Improving *In Vitro* Somatic Embryos Production of Medjool and Khalas Date Palm Cultivars via Modification of Ammonium and Potassium Nitrate

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Abstract: Date palm is one of the most economically powerful fruit tree grown in Egypt. Although Egypt tops the list of date's product in the world. Egyptian date palm industry suffers from many constraints. Low-quality varieties and limited conventional propagation methods are the main obstacles. Extension of date palm plantations in various areas of Egypt and replanting trees to compensate loss due to diseases or human factors are reduced expected to lack adequate planting materials. The knowledge of the interplay between some fundamental medium parameters allows not only the optimization of the micropropagation system but also gives models to investigate and rationalize the process of induction and development of somatic embryogenesis itself. *In vitro* growth and development of produced embryos and plantlets has been improved by using of full MS salt strength. MS medium modification of some nutrients concentrations, i.e. NH_4NO_3 (1237.5 mg/l) which recorded (13.20 and 9.60 embryos/explant for Medjool and Khalas respectively) at the end of three subculture. KNO_3 which gave a higher score of mature somatic embryos (14.40 and 10.00 embryo/culture for Medjool and Khalas respectively) was obtained with 1425 mg/l KNO_3 after three subcultures as compared to those obtained from any other treatment combination. The morphological response of somatic embryos production is controlled by some internal factors that fall under the influence of the genetic make-up of the plant and specialized for each genotype that is responsible for the production of somatic embryos (Medjool and Khalas cultivars). The genetic make-up is a decisive factor during somatic embryos production. There are differences between the two cultivars named, Medjool and Khalas. The produced somatic embryos at germination stage were transferred from the two experiments namely ammonium nitrate and potassium nitrate to multiplication and rooting stages for more *in vitro* growth for 12 weeks at three re-cultures. Individual shoots were cultured on modified MS basal medium in addition to IBA (1.0 mg/l), sucrose (30 g/l) and solidified with phyto-agar (8.0 g/l) for more *in vitro* growth and development. *In vitro* plantlets were transferred to acclimatization stage in plastic pots 5*18 cm diameter containing peatmoss, perlite and washed sand at equal volume. *In vitro* date palm plantlets produced from rooting stage grow well in the greenhouse during acclimatization stage without morphological abnormality. The growth and development of Medjool cultivar were better than Khalas during *in vitro* culture and *ex vitro* acclimatization.

Keywords: Ammonium nitrate, Potassium nitrate, Somatic embryos, *In vitro*, Date palm.

1. INTRODUCTION

The date palm (*Phoenix dactylifera* L.) is reflected a symbol of life in the desert because it withstands high temperatures, drought, and salinity more than many other fruit crop plant species. It is one of the oldest trees from which man has derived benefit, and it has been cultivated since ancient times. It is only indigenous wild desert plant domesticated in its harsh native environments appears to be the date palm [1]. The date palm (*Phoenix dactylifera* L.) is a critical crop tree in a large section of North Africa, the Middle East, and South Asia. It is one of three commercially necessary palms (Arecaceae) that include the monoecious oil and coconut palms. The date palm is dioecious with separate male and female trees. Only the female bears the commercially important date fruit. Trees grown from seed require approximately 6–8 years to flower before gender can be determined and no sex chromosome has been identified [2]. The date palm is a halophytic plant that can tolerate high levels of salinity. In Egypt and largest of the Middle Eastern countries, the date palm is the oldest and greatly extensively cultivated tree that is commercially the most important tree in the life of its people and their heritage. The significance of the date palm occurs because the production of other fruit trees is limited in the harsh environment. Also, seed-propagated palms do not bear true to type fruits due to heterozygosis and need up to seven years to reach the adulthood fruiting step [3]. *In vitro* micropropagation thus became an essential and efficient means to assure the renewal and the increase of palm plantations [4]. Since 1970, intensive attempts have been joined into large-scale micropropagation of date palm using techniques such as somatic embryogenesis and organogenesis [5, 6, 7, 8]. Plant regeneration through tissue culture can provide technologies for large-scale propagation of healthy true-to-type plants. The most commonly used technology approach is somatic embryogenesis which

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presents a high possible for the fast propagation and genetic resource preservation of this species [9]. Somatic embryogenesis is considered the most efficient regeneration process for date palm micropropagation [10]. Palms are regarded as a recalcitrant species to be used in tissue culture. Thus, the *in vitro* regeneration of date palm using somatic embryogenesis still requires more refined and optimized protocols. Egypt is the world head in date production and cultivation. There are about 12261651 female palm trees (according to the statistics of Ministry of Agriculture 2012). Each year, this country produces approximately 1,373,570 Ton of dates. Palm area with palm trees currently 73.653 thousand acres, or about 6.32% of the total cultivated fruit total area [11]. This represents a little over 17% of global date production but only 3% of world exports. Although Egypt overran the list of date's production in the world, date palm sector in Egypt has faced several obstacles. Like, low fruit quality, the ineffectiveness of the conventional propagation, the weakness of marketing services, neglect of farmers from achieving post-harvest processes are other important challenges, diseases and pathogen pests particularly Red Palm Weevil [12]. Factors and mechanisms controlling cell differentiation in somatic embryos are relatively ambiguous. Certain compounds excreted by plant tissue cultures and found in culture media have been shown necessary to coordinate cell division and morphological changes [13]. These compounds have been identified by [14] as various polysaccharides, amino acids, growth regulators, vitamins, low molecular weight compounds and polypeptides. Several signaling molecules known to influence or control the formation of somatic embryos have been found and include extracellular proteins, arabinogalactan proteins and lipochitooligosaccharides. Temperature and lighting can also affect the maturation of the somatic embryo. Micropropagation of date palms is also simple to transport and plant. Micropropagation of date palm on the used MS media [15] faces problems. Such as little differentiation of somatic embryos, browning, and vitrification of embryo tissue, the recalcitrance of explants, blockage of growth due to non-availability of the proper kind and amount of the nutrient (proteins, sugars, and lipids) occurring in the production of weak embryos. Nitrogen is an essential element in modern mineral salt formulations and is present in the form of both nitrate (NO_3^-) and ammonium (NH_4+) ions. Nitrogen plays a significant role in growth and differentiation of somatic embryos. The strengths of NH_4NO_3 and KNO_3 significantly affect the differentiation of somatic embryos. Cell hyperhydrated tissues has been attributed to unfavored lignification [16]. Hyperhydricity (vitrification) is a tissue culture disorder affecting *in vitro* growth of date palm especially during somatic embryos formation [17]. Improving appropriate media is necessary for the efficient generation of embryos for the successful micropropagation of date palm employing *in vitro* methods. It is, therefore, important to suitably modify the media to ensure improved embryo production. There is a limit of research on the effect of ammonium and Potassium nitrate on maturation and germination of somatic embryos in date palm plants. To efficiently sufficient the induction of plants by indirect somatic embryogenesis, it is important to study the influence of potassium and ammonium nitrate on the maturation and germination of somatic embryos to plantlets *in vitro* than during the acclimatization stages.

2. MATERIALS AND METHODS

This study was carried out during 2013-2016 at the Laboratory of Tissue Culture Center. Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Egypt.

2.1. Plant Materials and Preparation

Two to three years old offshoots of date palm cv Medjool and Khalas were helpful provided by Prof. Ibrahim Abdel-Maksoud Ibrahim. For obtaining the shoot tip and leaf primordial explants. Offshoots 5 to 7kg in weight, were separated from adult date palm trees of cvs. Medjool and Khalas. Leaves were carefully separated and the terminal portion of the shoots composed of the apical meristem, many leaves primordial and subapical tissue, was excised. The leaves and fiber sheath were carefully removed from offshoot acropetally upwards with a hatchet, which saw, serrated knife and sharp knife (always cleaned with antiseptic solution, betadine 10 % w/v). The separation of leaves was carried out starting from the base until the shoot tip material reached 3- 4 cm in width, and 6- 8 cm in length. The plant materials were sprayed with ethyl alcohol (70%) then soaked in betadine for 30 minutes and then rinsed with distilled water. Then the explants were immersed in a cold sterilized antioxidant solution containing citric and ascorbic acids each at the concentration of 150 mg/l and kept in the refrigerator until the surface sterilization procedure is performed.

2.2. Surface Sterilization of Explant Materials and *In Vitro* Callus Establishment

Date palm explant materials were surface sterilized by soaking for 30 min in Clorox 40% (NaOCl 2%) containing two drops of Tween 20 as the wetting agent.

This process was again repeated once. It was then rinsed with sterile distilled water. Another sterilization treatment was used for 30 min by soaking plant material in Clorox 40% (NaOCl 2%) containing two drops of Tween 20 as the wetting agent. Following this one leaf primordium was thoroughly isolated from shoot tip explants. Followed by immersion in sterilized mercuric chloride solution $HgCl_2$ (0.1%) containing two drops of Tween 20. It was then rinsed five times with sterile distilled water and finally soaked in a filter sterilized antioxidant solution containing citric and ascorbic acids each at the concentration of 150 mg/l to avoid browning. The leaf primordial were carefully removed one by one and shoot tip explants were about 0.5 – 1.0 cm in length and consisted of the apical meristem, 4 – 6 leaf primordial, cut longitudinally into four sections and inoculated onto culture medium consists of [15] basal medium (MS) + 40 mg/l adenine-sulfate + 10 mg/l 2,4- diclorophenoxy acetic acid (2,4-D) + 3 mg/l isopenetyl adenine (2iP) + 170 mg/l NaH_2PO_4 + 30 g/l sucrose + 2 g/l activated charcoal (AC) and 8 g/l agar [18]. Cultures were incubated under complete darkness for eight months incubated at constant temperature 27±2 with annual transfer to fresh medium of the same components every four weeks. After this period, embryogenic callus was formed and then transferred to MS medium to use in the subsequent experiments to obtain somatic embryos.

2.3. Influence of NH_4NO_3 Ratio on Induction of Somatic Embryos and Hyperhydricity of Date Palm cv. Medjool and Khalas

The embryogenic callus produced from the previous stage was inoculated on MS basal medium modified. MS medium modified with NH_4NO_3 at various concentrations were tested (412.5, 825.0, 1237.5 and 1650.0 mg/l), with 0.1 mg/l naphthalene acetic acid (NAA) and 0.05 benzyl adenine (BA) for 12 weeks throughout 3 re-cultures (re-culturing interval every 4 weeks). Cultures were incubated at 25±1°C in a growth room. Each treatment was represented by five jars (replicates) after four weeks; the following data was shown for each studied treatment, i.e. Number of somatic embryos, somatic embryos formation percentage and Hyperhydricity were also scored according to [19].

2.4. Influence of Potassium Nitrate (KNO_3) Concentrations on Induction of Somatic Embryos and Hyperhydricity of Date Palm cv. Medjool and Khalas

Embryogenic callus was transferred on MS basal medium modified with different concentrations of KNO_3 (475, 950, 1425 and 1900 mg/l) were used with 0.1 mg/l (NAA) and 0.05 mg/l (BA) for 12 weeks throughout three recultures.

Data was calculated after each culture as follows:

1. Average number of mature somatic embryos
2. Somatic embryos formation percentage.
3. Hyperhydricity degree/explants (Scored visually according to [19].

The produced somatic embryos at germination stage were transferred from the two experiments namely ammonium nitrate and potassium nitrate to rooting medium as recommended by [20]. For more *in vitro* growth for 12 weeks at three re-cultures and subsequently to acclimatization stage in plastic pots 18*5cm diameter containing peatmoss, perlite and washed sand at equal volume [20].

2.5. Statistical Analysis

This experiment was carried in a Completely Randomized Design (CRD) with five replicates. The data was analyzed by one-way analysis of variance, and the mean values were separated using the Fisher's least significant difference test (LSD test at 5%) was used for means separation [21].

3. RESULTS AND DISCUSSIONS

3.1. Influence of NH_4NO_3 Ratio on Induction of Somatic Embryos and Hyperhydricity of Date Palm cv. Medjool and Khalas

There are several dissolved constituents in the medium, which could affect somatic embryos production and tissue hyperhydricity. It was concluded that alterations of some macro-nutrients level in the culture medium could affect embryo tissues hyperhydricity and increase number of somatic embryos. Data presented in Table **1** and Figure **1** indicated that Medjool cultivar after the third subculture recorded the best result for embryos formation than Khalas (9.95 and 7.15 embryos/explant for Medjool and Khalas respectively). Mean values also apparently observed that the embryos generation was gradually increased significantly when the strength was lowered from 1650 to 1237.5 the mean value was (5.30 and 11.40 embryos/explant respectively). The interaction between cultivar and ammonium nitrate concentrations, mean value was significant at 5% level. Embryos of Medjool after the third subculture using 1237.5 NH_4NO_3

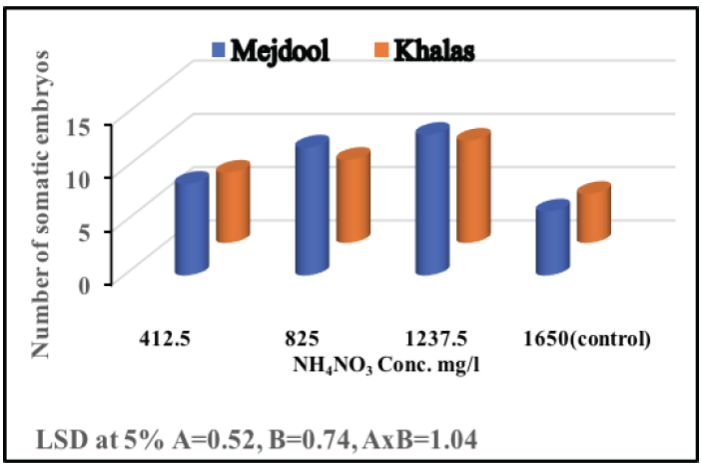

Figure 1: Influence of ammonium nitrate at different concentrations on number of somatic embryos formed from embryogenic callus of date palm cv. Medjool and Khalas.

Table 1: Influence of Ammonium Nitrate at Different Concentrations on Somatic Embryos Formation and Hyperhydricity of Medjool and Khalas Date Palm cvs

NH_4NO_3 Conc. mg/l	Cultivar			
	Medjool		Khalas	
	Somatic embryos formation %	Hyperhydricity	Somatic embryos formation %	Hyperhydricity
412.5	60	1.00	25	1.20
825	80	1.20	25	1.60
1237.5	85	1.80	30	2.20
1650 (control)	55	4.40	25	5.00
L.S.D at 5%		0.60		0.69

Hyperhydricity (vitrification) was determined as a score according to Pottino (1981) as the following; 1= No hyperhydricity, 2= Low hyperhydricity, 3= Moderate hyperhydricity, 4= High hyperhydricity, 5= Severe hyperhydricity.

in nutrient media recorded the best results (13.20 embryos/explant for Medjool) Figure **2**. Therefore, it could be concluded that, with a slight reduction in NH_4NO_3 concentration, there was a positive effect on increasing somatic embryos formation rate, which was also accompanied by reducing the degree of hyperhydricity to some extent. This might be related to lowering level of total N and ammonium ion in the culture medium. [22] who reported that the highest proliferation rate and the lowest vitrification incidence in chestnut shoots were observed on MS media containing low total N levels and low NH_4^+: NO_3^- ratios. Nitrogen (N) is one of the major basic nutrients present in the culture medium. It is essential for the biosynthesis of several compounds, such as amino acids, enzymes, proteins and pigments, all involved in a broad quantity of metabolic pathways [23,24,25]. A further increase in the concentration of NH_4NO_3 led to significant decrease in embryos induction and increased in hyperhydricity of the cultures. Nitrogen is accessible to plants either as nitrate (NO_3^-) or ammonium (NH_4^+) and depending on the style taken up by plants some changes might occur on its morphology [26]. Under *in vitro* statuses, likings for several nitrogen forms differ among species or also inside genotypes of the related species [27,28,29,30,31]. In this regard, [32, 33] found that reducing the level of NH_4^+ in the medium increased lignification and reduced vitrification. Evidently, N level could be directly related to their availability for the basic anabolic processes, which in turn could play a main role in the somatic embryos metabolism and subsequently their development. The level of NH_4NO_3 concentrations in somatic embryos culture medium could affect the C/N ratio and subsequently, could play an essential role in their growth and its multiplication. [34] who reported that, in cultures of *Castanea sativa*, the C/N ratio was lower in vitrified cultures. The high concentration of ammonium could be the cause for the decrease from the C/N ratio. Recently, [35, 36] stated the importance of MS medium

Figure 2: Effect of ammonium nitrate at 1237.5 mg/l on date palm somatic embryos cv. Medjool and Khalas. (Low hyperhydricity).

structure containing high nitrogen in the form of (NH_4^+, NO_3^-) in cell division, callus formation and the survival of the plants. Vital parameters and for producing somatic embryos, 1/2MS and 3/4MS were more efficient than full MS. Also, results were in harmony with those obtained by. [37] who demonstrated that the 3:1 ratio of (NO_3) : (NH_4^+) provided the best results for the *Eucalyptus grandis* × *Eucalyptus urophylla* cultivated in both cultivation systems, but greater biomass shoots were obtained in the bioreactor as compared to those from a semisolid medium. Increasing of organic nitrogen sources was much important in callus induction while it seemed different in plant regeneration [38]. The same result was obtained by [39] where hyperhydricity significantly increased in media containing 1650 mg/l NH_4NO_3.

3.2. Influence of Potassium Nitrate (KNO_3) Concentrations on Induction of Somatic Embryos and Hyperhydricity of Date Palm cv. Medjool and Khalas

Referring the special effect of different concentrations of KNO_3, Table **2** and Figure **3** display clearly that Medjool cultivar after third re-cultures gave the higher numbers of differentiated somatic embryos (10.75 and 8.05 for Medjool and Khalas respectively). Concerning the effect of KNO_3 concentrations on mature embryos number, data presented here showed that the presence of the ¾ level of KNO_3 (1425 ml) to culture medium enhanced significantly mature somatic embryos number compared with other treatments (12.20 embryo/culture) Figure **4**. Regarding the interaction between cultivar and KNO_3 concentrations, it is clear that the most favorable combination of these two factors was growing date palm explants. This combination of treatments gave a higher score of mature somatic embryos (14.40 and 10.00 embryo/culture for Medjool and Khalas respectively) was obtained with ¾ KNO_3 after three subcultures as compared to those obtained from any other treatment combination. The requirement for NH_4^+ (or of N in a reduced form) for embryogenic induction and differentiation was noticed by. Other authors in different species and culturing systems [40, 41, 42, 43, 44, 45]. Relating KNO_3 concentrations on hyperhydricity. It appeared that 1425 mg/l KNO_3 was superior in somatic embryos induction, but led to moderate hyperhydricity (2.20 and 2.40 for Medjool and Khalas respectively).

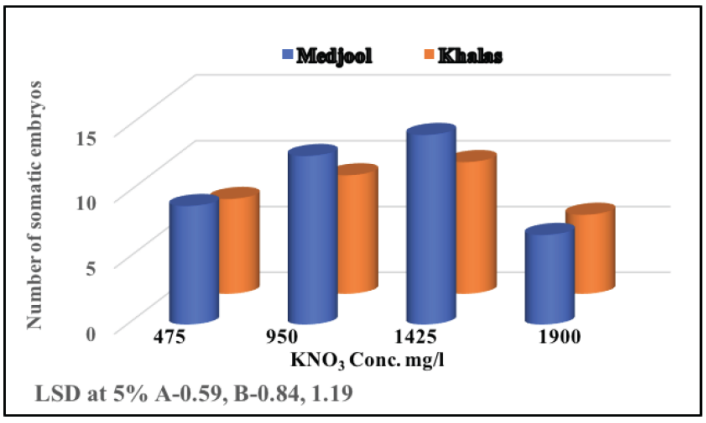

Figure 3: Influence of potassium nitrate at different concentrations on Number of somatic embryos formed from embryogenic callus of date palm cv. Medjool and Khalas.

Table 2: Influence of Potassium Nitrate at Different Concentration on Somatic Embryos Formation and Hyperhydricity of Medjool and Khalas Date Palm Cultivars

KNO_3 Conc. mg/l	Cultivar			
	Medjool		Khalas	
	Somatic embryos formation %	Hyperhydricity	Somatic embryos formation %	Hyperhydricity
475	45	1.40	25	1.40
950	55	1.60	35	2.00
1425	80	2.20	40	2.40
1900	50	3.40	25	4.20
L.S.D at 5%		0.77		1.02

Hyperhydricity (vitrification) was determined as a score according to Potino (1981) as the following; 1= No hyperhydricity, 2= Low hyperhydricity, 3= Moderate hyperhydricity 4= High hyperhydricity, 5= Severe hyperhydricity.

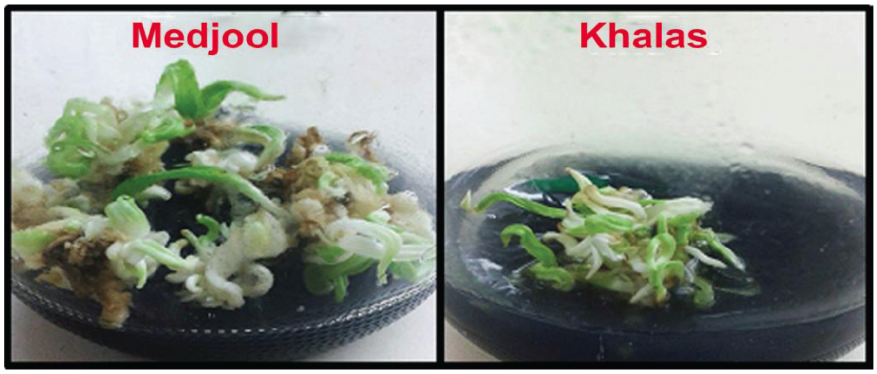

Figure 4: Effect of potassium nitrate (1425 mg/l) on somatic embryos formation of date palm cv. Medjool and Khalas.

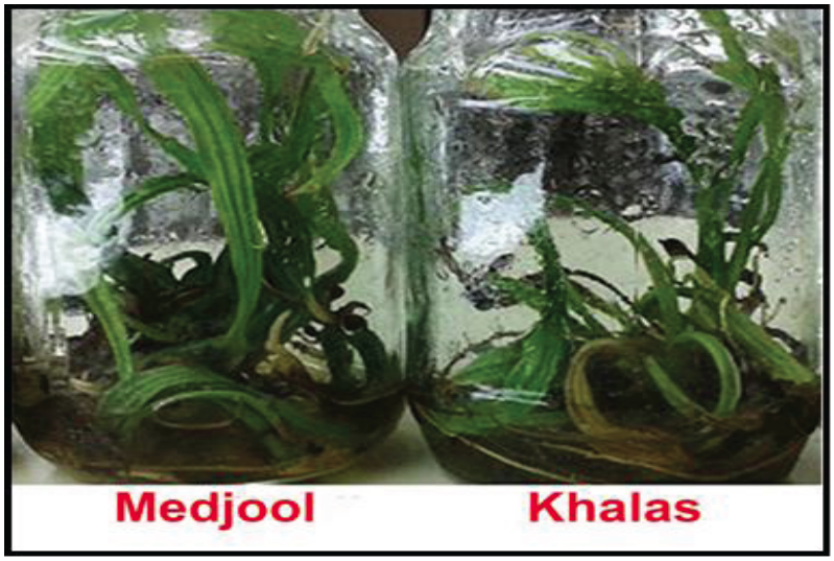

Figure 5: Root formation of date palm "Medjool and Khalas" shoots cultured in MS medium supplemented with 1mg/l IBA *in vitro*.

While the use of KNO_3 at 475 mg/l directed to the control of hyperhydricity, but led to the reduction of somatic embryos number (9.00 and 7.20 for Medjool and Khalas respectively). Nitrogen (N) is also a fundamental element for plant cell and tissue cultures, being essential for the synthesis of DNA, RNA, and proteins [46]. Although it is believed that nitrate has a main role in supporting the growth of plant tissues with influential low embryogenic capacity, the role of reduced nitrogen is not well understood. According to

Figure 6: Acclimatization of date palm "Medjool and Khalas" in greenhouse.

[47] reduced N is essential, especially in the induction phase. Somatic embryos induction also may be affected by a change in the pH of the culture medium. The absorption of the NO_3^- anion results in increasing the pH level due to an excretion of HCO_3^- from the explants. Conversely, absorption of the NH_4^+ cations results in a liberation of H^+ reducing the pH of the medium [48, 49]. Newly, [39] reported that the largest number of shoot buds per explant (18.7) introduced on the medium including 825 mg/l NH_4NO_3, 1900 mg/l KNO3, 220 mg/l $CaCl_2{\cdot}2H_2O$, 170 mg/l KH_2PO_4, 370 mg/l $MgSO_4{\cdot}7H_2O$, 1 g/l l-glutamine, 2 g/l myo-inositol and 30 g/l sucrose. Produced somatic embryos at germination stage were carried from the two experiments namely ammonium nitrate and potassium nitrate to rooting medium for more *in vitro* growth for 12 weeks at three re-cultures. And subsequently to acclimatization stage in plastic pots 18x5cm diameter containing peatmoss, perlite and washed sand at equal volume as recommended by [20] and [50]. Figure **5** illustrates *in vitro* growth of two cultivars in rooting medium and rooting percentage was 95% and 90% for Medjool and Khalas respectively and Figure **6** illustrates the acclimatization of two cultivars in the greenhouse after six months. Date palm plantlets produced from rooting stage for two cultivars grow well in the greenhouse without morphological abnormalities. From the previous results, we can conclude that potassium nitrate at 3/4MS (1425mg/l) and ammonium nitrate at 3/4 MS (1237.5 mg/l) improved *in vitro* growth and development of somatic embryos date palm cv. Medjool and Khalas and reduced hyperhydricty as the physiological disorder. The produced shoots are ready and suitable for *in vitro* rooting and subsequently to acclimatization stage.

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