

Chapter 5

Date Palm Micropropagation via Organogenesis

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Abstract The world demand for date palm *vitro*-plants has increased in recent decades. The use of all available techniques of rapid multiplication to fulfill this demand is an objective of great interest. However, the technique used should permit maximum fidelity in terms of genetic stability of regenerated plants. Hence, organogenesis presents an advantage of use of low concentrations of plant growth regulators and consequently the callus phase is avoided. Direct regeneration of vegetative buds minimizes the risk of somaclonal variation among regenerants. Moreover, the duration of culture period is limited by frequent renewal of the plant material. Actually, there are few laboratories that use this technique to produce date palm *vitro*-plants at the commercial level. Hundreds of thousands of date palm *vitro*-plants have been produced using this technique mainly in Morocco, Saudi Arabia and the United Arab Emirates. Research has permitted development of this micropropagation process and to adapt it to the most important date palm cultivars. In the present study, a review of the literature and our research results on date palm tissue culture, with emphasis on the organogenesis technique, are presented. The entire multiplication process from offshoot removal to plantlet acclimatization is fully described.

Keywords *In vitro* • Plant growth regulators • Regeneration • Tissue culture

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5.1 Introduction

Date palm, *Phoenix dactylifera* L. (Family: Palmae) is a perennial long-lived, dioecious, monocotyledon which is highly heterozygous. It is considered the key species in the preservation of oasis ecosystems. Date palm cultivation also is one of the most economically important activities in the arid zones of the Middle East and North Africa where it is grown not only for its valuable fruits but also to produce fuel, fiber and to provide shelter for ground crops. In this region, 62 million of the 105 million date palm trees worldwide grow on an area of over one million ha (1,264,611 ha). World production of dates is approximately 7.04 million metric tons and generates important commercial activities (FAO 2008).

In many countries, date palms suffer from serious biotic and abiotic constraints. Among the first are bayoud disease, a soil-borne fungus (*Fusarium oxysporum* f. sp. *albedinis*), and the most dangerous threat to date palm groves, mainly in North Africa. In this region, bayoud disease has destroyed, since its appearance in 1870, more than ten million palms in Morocco and three million in Algeria. It attacks the most renowned cultivars which are susceptible and produce high-quality fruit (Medjool, Deglet Noor, Boufegouss, etc.).

The second important biotic threat is the red palm weevil (*Rhynchophorus ferrugineus* Oliver) which has become the most serious pest of date palm in the world (Gómez and Ferry 1999). It is very difficult to detect early infestation, since the larva begins its life inside the palm and normally never comes to the surface. The rotting of tissue due to infestation by this pest produces a characteristic odor. In addition, wilting or yellowing of leaves can be easily observed on infected trees. The high rate of spread of this pest is human-caused, by transporting infested young or adult date palm trees and offshoots from contaminated to uninfested areas (Ferry and Gómez 2002).

Among the abiotic constraints, desertification is the most serious factor reducing palm groves in most of the countries where date palm is cultivated. This phenomenon is aggravated by drought conditions which hamper successful production of the crop. Taking into account those constraints, the need to select palms to replace senescent and destroyed date palm groves has been steadily increasing in decades. Hence, the exploitation of all available means of date palm propagation is required to fulfill the huge demand for palm vitro-plants.

5.2 Means of Date Palm Propagation

5.2.1 Conventional Techniques

Date palm is conventionally propagated sexually by seeds or vegetatively by offshoots. However, the two techniques are inefficient when huge numbers of plant material are needed or when some superior genotypes are selected and need to

be cloned vegetatively. Furthermore, attempts to micro-graft shoots on seedling rootstocks did not succeed to regenerate complete plantlets because no vascular connection between the two parts (scions and rootstocks) was established (Loutfi and El Hadrami 2005). As a result of these natural constraints, only biotechnologic tools (tissue culture) can be used to fulfill the increasing world demand of date palm vitro-plants. Advantages and disadvantages of each one of the conventional techniques are discussed below.

5.2.1.1 Sexual Propagation

The seed method is the oldest means of date palm propagation. Its main advantage is that it is simple in practice and also enlarges date palm genetic diversity. Hence, the technique is very useful in breeding programs and selection from among the progeny can lead to developing some elite palms with interesting traits. In some countries the number of date palm trees originating from natural hybrids is important; in Egypt, for example, there are 3.5 million and in Morocco more than two million. In other countries (United Arab Emirates, Kuwait, Pakistan, Yemen, etc.) propagation by seed is still practiced (Ferry et al. 1998).

However, this method cannot be used to propagate elite or select genotype since the progeny will be quite variable because of the highly heterozygous character of date palm (Tisserat 1982). Moreover, half of the progeny will be composed of male trees which cannot be distinguished before flowering. Female seed-derived plants will produce variable fruits which are generally of inferior quality (Al Khateeb 2006; Eke et al. 2005; Tisserat and DeMason 1980; Zaid and de Wet 1999).

5.2.1.2 Offshoot Propagation

Date palms produce shoots from axillary shoot meristems and inflorescences from floral meristems (Sudharsan et al. 2001). The use of offshoots for date palm propagation is the most conventional vegetative technique at the farm level (Al Khateeb and Ali-Dinar 2002). Use of this method permits the preservation of true-to-typeness of multiplied genotypes. However, the average number of suckers per palm per lifetime is very low and restricted to the juvenile stage (Tisserat 1983). Some cultivars do not produce suckers or produce limited numbers (10–30) of transplantable offshoots (Heselmans 1997). In addition, offshoots are difficult to root (Asemota et al. 2007; Eke et al. 2005) and the success in the field transfer is usually less than 60% (Saaidi et al. 1979). Some researchers have reported a high mortality of suckers transferred to soil (Al Khateeb 2006). One more disadvantage of this method is the spread of dangerous diseases and pests such as bayoud disease or red palm weevil which can be transported by contaminated offshoots.

The selection of suckers to be planted is of great importance since it determines the success in the field. The most important parameters to be considered are (Zaid

and de Wet 1999): (1) weight: 10–25 kg; (2) age: 3–5 years; (3) base diameter: 20–30 cm; (4) formation of own roots; (5) signs of maturity: production of own roots, first fruit set, production of offshoots of second generation (Nixon and Carpenter 1978); (6) well connected to the mother tree.

To enhance the rate of survival of transplanted offshoots, some researchers propose alternative solutions such as use of exogenous plant growth regulators mainly 25 mM indole butyric acid (IBA) (Qaddoury and Amassa 2004). They observed that IBA-treated offshoots rooted earlier and at a much higher frequency while untreated ones rooted poorly, later and tended to produce few roots. Hodel and Pittenger (2003a) reported that offshoots possessing more roots when removed from the mother palm have a greater ability to regenerate a root system and to become established more successfully and rapidly because over two-thirds of all new roots grow from existing cut roots. In addition, offshoots 10–35 cm in diameter have the highest survival rates, probably because they have more roots when initially removed from the mother palm, more stored carbohydrates to provide energy for root growth and increased levels of naturally occurring root-promoting substances (Hodel and Pittenger 2003b).

Ground offshoots of large size are usually used for date palm propagation. The use of small size and aerial (high and unrooted) offshoots is impractical due to their low rate of survival (Al-Ghamdi 1988). Therefore, they are usually discarded during the separation process of large-sized ones (Mohammed 1978). However, these offshoots could regenerate roots and be used successfully if an intermittent mist system is used (El Hamady et al. 1992). In fact, small offshoots, weighing 5 kg and less, can be used but they should initially be kept, for at least 2 years, in a nursery, mist bed, greenhouse or a shade net structure (Reuveni et al. 1972). Fungi are usually a serious problem in a mist bed, and offshoots must be treated twice a month with a wide spectrum fungicide (Zaid and de Wet 1999). In a study of the rooting of ground and aerial date palm offshoots, Al Mana et al. (1996), showed that rooting medium was an important factor in determining the extent of offshoot root formation. The highest rooting percentages were obtained using the following media: perlite: peat moss (3:1) medium followed by the wood shavings : peat moss (1:1) and perlite: peat moss (1:1) media. Sand medium was inferior to the others. It was also found that neither rooting percentage nor root weight of ground offshoots were increased by NAA and/or catechol treatments. On the other hand, NAA and/or catechol treatments appeared to be essential for good root formation and development of aerial offshoots.

5.2.2 In Vitro Propagation

Since conventional propagation techniques are limited in terms of providing insufficient numbers of date palm plants, biotechnology has provided a promising alternative to meet the increasing demand for date palm vitro-plants in the last decades. Plant tissue culture techniques have been used to clone a wide range of economically-important palms (coconut, oil palm and date palm) (Al Kaabi et al. 2001). Using these techniques, date palm can be micropropagated either by somatic embryogenesis in

which embryos are produced from embryogenic callus and then germinated to form complete plantlets (Letouze et al. 2000; McCubbin et al. 2000) or through organogenesis in which plantlets are produced from multiplied buds without passing through the callus stage (Al Khateeb 2006). Since buds come directly from mother plant tissue, the plantlets produced are identical to the mother tree (Aaouine 2000; Al Khateeb 2008b; Beauchesne 1983; Beauchesne et al. 1986).

The application of tissue culture techniques for date palm micropropagation has many advantages, particularly: (1) large-scale multiplication of commercial cultivars; (2) propagation of elite and select cultivars with desirable characters (bayoud resistance, males with superior metaxenia characteristics, high yielding, etc.); (3) production of homogeneous, vigorous and disease-free plants; (4) no seasonal effect on plant production; (5) enables exchange of plant material without any risk of the spread of diseases or pests (Zaid and de Wet 1999).

5.2.2.1 Somatic Embryogenesis

In this pathway, cells or callus cultures on solid media or in suspension cultures on liquid media form embryo-like structures called somatic embryos, which on germination media, produce complete plants. The primary somatic embryos are also capable of producing more embryos through secondary somatic embryogenesis (Ahloowalia et al. 2004). Somatic embryogenesis is one of the successful methods widely used for mass propagation of date palm throughout the world (Kunert et al. 2003). This technique can be started from any meristematic part of the date palm tree, particularly from axillary buds, shoot tips, immature inflorescences and immature embryos (Al Khayri 2003; Bhaskaran and Smith 1995). Many reported successful results in this field have been published: (Al Khayri 2003; Ammar and Benbadis 1977; Aslam and Khan 2009; Badawy et al. 2005; Bhaskaran and Smith 1992; Daguin and Letouze 1988; El-Bahr et al. 2004; Othmani et al. 2009; Sharma et al. 1984; Tisserat 1979; Zouine and El Hadrami 2007). For more details see chapter on somatic embryogenesis in this book.

5.2.2.2 Direct Organogenesis

The organogenesis technique, based on the exploitation of meristematic tissue potentialities to form new shoots, avoids callus formation and does not use 2,4-D. The growth regulators incorporated in the media are used at the lowest possible concentration. The organogenesis technique consists of four steps: initiation of vegetative buds; bud multiplication; shoot elongation; and rooting. The success of this technique is highly dependent on the success of the first step (initiation) which requires a well-trained staff. Furthermore, most of problems encountered in the succeeding stages (multiplication, elongation, rooting) may have their origin at the initiation phase (Zaid and de Wet 1999). Furthermore, since shoots are directly initiated from mother tissue without passing through a callus phase, the plantlets produced are supposed to be true-to-type (Kunert et al. 2003).

5.3 Organogenesis Protocols

5.3.1 Selection and Removal of Offshoots

Suitable offshoots for *in vitro* culture may have an average weight of 2.5–6 kg (Badawy et al. 2005; Beauchesne et al. 1986; Mohamed et al. 2001), 3–5 years old, 60–80 cm high or bearing 8–12 leaves (Badawy et al. 2005). Suckers must be disease free and selected from a well-known elite adult date palm tree. When removing offshoots, special care should be given to its attachment to the mother tree. In fact, seeds can germinate within adhering leaf bases and produce a small plant which cannot be distinguished from an offshoot until verification of the connection between the offshoot and the mother palm. Skilled laborers are required to cut and remove an offshoot properly without damage to its base. Once removed, the offshoot should be cleaned of soil then all roots and leaves severely cut back before transportation to the lab. The best time period for starting *in vitro* culture from offshoots is between the end of date fruit harvest and the start of the next flowering stage (Beauchesne et al. 1986).

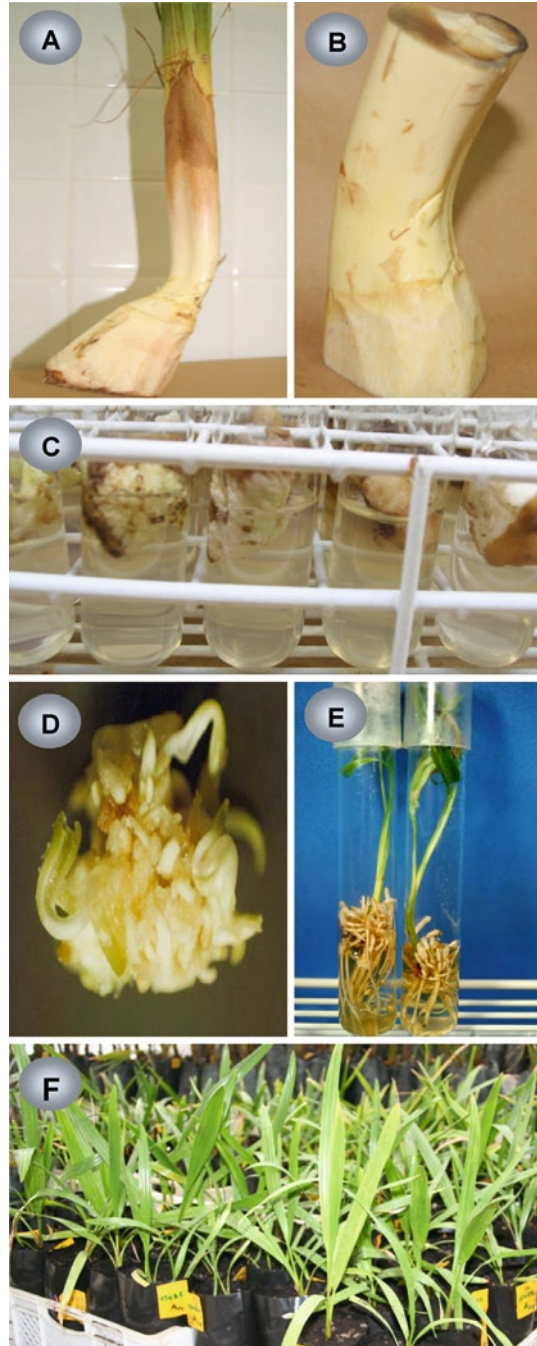
5.3.2 Offshoot Preparation

Offshoot preparation can be done with a sharp knife by removing, gradually (one by one), outer leaves and fibrous tissues at their bases until exposure of the shoot tip zone (Fig. 5.1 a, b). Careful handling is recommended to avoid damage to the brittle meristematic region. The shoot tip can then be excised by cutting a circle around the base of the cylindrical shoot tip at an angle of 45°. The ultimate size of the excised shoot tip should be about 3–4 cm in width and 6–8 cm in length. A sheathing leaf base enclosing the very young leaves of the heart of the offshoot should be left in place to protect it from disinfection solutions. A chain saw can also be used and makes easier the extraction of the shoot tip. Once removed, the shoot tip should be transferred to an antioxidant solution containing 100 mg ascorbic acid and 150 mg citric acid to avoid tissue browning due to the phenolic compounds.

5.3.3 Shoot Tip Disinfection

The excised shoot tips can be disinfected according to the following steps: (1) clean the shoot tips with distilled water to remove any organic debris; (2) soak in a fungicide solution (benomyl, mancozeb) for 10–15 min; (3) rinse three times with sterile distilled water; (4) soak again, for 20 min, in a commercial Clorox solution (sodium hypochlorite) supplemented with 0.3 g/l potassium permanganate; (5) rinse three times with sterile distilled water under aseptic conditions (Abahmane et al. 1999).

Fig. 5.1 Date palm micropropagation via organogenesis technique from offshoot shoot tip. (a) Offshoot after removing external leaves; (b) Shoot tip extracted from offshoot and ready to be disinfected; (c) Explants from shoot tip on culture medium in the starting stage; (d) Vegetative buds in the multiplication stage; (e) Well-formed plantlets ready for transfer under greenhouse; (f) Acclimatized vitro-plants under greenhouse



Other disinfection protocols are available and can be used according to Al-Khayri (2007), Badawy et al. (2005), Beauchesne et al. (1986), Bekheet and Saker (1998), Othmani et al. (2009), and Rao and Ganapathi (1993).

5.3.4 Explant Removal

After sterilization, the root tip can be dissected to extract cultured explants. Using scalpel and forceps, the young leaves surrounding the apical dome are gradually removed. The explants consist of the bottom of the excised leaves. The very young leaves closely surrounding the apical dome are difficult to separate; the entire shoot tip can be cut into 4–6 pieces and transferred to a culture medium. At the axils of young leaves, axillary buds can be found and are also suitable as explants. To avoid desiccation, explants must be immediately transferred to a prepared culture medium. In general, an average of 15–25 explants can be extracted from each offshoot shoot tip.

5.3.5 Incubation Conditions

After transfer into culture media, explants are incubated for 3–6 months in the dark so as to enhance bud initiation and also to prevent oxidation of phenolic compounds which occurs under light conditions. Explants should be transferred to fresh media each month. After initiation, shoots are transferred to lighted conditions with a photoperiod of 16 h. Air temperature in the growth chamber is maintained at $27 \pm 1^\circ\text{C}$ during the illuminated period and $22 \pm 1^\circ\text{C}$ during the dark period (Abahmane et al. 1999; Anjarne et al. 2005).

5.3.6 Culture Media

Generally a basal medium of Murashige and Skoog (1962) (MS) inorganic salts is used for micropropagation of date palm (Table 5.1). Based on the multiplication stage, it can be used at full strength or diluted usually to half strength due to its high level of mainly ammonium salts. A formulation for macro elements was proposed by Beauchesne et al. (1986) and can be used primarily at the initiation step. Beyond the previously-mentioned mineral salts, the following chemicals are usually added to the culture medium, especially NaH_2PO_4 (170 mg/l), myo-inositol (100 mg/l), adenine (30 mg/l), glutamine (200 mg/l), nicotinic acid (1 mg/l), pyridoxine-HCl (1 mg/l), Biotin (1 mg/l), calcium pantothenate (1 mg/l), sucrose (30 g/l), and agar (7 g/l) (Al Khateeb 2006; Beauchesne 1983). Plant growth regulators are added according to the multiplication stage (Table 5.1).

Table 5.1 Hormones added to MS medium during initiation and multiplication stages of date palm micropropagation (values in parentheses are in mg/l)

Date palm cultivars	Explant source	Initiation media	Growth responses	Multiplication media	References
-	Shoot tips	NAA(1), IBA (1), NOA(1-5.5), 2-iP(0.1)	Buds	-	Poulain et al. 1979
Boufegous, Bouskri, Black Bou Sthammi	Shoot tips, young leaves, axillary buds	2-iP(0.1), IBA(0.5), IAA(0.5), NOA(2)	Vegetative buds	-	Beauchesne 1983
-	Shoot tips	NAA(1), IAA (1), NOA(1-5.5), 2-iP(0.1-3)	Buds	NOA(2), NAA(1), IAA(1), BA(0.5), 2-iP(1), Kin(1-5)	Beauchesne et al. 1986
-	Shoot tips	2-iP(2), NAA(1)	Shoot buds	2-iP(3), NAA(0.5)	Taha et al. 2001
Maktoom	Shoot tips	2-iP(2), BA(1), NAA(1), NOA(1)	Buds	2-iP(4), BA(1), NAA(1), NOA(1) (Liquid medium)	Khierallah and Bader 2007
Sewy	Shoot tips, leaflets	2-iP(1), Kin (1) NAA(0.5-1)	Buds	MS + Zeatin (1), NOA(0.5)	Wanas et al. 1999
Khenezi	Shoot tips, apical meristem	IAA(1.6) or IAA(0.4), NAA(0.4) and BA(1.6) or 2-iP(3.2)	Bud generative tissue	IAA(0.4), NAA(0.1), Kin(0.1), 2-iP(1.5)	Al Kaabi et al. 2001
Yakubi	Shoot tips	BA(5), 2-iP(1), IAA(1.6), NAA(0.1)	Multiple shoots	-	Sharon and Shankar 1999
Barhi, Maktoom	Shoot tips	BA(1), 2-iP(1), NAA(1), NOA(1)	Vegetative buds	-	Bader and Khierallah 2007
Zagloul Sukry	Shoot tips	2-iP(2), NAA(0.1)	Shoot proliferation	2-iP(4), BA(4), NAA(0.5) Kin(0.2), 2-iP, BA, IAA, NOA, NAA(0.1 each)	Bekheet and Saker 1998 Al Khateeb 2006
Zaidi, Hussain, Asil	Shoot tips	IBA(0.1-6), BA(1)	Shoot proliferation	2-iP(1), TDZ(0.5)	Hussain et al. 2001
-	Axillary and apical buds, leaflets bases	NOA(3-5), NAA(1), 2-iP(0.5-1)	Buds from callus	-	Saka et al. 1998
Khalas	Shoot tips	-	Shoots from callus	BA(2)	Aslam and Khan 2009

The culture media are dispensed in test tubes (150 × 25 mm) at 15–20 ml or in culture jars (250 ml flasks, 170 ml baby food jars or magenta containers) at 50 ml. Media are then autoclaved at 121°C and under 1 bar pressure for 15–30 min according to the culture medium volume in the containers.

5.3.7 *Shoot Formation*

Depending on the genotype, shoot formation generally requires 6–12 months (Fig. 5.1 c, d). Bud initiation is controlled by several factors that may act in concert. Those factors in particular are the culture media components, genotype and time period of plant material collection. As shown in Table 5.1, different culture media are used to obtain shoots from explants, depending on the date palm cultivar. Concerning the time period of plant material collection, it was shown that the best period coincides with the dormancy stage in advance of flowering (Amin 2001). According to a study by Al Maari and Al Ghamdi (1998) on the effect of seasonal variation on date palm micropropagation, it was shown that the best growth and bud regeneration, and lowest rate of tissue browning were obtained when cultures were established between November and April (Northern Hemisphere).

5.3.8 *Shoot Multiplication*

Once initiated, vegetative buds should be transferred gradually to lighted conditions with a photoperiod of 16 h. Basal medium used for shoot multiplication is usually MS at full- or half-strength. Plant growth regulators are added in low concentrations as compared with the initiation stage and this is in accord with previous research on date palm micropropagation (Al Khateeb 2006; Zaid and de Wet 1999). Some authors have reported that shoot multiplication occurs on media characterized by auxin/cytokinin ratios > 1 (Loutfi and El Hadrami 2005).

According to a study of the multiplication stage of date palm cv. Sukry, Al Khateeb (2006) reported that low hormone concentrations promoted formation of new buds while high concentrations resulted in abnormal growth and without any observed sign of budding or shoot formation; the best combination that gave a good multiplication rate was (mg/l): Kin (0.2), 2-iP (0.1), BA (0.1), IAA (0.1), NOA (0.1) and NAA (0.1). Working on cv. Khalas, Aslam and Khan (2009) reported that the best shoot multiplication rate was obtained with 7.84 µM of BA. In addition, they reported that when the concentration of BA and KIN increased, respectively, to above 7.84 and 9.28 µM, the shoot regeneration rate decreased. In addition they showed that BA was more effective on shoot multiplication as compared to KIN. In their experiments, the authors found that the highest frequencies of shoot regeneration and number of shoots per an explant were obtained on solid MS medium as compared to liquid medium.

The influence of carbon sources and concentrations on *in vitro* shoot multiplication of date palm Khanezi cv. was investigated by Al Khateeb (2008a). He reported that 30 and 60 g/l of sugar was optimal for either qualitative or quantitative shoot growth, while abnormal growth was observed at 90 and 120 g/l, possibly due to osmotic stress. The author also reported that maltose, fructose or glucose were almost equally effective as a carbon source for date palm tissue culture as compared to sucrose. In addition, root formation was enhanced by increasing the sugar concentration above 60 g/l. This shoot rooting inhibits formation of new buds and hence decreases their ability to multiply during multiplication stage.

In general, the most common plant growth regulators used at this multiplication stage are auxins NAA, IAA and NOA and cytokinins BA, 2-iP and Kinetin (Table 5.1). However, other plant hormones can be used depending on the micropropagated genotype. Hussain et al. (2001) reported that TDZ plays an important role in the multiplication stage because it enhances horizontal rather than vertical growth.

As in the previous stage, the shoot multiplication rate is also genotype dependent (Beauchesne 1983; Hussain et al. 2001). In fact, the culture media of different compositions shown in Table 5.1 clearly elucidate this phenomenon encountered in date palm micropropagation. In addition, it was observed that *in vivo* behavior of date palm is also reflected *in vitro* i.e. cultivars producing more suckers in the field, show higher rate of multiplication *in vitro* (Hussain et al. 2001; Krikorian and Cronauer 1984). Concerning the transfer of cultures onto fresh media, it should be done at 4–6 week intervals depending on growth of genotypes in multiplication.

5.3.9 Shoot Elongation and Rooting

At this stage, the basal medium MS salts are used at either full- or half-strength (Fig. 5.1 e). Generally, shoot elongation requires transfer of shoots from the multiplication medium to another medium with a high auxin/cytokinin ratio (Beauchesne et al. 1986; Loutfi and Chlyah 1998). The most effective plant growth regulators at this stage are NAA or IBA, Kin and 2-iPA or BA. A combination of NAA (1 mg/l), BA (0.5 mg/l) and Kin (0.5 mg/l) enhances shoot growth and elongation. Gibberellins at 2 mg/l can also be incorporated into the culture medium in this stage but for no more than 15 days (Beauchesne et al. 1986). A study conducted on Zaghoul and Sewi cvs. showed that NAA at 0.1 mg/l has a pronounced effect on shoot length as compared with IBA and IAA (El Sharabasy et al. 2001). However, Aslam and Khan (2009) found that a rooting percentage of 87.34% was obtained on solid MS medium supplemented with 24.6 μ M of IBA. They also reported that root length was higher when MS liquid medium was used.

Among rooting auxins, NAA added at 0.1 mg/l gave the maximum percentage of root formation, numbers and length (Al Kaabi et al. 2001; Taha et al. 2001). They also reported that use of MS salts at three-fourths strength gave the best results on root formation as compared to one-fourth, one-half and full strength. In the same study, light intensity of 8,000 lx and sucrose at 40 g/l produced the best

results on root number and length. On the other hand, some authors have proposed alternatives to promote plant quality. Sidky et al. (2007) reported that plantlets transferred onto MS medium at half strength supplemented with 0.1 mg/l of NAA, 1 g/l of activated charcoal, 40 or 50 g/l of sucrose and 4 mg/l of paclobutrazol, increased thickness of plantlets, accelerated root formation and promoted secondary root formation.

Depending on the protocol used and genotype multiplied, some authors recommended media free of hormones for 1 month during the elongation stage (Al Kaabi et al. 2001). In the subsequent rooting stage, they used NAA at 1 mg/l coupled with sucrose at 30 g/l.

5.3.10 Plant Acclimatization

The specialized conditions which exist during *in vitro* culture can result in the formation of plantlets of abnormal morphology, anatomy and physiology (El Bahr et al. 2003; Saker et al. 2000). After *ex vitro* transfer (Fig. 5.1f), they may be easily impaired by sudden changes in environmental conditions and so need a period of acclimatization to overcome these abnormalities (Pospisilova et al. 1999). Accordingly, the acclimatization phase is the most important stage in the protocol of date palm micropropagation, because if not optimized, the whole process will be inefficient. Hence, it is not acceptable to produce a huge number of plantlets and lose them at this final stage. In fact, major differences exist between the environment of plants growing in tissue culture and those in the greenhouse; particularly, differences in light, both quantity and quality, relative humidity, nutrients and other growth promoters, the gaseous composition and the medium substrate (Seelye et al. 2003).

Previous studies have focused on optimization of factors such as soil mixtures, relative humidity, temperature and other greenhouse conditions. Studies in this field have led to significant enhancement (70%) in the percentage of survival of tissue culture derived plantlets (Quraishi et al. 1997). However, successful plant acclimatization of date palm should be started at the rooting stage. Plantlets to be transferred to the acclimatization stage should have certain important characteristics that enable them to succeed in the greenhouse. In fact, plantlets must be at least 12–15 cm in length with a well-formed and closed crown, two or three fully opened leaves and more than three roots. Such plantlets, if acclimatized under suitable conditions, have the maximum chance to survive when transferred to the greenhouse. However, plant quality is usually genotype dependant; some cvs. (Medjool, Boufeggous, Nejda, Aguellid, etc.) produce vigorous and well-formed plantlets of desired characteristics while others produce plants of inferior quality (open crown, curved leaves, weak plantlets, etc.). In such a situation, plantlets should be kept in the lab until they have acquired the desired characteristics. In fact, Al Salih et al. (1986) reported that death or failure of date palm plantlets during the acclimatization

stage may be attributed to the lack of root cell differentiation caused by a deficiency in growth regulators or sugar balance. In this context, El Bahr et al. (2003) pointed out that leaf, root and stomata morphology of date palm *in vitro* plantlets were different in structure and shape as compared with those produced from acclimatized ones.

In order to improve the survival rate at the acclimatization stage, some osmoticums are used prior to the transfer of plantlets to soil. The addition of polyethylene glycol (PEG) at 6 or 8 g/l to the culture medium enhances plantlet survival during the acclimatization stage. Histological studies have shown that PEG increased the epidermal wax layer of leaves as compared to control plants (Sidky et al. 2007). In fact, non-acclimatized *in vitro* plantlets had an average of 15% of the amount of wax of greenhouse plants. Treatment of *in vitro* derived plantlets with PEG during the acclimatization stage resulted in an increase of wax deposition and as a consequence water loss was drastically reduced (Zaid and Hughes 1995a). It was reported that PEG-treated plantlets showed a water loss of only about 27% (similar to that of greenhouse grown plants) as compared to an average of 40% in control plants (Zaid and Hughes 1995b).

In addition, the application of gamma aminobutyric acid (GABA) at 10 mM during the acclimatization stage increased the chlorophyll concentration and then improved the survival percentage of date palm plantlets (Awad 2007). Moreover, other substances were tested at the rooting stage. When the growth retardant paclobutrazol was added to the rooting medium, shoots developed fewer and smaller leaves. This reduction in plant growth makes them more wilt tolerant and increases their survival when transferred to the greenhouse (Seelye et al. 2003). On the other hand, a reduction in relative humidity leads to increased plant respiration with associated development of functional stomata for controlling plant water loss (Seelye et al. 2003).

Multiple soil mixtures have been used to transfer plantlets *ex vitro*. The main mixture characteristic that influences plant growth is moisture which should not be excessive to avoid fungi attacks (rots) and not too low to avoid plantlet desiccation. Tisserat (1981) reported that the best survival rate was recorded for 10–12 cm date palm plantlets transferred to peat moss: vermiculite mixture (1/1:v/v) and covered with transparent plastic. El Sharabasy et al. (2001) reported that the best results were obtained with a planting medium containing equal parts of peat, sand and vermiculite. The survival percentage was 80% after 18 months.

In order to maintain high relative humidity around plantlets newly transferred to the greenhouse, plastic micro-tunnels can be used during the first 3 weeks of acclimatization. In addition, during acclimatization plantlets should be protected against fungi that cause crown and leaf rot. Wide-spectrum fungicides should be used twice weekly to avoid plantlets lost. Insecticides also may be needed to control insects inside the greenhouse.

Water supply must be monitored very carefully during the first month of acclimatization. Too much can lead to plantlet rot and too little moisture in the substrate can decrease the relative humidity around the plants and cause their rapid wilt.

5.4 Date Palm Micropropagation from Inflorescence Tissue

In the case of certain rare or select date palm genotypes, micropropagation is hampered by the absence of suckers needed to start their multiplication. In such situation, the use of inflorescence tissue remains the only way to micropropagate those genotypes. Many publications exist about the use of this technique (Abahmane 1998, 2003, 2005a,b 2007; Abul-Soad 2007; Drira 1985; Drira and Benbadis 1985; Loutfi 1989, 1999; Loutfi and Chlyah 1998). The application of this technique to date palm micropropagation will be fully described in the chapter: “Micropropagation of date palm (*Phoenix dactylifera* L.) using inflorescence explants” in this book.

5.5 Problems Encountered in Organogenesis

Date palm micropropagation is hampered by certain constraints that affect its efficiency. Among these problems, tissues browning, vitrification, early rooting and bacterial contaminants are the most important factors affecting date palm tissue culture. Except for internal bacterial contamination, the other problems listed are encountered when the plant material source is from either offshoot shoot tips or inflorescence tissue.

5.5.1 Tissue Browning

Date palm tissue is known to contain high levels of caffeoylshikimic acids ranging from 190 to 430 $\mu\text{g/g}$ fresh weight depending on the cultivar (Loutfi and El Hadrami 2005). The released polyphenols accumulate in the culture medium which turns brown over time. These substances are oxidized by polyphenoloxydases and form quinons which are highly toxic to cultured tissue. Their secretion is enhanced by injuries due to explant preparation for *in vitro* inoculation or to their manipulation when transferred to fresh media.

Many suggestions have been made to reduce the incidence of this phenomenon. Among these are: pre-soaking of tissue in antioxidant solutions (100 mg/l ascorbic acid and 150 mg/l citric acid) before their transfer to culture media as reported by Murashige (1974) and Zaid and Tisserat (1983). Some researchers report that addition to culture media of some adsorbents like adenine; glutamine and citrate reduce date palm tissue browning (Rhiss et al. 1979).

Activated charcoal is among the compounds widely used to avoid tissue browning. However, a great amount of added growth regulators is also absorbed by activated charcoal and this is why high hormone concentrations are commonly used with this adsorbent (50–100 mg/l coupled with 3 g/l of activated charcoal). In addition, Beauchesne (1983) reported that use of Polyvinylpyrrolidone (PVP-40) in the culture medium at a concentration of 2 g/l reduced date palm tissues browning.

Some other measures have also been suggested including use of explants of small size, juvenile tissue (tissue with less lignification) and frequent transfers onto fresh media. Besides, a period of incubation in darkness during the first months in the starting stage is also recommended in date palm micropropagation in order to reduce the incidence of tissue browning, which is enhanced under light conditions. In addition, Al Khateeb (2008b) stated that culturing of explants during winter and spring seasons can reduce this phenomenon.

5.5.2 *Early Rooting*

During the starting stage, roots are formed on cultured explants instead of buds. The appearance of roots in this stage inhibits bud formation and leads to culture elimination. At the multiplication stage, early rooting decreases the rate of bud multiplication by diverting most tissue nutrients to root formation rather than shoot formation (Al Khateeb 2008b). Studies of this phenomenon have shown that root initiation requires high auxin concentrations, especially NAA (3 mg/l). In contrast, root elongation occurs on culture media with low auxin concentrations (0.1 mg/l). Moreover, the time of year when explants are introduced *in vitro* seems to have an important effect on root formation. Rooting percentages of 16% and 40% are observed when explants were cultured respectively in July and December (Northern Hemisphere) (Anjarne and Zaid 1993). Furthermore, low concentrations of mineral nutrients in culture media and incubation of cultures in darkness for a long period also lead to early rooting of buds (Al Khateeb 2008b).

5.5.3 *Tissue Vitrification*

Date palm tissue cultures are susceptible to a vitrification phenomenon. This physiological disorder is characterized by development of tissue with lignification deficiency. It is due to the accumulation of water in the cultured tissues (Al Khateeb 2008b). Many factors that enhance this disorder are cited in the literature. The more important among them are high levels of growth regulators (mainly cytokinins) and some mineral salts (especially ammonium ions) in the culture medium. A study conducted on the Aguellid cv. showed that media containing high concentrations of ammonium nitrate allowed rapid growth and consequently high levels of vitrification, rising from 46% to 53%. In contrast, on culture media containing a moderate concentration of ammonium nitrate, these percentages were reduced to 14–19% (Bougerfaoui and Zaid 1993). Other factors have been reported by Al Khateeb (2008b) including the presence of high humidity levels and gases, particularly ethylene, inside culture tubes and the use of liquid media. Measures to reduce this phenomenon are numerous. Among them the most effective are increasing agar concentration, use of container covers that allow proper release of gases, reduction of hormonal and ammonium concentrations and the use of solid instead of liquid media (Al Khateeb 2006).

5.5.4 *Tissue Contamination*

Date palm tissue cultures are sometimes highly contaminated with internal bacteria. These contaminants are introduced *in vitro* with cultured explants even if they were well disinfected. Many studies have confirmed the existence of internal bacteria in apparently healthy offshoot tissue. Isolation and identification of these contaminants have shown that they belong to the genus *Bacillus* (Leary et al. 1986). Their most important characteristic is formation of endospores which can survive at 80°C for 30 min! The presence of these contaminants in date palm tissues culture can lead to culture elimination at any time. In fact, their appearance is generally observed after 1 month of culture. However, this appearance can also occur even after three or five subcultures. The incidence of this contamination can increase from 20% to 50% of cultures. Control of these contaminants can be done by the use of some antibiotics: tetracycline (30 µg/ml), streptomycin (10 µg/ml), neomycin (20 µg/ml) and chloramphenicol (30 µg/ml) (Leary et al. 1986). To ensure the efficiency of bacterial control when using antibiotics, the following measures are recommended: cleaning contaminated tissue by washing in sterile distilled water, dipping in antibiotic solution before culturing in the physiological medium, sterilizing tools at 180°C and using young date palm tissue in culture (Benjama et al. 2001). However, in practice, the best way to overcome this problem is to screen for contaminated cultures at the beginning of the multiplication stages and discard them.

5.5.5 *Availability of Plant Material*

In the case of some rare or select genotypes, micropropagation is hampered by limited numbers of available offshoots. In fact, some selected genotypes with good fruit quality and presumed resistance to bayoud disease are usually represented by only one or two palms. Hence, few plant materials (shoot tip explants) are available for development of their micropropagation protocol and for plant distribution to farmers. In such a situation, explants excised from emerged inflorescences have been successfully used to multiply genotypes that no longer produce offshoots (Abahmane 2003, 2005a,b, 2007; Loutfi 1999; Loutfi and Chlyah 1998). In fact, inflorescences are produced almost every year and plant material is abundant. In our experience with this plant material, no internal bacteria contaminants have been observed in tissue cultures. In addition, the mother tree is preserved when plant material is removed if some technical cares are taken.

5.6 **Conclusion and Prospective**

Date palm tissue culture techniques have permitted successful micropropagation of a large number of commercial varieties worldwide. The availability of date palm vitroplants has had a tremendously positive impact on the agricultural sector, mainly in

oasis ecosystems. Normally, date palm can be micropropagated through two main methods. One is the embryogenesis technique in which somatic embryos are regenerated from embryogenic callus. By using this method, a large number of plants can be obtained in a short period. The second method is the organogenesis technique in which vegetative buds are regenerated from cultured explants without passing through a callus stage. Since plantlets are obtained directly from mother tissue, typically they will be identical to the mother tree. Hence, this method can be considered more secure and the risk of somaclonal variations can be easily avoided.

Explants are commonly excised from offshoot shoot tips. This plant material is the most suitable for micropropagation of date palm varieties. However, in the case of some select genotypes and rare cultivars, the availability of offshoot is quite limited. In such a situation, explants excised from inflorescences remain the only source of plant material for micropropagation.

The protocols described for date palm micropropagation by organogenesis, can be used both at the research and commercial levels. In fact, many research and commercial labs are actually micropropagating date palm using this technique, chiefly in Morocco, Saudi Arabia and the United Arab Emirates. In Morocco, this protocol has been developed by the National Institute for Agronomic Research (INRA) and transferred to the private sector. The collaborative partnership between INRA-Morocco and the private sector has permitted the production of more than 550,000 date palm vitro-plants. These plants have been distributed by the agricultural ministry extensive services to farmers of the southern oases free of charge.

In the present study, the entire organogenesis process has been described step by step and recommendations related to each stage have been formulated according to the literature and our experience in this field. Some problems facing date palm tissue culture are also discussed in order to help individuals using the technique to overcome them.

More research activities must be undertaken to reduce the time needed to produce date palm vitro-plants via organogenesis. The initial stage remains the main step where more research work has to be undertaken to produce vegetative buds in a shorter time. In addition, protocols have to be refined so as to obtain a routine procedure which can be used at the commercial level to produce more date palm vitro-plants at low cost within a short time.

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