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Callus induction, somatic embryogenesis, *in vitro* plantlet development and *ex vitro* transplantation of two date palm (*Phoenix dactylifera* L.) cultivars

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Abstract
The protocol has been developed and standardized in two date palm (*Phoenix dactylifera* L.) cultivars. Protocol was cultivar and plant growth regulators dependant. Embryogenic callus from excised shoot tips was induced on MS medium supplemented with 2,4-D 100 mg/l. A combination of ά-naphthalene acetic acid and 6-benzyladenine (NAA 0.1 + BA 0.05) in MS medium multiplied callus 3-4 times followed by abscisic acid (ABA, 0.5 mg/l) treatment matures callus and accelerated the initiation of somatic embryos in both the cultivars. Treatment combination of abscisic acid (0.5 mg/l) + indole-3-butyric acid (IBA, 0.05 mg/l) in medium found effective in maturation of somatic embryos. Somatic embryos germinated and converted into plantlets in half strength modified MS medium containing different concentrations of IBA @ 0.1, 0.2, 0.3 mg/l and NAA @ 0.1, 0.2, 0.3 mg/l. At a higher concentration (0.3 mg/l), *in vitro* roots and leaves (plantlets) were developed while at lower concentrations, response was comparatively poor. Secondary hardened plants after primary hardening were transplanted in the field for assessing their field performance. The findings of this paper indicate that enhanced production of somatic embryos and their conversion into plantlets can be a potential source of alternative mass propagation of date palm.

Keywords: Somatic embryogenesis, *in vitro* plantlet development, *ex vitro* transplantation, date palm cultivars

Introduction
Date palm (*Phoenix dactylifera* L.) is a perennial, monocotyledonous and dioecious tree belongs to family Arecaceae. This plant has immense socioeconomic, environmental and ecological values, particularly in the arid and semi-arid regions of the world [1]. It is one of the oldest fruit plants grown for its delicious fruit with high energy value. The plants can tolerate extremes of temperature and saline conditions of arid regions. It also has several nutritional and health benefits that have triggered a lot of biotechnological interventions related to its micropropagation, improvement and preservation of elite germplasm. Therefore, in Thar Desert and Kutch region of India several varieties were introduced and evaluated, which have shown promising response. However, availability of quality planting material is one of the major limiting constraints in its area expansion programme. So far, it is multiplied by suckers which is cumbersome and not an efficient method for its multiplication. Some organizations have made efforts to develop *in vitro* protocol but it is varietal specific and demands for planting materials of commercial varieties are huge. Indian government is also supporting to date palm growers to import the tissue culture planting material which is very expensive. Therefore, alternative seems to be have our own protocol for mass multiplication of commercial varieties of date palm.

Date palm is propagated by seeds and off shoots (vegetative means) which have been practiced from a long period of time. Several improvements were made through conventional means but they have their own merits and demerits. Due to the low rate of germination, long juvenility phage, progeny variations and strong heterozygosity seed propagation has often been undesirable [2]. The vegetative method of date palm propagation has also been reported to suffer from several inadequacies such as accumulation of numerous diseases (bacterial, fungal, viral and mycoplasmal) which decrease productivity and production of limited number of off-shoots by date plant during whole life span [3].

Tissue culture has become a preferred alternative over conventional propagation for mass multiplication of date palm to satisfy the world market demand. To overcome the propagation problems and mass multiplication of elite genotype in vitro micropropagation is the successful

technique which provides rapid production of genetically uniform, true-to-type, good quality and disease free planting materials of date palm round the year [4]. Potency of somatic cells to produce somatic embryos is known as somatic embryogenesis. It has been reported in several crop plants including date palm. It is the developmental process through which somatic cells develop into structures which resemble like zygotic embryos without fusion of gametes [3]. It is a special type of in vitro propagation tool which has many benefits *viz*., production of elite clones, artificial seed production *etc*. This process is regulated by several factors which are used in induction of somatic embryos. It was noticed that the presence of an auxin in medium was found important for induction of embryos and lowering concentration or complete absence of auxin supported maturation. Reduced nitrogen level in medium was also found important for somatic embryo initiation and maturation. Additionally, various requirements of plant growth regulators and different genotypes of date palm demand different basal media for callusing, somatic embryogenesis and plantlet formation [5]. Sucrose in the culture medium reported to be the most effective reduced carbon source for somatic embryogenesis. Environmental conditions like temperature, light, culture vessels *etc*. also have significant influence on somatic embryogenesis [6].

In the present investigation, we have studied callus induction, somatic embryogenesis and in vitro development of root-shoot in two date palm cultivars namely Halawy and Khalas growing throughout India and observed the effect of growth regulators on callus induction, callus maturation, somatic embryos and plantlets development. Observations were taken at different stages of somatic embryogenesis and plantlets development. To our knowledge, it is the first ever report compiling a detailed documentation on callusing, somatic embryogenesis and plant regeneration in these two commercially important date palm's cultivars in India. The developed and standardized protocol would be highly valuable to germplasm conservation and mass multiplication of date palm at commercial scale in the country.

Materials and Methods

Plant material

The off-shoots of the two date palm cultivars Halawy and Khalas were collected from germplasm block of ICAR-Central Institute for Arid Horticulture (ICAR-CIAH), Bikaner. The selected each off-shoot was 2-3 years old, weighing approximately 10-15 kg. These cultivars are being cultivated commercially and have good quality and higher economic position in the country.

Cleaning of explant

The off-shoots were washed with the tap water to remove the attached soil and other debris. The leaves and fibrous material were carefully removed with the help of secateurs and sharp knife until the shoot tip area was exposed. Shoot tips were then trimmed to approximately 15-20 cm in length and 5-10 cm in width.

Disinfection and surface sterilization

The excised shoot tips of both the cultivars were washed 2-3 times with double distilled autoclaved water. Thereafter, treated with potassium permanganate ($KMnO_4$ 500 mg/l) for 10 minutes and rinsed thrice with autoclaved water. Then, these shoot tips were sterilized with bavistin (2g/l) for 15 minutes and washed twice with double distilled autoclaved water and surface sterilized with mercuric chloride ($HgCl_2$ 1.0 g/l added with Tween 20^{TM} 500 µl/l) for 15 minutes. The shoot-tips rinsed thrice with autoclaved water under aseptic condition.

Explant inoculation

These shoot tips inoculated immediately on the callus induction media containing different concentrations of auxin (2, 4-D 0, 50, 100 mg/l) for about 100-120 days followed by cutting into pieces to make explants of about 0.5-1.0 cm in length.

Callus induction, multiplication, maturation and somatic embryogenesis

Explants were subcultured on high concentration of 2, 4-D medium (100 mg/l) followed by 2, 4-D (50 mg/l) in a half strength modified MS medium supplemented with activated charcoal 1.5 g/l at 5-6 weeks intervals. Callus were subcultured on hormone-free medium (MS) for about one month. The callus culture of both the cultivars of date palm was multiplied using MS basal medium supplemented with NAA 0.1 mg/l + BA 0.05 mg/l for 5-6 weeks followed by subcultured on hormone free media under culture room in darkness. Thereafter, the callus culture was subcultured into ABA 0.5 mg/l medium for callus maturation and initiation of somatic embryogenesis followed by treatment with a combination of ABA 0.5 mg/l + IBA 0.05 mg/l for somatic embryo maturation for about 45 days. Then cultures were subcultured in hormone free medium for about 5-6 weeks.

In vitro root and shoot formation in somatic embryos

Mature somatic embryos of date palm cultivars Halawy and Khalas were subcultured on half strength of modified MS medium supplemented with sucrose 40 g/l, agar 7.5 g/l, activated charcoal 1.5 g/l. Different concentrations of IBA and NAA (0.1, 0.2, 0.3 mg/l) were used for roots and leaves formation. Somatic embryos were inoculated vertically on these media in 100 ml capped culture tubes (40 ml/tube) after autoclaved for 20 minutes at 121 ^{0}C temperature and 15 pounds per square inch (*psi*). After inoculation, culture tubes were incubated at 27 ± 2 ^{0}C in 16 hours photoperiods and subcultured after 4-5 weeks into fresh (same) medium. The experiment was carried out in a complete randomized design (CRD). Ten culture tubes (replicates) were assigned per treatment with one somatic embryo per tube. The observations were recorded on daily basis and response was noted down after 45, 90, 120 and 150 days on days required to germinate somatic embryo, number of germinated embryo, germination percentage, number and length of roots and number and length of leaves.

Culture conditions

The pH of all the cultures media was adjusted to 5.8 before autoclaving. The all types of media were sterilized in autoclave for 28 minutes at 121 ^{0}C and 15 *psi*. Cultures were incubated at 27 ± 2 ^{0}C under 16 hour photoperiod with 2000 lux intensity provided by cool white fluorescent light and 70-80% relative humidity.

Preparation of plantlets for primary hardening

Well-developed plantlets having roots and leaves were taken out from media and washed gently with autoclaved water under laminar air flow cabination. Then these plants were placed into big culture tubes (170 ml capacity) containing autoclaved water for about 3-4 days for removal of remaining media from roots (Fig. 1).

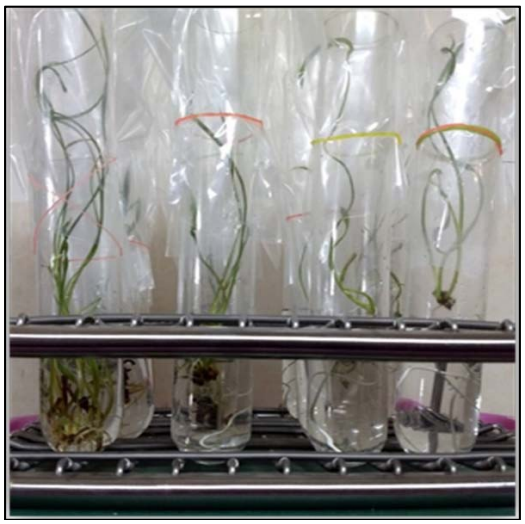


Fig 1: Preparation of plantlets for primary hardening in culture tubes (170 ml capacity) containing autoclaved water.

Primary and Secondary hardening of plantlets

Plantlets with well-developed roots and leaves were placed in culture room for primary hardening under 27 ± 2 ^{0}C temperatures and 3000 lux light intensity. Vermiculite based potting media was used for the purpose. Polythene bags with 04 holes were utilized as covering material to increase the relative humidity around the plants and kept the plants for about two months under such conditions. After that plantlets subjected to 28 ± 5 ^{0}C temperature, 70-80% relative humidity and 8000-10000 lux light intensity for better survival. Well survived plantlets then transferred to green house for secondary hardening under environment regime of 30 ± 2 ^{0}C temperature, 60-70% relative humidity and 10000-15000 lux light intensity for one year.

Results

Explant inoculation and callus initiation

The response of embryogenic callus initiation from explants of both the cultivars of date palm was successfully obtained with higher concentration of 2, 4- D (100 mg/l) after 3-4 months in Halawy and Khalas (Fig. 2). Lower concentration of 2, 4- D 50 mg/l did not showed any callus initiation activity in any of the cultivars of date palm even after 6 months of inoculation.

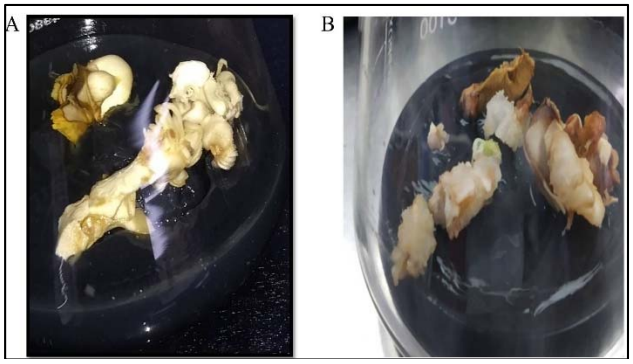


Fig 2: Shoot tips in modified MS medium supplemented with 2, 4-D 100 mg/l (a) and callus induction from cut pieces of explant in modified MS medium (b).

Callus multiplication, maturation and somatic embryogenesis

The callus culture of both the cultivars of date palm was multiplied 3-4 times after 5-6 weeks in NAA 0.1 mg/l + BA 0.05 mg/l MS medium. Initial weight of culture was 9.632g which multiplied and reached to 27.660g. The multiplied embryogenic callus cultures of date palm cv. Halawy and Khalas were treated with ABA which matures callus and accelerated the initiation of somatic embryos in both the cultivars. Treatment combination of ABA + IBA in medium matures somatic embryos and there were many proembryogenic masses of calli aroused from culture and so many somatic embryos have emerged from mature culture of both the cultivars (Fig. 3).

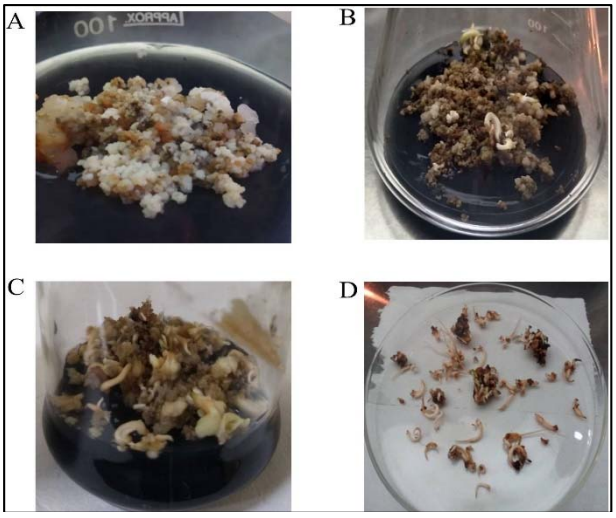


Fig 3: Callus multiplication in NAA 0.1 mg/l + BA 0.05 mg/l MS medium (a), maturation and initiation of somatic embryos from callus treated with ABA 0.5 mg/l (b), somatic embryo maturation in combination of ABA 0.5 mg/l + IBA 0.05mg/l in MS medium (c), somatic embryos in petri plate (d).

***In vitro* root and leaf formation in somatic embryos**

In both the cultivars, it was observed that somatic embryo started germination first at higher concentrations in plant conversion medium supplemented with IBA and NAA 0.3 mg/l while at lower concentrations (IBA 0.1, 0.2 mg/l and NAA 0.1, 0.2 mg/l) delayed and poor germination obtained (Fig. 4). In cultivar Halawy, average number of days taken for germination at 0.3 mg/l IBA media was 6.8 whereas it was taken 9.2 days in NAA containing media. Rapid and cent percent germination was recorded at higher concentration of IBA in cultivar Halawy while it was 90% in media supplemented with NAA. Similarly, germination percent of somatic embryos at higher concentrations in cultivar Khalas found 90 and 100% with IBA and NAA, respectively (Fig. 5 & 6).

It was observed that at a higher concentration (0.3 mg/l) of both the hormones (IBA and NAA) *in vitro* roots and leaves (plantlets) were developed in half MS basal medium and with activated charcoal while at lower concentrations, response was comparatively poor. Average number of roots and leaves in date palm cultivar Halawy after 150 days were noticed 3.8 and 5.6, respectively with hormone IBA 0.3 mg/l whereas they were observed 4.0 and 4.8, respectively with NAA 0.3 mg/l. In date palm cultivar Khalas, average number of roots and leaves after 150 days were observed 4 and 5.5 with hormone IBA 0.3 mg/l and 4.8 and 5.0 with NAA 0.3 mg/l respectively. Average length of leaves and roots in Halawy were recorded 17.44 and 8.2 cm with IBA 0.3 mg/l while 15.28 and 7.12 cm with NAA 0.3 mg/l, respectively. In cultivar Khalas, average leaf and root lengths were noted 16.20 and 8.08 cm with IBA 0.3 mg/l whereas 16.6 and 7.56

cm with NAA 0.3 mg/l, respectively (Graph 1a, 1b, 2a, 2b and Fig. 7 & 8). The interesting feature noticed during the study period was that in both cultivars in all the concentrations of NAA roots were slightly thin and brittle as compared to IBA where roots were somewhat thick and strong.

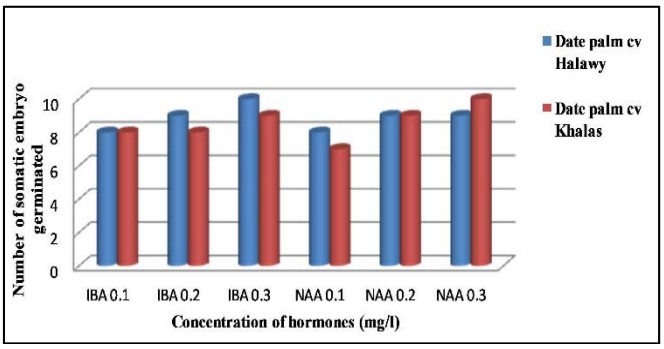


Fig 5: Response of different concentrations of auxins (IBA 0.1, 0.2, 0.3 mg/l and NAA0.1, 0.2, 0.3 mg/l) on number of germination of somatic embryos in date palm cultivars Halawy and Khalas.

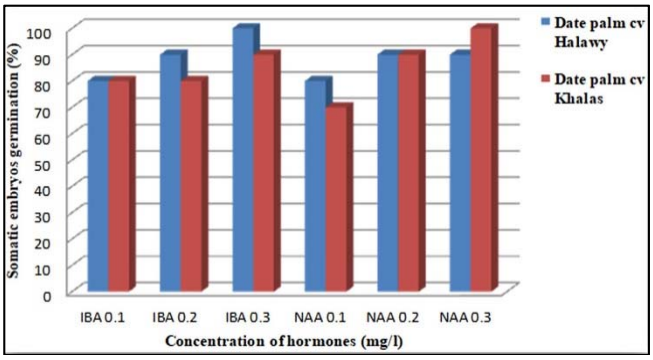


Fig 6: Germination percentage of somatic embryos of Date palm cultivar (cvs) Halawy and Khalas as influenced by different concentrations of hormones IBA 0.1, 0.2, 0.3 mg/l and NAA 0.1, 0.2, 0.3 mg/l.

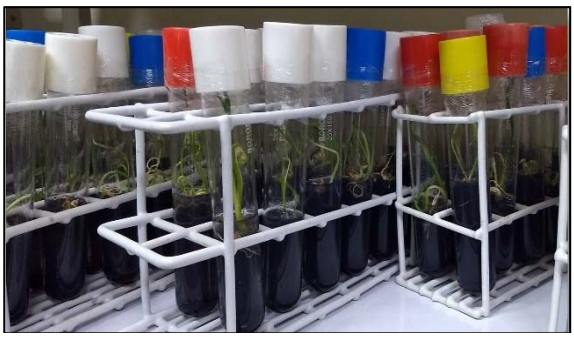
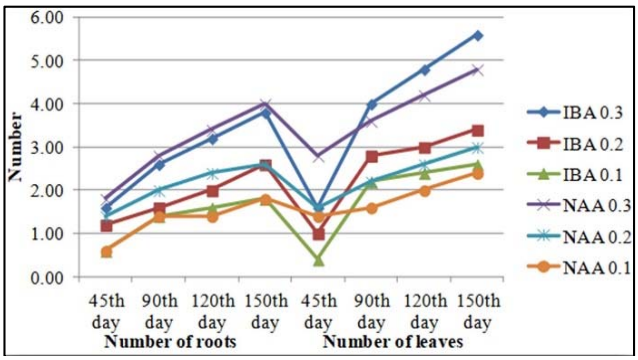
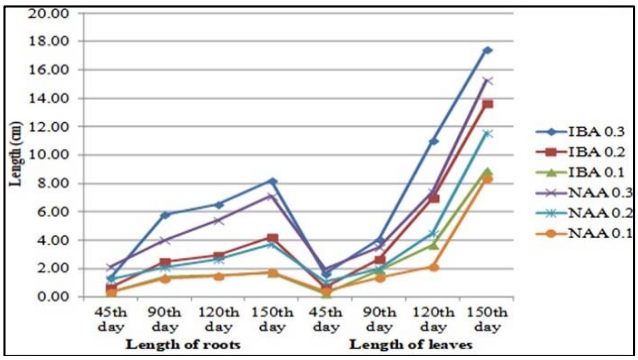


Fig 7: Conversion of mature somatic embryos into plantlets in ½ strength MS medium supplemented with different concentration of IBA 0.1, 0.2, 0.3 mg/l and NAA 0.1, 0.2, 0.3 mg/l.

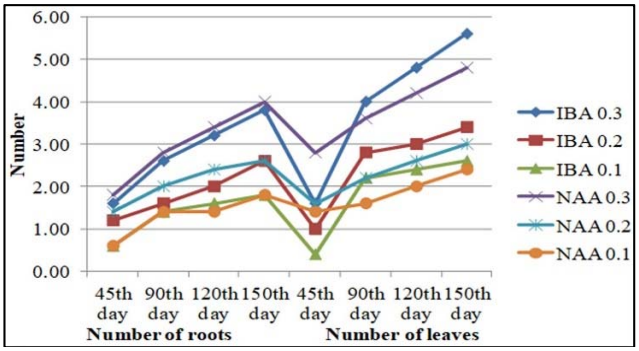
Fig 8: Well rooted plantlets of date palm ready for primary hardening.



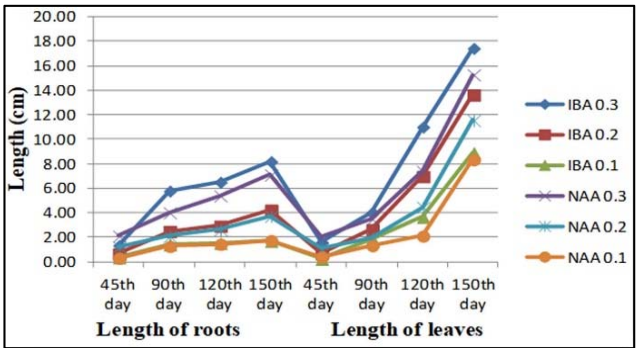
Graph 1a: Response of different concentrations of hormones (IBA 0.1, 0.2, 0.3 mg/l and NAA 0.1, 0.2, 0.3 mg/l) on in vitro root and leaf development from mature somatic embryos at 45, 90, 120 and 150 days in date palm cultivar Halawy.



Graph 1b: Response of different concentrations of hormones (IBA 0.1, 0.2, 0.3 mg/l and NAA 0.1, 0.2, 0.3 mg/l) on in vitro root and leaf lengths of plantlets at 45, 90, 120 and 150 days in date palm cultivar Halawy.



Graph 2a: Response of different concentrations of hormones (IBA 0.1, 0.2, 0.3 mg/l and NAA 0.1, 0.2, 0.3 mg/l) on in vitro root and leaf development from mature somatic embryos at 45, 90, 120 and 150 days in date palm cultivar Khalas.



Graph 2b: Response of different concentrations of hormones (IBA 0.1, 0.2, 0.3 mg/l and NAA 0.1, 0.2, 0.3 mg/l) on in vitro root and leaf lengths of plantlets at 45, 90, 120 and 150 days in date palm cultivar Khalas.

Primary and Secondary hardening

Plantlets with well-developed roots and leaves after attaining a height of about 10-15 cm were primary hardened in culture room for two months (Fig. 9). Well survived plants then transferred to green house and secondary hardening was done for about one year. There were five different steps involved during secondary hardening of plantlets. Primary hardened plantlets were placed under greenhouse near to cooling pad and polybags remain covered for about 15-20 days. Thereafter, Polybags were removed and after one month plantlets were shifted in small plastic pots containing with soil and vemicom-post (70:30). Hardening solution twice in a week was given to plantlets for proper hardening of plantlets up to 3 months. Then plants were shifted to medium and big sized plastic pots for optimum growth and development during secondary hardening. The plants have been attained 25-35 cm height after 6 months during hardening under green house (Fig.10).

Fig 9: Primary hardening of tissue culture derived plantlets of Halawy and Khalas in vermiculite based medium under culture room.

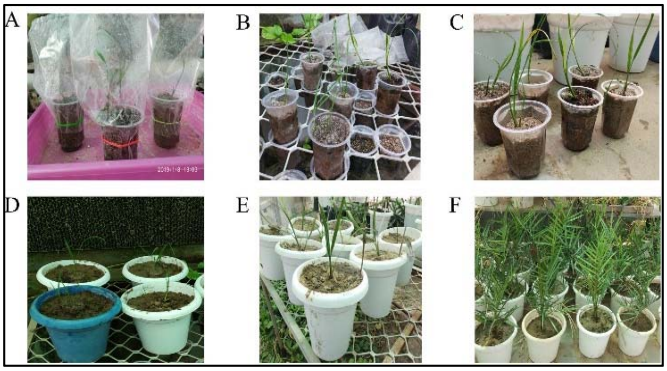


Fig 10: Different steps of secondary hardening of date palm plantlets of Halawy and Khalas under green-house unit; primary hardened plantlets shifted from culture room to hardening unit near to cooling pad (a), after one month polybags removed (b), plantlets ready to shift in soil + vermicompost medium (c), plantlets in small size (6 inch) plastic pots containing soil + vermicompost medium (d), plantlets in medium size (9 inch) plastic pots (e), plants ready to transplant in large size (12 inch) plastic pots (f).

Field transplantation of tissue cultured date palm plants

Tissue cultured date palm plants of both the cultivars Halawy and Khalas after secondary hardening were transplanted in open field condition for further evaluation purpose to evaluate establishment, survival, growth and their performance under field conditions (Fig. 11). After 18 months of transplantation, all plants established and surviving well under field conditions. The protocol developed needs up-scaling for improving efficiency of mass multiplication of date palm quality planting materials.

Fig 11: Notation a, b, c, d, e and f are different steps of field transplanting of tissue cultured date palm plants of cultivars Halawy and Khalas.

Discussion

The first callus appearance occurred after 120th day. The presence of endogenous auxins at higher concentration is the reason for the slow callus induction in both the cultivars. Boufis *et al.* [7] also reported callus initiation in date palm cultivars in the 4th month of culture and continue beyond 20 months. Our results are similar with those obtained by other studies[8-10]. The appearance of the first callus was observed at higher concentrations of 2, 4-D after four months while at lower concentrations of 2, 4-D callus did not initiated even after six months. According to Jimenez [11] obtaining good callusing requires auxinic pressure in order to maintain a high intra cellular concentration in endogenous auxin. In contrast to our study, several workers have reported fast callusing in date palm in the presence of 5 mg/l 2, 4-D medium [12, 13].

The initiation of somatic embryogenesis was achieved after the transfer of mature calli into hormone free medium. This result has supported those obtained by several workers [14-16]. In date palm cultivars Halawy and Khalas, the indirect type of somatic embryogenesis has been observed where embryos arose on mature callus culture. The similar result was also revealed by Aslam *et al.* [3] in six date palm cultivars namely Barhee, Zardai, Khalasah, Muzati, Shishi and Zart.

In the present study, embryogenic callus was observed from explants made from shoot tips of off shoots but both cultivars showed a significant variation at callus initiation and somatic embryogenesis. These variable responses may be due to the different levels of endogenous PGRs and other physiological aspects which are present in off shoots collected from Halawy and Khalas cultivars. Such kind of influence of PGRs at different stages of somatic embryogenesis has been reported earlier in some other plant species also [17-19]. Somatic embryos induction, maturation and germination showed varied response in both the studied date palm cultivars. Here, treatment with ABA followed by ABA+IBA was found very effective in maturation of somatic embryos before conversion into plantlets. The somatic embryo maturation followed by conversion in to plantlets showed a complex process. Aslam *et al.* [3] also found improved maturation of somatic embryos in six date palm cultivars when treated with thidiazuron.

Different concentrations of IBA and NAA were used on the hypothetical basis of Al-Khayri [20] with some modifications to study somatic embryo germination and leaf and root (plantlets) development. It was found that both the hormone were highly effective for high germination and plantlets development in studied cultivars of date palm. Our results are in conformity of the result of Al-Khayri [20]. Additionally, similar results were also reported by other authors when used

auxin alone or in combinations with cytokinin in date palm and other plants [3, 21, 22].

Plantlets with healthy roots were primary and secondary hardened and thereafter well survived plants transplanted for acclimatization purpose in open field conditions. Tissue culture raised plants from both the cultivars showed a good survivability percentage and normal morphological/vegetative growth in open field conditions. Successful transplantation of tissue cultured plants has also been achieved in date palm and several other plants [3, 23, 24]. In date palm cultivars Halawy and Khalas, we have been able to demonstrate an efficient somatic embryogenesis system originating from shoot tips (off shoots). Somatic embryos and plantlets were obtained in large numbers in both the studied cultivars. Somatic embryogenesis based plant regeneration reported in the present manuscript could be very useful for continuous production of somatic embryos/plantlets for *ex vitro* transplantation.

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