In Vitro Flowering Induction in Date Palm (*Phoenix dactylifera* L.)

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Abstract This article reports on a histological and morphological study on the induction of in vitro flowering in vegetatively propagated plantlets from different date palm cultivars. The study aimed to further explore the control of in vitro flower induction in relation to the photoperiodic requirements in date palm and to come up with a novel system that may allow for early sex determination through plant cycle reduction. In fact, the in vitro reversion of a shoot meristem from a vegetative to a reproductive state was achieved within 1–5 months depending on the variety considered. This reversion was accompanied by several morphological transformations that affected the apical part of the leafy bud corresponding mainly to a size increase of the prefloral meristem zone followed by the appearance of an inflorescence. The flowers that were produced in vitro were histologically and morphologically similar to those formed in vivo. The histological examination of the in vitro flowering induction process showed that the conversion into inflorescences involved the entire apical vegetative meristem of the plantlet used as a starting material and brought about a change in its anatomical structure without affecting its phyllotaxis and the leaf shape. Through alternating between hormone-free and hormone-containing media under different light/dark conditions, the highest flower induction rates were obtained with a basal Murashige and Skoog medium. A change in the architectural model of date palm was induced because unlike the natural lateral flowering, in vitro flowering was terminal. Such in vitro flower induction allowed a significant reduction in plant cycle and can, therefore, be considered a promising candidate to save time for future improvement and selection programs in date palm.

Keywords Date palm · In vitro flowering · Plant growth regulators · Light/dark alternation · Phyllotaxis · Photoperiodic requirements

Introduction

Date palm (*Phoenix dactylifera* L.) is a monocotyledonous, dioecious, perennial tree that belongs to the Arecaceae family. Its culture is of high economic importance for almost all the arid and warm regions in the Middle East and North Africa. However, this culture is currently subjected to a number of alarming dangers such as those caused by *Fusarium oxysporum* f. sp. *albedinis* and the brittle leaf disease (Triki and others 2003). The conventional method of date palm propagation is based on vegetative multiplication using offshoots, leading to seedlings that are closely similar to the mother plant. This method is very slow and risky, particularly because it can cause the dissemination of infectious diseases. The seed-dependent strategy actually generates a very heterogeneous population of male and female individuals in almost equal proportions (50% each). The plant's sex remains indiscernible until the flowering period which takes 6–10 years. To the best of our knowledge, no previous studies have yet clearly identified the photoperiodic requirements of date palm in relation to the flowering process.

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Several studies have been conducted to develop date palm micropropagation methods that could help to overcome the limitations associated with natural vegetative propagation techniques and to increase the number of plants produced while decreasing the period of plant production. Organogenesis or somatic embryogenesis starting from vegetative or inflorescential explants taken on adult plants or from vegetative explants cultured in vitro has been described (Drira and Benbadis 1985; Bhaskaran and Smith 1992; Fki and others 2003). However, only a few reports and little data are currently available on the control and induction of in vitro flowering in this species (Ammar and others 1987). Some studies have focused on a variety of factors involved in the in vitro flowering such as light (Heylen and Vendrig 1988), photoperiod (Floh and Handro 2001), pH of the medium (Jumin and Ahmad 1999), lipids (Groenewald and van der Westhuizen 2004), putrescine and silver nitrate (Bais and others 2000, 2001), and nutrients (Franklin and others 2000; Wang and others 2001; Zhang 2007). Moreover, several other studies have focused on the effect of plant growth regulators on the in vitro flowering process in some species. The requirement of cytokinin for the growth and development of the flower bud, for instance, has been reported in both monocots and dicots (Zhong and others 1992; Wang and others 2001; Zhou and others 2004; Zhang 2007). The effect of BA (6-benzyladenine) on early in vitro flowering for different plant species has also been reported (Jumin and Nito 1996; Jumin and Ahmad 1999; Wang and others 2001; Hee and others 2007; Sim and others 2008). Other reports described the combined effect of BA and other hormone species on the induction and stimulation of in vitro flowering (Galoch and others 2002). Tang and others (1983), Das and others (1996), and Wang and others (2002) have also reported on in vitro flowering by using BAP (benzylaminopurine) and GA3 treatment. The influence of cytokinins has also been reported (Zhang and Leung 2000; Kachonpadungkitti and others 2001; Taylor and others 2005). The effect of GA3 alone has also been reported on with respect to the induction of in vitro flowering in Gerbera (*Gerbera jamesonii* Adlam) (Ranasinghe and others 2006). Likewise, Saxena and others (2008) maintained that abscisic acid (ABA) and proline used alone or in combination influenced in vitro flowering in *Vigna aconitifolia*.

Floral induction in date palm was reported in only one study (Ammar and others 1987) in which the authors indicated that both male and female flowers were induced on 5-month-old seedlings (cv. Deglet Nour) in a 16-h day at 28°C by a combination of BAP, IAA, and glucose or sucrose. Accordingly, the present study sought to gather further information related to the control of in vitro flower induction in the date palm. To achieve this goal, flower development was induced in vitro on vegetatively propagated plantlets from several cultivars issued from shoot cultures. A series of histological analyses was then performed to identify the different transformations that the apical bud of the original plantlet underwent to produce flowers. A histomorphological comparison between in vitro and in vivo flowers was also performed. The effect of different factors on in vitro flower induction was investigated. Different growth regulators and light (16/8 h photoperiod)/dark conditions were tested. The results obtained indicate that in vitro flower induction was effective, thus providing a valuable system for further basic studies on flowering regulation mechanisms. It can also be considered a promising system that can open new prospects for date palm selection and improvement programs by controlling in vitro flowering induction under particular tissue culture conditions.

Materials and Methods

In vitro flower induction was carried out on 1-year-old plantlets regenerated in vitro from bud cultures via the organogenesis strategy using juvenile leaves taken from offshoots (Drira 1983) of several date palm (*Phoenix dactylifera* L.) cultivars: the elite Deglet Nour, Arichti, Bousthammi Noir, and Boufeggous. The offshoots taken from Deglet Nour and Arichti cultivars were collected from plants growing in the oases fields in southern Tunisia. Those from Bousthammi Noir and Boufeggous cultivars were collected from plants growing in oases in Morocco.

In Vitro Culture Conditions

The experiments of the present study were conducted on basal MS (Murashige and Skoog 1962) medium supplemented with sucrose (50 g l^{-1}), adenine (0.03 g l^{-1}), L-glutamine (0.1 g l^{-1}), and agar (8 g l^{-1}). In some cases, half-strength major salts of Quoirin and Lepoivre's nutrient solution (QL, Quoirin and Lepoivre 1977) were added to the basal MS medium (M1 and M2, Table 1). The plantlets were subcultured every 30 days alternately on M1 (hormone-rich) and M2 (hormone-free) medium or on M1 and M3 (hormone-free) medium (Table 1). In some cases, the subculture period was extended to 45 days. The pH of all the culture media was adjusted to 5.8. The experiments were performed at 28 ± 2°C during the day and at 24 ± 2°C at night. The present study involved three sets of experimental series. The first set was exposed to light (15 μM m^{-2} s^{-1}) using a 16/8-h photoperiod, the second was exposed to continuous dark, and the third to sequential 4-week light/dark treatments. Each treatment was applied to 24 explants of each cultivar (1 explant = 1 replicate) and

Table 1 Composition and concentrations of plant growth regulators of M1, M2, and M3 media

Culture medium	M1	M2	M3
Nutrient solution	MS + ½ QL major salts	MS + ½ QL major salts	MS
Plant growth regulators (μM)	NAA: 2.68 BAP: 4.44 Kin: 4.64 IPA: 5.28	No growth hormones	

the number of plantlets that showed initiation and development of inflorescences was recorded on a monthly basis.

Histological Analysis

For histological studies, the samples (flowering apical buds, in vitro and in vivo flowers) were fixed in Svaloff Navashine solution (chromic acid 0.5%, glacial acetic acid 5%, formaldehyde 15%, and ethanol 5%) at room temperature, washed with running water for 24 h, dehydrated in ethanol solution series (from 50 to 100°C), and then immersed in xylene–ethanol baths as described by Masmoudi-Allouche and others (2009). Finally, paraffin inclusion was performed and 10-μm-thick sections were made using a rotary microtome. The sections obtained were stained with a Regaud ferric hematoxylin solution (Regaud hematoxylin solution 10%, glycerol 10%) (Masmoudi-Allouche and others 2009) and then observed under a light microscope.

Results

Identification of the Best Flower Induction Conditions

Several physicochemical factors were evaluated for their influence on the induction of the reproductive process, including mainly the growth regulators and the light/dark conditions which were identified as having a direct implication in both vegetative and floral developmental processes. A hormonal alternation between M1 and M2 or between M1 and M3 was tested. The effect of a 16/8-h light/dark photoperiod, a continuous dark, or a light/dark alternation condition was also investigated.

The data analysis showed that in the absence of hormonal alternation (M1/M2 or M1/M3), none of the tested light/dark conditions allowed floral induction. However, regardless of the light/dark conditions used, the application of a hormonal treatment corresponding to monthly subculture alternation on M1/M2 or M1/M3 media allowed the induction of an important flowering capacity for the different varieties tested. Nevertheless, the percentages of the neoformed flowers and the initial response of explants were very dependent on the variety of the date palm used

Table 2 Cumulative means over 1 year of flowering percentages of date palm plantlets from the different varieties tested when cultured under light/dark alternations and submitted to alternative subcultures of M1/M2 and M1/M3 media

Variety	M1/M2 alternation	M1/M3 alternation	Time of initial flowering response (month)
Arichti	48	58	1
Deglet Nour	25	32	3
Boufeggous	25	28	3
Bousthammi Noir	10	13	5

The values represent the cumulative 1-year percentages of monthly means of 24 replicates each

(Table 2). In fact, the best in vitro flowering potential was obtained with the Arichti variety; therefore, this variety was selected for use in the subsequent elucidation (Table 3).

More accurately, when the plants were cultured under light/dark alternations and submitted to alternative subcultures of M1/M2 media, the cumulative 1-year flowering rates reached 48% for the Arichti variety whereas they did not exceed 25% for the Deglet Nour and Boufeggous cultivars and 10% for the Bousthammi Noir cultivar (Table 2). However, when the light/dark alternation conditions were combined with a hormonal alternation between M1 and M3 media, the Arichti variety had the best flowering rates; its cumulative 1-year flowering rate increased to reach 58% (Table 3). Furthermore, when the hormonal alternation of M1 and M3 media was coupled with light (16-h photoperiod) and continuous dark conditions, the flowering rates for the Arichti cultivar were 51 and 53%, respectively. These flowering rates are actually much more important when compared to those produced in light (25%) and dark (40%) conditions in the case of a monthly alternation between M1 and M2 media (Table 3).

These results suggest that an important flowering capacity was acquired in plantlets that were subjected to sudden change from hormone-rich to hormone-free media. This flowering ability increased when the plants were subjected to both a hormone alternation and a nutrient concentration deviation involving both a medium that was rich in hormones and MS and QL nutrients (M1) and a medium that was hormone-free and contained only the MS major salts (M3).

Table 3 Effect of various physicochemical treatments on the flowering percentage of date palm cultures of Arichti cultivar that underwent a monthly alternative subculture on M1/M2 and M1/M3 media

| | Month | | | | | | | | | | | | Cumulative 1-year flowering percentage |
	1	2	3	4	5	6	7	8	9	10	11	12	
M1/M3 alternation													
16-h daylight	5 ± 0.58[a]	0	5 ± 0.58	0	10 ± 1.15	5 ± 0.58	5 ± 0.58	10 ± 1.73	5 ± 1	6 ± 1.15	0	0	51
Dark	0	0	10 ± 0.58	0	0	0	20 ± 0.58	18 ± 1.15	0	0	5 ± 0.73	0	53
16-h daylight/dark alternation	0	0	5 ± 1	0	0	20 ± 2	0	0	15 ± 1	10 ± 1	8 ± 0.59	0	58
M1/M2 alternation													
16-h daylight	5 ± 1	5 ± 1	0	5 ± 0.73	0	0	5	0	0	5 ± 0.58	0	0	25
Dark	0	0	5 ± 0.58	0	0	20 ± 2	0	0	15 ± 1.15	0	0	0	40
16 h daylight/dark alternation	0	0	10 ± 1	0	0	0	20 ± 1.15	0	18 ± 1	0	0	0	48

[a] The values represent mean ± standard deviation of 24 replicates

It is also worth noting that in a number of cases in which the treated plantlets were subcultured for 45 instead of 30 days and subjected to hormone alternation between M1 and M2 media, the percentage of flowering explants obtained was even more important. For the Arichti variety, the cumulative 1-year flowering rate increased from 25 to 43%. Indeed, although the pH of the culture media was first adjusted to 5.8, it decreased during the culture period. In fact, after 30 and 45 days of culture, the pH decreased 0.4 and 1.8, respectively. This phenomenon revealed that active exchanges occurred between the culture media and the explant's tissues during the culture period and, therefore, might have created a stress condition due to the increase of the subculture period.

Evolution of the Vegetative Apical Meristem Toward an Inflorescential-Type Development

The transition of an apical bud from a vegetative to a reproductive state under in vitro culture conditions was accompanied by a series of morphological transformations that affected leaf growth. These modifications were manifested by the appearance of several small leaves that were long, thin, and strap-shaped along with and an increase in the size of the prefloral meristem zone. Then a pearly white inflorescence emerged (Fig. 1). At this point the vegetative flower organs (sepal + petal) were completely differentiated. Later, the inflorescence maturation process occurred and was followed by the appearance of green carpels. Starting from female plant material, the corresponding induced flowers were the same sex. Despite their unusual origin, the flowers that were induced in vitro were morphologically similar to the in vivo female flowers (Fig. 2).

A histological analysis was conducted on the floral initiation process to spot the morphological modifications that the apical bud underwent to generate a floral state (Fig. 3a, b). The analysis revealed that the apical vegetative meristem of the in vitro plants that were subjected to the flower-inducing treatment continued to form leaf primordia (Fig. 3a) according to the usual phyllotaxis of 2/5 index (Bouguedoura 1980). It then suddenly changed, without any transition, into a structure of inflorescential type (Fig. 3b). The latter had a unique axis that carried several spathe-lacking nude flowers (Fig. 1a). Two other structure types also were observed but only in a few cases. They corresponded to a typical inflorescence consisting of a spadice ramified into several branches (Fig. 1b), as is the case in nature, or to an inflorescence reduced to a unique flower (Fig. 1c).

Accordingly, the in vitro flower induction showed that the reversion of shoot buds from a vegetative to an inflorescential state affected the entire apical bud and brought about a change in the latter's anatomical structure but

Fig. 1 In vitro flowering of date palm plantlets. **a** Inflorescence reduced to a single floral axis. **b** Ramified inflorescence (typical inflorescence). **c** Inflorescence reduced to a single flower. The photo shows two flowers induced on two plantlets cultured together in the same test tube. L, leaf; ia, inflorescential axis; f, flower. Scale bars: 10^4 μm

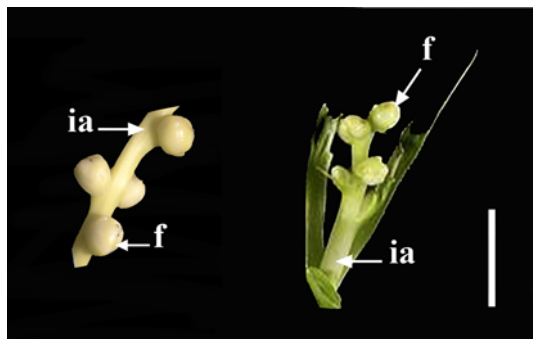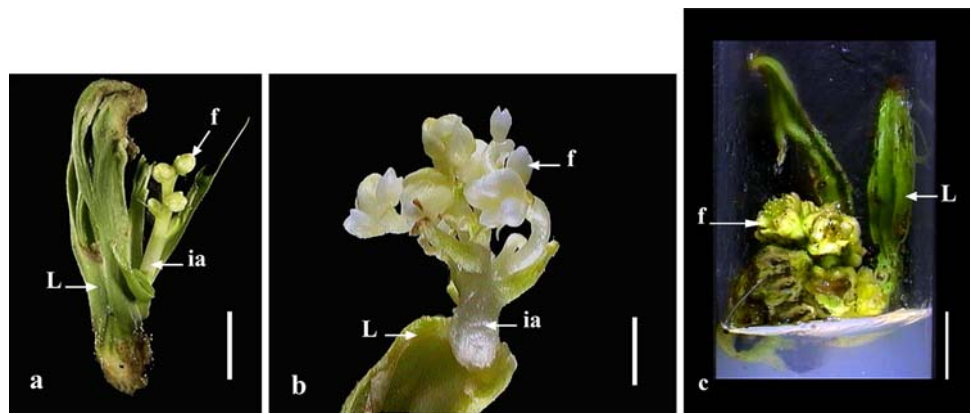**Fig. 2** In vivo (*left*) and in vitro (*right*) mature female flowers of date palm. ia, inflorescential axis; f, flower. Scale bar: 10^4 μm

without affecting the leaf shape and the phyllotaxis concerning the first flower initiation process.

Histological Comparison Between In Vitro and In Vivo Flowers

The longitudinal sections analysis that was performed on in vitro and wild-type date palm flowers showed that the in vitro flower structures (Fig. 4) were similar to those of the natural female ones (Fig. 5). In fact, both types of flowers were globular and consisted of three welded sepals, three free petals, six staminodes (vestigial stamens), and three carpels that harbor ovules, which confirmed the mature state of both flower types.

Discussion

Our results demonstrated that flower induction can be achieved on plantlets regenerated from shoot cultures of different date palm cultivars under certain in vitro culture conditions. They also showed that new flowering potential of date palm vegetative buds can unfold with the application of specific physicochemical conditions. These findings corroborate those of Ammar and others (1987) who reported on the sexual induction of young seedlings
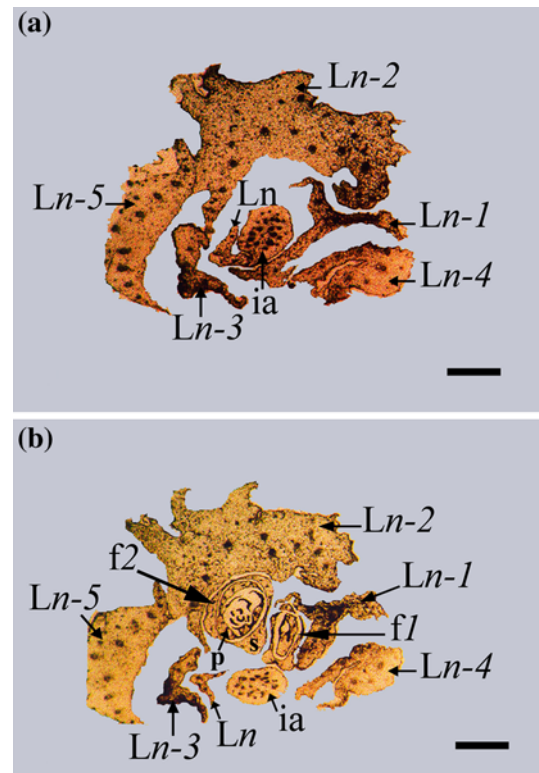
Fig. 3 Transverse sections on two different levels of the apical bud of date palm vitroplant cv. Arichti that have reversed to the floral growth. **a** Transverse section on the basal level of the apical bud showing the aspect of the last leaf structures formed at the time of the reversion to the floral state. **b** Transverse section on the upper level of the apical bud showing the development of the inflorescential axis on which two flowers were initiated. ia, inflorescential axis; f1, flower 1 in longitudinal section; f2, flower 2 in transverse section showing, from the external to the internal part, the three welded sepals (S), the three free petals (P), and the primordia of the reproductive organs of the flower. Ln, leaf of rang n (last leaf that was formed before the reversion process). Ln-1, Ln-2, Ln-3, Ln-4, and Ln-5, leaves of rangs n-1, n-2, n-3, n-4, and n-5, respectively. Scale bars: 1200 μm

obtained from the germination of Deglet Nour seeds. The present study also shows the potential gain obtained by the application of new methods of sex induction in date palm. This opportunity of sex induction could not be achieved in
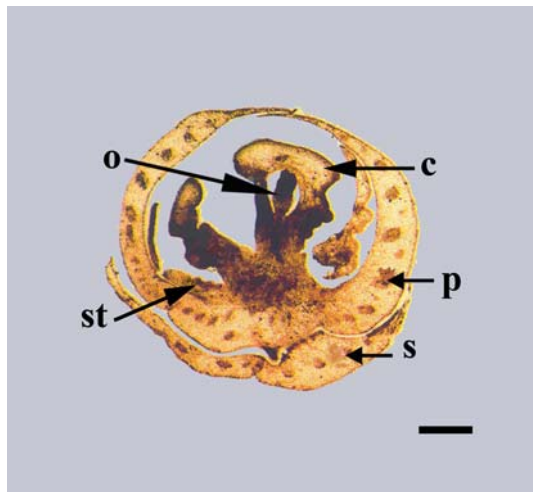
Fig. 4 Date palm female flower resulting from in vitro culture in longitudinal section. s, sepal; p, petal; c, carpel; st, staminode, o, ovule. Scale bar: 666 μm
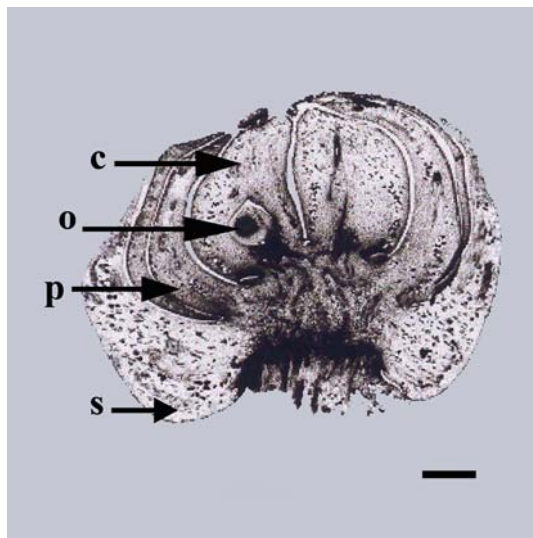

Fig. 5 Mature in vivo female date palm flower in longitudinal section. s, sepal; p, petal; c, carpel; o, ovule. Scale bar: 500 μm

all plant species, such as *Murraya paniculata* (L.) as reported by Jumin and Ahmad (1999) and Lang (1987).

Moreover, the current study showed that typical female flowers were induced in vitro. In fact, they were morphologically and histologically identical to the wild mature female flowers. They were able to complete their maturation under in vitro culture conditions and become ready for pollination. In contrast, the in vitro flowers of other plant species did not reach maturity, were undersized and malformed, and senesced prematurely (Tran Thanh Van 1973; Chang and Hsing 1980; Scorza and Janick 1980; Ramanayake and others 2001; Taylor and others 2005). Accordingly, our data are quite promising and could open new

prospects for future date palm improvement programs via the control of the in vitro fertilization tests.

Our data also revealed that the completely uncovered inflorescences that were produced in vitro can be either reduced to a single axis or ramified or reduced to a single flower. They usually resulted from the transformation of apical vegetative meristems that were devoid of bracts. Our results suggest that the in vitro shift from a vegetative to a reproductive state resulted in a change in the anatomical structure and shape of the bud but did not affect the phyllotaxis of the transitional bud of the first flower formation. In contrast, Bernier and others (1981) claimed that flower evocation in the shoot meristem was accompanied by a number of events that affected the growth habit of the plant, including changes in phyllotaxis and leaf shape.

The data presented here indicated that whatever the light/dark conditions used, a hormonal alternation that corresponded to the monthly subcultures of the plantlets treated on M1/M2 or M1/M3 media allowed the induction of an important flowering activity for the different varieties tested. Similar results were described by Galoch and others (2002) in the case of *Pharbitis nil* by using BA and GA3 during a completely noninductive photoperiod. They attributed the influence of phytohormones on the flowering process to a change in the growth correlations within the meristem of the apical shoot.

The present study also explored the mechanism of in vitro flower induction control in relation to the photoperiodic requirements of the date palm. The photoperiodic requirements for in vivo date palm flowering are not yet fully known. Nevertheless, under in vitro conditions, flowering induction was obtained by varying the nature and concentration of the hormonal factor in both continuous dark and 16-h light photoperiod exposure. These data provide additional evidence that date palm flowering occurred in both long-day (16-h photoperiod) and short-day conditions. Similarly, in vivo date palm flowering usually lasts from March to May but, in some cases, can extend from January to the end of September, which includes both short- and long-day conditions. These observations provide further evidence that date palm is able to flower, whatever the photoperiod, as long as sufficient photosynthetic activity is available. Under the present study's in vitro culture conditions, the availability of organic nutrients in the medium was sufficient to ensure flowering in continuous darkness. The in vitro flowering efficiency observed in this study varied with the date palm varieties used as described by Kenza and Chlyah (1998), who correlated the in vitro difference of plant tissue responses with a genotype effect.

The experimental essays of the present study used a combination of cytokinins and auxin (M1) to evaluate their effect on flowering induction. In fact, the significant effect

of cytokinins on in vitro flowering was well documented and detailed in the literature (Wang and others 2001; Saritha and Naidu 2007). The combined effect of auxin and cytokinin on in vitro flower induction was also described in a number of previous studies (Handro 1983; Wang and others 2002). The effect of the combination of cytokinin and auxin on date palm flower induction was also investigated by Ammar and others (1987). In the same vein, Taylor and others (2005) reported that phytohormones affected flowering by mediating growth changes within the apical meristem and that cytokinins, in particular, played a key role in the initiation of mitosis and the regulation of cell division and organ formation. Similarly, Galoch and others (2002) suggested that in the case of *Pharbitis nil*, the floral transition involved a multifactorial signalling system (including photoperiodic conditions, endogenous phytohormone concentrations, and exogenous phytohormone application that includes different phytohormones) that acted sequentially to trigger various steps in the flowering process.

The present study demonstrated that the plantlets' exposure to an alternation of light (16/8-h photoperiod)/dark conditions at 4-week intervals enhanced the percentage of in vitro flower bud formation. This contrasts with an earlier finding by Jumin and Nito (1996) for *Fortunella hindsii* (Champ.), for which a more than 3-week exposure to darkness followed by re-exposure to light reduced in vitro flowering. Under the present study's conditions, the concurrent application of a prolonged subculture period that led to a decrease in both pH and the organic and mineral compounds of the medium with an alternation between hormone-rich and hormone-free media improved the percentage of in vitro flowering. This may be attributed to chemical stress that favored the flowering process, as previously suggested by Thorpe (1980) for *Cichorium intybus* which was subjected to hydrous stress. Similarly, Neelu (1997) showed that flowering induction can be achieved by chemical stress using a medium containing 100 mM NaCl and an appropriate hormonal composition. These stress conditions favored in vitro flowering induction on date palm tissues, which consequently allowed the expression of new potentialities that were not expressed under natural conditions. Moreover, because the joint alternation between hormone-rich and hormone-free media together with the light/dark changes gave the highest flower induction rates, this may be related to an amplified stress condition and tissue disturbance that might have ultimately generated a high ability to undergo a reproductive morphogenesis and flower formation, as previously suggested by Tang and others (1983). All these findings are in good agreement with those of Bernier and others (1999) who indicated that the flowering process is controlled by several tens of genes. These authors suggested the

existence of an inhibitory system controlled by a flowering repressor gene (embryonic flower1, *EMF1*) that is able to block the expression of floral identity meristem genes. The activity of this system induces an obligatory vegetative growth in most plants, but when it is blocked, the plants would be able to flower spontaneously after germination. The authors added that this gene complex is present in all plants. The characterization of *Arabidopsis thaliana* flowering mutants suggested that a very complex system controls the flowering process in plants.

Drira and Benbadis (1985) showed that hormonal treatment at precocious stages of floral differentiation can induce other morphogenetic programs into the floral meristem, leading to the emergence of axes from the staminodes and the carpels of the flower. In the present study, however, the use of hormonal and physicochemical factors combined induced the reduction of the inflorescential system, which became limited to the differentiation of either a single flower or a unique axis carrying many flowers. These findings can confirm the hypothesis of Nozeran (1954) that the trimeric flower of date palm in fact corresponds to one group that is more ramified but highly contracted by phylogenesis. Therefore, its original structure is completely masked. This compacted group can appear only accidentally and in particular situations such as in vitro culture conditions.

The results of the current study revealed that contrary to natural flowering development, in vitro neoformed inflorescences were completely uncovered, that is, lacking spathe (inflorescence envelope). This distinct morphogenesis can be attributed to the inflorescential ontogenetic mechanism. In fact, under in vivo conditions, date palm flowering is pleonantic because, according to the Tomlinson model (Tomlinson 1961), the inflorescences evolve from lateral buds that develop at the leaf armpits, and the bract situated at the axillary position of the inflorescential bud develops into a spathe. However, under in vitro conditions, the first leaf armpits of the plantlets are empty (devoid of lateral buds), as is the case for plants that are derived from seedlings (Bouguedoura 1980). In the present study, the in vitro flowering was terminal (hapaxantic), resulting from the development of the apical bud which was devoid of any bract, which consequently gave rise to uncovered inflorescences. Once again, this confirms that in date palm, the inflorescence envelope evolved from bract development.

The in vitro flowering presented in this study can be useful for the in vitro rejuvenation process which was reported to be based on changes in vegetative characteristics (Hackett 1985; Pierik 1990) and on the flowering ability of cultured shoots (Harada and Murai 1998). These findings suggest that the use of in vitro flowers as starting material to reinitiate the micropropagation process can be

the basis of future renovation programs that aim to save date palm cultures that declined toward senescence. The significant shortening of the plant cycle by controlling in vitro flowering induction also provides a valuable and promising system for early sex determination. Accordingly, in vitro flowering can also be a potential way to save time in future genetic improvement programs using intra- or interspecific crosses and can help reveal novel properties and characteristics pertaining to fruit quality and stress resistance.

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