** Seeds of argan before culturing **(B)** Germinated seeds after 15 days, **(C)** after one month and **(D)** after two months of culture on MS medium supplemented with 4.44 µM BA in combination with 0.537 µM NAA and 1.445 µM GA_3.

2. In vitro culture establishment

Table (2) represents the results of *in vitro* culture establishment from shoot tips and stem node segments of argan seedlings, when cultured on MS medium supplemented with 0.289 µM GA_3 and 2.325 µM Kin in combination with different concentrations of BA. For both explants, growth initiation percentage was increased with increasing BA concentration and reached 90% using 8.88 µM BA with the highest mean number of axillary shoots/explant (Fig. 2). On the other hand, higher concentrations of BA (13.32 and 17.76 µM) decreased the percentage of growth initiation and the mean number of axillary shoots. However, the mean length of axillary shoots increased up to the maximum values, reaching 2.3 cm using 17.76 µM BA for both explants.

Table 2: Influence of MS medium supplemented with 0.289 µM GA_3 and 2.325 µM Kin in combination with different concentrations of BA on *in vitro* culture establishment of shoot tip and stem node segment of argan.

BA conc. (µM)	Shoot tip			Stem node segment		
	Growth initiation (%)	No. of axillary shoots/explant	Length of axillary shoots/explant (cm)	Growth initiation (%)	No. of axillary shoots/explant	Length of axillary shoots/explant (cm)
0.00	70^{ab}	1.14^{e}	1.75^{d}	70^{ab}	1.70^{c}	1.90^{d}
4.44	80^{a}	1.25^{d}	1.69^{e}	80^{a}	1.75^{b}	2.13^{c}
8.88	90^{a}	1.60^{a}	1.90^{c}	90^{a}	2.10^{a}	1.88^{e}
13.32	60^{ab}	1.48^{b}	2.10^{b}	60^{ab}	1.60^{d}	2.20^{b}
17.76	30^{b}	1.30^{c}	2.30^{a}	30^{b}	1.20^{e}	2.30^{a}

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$.

Benzyl adenine (BA) showed to be the most effective and affordable cytokinin for the induction of bud formation on primary explants (Malá *et al*., 2009). This may be contributed to the capability of tissues to metabolize BA more than other PGRs and for its ability to induce the production of natural hormones; such as zeatin (Lamaoui *et al.*, 2019). The negative effect of high concentrations of BA could be contributed to that BA is produced and stored at the plant base and fails to transport to the shoots for multiple shoots initiation (Bairu *et al*., 2007). The growth initiation of argan explants using BA in addition to an auxin with or without GA_3 was reported previously by Marfil *et al*. (2011), who established argan shoots on MS medium supplemented with different concentrations (6.66, 13.32 and 22.2 µM) of BA in combination with 0.049 µM IBA and 0.217 µM GA_3. However, Lamaoui *et al.* (2019) induced argan shoots (93%) on MS medium supplemented with 11.1 µM BA combined with only 5.71 µM IAA.

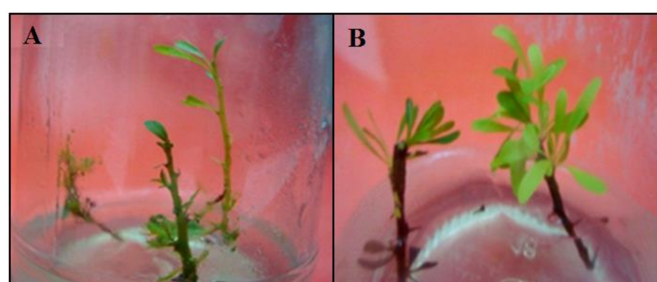

Fig. 2: In vitro culture establishment from argan seedling explants cultured on MS medium supplemented with 2.325 µM Kin, 0.289 µM GA_3 and 8.88 µM BA. **(A)** Shoot tips establishment. **(B)** Stem node segments establishment.

3. Multiplication of axillary shoots

Argan axillary shoots were examined for multiplication on MS medium supplemented with different concentrations of BA in combination with 0.537 µM NAA or on MS medium supplemented with different concentrations of TDZ in combination with 0.537 µM NAA or 2.89 µM GA_3 or both (Table 3). Both BA and TDZ were tested as cytokinins for multiplication, because bud break and axillary shoot multiplication is a cytokinin function as reported for other woody plants (Lamaoui *et al.,* 2019). From Table (3), it is clear that the highest mean number of axillary shoots/explant of 4.6 was achieved by culturing argan axillary shoots on MS medium supplemented with 8.88 µM BA and 0.537 µM NAA with 100% growth induction percentage and 0.86 cm mean length of axillary shoots/explant (Fig. 3). However, by increasing or decreasing BA concentration, the mean number of axillary shoots/explant decreased. The control medium without BA achieved the fewest number of axillary shoots/explant (1.72) with a mean length of 1.1 cm. The addition of an auxin to the medium promotes shoot growth and counteracting the inhibitory effect of BA on axillary shoot elongation (Lamaoui *et al*., 2019). This can explain the increase of mean length of axillary shoots at high BA concentrations, which may contribute to the presence of an auxin (NAA) in the medium. Using BA as a cytokinin with an auxin to multiply argan axillary shoots was also reported by Marfil *et al.* (2011), who multiplied argan shoots on MS medium supplemented with 3.996 µM BA in addition to 0.049 µM IBA and 0.217 µM GA_3 and also reported that, MS medium was found to give the better growth and multiplication of shoots, compared to other tested media. However, Lamaoui *et al.* (2019) multiplied argan shoots on MS medium supplemented with only 6.66 µM BA.

Table 3: Influence of MS medium supplemented with different concentrations of BA in combination with 0.537 µM NAA on multiplication of argan axillary shoots.

BA (µM)	Explants forming growth (%)	No. of axillary shoots/explant	Length of axillary shoots/explant (cm)
0.00	90^{a}	1.72^{b}	1.10^{b}
4.44	100^{a}	4.40^{a}	1.06^{c}
8.88	100^{a}	4.60^{a}	0.86^{e}
13.32	90^{a}	4.00^{ab}	1.20^{a}
17.76	90^{a}	3.10^{ab}	1.10^{b}
22.20	70^{a}	2.60^{b}	1.00^{d}

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$.

On the other hand, axillary shoots cultured on MS medium supplemented with different concentrations of TDZ (Table 4) were found to give higher mean number and length of axillary shoots/explant than MS medium supplement with BA (Fig. 3). Axillary shoots cultured on MS medium supplemented with 2.27 µM TDZ and 0.537 µM NAA gave the highest mean number (5.1), followed by the same concentration of TDZ (2.27 µM) in combination with 2.89 µM GA_3. This result is in harmony with that of Justamante *et al*. (2017), who promoted shoot multiplication of argan on MS medium supplemented with 2.27 µM TDZ and 2.89 µM GA_3. The second medium (with GA_3) gave the longest axillary shoots/explant of 2.1 cm. The control medium and that supplemented with 6.81 µM TDZ and 0.537 µM NAA ranked next concerning the mean length of axillary shoots/explant (2.0 cm), but with the lowest number of axillary shoots on the control medium. From the two multiplication experiments, as the main purpose is obtaining the largest number of axillary shoots, therefore, TDZ was more effective in axillary shoots multiplication than BA and MS medium supplemented with 2.27 µM TDZ and 0.537 µM NAA is considered the optimum for mulriplication of argan axillary shoots. Guo *et al*. (2011) reported that TDZ has shown both auxin and cytokinin like effects. Lamaoui *et al*. (2019) have also reported the cytokinin-auxin synergetic effect on the multiplication rate of argan shoots.

Table 4:. Influence of MS medium with different concentrations of TDZ supplemented with NAA and/or GA_3 on axillary shoots multiplication of argan.

PGRs Conc. (µM)			Explants forming growth (%)	No. of axillary shoots/explant	Length of axillary shoots/explant (cm)
TDZ	**NAA**	**GA_3**			
Control			90[b]	1.72[l]	2.0[b]
2.270	0.537	0.00	100[a]	5.10[a]	1.8[d]
3.405	0.537	0.00	100[a]	4.10[c]	1.3[h]
4.540	0.537	0.00	100[a]	3.20[f]	1.2[i]
6.810	0.537	0.00	100[a]	3.90[d]	2.0[b]
2.270	0.000	2.89	100[a]	4.80[b]	2.1[a]
3.405	0.000	2.89	100[a]	2.60[h]	1.5[g]
4.540	0.000	2.89	100[a]	3.60[e]	1.2[i]
6.810	0.000	2.89	100[a]	3.00[g]	1.6[f]
2.270	0.537	2.89	100[a]	2.60[h]	1.8[d]
3.405	0.537	2.89	100[a]	2.40[i]	1.9[c]
4.540	0.537	2.89	100[a]	2.30[j]	1.7[e]
6.810	0.537	2.89	100[a]	2.00[k]	1.8[d]

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$.

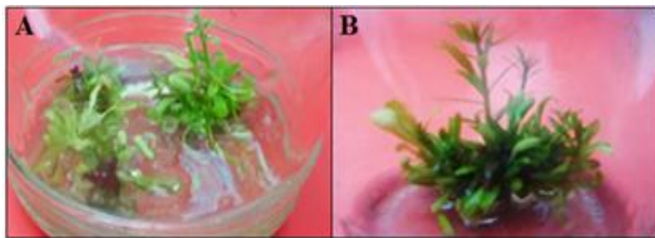

Fig. 3: Multiplication of argan axillary shoots cultured on **(A)** MS medium supplemented with 8.88 µM BA in combination with 0.537 µM NAA and **(B)** MS medium supplemented with 2.27 µM TDZ in combination with 0.537 µM NAA.

Influence of nanoparticles on multiplication

Table (5) represents the influence of TiO_2 and SiO_2 NPs on the multiplication of argan axillary shoots. Axillary shoots were cultured on the optimum medium for axillary shoots multiplication (MS medium supplemented with 2.27 µM TDZ and 0.537 µM NAA), in addition to 5, 10 and 20 ppm of TiO_2 or SiO_2 NPs. All axillary shoots (100%) formed growth on all tested treatments. However, using 10 ppm of TiO_2 NPs gave the highest mean number of axillary shoots of 6.2 with a mean length of 2.8 cm, compared to the control and other treatments. The second-best treatment was 5 ppm SiO_2 NPs, which gave 5.75 and 1.9 cm mean number and length of axillary shoots/explant, respectively. It is noticed from the results in Table (5) that the mean number and length of axillary shoots decreased by increasing or decreasing the concentration of TiO_2 NPs. However, increasing the concentration of SiO_2 NPs significantly decreased the mean number of axillary shoots. On the other hand the mean length significantly increased, giving the highest mean length of axillary shoots/explant of 2.92 cm.

To conclude, TiO_2 NPs was the best treatment for enhancing the multiplication of argan axillary shoots (Fig. 5A), compared to SiO_2 NPs. The concentration of 10 ppm of TiO_2 NPs was the optimum treatment that increased the mean number of axillary shoots up to 6.2. It was reported that, TiO_2 NPs play a vital role similar to PGRs; such as cytokinin and gibberellin (Davies, 2010 and Sauret-Güeto *et al*., 2012). Therefore, TiO_2 NPs were suggested to be alternative to PGRs for their ability to induce cell division and expansion (Mandeh *et al*., 2012). Jalill and Yousef (2015) mentioned that, application of TiO_2 NPs promotes plant growth, increases the photosynthetic rate, reduces disease severity and enhances yield by 30% in rice *in vitro*. Also, Dehkourdi and Mosavi (2013) reported an increase in the percentage and rate of germination, root and shoot length, fresh weight, vigor index, and chlorophyll content of *in vitro* grown *Petroselinum crispum* by application of TiO_2 NPs. They facilitate the absorbance of minerals that promote the formation of chlorophyll and activate key enzymes responsible for carbon fixation.

Table 5: Influence of TiO_2 and SiO_2 NPs on the multiplication of argan axillary shoots cultured on MS medium supplemented with 2.27 µM TDZ and 0.537 µM NAA.

Nanoparticles		Explants forming growth (%)	No. of axillary shoots /explant	Length of axillary shoots/explant (cm)
Type	**Conc. (ppm)**			
Control		100[a]	5.10[f]	1.80[g]
TiO_2	5.00	100[a]	5.50[c]	2.20[e]
	10.0	100[a]	6.20[a]	2.80[b]
	20.0	100[a]	4.80[g]	2.51[d]
SiO_2	5.00	100[a]	5.75[b]	1.90[f]
	10.0	100[a]	5.40[d]	2.62[c]
	20.0	100[a]	5.20[e]	2.92[a]

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$.

4. Rooting of axillary shoots

Elongated shoots were transferred to ½ MS medium supplemented with IBA and NAA at different concentrations, either individually or in combination, for root induction (Table 6). Using IBA or NAA individually gave negative results, while the combination of IBA with 5.37 µM NAA induced roots. Half strength MS medium supplemented with 9.8 µM IBA and 5.37 µM NAA gave the highest root induction percentage (30%), while using ½ MS medium supplemented with 4.9 or 14.7 µM IBA in combination with 5.37 µM NAA gave 10% of root induction. It was reported that in woody plant species, low mineral concentrations are better for rooting (Lamaoui *et al.*, 2019). Concerning previous researches on argan, Justamante *et al.* (2017) promoted root induction in argan shoots on MS medium supplemented with NAA and IBA. Recently, Lamaoui *et al.* (2019) achieved better rooting of argan shoots on ¼ MS supplemented with 24.5 µM IBA combined with 5.37 µM NAA. Nouaim *et al.* (2002) achieved the highest rooting percentage of argan of 17%, when IBA was added to the medium, 14.8% in the presence of IBA and BA and 6.9% with IBA and phloroglucinol. It is well known that rooting of argan is highly genotype-dependent (Nouaim *et al.*, 2002). In conclusion, in the present study, the best rooting medium of argan axillary shoots was ½ MS medium supplemented with 9.8 µM IBA and 5.37 µM NAA, achieving 30% rooting of 0.3 roots of 1.1 cm/shoot (Fig. 4).

Influence of nanoparticles on rooting

Despite the promising shoot multiplication efficiency, the achieved rooting percentage was still not sufficient. Table (7) represents the influence of TiO_2 and SiO_2 NPs on the rooting of argan axillary shoots. Axillary shoots were cultured on the best rooting medium (½ MS medium supplemented with 9.8 µM IBA and 5.37 µM NAA), in addition to 5, 10 and 20 ppm of TiO_2 or SiO_2 NPs. Table (7) shows that, there is a positive effect of SiO_2 NPs at 5 ppm on root induction percentage, and mean number and length of roots/explant, compared to the control, they reached 40%, 1.7 and 2.5 cm, respectively (Fig. 5B and C). However, TiO_2 NPs at the same concentration (5 ppm) showed an inhibitory effect, compared to the control. Using SiO_2 NPs at higher concentrations (10 and 20 ppm) showed a decrease in the percentage of root induction to 10% at 10 ppm with a significant decrease in the mean number and length of roots/explant, while no rooting was observed at 20 ppm. Also, higher concentrations of TiO_2 NPs (10 and 20 ppm) did not induce rooting. Nanoparticles of SiO_2 play an effective role in regulating diverse mechanisms involved in plant abiotic stress and they possess great surface area and solubility (Monica and Cremonini, 2009 and Manzer and Mohamed, 2014). The results of the present study agree with Lin *et al.* (2004) and Lee *et al.* (2010), who reported that, SiO_2 NPs promote root elongation at low concentrations and show toxicity effect at high concentrations. However, El-Boray *et al.* (2017) indicated the positive effect of SiO_2 NPs on inducing adventitious root number and length of *in vitro* propagated banana and also improving growth and photosynthetic pigments.

5. Acclimatization of plantlets

Rooted plantlets were transferred to be acclimatized into greenhouse with 60% of survival percentage (Fig. 5D). It is known that the acclimatization of plantlets is a challenge and required about six months to establish under greenhouse conditions.

Table 6: Influence of ½ MS medium supplemented with different concentrations of IBA and/or NAA on root induction of argan axillary shoots.

Growth regulators Conc. (µM)		Rooting (%)	No. of roots /explant	Length of roots/explant (cm)
IBA	**NAA**			
Control		0	0.0	0.00
4.9	0.00	0	0.0	0.00
9.8	0.00	0	0.0	0.00
14.7	0.00	0	0.0	0.00
19.6	0.00	0	0.0	0.00
24.5	0.00	0	0.0	0.00
0.0	5.37	0	0.0	0.00
0.0	10.74	0	0.0	0.00
0.0	16.11	0	0.0	0.00
0.0	21.48	0	0.0	0.00
0.0	26.85	0	0.0	0.00
4.9	5.37	10^b	0.1^b	0.30^b
9.8	5.37	30^a	0.3^a	1.10^a
14.7	5.37	10^b	0.1^b	0.25^b
19.6	5.37	0	0.0	0.00
24.5	5.37	0	0.0	0.00

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$.

Fig. 4: Rooted axillary shoots of argan on ½ MS medium supplemented with 9.8 µM IBA in combination with 5.37 µM NAA.

Table 7: Influence of TiO_2 and SiO_2 NPs on the rooting of argan axillary shoots cultured on ½ MS medium supplemented with 9.8 µM IBA and 5.37 µM NAA.

Nanoparticles		Root induction (%)	No. of roots/explant	Length of roots/explant (cm)
Type	Conc. (ppm)			
Control		30^{ab}	1.0^{b}	1.1^{d}
TiO_2	5.0	20^{ab}	1.0^{b}	2.0^{c}
	10.0	0	0.0	0.0
	20.0	0	0.0	0.0
SiO_2	5.0	40^{a}	1.7^{a}	2.5^{a}
	10.0	10^{ab}	0.5^{c}	2.2^{b}
	20.0	0	0.0	0.0

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$.

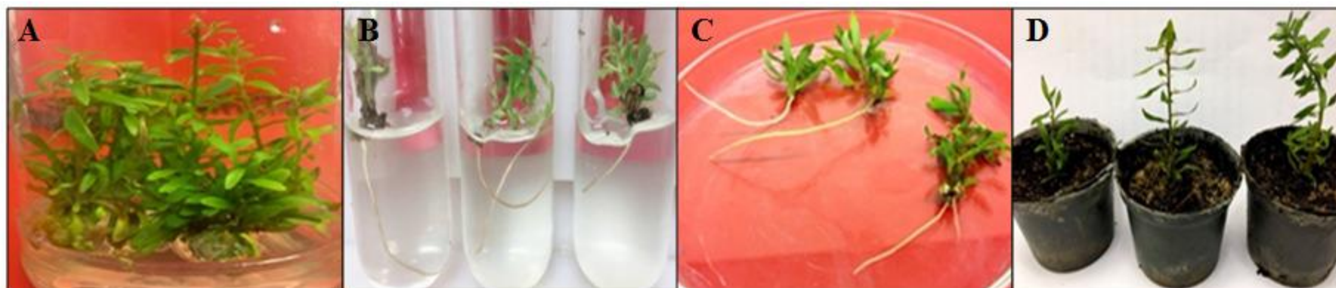

Fig. 5: Influence of NPs on the multiplication and rooting of argan. **(A)** Multiple axillary shoots on MS medium supplemented with 2.27 µM TDZ, 0.537 µM NAA and 10 ppm TiO_2 NPs, **(B)** and **(C)** Rooted axillary shoots on ½ MS medium supplemented with 9.8 µM IBA, 5.37 µM NAA and 5 ppm SiO_2 NPs, **(D)** Transplants acclimatized in the greenhouse.

Conclusion

A successful *in vitro* culture protocol for *ex situ* conservation of the endangered argan plant was carried out in the present study. This protocol could also be applied for the production of true to type plants of the superior genotypes for agricultural programs, for large scale production. Using TiO_2 and SiO_2 NPs at low concentrations exhibited better multiplication and rooting of *in vitro* derived axillary shoots. Therefore, using NPs at appropriate concentrations is promising in improving *in vitro* plant growth. The present study could be applied for the plantation of argan in the arid regions in Egypt as a drought tolerant valuable oil producing plant.

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