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Research Article
Effect of $AgNO_3$ and BAP on Root as a Novel Explant in Date Palm (*Phoenix dactylifera* cv. Medjool) Somatic Embryogenesis

[1]Marjan Roshanfekrrad, [2]Reza Zarghami, [1]Hassan Hassani, [1]Hedayat Zakizadeh and [3]Ali Salari

[1]Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran
[2]Agricultural Biotechnology Research Institute of Iran (ABRII), Karaj, Iran
[3]National Research Center on Plant Biotechnology (NRCPB), New Delhi, India

Abstract

Background and Objective: Somatic embryogenesis techniques are used for cloning a wide range of varieties of date palms around the world. The aim of the present study was to develop an efficient method with the lowest cost and the greatest potential to obtain *in vitro* plantlets of date palm cv. Medjool. Also, produce embryogenic callus and somatic embryos without using 2,4-dichlorophenoxyacetic acid (2,4-D). **Methodology:** In this study, produced plantlets through somatic embryogenesis were used *in vitro* roots as explant cultured on Murashige and Skoog (MS) media containing three level of Silver Nitrate ($AgNO_3$) (0, 3 and 6 mg L^{-1}) plus two level of 6-benzylaminopurine (BAP) (0 and 2 mg L^{-1}) plus 0.1 mg L^{-1} 1-naphthylacetic acid (NAA) for callus induction. After 12 weeks of culture, callus induction and after 16 weeks, production of embryogenic callus and embryos were occurred from root explants. **Results:** According to the results, medium containing 2 mg L^{-1} BAP and 3 mg L^{-1} silver nitrate+0.1 mg L^{-1} NAA showed the highest amount of embryogenic callus fresh weight (1.38 g). This treatment also cause the highest number and length of embryos by production of 90.04 embryogenic callus with length of 11.18 mm. On the other hand, shoots were appeared from germinated embryos and white roots began to appear within 8 weeks. Medium contains 3 mg L^{-1} BAP and 0.1 mg L^{-1} NAA with average of 12.27 cm shoot length and 15.48 cm root length was the best. Control treatment had the lowest average shoot (3.71 cm) and root (5.03 cm) length. **Conclusion:** This study showed that certain concentration of silver nitrate and BAP has stimulating effect on growth of produced embryonic callus from root segments of Medjool cultivar of date palm.

Key words: Date palm, plant tissue culture, micropropagation, somatic embryos, embryogenic callus, NAA, silver nitrate, root segment

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Corresponding Author: Reza Zarghami, Agricultural Biotechnology Research Institute of Iran (ABRII), Karaj, Iran

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is one of the most important fruit trees native to the desert countries. Dates have been a staple food of the West Asia and North Africa for thousands of years, where its cultivation is the main source of income for farmers[1]. Date palm growers can use all parts of the date palm trees like sap, seeds, leaves and trunk for finding additional income[2]. Vegetative propagation by traditional methods in *Phoenix dactylifera* L., is challenging and time-consuming due to the slow growth behavior of these plants[1,3]. Therefore, micropropagation is a useful alternative for propagation of elite cultivars of date palm as well as a rapid method for providing pathogen free and true-to-type plants[4].

Plant tissue culture techniques are used for cloning a wide range of varieties of date palms around the world. Somatic embryogenesis is one of these techniques in which embryos are produced from embryogenic callus and have ability to produce the whole plant[5,6]. This method is based on callus proliferation as an induction phase in the medium containing different values of auxin and elongation in the medium without auxin[7].

The other technique is direct organogenesis in which the plantlets are produced by the number of vegetative buds, directly from mother plant tissues, without passing callus phase[8,1]. The effect of different hormonal treatments have been tested in this method which of BAP as a cytokinin were shown significantly effective in different studies[9-11].

For plant regeneration through these techniques, different part of tissues can be used. Because, plant cells have totipotency property and can produce a new plant under appropriate nutritional and hormonal balance[12]. In some studies, the meristem and lateral buds were used as explants for micropropagation and production of callus and vegetative buds[8,13-15]. In the other studies, other organs were used for micropropagation of date palm such as, root segments[16], young leaves[17], stem segments[18] and inflorescences[19].

Staritsky[20] and Schroeder[21] did the first research on *in vitro* culture of root segments in palms and found that oil palm roots and roots primordia showed no signs of proliferation. Schroeder[21] also reported that cultured root segments of date palm produced secondary rootlets but did not produce buds. Some investigators reported that apical root region of young date palm plantlets can produce callus and the shoots and finally the plantlets can form from callus[22,23]. Zaid and Tisserat[24,25] observed formation of callus from asexual plantlets roots but the callus did not show any sign of morphogenic response. Some researchers used obtained roots of the other tissue culture techniques as explants and achieved positive results[26,16]. This is because, most of plant tissues can grow and have micro-propagation when the conditions such as growth regulators are appropriate.

In tissue culture, closed vessels are used with the purpose of avoiding contamination, therefore, compounds such as ethylene (C_2H_4) produced by tissue and accumulated in the media that has avoiding effects on callus growth, shoots and embryo initiation cause abnormalities in plantlets[27]. To avoid the effects of this growth regulator, chemical compounds such as silver nitrate ($AgNO_3$) are used in the media[28]. By adding 3 mg L^{-1} silver nitrate to the media cause enhancing shoot elongation in lemon[29].

Practically, date palm micropropagation method through somatic embryogenesis is time consuming by using meristem as explant. The procedure, force us to use high amount of 2,4-D during the different stages of callusing formation which cause the probability of mutation and abnormality. In addition, spending repetitive sub-culturing found to be negatively effective to the callus and embryo formation potential of date palm using the above explant. Therefore, the aim of the present study is to develop an efficient method using *in vitro* roots as an explant for the first time in this variety, with the lowest cost and time and the greatest potential to obtain date palm cv. Medjool *in vitro* plantlets and produce embryogenic callus and somatic embryos with the lowest number of sub-culture treatments without using 2,4-D and make the entire study unicultural up to the embryos formation.

MATERIALS AND METHODS

Effect of $AgNO_3$ and BAP on somatic embryos production in root segment explants: To study the effect of different concentrations of $AgNO_3$ and BAP on somatic embryos production in root segment explants, the produced plantlets through somatic embryogenesis were removed from their culture media under laminar air flow and were immersed in distilled water by sterile forceps to remove medium debris between the roots. Then, roots of the plantlets were cut into 1-2 cm segments by sterile scalpels in sterilized petri dishes and each four segments were cultured in petri dishes consists of MS media[30] supplemented with myo-inositol (100 mg L^{-1}), glutamine (200 mg L^{-1}), thiamine-HCL (1 mg L^{-1}), nicotinic acid (1 mg L^{-1}), pyridoxine-HCL (1 mg L^{-1}), sucrose (30 g L^{-1}), activated charcoal (1.5 g L^{-1}) and agar (7 g L^{-1}) in addition of three concentration of $AgNO_3$ (0, 3 and 6 mg L^{-1}) and two concentration of BAP (0 and 2 mg L^{-1})+0.1 mg L^{-1} NAA (Table 1). Then, cultures were incubated at 16/8 light/dark day. This experiment was conducted in the factorial form and in a completely randomized design with six replicates (each

replication consisting of 3 petri dishes). About 16 weeks after callus induction, embryogenic callus fresh weight, number and length of embryos which were outcome of 100 mg of 20 days old embryogenic callus were recorded.

Shoot and root production: The germinated embryos were transferred to MS media in five treatments containing control, 0.5, 1, 2 and 3 mg L^{-1} BAP with 0.1 mg L^{-1} NAA for shoot production and after 8 weeks (two sub-culture) the plantlets were individually cultured in the same treatments to produce adaptable and strong roots. The experiment was set up as a completely randomized design with six replicates (each replication consisting of three explants). The media were incubated at normal light condition and after 12 weeks, shoot and root length (cm) of each treatment was recorded.

Culture conditions and statistical analysis: Tissue culture media were adjusted to pH 5.7±0.1 using 1 N. The NaOH before autoclaving at 121°C and at 1.1 kg cm^{-2} pressure for 20 min. Cultures were incubated at 27±1°C and a photoperiod of 16/8 with light intensity of 2000 lux. The explants were sub-cultured every 4 weeks. The data were statistically analyzed using SAS version 9.1 and means were compared by Duncan's multiple range test at 1%.

RESULTS AND DISCUSSION

Effect of $AgNO_3$ and BAP on somatic embryos production in root segment explants: After 12 weeks of culture, callus induction and after 16 weeks, production of embryogenic callus and embryos were occurred from root segment explants. In this experiment, the effect of silver nitrate and BAP was evaluated on production of embryogenic callus and somatic embryos after 16 weeks. Variance of analysis (Table 2) of different concentration of $AgNO_3$ (A) and BAP (B) on average embryogenic callus fresh weight and number of embryos showed that the interaction between different treatments were significant at 1%.

Figure 1 shows the interaction between silver nitrate and BAP levels in which medium with 2 mg L^{-1} BAP and 3 mg L^{-1} silver nitrate+0.1 mg L^{-1} NAA had the greatest amount of embryogenic callus fresh weight (1.38 g) and after that, treatment without BAP and with 3 mg L^{-1} silver nitrate+0.1 mg L^{-1} NAA with 1.15 g embryogenic callus fresh weight was the best. Results revealed that silver nitrate has stimulating effect on growth of embryonic callus which is depend on its concentration. However, it could be suggested that among the silver nitrate levels (0, 3 and 6 mg L^{-1}) applied, middle concentrations are comparatively better than lower and higher ones in date palm cv. Medjool.

The BAP is one sort of cytokinins which promote cell division in plants and have effective role on maturation of callus and embryos. Sub-culture of the tissue onto a medium containing a cytokinin can then cause the cells to divide synchronously after a lag period[31]. Therefore, cytokinins like BAP have essential role in tissue culture techniques like somatic embryogenesis. As Kurup *et al.*[32] depicted, BAP is

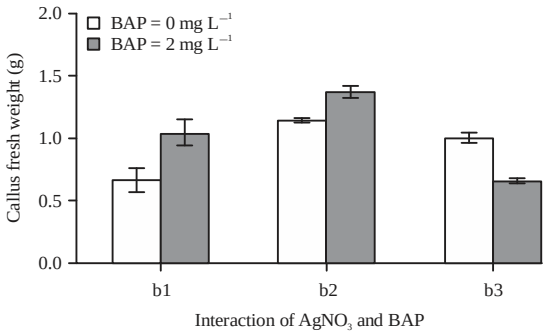

Fig. 1: Interaction of $AgNO_3$ and BAP on embryogenic callus fresh weight. b1: 0 mg L^{-1} $AgNO_3$, b2: 3 mg L^{-1} $AgNO_3$, b3: 6 mg L^{-1} $AgNO_3$ (each treatment contain 0.1 mg L^{-1} NAA)

Table 1: Treatments of $AgNO_3$ and BAP on somatic embryos production (each treatment contains 0.1 mg L^{-1} NAA)

Treatments	$AgNO_3$ (mg L^{-1})	BAP (mg L^{-1})
1	0	0
2	3	0
3	6	0
4	0	2
5	3	2
6	6	2

Table 2: Analysis of variance of the effects of different concentration of $AgNO_3$ and BAP on root segments through somatic embryogenesis (each treatment contain 0.1 mg L^{-1} NAA)

Source of variation	df	Average callus fresh weight (g)	Average No. of embryos	Average length of embryos (mm)
LSD: 0.01 mean square				
PGR treatment (A)	2	0.631**	2217.81**	51.43**
PGR treatment (B)	1	0.431**	1454.15**	52.80**
AB	2	1.170**	6796.20**	30.98**
Error	30	0.003	2.21	0.09
Total	35			
		CV% 5.26	CV% 2.16	CV% 4.40

**Significant at 1%

considered to be a potential cytokinin in rapid cell division process to accelerate the differentiation and development process. Likewise, many reports have shown that combination of BAP with auxins like NAA has significant effects on plant regeneration. Ezeibekwe *et al.*[33] demonstrated that BAP (0.2 mg L^{-1}) in combination with NAA (0.5 mg L^{-1}) has more increasing effects in almost all of the measured parameters in compare to other concentrations in *Dioscorea rotundata*. As well as, Sharma *et al.*[34] illustrated concentration of BAP (1.0 mg L^{-1}) and NAA (0.1 mg L^{-1}) motive *in vitro* generated callus and subsequent shoot proliferation in *Eclipta alba*. In addition, Aghaei *et al.*[35] represented that treatments containing BAP in the medium produced the greatest amount of callus fresh weight and dry weight of callus.

Smith and Thomas[22] produced callus by cultivating coconut roots. Eshraghi *et al.*[36] showed the effect of (2,4-D and BAP) on callus and asexual embryos induction in date palm. In fact, in Khanizi cultivar, embryogenic callus was induced on media containing 4.6 mg L^{-1} BAP and 3.4 mg L^{-1} 2,4-D. In return, in Mordarsing cultivar, using high concentrations of 2,4-D (150 mg L^{-1}) is necessary for embryogenic callus induction. Aasim *et al.*[37] observed that callus induction was more on MS medium containing BA-NAA compared to MS medium devoid of NAA. In other study, Aghaei *et al.*[35] showed that highest percentage of callus induction was in the medium containing 1 mg L^{-1} BAP (85%). Somatic embryogenesis has also been accomplished by Kurup *et al.*[32] who reported that the combination of BAP with NAA is considered to be the potential factor to elicit a rapid response in callus induction through somatic embryogenesis.

Ethylene accumulation *in vitro* strongly inhibits the growth of some plants, like date palms. To remove ethylene from date palm culture vessels, forced ventilation and the use of some chemical compounds have been reported. Among the different chemicals, silver nitrate ($AgNO_3$) has been widely used also for enhancing tissue culture growth. The $AgNO_3$ was also used in order to reduce the occurrence of hyperhydricity in tissue culture of sunflower[38]. In this study, Al-Khayri and Al-Bahrany[39] reported that embryogenic callus weight significantly influenced by the reaction between silver nitrate and 2ip, such that in the absence of 2ip and just in the presence of silver nitrate (75 μM), the highest embryogenic callus weight was achieved. However, in the present study, the highest amount of embryogenic callus was achieved in presence of both BAP and silver nitrate. Al-Khayri and Al-Bahrany[40] obtained Khusab cultivar exhibited significant increase in callus weight at 12.5 μM $AgNO_3$ but maximum growth occurred at 62.5 μM. Therefore, silver nitrate increased embryogenic callus proliferation, which is parallel results with our study.

Results of interaction between treatments (Fig. 2, 3) on number and length of embryos clearly show that, the best treatment was the medium containing 2 mg L^{-1} BAP and 3 mg L^{-1} silver nitrate+0.1 mg L^{-1} NAA, by production of 90.04 embryogenic callus with length of 11.18 mm. On the other hand, media with 2 mg L^{-1} BAP and 6 mg L^{-1} silver nitrate+0.1 mg L^{-1} NAA had produced the lowest number of somatic embryos (42.32) and media without BAP and silver nitrate had the lowest growth in length of somatic embryos. According to the obtained results, the effect of silver nitrate on increasing the length of embryos is obvious, especially when it is combined with BAP has greater effect. On the other hand, high concentration of silver nitrate doesn't have a significant effect on embryo length. These results are in agreement with those of Al-Khayri and Al-Bahrany[39] in which it was represented the positive effect of silver nitrate on increasing the length and number of asexual embryos in date palm that

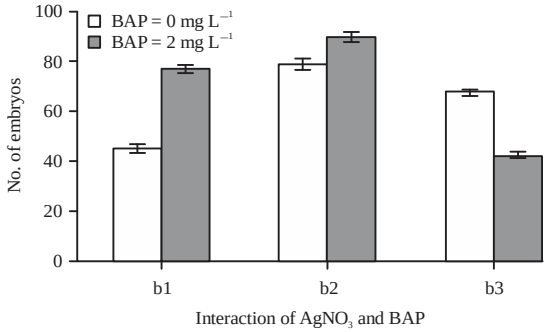

Fig. 2: Interaction of $AgNO_3$ and BAP on No. of embryos. b1: 0 mg L^{-1} $AgNO_3$, b2: 3 mg L^{-1} $AgNO_3$, b3: 6 mg L^{-1} $AgNO_3$ (each treatment contain 0.1 mg L^{-1} NAA)

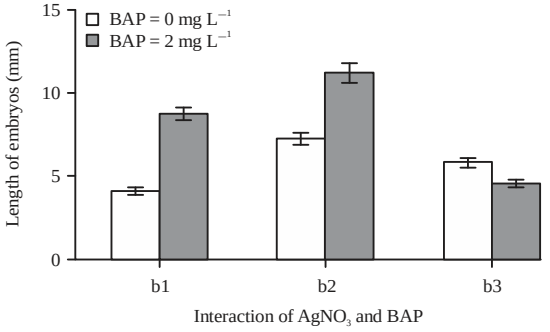

Fig. 3: Interaction of $AgNO_3$ and BAP on length of embryos (mm). b1: 0 mg L^{-1} $AgNO_3$, b2: 3 mg L^{-1} $AgNO_3$, b3: 6 mg L^{-1} $AgNO_3$ (each treatment contain 0.1 mg L^{-1} NAA)

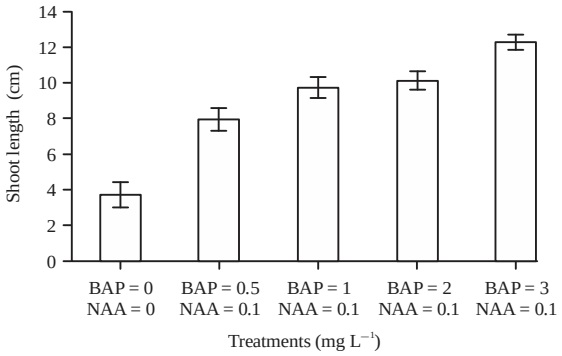

Fig. 4: Effect of BAP with 0.1 mg L^{-1} NAA on length of shoots (cm)

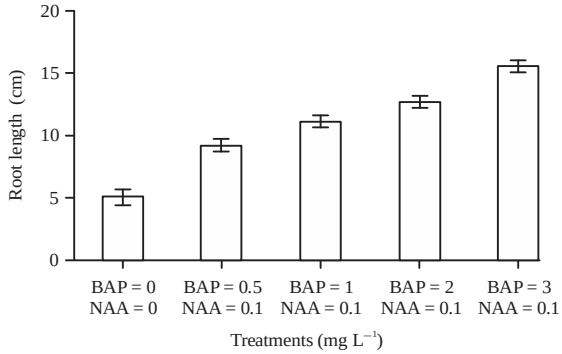

Fig. 5: Effect of BAP with 0.1 mg L^{-1} NAA on length of roots (cm)

Table 3: Analysis of variance of the effects of different concentrations of BAP with NAA on shoot and root length

Source of variation	df	Average length of shoots (cm)	Average length of roots (cm)
LSD: 0.01 mean square			
PGR treatment (A)	4	61.94**	91.96**
Error	25	0.21	0.14
Total	29		
		CV% 5.28	CV% 3.55

**Significant at 1%

the best treatment was 25 μM silver nitrate in combination with 0.5 μM 2ip. Furthermore, the action of silver nitrate was clearly modified by the addition of 2ip and in the present study the action of silver nitrate was completed with BAP. Also, Al-Khayri and Al-Bahrany[40] obtained that the number of embryos in cvs., Naboot Saif, Ruzaiz and Barhee were increased by 75, 12.5 and 37.5 μM $AgNO_3$. Hillali and Khusab cultivars were unchanged by $AgNO_3$ and at higher concentrations (50 μM), number of embryos was decreased. On the other hand, Ibrahim *et al.*[16] reported that treatment contains different concentration of BAP and NAA cause embryogenic callus and embryos production. Kurup *et al.*[32] obtained that transferring embryogenic callus to MS media containing 0.5 mg L^{-1} NAA and 0.25 mg L^{-1} BAP were resulted in the initiation of higher number of somatic embryos with roots (40.36) in a period of 10 weeks.

Shoots and roots production: In this study, the effect of BAP in combination with NAA was evaluated on production of shoots and roots after 12 weeks. According to the results presented in variance of analysis (Table 3), the effect of different concentration of BAP (A) with 0.1 mg L^{-1} NAA is significant (1%) and showed a different effect on length of shoots and roots.

Shoots were first appeared from germinated embryos and then white roots began to appear within 8 weeks. Sub-culturing the produced plantlets on individual same media and incubation for a period of 12 weeks resulted in the development of strong and healthy shoot and root system with plenty of lateral roots. The effect of five treatments on shoot and root length. According to these, the medium containing 3 mg L^{-1} BAP and 0.1 mg L^{-1} NAA with average of 12.27 cm shoot length and 15.48 cm root length was the best and control treatment had the lowest average shoot (3.71 cm) and root (5.03 cm) length (Fig. 4-6). The produced date palm seedlings of embryogenic callus have inferior root system that is due to the absence of adventitious roots. Production of adaptable and strong *in vitro* root system is necessary for succession in adaptation stage. El Sharabasy *et al.*[41] observed that NAA had a significant effect at 0.1 mg L^{-1} on root formation in compare with IBA and IAA. Al-Khayri[42] found out that embryos which are cultured in a medium without PGRs,

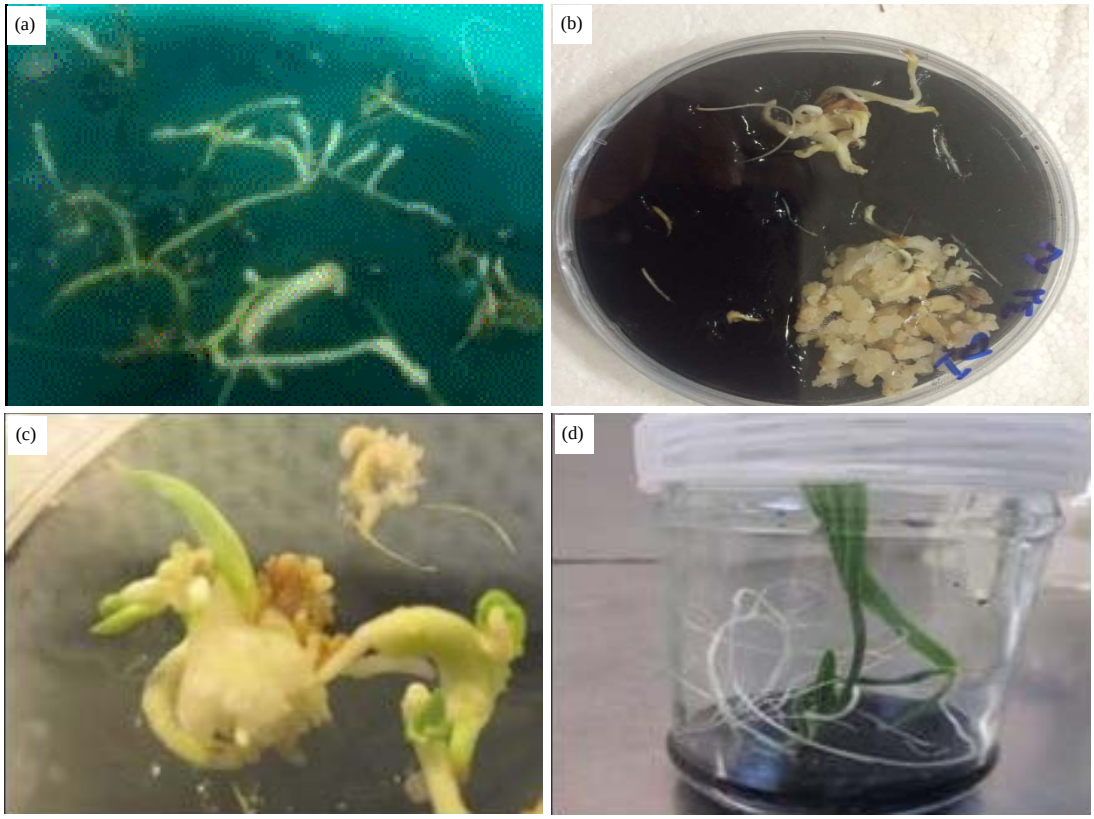

Fig. 6(a-d): Somatic embryogenesis from root segment explants of date palm cv. Medjool (a-b) Callus induction from root segments, (c) Somatic embryos production and (d) Shooting and rooting on MS medium

just producing shoots and need another step for rooting and shoot elongation and this step often has been completed using NAA. Eke *et al.*[43] mentioned that regenerated shoots are producing roots on a medium containing 0.1 mg L^{-1} NAA. Ghanati and Ishka[44] indicated that the transferred calli to the B_5 medium supplemented with ABA (2 mg L^{-1}) and high level of BA (400 mg L^{-1}) cause conversion of globular and heart embryos to shoot. Also, Yan *et al.*[45] reported that NAA has an important role on production of adventitious roots with the ability of shoot induction. According to observation of this study, although NAA has an important role on shoot and root elongation but in combination with 3 mg L^{-1} BAP has a clearly great effect on increasing the length of shoots and roots and also cause amplification in lateral roots which their presence is very important in acclimatization stage to absorb the nutrients from the soil.

CONCLUSION

This study introduced an efficient and low cost and time method for mass propagation of date palm Medjool cultivar through somatic embryogenesis. Results showed that silver nitrate has stimulating effect on embryonic callus growth, which is dependent on the concentration. However, it could be suggested that among the silver nitrate levels (0, 3 and 6 mg L^{-1}) applied, middle concentrations are comparatively better than lower and higher ones in date palm cv. Medjool. On the other hand, BAP together with NAA individually can prepare perfect shoot and root system ready for *in vivo* acclimatization. Also, this root formation can be used as explant for further callusing which introduced and used in this study for the first time in Medjool cultivar. Thus, the results of the present study can be used for micro-propagation of Medjool cultivar of date palm.

SIGNIFICANCE STATEMENTS

Plant tissue culture techniques are used for cloning a wide range of cultivars of date palms around the world in which more usefulness of somatic embryogenesis isn't dissembled. In present study, *in vitro* roots were used as explants in MS media containing silver nitrate and BAP+0.1 mg L^{-1} NAA, to produce somatic embryos. The reason behind this is fast growing *in vitro* root part and multitude number of them,

producing plantlets from this organ is more low cost and convenient for mass propagation of date palm Medjool cultivar.

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