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ESTABLISHMENT OF AN EFFICIENT PROTOCOL FOR MICROPROPAGATION OF SOME PAKISTANI CULTIVARS OF DATE PALM (*PHOENIX DACTYLIFERA* L.) USING NOVEL INFLORESCENCE EXPLANTS

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Abstract

An efficient protocol for rapid and large scale *In vitro* propagation of some Pakistani cultivars of date palm has been established using inflorescence explants at Date Palm Research Institute (DPRI), Shah Abdul Latif University (SALU), Khairpur, Pakistan. Immature inflorescences of desired cultivars of date palm detached from mother palms followed by surface sterilization with low torrent of current tap water and then 30% $NaOCl_2$ solution, the outer cover were removed in order to get spike explants and cut into the 2-3 cm small pieces and cultured on modified MS medium supplemented with 0.1 mg l^{-1} 2, 4-D + 0.1 mg l^{-1} IAA + 5.0 mg l^{-1} NAA for initiation and establishment of cultures. The obtained somatic embryos were subjected to multiplication medium involved 0.1 mg l^{-1} NAA + 0.05 mg l^{-1}BA. Rooting was achieved using quarter strength MS medium containing 0.1 mg l^{-1}NAA without activated charcoal (AC) initially and then with 3 g l^{-1} AC. Strong rooted plantlets with 2-3 leaves were transferred to pots contained sand and peat moss mixture (1:1 v/v) with more than 95% success in acclimatization. The acclimatized plants with at least one compound leaf were shifted to the open field conditions at SALU campus for further studying morphological and fruit characterization to ensure the true-to-type nature of tissue culture derived plantlets. High multiplication efficiency and survival percentage with no any somaclonal variation ensured the efficacy of the protocol developed for the production of elite cultivars of date palm of Pakistan and can be used to optimize production of other cultivars of date palm worldwide.

Key words: Acclimatization, Date palm, Inflorescence explants, Micropropagation, Multiplication, Rooting.

Introduction

The Date palm (*Phoenix dactylifera* L.) can be propagated naturally through seeds or offshoots and by Plant tissue culture artificially. Using seeds always brings heterozygosity due to its dioecious nature while offshoots usage for commercial propagation usually facing limitation of offshoot availability and often a source of spreading diseases in case if the offshoots taken from infected trees thus making tissue culture as only method enabling mega production with uniformity, round the year availability and transportation without a fear of spreading diseases.

The earliest attempts of tissue culture of date palm reported since 1970s but limited to callus & somatic embryos production and only few succeeded to produce plantlets (Schroeder, 1970; Smith, 1975; Tisserat, 1979; El-Hennawy *et al*., 1980). At present, several methods for commercial production through micropropagation of date palm have been reported by different researchers (Omar *et al*., 1992; Zaid *et al*., 2007; Abul-Soad & Mahdi, 2010).

The work on tissue culture of date palm is being done since long back using various explants like shoot tips (Veramendi & Navarro, 1996), immature zygotic embryos (Reynolds & Murashigue, 1979) lateral buds (Drira, 1983), lateral leaves of shoot tip (Bhaskaran & Smith, 1992; Fki *et al*., 2003) and immature inflorescences (Bhaskaran & Smith, 1992; Abul-Soad, 2003; Abul-Soad *et al*., 2004, 05, 07; Abul Soad & Mahdi, 2010).

A huge number of individual efforts for *In vitro* propagation of date palm from both dates producing & non dates producing countries have been reported but are limited to callus, somatic embryogenesis, multiplication, rooting, acclimatization while only few succeeded to transfer the plants into field conditions but with small scale. At present, a number of public and private sector laboratories concerned with date palm micropropagation on commercial scale such as; Date palm Developments (UK), Al-Rajhi tissue culture laboratory (Saudi Arabia), Al-Ain University date palm tissue culture laboratory (UAE), Marrionet G.F.A (France), Rahan Meristem (Israel), Sapad tissue culture date palm company (Saudi Arabia), Domaine Agricole el bassatine (Morocco), date palm research center (Oman), Green Cost nurseries, Fujairah (UAE), Al-Wathba Marrionet (UAE) producing thousands of tissue cultured plants annually (Zaid *et al*., 2011; Rajmohan, 2011; Jatoi, 2013).

The usage of the offshoots derived explants in tissue culture of date palm has been practicing since decades while the potential of inflorescence explants have been tested to develop direct (Abul-Soad *et al*., 2004) and indirect somatic embryos (Drira & Al-Sha'ary, 1993; Abul-Soad *et al.*, 2005) of date palm later on. Inflorescence explants exhibited numerous advantages over worldwide frequently used offshoot explants for date palm micropropagation such as: no or less browning& bacterial contamination, short production cycle and possibility to produce rare male and elite female cultivars of date palm in case of no offshoots availability (Bhaskaran & Smith, 1992; Abahmane *et al*., 1999; Zaid *et al*., 2007; Abul-Soad, 2011; Jatoi, 2013).

Pakistan always ranked among top 6 dates producing countries in the world having very rich date palm varietal structure and one of the strongest contenders among the countries claiming place of the date palm origin (Jatoi *et al*., 2009; Jatoi, 2013; Mirbahar *et al*., 2014; Haider *et al*., 2015; Abul-Soad *et al*., 2015). The growers of Pakistan still practicing centuries old date palm propagation method using offshoot transplantation for local cultivars and as well importing some international cultivars offshoots that often

incurred with some deadly pest and disease problem and thus, making the micropropagation of elite local and exotic commercial cultivars in Pakistan as need of the day. The efforts have been made for few decades through dispersed trials in the country to produce date palm plants by tissue culture technology. However, limited success has been achieved and trials weren't fruitful on large scale (Qureshi & Rashid, 1993; Rashid & Quraishi, 1994; Hussain *et al*., 1995; Quraishi *et al*., 1997; Hussain *et al*., 2001; Khan & Bibi, 2012).

On the other hand, Date Palm Research Institute (DPRI), SALU, Khairpur, Pakistan not only succeeded to established cultures of many elite local and exotic cultivars of date palm in the laboratory for commercial production range from juvenile to rooting stage and shifted few thousand tissue culture derived plantlets to Glass house (Jatoi, 2013) by using inflorescence explants within short period of time but, also shifted the tissue culture derived date palm plants into field conditions for field and fruit evaluation (Abul-Soad, 2011; Jatoi, 2013; Abul-Soad *et al*., 2015) and distributed a large number of plantlets among the growers of the region and other parts of Pakistan.

Material and Methods

This work was carried out in the biotechnology and tissue culture laboratory of DPRI, SALU, Pakistan during 2007 - 2013. The protocol was done as under:

Explant source: The immature inflorescences were excised from the mother trees of different date palm cultivars namely Gajar, Kashoo wari and Dedhi (Fig. 1) from Khairpur, Sindh, Pakistanin early spring. The excised inflorescences were kept in clean plastic cover and handled carefully from an open field to the laboratory.

Surface sterilization& explant preparation: The intact spathes were dipped into fungicide solution (2 gl^{-1} Topsin M 70) for30 seconds only without shaking followed by washing under current tap water for 30-60 seconds only. 30% sodium hypochlorite (NaOCl) solution was used as surfactant for 5 minutes and washed three times with sterilized distilled water for 30-60 seconds without shaking.

After sterilization, the outer protective sheath or cover was removed carefully without any damage to the spikes inside. The spikes were cut from their bases and cultured directly if 3-4 cm in length (Fig. 2A) while longer spikelet were cut and divided in to 2-3 cm each of which possessed 2-4 immature florets and laid in such a way that the entire explant is in contact with the surface of nutrient medium (Fig. 2B).

Cultural conditions: All cultured explants were incubated in a controlled growth room at 25 ± 2ºC under full darkness and re-cultured about 3-4weeks on same initiation medium as mentioned in Table 1. Well-responded explants were transferred on to maturation medium for 1-2 re-cultures. Matured and early differentiated explants under darkness were shifted onto differentiation medium under light conditions for 1-2 re-cultures. Subsequently the differentiated cultures were shifted to the multiplication medium to acquire desired number of shoots and then the elongated shoots were detached from multiplication stage and subjected to rooting medium. The individual plantlet with 2-3 leaves and thickened adventitious roots were selected and shifted to the glass house for acclimatization.

Acclimatization & field transference: The acclimatization protocol of date palm was followed as described by Abul-Soad (2011). Plantlets were taken out from test tubes and the roots were gently washed in luke-warm distilled water to remove any residual gel or medium. Before planting, plantlets were immersed in 0.5% (w/v) fungicide solution for 5 minutes. The plants were placed into 250 mm plastic pots containing soil mixture 1:1 of wash sand: peat moss (v/v) with little amount of perlite. Plants kept under natural day light and high relative humidity (90-95%) using a cover of white polyethylene sheet for one week and removed gradually to develop the plants under greenhouse conditions. The plants were watered once a week and sprayed with the fungicide if needed. The plants with at least one compound leaf were shifted to the field conditions at SALU campus and kept under observation for fruit and field evaluation to ensure the true-to-type nature of tissue culture derived date palm plants.

Gajar **Kashoo wari** **Dedhi**

Fig. 1. The fruit of studied cultivars used for micropropagation.

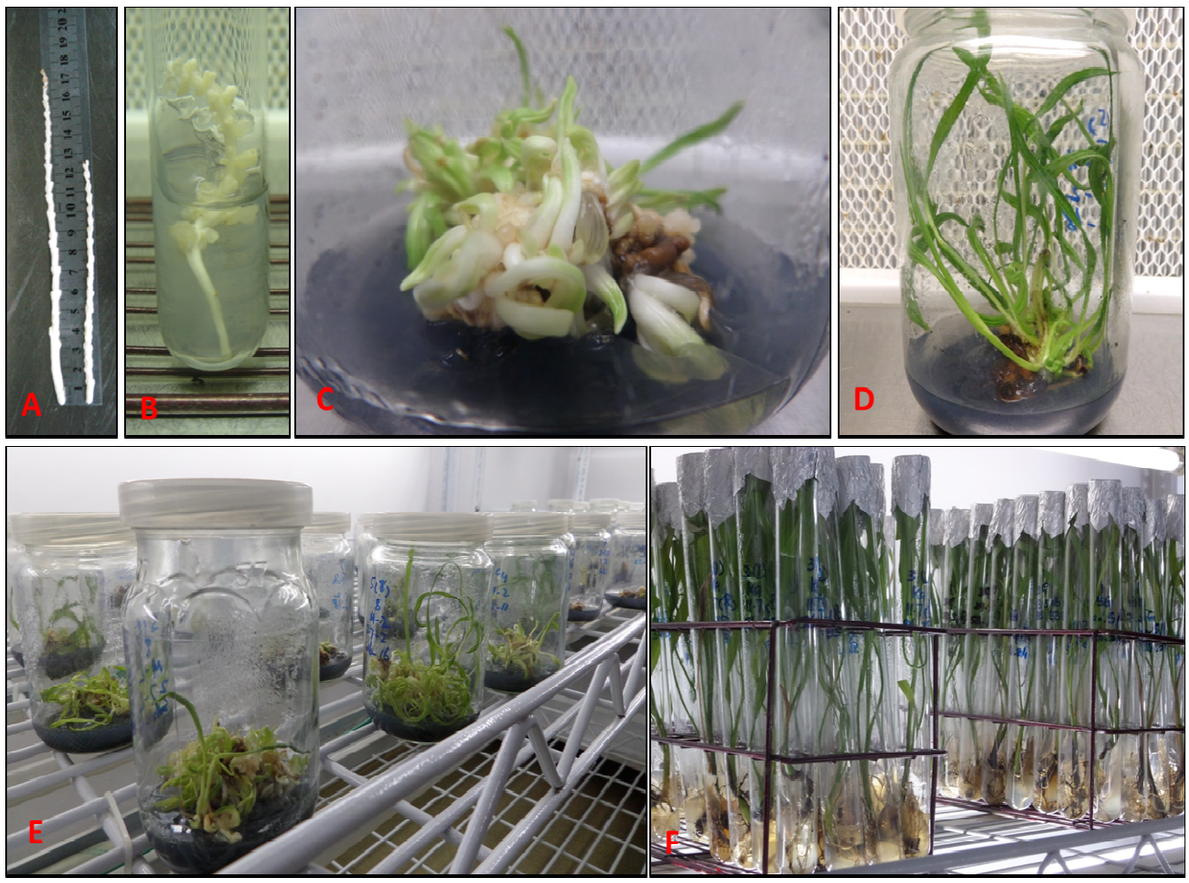

Fig. 2. Different growth stages of date palm micropropagation using Inflorescence explants in DPRI. **A.** Inflorescence Spikelets, **B.** Initiation stage, **C.** Shoots cluster with somatic embryos, **D.** Shoots elongation, **E.** Multiplication stage **E.** Rooting stage.

Table 1. Nutrient media composition for Inflorescence protocol and its sequence (Abul-Soad & Mahdi, 2010).

Medium	Composition (mgl^{-1})			
	Salts	Additives	Auxins	Cytokinins
Initiation	Macro of B5[z]+ Micro of MS[y]	30000 Suc.[x] + 2200 Agar + 1400 Gel + Vit.[w]of MS + 170 KH_2PO_4 + 100 Glutamine + 40 Ad.[v]	0.1 2,4-D + 0.1 IAA + 5.0 NAA	---
Maturation	Macro of B5+ Micro of MS	30000 Suc. + 2200 Agar + 1400 Gel + Vit. of MS + 170 KH_2PO_4 + 100 Glutamine + 40 Ad. + 1500.0 AC[u]	5.0 2,4-D	1.0 2iP
Differentiation	MS	30000 Suc. + 2200 Agar + 1400 Gel + Vit. of MS	0.1 NAA	0.1 Kinetin
Multiplication	MS	30000 Suc. + 2200 Agar + 1400 Gel + Vit. of MS	0.1 NAA	0.05 BA
Rooting	¼ MS	50000 Suc. + 2200 Agar + 1400 Gel + Vit. of MS + 0.1 Ca-panthothianate + with & without 3000.0 AC	0.1 NAA	---

$_z$B5: Gamborg *et al*. (1968) nutrient medium. $_y$MS: Murashige & Skoog Medium (1962). $_x$Suc.: Sucrose. $_v$Vit.: Vitamins. $_w$AC: Activated Charcoal. $_u$Ad.: Adenine sulfate

Table 2. Production capacity of three different cultivars of date palm from a single inflorescence after 1, 7, 12 subcultures during the multiplication and rooting stages.

Variety	Sub 1*				Sub 7				Sub 12		
	Callus	Embryo	Shoot	Total	Callus	Embryo	Shoot	Total	Shoot	Plantlet	Total
Kashoo Wari	110	38	28	176	5	111	408	524	419	773	1716
Gajar	24	---	2	26	---	44	119	163	299	295	757
Dedhi	70	---	17	87	6	31	86	123	50	257	430
General total	204	38	47	289	11	186	613	810	768	1325	2903

*Sub 1considered during multiplication stage

Each culture vessel (350 ml jar or 250 × 25 mm long tube) contained 1 gram of callus, or 10 embryos or shoots in average. Each long tube contained 1 intact plantlet with shoot-root system

Results and Discussion

The experiments were resulted in successful large scale micropropagation protocol of date palm using inflorescence explants. Date palm cultures during initiation process have been commonly observed to release phenolic compounds into the nutrient medium, which inhibit and often cease their own growth (Reuveni & Kipins, 1974). No browning and bacterial contamination observed during initiation phase which is commonly found using offshoot shoot tip explants. After initiation process, the nutrient medium protocol decides that whether the explants turned into callus or somatic embryos categorized into indirect and direct somatic embryogenesis respectively. All inflorescence spike explants responded well to the starting nutrient medium. Shining globular creamy structures formation was obtained within 2 months through 1-2 re-cultures. Maturation of initial structures occurred within 2-3 months through2-3 re-cultures. After the differentiation process, three types of cultures were obtained e.g., embryogenic callus, somatic embryos and green shoots. Somatic embryos can be generally divided into two categories. First category is the individual somatic embryos and second is a cluster of embryos (multiple embryos) (Fig. 2C). The growth behavior of the individual embryo is to grow vertically to produce more leaves and roots (Fig. 2D) while the multiple embryosis usually proliferating to additional shoots and somatic embryos which suits the multiplication stage (Fig. 2E). Hussain *et al*. (2001) reported while working on micropropagation of three Pakistani date palm varieties using shoot tip explants that multiplication rate is variety dependent response. Al-Khateeb (2006) while working on role of auxin and cytokinin concentrations on shoot proliferation of date palm var. Sukary found that lower concentrations of PGRs enhanced bud and shoot formation whereas higher concentrations inhibited buds and shoots production and resulted in abnormal development. Zaid *et al*. (2006) also obtained a high rate of multiplication when the cultures were transferred to media supplement with low levels of hormones.

In the first subculture of multiplication stage, 110, 24 & 70jars were having embryogenic callus in cvs. Kashoo wari, Gajar & Dedhi, respectively. While, with 38 jars having multiple embryos cv. Kashoo wari appeared the only cultivar produced embryos. However, 28, 2 & 17 jars of cvs. Kashoo wari, Gajar & Dedhi were appeared with shoots respectively (Table 2). All of these cultures were transferred onto the proliferation medium (Table 1). The embryogenic callus exposed high morphogenetic potentiality to differentiate to intact somatic embryos. During this process very little callus formation was occurred till subculture 7 of multiplication stage where the callus jars decreased to 5, 0 & 6 jars in cvs. Kashoo wari, Gajar & Dedhi respectively. While, with 111 and 408 jars of embryo and shoot cv. Kashoo wari produced prominent number of cultures as compared to cv. Gajar (44 embryo and 119 shoot jars) and cv. Dedhi (31 embryo and 86 shoots jars). During multiplication stage some shoots were growing up and reached to an appropriate height for rooting stage and subsequently subjected to the rooting medium (Table 1) (Fig. 2F). It was observed that removing the initial roots completely or trimming to 1-2 mm enhanced thicker-white adventitious root formation. Leaving the primary roots without trimming during rooting stage inhibited the adventitious roots formation which is important for the further growth in the acclimatization stage (Abul-Soad & Jatoi, 2014). Tisserat (1982) found the optimum adventitious rooting and subsequent plant survival using medium supplemented with 0.1 mg/l auxin for 8 to 16 weeks before transfer of plantlets to greenhouse for acclimatization. Hassan *et al*. (2008) studied the interaction between sucrose concentrations and MS strength during *In vitro* rooting stage of date palm var. Bartamouda. They observed that sucrose plays a significant role in root initiation. They found that ¾ MS salt strength in combination with 45 g/l sucrose enhanced number and length of roots, number and length of shoots, and thickness of roots and leaves.

Finally, all callus and embryos differentiated into shoots and rooted plantlets on rooting medium. Where number of shoots and plantlets were reached at 419 and 773 in cv. Kashoo wari, 299 and 295 in cv. Gajar, and 50 and 257 in cv. Dedhi, respectively. The shoot jars increased from 28, 2 & 17 in subculture 1, then 408, 119 & 86 in subculture 7, and 419, 299 & 50 in subculture 12. Each jar maintained 20-30 shoots, 5 of the mat least in the size of rooting stage while 1325 plantlets were in rooting stage in sub culture 12.

Rooting quality of the *ex vitro* plantlets of date palm was the vital factor increased the survival percentage in the greenhouse. Most of the reports indicated low survival percentage 25-35% during acclimatization stage rather than it used to be a big obstacle in the whole micropropagation protocol (Abul-Soad *et al*., 1999; Hegazy *et al*., 2006; Taha *et al*., 2007). Date palm tissue culture derived plants do not establish easily when transferred to *ex vitro* conditions (Abul-Soad *et al*., 1999). Miller (1983) and Ziv (1986) suggested that removal of lids or caps from culture vessels and placing in the area where they will be acclimatized like in greenhouse for few days may have positive effects when the plantlets are transplanted into the soil medium and kept in green house for acclimatization. On the contrary, Preece & Sutter (1991) recommended that uncapping of culture vessels should be done in stages, first by loosening the lids for a day or two, then by partially removing the cap for another day or two followed by complete removal. But the caps of larger vessels such as mouth mason jars or aluminum foil trays should be removed more slowly than narrow mouthed vessels such as culture tubes. Gabr & Abd-Alla (2010) reported that pre-acclimatization is an important step to complete micropropagation process. Plantlets grown in lab under optimum conditions (moisture, salts, sucrose and water) lack cuticle layer in leaves with high transpiration rate. They observed that the presence of PEG in MS medium increased the cuticle formation in leaves & root thickness and decreased transpiration rate that made a balance between transpiration and salts uptake from nutrient media. They established pre-acclimatization stage by gradual removing caps of culture vessels that resulted in high survival rates after five weeks of transplanting the plantlets.

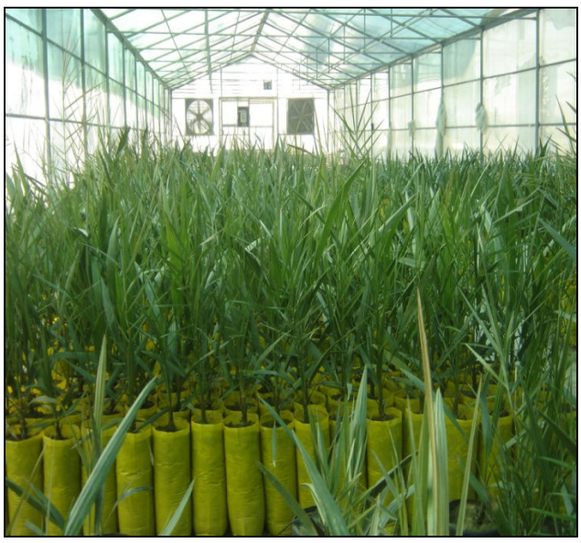

Fig. 3. Date palm plantlets acclimatization process in DPRI Glass house.

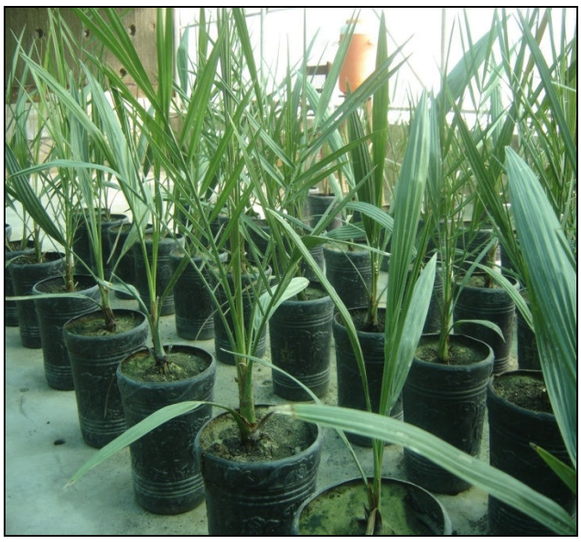

Fig. 4. Tissue cultured date palm with compound leaves ready to be shifted to field conditions.

But in current study and based on the utilization of high sugar concentration, AC after adventitious roots formation and proper handling for the plant material, the survival percentage reached more than 95%. The used soil bed was a simple mixture of washed sand and peat moss (1:1 ratio) with little amount of perlite. The acclimatized plants with at least one compound leaf were shifted to the field conditions (Figs. 3-5) and are under fruit & field evaluation studies to check the genetic stability and phenotypic nature of the produced plantlets with their mother plants and till now no any published symptoms of somaclonal variations has been observed. It is worth to mention that no any study has been conducted on these date palm cultivars before and offshoots were only the source of traditional propagation method in the region.

Fig. 5. Tissue culture derived date palm plants in field conditions, DPRI, SALU, Khairpur, Pakistan.

High multiplication efficiency and survival percentage ensures the efficacy of presented protocol developed for the production of elite cultivars of date palm of Pakistan that could be applicable for other cultivars of commercial importance worldwide.

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