Micropropagation of Date Palms

Asia-Pacific Consortium on Agricultural Biotechnology (APCoAB)
Asia-Pacific Association of Agricultural Research Institutions (APAARI)
Association of Agricultural Research Institutions in the Near East and North Africa (AARINENA)

MICROPROPAGATION OF DATE PALMS

Dr. Nasser S. Al-Khalifah

Dr. Shanavaskhan A. E.

National Center for Agriculture Technologies
King Abdulaziz City for Science and Technology
Riyadh, Saudi Arabia

Asia-Pacific Consortium on Agricultural Biotechnology (APCoAB)
Asia-Pacific Association of Agricultural Research Institutions (APAARI)
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Coordinator
Asia-Pacific Consortium on Agricultural Biotechnology (APCoAB)
C/o ICRISAT, NASC Complex
Dev Prakash Shastri Marg, Pusa Campus
New Delhi - 110 012

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FOREWORD

Date palm is one of the most important tree crops for the deserts, being highly resilient, requiring limited inputs and remaining productive for a number of years. Worldwide, nearly 14 billion tons of dates are produced annually which are consumed in numerous ways and in almost all the countries. Besides, its tree sap, seeds, leaves and trunk find other uses that bring additional income to date palm growers. Date palm plantations create suitable microclimate for growing other intercrops thus adding to farmers' income and helping in agricultural diversification. Date palm can change the livelihoods of resource poor farmers in the deserts world over. Best example is of California in this context.

One of the major problems in extending date palm cultivation is the slow rate of its vegetative propagation. The seeds do not produce true to type progeny and half of these turn out to be male, therefore, useless for fruit production. Hence, since long the need has been felt for a quicker method of propagation. The advent of tissue culture technology opened doors for mass propagation, thus enabling rapid coverage under improved high yielding varieties. Micropropagation of date palm has now become a commercial reality, made possible by dedicated efforts of several scientists, includuding those in the Arab world.

The Asia-Pacific Consortium on Agricultural Biotechnology (APCoAB), a program of Asia-Pacific Association of Agricultural Institutions (APAARI), has been promoting appropriate use of different biotechnological tools and techniques for improvement of agriculture in the region. One of the activities of APCoAB is to bring out status reports and success stories of biotechnological applications in the region. Earlier publications on micropropagation of banana, potato and sugarcane have been well appreciated for their practical details, impact assessment and relevant suggestions for technology adaptation and dissemination. I congratulate Prof. Al-Khalifah and Dr. Shanavaskhan for having authored this useful and very informative publication. It provides detailed information on date palm micropropagation techniques, field level success stories of technology adoption and suggestions for further benefitting from the technology. It is our expectation that Micropropagation of Date Palms will be found useful by diverse stakeholders comprising scientists, policy makers, extension workers and farmers in the Asia-Pacific region.

Raj Paroda
Executive Secretary
APAARI

PREFACE

Micropropagation is the true-to-type propagation of a selected genotype using *in vitro* culture techniques. The production of higher plants *in vitro* is one of the most important aims of tissue culture. Tissue culture technique has many advantages over conventional propagation and the plants produced through tissue culture have now wide acceptability from the farmers.

With its ability to accumulate exceptionally high levels of metabolites under extreme arid conditions, date palm (*Phoenix dactylifera* L.) is a unique physiological entity and is being considered as a keystone species in the harsh environmental conditions of deserts. Date palm cultivation is the main source of agricultural income in many countries of the desert region. Demand for highly popular and economically viable cultivars of date palm is mounting and more and more tissue culture laboratories are now engaged in date palm tissue culture.

In this book the authors wish to extend the knowledge about date palm especially to the non-Arabic readers. We have collected information from many Arabic and non-Arabic references and added our knowledge that we gained during the last three decades. We hope that the readers will find it valuable to their research and practice.

The aim of this book is to present the various aspects of date palm tissue culture. Different pathways used in tissue culture and the most common problems encountered are discussed. Somatic embryogenesis and direct organogenesis in date palm tissue culture and the effects of various components of nutrient media are also discussed in detail.

We wish to express our sincere thanks to all scientific organizations that were behind publishing this book: King Abdulaziz City for Science & Technology (KACST), Riyadh, Saudi Arabia, the Association of Agricultural Research Institutions in Near East and North Africa (AARINENA), Asia-Pacific consortium on Agriculture Biotechnology (APCoAB) and the Asia-Pacific Association of Agricultural Research Institutions (APAARI).

Nasser Saleh Al-Khalifah
Shanavaskhan A.E.

ACRONYMS AND ABBREVIATIONS

AARINENA	:	Association of Agricultural Research Institutions in Near East and North Africa
AC	:	Activated charcoal
Ad.	:	Adenine sulphate
APAARI	:	Asia-Pacific Association of Agricultural Research Institutions
APCoAB	:	Asia-Pacific Consortium on Agriculture Biotechnology
BA,BAP	:	6-benzylaminopurine
BC	:	Before Christ
BNOA	:	B- Naphthoxyacetic acid
EDTA	:	Ethylenediaminetetraacetic acid
Dicamba	:	3,6-dichloro-2-methoxybenzoic acid
DNA	:	Deoxyribonucleic acid
FAO	:	Food and Agriculture Organization of the United Nations
GA3	:	Gibberellic acid
Gel.	:	Gelrite
IAA	:	Indole-3-Acetic acid
IBA	:	Indole-3- Butyric acid
INRA	:	Institut National de la Recherche Agronomique
KACST	:	King Abdulaziz City for Science and Technology
Kinetin	:	6-furfurylaminopurine
KISR	:	Kuwait Institute for Scientific Research
MS	:	Murashige and Skoog
NAA	:	α-Naphthaleneacetic acid
NCAT	:	National Center for Agriculture Technologies
Picloram	:	4-Amino-3,5,6-trichloro-2-pyridinecarboxylic acid
PVP	:	Polyvinylpyrrolidone
sp.	:	Species

spp.	:	Species
Suc.	:	Sucrose
UAE	:	United Arab Emirates
UN	:	United Nations
USA	:	United States of America
Vit.	:	Vitamin
2,4-D	:	2,4-Dichlorophenoxyacetic acid
2iP	:	6-(γ, γ-Dimethylallylamino) purine (2-isopentenyladenine)

1. INTRODUCTION

Date palms

Date palm (*Phoenix dactylifera* L.) of the family Arecaceae is a key plantation crop of many countries of arid regions of West Asia and North Africa (Al-Khalifah *et al.*, 2012). Distributed throughout the Middle East, North Africa and South Sahel areas of East and South Africa, South West USA, Central and South America and even in Southern Europe, this species produces 7.4 million tons of dates worth US dollar 3.6 billion per annum (FAOSTAT, 2011). About 70% of the total production of dates is from Arab world. Worldwide about 3000 named date palm cultivars exist, though some names are probably synonyms, the result of a local or national name given to one cultivar which also exists in another location under another name (Johnson, 2011). This may be reflection of the vast genetic diversity in the plant having arisen due to its highly out-breeding, dioecious nature.

About 15 wild species of *Phoenix* are native to tropics and subtropics of the Old World from Canary Islands through Africa and the Near East to Southern Asia to East Indies (Sauer, 1993). Written history of the ancient Near Eastern cities, including Egypt shows dates were an important major crop from about 3000 B.C. *P. dactylifera* is interfertile with its allied species (Muirhead, 1961) and is successfully pollinated with *P. reclinata* Jacq. and *P. atlantica* A. Chev. in Africa. In India and Pakistan it is pollinated with *P. sylvestris* (L.) Roxb. and in Spain with *P. canariensis* Hort. ex Chabaud (Benbadis, 1992).

Dates have been a staple food of the Middle East and North Africa for thousands of years. They are believed to have originated around the Arabian Gulf, and have been cultivated since ancient times from Mesopotamia to prehistoric Egypt, possibly as early as 4000 BC. The date palm is recorded in ancient history extending over an area from the Indus Valley to Mesopotamia, the Nile Valley, Southern Persia, Eastern Mediterranean and Africa. Such a wide distribution implies that *P. dactylifera* either evolved as a plant covering quite extensive geographic, soil and climatic conditions, which is unlikely, or it spread with the help of man after originating in a more limited geographic region. Within the Indus Valley is found a sister species, *P. sylvestris* (Sugar Date Palm, or Toddy Palm) which still occurs in the wild.

Date fruits are of high nutritional value with about 70% sugar in the form of glucose, sucrose or fructose. Dates are also good sources of iron, potassium, calcium,

magnesium, sulphur, copper and phosphorus, along with various vitamins, including thiamine, riboflavin, biotin, folic and ascorbic acid (Table 1). Its additional use as a livestock feed supplement gives the tree much added value. The value-added products produced from fruits are syrups, jams, ice creams, baby foods, alcoholic beverages and soft drinks which are of a great demand in the market.

Almost every part of this tree is used and its food and industrial products play an important role in the rural communities and economies of many developing countries. Dates are marketed worldwide as high value confectionary (Mahmoudi *et al.,* 2008). In addition, valuable by-products such as building material, handicrafts from its leaves and trunk make it an important multiple purpose tree and a significant earner of foreign revenue for both small and large scale farmers. Date palm also makes a significant contribution towards the creation of equable microclimates within oasis ecosystems, thus enabling other agricultural crops to be grown as intercrops in otherwise harsh environment conditions of deserts. Date palm trees are essential integral components of farming systems in dry and semi-arid regions and can be produced equally well in small farm units or as large scale commercial plantation units. The tremendous advantage of the tree is its resilience, its requirement for limited inputs, its long term productivity and its multiple purpose attributes.

Table 1. Components of date fruits

Components	Percentage
Water	22.5-24.5%
Protein	2.3-5.6%
Energy	274 calories
Glucose	44-88%
Fibres	6.5-11.5%
Ash	1.9 gm
Calcium	59 mg
Phosphorus	63 mg
Iron	3 mg
Sodium	1 mg
Potassium	5.90%
Fat	0.20-0.5%
Vitamin A	50 IU
Thiamine	0.09 mg
Riboflavin	0.10 mg
Niacin	2.2 mg

Date palm has an importance in ceremonies of Judaism, Christianity and Islam. Date is also a traditional food for pilgrims to Makka and a major breakfast food during the fasting of Ramadan. Because of its high nutritional value, productivity and long life (about 100 years), the date palm was referred to as 'tree of life' in the Bible (UN, 2003). The Moors introduced the species into Spain in medieval days. The Spanish voyagers spread the dates to America and neighbouring areas. Now, apart from their natural distribution areas they are widely cultivated in many countries including United States of America and Australia.

Date palm is of great socio-economic importance in many countries in Middle East and North Africa where its cultivation generates main income for farmers. Iraq,

Iran, Saudi Arabia, Pakistan and Egypt are leading producers and share 69% of the world date production. Morocco, Tunisia, Algeria, United Arab Emirates, Sudan, USA, Israel and Oman are also some of the leading date producers of the world.

Date palm breeding is hampered by its long generation cycles. It usually takes more than 30 years to complete three backcrosses and to obtain the first offshoots from an inter-varietal cross. To produce sufficient offshoots for testing in the field, large numbers of mother plants are required and if the breeding target is yield or fruit quality even more time will be needed as the plant does not reach full commercial production for 10 years. It is, therefore, not surprising that date palm improvement has been achieved using traditional breeding approaches. However, chance hybrids develop spontaneously in different farms through wind pollination and hybridization. Most of the farmers growing date palms are good observers and if they find any variation in the fruit and vegetative characters of a tree, they preserve it and give a new name to it. It is presumed that much of the present day cultivars of date palms evolved through natural hybridization with several wild species of *Phoenix*. Human selection also helped in maintaining superior cultivars through vegetative propagation. Most of these cultivars are known only to some specific localities and their distribution is confined to these areas. In order to speed up the progress of date palm breeding programmes, particularly in those cases where the plant is being threatened by devastating diseases like the Bayoud in Morocco, Tunisia and North African countries, biotechnology can play a leading role.

Date palm is dioecious having separate male and female plants. It can be easily grown from seed, but only 50% of seedlings will be female and hence bear fruit. In addition, dates from seedling plants are often small and of poor quality. Therefore, the most common method of cultivation is the planting of offshoots. Over the last 20 years there have been further refinements in propagation methods including production of tissue culture date palms.

Tissue culture enables rapid reproduction of selected clones which is far more reliable than reproduction from seeds and much quicker than the sucker method, especially when most date palms produce less than 20 suckers in a lifetime. With the advent of tissue culture techniques in date palms, massive expansion of elite cultivars was made possible and transportation of the planting materials of these cultivars to distant places became easier. In the last decade, several small and large scale growers have adopted this innovative technique of propagation to expand their farming. This eventually helped in spreading the cultivars to faraway places, which were otherwise confined to certain localities only.

2. DIFFERENT PATHWAYS OF REGENERATION

Sexual propagation

Sexual propagation is the most convenient method to propagate date palm. Seeds can be stored for years; they germinate easily and are available in large numbers. Mature seeds can be collected from the fruits of *rutab* (half ripened) or *tamar* (full ripened) stage. Seeds are soaked in cold water for 24 hours, and then washed thoroughly in running water to remove any viscous matter. Washed seeds can be sown in raised sand or loamy soil beds or 30 cm high polythene pots. Seeds have to be placed 3-5 cm deep from the top of the soil and watered daily. The juvenile leafy shoot appears above the soil after one month. Six month old seedlings can be transplanted to polythene pots for the ease of transferring. However, this method cannot be used commercially for propagating the cultivars of interest in a true-to-type manner for several reasons. In a seed born population, half of the progeny is generally male, and at present no methods are available for detecting sex at seedling stage. Furthermore, the highly out-breeding nature of the species results in wide genetic variations in the following generation. Another important drawback of seed propagation is that the growth and maturation of seedlings is extremely slow. A date palm seedling may take 8 to 10 years or more for fruiting. For all these reasons, propagation by seed is practiced only in exceptional cases when supplies of offshoots are unavailable.

Propagation from offshoots

For centuries, propagation by offshoots was the only commercial method of vegetative propagation in date palm. These offshoots are produced from axillary buds developing from the base of the trunk during the juvenile life of the palm. Offshoots develop slowly; their numbers are limited and are produced only within a certain period in a palm's lifespan. The number of transplantable offshoots produced by a tree varies from 10 to 30 depending on the cultivar and the cultivation practices used. No field-based methods are as yet available to increase the number of offshoots. Offshoots have to be large enough (i.e. 10 to 12 kg) to survive when transplanted in the field, a process of regeneration that can take several years.

Date palms produce two types of shoots: lateral and aerial. Both of them originate from the leaf axils but the former arise from the older ones which are close to the soil

level or mostly from below the soil level (Fig. 1). Aerial offshoots are formed from comparatively new leaf axils, either from leaf crown or from main trunk (Fig. 2). Depending on cultivar, soil type and moisture, a palm produces a few to many offshoots. It is advisable to allow 5-6 offshoots to grow per tree and discard others by regular pruning. The lateral offshoots are more prone to root development than the aerial offshoots. A healthy offshoot of 20-25 cm diameter can be detached from the mother plant and nurtured in nursery for at least one year to develop enough rooting. Detachment of offshoots needs special skill and experience. Any damage to the apical meristem of the detached offshoot may lead to death, and serious wounds on the mother plant may attract insects and pests.

Fig. 1. *Lateral offshoots.*

Fig. 2. *Aerial offshoots.*

Micropropagation

Demand for date palms in the world market is mounting up to the tune of 1-2 million plants per year (Jain, 2007), but the small number of offshoots (10-20) that a female date palm produces in its life span (Zaid and deWet, 1999) is a major constraint for propagating desirable cultivars. Also, propagation through off-shoots involves the risk of mortality and transmission of diseases. Micropropagation of date palm has become a preferred alternative over offshoot propagation for large scale production to satisfy the market demand (Saker and Moursi, 1999). To satisfy increasing international demand of high quality propagating material of elite genotypes, it is necessary to develop alternative methods of vegetative propagation.

Plant tissue culture technology provides a means for rapid, large scale multiplication of plants and has been exploited for the propagation of crop and ornamental plants

(Hussey, 1983). Furthermore, it can also be the basis of agriculture applications which include improvement and pathogen elimination (Short, 1986). The mechanism of vegetative propagation is based on the induction and multiplication of shoot meristems as potential plants. In plant tissue culture this may occur from the existing meristems (apical or axillary) or from the organs suitable for inducing adventitious meristems (shoots or embryos) either directly within the parent tissue or via an intermediate callus stage.

Axillary bud proliferation

The simplest type of *in vitro* plant propagation is the stimulation of axillary bud development. An excised shoot tip cultured on a nutrient medium devoid of any plant growth regulator results in a single seedling-like shoot which exhibits strong apical dominance. When shoots are cultured on a medium containing cytokinins, apical dominance decreases and clusters of axillary shoots develop. Once these axillary shoots have sufficient growth, they can be separated and grown as independent plants. Many ornamental plants and woody plants are propagated commercially by axillary bud proliferation (Chu, 1992). In many instances it may produce tenfold shoot multiplication per monthly culture passage and within a short period, thousands of plants can be produced from a single explant.

Adventitious shoot proliferation

Plant regeneration from cultured tissues can also be achieved by culturing tissue sections lacking a performed meristem (adventitious origin) or from callus and cell cultures (*de novo* origin) (Phillips and Hustenberger, 1995). Adventitious regeneration occurs at unusual sites of cultured tissues such as internodes, leaf blades, and cotyledon or root elongation zone, where meristems normally do not exist. It is believed that all plant cells have ability to express their genetic make-up, but totipotency is confined to certain few cells called meristemoids (Torrey, 1966). These are morphologically competent cells that respond under appropriate nutritional and hormonal balance giving rise to shoots, roots or embryos. Adventitious regeneration occurs through either organogenesis, *i.e.* the development of organs such as roots or shoots or by somatic embryogenesis, *i.e.* the development of bipolar structures with root and shoot meristem similar to zygotic embryos.

Regeneration through direct organogenesis

Formation of organs directly from the cultured explants is referred as direct organogenesis. The process does not involve callus formation. African violet is conventionally propagated from leaf explants. Leaf and stem explants have been used for mass multiplication of *Begonia* (Takayama and Misawa, 1982), *Kalanchoe* (Smith and Nightingale, 1979) and potato (Webb and Henshaw, 1982).

Regeneration through indirect organogenesis

In this case, instead of forming a direct organ from the explant they produce a mass of unorganized cells called callus. On lowering the hormone levels in the culture medium or appropriately adjusting the auxin/cytokinin ratio many calli will differentiate into organs or somatic embryos. Large scale production of callus for the purpose of micropropagation has attracted the interest of commercial producers. But through repeated subcultures many calli exhibit reduction in morphogenetic potential and at least some of them exhibit somaclonal variations.

Somatic embryogenesis

Adventive or asexual embryogenesis is the development of embryos from cells that are not the product of gametic fusion. The initiation and development of embryos from somatic tissues in plant culture, rather than the maturation of excised zygotic embryo was first recognized by Steward *et al.* (1958) and Reinert (1958, 1959) in cultures of *Daucus carota* tissue derived from storage taproot. Tisserat (1979) reported somatic embryogenesis in 32 families, 81 genera and 132 species. Although the list of species for which somatic embryogenesis has been reported is long, the number of clear-cut examples was somewhat small. Somatic embryo should closely resemble their zygotic counterparts with appropriate root, shoot and cotyledonary organs and there should not be any vascular connection with the mother plant and should be able to grow into an independent plant. Somatic embryo production has been reported in many plants including banana (Sidha *et al.*, 2006); sorghum (Girijashankar *et al.*, 2007); grapes (Cutanda *et al.*, 2008) and many other crop plants.

Problems associated with plant tissue culture

Many laboratories refer to tissue culture as "black art", due to the numerous variables that make it difficult to determine solutions when problems arise. A systematic approach that examines the symptoms and meticulously retraces each step in the culture process will help to narrow down a problem to one material or procedure that has created the issue. Contamination of cultures, blackening of tissues and media, vitrification and shoot-tip necrosis are the main problems encountered in tissue culture.

Microbial contamination

Microbial contamination is a constant problem, which often compromises development of all *in vitro* techniques. Tissue culture techniques usually involve growing stock plants in ways that will minimize infection, treating the plant material with disinfecting chemicals to kill superficial microbes and the sterilizing the tools used for dissection, the vessels and media in which cultures are grown (George, 1993). However, contamination has been reported as constant problem which seriously jeopardizes

a tissue culture program (Enjalric *et al.*, 1988). About thirty-one microorganisms from ten different plant cultivars growing in micropropagation have been isolated, identified and characterized, with yeasts, *Corynebacterium* spp. and *Pseudomonas* spp. being predominant (Leggatt *et al.*, 1994). *Bacillus* sp., *Corynebacterium* sp. and an Actinomycete have also been found contaminating the *in vitro* culture of apple rootstocks (Hennerty *et al.*, 1988). Bacteria have been detected in a wide range of plant tissues, and in cultured tissues they may appear frequently or in later stages after a series of subcultures. For effective control of bacteria, surface sterilization is not enough and it is necessary to use antibiotics in the medium. Selection of suitable antibiotic and fixing the required concentration in the medium requires research.

Blackening of the tissues and medium

Mechanical injuries to the tissues stimulate the production of phenolic compounds, *i.e.* when explants are excised from mother plants or during subcultures. Oxidation of phenolic compounds can lead to the formation of phytotoxic compounds and blackening of explants and media. The oxidized products bind to proteins thereby inhibiting enzyme activity and leading to lethal decline of explants (Alderson, 1986). In order to reduce phenolics the excised explants can be stored in darkness for some days to inactivate phenoloxidase activity. Washing of explants for 2-3 hours in running water helps to leach out some phenols from the explants. Frequent subcultures in liquid medium at 2-3 days interval is recommended for the species rich in phenolic compounds. The excreted phenol from the medium can be removed by adding activated charcoal in the medium. Reducing agents such as ascorbates, citrates and glutathionate can also be used to prevent blackening. Adding chelating agents like EDTA and lowering the pH of the medium using ascorbic acid and citric acid may also inhibit the activity of phenol oxidase and thus reduce blackening.

Vitrification

Vitrification, now termed as hyperhydricity, sometimes referred to as water soaking or hyperhydration (Paques and Boxus, 1987) or glass formation (Williams and Leopold, 1989) is a serious problem in plant tissue culture, particularly in micropropagation (Rasco and Patena, 1997). Vitrification is a physiological abnormality of tissue cultured plants. This aberrant growth causes significant losses to the tissue culture industry because of poor survival of plants when they are transferred from *in vitro* to *ex vitro* (Gribble, 1999). Vitrification is associated with abundant water supply available in the medium. Hyperhydric plants exhibit higher water content than normal plants, lower chlorophyll content and defective deposition of epicuticular waxes (Debergh *et al.*, 1992). The severity of vitrification can be reduced through manipulating water content by increasing agar concentration (Casanova *et al.*, 2008) and effective

ventilation (Majada *et al.*, 2000) or by bottom cooling of the container vessels. High levels of ammonium ion in the medium have been attributed to the vitrification in *Salix babylonica* (Daguin and Letouze, 1986). Reducing the ammonium ion content in the medium also helped to resolve vitrification problem.

Shoot-tip necrosis

Apart from vitrification, shoot tip necrosis is a serious problem in the micropropagation of many plants (Abousalim and Mantell, 1994). This physiological disorder is persistent through the *in vitro* seedling stage, multiplication stage, and rooting stage. Many reasons have been attributed to this phenomenon but deficiency of boron (Mason and Guttridge, 1974) and calcium (Sha *et al.*, 1985) have been reported as the most likely causes. Movement of calcium ions in the transpiration stream of cultured plant is restricted by the high humidity in the culture vessels and poor water uptake associated with callus formation and browning of tissues. Increased air exchange in the culture vessels and increased calcium content of the medium can resolve the problem.

The next chapter details the tissue culture based propagation method used in date palm.

3. DATE PALM MICROPROPAGATION

Tissue culture refers to the aseptic growth of cells, tissues or organs in artificial media. Although the culture of plant cells and tissues has long been a tool of the plant physiologists, this technique is now increasingly used as a means of rapid plant propagation (Janick, 1979). *In vitro* culture or micropropagation of plants involves three distinct steps, each of which requires specific set of conditions: (1) establishment of aseptic culture, (2) multiplication of the propagule, and (3) hardening and acclimatization of plantlets. These steps involve the use of different chemicals, management of light, humidity, temperature, etc. Because of inherent limitations associated with conventional date palm propagation using offshoots, tissue culture has become an attractive alternative option for mass propagation of commercial cultivars (Al-Khayri, 2005, 2007).

Since 1970, many workers (Schroeder, 1970; Reuveni and Kipnis, 1974; Reuveni, 1979; Ammar and Benbadis, 1977; Eeuwens, 1976; El-Hannawy and Wally, 1978; Eeuwens and Blake, 1977; Poulain *et al.,* 1979; Reynolds and Murashige, 1979; Tisserat, 1979) attempted micropropagation of date palms using different types of explants. In 1980, Tisserat and DeMason successfully produced date palm plantlets through tissue culture. Later many workers (Sharma *et al.,* 1980; Zaid and Tisserat, 1983, 1984; Zaid, 1987; Al-Ghamdi, 1993; Al-Khalifah, 2000, 2006; Al-Khayri, 2003, 2005; Eshraghi *et al.,* 2005; El-Din *et al.,* 2007; Khierellah and Bader, 2006; Al-Khateeb, 2008; Aslam and Khan, 2009, Kamla Ibrahim *et al.*, 2009; Asemota *et al.*, 2010; Hegazy and Aboshama, 2010; Sani *et al.*, 2010; Chabane *et al.*, 2010) made significant contributions to date palm micropropagation protocol development which resulted in the standardization of techniques widely applied across cultivars. Two such techniques successfully employed in the tissue culture of date palms are somatic embryogenesis, and organogenesis. In 1988, Bhansali *et al.* made mass clones of date palm plantlets through repetitive somatic embryogenesis. This method is still widely used with success. However, it involves the possibility of some abnormal vegetative traits in the desired plants as well as unusual flowering and fruiting habits, which are not apparent until the fruiting stage. On the other hand, direct organogenesis method is expected to reduce the number of steps in culture, shortening the duration of culture.

Somatic embryogenesis

Regeneration of date palm by somatic embryogenesis has been reviewed by Tisserat (1979, 1984), and Branton and Blake (1989). It was demonstrated that the presence of an auxin was critical for embryo initiation, and lowering of the concentration of

auxin or its complete absence fostered maturation (Steward *et al.*, 1967). Reduced nitrogen was also important for initiation and maturation (Ammirato and Steward, 1971). Thus a primary medium with an auxin and a secondary medium without plant growth regulators, both containing a substantial supply of reduced nitrogen are required for successful somatic embryogenesis. Halperin and Jensen (1967) suggested that embryo initiation probably occurred during primary culture and the presence of auxin in the medium prevented their maturation. Embryos mature when the auxin is removed from the subsequent culture media. Sharp *et al.* (1980) described two routes to somatic embryogenesis. The first is direct embryogenesis where embryos initiate directly from tissue in the absence of callus proliferation, and second is indirect embryogenesis where some calli proliferation is required.

Evans *et al.* (1981) noted that about 70% of explants are cultured on MS or modified MS medium. A key element of the MS medium is the presence of high levels of nitrogen in the form of ammonium nitrate. The source of reduced nitrogen can be in the form of complex addenda such as casein hydrolysate (Ammirato and Steward, 1971), a mixture of amino acids (Kato and Takeuchi, 1963), single amino acid such as glutamine (Wetherell and Dougall, 1976) or the presence of ammonium ion (Halperine, 1966; Ammirato and Steward, 1971). In all these cases there is also nitrate in the medium. Another important requirement in the media for somatic embryogenesis is the presence of chelated iron, often in the form of iron EDTA. In *Atropa belladonna*, Heberle-Bors (1980) observed that in the absence of iron EDTA embryo development fails to pass through to the globular to heart-shaped stage.

Of all the auxins or auxin-like plant growth regulators, 2,4-D has proven extremely useful, being used in 57.1% of successful embryogenic cultures (Evans *et al.*, 1981). Effective concentration ranges are 0.5-27.6 μM for 2,4-D and 0.5-10.7 μM for NAA. Unusual high levels have been successful in certain species like *Phoenix dactylifera*, e.g. 452 μM (Reynolds and Murashige, 1979). Evans *et al.* (1981) also reported that cytokinins were used in the primary medium for 65.4% of the crop species and 21.4% of the non crop species. The effective concentration range for kinetin is 0.5-5 μM. Cytokines are important in fostering embryo maturation (Fujimura and Komamine, 1975) and especially cotyledon development (Ammirato and Steward, 1971).

In addition to the varied requirements of growth regulators, different genotypes of date palm demand different basal media for better callusing, embryogenesis and plantlet formation (Al-Khayri, 2011). The addition of activated charcoal to the medium has proven useful for somatic embryo development in many cultures including date palm (Tisserat and DeMason, 1980). Analysis of its effect shows that media with activated charcoal has substantially low level of phenolic compound when added to the cultures (Fridborg *et al.*, 1978). Sucrose appears to be the most effective reduced

carbon source for somatic embryogenesis, although many other mono and disaccharides can be successfully employed (Verma and Dougall, 1977). Environmental conditions like light, temperature, type of culture vessels and density of the medium also have significant influence on somatic embryogenesis.

Explant selection and isolation

The apical meristematic tissues extracted from the axillary and lateral offshoots are the most widely used explants for date palm tissue culture. Two to three year old offshoots can be used as explant for culture initiation, very young or over-matured offshoots are not recommended. Healthy offshoots (Fig. 3) are separated from field-grown trees using sharp tools. Older leaves are trimmed off and the remaining ones tied in one bundle. Offshoots are now transferred to the laboratory for dissection. Leaf fibers are cleaned from the base and the leaf-sheaths are removed one by one from the outer ring towards the center. Care must be taken to avoid any kind of shock or damage to the internal soft tissues. The final size of the offshoot heart is about 2 to 3 cm in width and 4 to 6 cm in length. At this stage the explant is transferred to a sterilized bottle containing chilled aqueous solution of citric acid and ascorbic acid (150 mgl^{-1} each) used as antioxidant (Fig. 4). Sterilization and final dissection are carried out under a sterile laminar air flow hood.

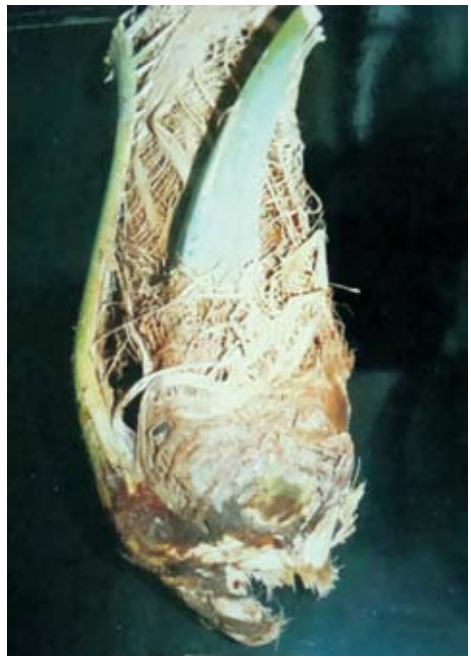

Fig. 3. *An excised offshoot ready for dissection.*

Fig. 4. *Excised offshoot-heart in antioxidant solution.*

Sterilization

Efficient sterilization is essential to eliminate contamination and maximize success of explants regeneration. The explant is kept in one percent sodium hypochlorite solution

mixed with one drop/100 ml of Tween-20 for 20 minutes followed by 4-5 rinses in sterilized water. It is recommended to give sterilization for 10 minutes with fresh sodium hypochlorite solution. Then the explant is immersed in 0.1% mercuric chloride solution for 5 minutes, followed by 4-5 washes in sterilized distilled water. Sterilized explants are kept in a cold sterilized solution of ascorbic and citric acid (150 mgl^{-1}) to avoid browning.

Media and culture conditions

Modified Murashige and Skoog (MS) medium (Table 2) with 3% sucrose supplemented with 2,4-D (100 mgl^{-1}), NAA (3 mgl^{-1}), 2iP (3 mgl^{-1}) and kinetin (3 mgl^{-1}) is being successfully used for date palm tissue culture. pH of the medium should be adjusted to 5.6 and solidified with 8.0 gl^{-1} of agar. Twenty five ml of medium is dispensed into 40 mm culture tubes and sterilized for 15 minutes at 121° C. Activated charcoal (1.5 gl^{-1}) is added to the medium to avoid browning.

Most of the date palm cultivars require specific protocols for large scale micropropagation. For example, some cultivars require more sugar in the medium while others need more vitamins, nitrogen, calcium and different growth regulators. Carbohydrate source, total nitrogen, ammonium to nitrate ratio, and auxin type and level have been identified as major factors affecting proliferation of embryogenic cultures (Merkle *et al.*, 1995). Table 3 shows the specific media requirements of some date palm cultivars as reported by earlier workers. Depending on the cultivar and culture type, conditions and growth regulator requirements also vary.

Culture initiation

The term somatic embryogenesis refers to the development of a complete embryo from vegetative cells. In the case of date palm, somatic embryos are produced from *in vitro* produced friable callus cells. The selection of the explant source material is the most critical decision and may require a systematic analysis of embryogenic potential of different explant sources within the plant. The first step involved is the culture of the selected explant in a suitable callus inducing medium. Induction of embryogenic potential in the callus is achieved through the transfer of cells to a basal medium (Modified MS, Table 2) with high concentration of auxin. The most effective auxin used in date palm is 2,4-dichlorophenoxyacetic acid (2,4-D).

Apical meristems (shoot tips), leafy buds and leaf primordial bases, the most are the most suitable explants to be dissected from the excised offshoot-heart for callusing and somatic embryogenesis. Modified MS medium supplemented with 100 mg^{-l} 2,4-D and 3 mg^{-l} 2iP and 1.5 g^{-l} activated charcoal is widely used for initiating callus in many cultivars of date palms. Addition of an equal proportion of kinetin (3 mg^{-l}) in the medium promotes speedy differentiation of callus in some

Table 2. Modified MS medium for date palm tissue culture

Macronutrients (Stock solution 10x)		**gl^{-1}**	**Working solution (1x)**
Potassium nitrate	KNO_3	19.0	
Ammonium nitrate	NH_4NO_3	16.5	100 ml
Calcium chloride	$CaCl_2.2H_2O$	4.4	
Magnesium sulfate	$MgSO_4.7H_2O$	3.7	
Sodium monophosphate	NaH_2PO_4	1.7	
Potassium phosphate	KH_2PO_4	1.7	
Micronutrients		mgl^{-1}	
Boric acid	H_3BO_3	620	
Manganese sulphate	$MnSO_4 H_2O$	2230	
Zinc sulphate	$ZnSO_4.7H_2O$	860	
Sodium molybdate	$Na_2MoO_4. 2H_2O$	25	
Copper sulphate	$CuSO_4.5H_2O$	2.5	
Cobalt chloride	$CoCl_2.7H_2O$	2.5	10 ml
Potassium iodide	KI	83	10 ml
Sodium EDTA	Na_2EDTA	3730	10 ml
Ferrous sulphate	$Fe SO_4.7H_2O$	2780	
Vitamins		mg/100ml	
Nicotinic acid		25	
Pyridoxine-HCl		25	10 ml
Thiamine-HCl		50	
Pantothenic acid		25	
Myo-inositol		$6.25gl^{-1}$	20ml
Amino acids			
Glutamine		$10gl^{-1}$	20 ml
Glycine		$1mgml^{-1}$	3ml
Sucrose		$30gl^{-1}$	
Agar		$8gl^{-1}$	
Activated charcoal		$1.5gl^{-1}$	

Table 3. Media requirements for micropropagation of different date palms cultivars

Sl. No.	Media (mgl^{-1})	Growth regulators	Purpose	Cultivar	Reference
1.	MS + glutamine 200 + biotin 0.5 + adenine sulphate 20 + thiamine 10 + Ca-pantothanate 10 +40 g sugar (pH 5.6)	BNOA 0.5 + BA 1 + K 1 + 2iP 1	Axillary bud proliferation from shoot tip	Medjhool	Hegazy and Aboshama, 2010
2.	MS + glutamine 200 + biotin 0.5 + adenine sulphate 20 + thiamine 10 + Ca-pantothanate 10 + 40 g sugar (pH 5.6)	Putrescine 150	Bud formation	Medjhool	
		Putrescine 100	Embryo multiplication		
		IBA 1	Rooting		
3.	Modified MS + KH_2SO_4 170	2,4-D 200uM	Callusing	5 Nigerian vars.	Sani *et al.,* 2010
4.	MS + Morel Vitamins	2,4-D 2+IBA 3	Callusing	D. Noor	Chabane *et al.,* 2010
5.	MS + vitamins	2iP 2 + BA 1 + NAA 1 + BNOA 1	Bud formation	Maktoum	Khierella and Bader, 2007
	Liquid MS	2ip 4 + BA 2 + NAA 1 + BNOA 1	Bud multiplication		
	MS	GAA 0.5 + NAA 0.1	Elongation		
	MS	NAA 1	Rooting		
6.	MS + $Na_2PO_4.2H_2O$ 170 + inositol 100 + sugar 30g + charcoal 3g	NAA 1 + 2iP 6	Callus multiplication	Sefri	Al-Ghamdi, 1993
7.	MS + $Na_2PO_4.2H_2O$ 170 + inositol 100 + adenine sulphate 0.04 + thiamine HCl 0.4+ glutamine 100+sugar 30 g + charcoal 3 g + agar 6g	2,4-D 100 + 2ip 3	Callus formation	Bartamuda Gondila Shamia	El-Din *et al.,* 2007
		2,4-D 10 + 2ip 3	Friable callus		
		NAA 0.1 + BA 0.05	Embryogenic callus		
		NAA 0.1 + BA 0.05	Germination of embryo		
	¼ strength MS	NAA 2	Rooting		
8.	MS + meso- inositol 100+thiamine-HCl 4+ agar 8g+ charcoal 3g	2,4-D 100 + 2ip 3	Callus production	Excised zygotic embryos from different cultivars	Tisserat, 1983
	Modified MS	No PGR	Embyogenesis		
	Modified MS	NAA 0.1	Rooting		

Contd...

Sl. No.	Media (mgl^{-1})	Growth regulators	Purpose	Cultivar	Reference
9.	MS + sugar 30g + agar 8.5g + inositol 100 + aneuric hydrochloride 0.002% + charcoal 3g	NAA 100 or 2,4-D 100 + 2iP 3mg	Callus induction	Different cultivars	Asemota *et al.,* 2010
		NAA 0.05 + 2iP 1	Embryo induction		
		GA3 2	Shoot elongation		
		NAA 0.05-0.1	Rooting		
10.	MS + $Na_2PO_4.2H_2O$ 170 + inositol 125 + glutamine 200 + nicotinic acid 1 + pyridoxine HCl 1 + thiamine 5 + sugar 30g + charcoal 1.5g + agar 7g	2,4-D 100 + 2iP 3	Callus induction	Khanizi Mordarsing	Eshraghi *et al.,* 2005
		NAA 10 + 2iP 30	Regeneration		
11.	MS + $Na_2PO_4.2H_2O$ 170 + inositol 125 + glutamine 200 + ascorbic acid 100 + citric acid 100 + nicotinic acid 1 + pyridoxine HCl 1+thiamine 1+ ca-pantothenate 1 + biotin 1 + sugar 30g + charcoal 1.5g + agar 7g	NAA 10 + 2iP 1.5	Callus multiplication	Not mentioned	Al-Khayri, 2005
		–	Somatic embryogenesis		
	½ strength MS	IBA 0.2-0.4	Regeneration		
12.	MS	2,4-D 100 + 2iP 3	Culture initiation	Sukary	Al-Khateeb, 2008
		NAA 10 + 2iP 30	Culture swelling		
		NAA 10 + 2iP 6	Embryogenic callus		
13.	MS	2,4-D 100	Callus induction	Khalas	Aslam & Khan, 2009
		BAP 2 or K 2	Regeneration		
14.	MS	NAA 0.1	Rooting	Kapkap Tharlaj	Kamla Ibrahim *et al.,* 2009

cases. Cultures have to be incubated under darkness at 25±2°C. Subculturing is done at 6 weeks interval for a period of 6 months. Within this period most of the cultures develop callus either from the base of the explants or from the apical portions (Fig. 5a and b).

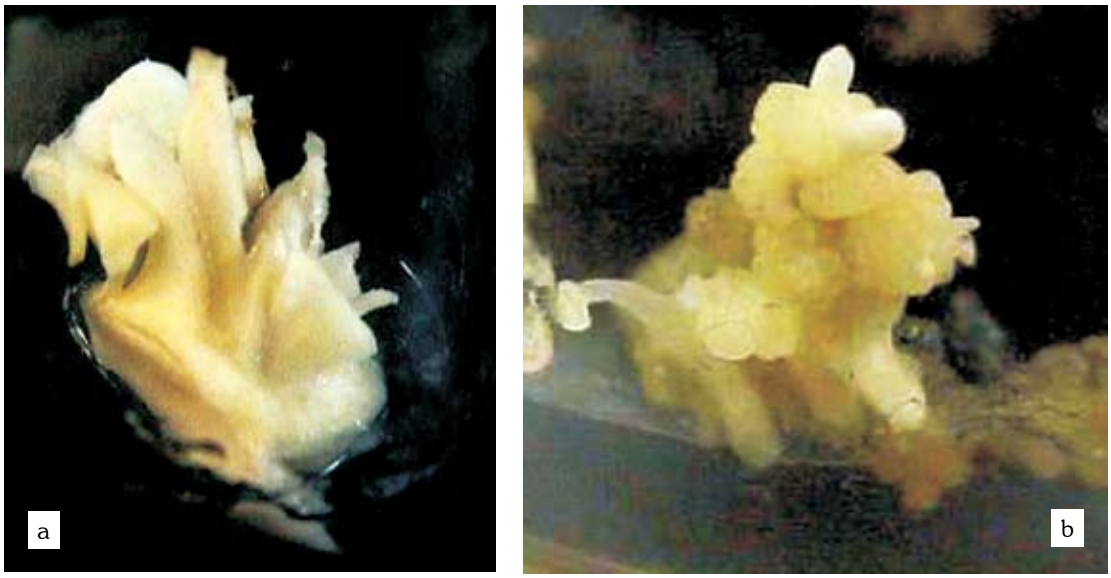

Fig. 5. *Callus initiation from explants. a. stage 1; b. stage 2.*

Proliferation of callus

Three to four weeks after callus induction, primary callus is transferred to another medium for development of embryogenic callus. The medium is supplemented with hormones comprising 3 mgl^{-1} α-naphthalene acetic acid (NAA), 3.0 mgl^{-1} 2-iP. Requirement of kinetin (3.0 mgl^{-1}) and the concentration of NAA vary according to the genotype. Cultures are incubated under darkness at 25±2°C and subcullturing is done every 6-7 weeks for further production of embryogenic calli. White, shiny globular structures (Fig. 6) formed from the calli are harvested and cultured on another medium meant for embryogenesis.

Fig. 6. *Embryogenic callus.*

Embryogenesis and germination

Differentiated proembryo masses are cultured in an auxin-free basal medium. Cultures are incubated in growth rooms at 26±1°C and light intensity of 2500 lux (lx) with a photoperiod of 16 hours. Somatic embryos develop from single cells

Fig. 7. *Somatic embryos developing from the embryogenic callus.*

in clumps or small masses, develop polarity and grow like zygotic embryos (Fig. 7). Regeneration of the somatic embryo is the next step, for which an agar medium devoid of auxin but containing a low level of cytokinin is required. This technique has been employed for mass production of many date palm cultivars. The embryos measuring 5-10 mm are separated from the embryogenic callus and cultured in fresh media for multiplication and germination. This stage is the most rapid phase in date palm culture where very active growth and multiplication of embryo takes place. Very frequent subculturing, division of the clumps and separation of embryos are often required in this stage. Subculturing is done in the same media supplemented with a little cytokinin. When the germination of embryos begins, light intensity must be increased to 3000 lx.

Embryo multiplication and shoot elongation

Rate of multiplication and germination of somatic embryos are important aspects of micropropagation for a better and balanced output at commercial level. Fully developed embryos (Fig. 8) are shifted to a hormone-free medium for multiplication and shoot formation. The embryos measuring 5 to 10 mm are harvested and cultured on the same medium without ascorbic acid and citric acid for multiplication and germination (Fig. 9). The young shoots measuring 2 cm or above in length are cultured on hormone-free medium for elongation (Fig. 10). One or more subculturings are required in hormone-free medium for the healthy growth of shoots and sufficient elongation before transfer to a rooting medium.

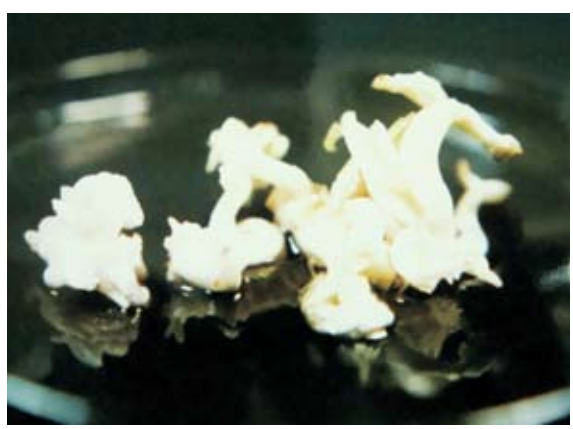

Fig. 8. *Fully developed embryos.*

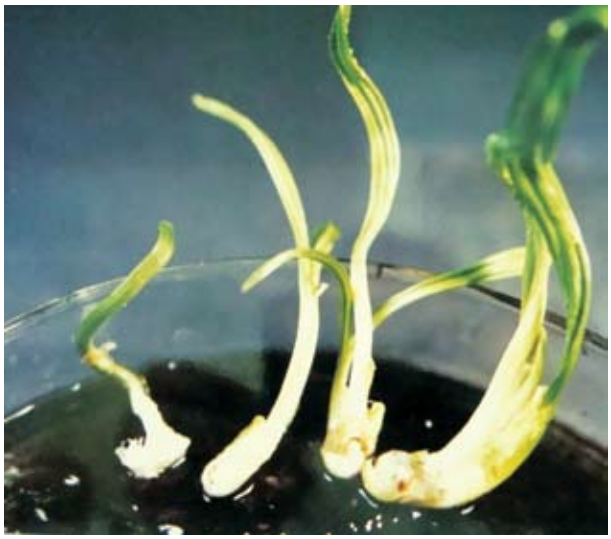

Fig. 9. *Germinated embryos in the multiplication medium.*

Fig. 10. *Plantlets in the elongation medium.*

Root development

For root induction, shoots measuring 5 cm or above in length are cultured on half strength of MS salts, full strength of vitamins and organics, 30-50 gl^{-1} sucrose supplemented with 0.1- 0.2 mgl^{-1} NAA (Fig. 11). In some cases direct rooting from the germinated embryos also occurs, which may be due to residual auxin carried over to the germinating embryos from the embryogenic callus. Usually, rooting starts after two weeks in the rooting medium. Nearly 100% rooting has been recorded by many workers in different genotypes.

Fig. 11. *Plantlet in the rooting medium.*

Organogenesis

This term refers to the development of adventitious shoots from the undifferentiated callus masses or directly from the explants (Fig. 12). The process usually occurs after an intervening period of callus growth. Organogenesis can be induced by transferring callus or explants to

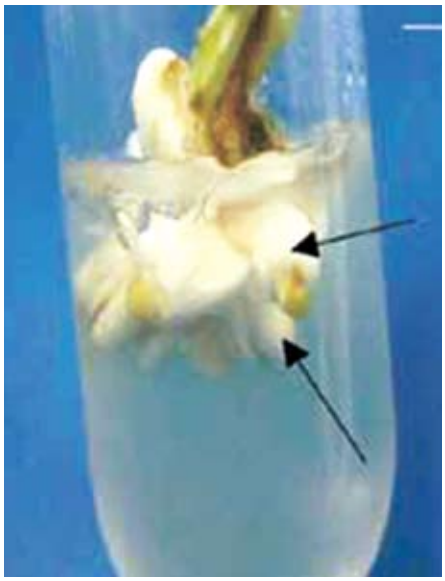

Fig. 12. *Direct shoot formation from leaf explant.*

a suitable medium or sequence of media that promote proliferation of shoot, root or both. Meristemoids are differentiated in the callus and are later transformed into cyclic nodules from which shoots or roots develop. Shoot formation followed by rooting is the general characteristic of organogenesis. Generally high concentration of cytokinin favours shoot-bud formation whereas high level of auxin promotes rooting.

Beauchesne and Rhiss (1979) established organogenesis as an alternative to somatic embryogenesis for date palm tissue culture. Many other explants like leaves (Zaid, 1981; Sharma *et al.,* 1980), inflorescences (Tisserat and De Mason, 1980; Drira, 1981), roots (Smith, 1975; Eeuwens, 1976) and zygotic embryos (Reuveni, 1979; Zaid and Tisserat, 1984) have been experimented for these purpose. Important successes of direct organogenesis were reported by some researchers in axillary branching of shoot tips (Tisserat, 1984). Al-Maari and Al-Ghamid (1997) and Al-Khateeb *et al.* (2002), were successful in enhancing adventitious bud formation on shoot tips. Direct organogenesis has also been successfully established from the shoot tip explants of date palm cultivars (Khierellah and Bader, 2007; Hegazy and Aboshama, 2010). Hence, direct organogenesis presents an advantage of use of low concentrations of plant growth regulators and consequently callus-phase is avoided. Direct regeneration of vegetative buds minimizes the risk of somaclonal variation among regenerants. Moreover, duration of culture period is limited by frequent renewal of the plant material.

Sterilized explants (3 cm long apical meristem with soft inner leaves) are cultured on MS medium supplemented with thiamine 1 mgl^{-1}, pyridoxine 1 mgl^{-1}, adenine sulphate 50 mgl^{-1} , myo-inositol 100 mgl^{-1}, sodium dihydrogen orthophosphate 170 mgl^{-1}, glutamine 200 mgl^{-1}, sucrose 3gl^{-1}, activated charcoal 2gl^{-1} and 7gl^{-1} agar. The pH of the medium is adjusted to 5.7 with 0.1 N NaOH or HCl, before the addition of agar. After one month long incubation under darkness in the above medium, the apical meristem is vertically cut into 4 pieces and cultured in the same medium supplemented with 2 mgl^{-1} 2iP and 1 mgl^{-1} BAP and 2 gl^{-1} of polyvinylpyrrolidone (PVP) substituting charcoal. Cultures are incubated in a culture room under low light intensity of 1000 lx for 16 hours daily at 27±1°C for four weeks. After 3-4 subcultures at 4 weeks intervals, axillary bud initiation can be seen in explants. Small clumps of explants having 3-4 initial buds are subcultured in the same medium supplemented with

4 mgl^{-1} 2iP, 2 mgl^{-1} BAP and 1 mgl^{-1} NAA. On subsequent subculturing in elongation media and rooting media, healthy plantlets are obtained.

Micropropagation from flower explants

Use of offshoots as explants for plant regeneration in date palm involves sacrificing one complete offshoot with no certainty of the desired results. Additionally, offshoots are expensive and involve substantial manual work to excise the actual explant - the apical dome. Contamination, browning of explant and delayed differentiation of embryonic callus are major limitations in the micropropagation of date palm using offshoot explants (Sharma *et al.*, 1980; Bhaskaran and Smith, 1982; Zaid, 1984). Inflorescences have been used as an alternative source of explants in date palms by many researchers (El-Kosary *et al.*, 2007; Masmoudi *et al.*, 2007; Feki and Drira, 2007; Abul-Soad, 2007; Tisserat, 1979; De Mason, 1980; Bhaskaran and Smith, 1982; Zaid *et al.*, 2007, Al-Khairy *et al.*, 2007; Kriaa *et al.*, 2007; Abahmane, 2007; Abul-Soad and Mahdi, 2010). However, only female inflorescences respond well to culturing since the use of male flower as explants either fails to induce embryogenesis (Al-Khairy, 2007) or yields less than 5% success (Zaid *et al.*, 2007).

Explant preparation and sterilization

Unopened female inflorescences are collected from mother plants, disinfected with 70% alcohol and sealed in fresh polythene bags to avoid moisture loss. The spathe is opened in the laboratory and rachillae bearing 5-6 flowers are separated and washed thoroughly in running tap water for 15 minutes followed by washing with sterilized distilled water and kept in sterilized solution of ascorbic acid and citric acid (150 mgl^{-1} each) to prevent browning.

Sterilization of explants is carried under laminar air flow. Explants are treated with 1% solution of sodium hypochlorite containing 1-2 drops of Tween-20 per 100 ml for 10 minutes. The explants are then treated with 0.1% solution of mercuric chloride for 5 minutes and are finally washed 5 times with sterilized distilled water. These explants are kept in sterilized solution of ascorbic acid and citric acid (150 mgl^{-1} each) to prevent browning.

Nutrient media

The basal medium consists of MS (Murashige and Skoog, 1962) salts and vitamins supplemented with 170 mgl^{-1} sodium monophosphate, 200 mgl^{-1} glutamine, 125 mgl^{-1} inositol, 3.0 mgl^{-1} glycine, 1.0 mgl^{-1} calcium pantothenate and 30 gl^{-1} sucrose. Plant growth regulators are added according to the stage of cultures. The medium is brought to pH of 5.8 before autoclaving and solidified with 8 gl^{-1} agar. Activated charcoal (1.5 gl^{-1}), ascorbic acid and citric acid (75 mgl^{-1} each) are used

in the medium for explant culture, development of callus and embryogenic callus to avoid browning. For the development of callus, the medium is supplemented with 2,4-D (100 mgl^{-1}), 2iP (3 mgl^{-1}). The medium is supplemented with 2iP (3 mgl^{-1}) and NAA (3.0 mgl^{-1}) for the development of embryogenic callus. For embryogenesis and embryo multiplication the same medium is used without any growth regulators. Shoot elongation is achieved on modified MS medium devoid of growth regulators.

Abul-Soad and Mahdi (2010) proposed a detailed protocol, starting from excising the immature inflorescence from the axils of the leaves and their culture in suitable medium. After sterilization, small pieces of spikelets are cultured in starting medium and subsequently transferred to maturation, differentiation, and proliferation and rooting media. The media proposed by these authors are given in Table 4.

Culture process

Flowers are removed carefully from the axis using a sterilized blade and are cultured immediately. The cut-part of the flower is placed in the medium to facilitate the nutrient uptake. Cultures are incubated in darkness at 26 ± 1°C from initial

Table 4. Nutrient media composition for inflorescence protocol and its sequences. (Abul-Soad and Mahdi, 2010, with consent).

Sl. No.	Medium	Composition (mg l^{-1})			
		Salts	Additives	Auxins	Cytokinins
1.	Starting	Macronutrients of B5 + micro nutrients of MS	30000 Sucrose + 2200 agar + 1400 Gel + vit. of MS + 170 KH_2PO_4 + 100 glutamine + 40 Ad.	0.1 2.4-D + 0.1 IAA + 5.0 NAA -	
2.	Maturation	Macro nutrients of B5 + Micronutrients of MS	30000 Sucrose + 2200 agar + 1400 Gel + vit. of MS + 170 KH_2PO_4+ 100 glutamine + 40 Ad. + 1500.0 AC	5.0 2,4-D	1.0 2iP
3.	Differentiation	MS	30000 Sucrose + 2200 agar + 1400 Gel + vit of MS	+ 0.1 NAA	0.1 Kinetin
4.	Proliferation	MS	30000 Sucrose+ 2200 agar + 1400 Gel + vit. of MS	0.1 NAA	0.05 BA
5.	Rooting	¾ MS	50000 Sucrose + 2200 agar + 1400 Gel + 0.1Ca-panthothenate +vit. of MS + with and without 3000.0 AC	0.1 NAA	

Suc - sucrose; Ad - adenine sulphate; AC - activated charcoal; vit - vitamins; Gel - gelrite

explants stage to the development of callus and embryogenic calli. First subculture of the explants for callus development is made at the end of two weeks using healthy and growing cultures and discarding the contaminated ones. Further subcultures are made at intervals of four weeks in the same medium till the appearance of soft callus. During the subculture, orientation of the explants with respect to the medium is kept same to avoid browning and mortality of the flowers. The calli developing from the flower-explants are cultured on modified MS medium supplemented with 3 mgl^{-1} α-naphthalene acetic acid (NAA) and 3 mgl^{-1} 2-iP for the differentiation of embryogenic calli. The embryogenic calli are transferred to another modified MS medium having same supplements except the growth regulators for embryogenesis. After 6 weeks, embryos measuring 5 to 10 mm are harvested and cultured on the same medium except ascorbic and citric acid for multiplication and germination. The young shoots measuring 2 cm or above in length are cultured on hormone free medium for elongation. For root induction, shoots measuring 5 cm or above in length are cultured on half strength of MS salt, full strength of vitamins and organics, 30 gl^{-1} sucrose supplemented with 0.2 mgl^{-1} NAA.

For embryogenesis, embryo-multiplication, embryo-germination, shoot elongation and root development the cultures are incubated under 16-hour photoperiod (3000 lx) at 25±1°C.

Development of callus, embryogenic callus and embryogenesis

The success in development of calli from explants depends on their meristematic potentiality and their orientation in the successive subculture cycles. In the offshoot, the potentiality of the tissue developing into callus is centralized at the shoot tip and adjoining leaf primordia and young leaves. On the other hand in flower culture, each young female flower behaves as a unit of meristematic tissue and produces profuse callus.

The first subculture made after two weeks of initial culture helps to discard the contaminated ones and retain only the apparently healthy cultures. After four weeks of culture, carpels become apparent in the female flower explants (Fig. 13A). At the end of ten weeks, the carpels enlarge considerably and tend to rupture (Fig.13B). After the third subculture, i.e. after 12 weeks, the enlarged carpels get ruptured by the growing shiny callus from the internal meristematic tissues (Fig. 13C). The frequency of callus development in the cultured female flower is significantly higher (65.5%) as compared to that of the offshoot source (32.5%). Granular and creamy embryogenic callus is differentiated at the end of 20 weeks in the callus cultures from the flower source (Fig. 13D) and 32 weeks in the callus cultures from the offshoot source. The frequency of embryogenic callus differentiation from the flower source is significantly higher (71.4%) than that from offshoots (42.6%). A significant superiority of the flower

explants over the offshoots is also recorded in terms of percentage of embryogenic callus cultures producing embryos (Fig. 13E and F). In all other developing stages (Fig. 13 G-I) plantlets produced from flower explants perform equal to or better than the plants from offshoot explants (Fig. 14).

Although many workers have reported success in producing plantlets from flower explants, commercially viable protocol is still awaited.

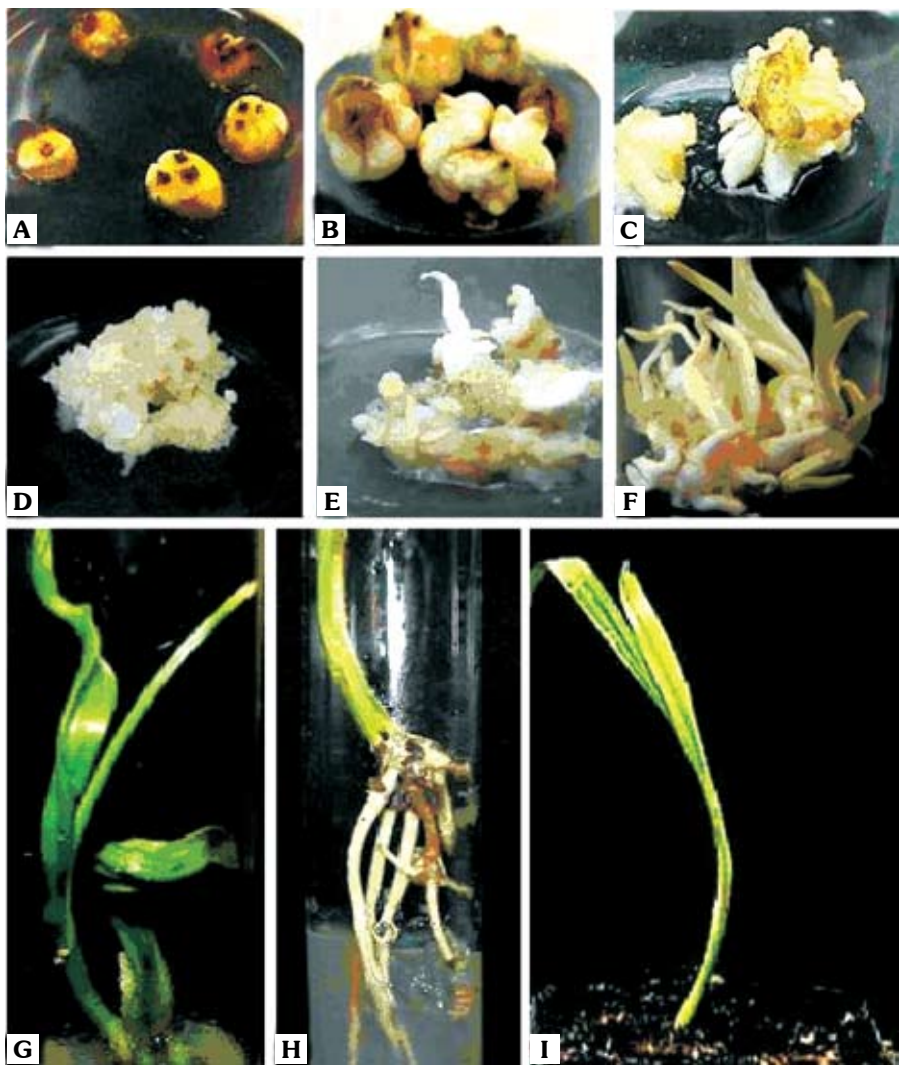

Fig. 13. *A-I. In vitro stages of development of date palm from female flower explants. A & B. Flowers at different developmental stages; C. Callus development; D. Embryogenic callus and embryogenesis; E. Embryo multiplication; F. Germination; G. Shoot elongation; H. Rooting; I. Potted tissue culture plant.*

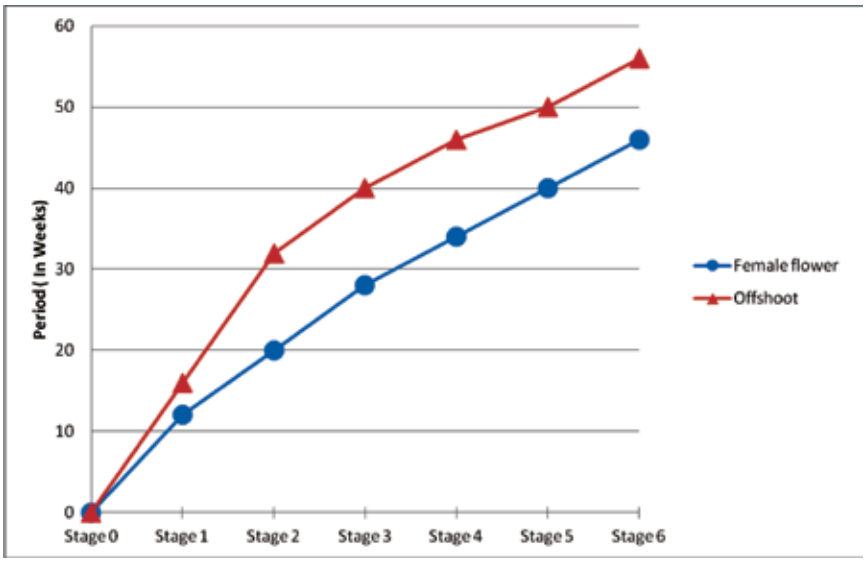

Fig. 14. *In vitro growth response of two explant sources (female flower and offshoot) of date palm cv. Barhy. Stage 0 explant stage; Stage 1 callus; Stage 2 embryogenic callus; Stage 3 embryogenesis; Stage 4 embryo multiplication and germination; Stage 5 shoot elongation; Stage 6 root development.*

Acclimatization

Healthy plantlets of 10-15 cm length, with shoot base of 15 mm or more diameter and adequate rooting should be transferred to pots. Planting operation should be completed as quickly as possible after washing off all traces of culture media and disinfecting with broad spectrum fungicides (1 gl^{-1} metalaxil or any other fungicide solution 5 minutes before planting). A sterilized medium comprising peat moss, vermiculite and coarse sand in the ratio 1:1:1 is ideal for soil transfer. It is recommended to irrigate immediately with 50% Hoagland solution or 10% MS solution to avoid dehydration of plants. Containers and potting media must be adequately cleaned and sterilized. Moisture content of the medium must be regulated to minimum, and relative humidity of the growth tub must be maintained high.

After progressive reduction of humidity to ambient levels, plants are exposed to the greenhouse conditions for further hardening. Later the plants are transferred to bigger pots before transferring to nursery. Successful acclimatization (about 90% survival) has been reported for the tissue culture derived date palm cv. Barhy (Smith and Aynsley, 1995).

Micropropagation and acclimatization of date palm are lengthy processes, which require several subculture steps and careful attention. Otherwise, casualty rate will be very high. Fifty to ninety percent loss has been reported in date palm culture during acclimatization.

Planting and rearing

Date palm has adapted to areas with long, dry summers and mild winters. It thrives well in different types of soils but the most preferred is sandy-alluvial-clayey soil. Date palm requires surplus good quality irrigation for luxuriant growth and quality production. Some varieties can survive with water having 22,000 ppm of total dissolved salts. However their fruit productivity would be very low. Water with salinity level of 3,000 ppm is the preferred upper limit for good productivity. An adult date palm requires an average of 70 liters of water in every 10 days in winter and an equal amount is required every two days in summer (Sanderson, 2001). Adequate source of water and irrigation facilities must be ensured before any planting operation. A thorough plowing of the field helps to eliminate big stones and weeds from the planting area. Spacing between the trees and rows depends upon the cultivar to be planted. Most of the date palm cultivars have a leaf-length of 3.0-4.5 meters. As a general thumb rule, double the leaf length can be fixed as distance between two trees. It is advisable to provide a little more space after two rows so as to facilitate the easy accessibility of vehicles and other equipment to the trees.

For transplanting tissue culture-derived plants it is advisable to have a pit of 75 × 75 × 75 cm size. The pit should be kept open to sunlight and aeration for a period of one week and then filled with organic manure, which should be covered by 10 cm thick layer of sand before placing the plant. After planting, daily watering is required for a period of 40-45 days and later twice or thrice per week. It is advisable to keep the basin clean from weeds to avoid insects, pests and rodents. As the tree grows, increase the diameter of the bund and quantity of organic manures. A mature tree requires an average of 25 kg of organic manures with 100 g nitrogen, 100 g potassium and 75 g phosphorus. Date palms begin their productivity between 5 and 8 years, however tissue culture-derived plants start yielding at the age of 4 years.

Axillary shoot formation from tissue culture-derived plants

A majority of the tissue culture-derived palms start to produce axillary shoots during the second year, whereas a few produce them in the third year of planting (Sudhersan and Abo El-Nil, 2004). Axillary shoots are of two types: normal and hapaxanthic (HAS). All the hapaxanthic shoots produce terminal inflorescence and die afterwards. Normal axillary shoots develop into offshoots and the number increases during the third year of growth. HAS are common in seedling and micropropagated palms while rare in the palms propagated via suckers. It is recommended to remove the axillary shoots developing on micropropagated date palms in order to enhance the growth of mother trees.

Media supplements and their effects on date palm tissue culture

Sucrose

Plant cell, tissue or organ culture normally requires a carbohydrate supply in order to satisfy energy demands. It is well known that in plant tissue culture a continuous supply of carbohydrates is essential, because the photosynthetic activity of *in vitro* plant tissues is reduced due to low light intensity, high relative humidity and limited gas exchange (Kozai, 1991). In tissue culture, carbohydrates act as osmotic agents to support growth of tissues and are generally regarded as a carbon source needed to sustain growth in culture medium. Varying the carbohydrate supply in the culture medium has a marked effect on the proliferation of cell suspension culture in date palm (Zouine and Hadrami, 2004).

Among the different carbohydrates, sucrose is most frequently used in tissue culture (Iraqui and Tremblay, 2001). Sucrose is essential for embryo development and its concentration has strong influence on embryogenesis. Three percent sucrose is invariably used in date palm micropropagation medium. Veramendi and Navarro (1996) reported that sucrose starvation of nodular callus has an important role on embryogenesis. They found that the best conditions for somatic embryo development in callus cultures were in shaken-liquid medium with a period of 2 weeks sucrose starvation followed by culture on 3% sucrose. Under these conditions 40-60 mature embryos per gram of callus were obtained after 4 months in culture using different genotypes.

Asemota *et al.* (2007) reported that date palm callus could be induced at different sucrose concentrations in combination with required growth regulators. Callus fresh weight increased with sucrose concentration up to 0.1 M and then declined. Eeuwens (1976) reported that fresh weight of explants from coconut and date palms cultured *in vitro* increased with sucrose concentration up to 0.2 M and then declined, whereas dry weight continued to increase up to 0.4 M.

Date palm syrup

Al-Khateeb (2008) used date syrup as a substitute for sucrose in the culture medium and concluded that date palm tissues are capable of utilizing date syrup as the sole carbon source for vegetative growth. Furthermore, date syrup at a concentration of 6% can be used as a total replacement of 30 or 60gl^{-1} sucrose which is the normal sugar concentration used in most of plant tissue culture including date palm.

Coconut water

Coconut water contains mainly water (94%) and growth promoting substances including inorganic ions, amino acids, organic acids, vitamins, sugars, sugar alcohols, lipids, nitrogenous compounds and phytohormones that can influence *in vitro* cultures

(Yong *et al.*, 2009). Coconut water has significant influence on the embryogenesis even at a lower concentration (5% v/v) and when the concentration is increased to 15%, rate of embryogenesis also increases (Al-Khayri, 2010)

Vitamins

Vitamins are nitrogenous substances required in trace amounts to serve catalytic functions in enzyme systems. Specific media components such as amino acids and vitamins have been found to exert a profound effect on tissue culture systems of certain species and optimization of such compounds can stimulate regeneration in recalcitrant cultivars (Benson, 2000). Plant cells grown *in vitro* are capable of synthesizing essential vitamins in suboptimal quantities; thus culture media are often supplemented with vitamins to enhance growth (Al-Khayri, 2001). Thiamine (vitamin B) at concentration ranging 0.1-5.0 mgl^{-1} is an important cofactor in carbohydrate metabolism, and is generally considered as an important ingredient in culture media. Biotin is important in carboxylation reaction and is less common in culture media, usually added at 0.01-1.0 mgl^{-1} (Bhojwani and Razdan,1983; Pierik, 1987). Both biotin and thiamine biosynthesis pathways utilize transfer of sulphur from cysteine to cofactor precursors (Begley *et al.*, 1999). Culture media of existing date palm regeneration systems are supplemented with arbitrarily selected vitamins at variable concentrations, 'biotin', including inositol, calcium pantothenate, pyridoxine, nicotinic acid, thiamine, 'biotin' etc. (Omar *et al.*, 1992). A study conducted by Al-Khayri (2001) on date palms has shown that callus growth and embryo formation were best achieved on a medium containing 0.5 mgl^{-1} thiamine combined with 2 mgl^{-1} biotin.

Glutamine

Organic nitrogen, particularly glutamine, is known for its positive effect on somatic embryogenesis. In alfalfa, addition of amino acids to the medium increased the number of somatic embryos (Skokut *et al.*, 1985) and yielded the best embryo conversion into plantlets (Stuart and Strickland, 1984). Under *in vitro* conditions, glutamine was shown to be important in maintaining cell division in Douglas-fir suspension culture (Kirby, 1982) and maritime pine callus (David *et al.*, 1984). Khlifi and Tremblay (1995) studied the effect of glutamine on the embryogenesis of black spruce varieties and showed that glutamine can be used as a sole source of nitrogen in the medium but when it was used as a supplement to inorganic nitrogen the resulting increase was two fold. Lea *et al.* (1979) demonstrated that glutamine and/or aspergine are required to maintain protein synthesis in cultured cotyledons of bean.

Comparing the effect of amino acid supplementations on the callus growth of date palm, Abdel-Rahim *et al.* (1998) concluded that tryptophan and methionine had

inhibitory effect on callus growth while arginine, alanine, asparagine and glutamic acid gave the highest values of both fresh weight and growth rate. However, their control experiment without any of these amino acids provided higher rate of callus growth. Mohamed (1996) reported that glutamic acid, methionine, tryptophan, phenylalanine and arginine inhibited the growth of callus tissue cultures of fenugreek. In contrast, Abou El-Nil (1989) reported that, growth of date palm callus tissue was stimulated by adding of amino acids, specifically glutamine.

Sodium chloride (NaCl)

Al-Khayri (2002) studied the effect of various concentrations of sodium chloride on callus induction and proliferation of date palm. He found that NaCl at a concentration of 25 μM had a stimulatory effect on the proliferation of callus and a concentration of 125 μM was inhibitory and detrimental.

Proline accumulation in response to salt stress has been perceived as symptomatic of salt-stress damage that provides a compatible cytoplasmic osmoticum protecting the cytosol from dehydration (Hasegawa *et al.*, 1986). Endogenous free proline accumulation with increase in medium salinity has been corroborated in various *in vitro* culture systems subjected to salt stress (Kumar and Sharma, 1989; Shah *et al.*, 1990; Patnaik and Debata, 1997). In date palm cultures, proline content increased gradually as the external concentration of NaCl increased. At 25 mM NaCl, proline content was unaffected in relation to the NaCl-free control. This suggests that low NaCl concentration was not sufficient to cause salt stress and consequently proline over production was unnecessary. However, when the level of NaCl was increased to 50 mM and higher, significant accumulation of proline occurred. Proline accumulation appears to be related to callus growth inhibition. This relationship holds true at low NaCl concentrations (25-100 mM), but at higher levels of NaCl callus growth was halted while proline accumulation continued to rise (Al-Khayri, 2002).

Auxins and cytokinins

Studies have shown that maturation and germination of somatic embryos can be influenced by various *in vitro* factors, including auxin and cytokinin concentration (Iraqui and Tremblay, 2001; Gonzalez *et al.*, 2001; Corredoira *et al.*, 2003). In most cases, 2,4-D and BAP have been used to support growth of somatic embryos (Ammirato, 1983). In *Phoenix canariensis*, the use of 0.2 mgl^{-1} BAP stimulated the germination of about 76% of somatic embryos (Huong *et al.*, 1999). In date palm, El Hadrami and Baziz (1995) reported the beneficial effect of 2,4-D to induce the embryogenic capacity of callus. In date palm, cultivar 'Deglet Nour', Feki *et al.* (2003) showed that the use of 1 mgl^{-1} 2,4-D favoured the differentiation of a large number of somatic embryos. Eshraghi *et al.* (2005) indicated that the effect of growth

regulators (2,4-D and BAP) on callus and on somatic embryos induction in date palm is cultivar-dependent. In fact, in 'Khanizi' cultivar, the embryogenic callus was induced on medium containing 4.6 mgl^{-1} BAP and 3.4 mgl^{-1} 2,4-D. In contrast, for Mordarsing cultivar it is necessary to use a higher concentration of 2,4-D (154 mgl^{-1}) for inducing the embryogenic callus.

Effect of auxin concentration and strength of MS salt was studied on *in vitro* germination of somatic embryos in date palms (Alkhayri, 2003). When full-strength MS medium was used, the percentage of complete plantlets decreased as the concentration of NAA increased. At low NAA concentration of 0.2 mgl^{-1}, germination inhibition was not significant; however, as the concentration was increased to 0.4 mgl^{-1}, a significant reduction in the number of complete plants was observed. Further, increase of NAA to 0.6 mgl^{-1} caused no further reduction, but at 0.8 mgl^{-1} NAA additional decrease occurred and then leveled off at 1 mgl^{-1}. These observations suggest that NAA was inhibitory to the germination processes of date palm somatic embryos at the levels tested. Germination inhibition associated with NAA was also observed with half-strength MS medium. In contrast, stimulation of embryos to produce complete plants was observed in response to increasing IBA concentration with full-strength MS salt. The best results were obtained when half-strength MS medium was augmented with 0.2 to 0.4 mgl^{-1} IBA. Compared to the hormone-free control, addition of 0.2 mgl^{-1} IBA resulted in a significant increase in the formation of whole plants. Further, increase in the concentration of IBA to 0.4 mgl^{-1} slightly increased the percentage of complete plant formation reaching to a maximum of 86%.

The effect of auxins, dicamba (3,6-dichloro-2-methoxybenzoic acid) and picloram (4-amino-3,5,6-tri-chloropicolinic acid) on callus growth and embryogenesis in date palm was investigated by Omar and Novak (1990). Maximum callus fresh weight was obtained in nutrient medium enriched with 200 μM picloram. Somatic embryogenesis and subsequent plant regeneration was achieved following transfer of such calli to hormone-free medium. Picloram and dicamba have been used successfully in tissue culture of various plants with no adverse effects on the callus or in regenerated plants (Conger *et al.*, 1983; Gray *et al.*, 1984).

Agar

In vitro cultures require a surface to grow on. For this reason agar is widely used to gel plant tissue culture media and provide a physical support to the growing cultures. Agar possesses many peculiar properties that suit its use as gelling agent in tissue culture media. It is chemically inert in the medium and is not digested by plant enzymes (Kinnersley and Henderson, 1988). It also forms a uniform gel that remains stable over the range of pH, temperature, and light conditions maintained during incubation.

However, despite the above characteristics, performance of agar as a gelling agent is not always consistent. Quality of agar and corresponding performance of cultures has been found to vary from brand to brand presumably due to varying level of impurities (Scholten and Pierik, 1998; Nairn *et al.*, 1995; Debergh, 1983). Agar also hinders aeration in the medium and may curtail availability of oxygen to the cultures (Newell *et al.*, 2003; Anon., 1988). Besides its qualitative shortcomings, agar constitutes the single costliest component of tissue culture media (Puchooa *et al.*, 1999; Bhattacharya *et al.*, 1994). There might be variation in the price of agar of different brands at different locations, but the predominance of its cost would remain overbearing.

Due to its qualitative deficiencies and high cost, several attempts have been made to find cheaper alternatives to agar that should put up better or similar performance of cultures as well. Starches from cassava (Maliro and Lameck, 2004; Gerbe and Sathyanarayana, 2001), corn (Puchooa *et al.*, 1999; Kinnersley and Henderson, 1988), sago (Bhattacharya *et al.*, 1994), and potato (Calleberg *et al.*, 1989) have proved their variable efficiencies as gelling agents in culture media. Some gums like gellan gum (Puchooa *et al.*, 1999; Calleberg *et al.*, 1989) obtained from *Pseudomonas elodea* and gum-katira obtained from bark of *Cochlospermum religiosum* have also been found to impart good gel strength to the medium (Jain and Babbar, 2002). Among other natural products, microcrystal cellulose (Gorivnova *et al.*, 1993), parenchymatic solidifier from apple (Titel *et al.*, 1987), an agar-like polysaccharide obtained from *Pseudomonas* (Kang *et al.*, 1982), and husk of *Plantago ovata* seed (Babbar and Jain, 1998; Bhattacharya *et al.*, 1994) have been tried. These materials have ample potential for use as gelling agent and are comparatively cheaper. But their performance has been inconsistent, perhaps due to lack of standardization and presence of impurities.

Synthetic solid matrices offer some distinct advantages over agar and other gelling agents. Their quality and properties can be precisely controlled for consistent performance; they are comparatively very cheap, can be reused and are more convenient to handle. Glass beads and glass beads with filter paper (Puchooa *et al.*, 1999) have shown limited superiority over agar. While some synthetic matrices like glass wool cloth, nylon cloth, polystyrene foam (Bhattacharya *et al.*, 1994), polyesteracetate membrane (Matsumoto and Yamaguchi, 1989), polypropylene membrane (Hew *et al.*, 1990; Tanny *et al.*, 1993; Desamero *et al.*, 1993; Adelberg *et al.*, 1992), and polyurethane foam (Conner and Meredith, 1984; Alkhalifah *et al.*, 2010) have shown promising results.

Charcoal

Different types of media-supplements and support-matrices are used in plant tissue culture to enhance growth and development of explants. Activated charcoal (AC) has been used in tissue culture media to improve culture growth and promote

morphogenesis in a wide variety of species (Wann *et al.*, 1997). Activated charcoal is often used in plant tissue culture to improve cell growth and development (Pan and van Staden, 1998). It plays a critical role in the micropropagation of date palms by adsorbing inhibitory compounds in media and decreasing toxic metabolites, phenolic exudation and brown exudate accumulation. However, there are reports that, in addition to adsorbing unwanted substances, it may also adsorb needed hormones (Ebert and Taylor, 1990; Ebert *et al.*, 1993; Nissen and Sutter, 1990), vitamins (Weatherhead *et al.*, 1979; Pan and van Staden,1998), or metal ions such as Cu++ and Zn++ (Van Winkle *et al.*, 2003). Addition of activated charcoal to the culture medium for date palm apparently overcame inhibition exerted by a dark coloured substance normally released into the medium by explants (Reuveni and Lilien-kipnis,1974).

During micropropagation, the exudation of phenol is very common and it often influences the result. Thomas (2008) compiled some recent reports on application of activated charcoal in plant tissue culture of 105 crops. In ninety cases it was found positive, in twelve cases found negative, and in three cases reported as positive and negative. Two important negative aspects of activated charcoal are catalyzed hydrolysis of sucrose into fructose and glucose (Druart and De Wulf, 1993) and drastic reduction of pH after autoclaving (Wann *et al.*, 1997). Phenolic exudation is a matter of serious concern in the micropropagation of date palms. In the explant culture for callus induction, incorporation of activated charcoal in the medium is inevitable; otherwise it may lead to considerable loss (Al-Khalifah *et al.,* 2010). On the other hand, activated charcoal delays morphogenesis and reduces success rate. Alternative use of antioxidants and peroxidases may help to eradicate the problems associated with phenolic exudation in date palm tissue culture and provide the full efficacy of growth hormones and vitamins used in the medium.

4. PROBLEMS AND PROSPECTS OF DATE PALM TISSUE CULTURE

Economic prospects of date palm tissue culture

Date palm ranks among of the first fruit trees that were brought into domestication in the Old World. It is considered as an economically viable resource, which is characterized by the multiplicity of uses in food, medicine, and other industries. The Arab world produces about 70% of global date palm which is the main source of income from agriculture sector in this region. In modern times, significant progress has been made in the development of direct and derived date fruit products and the utilization of by-products from packing and processing. However, comparatively minor attention has been given to date palm products other than the fruits. Once established, palm plantations can provide a sustainable and reliable supply of fruits and other products for decades.

Date palm has great socio-economic importance, especially in North Africa and the Middle East. Modern commercial palm plantation development has focused almost exclusively on a single economic product; as a result, insufficient attention has been given to secondary products from the fruits and other parts of the palms. In addition to its valuable fruit, the tree is cultivated for fuel, fiber and as shelter for ground crops. The trees create a shady microclimate which makes it possible to cultivate other cash crops such as alfalfa, oats, citrus and banana. The tree is an important source of wood for furniture and firewood and also leaves for matting, roofing, baskets, etc. Many value-added products are made and marketed from the fruit which create employment and income to thousands.

The plant tissue culture industry represents an estimated $15 billion market with 500 million to 1 billion plants produced annually and an annual growth rate of about 15%. In comparison to traditional plant production, micropropagation of plants via *in vitro* techniques currently represents only a small section of total plant production and is executed predominantly by small to medium-sized commercial companies. Typically, these companies have an annual production in the range of several thousands to several million plants.

Traditionally, date palms are propagated by offshoots. They develop slowly, and the availability of sufficient number of offshoots is always a constraint for raising large scale plantation of a desired cultivar. Use of tissue culture is the most

suitable approach for large scale multiplication of vegetatively propagated crops in horticulture and forestry (Jain and Ishii, 2003). The estimated need of date palm plants is about 1-2 million per year (Jain, 2007). With somatic embryogenesis it is easy to produce date palms on large scale but the use of high level of hormones in the media may cause somaclonal variations. Micropropagated plants of known origin, uniform size and of superior quality can be available throughout the year. It is thus easy to develop large scale plantations of uniform-sized plants. Tissue culture-derived plants are early bearing and hence returns can be expected within 3-4 years. This technique also provides a means for multiplying rare and superior quality varieties. Transportation of tissue culture derived plants is easy compared to offshoots, facilitating dissemination of the propagules of rare cultivars to faraway places where higher marketing demands exist.

Somaclonal variations

Vegetative propagation by means of either offshoots or tissue culture should produce progenies genotypically and phenotypically identical to their mother plants. However, abnormalities due to somaclonal variations are generally detected in tissue culture derived date palm. McCubbin *et al.* (2000) reported abnormal leaves with wide leaflets, slow growth rate and development, variegated leaves, non-flowering and low fruit setting in tissue culture derived date palms. Abnormal multi-carpel flowers and fruits with 6-7 carpels (Fig. 15 a-g) were also reported (Al-Khalifah *et al.*, 2007; Al-Wasel, 2000, 2001; Djerbi, 2000; Cohen *et al.*, 2004) from Israel, Jordan, Namibia and Saudi Arabia. A survey conducted by Hassanpour-Estahbanati and Hamidian (2007) on the micropropagated date palms in Iran showed excessive vegetative growth, dwarfing, leaf bleaching, leaf malformation, single leaf chlorosis, twisted leaf, lower leaves spreading on the soil, necrosis on midrib, bastard offshoot, abnormal terminal bud, twisted inflorescence, bending of whole plant, etc. An extensive field survey conducted by Al-Kaabi *et al.* (2007) in the tissue culture derived populations of date palms in United Arab Emirates also revealed many of these abnormalities. Al-Khalifah *et al.* (2009) reported an unusual spear-head shaped trunk formation in tissue culture-derived populations of date palms. Abnormal fruit formation is a serious concern of date palm fruit productivity in Saudi Arabia.

Various explanations have been put forward for the observed somaclonal variations in the tissue culture-derived plants. Kaeppler *et al.* (2000) attributed it to genetic and epigenetic alterations generated during the *in vitro* process. Phenomena like oxidative stress (Cassells and Curry, 2001; Joyce *et al.*, 2003), altered level of DNA methylation (Jaligot *et al.*, 2000, 2002; Bender, 2004; Xu *et al.*, 2004), and activation of transposable elements (Peschke *et al.*,1991; Courtial *et al.*, 2001) are associated with epigenetic variations.

A common observation in tissue culture-derived plants is higher abnormal fruit setting during the initial years and its decline during subsequent years (Gurevich *et al.*, 2005; Cohen *et al.*, 2004). This observation points to the epigenetic nature of this abnormality. Gradual elimination of epigenetic changes is believed to be due to correction of DNA methylation pattern and actions of transposons/retrotrasposons by cellular repair mechanism (Hirochika *et al.*, 1996; Brettal and Dennis, 1991). Plant growth regulators like 2,4-D are known to cause mutations. Higher concentration of plant growth regulators in the culture medium has been found to affect DNA methylation level of callus cells (Arnholt-Schimitt, 1995; LoSchiavo *et al.*, 1989) and perhaps resulting epigenetic variations (Corley *et al.*, 1986). DNA methylation is reported to increase with increase in concentrations of auxin (2,4-D) and decrease with increase of cytokinins (kinetin) (LoSchiavo *et al.*, 1989). Most of the somaclonal variations are found in plants derived from callus tissues (Al-Wasel, 2000, 2001; Ramage *et al.*, 2004). Unfortunately, many of these off-type phenotypes appear in plants at maturity stage thus escaping selection and screening during early growth and resulting in economic loss to the farmers.

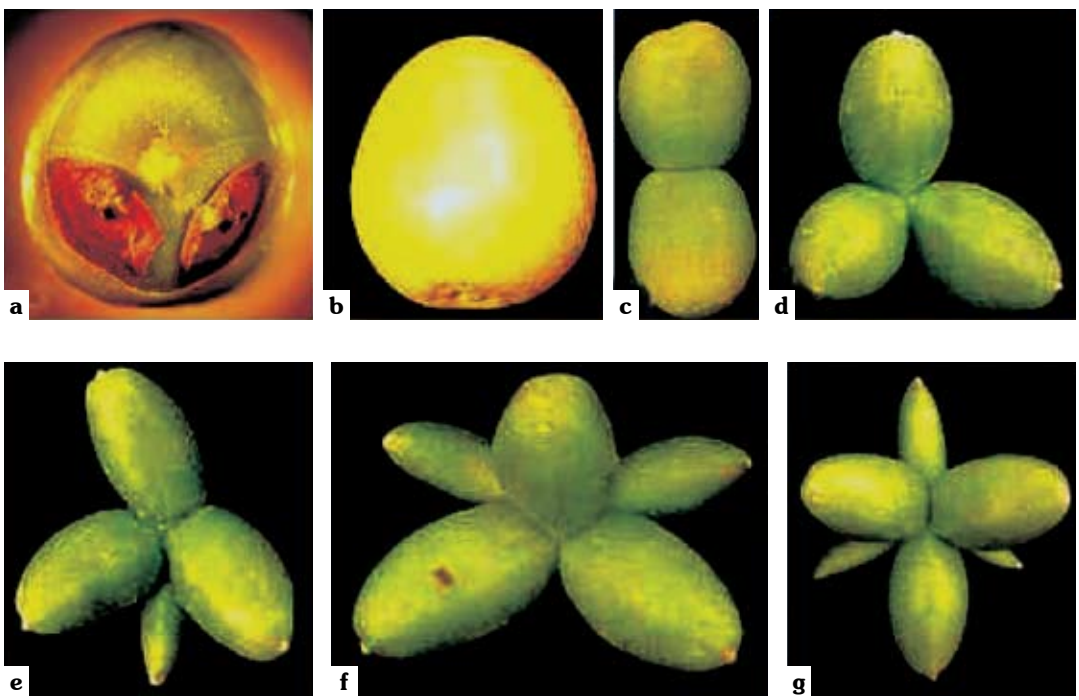

Fig. 15. *Normal and abnormal fruit developments. a & b. Normal development; a. Young normal fruit; b. Normal mature fruit; c-d. Abnormal development; c. Young fruit with two carpels; d. Young fruit with three carpels; e-g. Fruits with carpels and developed staminodes.*

Until recently micropropagation of date palm cultivars was carried out through somatic embryogenesis that originated from callus tissues induced by the higher concentration of auxins and cytokinins. The long phase of callus induction and somatic embryo formation with growth regulators might be responsible for genetic and epigenetic changes in the resulting plants.

Saudi Arabian achievements in date palm tissue culture

Date palm is the main fruit crop of the Kingdom of Saudi Arabia covering approximately 72% of the total area under permanent crops. With an estimated 25 million date palms, the Kingdom produces nearly a million tons of dates annually accounting for about 15% of the global production. More than 400 different date palm cultivars are reported to exist in Saudi Arabia (Anonymous, 2009a, b). Despite surplus production of dates in the Kingdom, marketing of the produce at national and international markets is not up to the expected level. Modern processing factories equipped with sorting, cleaning and packing facilities have to be encouraged. The Kingdom currently has only 64 date-processing factories (Anonymous, 2009b; Al-Shuaiby and Ismael, 2007).

Date palm Research Institute at Al Hassa has made tremendous contributions in the micropropagation of date palm and related research. Ministry of Agriculture has established a tissue culture laboratory in Riyadh which is mainly involved in mass multiplication of date palms through tissue culture. National Center for Agriculture Technologies in the King Abdulaziz City for Science and Technology has been involved in micropropagation and related research in date palm. The plant production departments of King Saud University and Al-Qassim University also have made considerable contributions in tissue culture researches on date palms. In addition to these government institutions, many private sector organizations are actively involved in large scale multiplication of date palms through micropropagation. Some of these organizations are currently selling their products to the national and international markets.

Application of tissue culture techniques in the development of date farming in Arab world

Past experiences from the developed countries have clearly shown that science and technology can contribute significantly to increase in agriculture productivity, stimulate economic growth and provide more opportunities for participation in global markets. However the technological challenges facing agriculture in the Arab world are considerable due to the increasing scarcity for arable land and water. To meet the increasing food demand and to raise the rural and urban income Agricultural biotechnology in the developing countries must grow more rapidly than in the past decades. Investments in agricultural research and development by both private and public sectors have resulted

in high level of productivity. Productivity increases occurred because of innovations in machinery, pesticides, fertilizers, information technology and breeding. Innovations in food storage, processing, packaging, transportation and increasing shelf life resulted in wide variety of products available throughout the year.

In the last few decades, farmers of Middle East have shown great interest in accepting tissue culture derived palms for their crop expansion, though in the beginning they showed some reluctance. But some entrepreneurs showed courage and planted tissue culture derived palms in their orchards. Of course, some abnormalities were observed in these plants, but the increased production, yield and many other advantages have attracted the farmers to accept the technology. The increasing number of tissue culture laboratories in the Middle East corroborates this acceptance.

In Saudi Arabia alone there are about ten laboratories representing government and private sectors, engaged in date palm tissue culture. A tissue culture laboratory was established at UAE University in 1989, which in 2003 produced around 100,000 transplants of different varieties. The Ministry of Agriculture and Fisheries in collaboration with El-Raghy Group started a tissue culture laboratory for producing date palm transplants and carried out research for true-to-type date palm from tissue culture. Date palm tissue culture laboratory established by United Arab Emirates University is one of the leading date palm tissue culture laboratories in this field. The very elaborate infrastructure developed here currently produces thousands of date palms through tissue culture. In addition, hundreds of private and government sector laboratories are engaged in date palm production.

In Oman, Ministry of Agriculture established a tissue culture laboratory in 1992 at Jemah Research Station. This laboratory was established to meet the long term needs of replacing 3.1 million trees with better performing cultivars. Currently it produces thirty thousand plants per annum. In 2000, another section was added at Rumais Research Center for molecular analysis. Later more laboratories were developed in different institutions in Oman. A tissue culture laboratory has been established in the state of Qatar under the Agriculture & Water Research Directorate (AWRD), Ministry of Public Work & Agriculture, with main objective of providing Date Palm transplants and preservation of natural resources.

Kuwait is a leading country in the developing world that has put in serious efforts to develop and apply biotechnological techniques for solving food security and agriculture related problems. The country has made considerable efforts to foster the development and application of biotechnologies for its economic and social well-being. During the past ten years of research, Kuwait institute for Scientific Research, (KISR) has developed tissue culture technologies for mass production of elite date palm cultivars. Currently, a pilot scale commercial delivery system of true-to-type plantlets of date palm and native plants through tissue culture is in operation.

At Regional Research Center, INRA, Marrakech, Morocco micropropagation of date palm is carried out using axillary buds and inflorescence as explants. National capacity of *in vitro* plants production is between 70,000 to 100,000 plantlets per year. This capacity is insufficient to fulfill the high demand for the plantlets. For instance, in year 2007, total demand for plantlets was more than three times the production capacity (total demand for plantlets 3,212,000 which includes variety 'Mejhool' 105,000 plantlets, other varieties 140,000 and other selected clones 1,075,000).

All other leading dates producing countries in the Middle East have started micropropagation units which are currently producing elite varieties of date palms in their respective areas. However a reliable account of such laboratories and their production capacity in each country is still lacking. Saudi Arabia and other Middle East countries offer tremendous scope for tissue culture industry, especially for date palm production. Uninterrupted power supply, availability of controlled greenhouses, comparatively cheaper labour, easy transportation to the east and west, favourable trading and quarantine rules are added attractions to the entrepreneurs in this region for starting tissue culture industry.

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APCoAB (Asia-Pacific Consortium on Agricultural Biotechnology) was established in 2003 under the umbrella of Asia-Pacific Association of Agricultural Research Institutions (APAARI). APCoAB has the mission to "harness the benefits of agricultural biotechnology for human and animal welfare through the application of latest scientific technologies while safeguarding the environment for the advancement of society in the Asia-Pacific Region". APCoAB's main thrusts are (i) to serve as a neutral forum for the key partners engaged in research, development, commercialization and education/learning of agricultural biotechnology as well as environmental safety in the Asia-Pacific region; (ii) to facilitate and promote the process of greater public awareness and understanding relating to important issues of IPR, sui generis systems, biosafety, risk assessment, harmonization of regulatory procedures, and benefit sharing in order to address various concerns relating to adoption of agricultural biotechnology; and (iii) to facilitate human resource development for meaningful application of agricultural biotechnology to enhance sustainable agricultural productivity, as well as product quality, for the welfare of both farmers and consumers.To know more about APCoAB, please visit: www.apcoab.org

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