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PLANT TISSUE CULTURE

Rapid *in vitro* regeneration of date palm (*Phoenix dactylifera* L.) cv. Kheneizi using tender leaf explant

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Abstract

Somatic embryogenesis has been accomplished from tender leaf base explant of date palm (*Phoenix dactylifera* L.). Three to five mm long tender leaf base explants derived from the meristamatic region of 2-3 year old offshoots of date palm cv. Kheneizi were cultured on Murashige and Skoog (MS) basal medium supplemented with 10, 50, 100 and 150 mg l^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D) and incubated in dark for 6 weeks to initiate callus. Callogenesis was obtained in all 2,4-D concentrations tested; however, callus growth was most significant in media supplemented with 100 mg l^{-1} 2,4-D. The leaf explants with callus were transferred to hormone-free MS medium for 4 weeks and then further sub-cultured to a medium supplemented with 0.5 mg l^{-1} α-naphthalene acetic acid (NAA) and 0.25 mg l^{-1} 6-benzyl amino purine (BAP) which was effective in inducing shoot and root primordia within 10 weeks. In another 12 weeks, two more sub-culturing of shoot clumps in the same medium resulted in the development of shoot with roots and gave whole plants by 8 weeks. The plantlets were hardened and acclimatized to the ambient conditions and planted in pots, containing 1:1:1 peat, sand and dehydrated cow manure, which resulted in over 60% ex vitro plant survival. Early plant regeneration was achieved by this technique.

Key words: Micropropagation, Somatic embryogenesis, Regeneration, Tender leaf base explant, Kheneizi

Introduction

Date palm (*Phoenix dactylifera* L.) improvement is slow due to their perennial, dioecious nature and lack of adequate vegetative propagation methods. Seed propagation does not produce true-to-type plants due to heterozygosity and equal number of male and female plants are produced which can be distinguished only at the flowering stage. Moreover seed-derived female progenies produce generally fruits of inferior quality (Jain, 2011).

Suckers or offshoots, produced during juvenile life cycle of the date palm tree in relatively small numbers, are the only viable type of vegetative propagules to produce true-to-type progeny (Jain, 2012). In certain cases it has been reported that the offshoots are difficult to root under field condition resulting in only 60% survival (Asemota et al., 2007; Eke et al., 2005).

In this context tissue culture may offer a plausible technique to produce large number of genetically uniform plantlets. Somatic embryogenesis is considered to be the most efficient method to produce uniform in vitro plants in date palm (Al-Khayri, 2007, 2011). Letouze et al. (2000) reported production of embryos from embryogenic callus and germinated to produce whole plants. Plantlets are also produced through organogenesis forming multiple buds without callus mediation (AlKhateeb, 2006) that are genetically identical to the mother palm (AlKhateeb, 2008).

Indirect somatic embryogenesis, where callus cultures form embryos which give rise to whole plants on appropriate media, is widely used for mass propagation of date palm (Kunert et al., 2003). The application of this technique in date palm has tremendous advantage particularly to produce large scale multiplication of true-to-type plants, propagation of elite cultivars, minimum space utilization and finally exchange of plant

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materials without any compromise on disease and pests incidence. Somatic embryogenesis can be started from any meristamatic regions of the date palm tree including axillary buds, shoot tips, immature inflorescences and mature embryos (Al-Khayri, 2003).

The regeneration technique used in the present study is unique in developing an alternate simple sterilization procedure, eliminating several steps involving the use of chemicals as sterilants and antioxidants in the existing sterilization procedure. The time consumed after collection of offshoot from the field till explant inoculation is substantially reduced, which might have led to cultures free of contamination, browning and rapid response of tissues in the culture media, thus reducing the total time taken for regeneration.

The present investigation focus on rapid clonal production of date palms from tender leaf base explants, extracted from offshoots, through somatic embryogenesis by initiating callus phase followed by formation of embryoids further differentiated to form whole plants.

Material and Methods

Plant material and explant preparation

Two to three year old offshoots of date palm cv. Kheneizi was selected from the field grown date palm plantation of the College of Food and Agriculture Farm, United Arab Emirates University at Al Ain. The central leaf column of the offshoots along with a covering of leaf sheath was dissected with the growing apex intact (Figure 1A). Leaf column was washed thoroughly in tap water and wiped with 70% ethanol as a precaution against possible contamination. It was then surface sterilized by flaming once in laminar flow chamber after a quick dip in 70% ethanol (Figure 1B). The cut ends were trimmed and the leaf sheath covering was removed using a sterile blade. Explants of size 3 to 5 mm length were isolated from the base of differentiated tender leaves 8 to 10 whorls away from the meristem which runs about 3 explants from each leaf base and 12 explants per offshoot.

Culture initiation

The explants prepared were inoculated on MS basal medium (Murashige and Skoog, 1962) supplemented with 200 mg l^{-1} NaH_2PO_4, 30 g l^{-1} sucrose 3 g l^{-1} charcoal and 10, 50,100 or 150 mg l^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D). The pH of all media was adjusted to 5.7 prior to autoclaving at 120°C and 1.4 Kg cm^{-2} for 20 min. The cultures were incubated at 25±2°C in the dark for 6 weeks.

Culture maintenance

After callus initiation, the cultures were transferred to MS medium but without 2, 4-D and the addition of 200 mg l^{-1} casein hydrolysate (CH) to promote proliferation. It was maintained in the same medium for 4 weeks, subcultured at 2 week intervals, and incubated under the same conditions.

Regeneration and shoot multiplication

Embryogenic calli were transferred to MS medium supplemented with NaH_2PO_4, 200 mg l^{-1}, sucrose 30g l^{-1}, charcoal 3g l^{-1}, casein hydrolysate 200 mg l^{-1} along with 0.5, 2.5 and 5 mg l^{-1} α – naphtalene acetic acid (NAA) and 0.25, 1.25 and 2.5 mg l^{-1} 6-Benzylaminopurine (BAP) (Table 2). The cultures were incubated under a 16-h photoperiod at 45µmol m^{-2} s^{-1} provided by florescent lamps and 28±2°C. Five subculturing were done at an interval of 6 weeks in a period of seven and half months.

Figure 1. Explant preparation. A) Trimmed offshoot; B) Sterilization by flaming.

Hardening

Resultant plantlets nearly 5 cm in length with shoot and roots were rinsed in tap water to remove the agar residues and potted in sterilized mixture of peat and vermiculite (2:1) in Styrofoam cups of uniform size. The plantlets were initially covered with polythene bag to maintain high humidity to prevent the plants from dehydration and maintained in the growth room at the laboratory (Figure 2E). The polythene bags were perforated and gradually removed in a span of 3 weeks (Figure 2F). The hardened plants were transferred to potting mixture consisting of 1:1:1 peat, sand and dehydrated cow manure in pots (12 inches) (Figure 3B) and maintained in a greenhouse under natural light at 27±2°C and 50 to 60% relative humidity.

Statistical analysis

The data were analyzed by one way analysis of variance and the mean values were separated using Duncan's multiple range test (DMRT) at $p \leq 0.05$.

Results and Discussion

Callus initiation

The tender leaf segments inoculated on MS media supplemented with different concentrations of 2,4-D (Figure 2A) exhibited differential responses in callus initiation. At 100 mg l^{-1} 2,4-D explants showed significantly higher frequency of embryogenic callus (73.33%) compared to 50 mg l^{-1} (33.33%) and 150 mg l^{-1} (33.33%) (Table 1). It was noticed that the medium containing MS+10 mg l^{-1} 2,4-D showed the least explant response (14.28%). Therefore it could be visualized that 2,4-D act as a crucial hormone in the development of embryogenic callus (Figure 2B). Abul-Soad (2012) reported the stimulation pulse of 100 mg l^{-1} 2,4-D in the initial medium for 6 weeks followed by subculture on to 10 mg l^{-1} for up to 24 weeks resulted in somatic embryo formation. In our experiment, initial exposure of the tissues to 2,4-D 100 mg l^{-1} for 6 weeks resulted in the rapid development of minimal callus. Duditis et al. (1991) reported induction of somatic embryogenesis by the use of auxin 2,4-D. In further subculturing it is presumed that the transfer of the callus to hormone free media help to nullify the residual hormonal effects of 2,4-D thereby better responses for embryogenesis could be obtained in the induction media. Eke et al. (2005) reported success with similar protocol, in conformity with the present results. The incorporation of casein hydrolysate 200 mg l^{-1} enhanced swelling and increased proliferation of the callus in the hormone free media. Shoot proliferation by using casein hydrolysate (CH) in MS medium indicated that the casein supplement could elicit maximum response which agrees with the present finding with respect to formation of callus in date palm (Al-Khayri, 2011; Khierallah and Hussein, 2013) and in dendrobium (Kurup et al., 2005). The callus became more compact and globular in hormone free media than in the initial induction phase in a period of 4 weeks incubation. This could be attributed to the addition of CH supplement as has been observed in previous reports.

Somatic embryogenesis

The transfer of embryogenic callus to MS media supplemented with NAA 0.5 mg l^{-1} and BAP 0.25 mg l^{-1} resulted in the initiation of significantly higher number of somatic embryoids with roots (40.36) in a period of 10 weeks. In respect of other treatments NAA 0.5 mg l^{-1} alone (3.48), NAA 2.5 mg^{-1}+BAP 1.25 mg 1^{-1} (5.62), and NAA 5.0 mg 1^{-1}+BAP 2.5 mg 1^{-1} (4.62) were on par in the production of somatic shoots (Table 2). Further subculturing on the same media and incubation for a period of 12 weeks resulted in the development of shoots and roots. The rooted shoot buds were found to produce whole plants, in a span of 8 weeks when subcultured and maintained in the same medium, suitable to undertake *ex vitro* hardening. The combination of NAA with BAP is considered to be the potential factor to elicit a rapid response in callus mediated somatic embryogenesis pathway. Somatic embryogenesis has been accomplished by Eke et al. (2005) using an induction media composition of 0. 5 mg l^{-1} of NAA along with 1 mg l^{-1} 2-ip. This pathway has been reported earlier by Letouze et al. (2000) where callus-somatic pathway could lead to production of somatic embryos and organogenesis with appropriate changes in the hormonal combination at induction stage. The dynamic equilibrium in the concentration of auxin and cytokinin in the medium would have resulted in the rapid shoot and root development which reduced the total time taken for the regeneration process which is very unique in this study (Figure 2C). BAP is considered to be a potential cytokinin in inducing rapid cell division process to accelerate the differentiation and development process. Aslam and Khan (2009) found that BAP was more powerful than kinetin in obtaining highest frequency of multiple shoots in date palm, supporting the results of the present study. NAA hormone is known for the ability to trigger the production of adventitious roots and thus could be the reason for the production of roots along with the shoot induction (Yan et al., 2014). The roots produced were adventitious in nature arising from the

hypocotyl regions which are whitish and thicker measuring about 5 to 8cm in length and in some cases more in length. These roots have lateral outgrowths and root hairs which are found to be hairy as has been reported by Tisserat (1982). This resulted in the production of whole plant, by-passing the subculturing for root initiation phase (Figure 2D).

Table 1. Mean responses of explants to different media combinations under different 2,4-D concentrations.

Medium composition	Number of explants inoculated	Number of explants forming friable callus	Mean percentage of cultures with callus
MS + 2,4-D 10 mg L^{-1}	14	2^{a}	14.28^{b}
MS + 2,4-D 50 mg L^{-1}	15	5^{b}	33.33^{a}
MS + 2,4-D 100 mg L^{-1}	15	10^{c}	73.33^{c}
MS + 2,4-D 150 mg L^{-1}	12	4^{b}	33.33^{a}

Means followed by different letters differ significantly at $P \leq 0.05$ according to Duncan's multiple range tests.

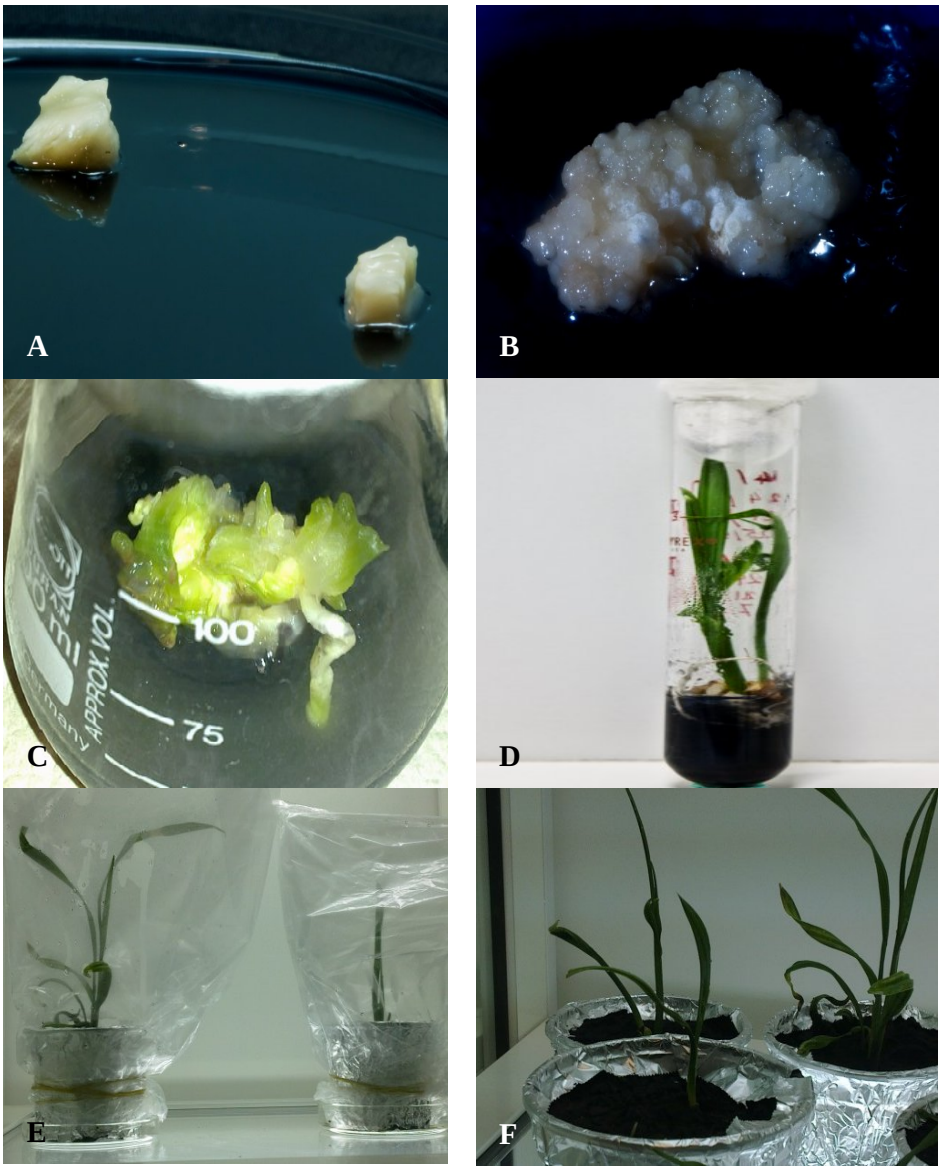

Figure 2. Stages of in vitro culture: A) Explants in culture establishment stage, B) Friable globular embryogenic callus, C) Somatic Embryogenesis, D) Whole plant, E) *Ex vitro* hardening, F) Hardened plantlets in peat.

Table 2. Mean number of somatic embryos initiated from callus cultures in different induction media composition.

Medium composition	Mean number of somatic embryos
MS + NAA 0.5 mg 1^{-1}	3.48[a]
MS + NAA 0.5 mg-1+BAP 0.25 mg 1^{-1}	40.36[b]
MS + NAA 2.5 mg-1+BAP 1.25 mg 1^{-1}	5.62[a]
MS + NAA 5.0 mg-1+BAP 2.5 0 mg 1^{-1}	4.62[a]

Means followed by different letters differ significantly at $P \leq 0.05$ according to Duncan's multiple range tests.

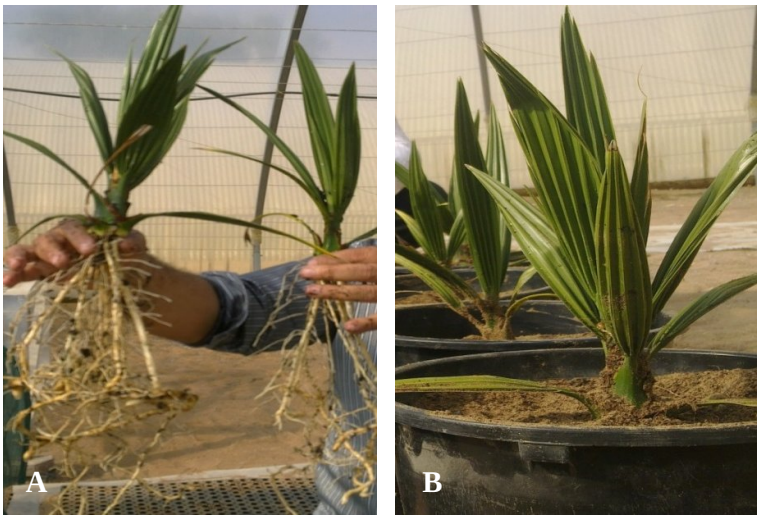

Figure 3. Well established tissue cultured date palm plants: A) Strong rooting to support growth, B) *In vitro* grown plants in pots for field planting.

Hardening and acclimatization

Rooted plants transferred to pots with peat, vermiculite mixture in the ratio of 2:1 showed nearly 60% survival under the methods described in the methodology for hardening (Figure 2E, F). These plants fertilized with ½ MS salts were found to pick up sufficient growth. However Raju et al. (1984) used Hogland's solution while potting, to establish strong plants of oil palm. The hardened plants were further transferred to potting mixture consisting of 1:1:1 peat, sand and dehydrated cow manure in garden pots (Figure 3A, B) before they are acclimatized for field planting.

Conclusion

To summarize, embryogenic callus has been achieved first in *Phoenix dactylifera* cv. Kheneizi from the explants extracted from differentiated tender leaf base. The whole plantlets were produced, hardened and potted under ambient conditions over a period of nearly 11 months facilitating rapid multiplication.

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