

REGULAR ARTICLE

## Influence of inflorescence explant age and 2,4-D incubation period on somatic embryogenesis of date palm

Adel Ahmed Abul-Soad\*

Horticulture Research Institute, Agricultural Research Center, Cairo University, Orman, 12619, Giza, Egypt.

### Abstract

The objective of this study was to determine the appropriate growth stage of inflorescence (spathe) explants among different cultivars of the date palm (*Phoenix dactylifera* L.) and to define the optimum period of incubation on high-auxin medium. Inflorescence explants were excised from seven Pakistani cvs. at different growth stages and cultured on a basal MS medium containing 100 mg l<sup>-1</sup> 2,4-D for 3, 6 and 24 weeks (Batches I, II and III) before being transferred to 10 mg l<sup>-1</sup> 2,4-D medium. The cultures were incubated at 25 ± 2°C in the dark and transferred onto fresh culture medium every 3 weeks. Results revealed that spikelet explants isolated from different cultivars with the same length were not necessarily at the same stage of development. Length varied according to cultivar, time of excision, location of the inflorescence in the crown and location of the spikelets in the inflorescence. Accordingly, the response differed among the cultivars tested. After 24 weeks from initial culture, some Batch II explants produced only unfriable callus and embryos. On the other hand, explants comprising Batches I and III failed to induce organs even after 42 weeks (14 subcultures); instead they turned blackish brown. Multiple somatic embryos were subjected to proliferation at the multiplication stage. Individual shoots were rooted and successfully transplanted in the greenhouse with about 90% survival.

*Key words:* Date palm, Inflorescence, Micropropagation, Embryogenesis, *Phoenix dactylifera*

### Introduction

The date palm (*Phoenix dactylifera* L.) is a staple food crop with high nutritional value and yield and can be cultivated under unfavorable soil and water conditions. Most of date palm cultivation is in North Africa, the Middle East and similar regions in the world (Botes and Zaid, 2002). It is traditionally propagated by offshoots which are limited in number, particularly in some elite cultivars which restricts agricultural expansion and land reclamation. To overcome this problem, tissue culture techniques have been utilized for mass propagation of elite date palm cultivars. Tissue culture of the date palm was initiated four decades ago and depended mainly on offshoot tip explants (Reuveni et al., 1972). Explants derived from offshoots and use of a high-auxin medium caused many technical problems such as endogenous bacterial contamination, browning, somaclonal variation and long-term duration of production.

Micropropagation by shoot tips of offshoots takes about 3 years, if the protocol is well-known (Abul-Soad et al., 2004a). A viable alternative explant is needed to overcome the above mentioned problems. Most of the commercial laboratories worldwide are using somatic embryogenesis from shoot tip explants (Tisserat, 1979; Al-Khayri, 2001; Abul-Soad et al., 2002b; Abul-Soad, 2003).

Use of inflorescence explants avoids all the obstacles cited. Since 1973, several researchers have attempted to culture palm inflorescences. Inflorescences of several species have been micropropagated in vitro (Smith and Thomas, 1973; Eeuwens, 1978). Subsequently the high probability of inflorescence explants to produce direct (Abul-Soad et al., 2004b) and indirect shoot formation of date palm (Drira and Al-Sha'ary, 1993; Abul-Soad et al., 2005; Sidky et al., 2007) were investigated with variable success.

In all forms of plant embryogenesis certain criteria have to be fulfilled before initiation. The species or genotype has to have the genetic potential to form embryos from somatic cells and one or a few cells of the plant/explant have to be receptive to a signal (endogenous or exogenous) that triggers the pathway of embryogenic development (commitment), leading to embryo formation even in the absence of further signals (Feher, 2005). Among all auxins, 2,4-D especially

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\*Corresponding Author

Adel Ahmed Abul-Soad  
Horticulture Research Institute, Agricultural Research Center,  
Cairo University, Orman, 12619, Giza, Egypt

Email: adelabelsoad@gmail.com

was found effective to trigger that pathway (Gaj, 2004). Date palm explants derived from shoot tip re-subculture onto high auxin medium led to friable callus formation. After that, friable callus is transferred onto free plant growth regulator medium to differentiate into somatic embryos. This cycle takes about 1-2 years but carries the risk of somaclonal variation because of prolonged sub-culturing of the explants on a high-auxin medium (Abul-Soad et al., 2002a).

A novel approach was adopted in this study by using a high auxin medium for a short period of time at the beginning of culture in order to trigger the pathway of embryogenic cell program, then shifting to a lower-auxin medium to allow the growth and development of induced embryos. A pertinent question in the current study was the length of the time period of a high auxin medium (the first objective). The second objective was to determine the factors affecting spikelet length among different cultivars which may lead to defining the appropriate age of the inflorescence explant.

### Material and Methods

This work was carried out in the Biotechnology Laboratory, Date Palm Research Institute, Shah Abdul Latif University, Khairpur, Sindh, Pakistan, in the period 2009-2011.

### Plant materials

Single immature inflorescences were excised from five Pakistani cvs. (Gajar, Kashoo-Wari,

Khar, Karbline, and Khormo) growing in a farm field in Khaipur District, a major date-palm area of the country (Table 1). In addition, inflorescence excisions were made at different time intervals from trees of two other cvs., Dedhi and Aseel; three excisions from Dedhi and four from Aseel, and numbered consecutively. The inflorescence excisions were made during the period 19 Jan. – 7 Feb., 2009. The immature spathes were excised within a period of a couple of weeks prior to the flowering initiation (emergence of the brown tip of the first spathe) and ceased after about 1 week. The excised inflorescences were stored in clean plastic bags and within a couple of hours carefully carried to the laboratory with a room temperature of around 30°C.

### Surface sterilization

The entire inflorescences were washed under running tap water for a few minutes and then surface sterilized by immersion for a minute in 1% sodium hypochlorite solution with a few drops of Tween-20, then the outer protective sheath cleaned with a piece of cotton immersed in 70% alcohol under aseptic conditions. Thereafter, the intact inflorescence was washed gently with sterilized distilled water three times. In the next step, the outer cover of the inflorescence was partially removed (Figure 1) and the spikelet explants were cut and cultured onto different treatments. Each treatment consisted of 72 tubes, each involved an explant.

Table 1. Spikelet explants length, excision date from the mother tree and obtained response of seven Pakistani cultivars cultured in Batch II<sup>z</sup>.

Cultivar name	Spikelets Length (mm)	Inflorescence excision date (d-m-y)	Type of response
Aseel-1	5 – 15 <sup>y</sup>	19 – 1 – 2009	Browned and died
Aseel-2	15 – 20	24 – 1 – 2009	Pro-embryos
Aseel-3	25 – 30	27 – 1 – 2009	Pro-embryos
Aseel-4	40 – 45	06 – 2 – 2009	Unfriable callus
Dedhi-1	9 – 12	27 – 1 – 2009	Shrunken and browned
Dedhi-2	22 – 26	06 – 2 – 2009	Pro-embryos
Dedhi-3	19 – 21	06 – 2 – 2009	Swollen florets
Gajar	25 – 30	22 – 1 – 2009	Browned and died
Kashoo-Wari	40 – 50	23 – 1 – 2009	Pro-embryos and roots
Khar	22 – 25	27 – 1 – 2009	Swollen florets
Karbline	25 – 30	03 – 2 – 2009	Swollen florets
Khormo	50 – 55	07 – 2 – 2009	Swollen florets

<sup>z</sup>The explants cultured onto 100 mg l<sup>-1</sup> 2,4-D for 6 weeks then transferred onto 10 mg l<sup>-1</sup> 2,4-D up to 24 weeks. <sup>y</sup>The minimum and maximum spikelets length within a single inflorescence.

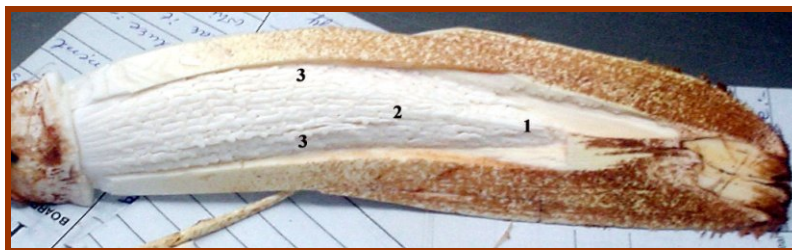


Figure 1. Initial spikelet explants of Kashoo-Wari cv. female inflorescence of a pyramidal structure, No. 1 is the longest spikelets (50 mm), No. 2 is the middle (45 mm) and No. 3 is the shortest (40 mm).

### Media composition and treatments

The basal nutrient medium contained Murashige and Skoog (1962) basal salts supplemented with (in mg l<sup>-1</sup>): 100.0 myo-inositol; 0.5 nicotinic acid; 0.5 pyridoxine-HCl; 0.4 thiamine-HCl; 2.0 glycine; 2100.0 agar (Agar Technical, Oxoid, Inc.); 1300.0 Gel (Gellan Gum, Caisson Laboratories, Inc.) and 30000.0 sucrose. Regarding the plant growth regulators and Activated Charcoal (AC), this basal nutrient medium was supplemented with 100 mg l<sup>-1</sup> 2,4-D + 3 mg l<sup>-1</sup> 2iP and 3 g l<sup>-1</sup> AC (Tisserat, 1979) at zero sub-culture (initial culture). This medium was utilized for 3, 6 or 24 weeks; after that the explants were transferred onto the lower-auxin medium with 10 mg l<sup>-1</sup> 2,4-D + 3 mg l<sup>-1</sup> 2iP + 1.5 g l<sup>-1</sup> AC. Cultures of the three time periods were designated Batches I, II and III.

All three batch cultures (three treatments) were subjected to a subculture process every 3 weeks up to 42 weeks initially (14 subcultures). After preparation of the medium, the pH was adjusted to 5.7 ± 0.1 and was dispensed into small culture tubes (25 × 150 mm) in aliquots of 15 ml per tube and the tubes capped with aluminum foil. Media were then autoclaved for 20 min at 1.11 kg/cm<sup>2</sup> at 121°C.

### Incubation conditions

In vitro explants were incubated in the dark in a temperature-controlled chamber at 25 ± 2 °C. Some observations were made and the following data were recorded at initial culture time and after 24 weeks (8 subcultures):

1. Spikelet length (age) and spathe excision date from mother tree (Table 1).
2. Browning, swelling and somatic embryo formation. This is expressed as +, ++, +++, and - ; representing poor, moderate, high and no response, respectively. This method was described by Mujib et al. (2005) and Abul-Soad et al. (2002b) (Table 2).

### Results and Discussion

#### The explant length

Based on evaluation of 84 date palm cultivars growing in Khairpur district, Sindh, Pakistan (Markhand and Abul-Soad, 2010), seven prominent cultivars were selected to be micropropagated. Propagation was by tissue culture using inflorescence explants to carry all possible trials to investigate the factors controlling the length of the immature spathe during the critical time period before flowering. In this regard, it was revealed that the length of intact spikelets within all excised spathes during this period exhibited a wide measurement range of 5-55 mm (Table 1). This range was recorded for all immature spathes before and during the flowering season (19 Jan. – 7 Feb., 2009). It is important to point out that immature spathes at a very early stage are difficult to excise because they are hidden in the crown and excision carries the risk of lethal damage to the parent tree. Thus, the innovative method for immature spathe excision from a parent plant was followed, as recommended by Abul-Soad (2011).

The time of immature spathe excision affected the inflorescence length, i.e. spikelet length increased according to the later date of excision (Table 1). The length range of Aseel-1cv. explants was 5-15 mm (minimum and maximum spikelet length within this inflorescence) on 19 Jan. and increased steadily by delaying the time of excision. After 18 days, the spikelet explant length of Aseel-4 cv. reached 40-45 mm on 6 Feb. Similar results were obtained with the three inflorescences of Dedhi cv. where the length of Dedhi-1 (9-12 mm) increased in Dedhi-2 (22-26 mm) and Dedhi-3 (19-21 mm) at later dates of excision. This indicated the rapid development of the inflorescence within about 3 weeks. Apparently the spikelet explant length is related to the ability of the tissue to differentiate into organs at the time of excision. The appropriate physiological age of the explant is necessary information to meet the targeted response

of friable or unfriable embryogenic callus formation. Spikelet explants of the same length from different cultivars varied in response to the initial nutrient medium and to form direct pro-embryos. This is due to the different developmental stages, i.e. organization of those tissues although they were the same length.

Therefore, the very small early-age spikelet explants of only 2.5 cm in length did not have the capacity to produce friable callus (pro-embryos) as the spikelet 7 cm explants achieved. Moreover, very small explants produced little of the unfriable callus needed to mature and reach a friable callus stage. The very long spikelet explants, 29 cm in length, once they have emerged from the crown, had a low capacity to change and required a dedifferentiation process to produce the meristemoid progenitors. This process may require more than a year and undergo elevated stress from the high concentration of 2,4-D (Abul-Soad et al., 2003; Abul-Soad, 2008). Early in their development, meristemoids are thought to be morphogenetically plastic and capable of developing into a number of different primordia (i.e., shoot, root, embryo, etc.). A developmental sequence involving an intervening callus stage is termed *indirect* embryogenesis. De novo organ formation via indirect embryogenesis may increase the possibility of introducing variation in the chromosomal constitution (e.g. ploidy change) of the cells in the callus stage and both physiological and morphogenic variation in the organs produced (Schwarz and Beaty, 2000). The current study recommends avoiding long explants in terms of late age to avoid indirect embryogenesis. The length of the explant in terms of age varied widely within the short period of time before flowering.

Spikelet lengths differed due to certain other factors such as the location of the immature spathe on the palm crown, which was constant for all four trees of Aseel cv. used in this study. In the case of Dedhi-2 cv. and Dedhi-3 cv. which were excised on the same day (6 Feb.), the location from which the spathe was excised was different. Consequently the inflorescence length differed and measuring 22-26 mm and 19-21 mm, respectively. This result confirms the significance of location in the palm crown when excising immature spathes. Spathes always emerge in three groups appearing one after the other. The earliest set of spathes emerge during the middle of the flowering season, followed by an

outer set and finally the upper set near the central apical meristem (heart) of the date palm tree (Abul-Soad, 2003; Abul-Soad et al., 2004b).

Spikelet explant length also was found to be different among the cultivars studied when subjected to excision on the same day from a particular location in the crown. On 27 Jan. the excised immature spathe of Aseel-3 cv. was 25-30 mm long, Khar cv. was 22-25 mm while Dedhi-1 was 9-12 mm. It was found that each cultivar exhibited a distinctive length/time frame (Table 1).

The length of intact spikelets in each immature spathe was measured and found different within a single spathe (Figure 1). This result is in agreement with the findings of Abul-Soad (2011) who reported the spikelets were located within the spathe in a pyramidal shape. The longest spikes are found in the center surrounded on the periphery by shorter ones.

Based on the above results obtained, the factors controlling the excised immature spathe lengths varies during the period before and after flowering according to the time of excision, location of the spathe in the crown, cultivar and the location of the spikelets in the inflorescence. Accordingly, the response differed among the cultivars tested (Table 1).

Observations recorded on the initial response of the explants after 3 weeks in culture varied significantly among the different cultivars (Table 1). Explants of Khar cv. were much faster to produce swollen florets than other cultivars lying on the spikelet stalks (Figure 2). The swollen florets were bright and larger in size as compared to other cultivars. These explants remained white in color during the 24 weeks. This result indicated the genetic potential (genotype) of this cultivar to react with the 2,4-D medium and quickly form such structures as compared to other cultivars.

The color of most of swollen florets was bright creamy or pale yellow and the florets appeared filled with water, i.e. watery, and mostly retained their shape through the subsequent subcultures until forming brownish balls or shrinking and dying. Rarely, these brownish balls, after many subcultures, were found to contain clusters of globular structures (pro-embryos) within them. These results are in accord with Abul-Soad et al. (2005) that such structures are believed to produce globular pro-embryos by re-culturing for further sub-cultures when maturation will take place.

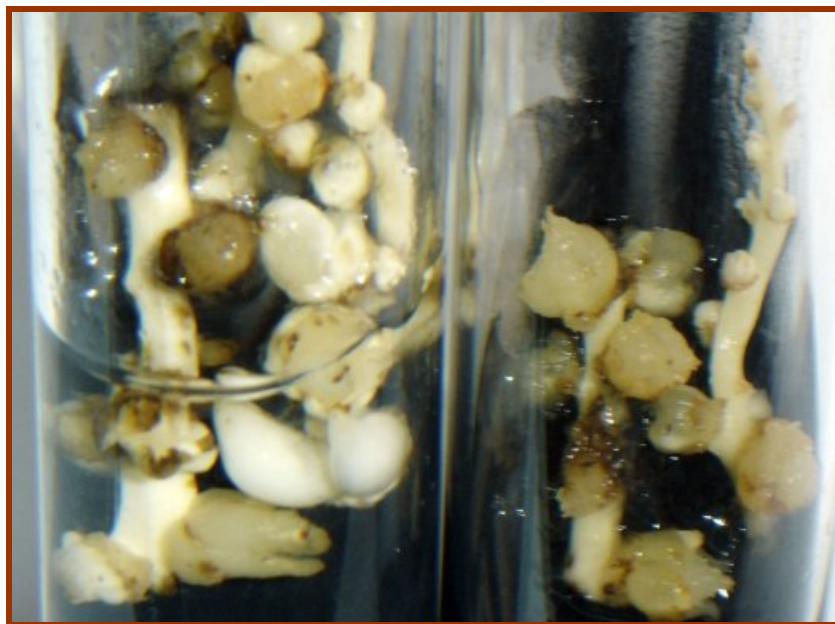


Figure 2. The vitrified and swollen florets on spikelet explants on the high-auxin medium ( $100 \text{ mg l}^{-1}$  2,4-D) after 24 weeks.

The late growth stage of Aseel-4 cv. explants often produced swollen florets. However, these later developed into white-callused carpels. Very few of these explants produced white unfriable callus. The exact origin of produced callus was not clear. However, it was clear that the callus emerged from the core of the swollen floret. It appears that the callus originated from a newly growing meristem which is presumed to produce the remaining different parts of the flower in the future. Sometimes callus was observed emerging from the basal part of the floret and the remaining brown floret parts are left floating on the callus.

Three inflorescences were excised from the mother trees of Dedhi cv. at three different times (Dedhi1-3). Data recorded after 24 weeks and observations indicated that the middle time was relatively suitable for culturing as some explants produced pro-embryos (Table 1). The spikelet explants of Dedhi-1 resulted in shrunken and brown florets. Although, some of these explants produced initial signs of callus formation, others remained without observable change and became entirely brown.

The phenomenon of burned terminal parts of spikelet explants was recorded for the majority of the cvs. studied after 6 weeks in culture (Figure 3). This effect could be the result of quick degradation of the tissues on unsuitable medium composition and/or depletion of endogenous hormones within the explant by the larger florets located in the

middle of spikelet explant. It has been reported that some of florets of the intact spikelet explants react to the medium formula used, but not for all the florets; it occurs mostly with those found in the middle (Abul-Soad et al., 2004b; Abul-Soad, 2011). Using  $100 \text{ mg l}^{-1}$  2,4-D medium may be causing burning of the terminal parts of the spikelet explants such that they become dark brown in color. However, in the responding explants (induced pro-embryos), fewer brownish tips were observed. Moreover, the explants of some cultivars were more sensitive to the phenomenon of burned terminal parts. The competence among the florets lying on the spikelet stalk explant may be caused by depletion of the endogenous hormone and subsequent browning and drying of terminal parts bearing the lying florets on the stalk occurred. This impact could be twice as significant in the case of using high concentration of 2,4-D, and the susceptibility of the cultivar to burned terminal parts of spikelet explants. Sensitivity was found to be cultivar dependent on the same nutrient medium composition.

As much as the length of the date palm inflorescence is a cultivar-related trait, determination of the appropriate age in terms of length routinely needs to be investigated for each cultivar within a particular area. This will provide only an approximation of the appropriate time to excise the immature spathe from a parent palm, which may differ, but not widely, according to the

general conditions such as climatic conditions and nutritional state of the date palm tree after the previous fruit harvest. For instance, the best time period of time to excise inflorescences of Aseel cultivar was 24 Jan. and Dedhi on 6 Feb. in Khairpur district. When excision was repeated in subsequent years in same area, little deviation was observed. Thereafter, other factors need to be adjusted, such as plant growth regulators, basal salts and sucrose concentration (Abul-Soad et al., 2007).



Figure 3. Burned terminal parts (tube at right side) compared to normal white tips (tube at left side) of spikelet explants after 6 weeks in culture.

### The initial 2,4-D period

In more than 80% of 124 recently-published protocols, induction of somatic embryogenesis required the presence of auxins alone, or in combination with cytokinins (Gaj, 2004). A high auxin level was thought to be necessary to disrupt normal development. This was subsequently confirmed in the date palm (Eeuwens and Blake, 1977). The current study aimed to avoid prolonged cultures on a high 2,4-D medium, then investigated the proper initial time period necessary for a high 2,4-D medium ( $100 \text{ mg l}^{-1}$ ) to disrupt the normal development of the floral buds and finally induce organs from the initial inflorescence explants.

The overwhelming majority of Batch I and III explants produced swollen florets which appeared vitrified (Figure 2). It is noteworthy that such a shape was dominant over all the explants cultured onto the high-auxin medium, where no growth progressed after 24 weeks from the initial culture (Table 2).

Although the medium used by (Tisserat, 1979) of  $100 \text{ mg l}^{-1}$  2,4-D provided viable callus formation medium for the shoot tip explants, the current study indicated that the same medium would not be effective for inflorescence explants (cultures of Batches I and III). Nevertheless, a stimulation pulse of the modified high-auxin medium used in this study for an initial period of 6 weeks was only partially able to trigger the embryogenic pathway of the cells and to form unfriable and friable callus (cultures of Batch II). Unfriable callus was mostly mixed with roots (Figure 4). Therefore, the induction medium of  $100 \text{ mg l}^{-1}$  2,4-D used for only 6 weeks successfully induced the direct somatic embryos after 24 weeks, compared to continuity with  $100 \text{ mg l}^{-1}$  2,4-D.

Table 2. Effect of initial-medium supplemented with  $100 \text{ mg l}^{-1}$  2,4-D for 3, 6 and 24 weeks (Batches I, II and III, respectively) on browning, swelling and somatic embryo formation of the female spikelet explants of date palm, after 24 weeks in culture.

Duration on initial medium	Browning	Swelling	Somatic embryos formation
Batch I	+++	+++	-
Batch II	+	++	++
Batch III	++	+	-

Each treatment had 6 replicates (72tubes). +, ++, +++ - represent poor, moderate, high and no response, respectively.



Figure 4. Unfriable callus (at left photo) and mixed with roots (at right photo) formed from the florets of spikelet explants of Kashoo-Wari cv. after 3 subcultures.

#### The length vs. 2,4-D initial period

The response obtained was a result of the interaction between the explant length and 2,4-D, as in all the explants disseminated among the three batches (Table 1).

The interaction effect between the cultivar and media was detected after 24 weeks in culture. Some of the explants produced embryogenic callus (nodular callus or friable callus) which was white and loose. Sometimes it was mixed with the differentiated somatic embryos and roots (Figure 5). The somatic embryos produced occurred in very few explants belonged to certain cvs. studied: Aseel-2 and Aseel-3, Dedhi-2 and Kashoo-Wari cvs. explants only (Table 1). The majority of explants which responded belonged to Batch II (Table 2).

The predominant response was the swollen florets some of which induced pro-embryos. Longer explants of late excised spathes such as Aseel-4 and Khormo cvs., possessed florets of about 5 mm in diameter. Very few of the swollen florets of Kashoo-Wari cv. of Batch II which produced pro-embryos developed into green shoots when shifted to the light conditions ( $2,000 \text{ l m}^{-2}$ ). Other explants, because of the media composition of 2,4-D, turned brown and died at the end (42 weeks). However, very few small explants of Aseel-2 cv. and Dedhi-1cv. produced only a small amount of unfriable white callus from the tiny florets. The callus produced was not from the stalks bearing these florets. The most appropriate

age/length of the inflorescence in terms of spikelet length was 15-50 mm; most of the organs induced on Batch II were in this range.

Morphogenesis began with swollen florets, some of which were watery (vitrificated). Then, after 24 weeks, some of the watery florets produced friable callus in which the synchronized embryos developed. White somatic embryos at different growth stages were produced (Figure 5). The other swollen florets developed aggregates of unfriable white callus. A small amount of unfriable callus formation occurred in the initial subcultures. The impact of 2,4-D to induce the unfriable callus was limited to a very few explants. The explants were sub-cultured for up to 42 weeks but without further development. Further exposure of spikelet explants to the higher-auxin medium for a longer period had an unfavorable impact on the explants.

The initial medium used in the current study is inappropriate for one hundred percent induction, and therefore this study focused on the 2,4-D role in triggering the embryogenic pathway. Nevertheless, the impact of interaction between the explant length and the incubation period on 2,4-D, and on embryogenesis was recorded for the few explants which only produced organs. An improvement for the initial media formula is needed for total explants inducement.

These cultures were transferred to a free-auxin medium under light conditions  $9,000 \text{ lm m}^{-2}$  (Abul-Soad et al., 1999) to effect differentiation into the intact plantlets (Figure 6).



Figure 5. Differentiated synchronized embryos with shoots and roots of Batch II cultures of Kashoo-Wari cv. after 24 weeks in culture.



Figure 6. Produced ex vitro plantlets from female inflorescence explants of 7 Pakistani cultivars after acclimatization in the greenhouse.

### Conclusions

The immature spathe could be useful in date palm tissue culture if excised a couple of weeks before the brown tip of the first spathe appears in the crown and for a week thereafter. During this time period, the length of the immature spathe varies and subsequently the spikelet explants within

it varies. The length of spikelet explants varied among different cultivars when excised from a particular location on the crown on the same day. In addition, spikelet explants were variable within a cultivar according to the time of excision, location on the crown and within a single spathe.



The impact of 2,4-D on inflorescence embryogenesis was studied. Using a stimulation pulse of 100 mg l<sup>-1</sup> 2,4-D in the initial medium for 6 weeks, followed by transfer of the explants onto 10 mg l<sup>-1</sup> 2,4-D medium for up to 24 weeks resulted in somatic embryo formation (Batch II). On the other hand, the initial periods tested of 3 weeks or continuous sub-culturing on the higher-auxin medium for 24 weeks proved ineffective for embryogenesis (Batches I and III, respectively). The morphogenesis of induced and failed spikelet explants is described. Only a few explants of certain cultivars developed synchronized embryos, while the overwhelming majority of the explants became burned. The interaction effect between the explant length and the media used could not be properly traced due to that limited impact of 2,4-D included-medium on the inflorescence embryogenesis.

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