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EDITORIAL

Special issue on Date Palm Current Research

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Date palm, *Phoenix dactylifera* L., a monocotyledonous angiosperm diploid species belonging to the Arecaceae (Palmae) family, is considered as one of the world's first cultivated fruit trees. Along with the olive and the fig, the three represent an ancient group of fruit trees closely associated with the beginnings of agriculture and the sustenance of humankind in the arid regions of the Old World.

For the last century, scientists have been engaged in research to delineate agricultural challenges, find suitable solutions, and continue to expand awareness and utilization of this majestic species. As part of an ongoing effort to provide researchers with periodic updates on date palm research, future special issues on the subject are planned, the frequency to depend upon the proliferation of relevant research results. Recently, a Special Issue on The First Date Palm and Dates Conference (held at King Abdulaziz City for Science and Technology, Riyadh, Saudi Arabia on December 4-7, 2011) was published (Emir. J. Food Agri., 2012, Volume 24, Issue 5).

This Special Issue on Date Palm Current Research provides a valuable source of current research achievements relevant to this important fruit tree crop. In addition to six review articles highlighting progress and current status of several subjects, four original research articles present the latest results obtained by scientists actively working with date palm. Aspects covered in this compilation are: Nutritional importance, Micropropagation, Abiotic and biotic challenges, Genetic resources, and Molecular biodiversity.

Date palm is mainly grown for its fruits, but the whole tree is utilized. Dates are a highly nutritious source of sugars, minerals, vitamins and antiatherogenic nutrients. Dates are used as a sweetener in numerous traditional desserts and contemporary baked goods, Manickavasagan et al. (2013) article recommends date fruits as a sugar-substitute source in traditional foods.

Propagation of date palm is traditionally by offshoots; however, increased demands for offshoots to expand agricultural areas have necessitated the use of tissue culture. Remarkable progress has been made in date palm micropropagation since it was first achieved in the early 1970s. At present, commercial micropropagation is becoming commonplace for commercial date palm production. Researchers continue to improve this process through empirical assessment of various tissue culture factors in relation to the wide array of available cultivars. An article exemplifying these efforts is presented by Ibraheem et al. (2013) where proliferation of somatic embryogenesis of cv. Zaghlool was investigated in response to solid and liquid cultures. Culture contamination, perhaps the oldest and most complex challenge of tissue culture success, is revisited in the article by Abass (2013) who focused on microbial contaminants tissue culture laboratories in Iraq.

Another restriction of date palm tissue culture is the scarification of the tree through the excision of shoot tips for utilization as explants source. An alternative source of explants is of paramount importance when none or a low number of offshoots are available. The in vitro response of various explants is influenced by genotype and numerous culture factors. Researchers have achieved in vitro regeneration of date palm using florescence explants as a less invasive alternative. Abahmane (2013) reviewed achievements and limitations of date palm inflorescence explants.

The main date-producing countries are found in regions where extremely high temperatures, a limited water supply and saline soils represent major agricultural constraints. Date palms are resilient in the face of these adverse environmental conditions. However, fruit quality and yield are affected by soil salinity and water stress. In light of global climate warming these problems are expected to be exacerbated in the date palm

growing regions, thus intervention strategies are needed to overcome these impending challenges. Al-Karaki (2013) has addressed in his review article about improved water utilization using mycorrhizae.

A current biotic challenge for date palm agriculture is the devastating impact of widely-disseminated pests and diseases. The most serious pest of date palm, as well as many other palm species in the world, is the red palm weevil. This aspect is well covered by Hazir and Buyukozturk (2013) highlighting the status and control methods of red palm weevil and red palm scale insects mainly focus on Turkey.

Breeding efforts to improve tolerance to abiotic as well as biotic stress conditions is of paramount importance for sustainable agriculture of date palm. Conventional date palm breeding is restricted by a long juvenile phase and high heterozygosity. Relevant to this aspect, Johnson et al. (2013) reviewed reflecting a contemporary look at the seedling date palms as a potential germplasm. Moreover, modern biotechnological approaches including in vitro embryo rescue techniques offer indispensable tools for date palm breeding through the production of interspecific hybrids. The prospect of interspecific hybridization in relation to date palm conservation and genetic improvement is reviewed by Gros-Balthazard (2013).

In support of breeding efforts, biotechnology offers effective approaches for date palm improvement and germplasm conservation and characterization. Molecular tools are being used in in different crops for studying genetic diversity, genomics, markers assisted selection and breeding. We have included two articles on the use of molecular techniques based on DNA analysis to study date palm biodiversity. Srivashtav et al. (2013) addressed genetic diversity in the Kutch region of India and El Kichaoui et al. (2013) described genotyping of date palm cultivars of the Gaza Strip of Palestine.

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REVIEW ARTICLE

Seedling date palms (*Phoenix dactylifera* L.) as genetic resources

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Abstract

An accelerating worldwide trend toward planting elite cultivars is leading to genetic erosion and a narrowing of the gene pool upon which the date-palm industry is based. Large numbers of seedling dates are known in many major date-growing countries, as well as in naturalized populations in Spain and the Americas where the palm was intentionally introduced. Seedling dates growing under different climatic conditions from those of the major production areas represent potential genetic resources that should be evaluated for desirable traits. Utilizing modern biotechnology, traits such as disease and pest resistance, hardiness, tolerance of salty soils and improved fruit quality and quantity potentially can be transferred to elite cultivars to sustain and further improve fruit production. Specific examples of important seedling date palm populations in Spain, Peru and Mexico are discussed, as well as new cultivars derived from seedlings in the United States. Research on seedling date populations is recommended, along with the establishment of ex situ germplasm collections of promising specimens as living plants, cold storage of seeds or cryopreservation.

Key words: Artificial ripening, Cultivar, Ethnovariety, Germplasm, Khalt, Mexico, Offshoot, Peru, Spain, USA

Introduction and Background

Based upon archaeological evidence, the date palm (*Phoenix dactylifera*) was domesticated some 6,000 years ago in the Mesopotamian Region (Zohary and Hopf, 2000); present-day Iraq, along with adjacent portions of northeast Syria, southeast Turkey and southwest Iran.

Tree-crop domestication appears to have taken place at the advent of agriculture and is presumed to have been a slow and incremental process. The initial stage of domestication involved wild populations, with fruit gatherers preferentially selecting fruits from highly-productive trees bearing large fruits of good quality. Fruit gatherers likely returned to the same more productive trees season after season and encouraged the natural regeneration of seedlings beneath them by eliminating competing vegetation. Under such circumstances of limited management, the trees are considered at the stage of semi-domestication. To attain full domestication, date seeds had to be retained from the harvests and germinated to establish intentional new plantings at a chosen location. Studies of plant domestication in general have speculated that the initial example of seed planting may have been inadvertent; seeds discarded near a dwelling place which germinated spontaneously, and were recognized as desirable plants, protected, given some care and ultimately provided fruit. Alternatively, seed planting may have been intentional, iterative of what was observed in the wild. Intentionally or unintentionally, domestication is achieved when cultivated plants begin to exhibit, over succeeding generations, characteristics which distinguish them from their wild progenitors. Larger fruit size would have been the most desirable trait in the case of the date palm.

Seeds were the means to intentionally reproduce date palms for millennia in Mesopotamia, and later in North Africa, from native wild species or through seed introductions from Mesopotamia. From these two areas, seeds were transported, in association with human migrations or invading armed forces, east to present-day Pakistan, into southern Europe, and later to South and North America, into southern Africa, and to distant Australia and New Caledonia (Johnson, 2010). Wherever environmental conditions were favorable, date palms flourished

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and provided fruit for subsistence purposes or for commerce. Untended or abandoned plantings spontaneously reproduced by seeds and offshoots and became naturalized. In some locations, date palms were planted as ornamental trees.

Two biological characteristics of the date palm played key roles in domestication. Early on, Mesopotamian farmers recognized that date palms were dioecious: female plants produced fruit while male plants produced only pollen. Seed propagated date palms yield approximately equal numbers of male and female offspring. During the early years after reaching sexual maturity, both male and female palms produce basal offshoots (also known as suckers) (Figure 1), which if left intact grow into mature trees. At some point, perhaps in southern Mesopotamia, an astute farmer discovered that it was possible to separate basal offshoots from a mother palm and transplant them to another location. Priority in implementing this technology would certainly have been given to female palms known to produce large good-quality fruits. Apparently, no historical record exists about the time and place for this important technological breakthrough. It was a momentous step for the date palm, nonetheless, giving rise to its vegetative propagation and the ability to carry forward to succeeding generations desirable fruit characteristics (Johnson, 2011). However, this practice has a negative consequence as well, for over time it has reduced the diversity of the general date germplasm pool. Without sexual reproduction, there are no opportunities for new genotypic combinations to occur.

Figure 1. Date palm cultivar with several offshoots. Indio, California.
Photo: D. Johnson.

Another undocumented event in date palm development was the innovation of assisted pollination of female trees to enhance fruit production. In the process, observant farmers noticed that pollen from certain male trees was superior in bringing about enhanced fruit production. When it was learned that a single male palm produced enough pollen to fertilize up to 50 female trees, the number of male trees in fields could be significantly reduced, allowing more space to grow female trees. The significance of certain male palms as pollen sources also led to their propagation by offshoots.

There emerged from these innovations certain types of female and male cultivars (cultivated varieties) recognized to possess desirable characteristics and those palms came to be assigned distinctive names for identification purposes. According to tradition, in what is now southern Iraq, Zahidi was the first named female date cultivar and Khikri the first male (Dowson, 1923). The naming of cultivars proliferated wherever dates were grown from offshoots and in some instances the same cultivar was assigned a different name when cultivated in a new area where a distinct dialect or language was spoken. It has been estimated that there are 3,000 date cultivars in the world (Zaid, 2002), but it is widely suspected that many of the names represent synonyms.

Thirteen species of *Phoenix* are recognized by scientists; all are dioecious and hybridize easily (Barrow, 1998). Therefore it is possible, although not common, that seedlings in date-growing areas thought to be offsprings of *dactylifera* may in fact represent natural hybrids between different species. This may occur if other *Phoenix* species are growing (wild or cultivated) in the vicinity of a plantation. A natural hybrid between *P. canariensis* and *P. dactylifera* was observed near Winterhaven CA, exhibiting the typical crown and thick trunk of *canariensis* but bearing basal suckers (D. Johnson, pers. comm., 2012) (Figure 2). Evidence of the commonplace occurrence of hybrids in *Phoenix* is given by Bergman (2005) who described and provided photographs of four spontaneous interspecific *Phoenix* crosses among four different species. This ease of crosspollination within the genus may have potential value in date improvement. Sudhersan and Al-Shayji (2011) used male pollen of the dwarf date palm (*P. pusilla*) to fertilize female date palm (*P. dactylifera*) flowers and create a short-stature date palm. Offsprings derived through embryo culture techniques are under field evaluation in Kuwait.

Figure 2. Natural hybrid of *Phoenix canariensis* and *P. dactylifera*, near Winterhaven, California.
Photo: D. Johnson.

This study examines relevant historical aspects of seedling date palms and their contemporary role for fruit production, as well as their germplasm potential and conservation, for the future sustainability and development of date palm cultivation. The worldwide trend in commercial date palm production toward planting elite cultivars inadvertently contributes to genetic erosion and the narrowing of the gene pool. Greater recognition of the potential role of seedling date palms will strengthen the sustainability of this important tree crop. Jaradat (2010) pointed out that sustainability of date palm oases is largely dependent upon a highly diverse genetic base of the date palms.

Standard date-industry terminology is used in this paper with regard to seedling date palm fruits. Date palm fruits on the tree pass through four stages of development as they mature; *kimri* is the first stage when the fruits are small, green, hard and immature; at *khalal* stage fruits attain their full size and take on a yellow or red skin coloration, but are still somewhat hard; upon reaching *rutab* stage the fruit begins to soften and take on a typical amber or brown color; at the *tamar* stage the fruits are firmer, cured and the skin becomes wrinkled, and can be stored without fermenting or souring. Cultivars are traditionally classified as *soft*, *semidry* or *dry*, based upon fruit consistency when ripened normally.

A seedling date palm may also be referred to as a marginal date palm, *khalt* or *deglet*, according to the fruit qualities. The meaning of the Arabic term *khalt* may be "mixed," "dry," or "seedling." The Arabic term *deglet* (*deglat*, *deglah*) may refer simply to a "date," "a date of poor quality of unknown origin" or a "seedling." Khalt and deglet usage and meaning vary from country to country; both appear as part of cultivar names, particularly in North Africa, with different meanings altogether. For example, In Tunisia, Khalt Mouachem is a soft cultivar date, neither dry nor a seedling (Kearney, 1906; Rhouma, 2005). In the Jerid oasis of Tunisia, all seedling dates with dry fruits are called "khalt" while all seedling dates with soft fruits are referred to as "chekken" (A. Othmani, pers. comm., 2012). As for the term "deglet," the well-known Deglet Noor of Algeria is a semidry cultivar date, neither of poor quality nor a seedling. Clearly, these Arabic terms must be interpreted with care.

Included in this study is a discussion of modern seedling-derived dates propagated by offshoots for research purposes (i.e. germplasm collections) and commercial fruit production. Clear examples of the latter are the American cultivars grown on a small scale in California and Arizona (Hodel and Johnson, 2007).

It could be argued that it is the seedling date that is emblematic of this important world commercial palm species, for no single named cultivar can fulfil that role. A direct link between past and present date palm growing became known in 2005 when a 2,000-year-old date palm seed, recovered from an archaeological site in the Judean desert of Israel, was germinated successfully. In late 2011, the Judean date palm was transplanted to an outside location at the research station where it was grown and can now be viewed by the public (Figure 3). Initial genetic studies of the palm reveal that it shares about 50% of its DNA with contemporary date palms. Unfortunately, the Judean date palm appears to be a male plant, requiring time consuming backcrossing experiments to incorporate its traits into other date palms (AAAS News, 2008; The Daily Plant, 2012).

Figure 3. Judean date palm (*Phoenix dactylifera*), Borick Natural Medicine Research Center, Jerusalem.
Photo: B. Glazner.

Seedling date palms

Seedling date palms occur spontaneously wherever date palms are grown; when they occur in modern plantations they are considered weeds and eliminated. There is an opinion shared among most date palm specialists that seedling dates are of no significance for commercial fruit production, owing to the heterogeneity of open-pollinated female palms and the great assortment of fruit size, shape, color and flavor. Standard sources on date palm cultivation discuss the value of seed propagation for breeding experiments, as well as ornamental use, but recommended against them for commercial fruit production because of the advantages of offshoot propagation in terms of genetic fidelity as well as greater fruit quantity and good quality.

Assessments of seedling date palms typically fail to take into account their value at the local level as a source of subsistence food and for animal rations. Nixon (quoted by Dowson and Aten, 1962), agreed that seedling dates were inappropriate for fruit production in the established date palm countries of the Old World, with the exception of those grown for research. However, Nixon strongly believed that in marginal areas elsewhere, planting seeds of selected varieties would be beneficial. He also held the strong view that seedling genotypes could profit from a testing and breeding program for marginal areas.

Relative to whether seedling date palms were worth cultivating, an interesting exchange occurred between two of the leading twentieth century date palm specialists, V.H.W. Dowson (1896-1980) and Roy W. Nixon (1895-1976), regarding the degree to which seedling dates resembled those of the mother palm. Dowson and Aten (1962) stated that a female date seed would "bear dates of unpredictable qualities. The chances are many thousands to one against their resembling those of the mother. Moreover, it is exceedingly unlikely that the dates borne by the seedling will be any good at all..." Nixon, who appears to have reviewed Dowson's manuscript before publication, responded: "There are certainly very large odds against getting a seedling identical with the parent variety, but there are often strong resemblances of seedlings to the parent variety. ...we have [in the USA] at least half dozen new varieties that originated from seeds...that are definitely superior to 90 percent of the varieties we imported from the Old World..." To his credit, Dowson included in his book Nixon's dissenting view.

Despite the disadvantages of variable fruit quality and quantity, seedling date palms are grown for fruit production, apparently even in areas where cultivar date palms predominate. In the Nizwa groves of Oman, for example, small quantities of seedling date fruits called Qash Nabhani are produced. These reportedly delectable fruits are large, soft, red-orange in color and have white flesh streaked with yellow (Nabhan, 2008).

Date palm seeds of undocumented origin were planted successfully in Australia for fruit production, as early as the 1880s. A few seedling palms from the early plantings reportedly have been preserved at the Mecca Date Garden near Alice Springs in Central Australia (Tanswell, 1999).

Small commercial quantities of seedling-derived dates are marketed in Spain, Mexico and Peru. In the USA, date fruits from American cultivars, derived from seedlings of introduced cultivars, are sold. These will be described in detail later in this paper.

In date palm plantations, viable unharvested fruits may fall to the ground and germinate to produce seedlings near the base of the mother tree. Seedling date palms growing at the base of trees may be mistaken for mutant off-type suckers. Nixon (1953a) recounts the instance of a farmer who had a female tree of cv. Deglet Noor which had two large offshoots bearing male flowers, which he believed were off-types, because the offshoots had no obvious leaf differences from the mother tree. However, when the soil was removed to expose the roots it became apparent that they were seedling date palms. Off-type suckers, nonetheless, do occur on occasion and may be of interest to breeders if they exhibit desirable characteristics. Off-types may originate as bud variations; Mason (1930) suggested that in date palm they are best termed *sectorial mutations*.

Seedling date palms can be distinguished from offshoots during the first year or two of growth by their different leaf form, but later they may be mistaken for offshoots.

Conducting a census of date palms grown on a formal plantation is simple because the trees are planted at a regular spacing; offshoots are removed for transplanting or simply eliminated to maintain spacing and to facilitate agricultural operations, leaving only single stems to count. In dealing with untended or feral seedling date palm populations, however, the situation becomes complicated if offshoots remain attached to the mother palm until they mature and produce fruit. In such cases, one original tree may multiply into several. In making estimates of seedling date palm population numbers, the most common practice is to enumerate producing trees as individuals, even if they are still attached to the mother tree.

In Egypt, seedling date palms account for some two-thirds (or 2.3 million) of the date palms grown in the Fayoum depression, the largest oasis in the country, and in the valley of the Nile from above Cairo to Aswan (Riad, 1996). These numbers are noteworthy in that currently Egypt is the world's leading date producer. Seedling date palm populations also predominate in countries such as Mauritania, where the ancient Adrar palm groves contain about 120,000 producing trees (Bonte, 2010; Munier, 1955).

Two ancient cities are celebrated for their old date palm groves. One is Marrakech, Morocco, at the northern foot of the Atlas Mountains, with its historic date palm grove of about 100,000 trees (Elhoumaizi et al., 1998). A study of Moroccan palm groves found that nearly 80% of the Marrakech trees were of seedling origin. The same study found that on average more than 50% of all 11 groves included in the study were made up of seedling date palms (Bendiab et al., 1998). The climate of Marrakech is marginal for ripening dates and therefore palms that bear fruit early in the season are preferred (El Houmaizi et al., 1993).

A second example of a historic date grove is in Tadmor (or Palmyra), Syria, the "city of palms," located in an oasis northeast of Damascus. An estimated 130,000 seedling date palms are growing in Tadmor. Studies are underway on seedling propagation to select superior Syrian genotypes for offshoot propagation (Ibraheem, 2012). These examples strongly suggest that other traditional date-growing countries also hold significant numbers of seedling dates.

Although not native, date palms have been cultivated since ancient times in what is now Pakistan and India. Introduction of date palm seeds probably took place in the eighth century or even earlier (Jatoi et al., 2009). Bonavia (1885) describes 12 locations where seedling date palms were present in the Awadh (Oudh) district of what is now Uttar Pradesh state in North India. He described these populations as fully acclimatized and bearing fruit. Traditional date palm cultivars in both countries originated from these early seed-propagated date palm populations. A large area of seedling date palm groves exists in the western part of the India, in the Kachchh region of Gujarat state. Established some 200 years ago, the planted area contains about 1.4 million seedling date palms. Fruit production is low and variable. Because the monsoon rains occur before the fruits can fully ripen, farmers have selected certain date palms which yield non-astringent fruits at the khalal stage, which are sold in Mumbai. Vashishtha (2003) states that the seedling date palms of Kachchh represent a rich genepool.

In general, information about the fruit and other characteristics of seedling date palm groves and of individual seedling date ethnovarieties is variable and incomplete. It is obvious that they have not received much research attention.

Following Rivera et al. (2006) the term *ethnovariety* in reference to seedling date palms is used to refer to infra-specific diversity in cultivated plants as understood and managed by farmers.

Seedling date palm propagation

Once established, female date palms are able to reproduce naturally by both seeds and offshoots, male palms only by offshoots. When left untended, the palms develop dense thickets composed of seedlings and offshoots. These feral date palm stands provide shade, habitat and food for birds and small terrestrial animals, and their fruits may be harvested by local people for food or to feed to livestock. Date palm thickets can be thinned, most male palms eliminated and minimal management adopted to enhance fruit production.

Seedling date palms are cultivated for two major reasons: breeding and germplasm conservation. The U.S. Department of Agriculture supported a date palm breeding program in the period 1948-1978. In 1965-1975, backcrossed male palms were bred and selected for promising metaxenia effects, followed by a major effort in the 1970s to produce female clones bearing fruit which could be harvested and processed mechanically. The latter experiments involved 12 imported and 2

American cultivars, each with desirable characters. However, none of the nine selections proved to be an appropriate substitute for Deglet Noor, the most common cultivar grown in the USA. The breeding program was concluded in 1978 in anticipation of the closing of the U.S. Date and Citrus Station in Indio, which occurred in 1982. The most promising commercial female and male selections from the research station are conserved in the National Date Palm Germplasm Repository in Thermal, California (Carpenter, 1979; Hodel and Johnson, 2007).

An investigation in Oman is making use of seedling date palms as germplasm sources to enrich the local genepool (El Kharbotly et al., 2010). Another example of date palm breeding began in Morocco and Algeria, also in the 1970s, seeking to identify genotypes with resistance to bayoud disease, caused by *Fusarium oxysporum* f. sp. *albedinis*, as well as producing high quality fruit. Selected wild male and female genotypes with desirable characters were crossed to create new genotypes for field testing, but these have not given anticipated positive results. The major difficulty is that backcrossing to generate F1 and F2 progeny requires as many as 30 years (El Hadrami et al., 2011). Conventional breeding has been eclipsed by dramatic developments in plant biotechnology over the past decade which have opened the possibility for molecular breeding of date palms (Abdulla and Gamal, 2010; Al-Dous et al., 2011; Arabnezhada et al., 2012; El Hadrami and El Hadrami, 2009; Hider and Nabulsi, 2012; Jain, 2012; Khanam et al., 2012; Yang et al., 2010).

A second reason for cultivating seedling dates is to maintain living germplasm collections of certain male and female seedling types with desirable traits for conventional breeding purposes and as sources of genetic material for molecular breeding. A review by Bettencourt et al. (1992) found that worldwide there were only 15 formal field date palm genebanks, the largest in Algeria, India, Iraq, Nigeria and the USA. A more recent study by Krueger (2011) notes the existence of a field genebank in UAE to study salt-tolerant varieties, along with collections in Saudi Arabia at Al Qasim University and at the Wadi Quriyat Date Palm Research Station in Oman. Most of the accessions in these collections appear to be elite cultivars. An exception is Nigeria where five field genebanks were established in the 1980s, with accessions of seedling date palms from different parts of the country's date palm belt. Fruit produced by these palms compare favorably with named cultivars from elsewhere; the palms should be fully evaluated to assess their germplasm value (Ataga et al., 2012). Date palm germplasm collections in the USA are also an exception because they contain elite cultivars along with 11 American cultivars derived from seedling dates (Hodel and Johnson, 2007).

The value of seedling date palms in Morocco was highlighted by Baaziz et al. (2000) and Baaziz (2003), who pointed out that encouraging commercial cultivars and discouraging seedling date palms leads to a loss of genetic diversity within date palm groves; they also reasoned that seedling date palms should be conserved because they may contain genes resistant to bayoud disease, as well as other diseases and pests. It was estimated that Morocco overall has more than 2.5 million seedling date palms bearing good fruit quality which could contribute genetic material to solve the bayoud disease problem.

Ornamental use is a third way in which seedling date palms may be utilized, taking advantage of existing mature palms and transplanting them to landscape shopping centers, buildings and as street trees. However, because of the variability they exhibit, preference is given to cultivar dates where the goal is to achieve a uniform appearance of palms in a landscape setting. Seedling and cultivar date palms are important components of the vegetation landscape in all of the traditional date-growing countries of the Middle East, North Africa and the Persian Gulf countries (Figure 4). In California and Arizona, where seedling date palms are rare, cultivar date palms are used as ornamentals, with a preference for cvs. Medjool and Zahidi.

Figure 4. Date palm cultivars used in landscaping, Hofuf, Al-Hassa, Saudi Arabia.
Photo: J. M. Al-Khayri.

Published references on date palm provide only a minimal of detail about seed propagation for whatever purpose. Date palm seeds can be germinated by direct planting in the field or in a nursery with subsequent selection of the healthiest seedlings to transplant to the field. Popenoe (1913)

recommended collecting seeds from trees with desirable fruit characteristics, discarding small seeds, and then soaking them in water for a week to accelerate germination. In the field, seeds are planted 3-5 cm in depth, at distances of 10 x 10, 10 x 9 or 10 x 8 m, giving densities of, respectively, 100, 121 or 125 palms per ha; the position of the seed in the hole is not important. Seed germination in the nursery commonly consists of a single seed in a polybag to facilitate potting up as the plants develop. Holding seedlings in the nursery for a year or two permits a useful selection and culling prior to transplanting into the field.

In vitro seed germination provides a controlled environment to examine the response of date palm seedlings to various growth-limiting factors as well as to study the response to stress conditions like drought and high soil salinity (Sané et al., 2005). Excised embryos from mature seeds also have proved useful for screening for abiotic stress (Ibraheem et al., 2012).

For successful in vitro germination of date palm seeds, Sané et al. (2005) recommended scarification of the seed with 96% sulfuric acid for 5 min and washing 5 times with sterile distilled water prior to densification in 1% mercuric acid for 3 min and then rewashing with sterile distilled water. The seeds were soaked in water for 48 h and then sterilized a second time with 5% calcium hypochlorite for 4 min and rewashed with sterile distilled water. The seeds were cultured in Murashige and Skoog (1962) basal salts supplemented with Nitsch and Nitsch vitamins (1969), 0.2 mg/l glutamine and 30 g/l sucrose. The medium was adjusted to pH 5.7 and solidified with 8 g/l of Agar. Recommended incubation was 80 μE s^{-1} m^{-2} with a photoperiod of 12 h at 30°C.

Spontaneously occurring date palms are a dubious seed source. If the seedlings are found beneath a female palm, they are most likely from that palm. However, wild animals may transport fruits to other locations, consuming only the mesocarp pulp and leaving the viable seed to germinate. Similarly, whole dates are eaten by domestic animals such as goats, sheep, cattle and camels, with the seeds passing through their digestive system and deposited in random locations (Barreveld, 1993).

Hypothetically, date palm seeds will yield equal numbers of male and female plants, but gender cannot be determined until the palm flowers at 4-6 years of age or later. Hence, a high percentage of male palms must be removed from a directly-planted field. It is more practicable to make a selection in the nursery of healthy females, along with a few healthy males, to establish a seedling-date plantation. Current molecular studies (Aberlenc-Bertossi, et al., 2010; Ageez and Madboly, 2011; Bekheet and Hanafy, 2011; Cherif et al., 2012; Elmeer and Mattat, 2012; Moghaieb et al., 2010; Younis et al., 2008) are expected to provide the means to identify the gender of seedlings at an early stage and thereby simplify propagation and breeding. Early selection of young seedlings could enhance date palm breeding programs and generate experimental male and female genetic stocks (Siljak-Yakovlev et al., 1996). Molecular markers would facilitate the identification and selection of good male pollinators for use in breeding programs and could be used to select date palm with desired traits such as high yield and improved physical and chemical fruit characteristics (Elmeer and Mattat, 2012).

Male seedling date palm

For centuries, it was presumed that all male date palm pollen was of equal quality and that the source of pollen was therefore inconsequential to the fruits resulting from natural or artificial pollination. However, it was suspected by some date specialists, that pollen quality influenced fruit production. Studies by Nixon (1926, 1927) and Swingle (1928) showed that the quality of pollen was indeed an important determinate of fruit quality and quantity; Swingle is credited with coining the term *metaxenia* to describe this effect.

In many areas, seedling date palms continue to play their most prominent role in commercial date cultivation through male palms serving as pollen sources; the pollen exhibiting a wide range of variation. In certain areas of seedling date palm cultivation, such as Marrakech, Morocco, farmers rely upon natural pollination by insects and wind within the groves of mixed female and male palms. Under natural pollination, fruit set is reduced, but in the Marrakech example, economic incentives do not justify the expense of artificial pollination (Zaid, 2002).

Research has been done in various countries on male seedling date palms. An Egyptian study involved 50 male seedling date palms which were evaluated for their pollen grain weight and viability, number of spathes produced, number of flowers and the length of the spathe burst (Moustafa et al., 2010a). A subsequent investigation took five of the selected males and compared the metaxenia effect in cv. Seewy in terms of fruit set, fruit quality and yield. Three of the male date palms produced the best overall results, confirming the importance of a superior

pollen source (Moustafa et al., 2010b). In Saudi Arabia, Taha et al. (1989) evaluated 600 male date palm seedlings to identify superior pollen providers, and a similar study in Pakistan involved 15 male date palm seedlings (Iqbal et al., 2009). The objective of these investigations was to identify superior male date palms for offshoot or tissue culture propagation to establish stable male cultivars.

Other uses of seedling date palms

Date palm seedlings are playing a role in biotechnology as laboratory test plants. In recent studies they were used to ascertain if magnetic fields (Al-Enezi et al., 2012) or X-radiation (Dhawi and Al-Khayri, 2011) can influence growth parameters of young palms. Positive results could bring about new protocols for more rapid growth of tissue-cultured plants. Moreover, mutations induced by irradiation of seeds could benefit the expansion of the gene pool available for selection in date palm breeding schemes. Male and female seedling date palms were used by Majourhat et al. (2002) in Morocco to study the diversity of leaf peroxidases and their potential as genetic markers.

Seedling date palms in Spain and the Americas

Important seedling date palm populations are found in Spain, Peru and Mexico. Date palms were introduced to those locations in historic times by seeds and the trees grown for fruit production. Date palms became naturalized in these locations, partially or fully ripening fruit depending upon climatic conditions, and reproducing by seeds and offshoots. In a number of other locations, such as the Caribbean Islands, fruit production failed because humid conditions during the ripening period caused fruit rot. In areas marginal for fruit production (e.g. Italy), date palms are grown for ornamental purposes comparable to the way the Canary Islands date palm (*Phoenix canariensis*) is cultivated.

Each of the three seedling date palm populations described in this study has a unique history. Also included is an account of the historic seedling date palms in the USA and how in the twentieth century new cultivars were derived from seedlings of imported cultivars.

The history of date palm introductions to the Americas, especially the provenance of the seeds the Spaniards brought to the New World, is being investigated by an informal international group of date palm scientists, under the leadership of Dr. Diego Rivera, University of Murcia, Spain. The research includes DNA analysis of samples from the naturalized populations in Baja California, Mexico and Peru and comparison to the DNA of date palms from Spain and North Africa, in an attempt to ascertain affinities and suggest origins. The results are not yet ready for publication.

Seedling date palms in Southeast Spain

Early in the period of Arabic domination of the Iberian Peninsula, which began in 713 A.D., Berbers from North Africa introduced the huerto (fruit and vegetable garden) oasis system to Spain. It was modelled on systems in use in Africa at that time, to produce fruits, vegetables and grains under irrigation and to sustain livestock. Planted from seeds, date palms became the keystone species of the huertos. Elche, located in coastal south-eastern Spain and enjoying the mildest climate in Europe, developed into the largest area for date palm cultivation. The huertos (each with an individual name) and their date palms were maintained for centuries and now form the contemporary Palmeral de Elche, all derived from seedling date palms. For unknown reasons, propagation by offshoot separation was not a tradition. Historically, in Elche, the practice of collecting seeds from female trees bearing fruit of good quality was used for replacement plantings in the huertos.

Figure 5. Elche, Spain, traditional palm grove, now a World Heritage Site.
Photo: iStockphoto.

The historic Palmeral de Elche contains about 180,000 adult date palms (Figure 5). Total date fruit production is reported by FAO at 5,200 mt in 2010; however, only about 100 mt are marketed for human consumption (Ferry et al., 2002). The area of the traditional Elche palm grove was inscribed as a UNESCO World Heritage Site in 2000 with the long-term objective to maintain it as a unique European example of an introduced oasis system, with the date palm as its key agricultural species.

The climate of Elche (at 38° 35' N. Lat.) provides only 792°C of heat units, making Elche marginal for ripening date fruits. Munier (1973) concluded that a minimum value of 1,000°C heat units were necessary to produce ripe fruits. Fruits produced by the ethnovarieties found in the Elche palm grove display a wide range of color, size, shape and flavor (Figure 6). Typically, they are harvested at the rutab stage and artificially ripened. Several ethnovariety fruits ripen naturally on the tree in Spain; these are referred to as *cándidos* and are characterized by balanced sugar content, are nonfibrous and have somewhat dry flesh not inclined to fermentation (D. Rivera, pers. comm., 2012). Ethnovarieties have not been described in detail, although the more important ones have been classified according to fruit consistency. Table 1 lists ten of them, their names typically derived from the huerto where they are grown.

Artificial ripening has been investigated in Elche to better utilize local seedling date fruit production. The studies referenced below are worth noting because they represent results from seedling date palms grown in Elche. Studies of the artificial ripening of cultivar date fruits have been done; for example on cv. Dkakki in Pakistan (Saleem et al., 2005) and cv. Khuneizi in Iran (Yektankhodaei et al., 2007), but those results may not be directly applicable to seedling date palm fruits.

One study of artificial ripening in Elche involved fruit at the khalal stage from the Caqui, Los Cherros and Los Olmos huertos. Experiments were conducted with acetic acid, freezing and vaporization treatments using parameters of fresh weight, firmness, total soluble solids, acidity, ripening index, color and sensory properties, before and after the treatments. The best results were found in fruit from the Caqui and Los Olmos huertos which ripened to good fruit quality using treatments of acidic acid and freezing (Amorós et al., 2007). Another study showed that Elche seedling date fruits consumed at the khalal stage have a high hydrophilic total antioxidant activity and are nutritional. The research involved seven ethnovarieties from Algorós and Caque huertos and included fruit measurements from medium khalal to full rutab stages. The range in weight was 6.1-12.8 g; length 33.9-43.6 mm and diameter 18-21.7 mm (Amorós et al., 2009). Gracia and Ortiz (1996) found that the date fruits in Elche, which represents 95% of Spanish date production, ranged in length from 26.9 to 52.6 mm. On average, the seed represented 7.5% of the fruit weight. These data indicate that Elche seedling date palm fruits are smaller than those borne by cultivar date palms, which is to be expected.

Figure 6. Seedling date palm fruits, Elche, Spain. Photo: S. Orts.

Table 1. Elche date palm ethnovarieties according to fruit consistency.

Ethnovariety name	Phenotypic groups based upon fruit consistency
De Aob	Soft
Bollior	Semidry
Candit	Semidry
Confitera[#*]	Soft
Léon[#]	Soft
Marraner	Semidry
Negre/Dora/Verdal	Soft
Tenat	Dry
Tendre	Soft
Tendre Dolz	Semidry

Considered to be a cultivar since it is propagated by offshoots.

*According to unpublished molecular studies conducted at Miguel Hernández University, Elche, Confitera is unrelated to local date palm ethnovarieties but correlates with North African material; therefore, it is not a traditional Elche date palm (D. Rivera, pers. comm., 2012). Source: Ferry, 1999; M. Ferry, pers. comm., 2012.

A study in Elche of physicochemical changes of Negros ethnovariety fruit at 16 ripening stages of maturity found that plant hormone ethylene was responsible for changes in color, firmness, soluble solids content and actidity (Serrano et al., 2001). Blanching is a ripening technique used in Elche with cvs. Medjool and Confitera fruits. Blanching water accumulates soluble solids and organic acids which can be used to reconstitute skim milk powder to produce yogurt (Trigueros et al., 2012).

In the early 2000s, a pilot study was conducted to select the seedling female date palms bearing the best quality edible fresh fruit. One hundred local ethnovarieties were selected for further study, based of fruit flavor, aroma and texture, as a fresh

perishable fruit at the khalal or rutab stages. When superior ethnovarieties have been ascertained they will be propagated by offshoots to create new cultivar palm groves to be established outside the boundary of the historic Palmeral. The overall objective is to produce attractive fresh dates for the European gourmet food market (Orts and Johnson, 2007).

Also since 2000, the Phoenix Research Station in Elche has produced about 30,000 tissue-cultured plants for commercial fruit cultivation, for use on surrounding farms not subject to regulations governing conversation of the historic Palmeral. Date palm cultivation beyond the protected Palmeral is forming a beneficial buffer zone and extending the palm landscape of Elche. Seedlings produced are of the introduced cv. Medjool and cvs. Confitera and Léon both derived from local ethnovarieties (Gómez and Ferry, 2010). All three produce soft dates. Fruits are harvested by cutting the entire bunches at the rutab stage for artificial ripening before being sold (M. Ferry, pers. comm., 2012)

In the Rio Segura Valley, just west of Elche, a comprehensive field study of seedling dates was carried out by Rivera et al. (1997). The findings, summarized in Table 2, represent the most detailed information about ethnovariety date fruits in Southeast Spain.

Efforts underway to improve upon the traditional seedling date area in Elche, Spain represent a significant step forward for the utilization of seedling date palms and may well serve as a model in other locations such as Marrakech, Morocco.

Table 2. *Phoenix* spp. seedling date palm ethnovarieties, Río Segura Valley, Spain*.

Species	Ethnovariety	Fruit characteristics and notes
*chevalierii**	De Adobo	Fruits ripen slowly, extending availability for table consumption. Edible only after being marinated in vinegar. Trees being replaced by more desirable cvs. Becoming scarce.
chevalierii	De Berberia	Fruits small ovoid 2 cm in length. Skin smooth to rugose, very dark reddish brown. Flesh dark, farinaceous, sweet and aromatic. Ripe from early November.
chevalierii	Verdal	Fruit small oval, elongated, 2.8-3.5 x 2 cm or more, apiculate. Skin smooth and fine, green, turning brownish green when mature. Ripe from mid November.
dactylifera	Cándidos	Fruits of average size, 4-4.5 x 2 cm, cylindrical. Skin smooth yellowish, at full maturity dark reddish brown. Flesh firm, smooth, granulose and farinaceous, taste sweet and pleasant. Used in desserts when fully mature but spoils easily. Ripe from early November.
dactylifera	Candíos Puntiagudos	Fruits of average size, 3.5-4.5 x 2 cm, cylindrical but narrowing toward the apex. Skin smooth yellowish turning reddish brown at maturity. Flesh firm, smooth granulose and farinaceous, taste sweet and pleasant. Harvested from mid November but if left until tamar stage become brownish.
dactylifera	Largos	Fruits large, 5 x 2 cm, cylindrical, smooth brownish yellow. Skin smooth, shiny, strongly adhering to the mesocarp. Flesh white, fibrous, sweet, and slightly sour. Consumed fresh and in dry sweet confectionery. Ripe from mid December. Similar to cv. Ghars (Rhars) of North Africa (Algeria).
dactylifera	Moscatel	Fruit of average size, cylindrical to 4 cm. Skin yellow, shiny, turning reddish-brown when mature, rugose. Flesh farinaceous and consistent, yellowish-green, very sweet when mature. Ripe from mid November. Dubious resemblance to cv. Deglet Noor of Tunisia and cv. Halawi of Iraq. Also, could be related to cv. Khalasa from the Arabian Gulf because the flesh is amber and without fibers.
dactylifera	Rojo	Fruits large 4.5-5 x 1.8-2.5 cm, elongated oval. Skin shiny red, turning brownish at maturity, smooth and shiny. Flesh white, fibrous-fleshy, juicy and sweet, taste a bit sour. Eaten fresh. Ripe from early November. Similar to cv. Aruri of Egypt, known for its large fruit size.

Table 2. Contd..

Species	Ethnovariety	Fruit characteristics and notes
dactylifera	Tiernos	Fruits spoil easily starting from the end, hence they are eaten before fully mature.
*iberica**	De Espiga	Fruits average size 4 x 2 cm. Skin yellow, straw-colored to shiny-reddish, smooth shiny and consistent, dense and strongly adhering to the flesh, which is firm, fibrous, somewhat dry, sweet-sour. Ripe from early December. Resembles cv. Fardh of Oman.
iberica	De Rambla	Fruits small 2.4-2.6 x 1.5-1.8 cm. Skin yellow, straw-colored shiny-reddish, smooth, consistent, firm and strongly adhering to the flesh, which is firm, fibrous, somewhat dry, sweet-sour.
iberica	De Sol	Fruits only suitable for livestock feed, although after freezing they are fit for human consumption.

Note: An expanded study is in preparation to cover all of southeastern Spain which is expected to name about 25 ethnovarieties (D. Rivera, pers. comm., 2012)

* Govaerts and Dransfield (2005) treat species chevalierii and iberica as synonymous with dactylifera, a classification which stands until further studies are done to prove otherwise.

Source: Rivera et al. (1997).

Seedling date palms in Canary Islands, Spain

Date palm seeds were introduced to the Canary Islands during the Norman-Castillian conquest of the fifteenth century, possibly originating from Spain; however, there is some evidence that the palm may have arrived earlier, with seeds brought in by the Phoenicians, Punics or Arabs. From those early introductions, small populations of naturalized *Phoenix dactylifera* still exist today near the ports of Fuerteventura, Gran Canaria and La Gomera. (Santana and Rodríguez, 1999). The climate of the island group is not warm enough to fully ripen date fruits. It is not documented, but some of the true date seeds carried to the New World may have come from trees in the Canary Islands, collected when Spanish explorer ships were provisioned for the last time at one of the aforementioned ports, before sailing west (Johnson et al., 2011).

Phoenix canariensis, the Canary Islands date palm, is endemic and the only palm native to the islands. It occurs as single palms or in small groups over the entire island group; also, it is a popular temperate zone ornamental in many places around the world. This *Phoenix* species does not produce basal offshoots and must be propagated by seed. The fruits are edible, but are small having only a very thin mesocarp and are sometimes fed to livestock (Rivera et al., 1997); birds consume the fruits and are an identified dispersal agent (Nogales et al., 1999). Near the stands of true date palm, some natural hybrids between the two *Phoenix* species have been observed (Morici, 1998). The emblematic tree of these Spanish islands, *P. canariensis* is depicted on the official flags of the islands of Gran Canaria and La Palma.

Seedling date palms in Peru

The Central Coast of Peru represents a location in the Americas where seedling date palms have been growing for four centuries. Ripe date fruits in the Rio Pisco Valley were observed by the Jesuit scholar Bernabé Cobo in 1612, writing in 1653 (Cobo, 1964). The Spanish Colonial capital of Lima was founded in 1535; date seed would have had to arrive no later than around the end of the sixteenth century for Cobo to make his initial observation. Cobo compared the date fruits in Peru to those brought in from the Barbary Coast of Africa, currently the countries from Morocco east to Libya, suggesting that region as the seed source**.**

The climate of Coastal Peru and the adjoining area of Northern Chile provide from 2,000-5,000°C heat units, more than enough to ripen date fruits. The total seedling date palm populations of Peru and Chile are not known, but it has been estimated that in the area of Pisco and Ica, Peru alone, there are 50,000 producing palms; however, 90% of them are abandoned and feral (Pavez et al., 2007).

According to the FAO, date fruit production in Peru in 2010 amounted to 337 mt. Production comes from seedling date palms and a modern cultivar date palm plantation, Huerto Alamein near Ica (Letts, 2003). It should be noted that seedling date palms in Peru receive no pruning, irrigation, assisted pollination or pest control (Figure 7). Fruits are harvested at the late khalal or early rutab

stages; fruit bunches are cut and taken for storage in an enclosure of adobe, palm trunk and leaves with a dirt floor. Fruit bunches are suspended on racks to ripen, being covered in foggy weather and at night. Midday temperatures reach 58-61°C within the structure. Ripening takes less than a week. Ripe fruits are removed individually from bunches and packed in baskets for sale. There is no tradition of eating tamar stage dates in Peru, but dry dates are fed to livestock. Most of the fruits of the seedling dates are consumed locally, a small amount reaching markets in nearby cities or Lima, but in general dates as fresh fruits are virtually unknown in Peru (Pavez, 2003).

Some efforts have been made in Peru to valorize the seedling date palms as well as to establish modern cultivar plantations, but thus far little has been achieved. Climatic, soil and water conditions in a number of areas of the country are very favorable to large-scale production; moreover, Peru is free of the major date palm pests and diseases (Pavez et al., 2007).

Figure 7. Feral seedling date palms Peru.
Photo: A. Pavez Wellmann.

Unpublished studies in Peru have generated some information about the more popular seedling date palm fruits. Table 3 presents the results of a survey in Ica of nine named ethnovarieties. Over four centuries of relatively isolated sexual reproduction, Peru's more than 50,000 producing seedling date palms must certainly contain a number of ethnovarieties with desirable traits.

Table 3. Seedling date ethnovarieties in Ica, Peru.

Spanish name (English translation)	Fruit description	Notes
Calvario (calvary)	Dark-red color, long and cylindrical, soft, translucent and shiny when mature. Weight 10-20 g, although some palms produce fruits of 35-38 g and 7-8 cm in length.	Only seedling date palm developed and propagated by local farmers.
Melacocha (molasses); Carmelo (caramel) or Meloso (honeyed)	Dark-red color, flesh caramel-like and translucent. Fruit only eaten fresh, perishable and subject to insect attack.	Names generally given to soft and syrupy dates that ooze honey when ripe.
Italia (Italian)	Fruit green color at rutab. Flesh thick, soft, translucent, aromatic and very sweet.	Name derived from its resemblance to Italian grapes. This date is renowned in Rio Grande and Ingenio.
Moscatel (muscatel)	Fruit purple at khalal, turning black when fully ripe, flesh soft and thick with thin seed and very aromatic. 7 cm in length, up to weight 40 g.	This seedling date palm is found exclusively in Rio Grande. Best date in Peru
Huando (seedless)	Fruit seedless; lack of pollination?	General name for seedless (parthenocarpic) fruit. Reported to be result of a genetic mutation. These palms are scarce.
De Mascar (chewy)	Fruit generally yellow, semidry, exhibits wide range of shapes and sizes. Sweet at khalal and can be eaten directly from the tree without further ripening.	Name applied to semidry dates that are sweet at khalal and can be eaten directly from the tree.

Table 3. Contd..

Spanish name (English translation)	Fruit description	Notes
Chocho or Pasa (raisin)	Fruit dry; neither softens nor becomes translucent at rutab, ripen directly to tamar when they wrinkle and dry, can remain unharvested on tree.	Despite self-preserving quality and extraordinary resistance to insects, fungi and diseases, fruits considered worthless and generally fed to livestock or palms cut down.
Chochón	Fruit dry, egg- or pear-shaped, with tough skin, weight over 25 g. Very difficult to ripen and dry, hence generally discarded.	No English translation for "chochón." If ripened, peeled and seeded, ideal for stuffing with sweetmeats and covered with chocolate, grated coconut or sugar to make a delicious sweet.
Colorado (red); Negrito (blackish) or Morocho (fresh)	Fruit blackish, reddish or crimson at khalal, turning dark red or velvet-colored at rutab, small size, weight 6-10 g. Some sweet and honeylike.	Names are general terms for date fruits of this type. Because of their size and color not considered to have commercial potential. Generally fruit not harvested or harvested for livestock feed.
Chuccos (pleasant)	Fruits small, flesh chewy with tough skin, weight 5-8 g. Fruits often infested with insects and gnawed by rodents.	General term for date fruits from wild palms growing on uncultivated land without any care. One advantage of this ethnovariety is that it ripens after falling on sandy or salty soil and is well adapted to industrial processing.

Source: Pavez, 2003.

Seedling date palms in Mexico

Along with Christianity, Spanish missionaries brought agriculture to Mexico's Baja California Peninsula, and the date palm was part of the crop assemblage. The native peoples of the peninsula did not practice agriculture, sustaining themselves by fishing, hunting and gathering. The first of a chain of missions, established at natural oases, was founded in Loreto in 1697. Sometime in the middle decades of the eighteenth century, date palms appear in the historical record at mission sites. The origin of the date palm seeds introduced to Baja California is not documented, but most likely it came from Mainland Mexico where there were some date palms at the time (Johnson et al., 2011); the mainland was the departure point for the Spaniards who colonized Baja California.

A field survey of seedling dates was carried out in 2004 covering the State of Sonora from Nogales at the US border south to Álamos. Only occasional seedling date palms were seen, typically at active or abandoned farm sites. Mature seedling date palms were observed as roadside plantings in beach areas west of Hermosillo; the palms had obviously been transplanted from other locations. The largest population found was in Madero Park in Hermosillo, which has a grove of over 100 seedling date palms, laid out as a plantation. The grove is said to have been established about a century ago for ornamental purposes. On a small scale, the Hermosillo grove resembles the ancient seedling date palm groves of North Africa (D. Johnson, pers. comm., 2012). The seedling date palms seen in Sonora are all likely descended from the original introductions during the Spanish Colonial Period.

In Baja California, Loreto, Mulegé (Figure 8), San Ignacio and Comondú are the major mission oasis sites for seedling date growing. In these locations, and several smaller ones, date palms naturalized and have flourished for about three centuries. Temperatures during the ripening season are sufficient to bring the fruits to maturity, although formal calculation of heat units appears lacking. Seedling date palms in Baja never have

been given much in the way of care, such as pruning and elimination of excessive male palms; offshoot propagation was not practiced. The date palms grow on their own in mixed production systems, with fruits harvested for local human consumption and for livestock feeding. As is typical of the fruit of seedling dates, they are varied in size and color. "The dates in the peninsula represent all types, large, small, large fat seeds, long narrow seeds, red, yellow, dark, black, caramel colored, sweet, astringent..." (Routson, 2012). Harvested fruits are dried in the sun before being packed in small baskets, typically woven from date palm leaves. Local people do not have names for the different seedling fruit types, but refer to them as *criollo carmelo* or *Mission* dates, to distinguish them from the fruit of cultivar dates, grown in two major locations: Datilera Del Desierto near San Luís Río Colorado in the extreme northwest corner of Sonora State and Ejido Bonfil near San Ignacio in Baja California Sur. Both plantations were established using offshoots from California.

Figure 8. Naturalized seedling date palms, Mulege, Baja California Sul, Mexico.
Photo: D. Johnson

As canopy trees of the Baja California oases, date palms provide welcome shade, and the leaves and stems are used for fencing, building materials and fuelwood. Prior to the introduction of date palms, the oases were dominated by the native Mexican fan palm (*Washingtonia robusta*). Because farmers more highly valued the date palms, the native palms have been significantly reduced in numbers in the oases, but still can be found, especially at the edges of the date palm areas. The historical importance of seedling dates in the peninsula is evident by the palm being depicted on the Coat of Arms of the Loreto municipality. Date palms have been present in Baja California for so long that local people are surprised when told that they are not native.

The number of seedling date palms in Baja California is not known with precision. Popenoe (1926) estimated that there were at least 100,000 palms in Baja California and Sonora. Nixon (1953b) made an arduous field trip down the peninsula in 1949 to study the date groves. He made a rough estimate of the number of trees producing fruits or pollen in the following locations: San Ignacio 50,000; Mulegé 25,000; Comondú 20,000; Purísima 15,000; Loreto 15,000 and possibly 20,000 elsewhere in the peninsula. The estimated total of 145,000 may be understated, for Nixon only visited the major seedling date areas mentioned above. Recent field research on the crops of the Baja oases in general, supplemented with satellite imagery, puts the number of producing seedling date palms at several hundred thousand (Routson, 2012)

Mexico's date fruit production in 2010, according to FAO, was 4,150 mt. Nearly all is from modern cultivar date palm plantations near the California border. Seedling date fruit production probably does not exceed a few hundred tons annually and is all consumed on the peninsula.

United States of America (USA)

Introduced cultivars

The history of seedling date palms in the United States began when the current states of California and Arizona were still part of the Mexican nation. It is documented that the Spanish missionaries introduced date palm seeds from Baja California to San Diego, California in 1769 (near the present US/Mexico border) and to Ventura in 1782 (300 km to the north), along with a few other mission locations. Because the missions were on or near the coast, the climate was unsuited to ripening fruit. The date palms apparently were maintained for their historical and ornamental value. Purportedly the last original seedling date palms from the mission period in San Diego survived until 1957 (Trent and Seymour, 2010) and until the 1960s in Ventura (Dolan, 2005).

After the U.S. - Mexican War ended in 1848, and the United States gained control of what is now California and Arizona, numerous American settlers moved to the new territory and an indeterminate number of date palm seeds were planted. In one documented introduction, seeds of the Amzi (probably Amri) cultivar were collected in 1878 from the Nile Delta of Egypt and sent to the

USA; there is no information about the result of the introduction. Plantings also originated from date fruits imported for food (USDA, 1878). Scattered plantings of date palm seeds began bearing fruit in the late nineteenth century (Nixon, 1955). A program by the USDA, begun in about 1900, brought date palm offshoots of named cultivars from North Africa and the Middle East, to establish the modern commercial date industry. In the same period (1909-1913), the USDA also distributed some 3.8 million imported date palm seeds for planting (Paul, 1924). These seed introductions took place during a time when it was still believed that seed propagation of desirable cultivars was a commercial option. Popenoe (1926) estimated that there were perhaps 150,000 seedling dates in Southern California and Arizona. In the early decades of the twentieth century, cultivar date palms began to replace seedling date palms.

At the first annual meeting in 1924 of the Date Growers' Institute in Coachella California, seedling dates were among the topics discussed. Reeves (1924) recommended seven criteria in selecting seedling dates for propagation: ability to tolerate climatic conditions; fruit size and shape; fruit-packing quality; heavy-bearing palms; palms easily propagated by offshoots; fruit color and fruit of high sugar content. The author was obviously considering offshoot propagation from candidate seedling dates. Suggestions also were made at the meeting by Swann (1924) on the selection of good seedling palms for seed propagation, and by Goar (1924) on seedling date fruit cleaning by dry or water methods, grading, vacuum fumigation and heating before being packed.

The first importation of date palm offshoots from Algeria and Egypt were infested with the date palm scale insect (*Parlatoria blanchardii*), which attacks the leaves. The insect was detected when the shipment arrived in Washington, D.C., and the offshoots were treated with chemicals before shipment to the American southwest for trial plantings (Shamblin, 1924). Unfortunately, neither the treatment in Washington nor subsequent attempts in California and Arizona were able to control or eradicate the pest. Subsequent offshoot imports in the 1920s were likewise infested with date palm scale, exacerbating the situation and the insect became a major pest problem for date growers for nearly four decades.

Beginning in the late 1920s, a joint California state and federal program was launched to eradicate date scale. The program focused on both seedling and cultivar date palms. The date scale eradication program targeted destruction of numerous seedling date palms in both states, to eliminate potential host reservoirs of the pest. In the cultivar date palms, success was finally achieved in 1936 by defoliation and blow-torching the palm stems to kill the pest and its eggs, followed by careful inspections to assure there were no recurrences. The measures taken were harsh but effective since there has been no reappearance of date scale. To prevent inadvertent introduction of it or other pests as well as diseases, California interior quarantine regulations prohibit all plant materials of the genus *Phoenix* from being taken into the counties of commercial date growing (CDA, 2010). Similar regulations are in force in Arizona (ACAH, 1923).

A few populations of seedling date palms, some distance from the commercial growing in Southern California, escaped destruction. A small stand of seedling dates established in 1857 near Winters, California (38° N. Lat.) began to bear in 1877 (Nixon, 1950). Seeds were reportedly obtained from fruit purchased at a food market in San Francisco (Klee, 1883). The surviving palms are now part of the University of California Davis's Wolfskill Ranch Experimental Orchard; they bear fruit (albeit seedless because there is no male among the 8 mature female palms) and appeared in good condition in 2008 (D. Johnson, pers. comm., 2012). Typical of unpollinated (parthenocarpic) date palm fruits, the Wolfskill fruits are small and not fully developed. The Wolfskill seedling date palms are likely the oldest extant in California.

A stand of seedling date palms can be seen at China Ranch Date Farm, near Tocopa, California (at 35° N. Lat.), where seedling and cultivar date palms are grown on a small scale for commercial purposes. The seedling date palms were planted in the 1920s; the ethnovariety fruits are marketed as China Ranch Dates.

In the present day, occasional ornamental date palms, male and female, can be seen along roads in the Indio, California area and also farther to the south in the state, and in the Yuma, Arizona area. It is highly likely that the male palms are of seedling date palm origin, the females could be of seedling or cultivar origin. Possibly, these seedling palms originate from after the eradication program of the 1930s.

American cultivars

Seedling dates can become cultivars via offshoot propagation, and that is what has occurred in the southwestern USA. Roy Nixon's research work provided much of the scientific basis for the development of the US date industry. He published a

detailed study describing 140 cultivars imported as offshoots into the country (Nixon, 1950). Although the study is on cultivar date palms, it is also relevant to seedling date palms because it includes some notes on them.

Figure 9. American date palm cv. Empress, Indio, California.
Photo: D. Karp.

Figure 10. American date palm cv. Empress fruit, Indio, California.
Photo: D. Karp.

Table 4. American date cultivars* grown in the USA.

Cultivar	Origin	Fruit characteristics	Notes
Abada	Uncertain, resembles cv. Deglet Noor	Khalal deep red; rutab and tamar glossy black with bloom. 48-52 x 20-22 mm. Flesh soft and melting with sweet flavor. Fruit subject to checking.	Early ripening. Has characters desirable for breeding (Carpenter 1979)
Blonde Beauty	Believed from cv. Deglet Noor seedling	Khalal yellow; rutab amber; tamar reddish brown. 45-50 x 20-24 mm. Flesh soft and melting, flavor pleasing.	Midseason ripening. Palm resembles cv. Deglet Noor. Exclusive cv. of single grower, Indio CA.
Brunette Beauty	Believed from cv. Deglet Noor seedling	Khalal medium red; rutab and tamar nearly black. 42-47 x 20-23 mm. Flesh soft with good flavor.	Late ripening. Exclusive cultivar of a single grower, Indio CA.
Empress	cv. Thoory seedling	Khalal yellow; rutab amber; tamar reddish brown. 46-54 x 21-24 mm. Flesh soft with pleasing flavor.	Midseason ripening. Has characters desirable for breeding (Carpenter 1979)
Honey	cv. Deglet Noor seedling	Khalal yellow; rutab amber; tamar reddish brown. 40-47 x 21-23 mm. Flesh soft and melting with pleasing mild flavor.	Midseason extending to late ripening.
McGill's No. 1	Uncertain, palm somewhat resembles cv. Kustawy	Khalal yellow; rutab amber; tamar reddish brown. Flesh soft and caramel-like, rich flavor.	Fruit size and fruiting season unknown.
Sphinx or Black Sphinx	Uncertain, possibly a cv. Hayany seedling	Khalal medium red; rutab and tamar dark brown to black. 41-48 x 26-28 mm. Flesh soft with good flavor. Fruit subject to severe checking.	Late ripening. Prolific offshoot producer.
Tabarzal	Uncertain, probably from imported offshoots	Khalal light yellow; rutab light amber brown; tamar reddish brown. 46-54 x 27-29 mm. Flesh thick, soft with mild flavor. Fruits subject to slight checking.	Early ripening.
T-R or Triumph	cv. Thoory seedling	Khalal pale yellow; rutab amber; tamar reddish brown. 43-52 x 19-22 mm. Flesh soft and melting, pleasing flavor.	Early ripening.

Source: Nixon, 1955.

* Specimens of all these American cultivars are in formal field germplasm collections in California and Arizona, USA.

In an unpublished manuscript, Nixon (1955) described the 40 major American cultivars found in the course of extensive field work in California and Arizona, all believed to have originated from seed of the imported cultivars. The nine American cultivars grown on a limited commercial scale in the two states are listed in Table 4. The cv. Empress date palm and its fruit are shown in Figures 9 and 10. The fruit quality of the American cultivars is on a par with the imported cultivar dates grown in the USA.

The most fascinating account of American cultivars concerns the Sphinx (or Black Sphinx). It was discovered near Phoenix, Arizona in 1928, gave rise to the establishment of stands of the palm, facilitated because Sphinx is a prolific offshoot producer. The land of the largest grove of Sphinx palms was sold for a housing development in the 1950s. Many of the Sphinx palms were left in place (Figure 11) as attractive ornamental trees for the housing tract (Johnson et al., 2002).

Figure 11. American date palm cv. Black Sphinx, *Phoenix*, Arizona.
Photo: D. Johnson.

A few American male cultivars have also been established in California as pollen sources; Zaid (2002) names six cvs. - Mosque, Mejhool BC3, Deglet Nour BC4, Fard No. 4*, Jarvis No. 1* and Boyer No. 11*. Three more cvs. are known: Crane*, Barhee A-19* and Tazizoot BC#3*. An asterisk indicates the cultivar is accessioned in one or more of the official government or university date palm field germplasm collections in California and Arizona (Hodel and Johnson, 2007).

Most if not all of the other American female cultivars Nixon described have been lost to cultivation, because they apparently were unpromising based upon early assessments and hence are no longer grown on research stations or by farmers, or through changing agriculture land use which eliminated the palms. Unfortunately they were lost before they could be studied fully to assess their germplasm potential.

Research priorities

A research program focused on the potential genetic value of seedling date palms is recommended and should consider two broad factors. One, there are large stands of seedling date palms which have persisted for hundreds of years. These stands, reproducing naturally within a restricted population, will have produced a wide range of different genotypes for evaluation. Second, seedling date palm stands occur under different conditions of latitude, elevation and climate, as compared to the traditional date growing areas of the Middle East and North Africa. Over time, palms growing under a different set of environmental conditions may, through sexual reproduction, develop traits to cope with such factors as abiotic stress.

It is suggested that research on seedling date palms be directed toward the following purposes:

1. Examine genetic fidelity among seedlings with respect to the mother tree.
2. Evaluate behavior of seedling growth, with respect to chemical and physiological parameters.
3. Determine yield quantity and fruit characteristics of adult seedling date palms.
4. Evaluate behavior of adult palm growth, with respect to chemical and physiological parameters.
5. Examine the resistance of adult seedling date palms to common biotic and abiotic stresses.
6. Explore modern technologies like mutagenesis and genetic engineering for the purpose of genetic improvement.

7. Establish and maintain a germplasm collection of seedling date palms (living or by cryopreservation) with desirable traits. Engelmann (2010) provides a review of the methods of conserving date palm germplasm.
8. Establish a date palm germplasm website.

Conclusion

As the original source of all cultivar dates, seedling dates continue to be present, to varying degrees, in all date-growing areas. As many as 500,000 producing seedling date palms may be growing in Spain, Peru and Mexico, with countless others in North Africa and the Middle East. Seedling date palms represent unevaluated germplasm resources that could hold new forms with superior fruit characteristics, hardiness and tolerance of saline soils as well as resistance to drought, diseases and pests.

Molecular breeding and the engineering of genetically modified date palm cultivars holds significant promise, but investigations should not be limited to the genetic materials in cultivar date palms. Inclusion of seedling date palms in the search for desirable traits will increase the likelihood of discovering superior genetic material to sustain and improve this ancient domesticated tree, and to transform it into a more highly productive genetically modified plant.

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REVIEW ARTICLE

Hybridization in the genus *Phoenix*: A review

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Abstract

The genus *Phoenix* is composed of 14 species naturally distributed in the Old World. This genus comprises the date palm, *Phoenix dactylifera* L., cultivated for its fruits, the dates, while other species are grown for food, ornament and religious purposes. *Phoenix* species were, for these reasons, spread out of their natural distribution area. It is therefore common to find species not naturally sympatric, growing together, in cultivation or in the wild. *Phoenix* species are interfertile and crossing distinct species leads to fertile hybrid offspring (interspecific hybridization). The introduction of a species in the wild generates gene flows leading to the creation of new hybrids and has conservation implications. In cultivation, such crossings may be spontaneous or are the result of artificial pollination, as several reasons impel doing so. Crossing gives rise to beautiful hybrids and is also useful for the conservation of old palm groves threatened by pests. Moreover, artificial pollination of date palms using another *Phoenix* species can be of interest given the metaxenic pollen effects. In addition, this process may have some potential benefits in date palm improvements, by the creation of hybrid cultivars. Thus, an increasing need of hybrid detection and characterization exists, particularly as morphology alone is not sufficient for this task. Besides new methods such as traditional and geometric morphometrics that may bring new clues, the advent of genetic and molecular markers helps to detect hybrids, especially based on the combination of nuclear and chloroplastic data. The application of methods such as near-infrared reflectance spectroscopy is currently under examination to estimate their potential use for hybrid characterization.

Key words: *Phoenix*, Date palm, Hybridization, Metaxenia, Varietal improvement

1. Introduction

The genus *Phoenix* belongs to the Arecaceae or Palmae family. It is composed of 14 species, the most famous being the widely cultivated date palm, *Phoenix dactylifera* (Figure 1a, WCSP, 2013). *Phoenix* palms fulfil various roles, from food and religion to construction and ornamentation (for review see Munier, 1973 and Barrow, 1998). The date palm is cultivated mainly for its fruits but it also provides a favorable environment for the cultivation of other species such as olives, figs, vegetables and so on in oasian agrosystems (Tengberg, 2012). The sap of *Phoenix sylvestris* (Figure 1b) is boiled down to produce a sweet juice (Newton et al., 2013). Several species are used for ornamentation especially *P. canariensis* and *P. roebelenii* (Figure 1c). Leaves of the date palm, *P. canariensis* or *P. theophrasti* are essential for Palm Sunday celebrations in the Christian religion.

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Phoenix species are naturally distributed in the Old World (Figure 2). According to their distribution area and habitat (Munier, 1957; Barrow, 1998; Henderson, 2009), some species may probably be found together in the wild (sympatric areas). Additionally, given the different purposes described above, the species were dispersed out of their natural ranges creating artificial sympatric areas. *Phoenix* species are interfertile and the crossing of two distinct species leads to fertile offspring, most of the time (Corner, 1966; Greuter, 1967; Hodel, 1995; Wrigley, 1995). Interspecific hybridization is the process whereby two distinct species cross, creating hybrid individuals. Gene flow, i.e. the incorporation of a gene from one species to the gene pool of another, is called introgression and is the result of interspecific hybridization.

In this review, an overview of the genus *Phoenix* is given with a focus on the natural and artificial distribution of the species. It is followed by the description of the different kinds of interspecific hybridization events that can occur in the genus. The implication in term of conservation and varietal improvement are discussed. To conclude, the different methods that can be employed to detect and characterize hybrids are described and appraised.

Figure 1. Some *Phoenix* species: (a) *P. dactylifera* (Photo: M. Gros-Balthazard), (b) *P. sylvestris* (Photo: S. Ivorra), (c) *P. roebelenii* (Photo: M. Gros-Balthazard), (d) *P. atlantica* (Photo: Henderson et al., 2003).

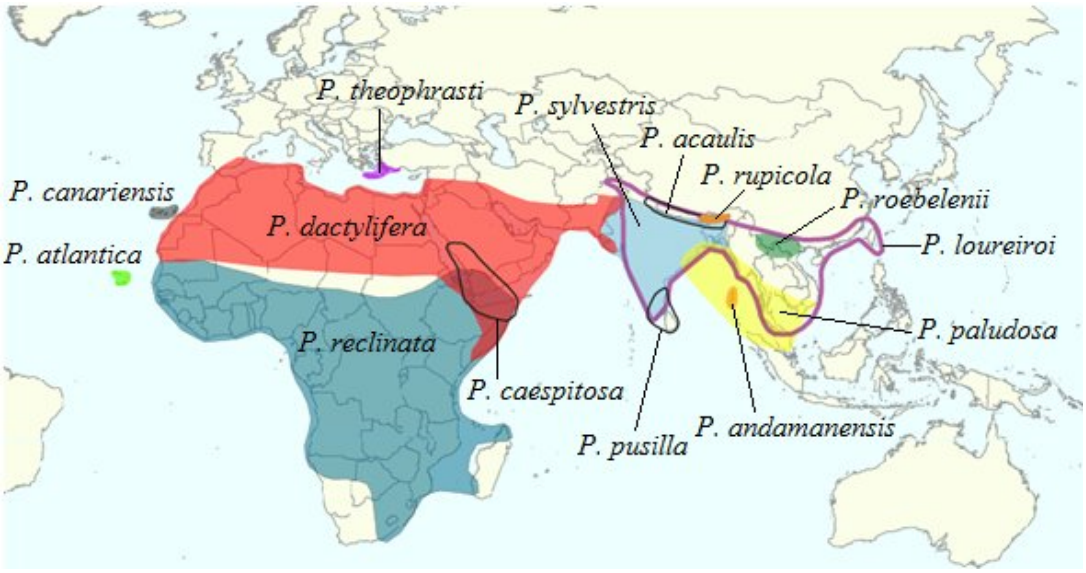

Figure 2. Distribution of the *Phoenix* species.
(Map by M. Gros-Balthazard based on Munier, 1973; Barrow, 1998; Henderson, 2009).

2. Overview of the genus *Phoenix*

The genus *Phoenix* belongs to the Arecaceae (Palmae) family, Coryphoideae subfamily and is the only genus in the *Phoeniceae* tribe. It is composed of 14 species (Govaerts and Dransfield, 2005; Henderson et al. 2006; WCSP, 2013) distributed in the Old World (Figure 2). All species are dioecious with male individuals bearing staminate flowers and female individuals pistillate flowers. The female flower is composed of 3 carpels. After pollination, only one will develop into a fruit while the other two abort. Without pollination, there is no fruit development or sometimes, fruit develops but it is parthenocarpic, that is it does not contain seed, is small and does not reach full maturity. *Phoenix* species share unique morphologic characteristics in the Arecaceae family, making the genus *Phoenix* the most distinctive of all palm genera. Leaves are pinnate; leaflets are V-shaped in cross-section (induplicate) and at the leaf base are modified as sharp spines called acanthophylls. At the molecular level, the genus *Phoenix* also appears highly divergent from other palms but it remains hardly classifiable in phylogenetic analysis (Asmussen and Chase, 2001; Hahn, 2002; Baker et al., 2009).

2.1. Interspecific relationships in the genus *Phoenix*

Within the genus, species are morphologically close to each other, sometimes hardly distinguishable (Barrow, 1998; Henderson et al., 2006; Pintaud et al., 2010). Thus, the number of recognized species has changed during recent decades. In the last monograph, Barrow (1998) identified 13 species. *Phoenix atlantica* (Figure 1d), which is morphologically very close to the date palm, was later distinguished as a distinct species (Govaerts and Dransfield, 2005; Henderson et al., 2006), bringing the number to 14 species. Two other species, supposedly endemic to southern Spain, have been described: *Phoenix iberica* and *P. chevalieri* (Rivera et al., 1997). However, these species are not recognized by the World Checklist of Selected Plant Family of Kew and considered synonyms of *Phoenix dactylifera* (WCSP, 2013).

The interspecific relationships within the genus *Phoenix* remain poorly understood. Based on morphological, anatomical and molecular data (5S spacer sequences), Barrow (1998) identified one clade comprising *P. dactylifera*, *P. sylvestris*, *P. canariensis* and *P. theophrasti*. Another study based on nuclear and chloroplastic microsatellites and minisatellites does not reach this conclusion and although it allows distinguishing species, affinities are still speculative (Pintaud et al., 2010). A new study based on

chloroplastic sequences (psbZ-trnM and rps3-rpl16) permits, for the first time, discussion of the phylogeny and the biogeography of the genus (Pintaud et al., 2013; Gros-Balthazard et al., unpublished data). The so-called date palm clade is identified as comprising *P. dactylifera*, P. *sylvestris*, *P. atlantica*, *P. canariensis*, *P. theophrasti* and *P. rupicola*. The date palm appears most closely related to *P. sylvestris* and *P. atlantica*. Although this study constitutes a great advance, the *Phoenix* phylogenetic relationships remain to be investigated with more variable molecular data such as nuclear sequences.

2.2. Distribution of *Phoenix* species

The genus *Phoenix* likely originates from Eurasia as attested by *Phoenix*-like fossils dating back to the Eocene (Munier, 1973; Dransfield et al., 2008). Today, *Phoenix* species are distributed in the tropical and subtropical regions of the Old World, in southern Europe, Africa and South Asia (Figure 2). They grow in a wide range of habitats, from sea level up to 2,000 meters, but all require moisture around the roots. For instance, *P. reclinata* and *P. roebelenii* grow in seasonally-inundated areas, *P. paludosa* is found in mangroves while *P. dactylifera* grows in oases in hot, arid deserts. According to the different reports of the geographic ranges and habitats of *Phoenix* (Barrow, 1998; Henderson, 2009), it is likely that contact zones between different *Phoenix* occur (Figure 2). For instance, *P. loureiroi* and *P. acaulis* are both reported in Northern India and Nepal in similar habitats (open forest and pine forest) according to Henderson (2009). Additionally, three species (*P. dactylifera*, *P. caespitosa* and *P. reclinata*) are mentioned in southern Arabia and the Horn of Africa (Barrow, 1998). The date palm is reported in mixture with *P. sylvestris* in India, sometimes in the same field (Newton et al., 2013). Nonetheless, it is important to note that the natural distribution of the date palm is unknown as the long history of its cultivation has likely extended its original distribution (Barrow, 1998). The area represented in Figure 2 corresponds to its *historical* or *traditional* cultivation area, where it has been cultivated for centuries or even millennia. Thereby, sympatric areas of date palm and other *Phoenix* should be regarded with caution as they may not be natural. To conclude, as far as I know, no natural sympatric area between *Phoenix* has been noted to date. This may be a reality or an artefact due to lack of intensive research and the difficulty to differentiate species.

Due to their many uses, *Phoenix* species have been dispersed from their original distribution area. As mentioned above, the date palm was introduced in many regions for fruit production, as far as California and Australia. For instance, the date palm was introduced in the Italian Riviera during the Middle Ages for religious purposes (Castellana, 1998). *Phoenix roebelenii* and *P. canariensis* are of great ornamental value and therefore are grown in many botanical gardens around the world (Figure 1c). In this way, species that are not sympatric in the wild are brought into contact; such areas are here referred as anthropogenic or artificial zones of contact. They exist in cultivation, especially in botanical and ornamental gardens such as in San Remo gardens and in the *Phoenix* collections of the U.S. Department of Agriculture in California (Figure 3). Additionally, *Phoenix* spp. may be introduced into the wild where a native species naturally grows. This is the case in the Canary Islands where *P. canariensis* is endemic, whereas the date palm was introduced, probably after the Spanish Conquest (Morales, 2007).

Figure 3. Artificial contact zones between *Phoenix*. (a) *P. dactylifera* and *P. acaulis* in the collection of the U.S. Department of Agriculture in California. (b) *P. theophrasti* and *P. rupicola* in San Remo.
(Photos: M. Gros-Balthazard)

3. Hybridization in the genus *Phoenix*

In the genus *Phoenix*, interspecific barriers appear to be very limited (Pintaud et al., 2010). When *Phoenix* species are brought into proximity, crossings occur (Corner, 1966; Greuter, 1967; Hodel, 1995; Wrigley, 1995). For most crossings, these hybrids appear fertile. However, this is not always the case, as reported by Sudhersan (2010): *P. dactylifera* fruits, obtained after pollination with *P. pusilla*, appear infertile as seed development is arrested at the Khalal stage (Figure 4). The ease with which *Phoenix* hybridize has produced a range of hybrids of different kinds. Crossing between very different species is possible such as the dwarf *P. roebelenii* and the imposing *P. canariensis* (Figure 5).

This ability to hybridize sometimes leads to misinterpretation of species. For instance, the cultivated date palm was thought to be of hybrid origin **(**Munier, 1973**)** as well as *P. atlantica* (Barrow, 1998) while we now know that they are distinct pure species (Henderson et al., 2006; Pintaud et al., 2010**).** On the contrary, many taxa (species or varieties) described are invalid and refer to putative hybrids of *Phoenix* (Barrow, 1998). This is the case of *P. macrocarpa* and *P. intermedia* which refer to hybrids between *P. dactylifera* and *P. canariensis* (Beccari, 1890). Introgression is so common that it is sometimes questionable what the true species is supposed to look like as reported by Bergman (2005) concerning *P. roebelenii*.

Here, we differentiate three types of interspecific hybridizations. First, the possibility of interspecific gene flows in natural sympatric areas is treated. In the second part, the spontaneous hybridization events between *Phoenix* in anthropogenic contexts are addressed. Finally, hybridization events achieved by artificial pollination are discussed.

3.1. Natural interspecific hybridization

As mentioned above, it is possible that contact zones of different *Phoenix* exist (Figure 2) but none has been clearly identified to date. Consequently, gene flows between different species have never been reported in nature. This may be due to an artefact induced by a lack of research to identify natural contact zones. Additionally, *Phoenix* species are sometimes hardly distinguishable and the identification of hybrids is therefore challenging (see section 4, Detection and characterization of hybrids). When species are sympatric, their integrity, that is the avoidance of interspecific gene flows, may also be preserved by temporal isolation. Indeed, even in case of contact, gene flows are impossible if males of a species and female of another do not flower at the same time. Interspecific hybridization events in natural context remain to be carefully assessed with first the identification of natural sympatric areas followed, if existing, by the identification of hybrids or by the processes limiting hybrid occurrence.

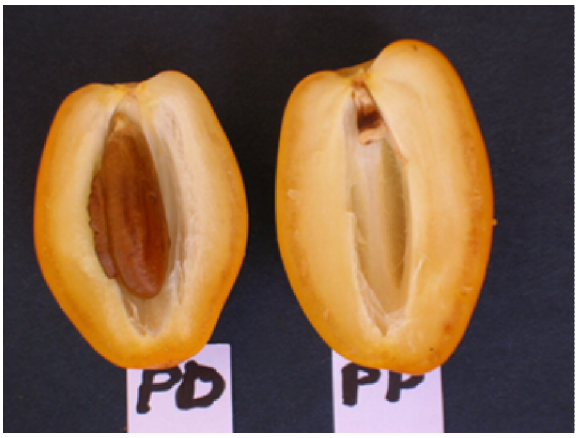

Figure 4. Fruits of Medjool cultivar. On the left, after pollination by *P. dactylifera* (normal seed). On the right, after pollination by *P. pusilla*. The seed appears very small and rudimentary.
(Photo: Sudhersan, 2010)

Figure 5. Hybrid *P. canariensis* x *P. roebelenii* in a garden of Medellin, Colombia.
(Photo: J.-C. Pintaud)

3.2. Spontaneous hybridization in anthropogenic contexts

Spontaneous gene flows are possible if one male of a species and one female of another flower at the same time and are close enough for natural pollination (wind and insect). The female thus bear fruits containing seeds of hybrid origins. This type of spontaneous event is possible in cultivation but also in the wild.

In the Italian Riviera, there are many hybrids (Figure 6) that appear to be adapted to local conditions (Pintaud, comm. pers.). For several years now, the ornamental groves of this region are under threat by the introduced red palm weevil (*Rhynchophorus ferrugineus*). The hybrid populations could be used to regenerate the plantations with resistant genotypes and therefore appear very useful for ornamental palm grove management.

In the wild, spontaneous hybridization events have been noted in the Canary Islands between the introduced date palm and the endemic *Phoenix canariensis* (Figure 7, González-Pérez et al., 2004a; González-Pérez and Sosa, 2009). The authors stipulate that this poses a clear threat to the survival and conservation of the endemic species. Indeed, the genetic purity of the local *P. canariensis* is endangered and introgressed populations are at risk of outbreeding depression (i.e. loss of adaptation) or genetic assimilation. Furthermore, gene flow impacts conservation programs, as not only introgressed population should be the target of conservation programs. In the Cape Verde Islands, the date palm has also been introduced and is a threat to the endemic *P. atlantica*, a subject that remains to be elucidated (Henderson et al., 2006).

3.3. Hybridization by artificial pollination: purposeful hybrids

Purposeful hybrids are obtained by artificial pollination. Manual pollination consists of collecting male inflorescences and pollen and their application to female flowers. Therefore, it may concern two individuals that are spatially close to each other but not necessarily given that pollen may be transported. Moreover, temporal interspecific barriers can also be broken (while this is not the case for spontaneous hybridizations) as the pollen can be stored for months. Several reasons impel artificial production of *Phoenix* hybrids.

The result of interspecific hybridization gives different phenotypes, and crossing of distinct species is a subject of curiosity. Hybrids are unique phenotypes and some may be of great ornamental values, demonstrated by their presence in private and public gardens (Figures 5, 6).

In fruit cultivation, female date palms are manually pollinated using pollen usually from the closest male tree. This improves the pollination efficiency and therefore fruit yield, and decreases the number of males in cultivation (2-3%) therefore giving more space in a plantation to the female fruit-bearing palms (Swingle, 1928). The pollen appears to have an influence on the characteristics of fruit and seed although these tissues are of maternal origin; this is called metaxenia (Swingle, 1928; Sedgley and Griffin, 1989). This is probably due to hormones or soluble substances produced by the embryo or the endosperm or both which diffuse into these maternal tissues and affect them (Swingle, 1928).

Figure 6. *Phoenix* hybrids in Bordighera, Italy (a) *P. canariensis* x *P. dactylifera*, (b) *P. reclinata* x *P. canariensis*.
(Photos: J.-C. Pintaud).

Figure 7. Presumed hybrid *P. canariensis* x *P. dactylifera* in the wild, in the Canary Islands. (Photo: P. Sosa)

Different metaxenia experiments have been carried out. They consist of pollinating different inflorescences of a female with pollen of different males and comparing the resulting fruits and seeds. The physical properties like size and shape may be evaluated, as well as the time of ripening, yield and chemical properties. Most of these experiments employ only male date palms (Nasir et al., 1994; Al-Khalifah et al., 2006; Al-Muhtaseb and Ghnaim, 2006). However, some used the pollen of other *Phoenix* spp. (Nixon, 1928a, 1928b; Ahmad et al., 1962; Sudhersan et al., 2010). Different species have been tested: *P. canariensis* (Nixon, 1928a; Ahmad and Ali, 1960), *P. roebelenii* (Nixon, 1928b), *P. sylvestris* (Ahmad et al., 1962), *P. loureiroi* (Ahmad and Ali, 1960) and *P. pusilla* (Sudhersan, 2010).

Pollen appears to have an effect on the size of fruits (Nixon 1928a; Ahmad et al., 1962). Nixon (1928a) showed that fruits and seeds of Deglet Noor are smaller when pollinated by *P. canariensis*. The same result was observed when the pollen of *P. loureiroi* was used (Ahmad et al., 1962). To the contrary, fruits are bigger when *P. sylvestris* pollen was used (Nixon, 1935). The shape of fruits differed from oval when pollinated by date palm to dumbbell-shaped when pollinated by *P. pusilla* (Sudhersan, 2010). Pollen also affects the shape of seeds (Nixon 1928a). According to Nixon (1928a), the seed color changes when *P. canariensis* is used as a source of pollen, compared to date palm pollen; whereas Sudhersan (2010) did not notice any color change in fruits pollinated by *P. pusilla*. Ripening time is also influenced by pollen. When pollinated by *P. roebelenii*, *P. canariensis*, or *P. pusilla*, fruit ripening is delayed (Nixon, 1928a; 1928b; Sudhersan, 2010) while the fruits ripen earlier when pollinated with *P. loureiroi* (Ahmed, 1962) compared to *P. dactylifera*-pollinated palms. It is important to note that the response to various pollens depends on the cultivar (Sudhersan, 2010). For instance, the pollen of *P. pusilla* induces smaller fruit in Barhee cultivar while they are of equal size in Medjool (Sudhersan, 2010). Very interestingly, the seed stops growth in fruits developed from *P. pusilla* pollen (Figure 4) and this result was observed in the three cultivars tested (Barhee, Medjool, Sultana) (Sudhersan, 2010).

It thus appears that the choice of the species from which the pollen is extracted for pollination is of importance in date palm cultivation. It is possible to have larger fruits (with *P. sylvestris*, Nixon, 1935). Additionally, the possibility to impact ripening time is of interest as it would be possible to speed up or delay the time at which dates are fully mature according to the market demand. Last but not least, the formation of seedless fruits using *P. pusilla* pollen (Sudhersan, 2010) appear very promising as consumers prefer these types of fruits since seeds are hard and inedible.

Date palm propagation is mainly achieved by cloning, which is the planting of offshoots that are clones of the mother plant and therefore give individuals bearing the same fruits. The planting of seeds presents several disadvantageous. Indeed, the sex ratio is 50/50 and the sex identification is possible only after the first flowering at 5 or 6 years of age. Moreover, the fruits are of the same quality or better than the mother plant in only 4% of the individuals (Peyron, 2000). This explains the preferred way of propagation using cloning. However, this decreases the genetic diversity within the species and thus its capacity of adaptation to disease or climate change. The creation and selection of new cultivars presenting great organoleptic characteristics or disease resistance necessitate sexual reproduction. When the date palm is pollinated by other *Phoenix* species, it yields fruits containing hybrid seeds that if planted give hybrid individuals. Interspecific gene flow is a major source of genetic variation that counterbalances the detrimental effects of genetic drift observed in clonally propagated crops (Lynch, 2010). Such gene flows have long been noticed in many crops (Ellstrand et al., 1999), especially perennial crops such as almonds (Delplancke et al., 2012) or grapevine (Myles et al., 2011). Concerning date palm, no hybrid cultivars have been reported so far. However, this could be due to the lack of genetic research involving not only the

date palm, but other *Phoenix* as well. We know that the cultivated date palm is not of hybrid origin but derived from wild populations of the same species (Pintaud et al., 2010; Gros-Balthazard et al., unpublished data); nonetheless, the role of other *Phoenix* species during the diffusion of the phoeniciculture remains to be investigated. Additionally, the potential beneficial role of interspecific gene flows from wild *Phoenix* to the date palm needs to be assessed.

4. Detection and characterization of hybrids

Phoenix hybrids, when spontaneous, are hard to characterize since even distinguishing species is sometimes difficult. Speculation about the parent species is easier when the origin of the seed from which the palm emerges is known as it may be possible to identify the parents. However, in botanical gardens when several *Phoenix* are close to each other, the origin of the seed does not help. Moreover, they could be hybrid of several generations complicating the parental identification. In most situations, the identification of parents is therefore straightforward.

The recognition of hybrids and the characterization of the parent species are of interest for different purposes. In cultivation, some hybrids are of great ornamental value and their characterization could be directed toward interesting interspecific mating. The characterization of hybrids is also important to implement appropriate conservation programs. For example, as previously noted, the endemic *Phoenix canariensis* of the Canary Islands is a threatened species and there is a need to differentiate pure and introgressed individuals in order to target for conservation pure plant while eliminating introgressed ones (González-Pérez et al., 2004a).

4.1. Morphological and morphometric studies

The morphology of hybrids is a mosaic of intermediate parental traits (Rieseberg and Ellstrand, 1993). The size of the trunk, the color and size of the leaves and the display of the leaflets may be useful to identify hybrids. Another criterion is the ability to sucker. For instance, *Phoenix canariensis*-like palms that are suckering may be identified as hybrids given that the pure species is not suckering (Bergman, 2005). In this way, it is likely that botanists or genus *Phoenix* experts can distinguish hybrids of first generation between two very different species, e.g. *P. roebelenii* and *P. dactylifera*. Nonetheless, the criteria listed above are subjective and hybrids of several generations must be even harder to distinguish by morphology alone. Moreover, the variability is important within species, especially in *P. dactylifera* thus differentiating hybrids from ecotypes of a pure specimen based on morphology alone is challenging (González-Pérez et al., 2004a). The characterization using genetic markers of cryptic hybrids *P. canariensis* x *P. dactylifera* in the Canary Islands, while they were morphologically described as pure *P. canariensis*, illustrate the difficulty of differentiating hybrids using morphology alone.

Characterizing hybrids based on morphology should therefore be based on quantitative traits rather than qualitative ones. This is the field of morphometrics. Morphometrics is the study of the size and of the shape of an object. Recently, it was proven that *Phoenix* species might be differentiated based on their seed size (traditional morphometrics) and shape (geometric morphometrics) (Terral et al., 2012; Gros-Balthazard et al., unpublished data). This method appears of great interest since seeds are easily sampled and stored. Studies are currently underway at the Center for Bio-Archaeology and Ecology in Montpellier, France to test the suitability of seed morphometrics to identify the parents of hybrids. First results indicate that hybrids have seeds of intermediate size between their parents (Figure 8). The effect of hybridization on seed shape remains to be investigated. This technique has limitations because only females could be identified using seed morphometrics. Moreover, it is not suitable to characterize hybrids of several generations or hybrids of more than two species.

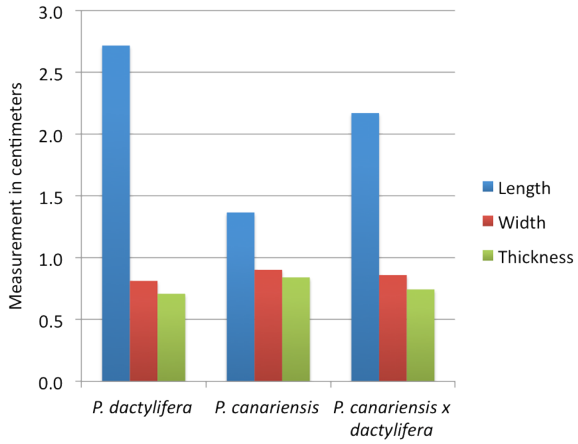

Figure 8. Seed measurement of *P. dactylifera* cv. Deglet Noor, *P. canariensis* and hybrid between *P. dactylifera* and P. *canariensis*. Length, width and thickness where measured over 20 seeds for each and averaged.

4.2. Molecular tools

A wide range of genetic markers are now available to detect hybrids based on DNA variations. They need to be variable between the potential parent species and to allow the characterization of pure species (exclusive molecular markers). For example, González-Pérez et al. (2004b) tried to identify *Phoenix dactylifera* x *P. canariensis* hybrids in the Canary Islands using allozymes but it appears unsuitable as both pure species have the same profile. Using Random Amplified Polymorphic DNA (RAPD) and isozymes, *P. canariensis* and *P. dactylifera* appear to have different profiles and hybrids were detectable (González-Pérez et al., 2004a; González-Pérez and Sosa, 2009).

Recently, there has been increased interest in the development of genetic markers for DNA barcoding in plants (for species and cultivars identification), especially microsatellite markers. These markers are highly variable and therefore suitable for hybrid identification and they have been widely used in the identification of hybrids in crop species such as rice (Gealy et al., 2002). A huge number of microsatellite primers for the date palm were described in recent years (Billotte et al., 2004; Akkak et al., 2009; Arabnehzad et al., 2012). They have not been tested so far for hybrid identifications but if they are transferable to other species (this is the case for microsatellites described by Billotte et al., 2004) they could be applied to achieve this goal. It is important to note that the combination of nuclear and chloroplastic data appears of interest for the following reason. The nuclear genome is inherited from both parents while the chloroplastic genome is maternally inherited. Thus, studying the nuclear profile allows identification of both parent species and reveals the degree of introgression (50% for a hybrid of first generation) while analyzing the chloroplastic genome allows identification of the mother species. There are some ongoing analyses regarding this subject at the Research Institute for Development in Montpellier, France (Pintaud, unpublished data). To conclude, the characterization of *Phoenix* hybrids using molecular markers, especially microsatellites, appears promising as primers are available and this is a lab routine work. However, it is essential to note that if one of the parent species is not included in the analysis, the hybrid cannot be detected. The implementation of hybrids detection through molecular markers thus necessitates inclusion of all potential parent species, or all Phoenix species when there is no prior information about parents and morphology is unhelpful.

4.3. Near-infrared reflectance spectroscopy (NIRS)

Molecular marker determination is expensive and requires hard work. Another possibility to identify hybrids relies on the Near-Infrared Reflectance Spectroscopy (NIRS) method which is less expensive. NIRS is based on the absorption of electromagnetic radiation by matter (Osborne et al., 1993). Leaves of a sample are dried and milled into powder and then subject to NIRS in order to obtain a spectrum. This spectrum is characteristic of individual species (Atkinson et al., 1997) and even varieties in coffee for instance (Downey and Boussion, 1996). The method appears useful for differentiating hybrids of *Betula pubescens* and *B. pendula* (Atkinson et al., 1997) and is being tested at the Research Institute for Development in Montpellier, France for characterization of *Phoenix* hybrids. Initial results appear very promising (Pintaud, unpublished data).

5. Conclusion and perspectives

Interspecific hybridization events in the genus *Phoenix* are very common and in most cases lead to fertile hybrids. The difficulties to distinguish species and their hybrids lead to misinterpretation of species and complication in resolving interspecific relationships.

In the wild and natural context, gene flows have not been reported so far but more research is required. In cultivation, when species are artificially brought into contact, spontaneous hybridizations may be useful, or at least not damageable. These events appear very common, so much so that according to Bergman (2005), to get a pure species, it is necessary to collect seeds in the wild and far from sympatric areas. Hybrids may be of great ornamental and conservational great value. In the wild, spontaneous gene flow from introduced to endemic species threatens endemic species and has conservation implications.

Crossing the date palm with other *Phoenix* is of great interest for its cultivation. Indeed, because of metaxenia, the selection of pollen from other species to pollinate females could improve yields, fruit size and even produce seedless fruits. More research and experiments in metaxenia are necessary to assess the effect of different male genotypes and understand the basis of these effects. This could lead to the selection of male cultivars specific to the pollination of given female cultivars. Moreover, other *Phoenix* appear as genetic

reservoirs and hybrid cultivars could be of great interest in terms of cultivation and disease resistance and therefore more research should focus on it.

The interest in developing methods for detection of hybrids was discussed here. Several methods are available, especially molecular tools such as microsatellites, and others are being developed. This will help understanding hybridization in the genus *Phoenix*.

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REVIEW ARTICLE

Phoenix spp. and other ornamental palms in Turkey: The threat from red palm weevil and red palm scale insects

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Abstract

Phoenix theophrasti is the only palm species native to Turkey and it exists in small numbers in only a few locations along the Mediterranean Coast. Other *Phoenix* species and other palms are mainly grown in Turkey for environmental and ornamental purposes in house gardens, public gardens, parks and as street plantings. The common usage of palms in landscaping highlights their economic importance for the country, especially in coastal tourism areas. Date palms grown in Turkey produce fruits which are small in size, of low nutritional value and with little sugar content due to insufficiently warm temperatures to ripen fruit. Adult date palm trees were imported into the country, mainly from Egypt, until 2005, when imports ceased to prevent further introduction of red palm weevil, *Rhynchophorus ferrugineus*, which has become a serious threat to Phoenix and other palms. The other major insect problem is red palm scale insect *Phoenicococcus marlatti*. Measures are being taken to control these two pests. The limiting of palm imports has stimulated seed propagation of palms within Turkey.

Key words: Datça date palm, *Phoenix dactylifera*, *Phoenix theophrasti*, *Rhynchophorus ferrugineus*, *Phoenicococcus marlatti*

Introduction

Palms (Arecales: Arecaceae) are a plant group of 183 genera and over 2,300 species in tropical and subtropical climatic regions of the world; the genus *Phoenix* has 14 formally described species (Kew, 2013). The palm tree is a symbol often depicting values such as victory, peace and productivity in many cultures throughout history. Today, the palm tree is a symbol of the tropics and of tourism. In Turkey, palms provide some shade but no marketable fruit; they are entirely ornamental and used as decorative plants. The palm trees are mostly planted along the touristic coastline regions of Turkey.

Only a single palm species (*Phoenix theophrasti* Greuter) and a subspecies (*P. theophrasti* spp. Gölköy) are native to Turkey. All of the other palm grown planted in the various provinces, but mainly along the Turkish coast, are exotic.

The purpose of this article is to summarize the current status of *Phoenix* spp. in Turkey, as well as other palms grown for ornamental purposes in the country, and to outline the threats posed by the red palm weevil and red palm scale insect and measures to control them.

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Phoenix spp. in Turkey

Native *Phoenix* palms

In 400 B.C., Theophrastus, the Greek "father of botany," noted the existence of palm trees in Crete. Thus, a second palm species was recorded in Europe in addition to the Mediterranean fan palm (*Chamaerops humilis*), native to the central and western Mediterranean. In 1967, the Swedish botanist Werner Greuter denominated this species in Crete as *Phoenix theophrasti* to honor its discoverer. The palm was subsequently found to occur in ten stands on Crete and nearby islands. It was not until 1982 that *P. theophrasti* was first found on the Datça Peninsula, Turkey by Melih Boydak (Boydak, 1985). In addition another occurrence was recorded in 1986 on the Kumluca coast of Finike Bay, Turkey (Boydak, 1986, 1987). Apparently, *P. theophrasti* also occurs on three islands of the Dodecanese group in the Southeast Aegean area of Greece. Typical common names for *P. theophrasti* are Datça date palm or Cretan date palm. The subspecies *P. theophrasti* ssp. *Gölköy* occurs on the Bodrum Peninsula-Gölköy in Turkey (Boydak and Barrow, 1995; Barrow, 1998; Esener, 1999; Anonymous, 2010). Turkish locations are shown in Figure 1 and photos of the palms in habitat in Figure 2.

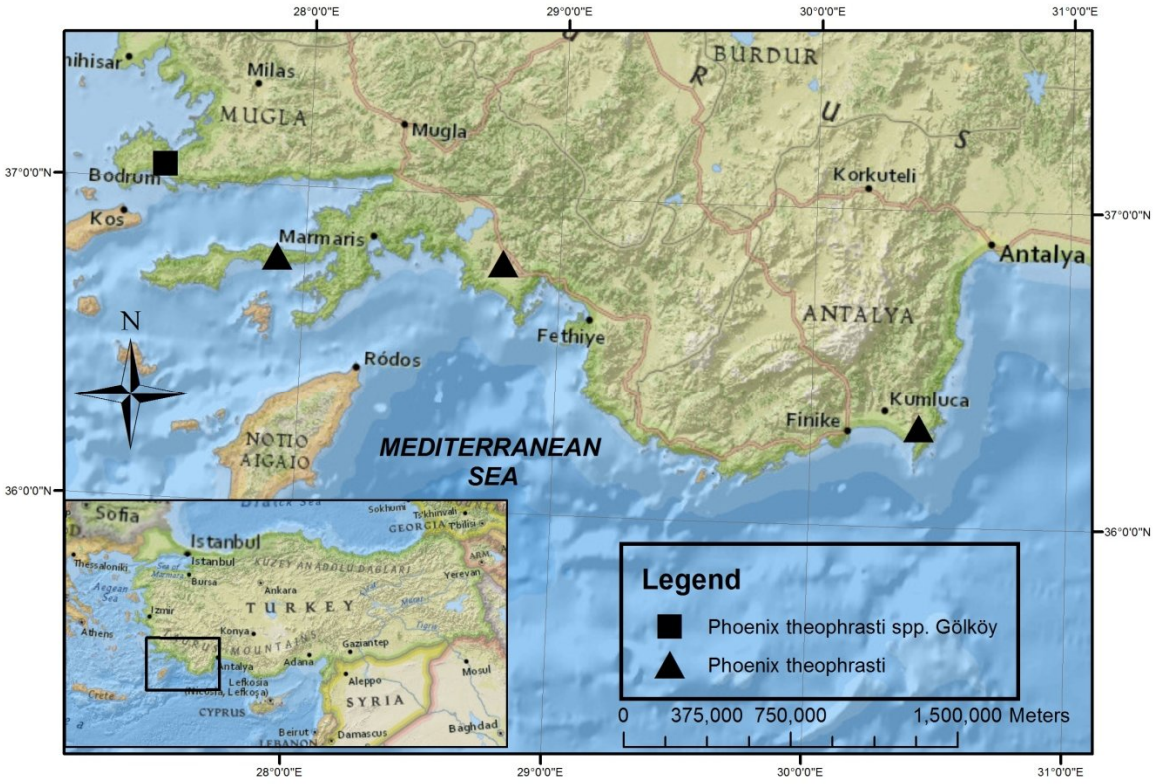

Figure 1.The native stands of endemic palms *Phoenix theophrasti* and *P. theophrasti* ssp. Gölköy in Turkey.

The Datça date palm (*Phoenix theophrasti*) has a clustering stem and can grow up to 17 meters while *P. dactylifera* can grow up to 30 meters. The leaves are composed of smaller, shorter and thicker leaflets when compared to *P. dactylifera.* The tips of the leaflets are pointed and very sharp and can cause a wound when touched. In addition, *P. theophrasti* has obliquely vertical bluish-colored leaves which turn brown when old. The old, dead leaves persist on the tree for a few years before eventually falling off. This species is resistant to fire and, even if all of the offshoots are burned, new shoots are produced. In order to survive in nature, the Datça date palm needs a warm climate and a ground water supply. The Datça date palm is dioecious and therefore there is a distinct difference between the male and female palm. The palm blooms in May and the flower pedicel length grows to 30 cm in female plants which is twice as long as in the male plant. Male plants generate a large amount of pollen which is carried by wind onto female flowers. After pollination, the fruit, which is smaller than *P. dactylifera*, matures in autumn. Immature (raw) fruit is bright orange in color and has an acrid taste. Mature fruit are dark brown in color, soft, and edible but have no commercial value. In Datça, the fruit matures at the end of September and into October.

The Gölköy date palm (*Phoenix theophrasti* spp. Gölköy) has been known to local people for centuries because of the presence of a date palm grove in Gölköy, northern Bodrum province. The first scientific survey of the region in 1988 was done by landscape professors from Ege University in Izmir. In 1990, M. Boydak realized that the Bodrum-Gölköy date palms differed from the *P. theophrasti* in Datça. In a study in collaboration with S. Barrow from Kew Gardens, Boydak determined that the date palm group in Gölköy was either a new species or a subspecies of the Datça date palm (Boydak and Barrow, 1995; Anonymous, 2010). The subspecies has not yet been formally described and published as a valid taxon.

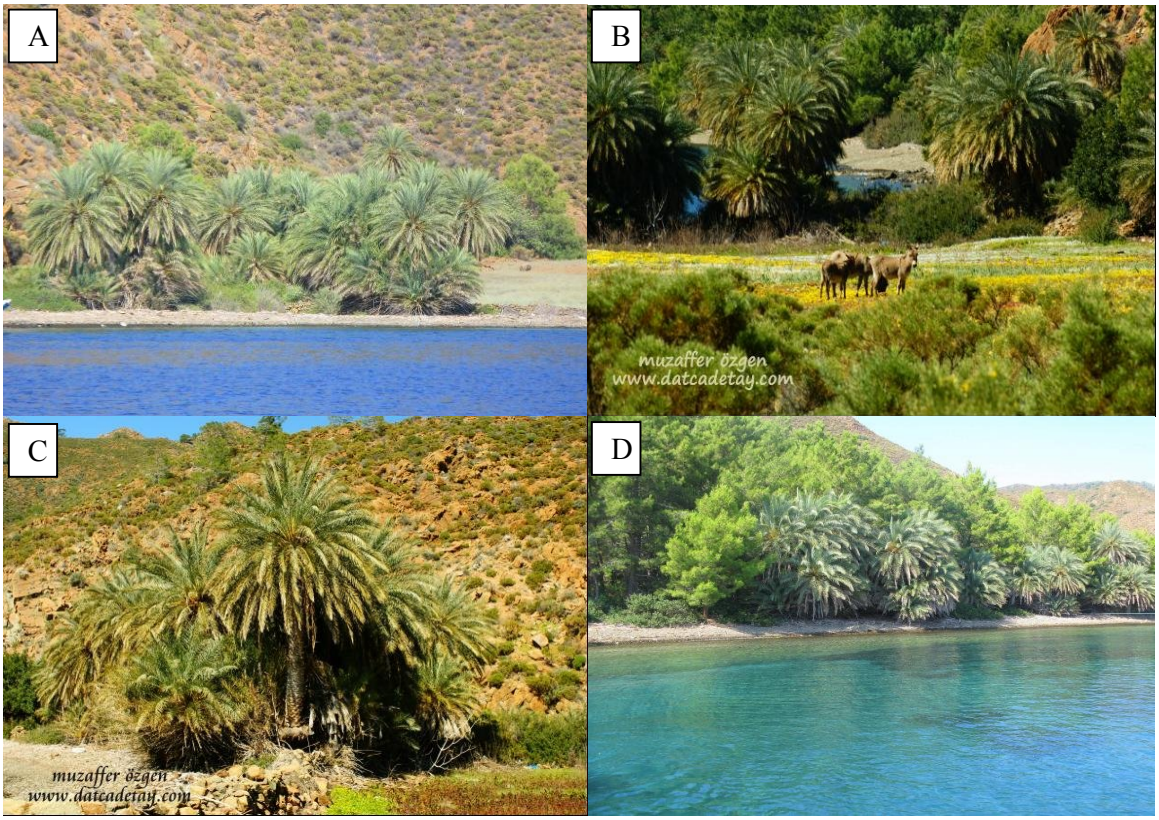

Figure 2**.** *Phoenix theophrasti* in Datça/Hurmalıbük, Turkey.
Source:
(A) www.agaclar.net/forum/agaclar-hakkinda-genel-konusmalar/27930.htm
(B) www.datcadetay.com/dogagezisi/datcahurmalibuk/datcahurmalibuk4.html
(C) www.datcadetay.com/dogagezisi/datcahurmalibuk/datcahurmalibuk5.html
(D) www.agaclar.net/forum/agaclar-hakkinda-genel-konusmalar/27930.htm

The aforementioned study found that the Gölköy date palms grow, at most, to 8 meters while the Datça and Finike date palms reach up to 17 meters. The pedicel length of the Gölköy species is 60-200 cm, while the Datça species bears a 30 cm pedicel. It was also found that the Datça date palms had larger seeds as compared to those of the Gölköy species. A small lake exists in the area where the Gölköy date palms are found. In the dry Mediterranean climate summer months, the palm trees are not water stressed because of the water sources that feed the lake. In winter, the region stands under water for 2 to 3 months (Esener, 1999). For a long time, the palm grove has been under pressure by expansion of the local village and from summer house settlers, chiefly through disrupted water drainage and fire. Endemic date palm trees can still be found in the gardens of the houses in the region. Because of the human population growth in this and the regions, the conservation status of *Phoenix theophrasti* is classified as Lower Risk / Near Threatened, according to the IUCN Red List of 2007.

Table 1. Enumeration of palm trees in Turkey in 2010.

Province	Number of *Phoenix* spp.	Number of other species	Number of young trees	Total
Muğla	13,425	26,090	35,850	75,365
Antalya	35,000	100,000	50,000	185,000
İzmir	9,554	47,015	155,465	212,034
Mersin	5,000	40,000	20,000	63,000
Adana	8,500	30,000	25,000	63,500
Hatay	2,000	5,000	5,000	12,000
Aydın	2,500	15,000	5,000	22,500
Total	73,979	263,105	296,315	633,399

Source: Republic of Turkey Ministry of Agriculture Provincial Directorate of Agriculture Report, 2010.

Introduced *Phoenix* palms

Climatic conditions in Turkey are favorable for growth of all 14 species of *Phoenix*, assuming there is adequate ground water. Coşkuner **(**2006) included eight species of *Phoenix* in his evaluation of palm species suitable for ornamental cultivation in Turkey: *acaulis, canariensis, dactylifera, pusilla, reclinata, robelenii, rupicola* and *sylvestris.* It is highly likely that the remaining accepted *Phoenix* species (*andamanensis*, *atlantica, caespitosa, loureiroi, paludosa*) could also be successfully grown.

The Canary island date palm, *Phoenix canariensis* is found along the Black Sea coastline, in Istanbul, and on the Aegean and Mediterranean coasts. This palm has a thick trunk, long and feathery leaves, and never produces suckers. *Phoenix dactylifera* is another ornamental species found in Antakya, Mersin, Adana, and some other southern provinces. This species has bluish-grey colored leaves and produces suckers in younger palms. The small attractive *P. roebelenii* is only found on the Mediterranean coastline. An enumeration of all palm trees grown in Turkey, carried out in 2010 (Table 1), revealed that *Phoenix* spp. represented 22% of all mature palm trees in the country.

The date palm (*Phoenix dactylifera*) is known in Turkey as the Arabic date palm. In addition to ornamental plantings, about 100 date palms are reportedly being grown for research purposes at the Citrus Research Station in Antalya (Dowson, 1982).

Information about worldwide date palm fruit production indicates that there is commercial fruit production in Turkey. This error can be attributed to language and possibly influenced by history.

Since 1961, FAO has included Turkey as a date palm growing country in their annual production statistics, and the error has been carried over into books such as Date Palm Cultivation (Zaid and Arias-Jiménez, 2002). Possibly related to this misunderstanding, is the fact that during the late 19th Century, Ottoman Turkey recorded date fruit exports. For example, in 1897, dates were ranked 16th in value of exports, amounting to 19.4 million Kuruş (Shaw and Shaw, 1977). Prior to the partitioning of the Ottoman Empire and the creation of the modern Republic, Turkey's, border extended farther south and included an area of known commercial date production, which is now within the boundaries of Syria. Ottoman Turkey date exports may have come from that area.

Language is the more obvious reason for the misreporting of date fruit production from Turkey. In the Turkish language, the noun "hurma" can refer to date palm or to the Japanese persimmon (*Diospyros kaki*). It is clear that persimmon production amounts are being given by mistake to FAO and reported as date production. A recent study of date fruit marketing and consumption in Turkey confirms that all commercial dates must be imported from neighboring date-growing countries because the fruits borne by trees grown in Turkey fail to ripen fruit (Karadeniz, 2010).

Palms in the Turkish landscape

Virtually all of the palm trees found in Turkish landscapes have been introduced. Most commonly they are found along the Black Sea coastline, in Istanbul, and in the Marmara region. A typical landscape palm with thin stems and fan-shaped leaves is the Chinese fan palm (*Trachycarpus fortunei*). This species is produced by tree nurseries in İzmir and sold under the name of "dwarf chamaerops." The former classification name of this species, which was believed to be an interior palm by many growers and landscaping architects, was *Chamaerops fortunei*, hence the common name. In comparison, the dwarf Mediterranean fan palm (*Chamaerops humilis*) has smaller leaves than

Trachycarpus fortunei. The leaves of *C. humulis* are bluish-grey in color and bear small thorns on the pedicel. The tree grows no more than 1.5-2.0 meters in height and has multiple stems. This species is seen on the Aegean and Mediterranean coastlines, at hotels and in seaside residential gardens in Istanbul.

The California fan palm (*Washingtonia filifera*) is another species frequently found along the Aegean and Mediterranean shorelines and in Denizli, but rarely in İstanbul or the Marmara region. The thick stem of this palm is relatively smooth and brown in color and the leaves are large and fan-shaped with thorns on the leaf stalk. A related palm, the Mexican fan palm (*W. robusta)* is grown in the Mediterranean provinces, in Köyceğiz, and Dalaman, but is not found in the Black Sea, Marmara and Aegean regions. This species is taller than *W. filifera* and has a thinner trunk.

In recent years, a popular new species have been imported, the Queen Palm (*Syagrus romanzoffiana*), which has a smooth trunk and pinnate leaves hanging downwards (Anonymous, 2010). All of the abovementioned species are commonly used along streets and in landscaped central parks of many provincial cities. These species are also planted in home and hotel gardens for the same purpose (Esener, 1999).

The preferred ornamental palms in Turkey are *Washingtonia filifera* and *Phoenix canariensis*; followed by *Washingtonia robusta*, *P. dactylifera*, *Trachycarpus fortunei* and *Chamaerops humilis.* Coskuner (2006) determined that a few species were produced from seed in significant quantities locally in and around İzmir province, while the other species were imported. *Phoenix dactylifera* (formerly) and *W. robusta* were the leading species imported. *Washingtonia filifera*, *Phoenix roebelenii, P. canariensis*, *Chamaerops humilis*, *Trachycarpus fortunei*, *Syagrus romanzoffiana, Livistona chinensis, Archontophoenix* spp., *Butia* spp., *Sabal* spp., *Brahea* spp. and *Ravenea rivularis* are other imported species. *Butia capitata*, *P. roebelenii* and *S. romanzoffiana* are produced in limited quantities from seed (Esener, 1999; Coskuner, 2006).

Palm species suitable for Turkey

The number of palm genera throughout the world is 183. According to cold hardiness criteria, 35 genera were determined to be able to grow in Turkey if the other ecological conditions such as soil pH, soil saltiness, air humidity, and wind were suitable. Coşkuner (2006) proposed that 112 species in these 35 genera were suitable for the country as a whole (Table 2.). İzmir province (9a climate zone) on the Aegean Coast is a major tourism area. There Coskuner (2006) suggested the following genera as most suitable: *Brahea*, *Butia*, *Chamaedorea*, *Chamaerops*, *Guihaia*, *Jubaea*, *Livistona*, *Nannorrhops*, *Phoenix*, *Rhapidophyllum*, *Rhapis*, *Sabal*, *Serenoa*, *Trachycarpus*, *Trithrinax* and *Washingtonia.*

Table 2. Exotic palm species suitable for Turkey.

Genus	Species
Acoelorraphe	*wrightii*
Acrocomia	*aculeata, hassleri*
Allagoptera	*arenaria, campestris*
Archontophoenix	*alexandrae, cunninghamiana*
Arenga	*engleri*
Attalea	*cohune*
Bismarckia	*nobilis*
Brahea	*aculeata, armata, brandegeei, calcaria, decumbens, dulcis, edulis, moorei, pimo*
Butia	*archeri, campicola, capitata, eriospatha, microspadix, paraguayensis, purpurascens, yatay*
Caryota	*mitis, obtusa, ochlandra, urens*
Chamaedorea	*cataractarum, costaricana, fragrans, klotzschiana, metallica, microspadix, oreophila, plumose, radicalis, seifrizii*
Chamaerops	*humilis*
Copernicia	*alba*
Dypsis	*decipiens, lutescens*
Guihaia	*argyrata, grossefibrosa*

Table 2. Contd..

Genus	Species
Hedyscepe	*canterburyana*
Howea	*belmoreana, forsteriana*
Jubaea	*chilensis*
Jubaeopsis	*caffra*
Livistona	*australis, carinensis, chinensis, decora, drudei, muelleri, nitida*
Nannorrhops	*ritchiana*
Parajubaea	*cocoides, sunkha, torallyi*
Phoenix	*acaulis, canariensis, dactylifera, pusilla, reclinata, robelenii, rupicola, sylvestris*
Ravenea	*rivularis*
Rhapidophyllum	*hystrix*
Rhapis	*excelsa, humilis, multifida, subtilis*
Rhopalostylis	*baueri, sapida*
Roystonea	*regia*
Sabal	*bermudana, causarium, domingensis, etonia, guatemalensis, maritima, mexicana, minor, palmetto, pumos, rosei, uresana, yapa*
Serenoa	*repens*
Syagrus	*coronata, romanzoffiana, sancona, schizophylla*
Trachycarpus	*fortunei, geminisectus, latisectus, martianus, nanus, oreophilus, princeps, takil*
Trithrinax	*brasiliensis, campestris, schizophylla*
Washingtonia	*filifera, robusta*
Wodyetia	*bifurcata*

Source: Coşkuner, 2006.

Entomological threats to palms in Turkey

The red palm weevil *Rhyncophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae)

The importation of ornamental palms led to the inadvertent introduction of the red palm weevil (*Rhynchophorus ferrugineus*) (RPW) around 2005. Subsequently, plant quarantine regulations were modified to prohibit imports of palms from 37 infested countries. The quarantine stimulated palm propagation within Turkey. Although there have been many difficulties encountered during the production of young palm trees, the entrepreneurs found production easier than attempting to importation palms from pest free countries. Propagation problems are related to slow development of young trees and adaptation problems mainly associated with inadequate or unfavorable ecological and climatic conditions.

The RPW is a serious pest of various palm species (e.g. coconut, *Cocos nucifera* and African oil palm, *Elaeis guineensis*), *Phoenix* palms in particular. The adult beetles are reddish-brown in color, 19-42 mm in length, 8-16 mm in width and have a long, curved rostrum. The head and rostrum comprise about one third of the total length of the insect. The rostrum of females is bare, while in males the dorsal apical half of the rostrum is covered by a patch of short, brownish hairs. Both the male and female adults have dark spots on the upper side of their thorax. Adult females lay an average of 204 eggs. The eggs average 2.62 × 1.12 mm in size and are creamy white, oblong and shiny. The larvae have a brown head and measure 36-47 mm in length and 15-19 mm in width (Figure 3). They have a slightly curved creamy white body composed of 13 segments; mouthparts are well developed and strongly chitinized (Anonymous, 2007, 2008a). The larvae develop within the palm trunk, destroying its vascular system, and eventually cause the collapse and death of the tree, such as in the *Phoenix canariensis* shown in Figure 4.

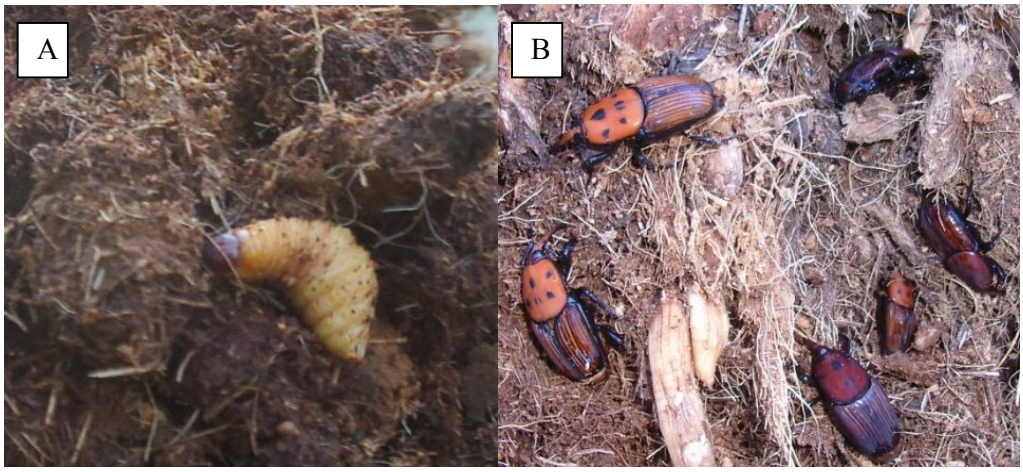

Figure 3. *Rhyncophorus ferrugineus* adults (A), larvae (B).
Photo by: D. Büyüköztürk.

Figure 4. *Phoenix canariensis* infested with red palm weevil, Pissouri village, Cyprus.
Source: http://www.about-pissouri.com/component/content/article/116-charities-directory/4140-charities-news.pdf

Red palm weevil infestation was first detected in Turkey in the summer of 2005 in parks and gardens of Mersin province along the Mediterranean Coast (Karut and Kazak, 2005).

Following discovery of the pest, integrated pest management (IPM) measures were initiated in June 2007 to include destruction and burning of infested plant material, applying prophylactic insecticide chemical treatments and by adult weevil trapping on palm plantations. During 2008-2010, approximately 2,000 infested palms were removed in Turkey. In order to monitor the adult beetles and the extent of pest infestation, traps containing a commercial aggregation pheromone were hung in dense palm tree areas. To reduce the weevil population by mass trapping, traps were placed at 100 m intervals in urban parks, gardens and on streets. The area where the traps were placed was estimated to contain about 640,000 trees in 2008-2010. A significant decrease in the number of trapped beetles and the destruction of infested plant material was observed in 2009 and continued the following years in several cities in Turkey.

A study was conducted by Zeki and Ozkan (2009) in Antalya during 2007-2008. In this study, "Rhyfer®" pheromone + Scandinavian-type trap system was used to monitor the RPW population. The results showed that pheromone + trap system was highly effective in attracting both males and females of the RPW. It was determined that the pheromone + trap system could be used for the survey studies indicating the presence of the pest on palms and cycads; the adult flight activity for its control and monitoring of population activity.

In a study conducted in Adana, natural infestations with the entomopathogenic nematodes (EPN), *Heterorhabditis bacteriophora* (Poinar, 1975) (Nematoda: Heterorhabditidae) on the weevil individuals was evaluated. Seasonal mortality rate in the total larval population of *Rhyncophorus ferrugineus* by the EPN was 50%. EPN caused a significant mortality rate (85%) in the pupae population and also killed a few adults, corresponding to 1-5% of the mortality rate (Atakan et al., 2012).

As to chemical control, pesticides were used in the infested areas as both preventive and curative treatments. Preventive treatments, which included stem spraying up to 2 m high, were implemented from 2008 to 2010 to prevent weevil attacks in highly infested areas. The application of pesticides was conducted each month from March until September with imidacloprid + betacyfluthrin. In the infested plantations, all young, offshoot-bearing trees were treated. Remedial actions against the RPW involved stem injection or soil application with imidacloprid SL 200 or SC 350 at different concentrations (Buyukozturk et al., 2011). According to Plant Protection Technical Instructions Book (Anonymous, 2008b), a pit with 20-30 cm depth is burrowed in the ground where the stem of the tree joins the soil then the systemic insecticide is applied with a dose of 35 ml/tree with 10-20 liters of water. Another application of systemic insecticide is stem injection. For this purpose, 10-15 cm long four holes are drilled in the stem of the tree at 1-1.5 m height from the ground and then 35 ml insecticide/tree is injected into these holes. After the application, the holes are closed with grafting wax. Soil or injection applications should be applied preferably in spring or summer and should be repeated every 25-30 days to prevent new infections.

It has been observed that control efforts including mass trapping, prophylactic and curative pesticide applications, and natural infestation of the EPN are effective in reduction of the RPW populations in palm plantations in Turkey.

There are certain other methods employed around the world to control this pest, such as to drench the base of palm fronds with the entomopathogenic fungus *Metarhizium anisopliae*, or *Beauveria bassiana* (Gindin et al., 2006). The Ecopalm method is another control tool developed in Italy which depends on the sterilization of infested trees by a microwave collar (Yamen Khatip et al., 2010). According to the researchers, the Ecopalm method has given promising results in the fight against the red palm weevil by eradicating all the individuals hosted in the infested trunk, in whatever stage of their development they are found. The microwaves destroy selectively the eggs, larvae, pupae and adults, breaking and interrupting their life cycle completely, without causing any collateral damage to the host palm, which is saved (Yamen Khatip et al., 2010; Anonymous, 2013).

The red date scale, *Phoenicococcus marlatti* Cockerell (Hemiptera: Phoenicococcidae)

The red date scale is native to North Africa and the Middle East and infests the bases of inflorescences or it occupies roots. It affects date palm, other *Phoenix* spp., and has been reported on tropical rattan palm species of *Calamus* and *Daemonorops* (Howard et al., 2001). Adult females are small, spherical-shaped insects with a body length of approximately 1.0-1.5 mm with legs that are reduced or absent. Adult females are red to reddish-brown in color and may be found embedded in a mass of white, cottony wax on plant tissue (Anonymous, 2011) (Figure 5). Wax forms around the body and often completely covers it as curly, shiny, white strands. Eggs are smooth, oval, and pinkish in color. Males and 1st instars of both

sexes have well-developed eyes, antennae and legs. Wax is denser on 2nd instar males, but present in sufficient quantity to completely cover the body of all instar males and females. Prior to emergence as adults, small, wingless, elongated white male cocoons may be visible (Anonymous, 2011). In Turkey, this pest was first detected in Antakya province in 2008 by Doğanlar et al. (2008). It was found in Antalya and Adana provinces during ongoing survey studies.

Red date scale insects usually establish themselves at the base of fronds near the trunk, leaf midribs or fruit stalks. Host plant leaves, stems, trunk, fruits, as well as exposed and underground roots may also be infested (Figure 6). Extensive damage may occur and be undetected until pruning occurs (Anonymous, 2011). Premature leaf aging, drying of the fruit, disruption of normal plant metabolic functions and even plant death may result from heavy infestations (Zaid et al., 2002). Cultural practices are recommended as control methods of this pest. For that purpose, cutting and burning of the infested leaves and applying a deep pruning in order to expose the base of the leaves and the pests at the base directly to sunlight are recommended.

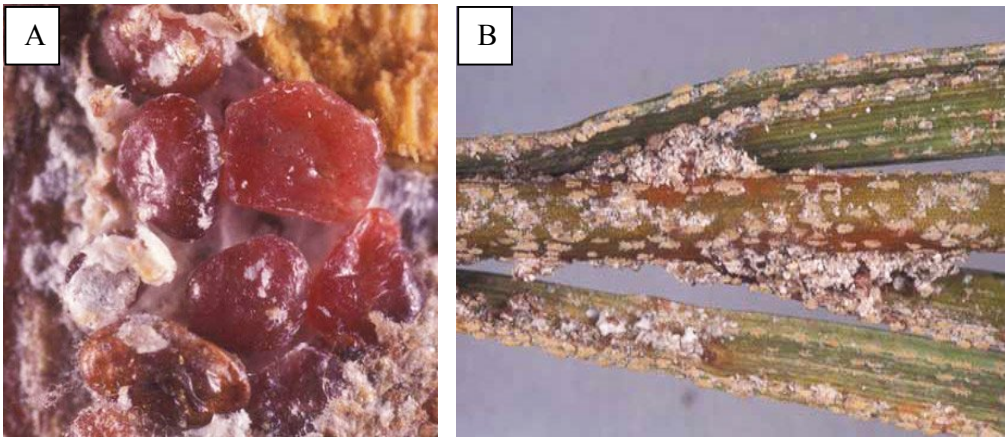

Figure 5. (A) Reddish-brown body of adult female red date scale insect.
(B) heavy infestation of red date scale insect, *Phoenicococcus marlatti* (Cockerell).
Photograph by Lyle J. Buss, University of Florida

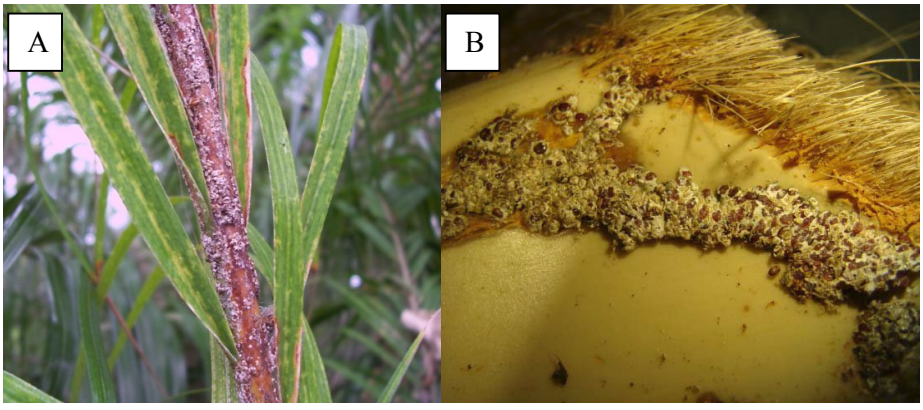

Figure 6. (A) Red date scale insect colony on the base of a date palm leaf pedicel.
(Photo by M. Doğanlar).

(B) Red date scale insect on Pygmy Date Palm.
Source: http://www.bug-master.com/PestAlert/tabid/100/EntryId/14/Red-Date-Scale.aspx.

Doğanlar et al. (2008), found two natural enemies on the nymphs of the pest, one is larvae of an undefined Cecidomyiidae (Diptera) species and the other adults of *Chilochorus bipustulatus* (L.) (Coleoptera: Coccinellidae). Further studies must be conducted on these natural enemies in order to determine their effect on the pest.

As to chemical control, Djerbi (1995) proposed to apply methidathion or dimethoate during the time active nymphs are seen. Zaid et al. (2002) proposed 80-100 g malathion per100 L water or 26 g parathion per 100 L water in infested nurseries (Doğanlar et al., 2008). Horticultural oil and insecticidal soaps also can provide good control, but must be treated like contact insecticides which require thorough coverage and repeat applications (Anonymous, 2011).

Conclusion and Prospects

Exotic and endemic palm species are present in Turkey. Large numbers of exotic species that were introduced to the country by importation are planted for decorative purposes in gardens, parks and along street in many provinces of the country. The endemic species are seldom seen.

Phoenix theophrasti and *P. theophrasti* ssp. Gölköy are of considerable scientific importance to the country's flora because they are the only representations of native palms. These endemic species should be conserved in terms of biodiversity against possible threats such as physical development on private property, tourism, cultivation and also against pests. The planting of imported exotic date palm trees should be forbidden in Datça date palm areas in order to prevent the endemic species from being infested with the red palm weevil, the red date scale, and some other exotic pests that may be inadvertently introduced. This prohibition will also prevent the genetic deterioration of the endemic *Phoenix* species through hybridization. In order to avoid importation, studies should be done by universities and research institutes on the propagation of the endemic and exotic palms adaptable to the country by seed or tissue culture**.** In addition, the planting of these locally-propagated species should be encouraged in fields, parks and gardens.

Preventing the spread of pests has proven difficult on palm trees grown in private and public gardens, in parks and along streets. Close cooperation is needed among the public sector, municipalities, communities and private property owners, so that a plant quarantine system can work properly in order to prevent new entomological problems.

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REVIEW ARTICLE

Application of mycorrhizae in sustainable date palm cultivation

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Abstract

Date palm (*Phoenix dactylifera*) is a significant and developing crop especially in the Arabian Peninsula, the Middle East and North Africa regions. The area under cultivation of this tree is increasing annually. Date palms usually grown under harsh and unfavorable growing conditions with low rainfall and high rates of evaporation as well as in soils with low organic matter and nutrient deficiencies. Hence, date palm cultivation becomes dependent on application of high levels of fertilizers as well as on irrigation. This may lead to salinization of soil and leaching of nutrients to deep soils that might affect ground water. Therefore, it is important that date palm plantations are managed in a sustainable way to reduce the impact of date palm cultivation on ecosystems while maximizing dates yield through using such practices as mycorrhizal fungi technology. The application of mycorrhizal fungi technology is an option that can benefit both agronomic plant health and ecosystems. Mycorrhizae confer numerous benefits to host plants including improved plant growth and mineral nutrition, water uptake, tolerance to diseases and stresses such as drought, temperature fluctuation, metal toxicity and salinity. Mycorrhizae may also play a role in the formation of stable soil aggregates, building up a macro porous structure of soil that allows penetration of water and air and prevents erosion. All of these beneficial effects on plant health and soil fitness mean that mycorrhizae have the potential to increase agricultural productivity and are crucial for the sustainable functioning of agricultural ecosystems. This study provides an insight into the application of mycorrhizae in date palm cultivation.

Key words: Arabian Peninsula, Dry lands, Jordan, *Phoenix dactylifera*, Sustainable agriculture

Introduction

Date palm (*Phoenix dactylifera* L.) is grown widely in hot, dry, desert regions of the Arabian Peninsula, North Africa and the Middle East, and its fruit used as important source of nutrition. During the past three centuries, dates were introduced for cultivation in many parts of the world like India/Pakistan, South America and United States (Chao and Krueger, 2007). Date production is a world agricultural industry producing about 6.8 million metric tons of fruit (FAO 2007). Over 100 million date palm trees are currently grown worldwide on an estimated area of 1 million ha (El-Hadrami and Al-Khayri, 2012). Date palms have been used also for ornamental and landscape purposes in all Arabian Gulf countries and anywhere freezing temperatures are non-existent or of brief duration (Chao and Krueger, 2007). The area of date cultivation in the Arabian Peninsula and the Middle East has increased dramatically during the past few decades and is expected to continue to increase. In United Arab Emirates, for example, there were about 1.5 million date palms in 1971 has increased more than 27-folds to an estimated 41 million date palms in 2005 (MEW, 2005). While in Jordan, the area cultivated with date palms has increased by about 20-folds during the last 15 years (from 108 ha in 1994 to 2200 ha in 2010) (Anonymous, 2011). This expansion may put pressure on limited natural resources (land and water) because date palms are planted under harsh and unfavorable growing conditions, such as low organic matter and nutrient deficiencies in soil, coupled with low rainfall and high rates of evaporation. Therefore, date palm cultivation becomes dependent on high levels of chemicals for production (fertilizers) and for protection (pesticides) from diseases and insects, as well as on irrigation which has led to the salinization of many soils. Hence it is important that new plantations (and even established ones) of

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date palms are managed in a sustainable way to maximize yields and reduce the impacts on ecosystems. The application of mycorrhizal fungi technology is an option that can benefit both agronomic plant health and ecosystems. This paper provides an insight into how mycorrhizae application might benefit date palm cultivation through more sustainable management and the practical use of mycorrhizal technology for date palm plantations.

Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi (AMF) are abundant and available in almost all natural communities and can form associations with over than 80% of higher plants (Smith and Read, 1997). AMF is considered an integral component of plant communities in both natural and agricultural ecosystems (Redecker et al., 2000). The AMF confers numerous benefits to host plants including improved plant growth and mineral nutrition, tolerance to diseases and stresses such as drought, temperature fluctuation, metal toxicity and salinity (Al-Karaki, 2000; Al-Karaki et al., 2004; Borowicz, 2001). Mycorrhizal plants have more nutrient and water uptake from soil in comparison with nonmycorrhizal plants, because AMF develop an extensive network of external fungal filaments (hyphae) that act as an extension of the root absorbing area. Furthermore, AMF may play a role in the formation of stable soil aggregates, building up a macro porous structure of soil that allows penetration of water and air and prevents erosion (Jeffries et al., 2003). All of these beneficial effects on plant health and soil fitness mean that AMF are crucial for the sustainable functioning of terrestrial ecosystems. The benefits of inoculating a wide array of agronomic plant species with AMF have been documented in numerous studies including date palms (Al-Whaibi and Khalil, 1994; Bouhired et al., 1992; Jaiti et al., 2007; Al-Karaki, 2000; Al-Karaki et al., 2004).

Occurrence of mycorrhizae in date palms

Since date palm grows mainly in areas that are characterized by harsh and unfavorable growing conditions, such as in soils with low organic matter and nutrient deficiencies coupled with low rainfall and a high rates of evaporation, mycorrhizal association could be helpful for the plant survival and growth. Many reports indicated that the presence of fungi that form mycorrhizae in date palm plants and their activities which is very important to these plants. One of the earliest observations about the presence of AMF in date palm was in the Crescent desert near Baghdad (Iraq) where it seemed to contribute in the plant mineral nutrition and supply water when it takes the place of root hairs as absorbing structures (Khudairi 1969). Similar observations were recorded for date palm growing in oasis of Qassim, Saudi Arabia indicating the presence of mycorrhiza (Khaliel and Abou-Hailah, 1985). A survey of AMF diversity and date palm tree root colonization in arid areas undertaken in Southwestern Morocco found a total of ten AMF species were trapped from palm groves (Bouamri et al., 2006).

In a preliminary survey conducted in Bahrain in 2008 (Al-Karaki, unpublished data), fresh, young roots collected from around well-established date palm were found extensively colonized by AMF (Figure 1A and B). Six AMF species were identified as present in the rhizosphere soils of date palm trees: *Glomus arenarium, Glomus etunicatum, Glomus versiforme, Glomus fasciculatem, Glomus pulvinatum* and *Acaulospora longula* (Figure 2). The outcome of these observations in regard to the presence of mycorrhizae in date palm could be a general case for this important tree in other areas and hence, this association speculatively increases the tolerance of this species to harsh environments.

How mycorrhizae may benefit date palms?

Many crops could potentially benefit from mycorrhizal symbiosis, although the degree to which plant benefit from mycorrhizae might vary greatly due to variation in plant species dependency on mycorrhizae (Janos, 2007). In the case of date palm, the limited development of the root system (low densities of root hairs), along with field observations of high levels of mycorrhizal colonization, suggest that they benefit greatly from mycorrhizal relationship. It becomes especially more important under harsh climatic conditions prevailing in arid and semi-arid regions like the Arabian Peninsula. The climatic conditions of Arabia are characterized by high temperature, low rainfall, poor soil conditions in terms of nutrients and organic matter and various biotic and abiotic stresses. These conditions may result in loss of indigenous mycorrhizal fungi that reside in the top soil. The combination of poor soil and low nutrient content means that the land is less capable to support the growth of plants without the application of high amounts of fertilizer and water. Although date palms can withstand long periods of drought under high temperatures, large amounts of water are required for vigorous growth, high yield and high quality fruit (Chao and Krueger, 2007). Often, slightly saline water is used for irrigation of crops in hot and dry regions of the world. Even though

drip irrigation is often used in newer plantations, flood irrigation is still highly practiced by farmers in many countries (e.g., Arabian Peninsula), which result in high water consumption and hence high accumulation of salts in the root zone of palms. This might lead to soil degradation and may negatively affect date palm production. Palms are susceptible to nutrient deficiencies (Downer, 2004). Large amount of mineral fertilizers (e.g., N, P) is added annually to the soils in order to enhance establishment of young plantations and/or adult trees to attain high yields and good quality of dates (Barreveld, 1993). However, much of the P and N may not be taken up by plants and thus large amounts are leached down escape into the ground water. This has led to environmental concerns which require attention.

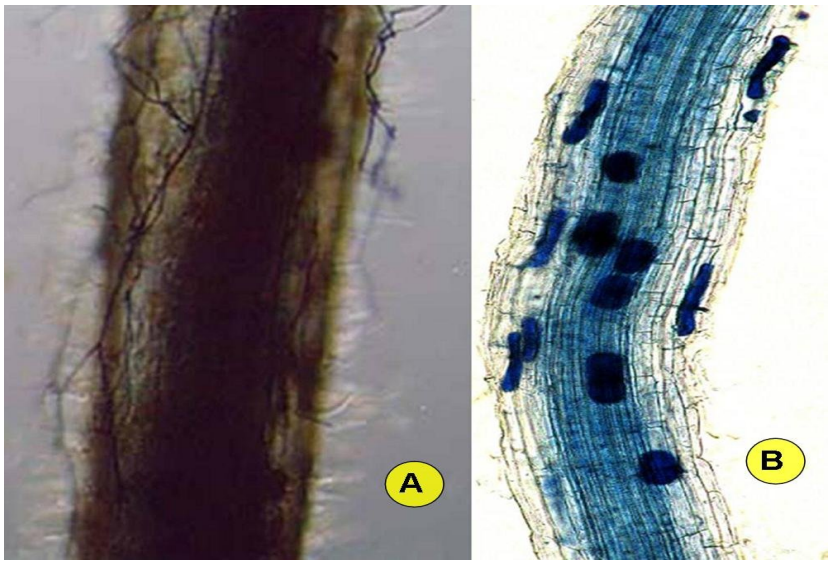

Figure 1. A: Stained root of date palm with associated soil mycelium of AMF. B: Stained root of date palm with intercellular mycelium and arbuscules of AMF.

Figure 2. AMF species found in the rhizosphere soils of a palm date in Bahrain. A: *Glomus arenarium*, B: *Glomus etunicatum*, C: *Glomus versiforme*, D: *Glomus fasciculatem* E: *Glomus pulvinatum* and F: *Acaulospora longula.*

Worldwide, there are efforts to include AMF technology into the processes of agricultural production. AMF may have the potential to make date palm cultivation more sustainable and reduce the impacts of ecosystems on their plantations. Since AMF could form an enormous hyphae network system in the rhizosphere, it could enhance the stability of soil aggregates, fix dune, and improve soil conditions physically and chemically (Bearden and Petersen, 2000). Some scientists explained the role of AMF as enhancement of establishing plants in soil by increasing the plant resistance to environmental stresses, enhance nutrient uptake and improe soil quality (Al-Karaki, 2000; Jeffries and Barea, 2000). Also, a main benefit of mycorrhiza is that the root-fungus network in the soil builds an excellent soil structure through glomalin, improving water storage as a reserve during times of no irrigation (Sieverding, 2008). Through mycorrhiza, roots are kept healthy and the soil is well structured helping plants resist short periods of water stress. In fact, AMF is considered a method for saving irrigation water through enhancing the efficient use of applied water (Sieverding, 2008).

Relatively few reports have been published on the interaction of AMF and date palm. Inoculation of date palm seedlings with AMF has been reported to enhance their growth (Bouhired et al., 1992) and increased the absorption of both P and K over uninoculated seedlings (Al-Whaibi and Khaliel, 1994). Desert soils are generally low in K, P and N where these ions are enhanced in the presence of mycorrhiza (Al-Karaki et al., 2007). In other palm species, Blal et al. (1990) reported that the utilization of P fertilizer in oil palm seedlings increased by 4–5-folds after mycorrhization. This finding is supported by results of experiments conducted by Blal and Gianinazzi-Pearson (1990) who showed that oil palm seedlings grow well and used phosphate fertilizer efficiently in the presence of AMF. In their study, AMF inoculation resulted in a 2.7–5.6 folds increase in P fertilizer use efficiency.

Organic material addition to soils been practiced in date desert areas as the soils are always lacking in organic matter. However, the uptakes of nutrients, fruit growth and yields of date palms have shown to increase when the chemical fertilizers were applied together with the organic fertilizers (Bacha and Abo-Hassan, 1983). Organic fertilizers are generally compatible with mycorrhizae, whereas phosphorus-rich inorganic fertilizers inhibit the fungi activity (Amaya-Carpio et al., 2009). Phosphate is the form of P that is directly utilized by plants which is usually added as inorganic P. The organic P is made available to plants mostly when hydrolyzed or after its mineralization into inorganic P. Hydrolysis and mineralization of organic P is mediated by the enzymatic activity of phosphatase. It has been reported that enzymatic activity of phosphatase has greatly enhanced in the roots of mycorrhizal compared to nonmycorrhizal plants (Fries et al., 1998; Tawaraya and Saito, 1994). However, studies indicated that AMF can have the ability to acquire N directly from organic sources by both enhancing decomposition of and increasing N capture from complex organic material in soil. Hyphal growth of the mycorrhizal fungi was found to increase in the presence of the organic material, independently from the host plant (Hodge et al., 2001). Therefore, AMF might be able to substitute for low fertilizer inputs in organic farming systems.

Mycorrhizal fungi as bioprotectants in date palms

Date palms are affected with many diseases and pests. Most reported diseases of date palm that can be associated with a pathogen are attributed to fungi (Zaid et al., 2002) and nematode (Eissa et al. 1998). In North Africa, bayoud disease incited by *Fusarium oxysporuym* f. sp. *Albedinis* was found as one of the most serious fungal diseases associated with date palms (Jaiti et al., 2007). The red palm weevil (*Rhynchophorus ferrugineus*) is considered as one the most important pests of date palms in the world especially in the Arabian peninsula (Abraham et al., 1998). It has been found that the chemical treatments are not efficient in controlling these diseases and pests. AMF has been demonstrated to have high biocontrol potential of plants against many pests (Quarles, 1999), especially for plant diseases caused by *Phytophtora* and *Fusarium* pathogens (Vigo et al., 2000). These observations suggest that AMF may affect plant and soil microbial activities by stimulating the production of root exudates, phytoalexins, and phenolic compounds (Morandi, 1996; Jaiti et al., 2007). Some studies indicated that existence of mycorrhiza in plant roots discourages their invasion by some pathogens by producing structures that prevent diseases from entering roots or producing antibiotics that attack diseases (Harrison, 1997). Other, more active, mechanisms are used by AMF to trap and strangle root-feeding nematodes.

Utilization of commercial AMF in date palm cultivation

Due to difficulties at the biological system (cannot be axenically cultured fungus), AMF application in agriculture are not possible until recently, when certain companies have totally or partially achieved a procedure for production of AMF inoculants commercially. However, AMF utilization on a commercial scale in Arabian Peninsula is still very limited because of inappropriate information regarding the advantages of mycorrhiza and of their incompatibility with the application of high rates of fertilizers and pesticides. Therefore, to promote utilization of AMF inoculants, it is important to determine beforehand the optimal conditions compatible with their utilization. BioMyc International Corporation (BioMyc) has registered in UAE as the first company in the region produces a range of commercial AMF inoculants (German BioMyc™ Vital) in a granular form using expanded clay as carrier, well adapted to be used in nursery and field, in different systems of plant production. To promote utilization of these inoculants, BioMyc has set up a wide range of experiments in cooperation with some technical centers in the region [e.g., International Center for Biosaline Agriculture (ICBA)/UAE, and Jordan University of Science and Technology, Jordan] to determine the potential of AMF application in enhancing the survival and development of nursery seedlings and hence the success of plantations under field conditions. The utilization of the commercial inoculant BioMyc in the nursery permits overcoming many problems such as low survival and low growth rate to ensure a better rate of success in plant production and guaranty plant survival along with more growth when transplanting into field. Albers (2009) found that inoculation of tissue-cultured seedlings of date palms with a commercial mycorrhizae inoculum (German BioMyc™ Vital) resulted in larger shoots and roots over that uninoculated seedlings (Figure 3).

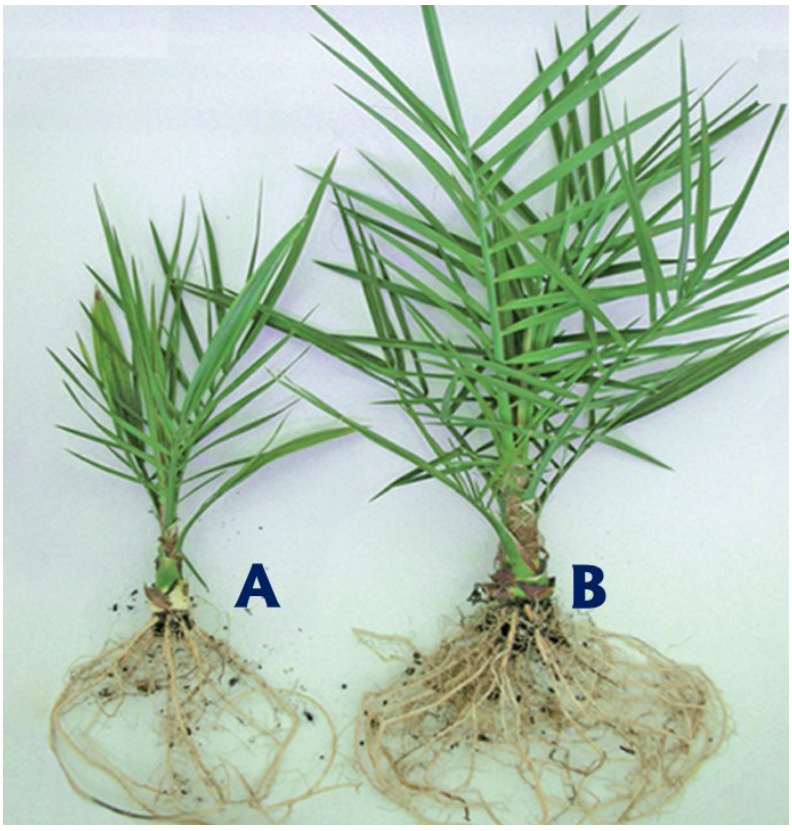

Figure 3. Date palm seedlings inoculated (B) or uninoculated (A) with a commercial mycorrhizae inoculum (German BioMyc™ Vital, Germany).
(Albers, 2009).

Table 1. Plant height and trunk diameter of date palm seedlings as affected by AMF inoculation after 60 days of transplanting.

AMF status	Plant height (cm)	Trunk diameter (mm)
AMF inoculated	30	31
Uninoculated	23	24
Significance	**	**

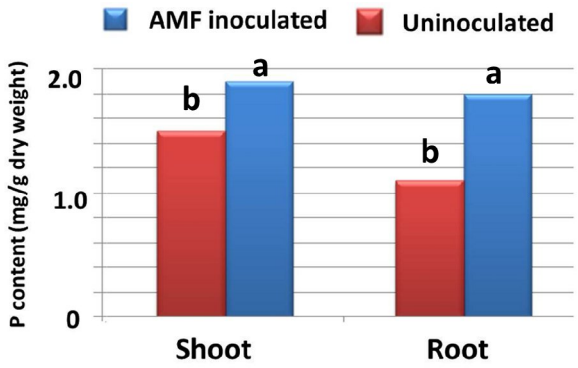

Figure 4. P content of roots and shoots of date palm seedlings after 6 months of AMF treatment. Columns with different letters within a plant organ are statistically different at P = 0.05.

An experiment has been conducted at ICBA to determine the potential effects of inoculation with commercial AMF (German BioMyc™ Vital) on the establishment and growth of date palms at various salinity and fertility levels (Shabbir et al., 2011). Results of this study indicated that growth of plants given low fertilizer rates was higher than plants fertilized with full fertilizer rates over salinity and mycorrhizae levels. However, seedlings inoculated with AMF could grow better than nonmycorrhizal seedlings under saline conditions. Another experiment conducted at Jordan University of Science and Technology (G. Al-Karaki, unpublished data), aimed to study the effects of AMF inoculation (German BioMyc™ Vital) on growth and nutrients uptake of date palm (cv. Mekfazy). A 10x10 cm planting hole was made in the center of the planting pot (20 L plastic pots), putting mycorrhizal inoculum at the bottom of the planting hole and covered with a thin layer of soil. Seedlings were then transplanted exactly at the top of inoculum, to ensure quick root colonization with mycorrhizae. Results revealed that AMF inoculated plants had higher plant height (30 cm) and trunk diameter (31 mm) than uninoculated plants (23 cm and 24 mm, respectively) (Table 1). Bouhired et al. (1992) reported that inoculation with AMF enhanced the growth of date palm seedlings. Moreover, it was found that P content in both roots and shoots of date palm seedlings increased significantly in seedlings inoculated by AMF over those not inoculated, 60 days after transplanting (Figure 4, unpublished data). Thus introducing mycorrhizal inoculum into date palm farming is considered a potentially positive feature in the development of this important tree.

Interaction of AMF and agricultural practices

Agricultural practices such as fertilizer and biocide applications, soil tillage, cropping with a non-host plant species and crop rotation have been reported to affect the potential of AMF for root colonization and hence plant growth. For example, mycorrhizal efficiency in enhancing plants have been found to be too slowed down or inhibited under high levels of P fertilization (Ezawa et al., 2000). Moreover, mycorrhizal development in subsequent crop has been found to be delayed with cropping of a soil with a non-host plant species (e.g. canola) (Gavito and Miller, 1998). Higher soil infectivity with mycorrhizae was reported under no or reduced tillage (Mozafer et al., 2000). Plant dependency on AMF was found to vary considerably from one crop to another which consequently affects the crop requirements of fertilization (Plenchette et al., 1983). For example, under field conditions, leek has a much higher mycorrhizal dependency than wheat. Therefore, plant response to AMF has to be considered when managing a cropping system. The presence of AMF in date palm plants with high root colonization indicate that date palm highly benefit from relationship with this fungi (Bouamri et al., 2006).

Application of AMF in nurseries and plantations establishment

Under normal nursery conditions, young plants do not develop the beneficial mycorrhizal symbiosis. Likewise, when they are planted out into desert soils, their roots may not be colonized with mycorrhizal fungi, and they will grow more slowly, have lower survival rates and lower yields, while needing more water and fertilizer to survive. The introducing of mycorrhizal fungi technology to these plants at early stages of their lives might reverse these problems, resulting in higher plant

establishment, better growth and yields, with complementary reductions in water and fertilizer use (Sieverding, 2008).

In the last three decades, there has been an increasingly high demand in several countries for good quality cultivars of date palm trees. This demand has encouraged use of tissue culture to produce large number of transplants (Awad, 2008). In spite of the great potential of tissue culture techniques for plants, there are great reductions in survival rates (e.g. 40%) of produced plants of some cultivars when planted under field conditions (Zaid and de Wet, 1999). Under these conditions, inoculation with AMF has been considered as an important factor in enhancement of establishment and survival of date plants. However, so far most of the work on AMF effects on date palm has been concentrated on the potential of AMF to improve date palm growth. Schultz (2001) working on oil palm reported that the survival rate of *post vitro* growth ranged between 83-100% after 3 months of *in vitro* inoculation with AMF. While in the non-inoculated plants, only 55% of plants has survived this critical growth stage during the measured experimental period. From the economic perspective, AMF enhancement effects on survival rate and growth of date palms could lead to enormous savings in investment in the nursery (Schultz, 2001). Schultz (2001) also showed that AMF inoculation has positive effects on palm growth (shoot and root), development and plant nutrition (N, P contents). Given the high costs in terms of loss of nursery plants as well as high mortality and slow growth at out planting, experiments are needed to accurately address the saving potential made by AMF inoculation in date palms. Therefore, efficiency of commercial AMF application in date palm nurseries should be evaluated, not only for beneficial growth effects, but also for water-fertilizer comprehensive system designed for date palm production under field conditions.

Application of AMF in established date palm plantations

When mycorrhizae are applied to mature trees grown in the field, benefits of mycorrhizal inoculation become much less apparent, especially when palm trees were heavily fertilized (Al-Karaki personnel observation). This might be attributed to the inhibition of mycorrhizal infection to growing roots by high fertilizer level especially P (Amaya-Carpio et al., 2009). However, as the P levels drop with root absorption as the plant grows, AMF from the surrounding soil will begin to colonize and develop in the growing root system thus negating the need for inoculum. The benefits of AMF application under field conditions with adult trees are clearly less effective to achieve than in the nursery situation with young plants.

Conclusions

There are continuous debates about whether mycorrhizal inoculation has a real potential to improve date palm plantations and especially if growers continue using high levels of fertilizers and pesticides. Nevertheless, the fact that date palm is very responsive to mycorrhizal inoculation. There is a clear rationale for mycorrhizal technology use in the nursery. This is especially important with tissue cultured date palms due to the great benefits of enhancing the health, survivorship and establishment when out planted under challenging environmental conditions. This is coupled with the enormous potential reduction in the overall costs due to avoiding high mortality of tissue cultured plants in addition to complementary reductions in fertilizer and water use.

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REVIEW ARTICLE

Recent achievements in date palm (*Phoenix dactylifera* L.) micropropagation from inflorescence tissues

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Abstract

The Moroccan demand for date palm plants over the next decade is estimated to be 2.9 million. To fulfill this huge demand, the use of rapid micropropagation techniques is an objective of great interest. Generally, date palm micropropagation is performed from offshoot tissue. However, in the case of some rare genotypes, this technique is inefficient because only limited numbers of offshoots are available. To overcome this problem, a new technique based on the use of emerged inflorescence tissue was developed. The research activities resulted in a completely successful process starting from plant material excision to plant acclimatization and field planting. The technique was used for the micropropagation of 16 date palm genotypes with good fruit quality. To date, hundreds of well-acclimatized plants belonging to 9 genotypes have been produced. In addition, clusters of buds of 3 selected genotypes and Medjool cv. were produced and transferred to a private laboratory for mass propagation. In vitro plants needed to test for bayoud disease resistance of 6 selected genotypes were produced using this technique. Since 2001, well-acclimatized in vitro plants were gradually transferred to the field to study their behavior. Inflorescence-derived plants have shown normal growth and no abnormalities. Fruit set of planted palms started in 2005. In the present paper, micropropagation with inflorescence tissue as well as the related major achievements are presented.

Key words: floral explants, in vitro, organogenesis, selected genotypes, vitro plant

Introduction

Date palm, *Phoenix dactylifera* L. is a perennial long-lived, dioecious, monocot which is highly heterozygous. It is considered the key agricultural species in the preservation of oasis ecosystems. Date palm cultivation is also one of the most economically-important activities in the arid zones of the Middle East and North Africa (El Hadrami and Al-Khayri, 2012). In this region, 62 million of the 105 million date palm trees worldwide grow on over 1.2 million hectares. World production of date fruits is approximately 7.5 million metric tons and generates important commercial activities (FAOSTAT, 2011).

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In many countries, date palm cultivation is suffering from serious biotic and abiotic constraints (Sedra, 2011a). In fact, bayoud disease, a soil-borne fungus (*Fusarium oxysporum* f. sp. *albedinis*), is the most dangerous threat to date palm groves in North Africa (Fernandez et al., 1995; Sedra, 2011b). In this region, the disease has destroyed more than 13 million palms in Morocco and Algeria (Sedra, 2011a; Djerbi, 1988). A second important threat is the red palm weevil *(Rhynchophorus ferrugineus* Oliver) which has become the most serious insect pest of date palm in the world (Gómez and Ferry, 1999; Faleiro, 2006). The high rate of spread of this pest is human-caused, by transporting infested young or adult date palm trees and offshoots from contaminated to uninfected areas (Ferry and Gómez, 2002**)**. Among the abiotic constraints, desertification is the most serious factor reducing palm grove production in most of the countries where date palm is cultivated. This phenomenon is aggravated by drought conditions which hamper successful production of the crop. Taking into account those constraints, the need to select palms with high commercial value to replace senescent and destroyed date palm groves has increased steadily in recent decades. Hence,

the exploitation of all available means of date palm propagation is required to meet the huge demand for date palm in vitro plants.

Plant tissue culture techniques have been used to clone a wide range of date palm cultivars worldwide. Using these techniques, date palm can be micropropagated either by somatic embryogenesis in which embryos are produced from embryogenic callus and then germinated to regenerate complete plantlets (Letouze et al., 2000; McCubbin et al., 2000; Fki et al., 2011; Al-Khayri, 2013) or through organogenesis in which plantlets are produced from multiplied buds without passing through the callus stage (Al Khateeb, 2006; Abahmane 2011a). The organogenesis technique is based on exploitation of the meristematic potentialities of shoot tip explants to form new shoots. The growth regulators incorporated into the media are used at the lowest possible concentrations. Since vegetative buds come directly from mother plant tissue, the plantlets produced are identical to the mother tree. However the success of this technique is highly dependent on the success of the first multiplication step (initiation) which requires a well-trained staff. Furthermore, date palm shoot tip explants are sometimes highly contaminated with internal bacteria. These contaminants are introduced in vitro with cultured explants even if they have been well disinfected. Many studies have confirmed the existence of internal bacteria in apparently healthy offshoot tissue. The incidence of this contamination affects 20-50% of cultures (Abahmane, 2011a). Moreover, in the case of some rare or select genotypes, micropropagation is hampered by limited numbers of available offshoots. In fact, some selected genotypes with good fruit quality and presumed resistant to bayoud disease are usually represented by only one or two individual palms. Hence, little plant material (shoot tip explants) is available for development of their micropropagation protocols. In such situations, the use of inflorescence tissue remains the only way to launch in vitro multiplication of those genotypes. The use of this technique permits preserving the multiplied trees, especially when rare genotypes are involved. The plant material is abundant and produced every year. In addition, floral plant material is free from internal bacterial contaminations usually associated with shoot tip derived explants. Recently, interesting research related to this technique has been published (Abahmane 1998, 2003, 2005a, 2005b, 2007, 2010, 2011b, 2011c; Abul-Soad and Mahdi, 2010; Abul-Soad, 2011, 2012; El-Korchi, 2007; Drira and Benbadis, 1985; Drira, 1985; Loutfi, 1989, 1999; Loutfi and Chlyah, 1998). However, more research must be undertaken before routine use of this technique at a commercial level is feasible for date palm mass propagation.

Micropropagation protocols via organogenesis from inflorescence explants

Plant material preparation

Most research on floral explants has been done on plant material excised from immature inflorescences before emergence. However, tissue excised from newly-emerged inflorescences, before spaths open, can be a valuable source of plant material, particularly in the case of rare or selected genotypes of unique palm trees or those of limited populations. In fact, inflorescences are easy to collect and can be removed without serious damage to the mother tree. The inflorescences are harvested during the flowering period, in the Northern Hemisphere from February to April (Figure 1a). The spathe dimensions are genotype-dependent and consequently are variable, measuring 15-25 cm. The florets are 1.5-2 mm in diameter (Figure 1a, c).

Disinfection of plant material can be done in several steps; first, the spathe is immersed for 10 minutes in a fungicide solution containing 3 g/l of Moncozan (mancozeb). Then, it is opened under aseptic conditions and the spikelets carefully immersed for 15 minutes in a solution of sodium hypochlorite 50% (Figure 1b). Finally the spikelets are washed three times with sterile distilled water before transfer onto culture medium. An antioxidant solution (ascorbic acid: 100 mg/l and citric acid: 150 mg/l) can be used to protect the plant material until its inoculation on culture media. Spikelet fragments with at least 2 or 3 flowers were used as explants to start the micropropagation process from inflorescence tissue (Figure 1c).

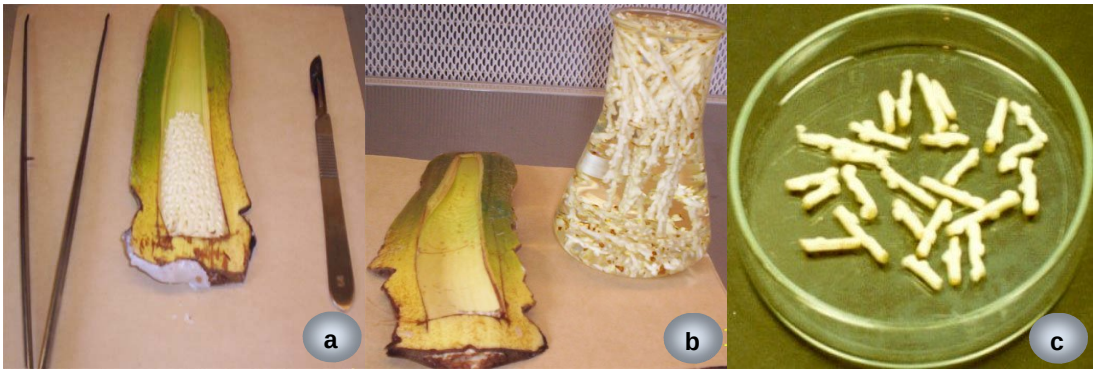

Figure 1. Plant material employed for date palm micropropagation protocols using inflorescence tissue: (a) Emerged inflorescence opened under aseptic conditions, (b) Plant material disinfection in sodium hypochlorite solution, (c) Inflorescence explants (1.5-2 cm) ready to be inoculated on culture media.

Table 1. Culture media used for date palm micropropagation from inflorescence explants.

Media components	Starting stage	Multiplication and elongation stages
Macro elements	Greshoff & Doy (1979)	Murashige & Skoog (1962) (1/2 strength)
Micro elements	Gamborg & Eveileigh (1968)	Murashige & Skoog (1962) (1/2 strength)
Iron source	Murashige & Skoog (1962)	Murashige & Skoog (1962)
Vitamins	Murashige & Skoog (1962)	Murashige & Skoog (1962)
Myo-inositol	100 $mg.l^{-1}$	100 $mg.l^{-1}$
Adenine	25 $mg.l^{-1}$	25 $mg.l^{-1}$
Sucrose	40 $g.l^{-1}$	30 $g.l^{-1}$
Agar	8 $g.l^{-1}$	8 $g.l^{-1}$
PVP-40	2 $g.l^{-1}$	0

Culture medium

After disinfection procedures, floral explants should be transferred immediately to a culture media. According to the multiplication stage, different mineral salts and other compounds are used in culture establishment, bud multiplication and shoot elongation (Table 1).

The culture media were dispensed in test tubes (150 x 25 mm) at 15-20 ml in the starting stage or in culture jars (170 ml baby food jars) at 50 ml in the multiplication stage. Media were autoclaved at 121°C and 1 bar pressure for 15 or 25 minutes according to the culture medium volume in the containers.

Incubation conditions

During the first 6 months of incubation, cultures were incubated under complete darkness to avoid polyphenol oxidation which is catalyzed under light conditions. In the succeeding stages, explants were transferred gradually under illuminated conditions with a photoperiod of 16 hours a day. Light intensity varied from 3,000- 6, 000 lux illumination according to the stage of multiplication. The growth room temperature was maintained at 27±1°C during the illumination period and 22±1°C during the dark period.

Establishment of initial cultures

Research results on plant material from date palm inflorescences have shown that this kind of tissue is very sensitive to growth regulators, particularly auxins. In fact, tolerable concentration of the most used auxins (NAA, 2,4-D and IBA) is about 2 $mg.l^{-1}$. In the case of NOA, a concentration of 1 $mg.l^{-1}$ causes 50% of culture necrosis during the first 3 months of culture. Furthermore, NAA has been shown to be the most effective auxin in bud formation. In the case of cytokinins, 2-iP was more effective than zeatin when combined with BA (Loutfi, 1999). Moreover, the combination of one auxin and two cytokinins was found to be more effective on tissue growth and bud formation (Abahmane, 2005a; Loutfi, 1999; Drira, 1985).

Concerning growth regulator balance, it was shown that high auxin/cytokinin ratios [NOA (0.5 $mg.l^{-1}$), IBA (0.5 $mg.l^{-1}$), BA (0.1 $mg.l^{-1}$)] permitted a high percentage of tissue reactions, but root formation was the most frequent. In the opposite situation [NAA (0.5 $mg.l^{-1}$), BA or 2-iP (2 $mg.l^{-1}$)], budding was obtained either directly from cultured

explants or following floral piece multiplication. Similar results were reported in the literature on inflorescence explants of some Moroccans cultivars (Abahmane, 2005b; Loutfi, 1999).

Regarding sugar concentration, 30 g l^{-1} has shown good growth and morphogenesis of the floral explants excised from emerged inflorescences. However, some authors reported that sucrose at 40 $g.l^{-1}$ was also optimal for growth and morphogenesis (Loutfi, 1989; Drira, 1985). Furthermore, the absence of sucrose in the culture medium leads to culture browning and necrosis. At concentrations of 10-90 g/l, sucrose permits tissue reversion in a proportion of 6-16% when immature inflorescences were used. Moreover, the percentage of cultures showing root formation increased when sucrose concentrations were augmented (Drira, 1985).

Types of observed responses

Different tissue responses were observed on floral plant material cultured in vitro. The kind of tissue reactions were influenced by many factors but mostly by medium composition and the level of tissue differentiation and lignification. In fact, culture media containing low ratios of auxins/cytokinins: NAA (0.5 $mg.l^{-1}$), BA or 2-iP (2 $mg.l^{-1}$) enhance floral piece multiplication, mostly petals, usually followed by shoot formation (Figure 2a,b). Explants showing petal multiplication were about 5% of the total cultured tissue. In contrast, the use of culture media with high auxins/cytokinins ratios: NOA (0.5 $mg.l^{-1}$), IBA (0.5 $mg.l^{-1}$), BA (0.1 $mg.l^{-1}$) leads to root formation (Figure 2c) and carpel development (Figure 2d). The percentage of explants showing carpel development was about 30%. This percentage decreased when cytokinins (BA or 2-iP) concentrations were augmented.

Figure 2. Floral explant responses on culture media used: (a) Shoot initiation, (b) Shoot multiplication, (c) Root formation and (d) Carpel development.

Similar results were reported in the literature. Drira (1981) reported that cultured floral explants, excised from emerged inflorescences taken from date palm trees 50 years of age, produced more roots, and a fast growth of carpels was observed on culture media containing NAA (2.5 10^{-6}M) and BA (5 10^{-8} M). Callus formation was also induced on floral explants excised from immature inflorescences when cultured on media containing 2,4-D (0.5 mg.l^{-1}), IBA (0.5 mg.l^{-1}) and BA (0.2 mg.l^{-1}) (Drira and Benbadis, 1985).

Reversion of date palm floral tissue to a vegetative state can take place under tissue culture conditions in one of the following pathways:

• Induction of inflorescence buds reversion: this method requires the use of floral tissue excised from immature inflorescences before spikelet formation. It has the disadvantage of the limited number of inflorescence buds available per tree (Drira, 1985).

• Induction of floral buds reversion: this method has the advantage of working with floral buds at different stages of inflorescence development (Drira, 1985).

• Induction of vegetative budding from floral parts: it presents the major advantage of working with plant material which is abundant and easy to collect from the mother tree (Abahmane, 2005a, 2007; Loutfi, 1989).

• Regeneration of vegetative buds (or somatic embryos) from callus induced from floral parts (Abahmane, 2010; Loutfi, 1999).

Shoot multiplication

Different culture media have been tested to multiply vegetative buds obtained from inflorescence tissue. Working on inflorescence-derived shoots regenerated from some selected female genotypes, the use of a culture medium containing MS salts at half strength supplemented with NAA (0.2 mg.l^{-1}), 2-iP (0.5 mg.l^{-1}) and BA (0.5 mg.l^{-1}) has attained a satisfactory rate of multiplication varying between 1.5 and 2.2 depending on multiplied genotypes (Figure 3). This culture medium was developed in a previous study on shoot multiplication of some selected genotypes (Abahmane, 2005a). According to recorded data, a different behavior was noticed among genotypes, but in general, the rate of multiplication was beyond 2.2 after the fourth subculture in the case of selected clones INRA-3003 and INRA-B11.

Furthermore, using the developed culture medium, clusters of buds of 3 selected genotypes (INRA-1443, INRA-1445 and INRA-1782) and Medjool cv. were produced and transferred to a private laboratory for mass propagation. From 980 clusters of buds, more than 30,000 vitro plants were produced and acclimatized. This work substantiated the utility of the process developed for date palm mass propagation at a commercial scale.

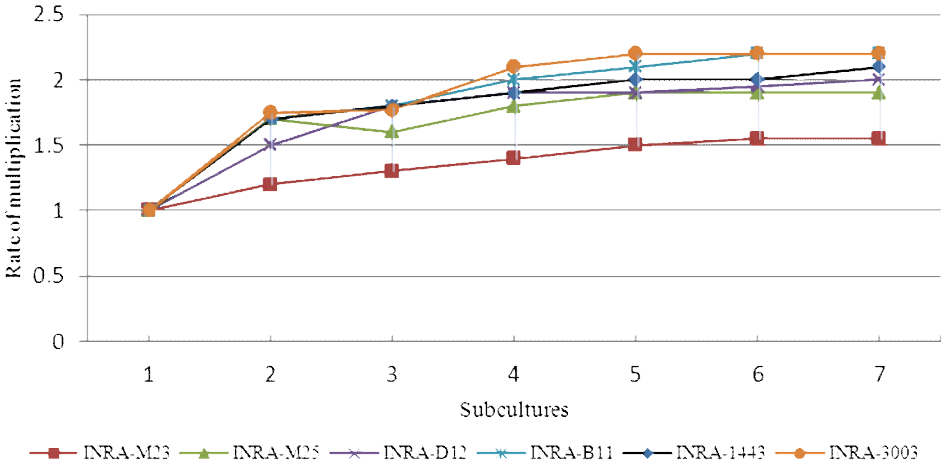

Figure 3. Evolution of multiplication rate of 6 selected date palm genotypes multiplied from inflorescence tissue.

Similar results were reported by Loutfi and Chlyah (1998) in date palm micropropagation from inflorescence tissue. In fact, working on several female cultivars and some palm males, they achieved a satisfactory rate of multiplication of 2 when culture medium containing MS salts supplemented with NAA (0.5 mg.l^{-1}), BA (2 mg.l^{-1}) and 2-iP (1 mg.l^{-1}) was used. However, a diminution in the rate of multiplication was observed after some subcultures. Reduction of BA concentration to 1 mg.l^{-1} permitted sustainable bud multiplication even after many subcultures. In addition, Drira (1985) used a culture medium containing NAA (0.2 - 0.5) mg.l^{-1} and BA (2-3 mg.l^{-1}) to multiply inflorescence-derived vegetative buds of the Tunisian cv. Allig. In contrast, a different behavior was reported in the literature for offshoots derived from bud proliferation. Hence, shoot multiplication (Sukary cv.) was found to be better on culture media containing low hormone concentrations, as new bud formation was promoted. In addition, high concentrations resulted in abnormal growth without any observed signs of budding or shoot formation. According to this study, the best combination that gave a good multiplication rate was (mg.l^{-1}): Kin (0.2), 2-iP (0.1), BA (0.1), IAA (0.1), NOA (0.1) and NAA (0.1) (Al Khateeb, 2006).

Shoot elongation and rooting

At this stage, the use of modified MS salts at half strength supplemented with BA (0.05 mg.l^{-1}) and NAA (0.1 mg l^{-1}) produced well-formed plantlets. Rooting of elongated shoots occurred in parallel with leaf elongation on the previous medium. In the case of immature inflorescences, Drira and Benbadis (1985) reported that well-formed plantlets can be obtained on MS medium supplemented with BA and NAA at 1 mg l^{-1} each, but good rooting of elongated shoots was obtained on culture medium containing BA at 1 mg.l^{-1} and IBA at 1 mg l^{-1}. Furthermore, Loutfi (1999) reported good shoot elongation on culture medium containing MS salts supplemented with 2 mg.l^{-1} NAA, 0.5 to 1 mg l^{-1} 2-iP. In addition, gibberellins (GA3) at 0.01 mg l^{-1} were used to enhance elongation of the shoots obtained.

In the case of shoot-tip derived shoots, the most effective plant growth regulators at the elongation stage are NAA or IBA for auxins and Kin and 2-iPA or BA for cytokinins. A combination of NAA (1 mg l^{-1}), BA (0.5 mg l^{-1}) and Kin (0.5 mg l^{-1}) enhances shoot growth and elongation. Gibberellins at 2 mg l^{-1} can also be incorporated into the culture medium in this stage but for no more than 15 days (Beauchesne et al., 1986).

Among rooting auxins, NAA added at 0.1 mg/l gave the maximum percentage of root formation, numbers and length (Al Kaabi et al., 2001; Taha et al., 2001). They also reported that use of MS salts at ¾ strength gave the best results on root formation as compared to ¼, ½ and full strength. In the same study, light intensity of 8,000 lux and sucrose at 40 g l^{-1} produced the best results in terms of root number and length.

Plant acclimatization

The regenerated plantlets were transferred in plastic bags under greenhouse conditions. The substrate consisted of a mixture of peat moss and small gravel at equal volume (v/v). The greenhouse temperature was maintained at 28 ± 2°C by using heat pumps. The relative humidity was maintained at 70% with a fog system. To increase relative humidity around the newly transferred plantlets, a micro-tunnel covered by transparent plastic was used. Every 2 to 3 days, plantlets were sprayed with a fungicide (Pelt 44) to prevent crown and leaf rot. Under such conditions, plantlets having 2 to 3 leaves, a well formed and closed crown and 3 to 4 roots showed a high survival rate of about 80-90%, depending on the multiplied genotype.

Using this protocol, hundreds of well-acclimatized vitro plants were produced in 9 genotypes: INRA-1007, INRA-1394, INRA-1782, INRA-1443, INRA-1445, INRA-B11 and INRA-3003, INRA-954 and Medjool cv. (Figure 4a,b).

During the acclimatization stage, water supply must be monitored very carefully during the first months. Too much water can lead to plantlet rot and too little moisture in the substrate can decrease the relative humidity around the plants and cause their rapid wilt. After 6-8 months, acclimatized vitro plants were transferred to a shade house for further hardening before transfer to open field.

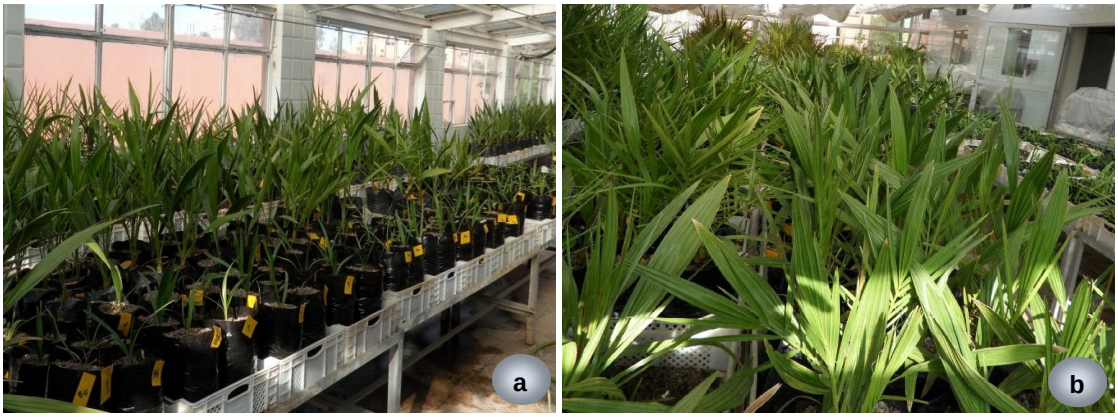

Figure 4. Well-acclimatized inflorescence-derived vitro plants regenerated from genotypes (a) INRA-B11 and (b) INRA-1443.

More details on date palm plantlets acclimatization data were reported previously (Abahmane, 2011a). In fact, the research done on date palm acclimatization has shown that plantlets to be transferred ex vitro should have some important characteristics that enable them to succeed in the greenhouse. Multiplied date palm genotypes produced plantlets of different quality. In fact, some selected genotypes produced vigorous and well-formed plantlets of desired characteristics (INRA-3003, INRA-B11, INRA-1394, INRA-1007) while others produce plantlets of inferior quality (open crown, weak plantlets, etc). In the latter situation, plantlets should be kept in the lab until they acquire the desired characteristics. In fact, the artificial conditions during in vitro culture resulted in formation of plantlets with abnormal morphology, anatomy and physiology (El Bahr et al., 2003; Saker et al., 2000). After ex vitro transfer, they may be easily impaired by sudden changes in environmental conditions and so need a period of acclimatization to overcome these abnormalities (Pospisilova et al., 1999). Accordingly, the acclimatization phase is the most important stage in the protocol of date palm micropropagation, because if not controlled, the whole process can be inefficient. Hence, major differences exist between the environment of plants growing in tissue culture and those in the greenhouse; in particular, differences in light (both quantity and quality), relative humidity, nutrients and other growth promoters, the gaseous composition and the medium substrate (Seelye et al., 2003).

Multiple soil mixtures have been used to transfer plantlets ex vitro. The main mixture characteristic that influences plant growth is moisture, which should not be excessive to avoid fungi attack (rot) and not too low to avoid plantlet desiccation. Tisserat (1981) reported that the best survival rate was recorded for 10-12 cm date palm plantlets transferred ro a mixture composed of peat moss and vermiculite (v/v) and covered with transparent plastic.

Regenerated plants have shown a normal behavior similar to that of shoot-tip derived plants in terms of growth and development. Cultures are usually kept in the lab for 18-24 months and then plant material is renewed. Within this period, no abnormalities were observed. Currently, only field evaluation is used to study genetic fidelity of produced vitro plants. However, some parameters (ploidy level, DNA analysis) must be examined to confirm field observations and confirm true-to-typeness of regenerated plants.

Uses of inflorescence-derived vitro plants

Bayoud resistance tests

One of the main objectives of the development of this multiplication process was the production of vitro plants needed to perform bayoud resistance tests on some selected genotypes. These tests are necessary to complete the studies on selected genotypes, mainly in terms of tolerance to bayoud disease, before their large scale propagation and distribution to farmers. For this reason, 40 well-acclimatized vitro plants, with 4 to 5 leaves and belonging to INRA-1007 and INRA-1394, were transferred to the plant pathology laboratory to complete those tests. This work was done under greenhouse-controlled conditions by artificial inoculation of vitro plants with *Fusarium oxysporum* f. sp. *albedinis* spores. Beyond the tested genotypes, vitro plants of Black Bousthami cv. were used as the

bayoud resistant control and Boufeggous cv. as the sensitivity control.

Currently, vitro plants of four genotypes: INRA-954, INRA-1782, INRA-1443 and INRA-B11 are available and ready to be tested against bayoud disease in the coming months.

Transfer of vitro plant to the field

Vitro plants, at a 1-3 pinnate leaf stage, belonging to INRA-954, INRA-1782 and INRA-1007 genotypes were transferred to the field of the experimental stations in Marrakech (March 2001), Zgora (March 2004) and Errachidia (April 2006). The main objectives of this work were:

- ***Study of agronomic behavior***: Field evaluation of planted vitro plants has shown normal growth in the three localities for all planted genotypes. Growth rate is slightly different among vitro plants in soil according to their origin genotypes (Figure 5). No abnormalities in growth or development were observed after 10 years in the field (Figure 6a). Morphological traits between regenerated plants and their origin trees were similar.

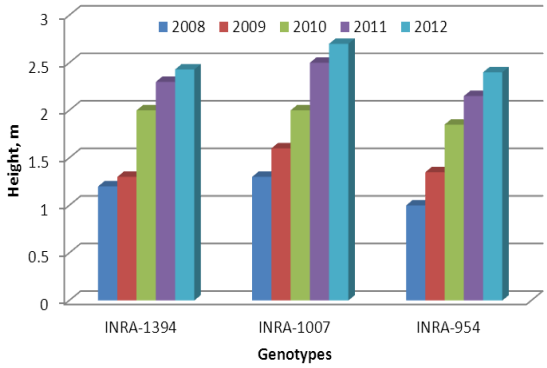

Figure 5. Growth over 5 years, of regenerated vitro plants in the field at Errachidia experimental station.

- ***Evaluation of true-to-typeness***: The vitro plants of INRA-1007, transferred to the field at Marrakech station, fruited for the first time in 2005 (Figure 6b). The vitro plants of the second genotype, INRA-1394, produced fruit in 2007 (Figure 6c). Fruit set of vitro plants transferred to an open field at Errachidia and Zagora experimental stations was observed, respectively, in 2009 and 2011. Actually more than 45% of planted vitro plants have produced fruit. In order to evaluate true-to-typeness of inflorescence-derived plants, their fruits were compared to those of the mother trees. No differences were found between vitro- plant derived fruits and fruit of the mother plants growing in Zagora and Errachidia experimental stations. All fruit traits (color, shape, weight, dimensions) were comparable. Results obtained provide strong indications that the inflorescence-derived plants are true-to-type. A previous study done on root tips of inflorescence-derived shoots, at the multiplication stage, showed that all samples studied were diploid (2n = 26). The regenerated plantlets have the same number of chromosomes as the mother trees (Loutfi, 1999).

- ***Study of planted vitro plants resistant to bayoud disease***: This work was undertaken at Zagora station which has soil which is highly contaminated with bayoud fungus. After 7 years in contact with fungus spores, no symptoms of bayoud disease were observed on planted vitro plants, suggesting bayoud tolerance of the genotypes studied. Bayoud tolerance confirmation must be done using artificial inoculation by fungal spores.

- ***Enrichment of date palm gene banks***: Two field gene banks were established at the Zagora and Errachidia experimental stations. They contain all selected date palm genotypes for their fruit quality and tolerance to bayoud disease. However, some interesting genotypes were lost because of their high sensitivity to bayoud disease. So, two other gene bank collections were newly-created at Saada and Tassaout experimental stations in Marrakech. Those new collections are in areas free of bayoud disease and hence will be suitable sites to preserve bayoud-sensitive genotypes. They were gradually enriched by newly multiplied genotypes. Currently, vitro plants belonging to INRA-1394, INRA-1443, INRA-1007, INRA-954, INRA-1782 and INRA-B11 are available and ready to be transferred to those gene banks (Table 2).

It is worth mentioning that this was the first time in Morocco that hundreds of vitro plants have been produced from inflorescence tissue, and derived plants transferred to the field and fruited. The fruiting palms give good indications of true-to-typeness and consequently attest to the possibility of using this new process for mass propagation of date palm cultivars.

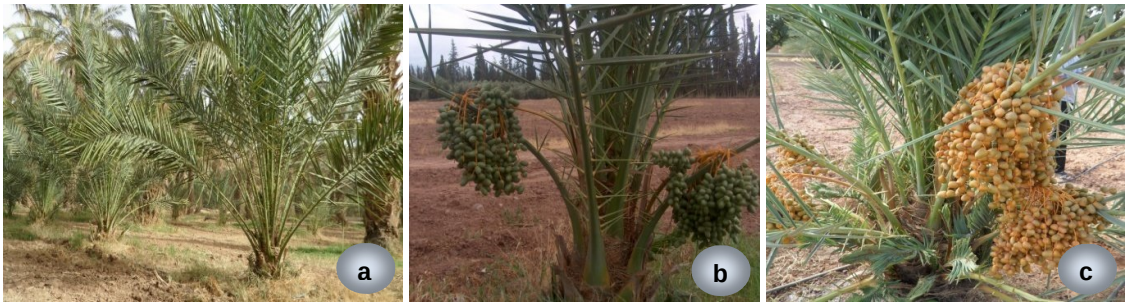

Figure 6. inflorescence-derived vitro plants at INRA experimental stations: (a) Vitro plants of good growth at Zagora station, (b) Fruit set of INRA-1007 vitro plants at Marrakech station and (c) Fruit set of INRA-1394 vitro plants at Errachidia station.

Table 2. Well acclimatized vitro plants regenerated from inflorescence tissue.

Genotypes	Produced numbers at:		Stage
	Research level	Commercial level	
INRA-1007	270	-	Hardening + field [b]
INRA-1394	300	-	Hardening + field [b]
INRA-0954	20	-	Hardening + field [b]
INRA-1782	100	3, 000[a]	Hardening
INRA-1443	150	4, 000 [a]	Hardening
INRA-1445	10	3, 000 [a]	Hardening
INRA-3002	180	-	Acclimatization (Greenhouse)
INRA-3003	500	-	Acclimatization (Greenhouse)
Medjool variety	20	20, 000 [a]	Hardening
TOTAL	1, 550	30,000 [a]	

a Vitro plants produced in partnership with a private laboratory.

b Samples of regenerated vitro plants were transferred to the field.

Conclusions and prospects

The use of emerged inflorescence tissue, as a novel source of plant material, is a powerful alternative method to micropropagate rare or selected date palm genotypes. In addition, the protocol used for inflorescence removal is very safe and does not affect adversely the mother tree. Results achieved make clear the possibility of shoot initiation from this kind of plant material. Shoots obtained can be mass propagated on suitable culture media in the same manner as offshoot-derived buds. Hundreds of complete plantlets were produced and successfully acclimatized under controlled conditions in the greenhouse. Well-acclimatized vitro plants were produced and transferred to the field in three experimental stations in Marrakech, Zagora and Errachidia, in order to study their agronomic behavior at different localities. After more than 10 years in the field, the inflorescence-derived plants have shown normal growth with no observed abnormalities. Moreover, fruits produced were exactly comparable to those of the mother trees. A thousand clusters of buds were transferred to a private laboratory that managed to produce from them about 30,000 vitro plants. All those encouraging results attest to this technique as a promising useful tool for future mass propagation of date palm in Morocco. Additional research is planned, in particular to determine the ploidy of in vitro regenerated plants via the flow cytometry technique.

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REVIEW ARTICLE

Microbial contaminants of date palm (*Phoenix dactylifera* L.) in Iraqi tissue culture laboratories

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Abstract

The date palm is one of the most important economic species of the palm family, grown mainly for its fruits (dates). Nowadays there is increased demand for date palm fruits around the world. To meet this demand, several propagation methods have been utilized, among them micropropagation which has been used in Iraq and many other countries for large-scale multiplication of date palm. Micropropagation faces several constraints; one is microbial contamination which represents a major challenge to the initiation and maintenance of date palm micropropagation laboratories. In recent years, two major groups of contaminants have been identified and isolated from different date palm tissue culture laboratories in Iraq. The first group is fungi. Several fungal species have been isolated and identified as contaminants; most predominant are: *Aspergillus niger*, *Alternaria alternata* and *Penicillium* spp. The second group is bacteria; predominantly of the genera Bacillus, *Staphylococcus* and *Proteus*.

Key words: Date palm, In vitro, Microbial contamination, Tissue culture

Introduction

Date palm is one of the most popular fruit trees in Middle Eastern countries. World production of dates was estimated to be more than 7.5 million metric tons in 2009, from a total harvested area of 1.3 million ha. Most of the world's production of dates is provided by countries in the Middle East and North Africa (FAO, 2011).

Two different explanations have been proposed to explain the meaning of the scientific name of the date palm (*Phoenix dactylifera* L.). The first is from the Phoenician term "Phoenix" which means date palm; while the species name "dactylifera" is derived from the Greek word (daktous) which means fingers (Linne, 1734). The second explanation is based on the mythological Egyptian bird, the Phoenix, said to live for more than 500 years, and then is reborn after a fire. Regarding the species name meaning, this is derived from Hebrew word "dachel" and describes the fruit shape (Popenoe, 1938).

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Nutritional value of dates

Dates are a good source of energy and are rich in nutrients. Date fruits consist of 70% carbohydrates; mostly as sugars, and 15-30% water. Dates are also a source of different minerals including iron, potassium and calcium, with low levels of sodium and fat (Al-Rawi et al., 1967; Thabet et al., 2010; Dayani et al., 2012).

Date palm production and consumption in Iraq

Iraq is one of the top ten date producers in the world; between 1991 and 2001 Iraq contributed a total of 7.5% of world date production (FAO, 2011).

Date palm has been of socioeconomic importance to the population of Iraq for a very long time. Many traditional and new date industries are widely distributed in Iraq, especially in the Middle and Southern regions of the country. Most of these industries are entirely dependent upon dry and soft date types. A majority of the dates produced are consumed locally because of an increased domestic demand for date palm fruit. Not only is the date palm fruit of economic importance, but date palm leaves are used in traditional industries to make products such as mats, fans, baskets and crates (El-Hadrami and Al-Khayri, 2012).

Propagation of date palm

Seed propagation

The oldest method of date palm propagation is by seed, also known as a sexual propagation. This method is not ideal for propagation because of the nature of date palm itself. Date palm is a dioecious species (male and female flowers are borne on different plants), meaning that the half of the progeny is male and the other half female (Jain, 2012). In addition, the quality of fruits produced by female seedling trees is of inferior quality, as compared to clonal plants. Finally, another disadvantage is the heterozygous nature of seedling date palms, which exhibit a high level of variation within progeny (Mater, 1986).

Off shoot propagation

A second method of propagation is by means of offshoots, asexual or vegetative propagation, which is true-to-type to the parent palm and with the capacity of flowering 2-3 years earlier than seedling date palms. The problem with this method of propagation is that each palm produces a limited number of offshoots, borne only at the early stage of life (10-15 years) after planting (Popenoe, 1938; Jain, 2012).

Tissue culture

The third procedure is the tissue culture propagation, also referred to as micropropagation or *in vitro* multiplication (Aaouine, 2003; Al-Khayri, 2005, 2007). The importance of this technique can be attributed to several advantages, including: (1) large scale of production (mass production), (2) no seasonal effect on the propagation procedures, because the entire propagation procedures are done under controlled conditions in the laboratory, (3) production of healthy females (disease and pest-free) and (4) production of genetically uniform plants, i.e. true-to-type (Mater, 1986; Omer et al., 1992; Al-Ghamdi, 1993; Letouze et al., 1998; Al-Wasel, 2001; Kriaa et al., 2011). Date plant tissue culture techniques are employed successfully to clone other economically important palms, such as coconut (*Cocos nucifera*) and African oil palm (*Elaeis guineensis*) (George et al., 2007).

In Iraq, several institutes, both commercial and governmental, are working on micropropagation of elite date palm cultivars, such as Sherafy, Al-Sayer, Hilawi, Khasab, Um Al-Dihin, Barhi, Kantar, Shwaythee, Breem, Alawaidy and Ashkar (Muhsen, 2006; Al-Khalifa et al., 2009; Al-Najm, 2009; Jasim et al., 2009; Al-Meer and Jameel, 2011).

Challenges to date palm micropropagation

Micropropagation of date palm is a difficult procedure and faces numerous challenges. Microbial contamination is rapidly becoming one of the most important constraints, which can occur in any stage of the tissue culture (Leifert and Waites, 1992). Date palm explants are exposed to microbial infection at all stages of tissue culture, contamination coming with the explants themselves, or occurring during the propagation procedures (Al-Mussawi, 2010).

Different explanations have been proposed about the causes of microbial contamination of date palm tissue culture. One explanation is the method used to sterilize the explants, tools and equipment. Improper methods and insufficient amounts of disinfectant are the most common problems in tissue culture. Another problem is external contamination of the explants, which comes from contaminated tools, equipment and workers in the preparing and culturing of media (George, 1993). Composition of the tissue culture medium is a good source of nutrients for microbial contaminants, with all the essential requirements to support their growth and development (Odutayo et al., 2007).

The major adverse effects of microbial contamination on date palm tissue culture are degradation and browning of infected tissues caused by the release of substances into the medium, such as degrading enzymes (cellulase, phenol oxidase and others) as well as toxins (Hameed and Abass, 2006). Microbial contamination leads to wasted time, effort and material, and contributes to severe economic losses. Total losses due to the microbial contamination are about 3-15% in most tissue culture laboratories around the world; chief contaminants being fungal, bacterial and yeast agents (Leifert et al., 1994).

The present review attempts to shed light on the major groups of date palm tissue culture contaminants in Iraq, their identification, negative effects and the best methods to minimize their impact on the growth and development of date palm tissue culture.

Microbial contamination of date palm tissue culture

Two major groups of date palm tissue culture contaminants have been identified in Iraqi date palm tissue culture laboratories: fungi and bacteria.

Fungal contamination

Fungi represent a major group of date palm tissue culture contaminants in Iraq, and this can be explained by their ability to grow anywhere, outdoors or indoors, when sufficient moisture is

available (at least 60% relative humidity) and there is a source of suitable food, which stimulates their growth and development. Therefore, one can expect to isolate fungi from contaminated building materials (wherever there are damp dust particles, paint, wallpaper, carpet and other substrates) (Flannigan and Morey, 1996). Fungal contamination (including the most common fungi and yeasts) cause various types of damage to tissue cultures such as increasing turbidity, changing the pH of the medium, as well as cell destruction (Hameed and Abass, 2006). A survey of fungal contamination of different date palm cultivars during embryogenic callus production, showed that the fungi *Aspergillus niger*, *A. clavatus* and *Alternaria alternata* were the most predominant species as contaminants of six different date palm cultivars, including Um Al-Dihin, Shwaythee, Breem, Barhi, Hilawi and Al-Sayer (Hameed and Abass, 2006). Another two-year study by Abass et al. (2007), showed that the fungi *Aspergillius niger*, *Penicillium* sp. and *Alternaria alternata* were the most commonly isolated contaminants with frequencies of 27, 25 and 18 %; respectively, while the lowest frequency was seen with *Aspergillius terreus*. A recent study conducted by Al-Mayahi et al. (2010) identified different fungal genera from contaminated date palm tissue cultures such as *Aspergillius niger*, *Chaetomium atrobrunneum*, *Penicillium* sp. and *Fusarium* spp.

A summary of all papers published in Iraq (Table 1) indicates that both *Aspergillius niger* and *Alternaria alternata* were the most predominant fungi in the contaminated tissue cultures of date palm. *Aspergillius niger* is a filamentous ascomycete fungus that is ubiquitous in different environments and the most common member of the microbial communities found in soil, air and many other environments (Samson et al., 2002). Hence, its saprophytic activity with a wide range of hydrolytic and oxidative enzymes enables this fungus to grow wherever there is a suitable source of food and moisture (Schuster et al., 2002). The *Aspergillius niger* fungus appears to be a strong contaminant of tissue culture in Iraq.

Alternaria alternata is the second most important contaminant of date palm tissue culture in Iraq. It is a cosmopolitan saprophyte fungus found in soil, plants, as well as in the air (Shelton et al., 2002). Results of a study by Hameed and Abass (2006) revealed a high level of hydrolytic activity of phenol oxidase and cellulase in vitro for several date palm tissue culture fungal contaminants including *Aspergillius niger* and *Alternaria alternata*. For phenol oxidase activity, *Aspergillius niger* showed the highest in vitro activity with a total of 18.60 mm as an activity zone, while the filamentous fungus *Alternaria alternata* showed the highest level of activity of cellulase enzyme with a total of 11.50 mm as a zone of extracellular activity. All of these features along with the saprophytic behavior of both contaminant fungi could be a good explanation for their devastating role on date palm tissue culture in Iraq (Figure 1).

Table 1. Predominant fungal species of date palm cultivars propagated *in vitro*.

Reference	Fungal species	Date palm cultivar
Hameed and Abass (2006)	*Alternaria alternata*	Al-Sayer,
	Aspergillus niger	Barhi
	Aspergillus clavatus	Breem
	Scytalidium lignicola	Hilawi, Um Al-Dihin, Shwaythee
Abass et al. (2007)	*Alternaria alternata*	Al-Sayer
	Alternaria citri	Ashkar
	Aspergillus niger	Barhi
	Aspergillus terreus	Breem
	Cladosporium sp.	Hilawi
	Epicoccum sp.	Um Al-Dihin
	Penicillium spp.	Sherafy, Shwaythee
Al-Mayahi et al. (2010)	*Alternaria alternata*	Alawaidy
	Aspergillus niger	Kantar
	Chaetomium atrobrunneum	Khatraway
	Eurotium amstelodami	Shwaythee
	Penicillium spp.	
	Fusarium sp.	

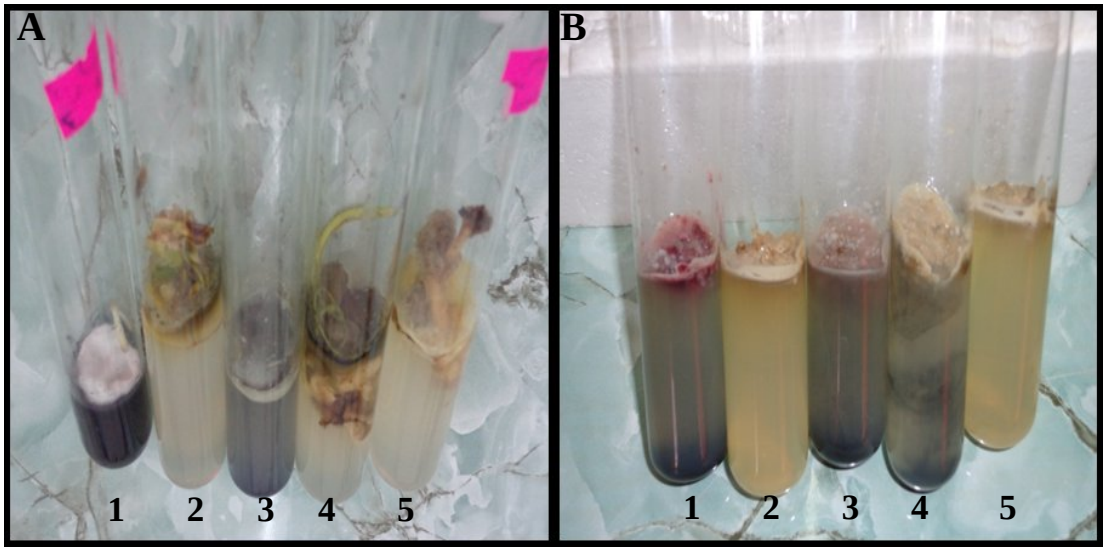

Figure 1. Fungal and bacterial contaminants of date palm tissue cultures.
A) Fungal species:1. *Penicillium* sp.; 2. *Alternaria* sp.; 3. *Chaetomium* sp.; 4. *Aspergillus niger*; 5. *Aspergillus terreus*.
B) Bacterial species: 1. *Staphylococcus aureus*; 2. and 3. *Proteus* sp.; 4. and 5. *Bacillus subtillis*.

Bacterial contamination

Bacterial contamination is an important problem in date palm tissue culture laboratories in Iraq, and is not easy to eliminate by surface sterilization, with some difficulties in determining the source of contamination. Several species of bacterial contaminants have been purified using general standard bacteriological methods and identified by biochemical tests such as Gram stain, motility, gelatinase, oxidation/ fermentation and oxidase (Al-Mussawi, 2010; Al-Dosary et al., 2011). The highest rates of occurrence of different genera of bacteria were reported with contaminated date palm tissue culture. The most unusual bacterial species were found to be *Bacillus subtillus*; *Staphylococcus aureus* and *Proteus* sp. (Al-Hadethy et al., 2007; Al-Mussawi 2010; Al-Dosary et al., 2011). The high percentage of occurrences for the abovementioned species were found to be associated with most tissue cultured date palm cultivars at different stages of micropropagation (Figure 1).

Regarding *Bacillus subtillus*, a gram-positive bacterium with the ability to produce a tough and protective endospore, this bacterium is able to tolerate extreme environment conditions (Nakano and Zuber, 1998). *Bacillus subtillus* is commonly found in different environments, such as soil, the normal human gut, in the air and so on. *Staphylococcus aureus* is widely known to be the second most serious bacterial contaminant of date palm tissue culture (Al-Hadethy et al., 2007; Al-Dosary et al., 2011); it is a gram-positive coccal bacterium, facultative anaerobic and considered as one of the most common skin and nasal passage flora. More than 20% of the human population have been recorded as long-tem carriers of this bacterium (Hong et al., 2009).

The genus *Proteus* belongs to the Proteobacteriaceae family; it is a gram-negative bacterium, and widely known as a contaminant of tissue culture laboratories around the world. Several species has been isolated and identified from contaminated tissue culture samples, such as *Proteus vulgarius* from the laboratories of tissue cultures in Southern Nigeria (Odutayo et al., 2007).

All of the abovementioned cosmopolitan bacteria can be isolated from plant debris, human skin, as well as the tables and walls of tissue culture laboratories (Odutayo et al., 2007). Therefore, different sources of contamination are present in the preparation and incubation rooms, and the indoor and outdoor air of date palm tissue culture laboratories. All of these factors together account for the high isolation frequencies of these bacteria from different laboratories in Iraq. Intensive care should be taken at each individual step of the date palm tissue culture process in order to prevent or avoid contamination.

Table 2. Antibiotic used in date palm micropropagation.

Reference	Type of antibiotic	Concentration mg/L	Date palm cultivar
Al-Kaby (2004)	Nystatin Griseofulvin Streptomycin	25-50	Ashkar
Al-Mussawi (2010)	Gentamycin Streptomycin	50	Al-Sayer Breem Kantar
Al-Dosary et al. (2011)	Amoxicillin Gentamicin Chloramphenicol	50	Al-Sayer Breem Kantar

Preventing and Reducing Contamination

Several approaches from around the world have been used to prevent or reduce fungal and bacterial contamination in date palm micropropagation. Approaches vary according to the incorporation of chemical agents or their tested concentrations that significantly prevent or reduce the growth and spread of microbial contaminants, and allows a substantially normal growth and development of date palm tissue (Hameed and Abass, 2006; Abass et al., 2007).

Different antibiotics and fungicides have been incorporated into the medium to control microbial contaminants (Table 2), sometime in combination, but otherwise alone. Most of chemical agents are effective at preventing bacterial and fungal growth when applied as prophylactics in date palm tissue culture medium, and other types of agents should be used to disinfect explants (Al-Mayahi et al., 2010).

For the selection of antibiotic and fungicide, care must be taken to best understand the effectiveness, regarding the type of antibiotic and fungicide, as well as the concentration. The most effective antibiotic and fungicide should be soluble, stable, suitable for combination treatment, inexpensive, a nonresistance inducer, produce high antimicrobial activity and not be harmful to human health (Al-Kaby, 2004; Hameed and Abass, 2006; Odutayo et al., 2007; Al-Dosary et al., 2011).

Selection of an antibiotic or fungicide is dependent on the type of microbial contamination present in the culture medium. Therefore, proper identification of the contaminant bacteria and fungi is essential and should be followed by preliminary testing for antimicrobial activity of both antibiotics and fungicides at different concentrations to select the most appropriate. Another test which may be necessary and needs to be explored, is the phytotoxicity (side effects) of chemical agents on date palm tissues, before adding them to the culture medium, especially for combination treatments which are more likely to be phytotoxic. Knowledge of the effects and side effects of both antibiotics and fungicides on bacteria and fungi, as well as on the date palm tissue cultures, is a crucial factor for controlling microbial contamination and recovering healthy plants.

Sterilization methods for in vitro cultures

Most of the attention and effort regarding sterilization has been carried out to obtain axenic explants. Several procedures and methods have been utilized in Iraq to avoid microbial contamination and produce contaminant-free cultures in Iraq. Unfortunately, the majority of these procedures are focused on the laboratory, whereas more attention should be paid to the donor plants in the farm field before selection, which is no less important. Treatment of donor plants with contact and systemic chemical agents may be necessary to obtain clean explants. The systemic fungicide Benlate is increasingly seen as a good option to decrease possible internal fungal contamination, either on the farm or in the laboratory. For surface sterilization of date palm explants, sodium hypochlorite (NaOCl) has been widely used as a disinfectant solution in Iraq; concentrations of 20% of NaOCl and 1 ml/l of Tween 20 were used successfully for this purpose (Muhsen, 2006; Al-Khalifa et al., 2009; Al-Meer and Yassen, 2010; Al-Mayahi et al., 2011).

Control of bacterial contamination

Several antibiotics have been used successfully to control bacterial contamination in date palm tissue culture (Table 2). Al-Kaby (2004) showed that the screening of three antibiotics (Nystatin, Griseofulvin and Streptomycin) at concentrations of 25 and 50 mg/l had no obvious effect on the embryogenic callus or somatic embryos of cv. Ashkar. Hence, a significant inhibition role was

seen in their effect to reduce and prevent contamination of date palm tissue culture from 20 % as a total contamination percentage to 0-10 % with antibiotic treatments. Also, treatments of Amoxicillin, Gentamicin and Chloramphenicol gave high protection efficiency for date palm callus. The most important results were seen with the treatment with Chloramphenicol at a concentration of 50 mg/l, which inhibits growth of *Bacillus*, *Staphylococcus* and *Proteus* bacteria, followed by the Gentamicin antibiotic (Al-Dosary et al., 2011).

Control of fungal contamination

Several fungicides have been used in Iraqi tissue culture laboratories to prevent or control fungal contamination. Al-Kaby (2004) showed that a concentration of 0.5-1 g/l of both fungicide Carbendazim and Score succeeded in decreasing the total percentage of contamination without any side effects on the growth and development of date palm tissue culture.

A study by Abass et al. (2007) revealed the positive effect of Benlate fungicide at a concentration of 1 g/l to inhibit the growth of contaminant fungi in vitro. Also, no obvious effects were seen on the callus of different date palm cultivars, such as Ashkar, Al-Sayer, Sherafy and Shwythee. Similar results were seen concerning the neutral effect of Benlate on both the growth and development of date palm tissue culture. Benlate treatment decreased the contamination percentage from 25% in control treatment to 1.65%. Similar findings were observed regarding the activity of Benlate in controlling the most common fungal contaminants including *Aspergillus niger*, *Alternaria alternata* and *Penicillium* spp. (Al-Mayahi et al., 2010).

Conclusion

Microbial contamination remains a serious threat to date palm tissue culture at different laboratories in Iraq. Several fungal and bacterial species have been isolated and identified during all stages of date palm micropropagation. Many practical steps are taken to maintain sterile techniques to greatly reduce the contamination of tissue culture, including use of antibiotics and fungicides. The cost of using fungicides and antibiotics in date palm tissue culture media, especially for large-scale propagation, should be considered along with their potential benefits in controlling microbial contamination and achieving better results.

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REGULAR ARTICLE

A comparative study between solid and liquid cultures relative to callus growth and somatic embryo formation in date palm (*Phoenix dactylifera* L.) cv. Zaghlool

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Abstract

Tissue culture techniques enable mass propagation of elite cultivars of date palm (*Phoenix dactylifera* L.). The main limitations for date palm in vitro multiplication are the low rates achieved using solidified medium and the long period needed to produce acclimatized plantlets. This research focuses on the comparison between different culture types and plant growth regulator (PGR) combinations on callus growth and somatic embryo formation of cv. Zaghlool. For callus growth, 200 mg friable embryogenic callus dispensed in Rasotherm and Phytacon flasks containing 200 ml liquid (100 rotations per minute) and in temporary immersion system, RITA® bioreactor (5 min immersion every 12 h), were compared with cultures grown on 200 ml solidified medium. For somatic embryo formation, 500 mg of friable embryogenic callus grown in Erlenmeyer flasks filled with 50 ml liquid or solid MS medium. The medium was supplemented with 0.1 mg l^{-1} Naphthaleneacetic acid (NAA), 1.5 g l^{-1} activated charcoal (AC) with or without 0.05 mg l^{-1} 6-Benzyl amino purine (BAP) compared to PGR-free medium. Results proved that cell suspension cultures produced the highest callus fresh mass as compared to the other systems tested, and the callus fresh mass reached 4 g after 16 weeks. The temporary immersion system did not significantly enhance the fresh mass of callus compared to the solidified medium. For somatic embryo induction, the number of somatic embryos increased in cell suspensions 6-16 fold compared to the solidified medium. Using liquid MS medium enriched with 0.1 mg l^{-1} NAA and 1.5 g l^{-1} AC gave rise to the highest number of somatic embryos formed from 500 mg initial callus: 160 embryos. The number of somatic embryos was also affected by the callus source. The calli induced from leaflet segments excised from converted somatic embryos resulted in a lower somatic embryo number than those of shoot tip origin with about 60 somatic embryos per 500 mg callus. The formation of somatic embryos using liquid medium required only 6 weeks, thus considerably reducing the previously reported period of 18 weeks which is required for somatic embryo formation using solidified media.

Key words: Cell suspension, Fresh mass, Growth curve, Leaflet segments, *Phoenix dactylifera*, Phytacon, RITA®, Secondary embryogenesis, Temporary immersion system

Introduction

Date palm treeshave high socioeconomic and nutritional value (ElHadrami and Al-Khayri, 2012); their seed propagation resultsin inferior quality. Offshoot use is restricted due to supply in limited numbers (Al-Khayri, 2012). Tissue culture techniques provide an alternative method to large-scale propagation of date palm (Zaid et al., 2011).

Current techniques for micropropagation require a large number of small containers, solid media and aseptic conditions, resulting in high cost of production (Berthouly and Etienne, 2005). The use of solid medium for commercial production is still hampered by low plantlet production rates, high labor cost and more space requirement (Soh et al., 2006). Our previous results on date palm cv. Zaghlool showed that the highest number of somatic embryos induced from initial 100 mg callus was about 7-8 embryos on a solid MS medium containing 0.1 mg l^{-1} NAA and 1.5 g l^{-1} activated charcoal (AC). Furthermore, the somatic embryos required about 18 weeks to develop (Ibraheem et al., 2010).

Liquid media have been used as an efficient method for mass propagation facilitating automation and a reduction in cost and time (Aitken-Christie, 1991; Etienne and Berthouly, 2002). Commonly, using liquid culture for plant propagation has been mainly reported either as cell

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suspension cultures or in bioreactors (Preil, 2005). The advantages of liquid culture systems are: uniform culture conditions, easy media replacement without changing the container, sterilization with ultra-filtration and easier container cleaning after use. In addition, with liquid culture media, containers of different volumes can be used, whereas agar media necessitate surface culturing of tissues (Berthouly and Etienne, 2005).

Cell suspension culture is applicable for efficient mass micropropagation, and provides a versatile tool for various in vitro studies (Al-Khayri, 2005). Numerous secondary metabolites of medicinal uses were detected in cell suspension cultures of various plant species (Preil, 2005; Wilken et al., 2005). Several reports have been published on the establishment of cell suspensions of African oil palm (*Elaeis guineensis*) (de Touchet et al. 1991; Teixeira et al., 1995; Aberlenc-Bertossi et al., 1999). Under the best conditions, the initial weight of cells increased about 4-fold in 1 month (de Touchet et al., 1991). Similarly, many researchers carried out work on cell cultures of different date palm cultivars including cv. Barhi (Bhaskaran and Smith, 1992; Al-Khayri, 2012), cv. Deglet Nour (Fki et al., 2003), cvs. Bousthami noir and Jihel (Zouine et al., 2005; Zouine and El-Hadrami, 2007) and cv. Khalas (Gadalla, 2007).

Cell suspensions cultures were used either for callus growth (Al-Khayri, 2012) or for somatic embryo formation (Fki et al., 2003; Zouine et al., 2005). The productivity of embryogenic cell suspension cultures increased 20-fold (from 10 to 200 embryos per month per 100 mg fresh weightof embryogenic callus) when embryogenic suspension were used instead of agar-solidified media (Fki et al., 2003). Zouine et al. (2005) reported that cell suspension resulted in higher somatic embryo number compared to solid medium; their induction period was reduced by 2 months. Normally the plant growth regulators are reduced or abandoned in the cell suspension cultures to induce morphogenesis leading to plant regeneration. Daguin and Letouzé (1988) reported cell suspension growing on half strength MS medium devoid of plant growth regulators. Yadav et al. (2001) initiated cell suspensions by transferring embryogenic callus to MS liquid medium containing 0.1 mg l^{-1} NAA. Fki et al. (2003) used ½ MS supplemented with 1 mg l^{-1} 2,4-D. Gadallah (2007) reported using ½ MS amended with 0.5 mg l^{-1} 2,4-D. Zouine and El Hadrami (2007) tried ½ MS containing 0.1 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BAP. Understanding the behavior of date palm (*Phoenix dactylifera*) cell suspension growth and differentiation would promote effective utilization for mass micropropagation and various in vitro investigations (Al-Khayri, 2012). In this context, Al-Khayri (2012) recently reported investigating the growth curves of cell suspension and their importance for evaluating its efficiency.

Currently, bioreactors play a crucial role in scaling up the production of somatic embryogenesis and multiplication of clusters of meristem- and bud-based plant micropropagation (Tahardi et al., 2003; Ducos et al., 2007). The temporary immersion system has positive effects on micropropagation as indicated for shoot proliferation in potato (Abdullateef et al., 2009), microtuberization (Jimenez et al., 1999) and somatic embryogenesis for coffee (Ducos et al., 2007). Furthermore, the performance of micropropagated plantlets in temporary immersion system was better during acclimatization over those cultured in solid medium (Berthouly and Etienne, 2005). Immersion time, i.e. duration or frequency, is the most decisive parameter for system efficiency (Etienne and Berthouly, 2002). To meet the increasing demand for date palms, Okere et al. (2010) recommended complementing the tissue culture techniques with temporary immersion bioreactor systems (TIBs) to enhance the commercial production of date palm plantlets. Tisserat and Vandercook (1985) developed an automated plant culture system and investigated it on some plant species and explants and they included date palm callus in their study. Their results showed better callus growth on this system compared to agar medium. The application of temporary immersion systems in date palm tissue culture was applied by Othmani et al. (2009) using the RITA® system (Vitropic-Cirad, France) for either somatic embryogenesis or for shoot proliferation of cv. Deglet Bye. They found that this system was suboptimal for callogenesis and somatic embryo formation; however, better yield of regenerated shoots from the shoot clusters was found with this liquid system compared to the agar-solidified cultures.

To the best of our knowledge there is no published report on the comparison between different liquid culture systems and its effect on date palm callus cultures. Furthermore, there is no specific literature on using liquid cultures for somatic embryo formation of cv. Zaghlool. The aim of this research was to investigate the effect of different liquid culture on the callus growth of date palm cv. Zaghlool and on the number of the induced somatic embryos.

Material and Methods

Plant material and explant preparation

Shoot tips of date palm cv. Zaghlool were separated from healthy offshoots (3-4 years old) of 5-7 kg in weight and about 50-70 cm in height, grown in the Central Laboratory of Development of Date Palm Research at Giza, Egypt.

Outer leaves were acropetally removed, exposing the hearts of the offshoots (15-20 cm length, 6-8 cm width) which were transported to Germany. To prevent browning, the hearts were immersed in a chilled antioxidant solution consisting of 100 mg l^{-1} ascorbic acid and 150 mg l^{-1} citric acid until the time of culture. The outer leaves of the offshoot hearts were removed under aseptic conditions exposing the shoot tip region (3-4 cm length, 1-1.5 cm width) with 3-4 primary leaves.

The shoot tips were disinfected by immersion in 0.3% $HgCl_2$ with 3 drops of Tween 20 for 5 min under agitation and then washed three times in sterile distilled water before dividing them to small squares (0.5-1 cm^2) which formed our initial explants.

Callus induction

The explants were cultured on a medium consisting of MS salts (Murashige and Skoog, 1962) supplemented with (per liter) 170 mg NaH_2PO_4, 100 mg myo-inositol, 200 mg glutamine, 2.5 mg thiamine-HCl, 0.2 mg biotin, 0.2 mg pyridoxine-HCl, 30 g sucrose and 7 g agar (Serva, Kobe I). The medium was enriched with 50 mg l^{-1} picloram, 3 mg l^{-1} N-(3-methyl-2-butenyl)-1Hpurin-6-amine (2iP) and 1.5 g l^{-1} activated charcoal (AC) during the first 8 months of culture, then the explants were transferred to a medium supplemented with 10 mg l^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D), 3 mg l^{-1} 2iP and 1.5 g l^{-1} AC for additional 2-3 months (Ibraheem et al., 2010). The pH was adjusted to 5.7 and distributed into 100-ml Magenta vessels (Sigma-Aldrich) containing 35 ml of the medium/vessel, caped with Magenta B-cap and autoclaved at 121°C for 15 min. Culture incubation conditions consisted of complete darkness and 24 ± 2°C. Resultant callus from the explants served as a source of callus for the following two experiments.

Experiment 1: The effect of culture system and medium composition on callus growth

Solid cultures

Portions of embryogenic friable callus (200 mg) of the same quality were chopped and cultured in 500-ml Phytacon tissue culture vessels (Figure 1a) (Sigma) containing 200 ml solid MS medium as described below.

Cell suspension establishment

Portions of embryogenic friable callus (200 mg) of the same quality were chopped and cultured in 200 ml liquid MS media dispensed in either 500 ml Phytacon™ tissue culture vessels (Sigma) or 500 ml Rasotherm flasks (Figure 1b). The suspension cultures were incubated on a rotary shaker set at 100 rotations per minute (rpm) using electric shaker (Certomat® R).

Temporary immersion system

A temporary immersion system (TIS) was conducted using RITA® (Vitropic-Cirad, France) bioreactor (Figure 1c). The lower compartment of the RITA® bioreactors was filled with 200 ml liquid medium. The calli were cultured in the upper compartment at 200 mg in each bioreactor. The immersion frequency was 5 min every 12 h controlled by a digital programming timer (REV Ritter GmbH, Germany). The calli in RITA® bioreactors remained in their culture vessels and a new liquid medium was supplied aseptically after removing the old one at 5-6 week-intervals. The fresh mass of callus was evaluated at every subculture.

Figure 1. Date palm callus cv. Zaghlool cultured in: a) Solid medium in Phytacon vessels, b) Cell suspension culture in Rasotherm and Phytacon vessels, c) RITA bioreactor.

Culture medium

Three medium compositions were chosen according to previously reported protocols for other date palm cultivars: ½ MS with 0.5 mg l^{-1} 2,4-D (Gadallah, 2007), ½ MS with 1 mg l^{-1} 2,4-D (Fki et al., 2003) and MS with 10 mg l^{-1} Naphthaleneacetic acid (NAA)+1.5 mg l^{-1} 2iP (Al-Khayri, 2012). The pH was adjusted to 5.7 before the addition of 30 g l^{-1} sucrose and further 7 g l^{-1} agar to the solid culture medium (Serva Kobe I). The media were autoclaved at 121°C for 15 min. The cultures were incubated under 16-h photoperiods of cool-white florescent light (35 µmol m^{-2} s^{-1}) at 24 ± 2°C.

Cell growth curve

The fresh mass of the cell suspension cultures was determined from the cell mass collected on 800-µm stainless steel sieve at each transfer. Cells were then transferred to a sterile petri dish and weighed aseptically, following the procedures used by Teixeira et al. (1995). For the calli grown in RITA bioreactors and on solid media, the cells were weighed aseptically at every subculture before transferring to the fresh medium. The growth curve was obtained using Excel for Windows 2007 depending on the fresh mass of the cells (g) evaluated at every transfer.

Starch identification

A few samples were taken from each treatment of the cell suspensions and treated with a tincture of iodine/ potassium iodide to identify starch. The samples were examined under a microscope (Axiovert 100 Carl Zeiss. Germany) and photographed by digital Camera (Olympus c3 040-ADU. Japan).

Somatic embryo induction

At the end of the 16th week the calli were transferred to a somatic embryo induction medium with 0.1 mg l^{-1} NAA and 1.5 g l^{-1} AC for 18 weeks. The calli were subcultured every 6 weeks and the number of formed somatic embryos was determined after 18 weeks on this medium.

Experimental design and statistical analysis

The experiment was set up as a 4x3 factorial in Completely Randomized Design (CRD) comprising two main factors: culture system as 4 levels and medium composition as 3 levels. Each treatment consisted of 2 replications and the experiment was repeated once. The data present an average of the repetitions. Data were subjected to analysis of variance (ANOVA) and the means were separated using Tukey test.

Experiment 2: The effect of culture system and medium composition on somatic embryo formation

Callus source

Callus was induced from either shoot tips or from leaf segments excised from converted somatic embryos. Embryogenic friable callus induced from shoot tips of cv. Zaghlool as described above.

The somatic embryos were induced from shoot tip-callus on MS medium with 0.1 mg l^{-1} NAA (Ibraheem et al., 2010) and converted on MS medium with 1 mg l^{-1} NAA under darkness. The white leaflet segments were cut out from the basal part of the first leaf appeared from these converted embryos. Embryogenic callus was induced from these leaf segments using MS medium enriched with 10 mg l^{-1} picloram and 1.5 g l^{-1} AC for 2 months with 1 month subculture.

Cell suspension establishment

For both callus sources, portions of embryogenic friable callus (500 mg) were finely chopped and cultured in 50 ml liquid MS media dispensed in 125 ml Erlenmeyer flasks. The suspension cultures were incubated on a rotary shaker set at 100 rpm using an electric shaker (Certomat® R). The cultures were transferred to new medium once after 3 weeks of initial culture.

Culture medium

The media supplemented with 0.1 mg l^{-1} NAA and 1.5 g l^{-1} AC with or without 0.05 mg l^{-1} BAP compared to PGR-free medium with 1.5 g l^{-1} AC as control. Sucrose was added at 30 g l^{-1} and agar for the solid media at 7 g l^{-1}.

Experimental design and statistical analysis

The experiment was set up as a 2x3 factorial in Completely Randomized Design (CRD) comprising two main factors: culture system as 2 levels and medium composition as 3 levels. Each treatment had 5 replicates and the experiment was repeated twice. The fresh mass and the number of somatic embryos were evaluated after 6 weeks of initiation of culture. The data present an average of the repetitions. Data were subjected to analysis of variance (ANOVA) and the means were separated using Tukey test by using the program SPSS 10 for Windows.

Results and Discussion

Experiment 1: The effect of culture system and medium composition on callus growth

The embryogenic friable callus continued to grow on all of the studied media and culture systems (Table 1). Data demonstrated differences in

callus fresh mass depending on the treatments.The callus reached between 4-fold and up to 20-fold mass after 16 weeks. Regardless of medium composition, the fresh mass of callus increased significantly in cell suspensions grown in Rasotherm flasks or Phytacon vessels. However, the calli grown in Rasotherm flasks showed a slight increase over those grown in Phytacon. The calli subjected to temporary immersion system showed less growth, but the lowest increase in mass was recorded on solid medium, however the difference was not significant compared to RITA (Table 1, Figure 2a, 2c). Regarding the difference between RITA® and suspension cultures, the fresh mass of calli grown in Rasotherm flasks was significantly higher than those of RITA® (Figure 3a, b). However, no significant difference was noted between the calli grown in Phytacon vessels and RITA® system (Table 1, Figure 2a, 2d).

Concerning the effect of the medium PGR contents, adding 0.5 mg l-1 2,4-D was slightly superior to 1 mg l-1 2,4-D, but without significant difference in all culture systems (Table 1). Both concentrations of 2,4-D showed higher callus mass than 10 mg l-1 NAA combined with 1.5 mg l-1 2iP. This increase was significant for two of the tested culture systems (solid and liquid in Phytacon vessels), but not significant for the RITA® and liquid culture in Rasotherm flasks (Table 1).

Similar to the current observation the positive influence of liquid medium on fresh mass development was reported for African oil palm (Teixeira et al., 1995; Kanchanapoom and Chourykaew, 1998) and date palm (Tisserat and Vandercook, 1985; Al-Khayri, 2012). A higher mass of African oil palm callus as compared to solidified medium was also reported using TIS (Sumaryono et al., 2008). The use of Phytacon vessels for cell suspension, to our knowledge, has not been reported for date palm cell suspensions. The use of Phytacon vessels were reported to maintain lower air exchange, and thus a higher CO_2 concentration inside these vessels as compared to other tissue culture vessels (Tisserat et al., 1997) and offer reduced cost and labor (Muralidharan, 1998). The lower callus mass in the Phytacon vessels compared to Rasotherm flasks may be due to better fluidity efficiency which may lead to better gaseous exchange of the Rasotherm flasks, nonetheless the difference was not significant.

The biomass was higher in suspension cultures than temporary immersion system (Table 1). This superior effect of suspension cultures was reported recently for African oil palm cultures (Sumaryono et al., 2008) who noted that the callus mass of African oil palm was lower in RITA® bioreactor than suspension cultures. It is worth mentioning that they immersed their calli for 3 min every 6 h. In contrast to our results, temporary immersion culture systems have proved to be more successful in achieving embryogenic tissue proliferation than conventional systems using an agar medium or suspension cultures for *Coffea arabica* (Berthouly et al., 1995; Berthouly and Etienne, 2005). The lower callus mass in a RITA® bioreactor (Table 1) might be due to a suboptimal immersion duration and/or frequency (5 min every 12 h). To our knowledge there is no optimal protocol for using RITA® system for date palm tissue cultures. Tisserat and Vandercook (1985) reported developing an automated plant culture system (APCS) for date palm callus with 5-10 min immersion every 2 h. They found superior growth of callus which reached up to a 4-fold increase as compared to those grown on agar medium. In our experiment callus increased only 1.5-2 fold compared to agar-medium (Table 1). The immersion system we used involved 5 min every 12 h. This duration was arbitrarily selected based on reports with other perennial crops which varied from 1 to 15 min immersion every 2 to 12 h (Etienne and Berthouly, 2002). Similar to our results, Othmani et al. (2009) recently reported that the embryogenic calli of date palm cv. Deglet Bey turned brown and died using a RITA® bioreactor with immersion frequency of 5 min every 8 h. They found that temporary immersion system was better than the solid medium only for shoot proliferation. The immersion frequency tested so far appeared to be suboptimal for date palm callus growth and could be optimized by testing different durations.

Table 1. The effect of different tissue culture systems and medium compositions on callus fresh mass of date palm cv. Zaghlool after 16 weeks of initial culture starting with 200 mg callus per culture The different letters indicate significant differences according to Tukey (P<0.05).

Callus growing medium	½ MS with 0.5 mg l^{-1} 2,4-D	½ MS with 1 mg l^{-1} 2,4-D	MS with 10 mg l^{-1} NAA, 1.5 mg l^{-1} 2iP
Solid	1.99 cde	1.57 ef	0.76 f
RITA®	2.86 bcd	2.05 cde	1.80 def
Suspension in Rasotherm	4.09 a	3.79 ab	3.02 abcd
Suspension in Phytacon	3.08 abc	3.48 ab	1.76 def

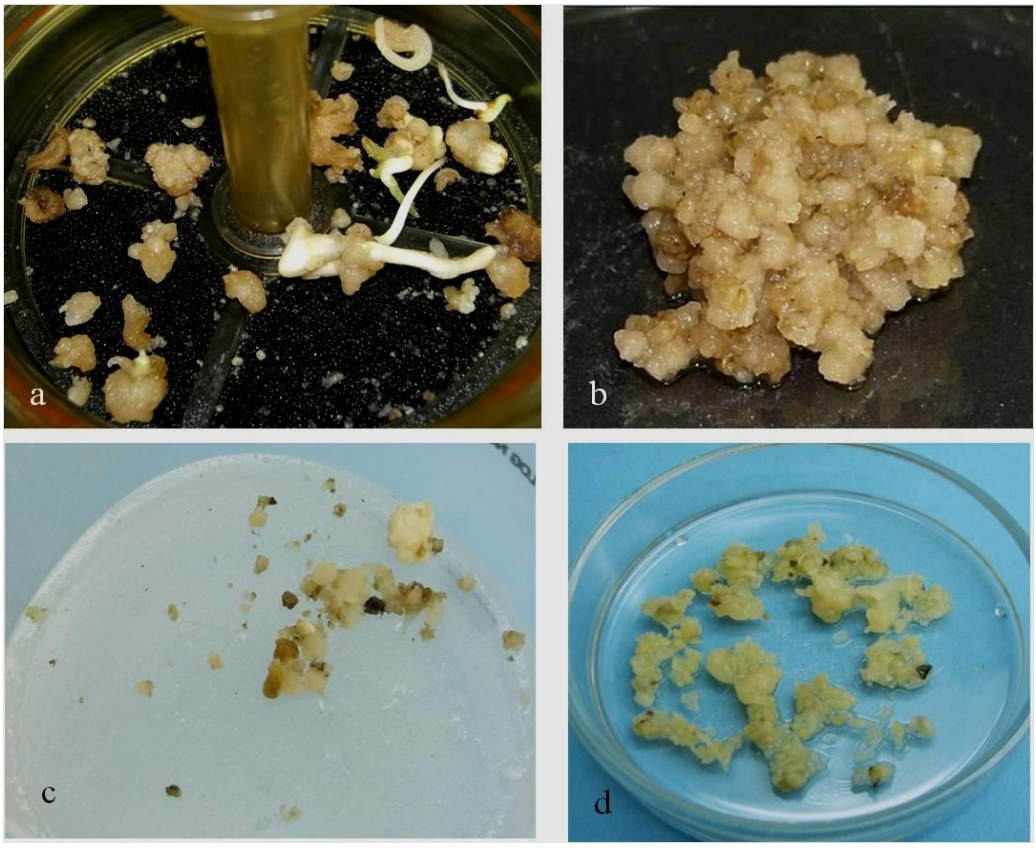


Figure 2. Callus of cv. Zaghlool after 16 weeks in ½ MS liquid medium enriched with 0.5 mg l-1 2,4-D a) In a RITA® bioreactor, b) In Rasotherm flasks, c) In Phytacon vessels with solidified medium, d) In Phytacon vessels with liquid medium.

Table 2. The growth of 0.2 g friable calli of date palm cv. Zaghlool in solid and liquid culture systems after 5, 10 and 16 weeks of incubation in ½ MS with 0.5 mg l^{-1} 2,4-D.

Callus growing medium	5 weeks	10 weeks	16 weeks
Solid	0.55	1.08	2.00 cde
RITA®	0.62	1.59	2.86 bcd
Suspension in Rasotherm	0.63	1.71	4.10 a
Suspension in Phytacon	0.69	1.55	3.08 abc

Callus growth curve

The trend of the callus growth curve was the same in all medium compositions tested. For instance the callus growth curve on ½ MS supplemented with 0.5 mg l^{-1} 2,4-D is demonstrated in Table 2. The fresh mass of callus or cell suspension showed no difference between all culture systems at the first subculture. This stage is called the lag phase in which the cells prepare themselves for the division (Al-Khayri, 2012). In the second subculture the growth curve differentiated between the liquid culture systems and the solid medium. However, no difference was found in the biomass at this point between the three studied liquid culture systems. The differencebetween liquid culture systems appeared only at the third subculture (between 10-16 weeks after inoculation). The cell suspension grown in Rasotherm flasks showed the best growth followed by those grown in the Phytacon vessels. Using PhytaconTM tissue culture vessels for liquid cultures was reported for the first time in date palm tissue culture. The calli grown in RITA® bioreactor showed lower mass but it remained higher than those developed on solid medium (Table 2). Generally, the callus fresh mass increased about 9 times in solid medium and between 13-20 times in the liquid cultures, clearly illustrating the advantage of liquid medium for plant micropropagation.

Various methods are employed to measure in vitro cell growth, including cell or colony counting, dry and fresh weight and packed cell volume (Dixon, 1985). The growth curve shape depends upon the measurement method used to evaluate cell growth (Yamamoto and Yamada, 1986). Studies on growth curves of date palm suspension cultures are scant. Recently, Al-Khayri (2012) studied the growth curve of date palm cell suspensions using a packed cell volume method which demonstrated five growth phases: lag, exponential, linear, declaration and stationary. Sumaryono et al. (2008) reported a similar growth curve trend in African oil palm cell suspension using the fresh mass measurement method. Growth curves are essential to assess culture performance and metabolic activities at various growth phases (Al-Khayri, 2012). The method we used showed no stationary and/or declaration phase as we reported for the cell suspension growth curves used the packed cell volume method (Abbade et al., 2010; Al-Khayri, 2012). That may be due to the long intervals between the readings we took (5-6) weeks. It is recommended for cell suspension culture studies that the evaluation of the mass must be evaluated many times during the same subculture to obtain more precise growth curves that may help in defining the best time for subculture to fresh new medium. Otherwise, using other growth curve methods such as packed cell volume may give a better understanding of the growth nature of the cell suspensions.

Somatic embryo formation

The calli grown on the aforementioned culture systems and media were cultured on somatic formation solid medium amended with 0.1 mg l^{-1} NAA and 1.5 g l^{-1} AC (Ibraheem et al., 2010). Somatic embryo number was evaluated after 18 weeks (Table 3). The cells obtained from Rasotherm flasks and Phytacon vessels containing 10 mg l^{-1} NAA and 1.5 mg l^{-1} 2iP produced the highest number of somatic embryos (Table 3, Figure 3a,b). The somatic embryo number was rather low from the calli grown in all culture types enriched with 0.5 and 1 mg l^{-1} 2,4-D and also from the calli grown on solid and in a RITA® bioreactor on medium enriched with 10 mg l^{-1} NAA and 1.5 mg l^{-1} 2iP (Table 3).

Table 3. The effect of callus growing medium on the number of induced somatic embryos/callus cv. Zaghlool after 18 weeks of transfer to MS medium supplemented with 0.1 mg l^{-1} NAA and 1.5 g l^{-1} AC

Callus growing medium	½ MS with 0.5 mg l^{-1} 2,4-D	½ MS with 1 mg l^{-1} 2,4-D	MS with 10 mg l^{-1} NAA, 1.5 mg l^{-1} 2iP
Solid	3.00 c	3.00 c	3.00 c
RITA®	5.00 c	3.00 c	4.00 c
Suspension in Rasotherm	2.00 c	2.00 c	35.00 a
Suspension in Phytacon	3.00 c	2.00 c	22.00 b

The different letters indicate significant differences according to Tukey ($P<0.05$).

Figure 3. a) Embryogenic nodular callus cv. Zaghlool grown in liquid MS with 10 mg l^{-1} NAA, 1.5 mg l^{-1} 2iP after transferring to somatic embryo formation medium with 0.1 mg l^{-1} NAA and 1.5 g l^{-1} AC for 9 weeks, b) Somatic embryo formation from callus grown in liquid MS with 10 mg l^{-1} NAA, 1.5 mg l^{-1} 2iP after transfer to MS with 0.1 mg l^{-1} NAA and 1.5 g l^{-1} AC for 18 weeks.

The induced number of somatic embryos was very low compared to our observations on callus of cv. Zaghlool by using solid medium. From about 3 g callus grown in liquid MS with 10 mg l^{-1} NAA and 1.5 mg l^{-1} 2iP only 35 somatic embryos were formed (Table 3) while we could obtain in previous findings 75 somatic embryos from only 1 g callus in solid MS enriched with 0.1 mg l^{-1} NAA and 1.5 g l^{-1} AC (Ibraheem et al., 2010). This difference in somatic embryo number may be due to lack of desiccation treatment of the calli grown in liquid media. Othmani et al. (2011) reported that a desiccation procedure before transferring the calli to agar medium enhanced the number of induced somatic embryos. Othmani et al. (2011) suggested that a complex interaction exists between the water content of embryogeniccalli and development of somatic embryos. They found that a desiccation treatment of 12 hours induced significantly more somatic embryos than 6, 24 or 48 hoursdesiccation. For Indica rice, Rance et al. (1994) assumed that the desiccation treatment might trigger rapid biochemical changes in the calli and under water stress specific enzymes or polypeptides probably appear in callus culture.

Starch content

To understand the difference in resulting somatic embryos between the callus grown in MS medium contained 10 mg l^{-1} and 1.5 mg l^{-1} 2iP and those grown in ½ MS enriched with 2,4-D at 0.5 and 1 mg l^{-1} (Table 3) samples of the cell suspensions were studied under the microscope (Figure 4). The histological observation for the cell suspensions indicated that the cell suspension contained densely cytoplasmic cells (Figure 4b, d). These cells were either individual or grouped in cell aggregates. Furthermore, they varied in shape from spherical to oblong. The diameter of these cells was between 40-60 µm and the length varied between 100-250 µm. The main difference between the cells grown in 10 mg l^{-1} NAA medium (Figure 4c, d) and those grown in 2,4-D media (Figure 4a, b) was the appearance of starch grains (S). These starch grains appeared clearly in the cells grown in MS medium enriched with10 mg l^{-1} NAA (Figure 4c, d) while no or very few starch grains existed in the cells grown in ½ MS enriched with 0.5-1 mg l^{-1} 2,4-D enriched media (Figure 3a,b). This difference possibly explains the variation noted in the number of induced somatic embryos from these media after transferring to somatic embryo formation medium (Table 3). In further experiments it should be clarified whether the auxin type or the macronutrient content was responsible for starch accumulation and the somatic embryo formation. Our results support those published by Sharma et al. (1986) who reported that the samples of cell suspensions of date palm contained a mixed population of actively dividing, cytoplasmically rich, globular cells, elongated cells and cell aggregates. In line with our results, Sané et al. (2006) observed a significant accumulation of starch and proteins in date palm liquid cultures. In African oil palm cell suspensions, de Touchet et al. (1991) reported that the proliferating embryogenic aggregates composed of meristematic cells in active division.

Experiment 2: The effect of culture system and medium composition on somatic embryo formation

Callus induced from shoot tips

The fresh mass was significantly higher on liquid media compared to solid media (Figure 5a); it mass increased in 6 weeks 2-3 fold: 1.5-2 g in solid media versus 4.5-5 g in liquid media. There was no significant difference among the PGR treatments for each culture system individually. However, a slight increase in the fresh mass was found on the medium containing only 0.1 mg l^{-1} NAA.

The somatic embryos appeared at the 3rd week after transferring the calli to the liquid medium and the number increased obviously in the next 3 weeks. The number of somatic embryos varied according to the culture system and the medium composition (Figure 5b). The culture system obviously had a crucial effect on the number of somatic embryo formation. For all media composition the number of somatic embryos increased dramatically and significantly on liquid medium (Figures 5b, 6). This number increased 8-16 fold: 5-10 somatic embryos on solid media versus 40-160 somatic embryos in liquid media (Figure 5b). There was also a significant difference among the media compositions used in liquid cultures. The medium enriched with 0.1 mg l^{-1} NAA and 1.5 g l^{-1} AC showed the highest number of somatic embryos (Figure 5b). The number of somatic embryos was significantly lower on medium enriched with 0.1 mg l^{-1} NAA, 0.05 BAP and 1.5 g l^{-1} AC. Nevertheless, this medium was significantly superior to the PGR-free liquid medium (Figure 5b).

The number of somatic embryos (Figure 5b) showed either better or worse results in comparison to other date palm genotypes cultivated in liquid cultures. On the same medium, Saker et al. (2007) induced 120 somatic embryos from 500 mg of friable callus of cv. Sewi. This difference may be due to the genotypic effect (Pinker et al., 2009). Gadallah (2007) and Al-Khayri (2012) initiated 69 somatic embryos from 500 mg callus of cvs. Khalas and Barhi, respectively. Badawy et al. (2009) induced 48 embryos from 200 mg callus, equivalent to 120 embryos from 500 mg, on cv. Sakkoty. However better results were reported by Fki et al. (2003) with 200 embryos per 100 mg initial callus weight from cv. Deglet Nour. Furthermore, Othmani et al. (2009) produced a yield of 501 embryos beginning from 500 mg callus on suspension cultures of cv. Deglet Bey.

The medium enriched with only NAA was better than that enriched with both NAA and BAP (Figure 5b). This ensured our previous results for solid cultures where addition of BAP to the somatic embryo formation medium was also unfavorable (Ibraheem et al., 2010). Adding BAP to the cell suspension cultures seemed to be controversial. Using liquid medium with NAA and without cytokinins as we found, was reported to be optimal in some date palm liquid cultures (Tisserat and Vandercook, 1985; Sharma et al., 1986; Saker et al., 2007). In contrast to our results, Gadallah (2007) reported higher fresh mass and somatic embryo number in date palm cv. Khalas suspension cultures after adding BAP at 1 mg l^{-1} to the medium enriched with 2,4-D. Adding BAP to date palm suspension cultures was also reported by Zouine et al. (2005) and Zouine and El Hadrami (2007). For other species, Yamamoto and Yamada (1986) reported that the hormonal combination of NAA and BA was the most suitable for cell suspension culture for snakeroot (Rauwolfia serpentine). Furthermore, Stafford (1996) stated that plant cell cultures are normally established and maintained on media containing an auxin and a cytokinin. Removal of either hormone from the medium would normally result in culture death.

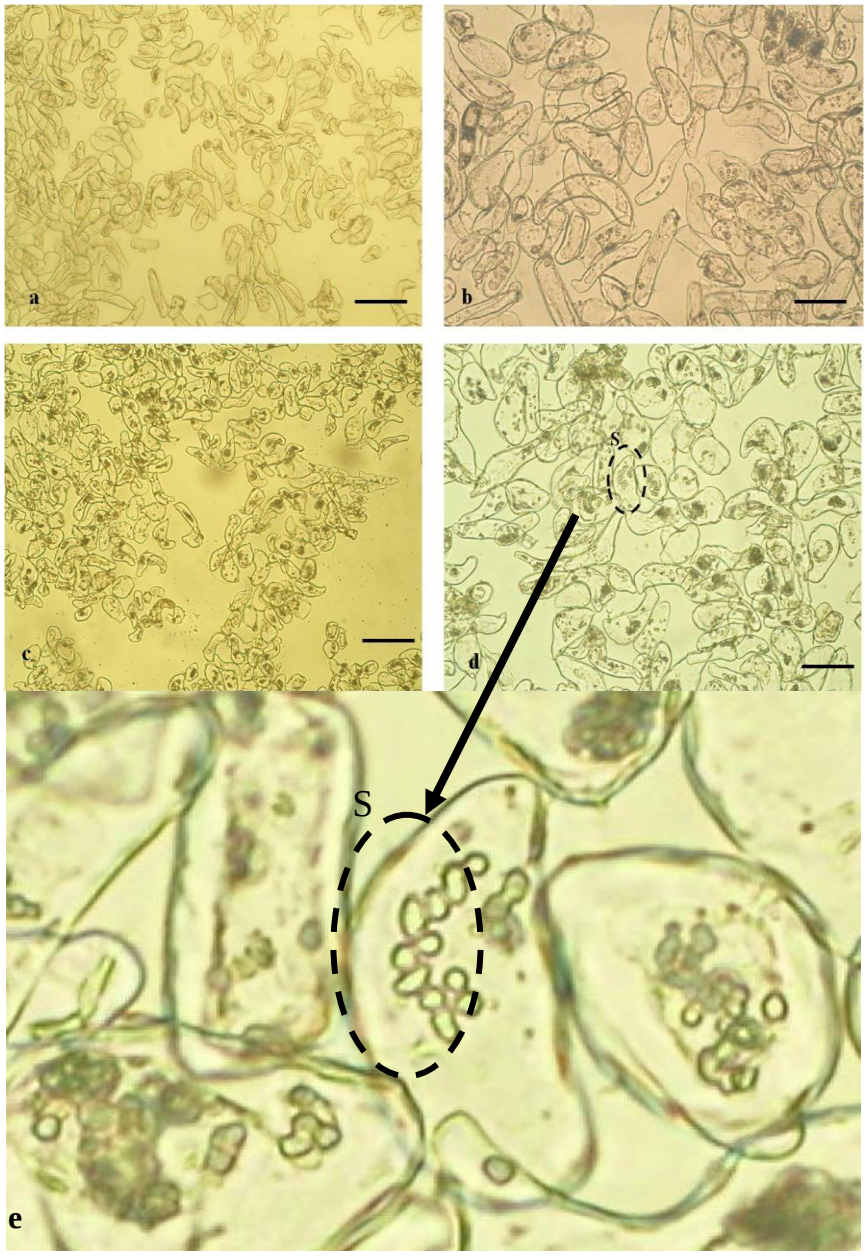
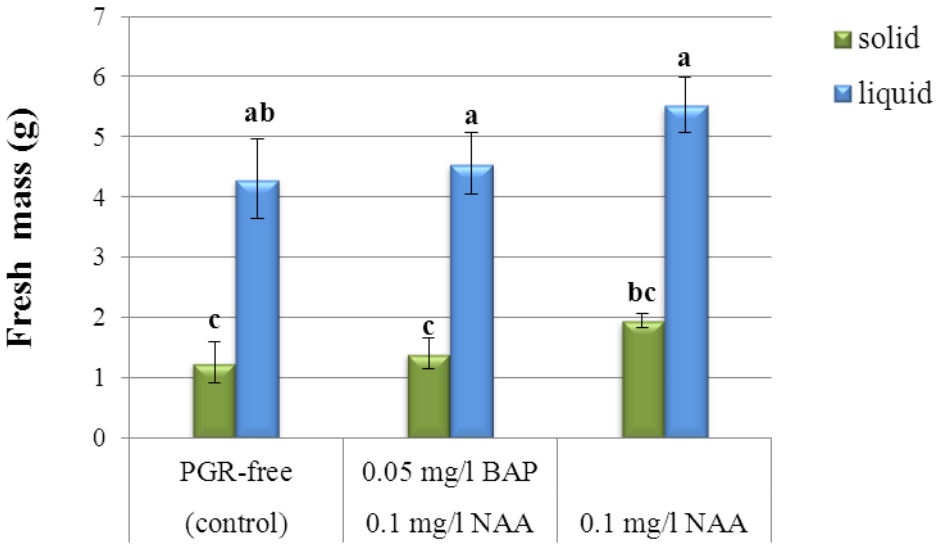
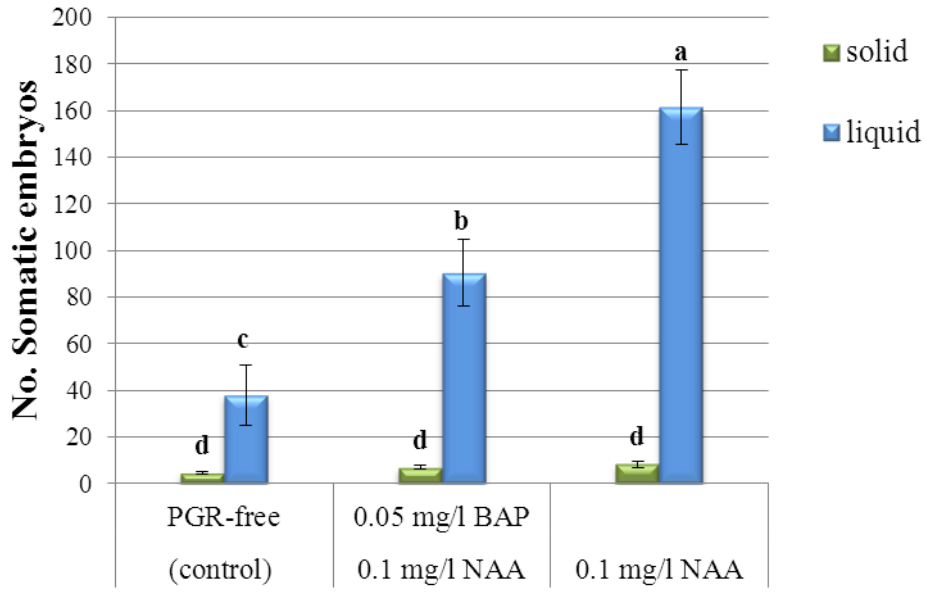


Figure 4. Microscopic view of date palm cv. Zaghlool cell suspension culture 16 weeks after transferring of friable embryogenic callus to a, b) Rasotherm flasks filled with liquid MS containing 1 mg l^{-1} 2,4-D under 4x,10x magnification, respectively, c, d) Rasotherm flasks filled with liquid MS containing 10 mg l^{-1} and 1.5 mg l^{-1} 2iP under 4x,10x magnification, respectively, E) Starch grains under 20x magnification, S= starch grains, for a, c: bar = 200 µM and for b, d: bar = 80 µM.



a.



b.

Figure 5. The effect of culture system and medium composition on a) The fresh mass resulted from 500 mg callus cv. Zaghlool after 6 weeks of transfer to solid or liquid medium with different medium compositions, b) The number of somatic embryos resulted from 500 mg callus after 6 weeks of transfer to solid or liquid medium with different medium compositions Means followed by different letters are significantly different using Tukey test at p=0.05.

Figure 6. Somatic embryo formation of cv. Zaghlool from 500 mg initial callus weight after 6 weeks of culture in: a) Solid PGR-free MS with 1.5 gl^{-1} AC, b) Cell suspension in Erlenmeyer flasks filled with liquid MS medium containing 0.1 mg l^{-1} NAA and 1.5 g l^{-1} AC, c) Cell suspension in Erlenmeyer flasks filled with PGR-free liquid MS medium and 1.5 g l^{-1}AC, d) Cell suspension in Erlenmeyer flasks filled with liquid MS medium containing 0.1 mg l^{-1} NAA, 0.05 mg l^{-1} BAP and 1.5 g l^{-1} AC

The large increase in the number of somatic embryos in liquid media may be due to the ease of available nutrients to the cells or perhaps due to the large surface area of the cells directly exposed to the nutrient medium (Duval et al., 1995). High numbers of somatic embryos can be produced in suspension cultures, which makes this technique ideal for large-scale micropropagation of healthy plant material. The liquid medium allows close contact of the tissue with the medium stimulating and facilitating the uptake of nutrients and phytohormones, leading to better growth (Mehrotra et al., 2007).

Liquid medium not only increased the number of resultant somatic embryos but also accelerated the formation of somatic embryos. The somatic embryos were formed within 3-6 weeks after inoculation in the liquid media (Figures 5b, 7b) while they were formed in 12-18 weeks on solid medium (Ibraheem et al., 2010). Similar to our results Zouine et al. (2005) reported that the initiation of somatic embryos in suspension culture within 2 months, while on solid medium somatic embryos were formed after 4 months. Recently, Al-Khayri (2012) found that cell suspensions accelerated significantly the appearance of somatic embryos in date palm cv. Barhi., Mehrotra et al. (2007) reported that the growth and multiplication rate is enhanced by forced aeration, since continuous shaking of the medium provides sufficient oxygen supply to the tissue, which ultimately leads to their faster growth. Furthermore, in addition to these advantages, the preparation of

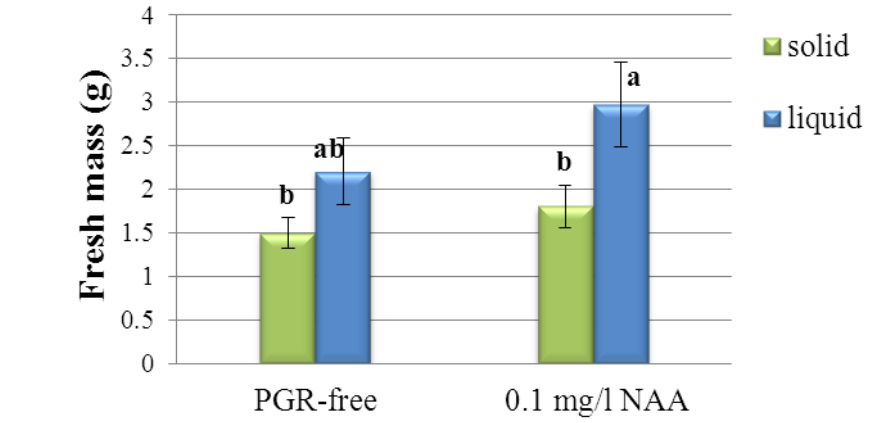
liquid medium and handling of shake cultures is comparatively easier to the solid medium.

Callus induced from leaflet segments

To ensure the previous results and to examine any effect of the source of callus another comparative study between liquid and solid media was carried out. The friable embryogenic callus induced on leaf segments of converted somatic embryos was the starting material. According to the above results on shoot tip-induced callus, the treatment of BAP was eliminated.

The fresh mass increased significantly in the liquid medium supplemented with 0.1 mg l^{-1} NAA and 1.5 g l^{-1}AC compared to the solid medium (Figure 7a). It increased also in the liquid PGR-free medium but without significant effect compared to the PGR-free solid medium. Also for solid medium adding 0.1 mg l^{-1} NAA enhanced the fresh mass but without significant effect compared to the PGR-free medium (Figure 7a).

Somatic embryos appeared in the 3rd week after transfer to the induction medium and increased in the second subculture (subculture=3 weeks). The number of somatic embryos varied according to the culture system and the medium composition (Figure 7b). The number of induced somatic embryos was enhanced significantly on liquid media (Figure 7b). The liquid medium enriched with 0.1 mg l^{-1} NAA and AC was the best treatment with about 65 embryos per callus. Also the PGR-free liquid medium enhanced the number of embryos significantly, compared to the solid media and the number of embryos was about 35 embryos per callus (Figure 7b).



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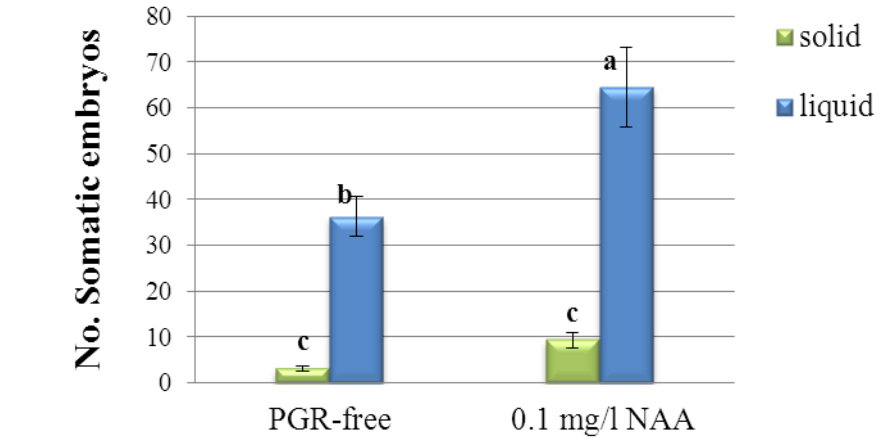

b

Figure 7. The effect of culture system and medium composition on somatic embryogenesis induced from callus of leaflet segments cv. Zaghlool: a) The fresh mass resulted from 500 mg callus after 6 weeks of transferring the calli to solid or liquid medium with 1.5 g l^{-1} AC with or without 0.1 mg l^{-1} NAA, b) The number of somatic embryos resulted from 500 mg callus after 6 weeks of transferring to solid or liquid medium with 1.5 g l^{-1} AC with or without 0.1 mg l^{-1} NAA Means followed by different letters are significantly different using Tukey test at p=0.05.

Generally, the number of somatic embryos was 7-12 times more in cell suspension cultures compared to solid cultures (Figure 7b). There are no published reports about using the callus induced from date palm converted somatic embryos as a source for cell suspension cultures. This method might be promising for commercial and scientific purposes because it enables the researchers to obtain callus and somatic embryos in a shorter time than the common pathways.

These results confirmed our previous results on the calli of shoot tip origin. The medium we developed previously for somatic embryo formation on solid media (Ibraheem et al., 2010) stimulated also the number of somatic embryos in liquid culture (Figure 5b, Figure 7b). However the number of somatic embryos formed from the leaflet segment calli was less than those of shoot tips calli. The number of induced embryos from 500 mg callus was about 160 from shoot tip calli and 65 from leaf segment calli. Nevertheless, this technique seems to be promising to have callus in a short time and to increase the number of produced somatic embryos by using liquid medium. The number of somatic embryos increased about 6-16 fold in liquid media compared to those developed in solid media as shown in Figure 5 and Figure 7.

Conclusions

Liquid culture system is a promising technique for rapid mass propagation of date palm. Somatic embryo production in liquid media is about ten times greater than that on solid media. The suspension cultures were superior for date palm callus growth than in RITA® bioreactors. Furthermore, the suspension cultures are technically easier and more economical than the bioreactors. Therefore we suggest the use of cell suspensions to produce high number of date palm somatic embryos and recommend it for a large scale production of date palm plantlets and should be studied for other cultivars. Adding low concentration of auxin 0.1 mg l^{-1}NAA and 1.5 g l^{-1} AC enhanced the number of induced somatic embryos in the liquid cultures of date palm.

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Dates as a substitute for added sugar in traditional foods – A case study with idli

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Abstract

Scientific evidences suggest that increased intake of added sugar is one of the major causes for dental caries, glucose intolerance, diabetes mellitus, cardiovascular diseases, obesity, hypertension and behavioural complications such as hyperactivity in children. In many parts of the world, consumption of added sugar is much higher than the dietary recommendation from health organizations. Several researchers have used dried fruits to sweeten the traditional foods as the functionality of the sugar incorporated within the structure of intact fruits is different than added sugar in human health. Date fruits have also been used in several forms such as syrups, spread, sugar and flour as a sweetener in food. The objective of this study was to develop acceptable idli (traditional Indian breakfast) with chopped dates, date paste and date syrup, and determine their sensory and chemical properties. Total phenol and vitamin C contents of dates idli were significantly higher than control idli with added sugar. The sensory properties of four idli products (idli with date paste, idli with date syrup, idli with chopped dates and control idli served with white sugar) were evaluated by 40 untrained panelists. The sweetness and aroma of the idli with chopped dates got significantly higher scores than other three idli products with no difference among them. Similarly the overall acceptability score of the idli with chopped dates was higher than other idli products. Panelists from a regular eaters group gave higher scores for the overall acceptability of developed idli products than first time consumers. In all sensory attributes and consumer types, idli with dates scored higher preference or at least equal preference with control idli and white sugar combination. There are ample opportunities to educate people and create awareness about preparation and consumption of traditional foods with dates in order to reduce added sugar intake.

Key words: Dates, Nitrification, Total phenols, Sensory, Added sugar

Introduction

Increased intake of added sugar might increase the risks for obesity, cardiovascular diseases, dental caries, glucose intolerance, diabetes mellitus, hypertension and behavioral complications such as hyperactivity in children (Anderson, 1997; Johnson and Yon, 2010). World Health Organization recommends limiting added sugar intake to <10% of total energy (Kranz et al., 2005; World Health Organization, 2003). The American Heart Association recommends limiting the daily added sugar intake to 100 calories for women and 150 calories for men (Johnson and Yon, 2010). The food guide from the United States Department of Agriculture (USDA) recommends consumption of added sugar in the range between 6% and 10% of total energy (Welsh et al., 1993). Although added sugar intake is higher in many Asian countries, the statistical information for per capita intake is not available in the public domain.

Utilization of fruits in food preparation while requiring sweet taste is a wise strategy to reduce the added sugar intake. Dates are ideal fruits to substitute added sugar in foods, and they play an important role in daily nutrition of many people in the arid regions (Jain, 2012). Date fruits have been used in several forms such as juice, syrups, and spreads (El Hadrami and Al-Khayri, 2012). Dates are excellent raw materials for the production of value-added products such as medical and industrial ethanol, bakery yeast, single-cell protein as a fodder yeast, citric acid, and date flavored probiotic fermented dairy products (Aleid, 2011). Dates are rich in dietary fiber, phenolic compounds,

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minerals, vitamins and antioxidant compounds (Al-Farsi et al., 2007; Biglari et al., 2008; Taha et al. 2012). Al-Shahib and Marshall (2002) reported that the dietary fiber content of dates ranged from 8.1 to 12.7% (w/w). It has been determined that date fruits have potent antioxidant and antimutagenic qualities (Vayalil, 2002).The presence of insoluble fibers such as cellulose, hemicellulose, pectin, and lignins in dates, reduces the chances of bowel cancer and increases cardiac vitality (Baliga et al., 2011).

Idli is an indigenous food of India and Sri Lanka, and consumed three to four times a week at breakfast and supper. It is preferred by many people around the world because of its spongy texture, appearance, mouth feel, taste and aroma (Iyer and Ananthanarayan, 2008). The ingredients of idli are milled rice (*Oryza sativa)* and dehulled black gram (*Phaseolus mungo)* (Ghosh and Chattopadhyay, 2011). Rice and black gram are soaked separately and ground in a mortar and pestle with adequate water to yield a coarse rice batter and fine black gram batter (Blandino et al., 2003). Both the batters are mixed together with an adequate amount of salt and allowed to ferment overnight naturally. The fermenting microorganisms present in the batter use the reducing sugars for their growth and help in the fermentation process. Lactic acid bacteria play an important role in fermentation by producing acid and gas in the idli batter (Iyer and Ananthanarayan, 2008). The fermented batter is then poured in special idli pans and steamed for a few minutes. The cooked idli has a soft spongy texture and a characteristic taste and aroma (Nisha et al., 2005).

Generally idli is consumed with accompaniments such as chutney and sambar (Sharavathy et al., 2001). In many regions, people, especially children, prefer to eat idli with refined sugar (added sugar) for sweetness. The consumption of added sugar by children in Asian countries is high. High intake of fatty fast food coupled with reduced physical activity has promoted childhood obesity in many Asian countries. For example, although the prevalence of overweight and obesity among Indian adults is relatively lower (1.3 to 2.8%) compared to many other developed countries, it is higher among Indian children (18 to 21%) (IASO, 2007). The objective of this study was to develop acceptable idli products with chopped dates, date paste and date syrup, and determine their sensory properties.

Materials and Methods

Batter preparation and fermentation

Four parts rice and one part black gram dal (w/w) were washed and soaked separately in potable water for 5 hours (Sridevi et al., 2010). After draining the water, rice and black gram were ground separately with water in a heavy duty mixer grinder (Preethi Blue Leaf mixer grinder, model – MG140E, 230 V, 750 W, 50 Hz, Preethi Kitchen Appliances (P) Ltd, Chennai, India). The black gram was ground to a fine, smooth paste, while the rice was ground coarsely. Both the batters were then incorporated together in a stainless steel container, 2% (weight/total weight of raw material) salt was added (Balasubramanian and Viswanathan, 2007) and mixed properly. Then the batter was allowed to ferment naturally for 18 hours at overnight room temperature ranging from 26 to 30°C (Murthy and Rao, 1997). Idli batters were prepared four different times (n=4).

Dates preparation for nutrified idli

Date paste

Fresh dried date fruits (cv. Khalas) were procured from the local market. The dates were soaked in warm water for 10 min to soften the flesh. The seeds were then removed manually and the flesh was ground in the mixer grinder (Preethi Blue Leaf mixer grinder, model – MG140E, 230 V, 750 W, 50 Hz, Preethi Kitchen Appliances (P) Ltd, Chennai, India) until a smooth homogenous paste was obtained (Sanchez-Zapata et al., 2011).

Chopped dates

A sharp stainless steel knife was used to remove the seeds and chop the dates (cv. Khalas) into small pieces, approximately 3 mm cubes.

Date syrup

Date syrup with 76 to 78% concentrate (Golden dates, United Dates Processing Company LLC, Azaiba, Sultanate of Oman) was purchased and used in this study.

Idli preparation

Normally, one idli is consumed with approximately one teaspoon of refined sugar (about 6 g). Hence, the date paste, date syrup and chopped dates were accordingly measured and mixed into separate fermented idli batters (presuming 60% sugar content in dates). The date products were blended with the fermented batter and used for idli preparation. The batter was poured separately into circular molded idli plates and steam cooked for 7 min in an electric idli steamer (Panasonic Automatic Cooker Warmer, model – SR/WA18H (T), 230V, 660W, 50Hz, Panasonic Home Appliances India Co. Ltd, Chennai, India). The steamed idli was carefully removed using a spatula and served to the panelists for sensory evaluation. In all the experiments, a control (fermented batter

without addition of dates) was also cooked and served with 6 g of sugar per idli.

Sensory evaluation

The sensory test was performed by 40 untrained panelists (N=40) selected randomly from the students and the staff members of Sultan Qaboos University and Hospital, Sultanate of Oman. There were 2 groups of panelists: first time eaters of idli (10 males and 10 females) and regular eaters of idli (10 males and 10 females). The sensory test was conducted in four batches (with idli products prepared from four different batters), and equal number of panelists participated from each group in each batch. A sensory evaluation sheet was designed and developed. The sensory attributes of idli were collected under four groups such as appearance (colour, surface smoothness), mouth feel and texture (softness, graininess, springiness), flavour and taste (sweetness, aroma) and overall acceptability. Four idli samples (idli with date paste, idli with date syrup, idli with chopped dates and idli served with sugar (control) were evaluated by the panelists using a 9-point hedonic scale (9 – like extremely, 8 – like very much, 7 – like moderately, 6 – like slightly, 5 – neither like nor dislike, 4 – dislike slightly, 3 – dislike moderately, 2 – dislike very much and 1 – dislike extremely).

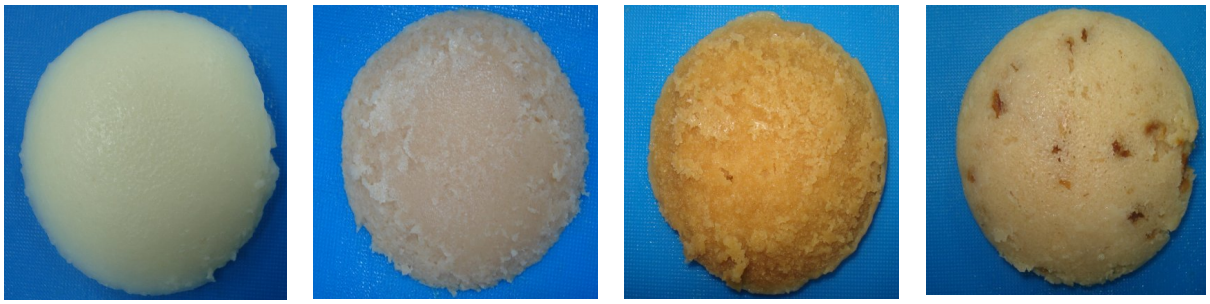
Statistical analysis

The effect of idli type and consumer type on individual sensory attribute was analyzed statistically using the Statistical Analysis System software (SAS, version 8.02, SAS Institute, Inc., Cary, NC). For each sensory attribute, the effect was studied by analysis of variance (ANOVA) using 2–factorial design models [4 product types (idli with sugar (control) × idli with date paste × idli with date syrup × idli with chopped dates) × 4 consumer types (male–regular eaters × female–regular eaters × male–first time eaters × female–first time eaters)] with the general linear model (GLM) procedure. In all analyses, the differences within the levels under each variable were tested at 95% confidence interval (type I error, $\alpha = 0.05$) using the least significant difference (LSD) method of comparison of means.

Results and Discussion

Idli products

A preliminary study was conducted to determine the behavior of idli batter while adding date fruits in different forms (date paste, date syrup and chopped dates) before and after fermentation. When the date products were added into the batter before fermentation, the cooked idli products produced an undesirable taste (without sweetness) and showed significant differences in the texture compared to the control. The products were not satisfactory during preliminary screening by the authors. When the dates were added with batter after fermentation, the products obtained were soft, spongy, sweet and comparable with control idli. Addition of dates into the batter probably affects the fermentation process and produce undesirable flavor in cooked idli. Further investigation is required to study the effect of dates in the fermentation process. Three idli products (idli with date paste, idli with date syrup, idli with chopped dates) were developed by adding dates in the batter after fermentation (Figure 1) and their sensory properties were evaluated.



Control idli | Idli with date paste | Idli with date syrup | Idli with chopped dates

Figure 1. Developed idli products.

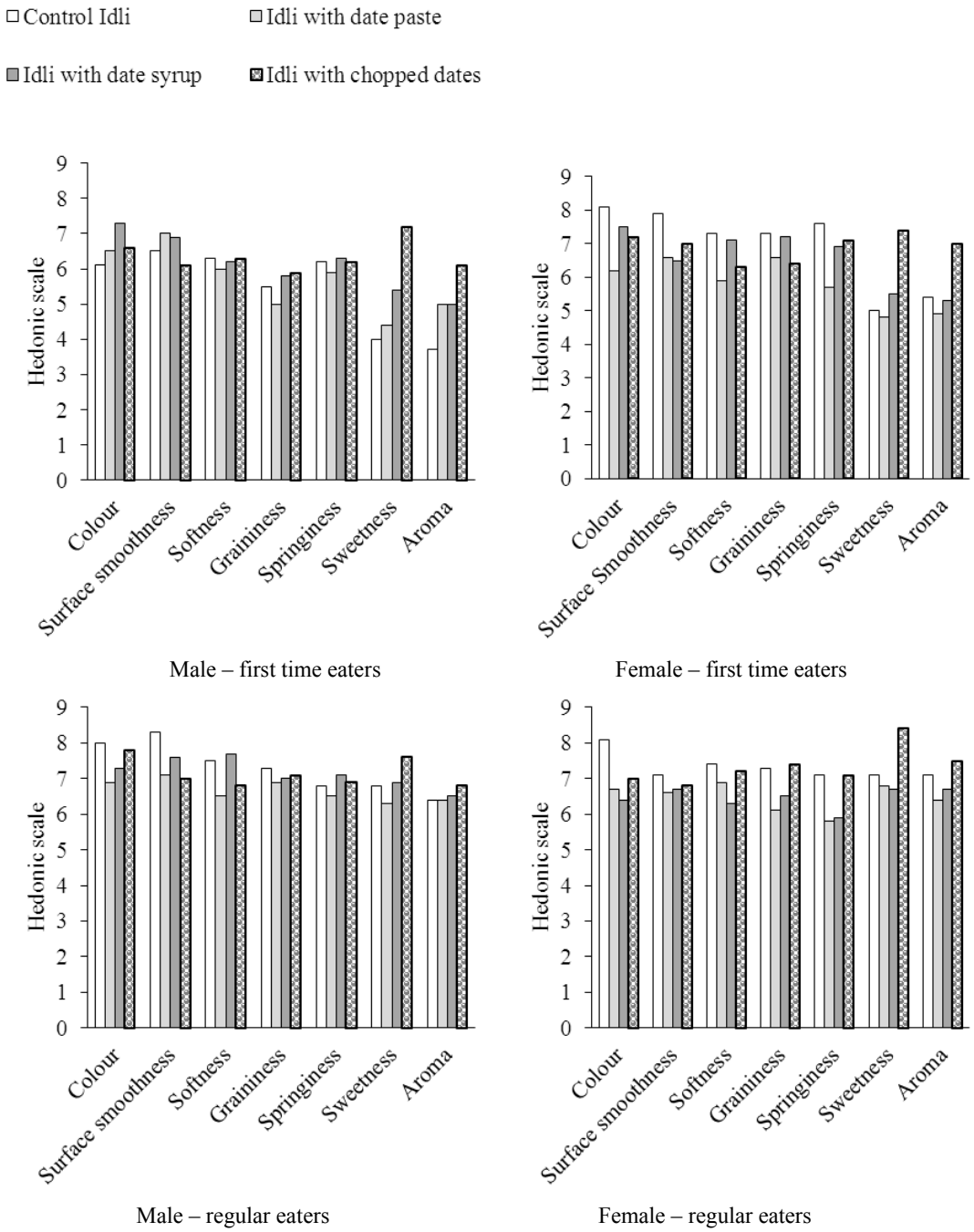


Figure 2. Sensory scores for developed idli products (n=40 panelists). Hedonic scale: 9-like extremely, 8-like very much, 7-like moderately, 6-like slightly, 5-neither like nor dislike, 4-dislike slightly, 3-dislike moderately, 2- dislike very much and 1-dislike extremely.

Sensory evaluation

In order to attain eating quality, it is essential to include all important sensory features such as appearance, texture and flavour (Auerswald et al., 1999; Ben Ismail et al., 2013). A descriptive study on the consumer acceptance of a new product or modified product is essential. The hedonic scores of various attributes of the developed idli products are given in Figure 2.

Appearance

Idli with date paste and date syrup had a homogeneous light brown colour whereas idli with chopped dates had dark specks wherever date pieces were present. The color of the control idli got the highest score and the idli with date paste got the lowest score. There were no differences in the surface smoothness scores of control idli, date paste idli and date syrup idli. However idli with chopped dates scored significantly lower in surface smoothness than control idli.

This suggests that, addition of dates in idli does not improve the appearance of the products. Date syrup has been widely used as sweetener in food. Aboubacar et al. (2010) determined uses of date syrup as a substitute sweetener for sucrose in food using muffin as a model product. Control muffin had lighter color compared to muffin with syrups. Muffins made with date syrup had significantly lower hedonic rate for appearance and color compared to the control muffin. It was concluded that color appears to be the major sensory attribute affecting consumer acceptability while adding more than 50% of date syrup.

Mouth feel and Texture

There were no differences between control idli, idli with chopped dates and idli with date syrup in the hedonic scores of softness and graininess. In all these attributes, idli with date paste scored lower than control idli. All panelists gave equal scores for the springiness of the developed idli products. The male- first time eaters gave the lowest scores for softness and graininess of the developed products, but there were no differences among the other three groups of panelists. The mouth feel and texture of modified products are dependent upon many factors including the amount of replacement, changes in the ingredients, and consumer's perception. For example, Rekha and Vijayalakshmi (2011) reported that fortified idli with soy residue okara was soft and spongy compared to control idli. While replacing white sugar with date syrup, Aboubacar et al. (2010) determined that up to 50% replacement of white sugar with date syrup produced similar texture and acceptance rating to that of control muffins.

Flavor and taste

The sweetness and aroma of the idli with chopped dates got the highest score (between 6 and 9) and there was no difference among the other three products. Similarly regular eaters of idli gave higher scores to these attributes than first time eaters for the developed idli products. The chopped dates probably maintained their freshness after steaming and were preferred by the panelists. The flavor and taste might have improved during steaming of idli. Whenever the date chunks were bitten, the panelists might have felt the fruit taste and liked the product. In terms of sweetness and taste, researchers have compared the products while replacing white sugar with date syrup at various levels. Gouhari et al. (2005) investigated the possibilities of using date syrup to replace sucrose in ice cream. They prepared five types of ice creams (0% (control), 25%, 50%, 75%, and 100% replacement of sucrose with date syrup) and determined that the sensory properties of the modified ice cream were not affected up to 50% replacement. Gad et al. (2010) used date syrup as part of water (2%, 4%, 6%, 8%, and 10% v/v) in reconstituting skim milk powder during the processing of yogurt with 14% total solids. Yogurt enriched with 10% date syrup had the highest rating for sweetness and taste. However the sensory attributes of any product developed with chopped dates are not available in the public domain.

Overall acceptance

The hedonic scores for the overall acceptability of the developed idli products by the panelists are given in Figure 3. All idli products scored between 5 and 9 for the overall acceptability. Idli with chopped dates scored the highest acceptability than other products without any difference among them. Similarly, regular eaters gave a higher score for overall acceptability than first time eaters. In sensory evaluation, although, the individual sensory attribute score varies among the products, overall acceptability is a good indicator about their choice and likeliness of a specific product. For example, Sidhu et al. (2003) used date syrup from two varieties in Kuwait (Birhi and Safari) to replace sucrose in pan bread formulations [0% (control), 50%, and 100% replacement]. It was determined that in spite of lower score in crumb color of date syrup pan bread, there were no significant differences in texture and overall acceptability of control and date syrup pan breads. Similarly in our present study, although the appearance of the idli with chopped dates scored lower, the overall acceptability was higher than other products.

Table 1. Total phenol and vitamin C content of developed idli products (n=3).

Product	Total phenol (mg/ 100 g)	Vitamin C (mg/ 100g)
Idli with added sugar (Control)	4.5	0.19
Idli with date syrup	82.1	0.63
Idli with chopped dates	75.1	0.58

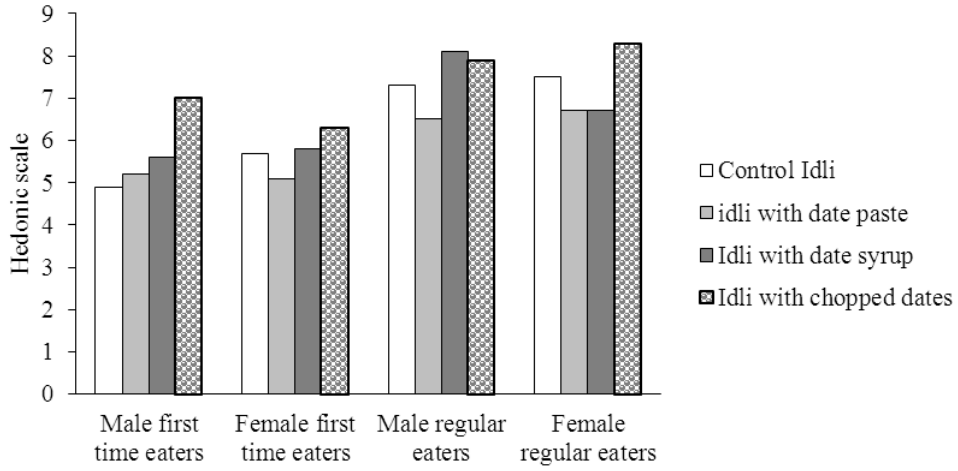


Figure 3. Overall acceptability scores of developed idli products (n=40 panellists). Hedonic scale: 9-like extremely, 8-like very much, 7-like moderately, 6-like slightly, 5-neither like nor dislike, 4-dislike slightly, 3-dislike moderately, 2- dislike very much and 1-dislike extremely.

Total phenol and vitamin C

The total phenol, vitamin C and total reducing sugar contents of control idli and dates idli (idli with date syrup and idli with chopped dates) were measured (n=3). The total reducing sugar content of control idli, and dates idli was in the range of 140 to 180 mg/g without significant difference among them. Total phenols and vitamin C content of developed idli products are shown in Table 1. Dates idli contained higher total phenol and vitamin C content than the control idli. Al-Farsi and Lee (2012) reported that the phenolics and vitamin C contents of dried dates of several varieties were 353 mg/100 g and 1.88 mg/100g, respectively.

Conclusions

In many Asian countries the consumption of added sugar is relatively higher through coffee, tea, milk, juices, sweets and many other ethnic foods. It has been scientifically proven that increased intake of added sugar increases the risk factor for various diseases. To reduce the consumption of added sugar, and enjoy the sweet taste, dates in various forms have potential to blend in ethnic food preparation. Nutrified idli with chopped dates were mostly preferred by all panelists in all groups with higher sweetness and aroma. Idli with chopped dates could be prepared easily by adding the required amount of chopped dates in the fermented batter just before cooking. In addition to health benefits, idli preparation with dates will improve the convenience of handling of idli in public places such as railway stations and food outlets without any separate accompaniments. Date fruits have great potential to use along with several traditional foods and reduce the consumption of added sugar.

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REGULAR ARTICLE

Genetic diversity analysis of date palm (*Phoenix dactylifera* L.) in the Kutch region of India using RAPD and ISSR markers

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Abstract

First line in abstract should be Date palm is an important fruit crop of the arid and semiarid region. In the present investigation two types of DNA based marker RAPD and ISSR were used to characterize 8 date palm genotypes grown in the Kutch region. Amplification of genomic DNA of 8 genotypes using 13 RAPD analyses yielded 88 fragments, of which 35 were polymorphic, with an average of 2.69 polymorphic fragments per primer. Two ISSR primers produced 13 bands of which 3 were polymorphic. RAPD markers were more efficient than ISSR assay with regard to polymorphic detection; RAPD markers detected 39.77% as compared to 23.07% of ISSR markers. Cluster analysis by UPGMA showed that the dendrogram obtained by RAPD and RAPD + ISSR were similar. Cluster A consisted of Early maturing, Ghanshyam and Late maturing female genotypes with 0.81 to 0.88 Jaccard's similarity range. Cluster B consisted of Seasonal female, Male-1, Male-2, Male-3 and Male-4 genotypes with 0.82 to 0.91 similarity range. Genotypes Male-1 and Male-2 were most closely related with the highest value in similarity for Jaccard's coefficient (0.91). Principal coordinate analysis differentiated one group of genotype Male-1 and Male-4 while other genotypes were randomly distributed.

Key words: Date palm, Diversity, Molecular markers, RAPD, ISSR

Introduction

The date palm (*Phoenix dactylifera* L.), 2n=36, is a dioecious long-lived monocotyledonous plant, which belongs to the family Arecaceae. It is one of the excellent candidate crops in arid and semiarid regions of the world with high tolerance to environmental stresses. In addition to its valuable fruit, the tree is cultivated for fuel, fiber and as shelter for ground crops. The annual world production of dates has reached 6-8 million mt (metric tons), representing a market exchange value of over 1 billion USD. The fruit is nutritionally rich; several products are made that generate employment and thus benefit the socioeconomic status of local people (El Hadrami and Al-Khayri, 2012). Moreover, this crop has great potential as a source of renewable energy due to the high carbohydrate content, 44-88%, of date palm fruit (Jain, 2012).

Date palm is one of the important horticultural crops of the Kutch region which enjoys the monopoly of commercial cultivation of this crop in India. The total area under cultivation has increased to 16,000 ha during 2010-2011, from 8,973 ha during 2000-2001, with production of 1.2 lakh tons. (Shandilya, 2012). The Kutch is the largest producer of date palm in India having the most suitable climate for date palm plantations. But available germplasm in the Kutch region lacks information about its genetic base. Due to the crosspollination mode of reproduction and seeds thrown by army camps and partly from the seeds and offshoots planted by the settlers, there is tremendous variation in the genotypes grown in Kutch. Hence, there is an urgent need to document these genotypes and identify characteristics of the best genotypes growing under Kutch conditions in order to expand/replace cultivars for higher and better-quality yield.

Morphological characterization of cultivars requires a large set of phenotypic data that are normally difficult to collect, and statistically variable due to environmental effects (Sedraet al., 1998). Biochemical markers (isozymes and

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proteins) have proven to be effective in cultivar identification as well (Bennaceuret al., 1991). However, they provide limited information and are usually an indirect approach for detecting genomic variation.

DNA fingerprinting in plants is primarily used for identification of genetic diversity, protection of biodiversity or germplasm conservation and identifying markers associated with specific traits (Khanam et al., 2012). Molecular markers based on RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequence Repeats) are powerful techniques, which can be used to identify and determine plant genomes or to estimate the phylogenetic relationship among genotypes of date palm (Cullis, 2011; Elshibli and Korpelainen, 2011). The RAPD technique has been used for cultivar genotyping (Ben-Abdallah et al., 2000; Trifi et al., 2000) and for analyses of phylogenetic relationships and genetic diversity (Al-Khalifah and Askari, 2003; Al-Moshileh et al., 2004; El-Tarras et al., 2007; Khalifah et al., 2012). Estimating the genetic distance assists in studying genetic diversity, a trait that is important for parent selection associated with genetic mapping and for marker-assisted selection in breeding programs (Lapitan et al., 2007; Trethowan and Kazi, 2008). The ISSR targets multiple genomic loci, and usually yields dominant markers (Zehdi et al., 2004; Khanam et al., 2012). Therefore, the present investigation was conducted to define the genetic diversity among female and male genotypes grown in the Kutch region using RAPD and ISSR markers.

Materials and methods

Date palm genotypes

Fresh juvenile leaf samples of date palm germplasm were collected from the Kutch district of Gujarat. This included 8 date palm genotypes comprising 4 females, cvs.: Early maturing, Ghanshyam, Late maturing and Seasonal (also known as Timely maturing) and 4 males plants of different characters designated as Male-1, Male-2, Male-3 and Male-4. Morphological characters of these genotypes are given in Table 1 and their growth locations are shown in Figure 1.

Table 1. Morphological characters of 8 date palm genotypes under study.

Sr. No.	Female trees	Fruit color	Fruit size	Resistance to rain water	Fruit Taste	Plant age (yr)
1	Early maturing	Red	Large	Resistance	Sweet	16
2	Ghanshyam	Red	Medium	Resistance	Much sweet	8
3	Late maturing	Yellow	Large	Low resistance	Medium sweet	17
4	Seasonal	Brown red	Large	Susceptible	Medium sweet	12
Sr. No.	Male trees	Location	Quality	Source	Plant age (yrs)	
5	Male-1	Bhuj (Kutch)	Medium	Offshoot	4 years	
6	Male-2	Bhuj (Kutch)	Medium	Main plant	15 years	
7	Male-3	Mandvi (Kutch)	Medium	Offshoot	15 years	
8	Male-4	Bhuj (Kutch)	Good	Offshoot	4 years	

Figure 1. Location of Kutch region in India. All genotypes were collected from Bhuj except male-3 which was collected from Mandvi.

DNA extraction

Total genomic DNA of 8 date palm genotypes was extracted from 500 mg of young leaf samples using the CTAB (Cetyltrimethylammonium bromide) method of Doyle and Doyle (1990) with a few modifications. The leaves were first ground to a fine powder in liquid nitrogen using autoclaved and pre-chilled mortar and pestle. It was then transferred into a prewarmed extraction buffer (1.5M NaCl, 100 mM Tris-Hcl pH-8, 40 mM EDTA, 1% PVP, 3% C-Tab and 1% β-mercaptoethanol) and incubated at 65°C for 1 hr. Equal volume of chloroform : isoamyl alcohol (24:1 v/v) was added, mixed well by gentle inversion and centrifuged. The supernatant was transferred to a fresh tube and DNA was precipitated by adding 0.6 volume of ice-cold isopropanol. After centrifugation, the pellet was washed in 70% ethanol, dried and dissolved in TE buffer. RNA was removed by RNase treatment. DNA was quantified using UV-spectrophotometer and diluted to 50-60 ng/µl and used in PCR.

PCR conditions and electrophoresis

Polymerase chain reaction (PCR) for both RAPD and ISSR analysis was performed in 20µl volume containing 1x PCR buffer (Banglore Genei), 1.5 mM MgCl2, 0.2 mM dNTPs, 0.1µM of primer, 50 ng of genomic DNA and 1 U Taq DNA polymerase(Banglore Genei). The reaction mixture was placed on a DNA thermal cycler (Biometra). RAPD program was performed as 1 cycle of 94°C for 5 min and 40 cycles of 94°C for 1 min, 38°C for 1 min and 72°C for 2 min, then, a final extension step 72°C for 8 min. The ISSR program was performed as 1 cycle of 94°C for 5 min and 35 cycles of 94°C for 1 min, 45°C for 45 sec, 72° C for 1.5 min and a final extension step of 72°C for 8 min. PCR products were electrophoreses on 1.5% (w/v) agarose gels, in 0.5X TBE Buffer at 80 V for 1 hr and then stained with ethidium bromide (0.5 µg/ml). Gels with amplified fragments were visualized and photographed under UV light.

Scoring and data analysis

The DNA bands were scored for computer analysis on the basis of presence or absence. If a product was present in a genotype it was designated "1", if absent it was designated "0" after excluding irreproducible bands. Pair-wise comparisons of genotypes, based on presence or absence of unique and shared polymorphic products, were used to generate similarity coefficients based on similarity matching. Using a distance matrix, a principal coordinate analysis to construct a three-dimensional array of eigenvectors was performed using a DCENTER module of NTSYS 2.2 program.

Results and discussion

The use of molecular markers, revealing polymorphism at the DNA level, has been playing an increasing part in plant biotechnology and their genetic studies. In this work, the utility of RAPD and ISSR markers for variation and identification of date palm germplasm was studied.

RAPD banding patterns and dendrogram analysis of germplasm

Eight date palm genotypes obtained from the Kutch region were amplified using 13 RAPD oligonucleotide (Table 2). Amplification products of 8 genotypes with these 13 RAPD primers yielded a total of 88 scorable bands, out of which 35 (39.77%) were found to be polymorphic. The size of the amplification product ranged from 200 to 4000bp. The highest number of bands (12) were obtained with primer OPE-5, while the lowest number of bands (3) were obtained with primer OPE-9. The percentage of polymorphism ranged from 16.66 to 80%. Primer OPE-10, revealed the highest polymorphism (80%) while primer OPJ-1 and OPL-3 exhibited the lowest polymorphism (16.66%). Total number of bands amplified ranged from 16 (OPE-9) to 63 (OPM-7). Similarly, Sedra et al. (1998) observed 66.07% polymorphism among 43 date palm accessions using 19 RAPD primers. The polymorphism was much higher than our study which might be due to a higher number of date palm accessions.

The primer OPE-17 showed 5 monomorphic bands among which 4 bands were in the range of 1.1 to 1.5kb and 1 band of 600bp (Figure 2). A band of~1kb was present only in late maturing and seasonal female genotype, while absent in other genotypes. A band of ~300bp was present in Seasonal, Male-2 and Male-4 while absent in the other 5 genotypes. The primer OPE-3 has amplified a total number of 8 scorable bands in the ranges from 300 to 1000bp. Genotypes from lane 1-4 (Female) and lane 5-8 (male) exhibited first 6 monomorphic bands. One band of ~475 bp was present in Late maturing, Ghanshyam and Early maturing female genotypes which are rain water resistance while absent in the other 5 genotypes. Bands of ~300bp was present in 4 female genotypes (lane 1-4) and absent in 4 male genotypes (lane 5-8) which may be for female specific. Similarly, 3 female specific markers were

identified in date palm by Younis et al. (2008). Male specific alleles were identified by Cherif et al. (2012) in 52 male genotypes of date palm using microsatellite markers. They found three genetically linked loci which were heterozygous only in males.

Table 2. Details of amplification obtained with different RAPD primers.

Sr. No.	Name of primer	Sequence (5'—3')	No. of total bands	No. of polymorphic bands	No. of monomorphic bands	Polymorphism percent (P%)	Total No. of bands amplified
1	OPE-3	CCAGATGCAC	8	2	6	25	55
2	OPE-5	TCAGGGAGGT	12	6	6	50	61
3	OPE-9	CTTCACCCGA	3	2	1	66.66	16
4	OPE-10	CACCAGGTGA	5	4	1	80	24
5	OPE-17	CTACTGCCGT	8	3	5	37.5	53
6	OPE-18	GGACTGCAGA	7	3	4	42.85	45
7	OPJ-1	CCCGGCATAA	6	1	5	16.66	45
8	OPJ-5	CTCCATGGGG	8	2	6	25	59
9	OPJ-18	TGGTCGCAGA	5	1	4	20	35
10	OPK-8	GAACACTGGG	5	2	3	40	35
11	OPL-3	CCAGCAGCTT	6	1	5	16.66	42
12	OPM-7	CCGTGACTCA	10	5	5	50	63
13	D07	TTGGCACGGG	5	3	2	60	26
Total			88	35	53	39.77	559

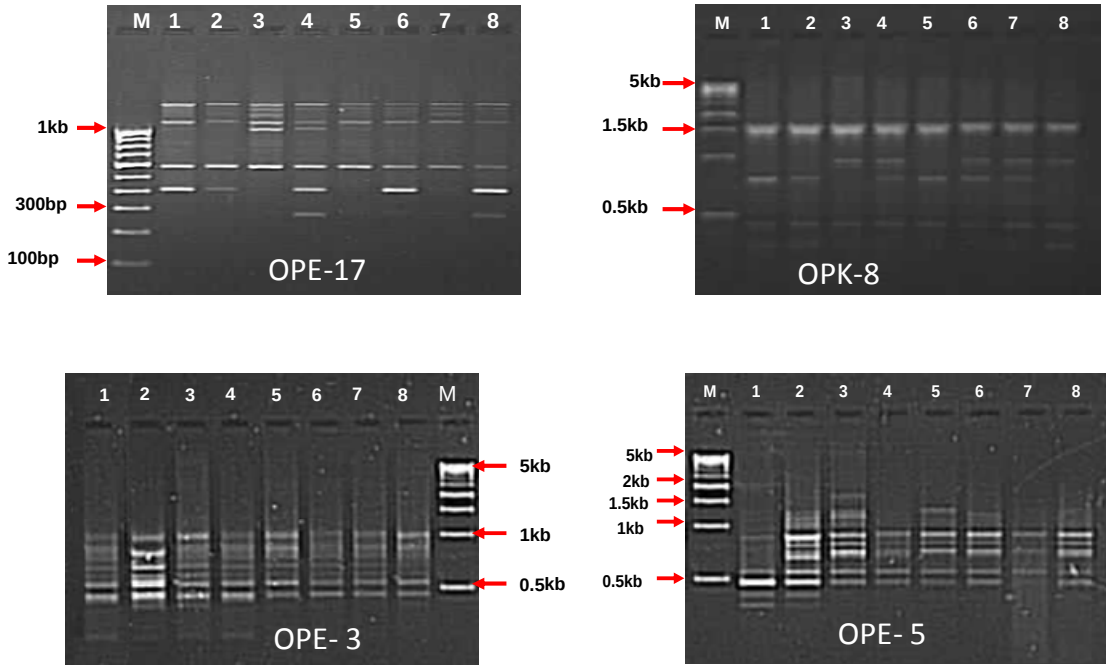

Figure 2. RAPD amplification pattern of 8 date palm genotypes obtained with 4 random primers. M is the DNA marker, lanes 1-4 are females (cvs. Ghanshyam, Early maturing, Late maturing and Seasonal) and lanes 5-8 are the males (M1, M2, M3 and M4).

The primer OPK-8 generated 3 monomorphic bands of size ~350, 400 and 1500bp while 1 band of 1kb was absent in Early maturing, Ghanshyam and Male-1, and present in the other genotypes. A band size of ~800bp was present in Late maturing and Male-4, while absent in the other 6 genotypes. In the case of primer OPE-5 it amplified a total number of 12 scorable bands. All the 8 genotypes reveal 6 monomorphic bands of which 4 bands ranged from ~750bp to 1000bp and the other 2 ranged from ~480 to 510bp. A band of ~700bp and 1.5kb was unique to Ghanshyam (very sweet fruit) and Late maturing (yellow color fruit) respectively. A band size of ~300 and 400bp was unique to Early maturing and Ghanshyam but absent in the other 6 genotypes and may be for red color or resistance to rain water.

A dendrogram (Figure 3) based on UPGMA analysis grouped the 8 genotypes into 2 main clusters, with Jaccard's similarity coefficient ranging from 0.78 to 0.93. Similarly, Al-Moshileh et al. (2004) observed similarity coefficient ranges from 0.70 to 0.84 in 6 cultivars of date palm using RAPD profiles. This higher similarity reflects less diversity of germplasm due to collections coming from the same geographic region. The dendrogram obtained 2 main clusters (A and B) with 3 and 5 genotypes respectively. The cluster A has 2 subclusters (A1 and A2). Subcluster A1 has Early maturing and Ghanshyam with red fruits and high resistance to rain water and subclusters A2 contains Late maturing with yellow fruit and medium resistance to rain water. Cluster B bifurcates into 2 subclusters. Subcluster B1 with 1 female genotype Seasonal (not resistance to rain water) while subcluster B2 contained 4 male genotypes (Male-1, Male-2, Male-3 and Male-4) in which Male-1 and Male-2 were the most similar genotypes and Male-4 was a good quality male genotype with most variable characteristics in subcluster B2. These results suggest that RAPD may differentiate genotype based on morphological characters and also gave some unique markers in some genotypes.

ISSR banding patterns and dendrogram analysis of germplasm

Eighteen ISSR oligonucleotides were used for amplification of all the 8 genotypes out of which only 2 primers gave reproducible amplification products (Table 3), while 16 primers did not give any polymorphism. Amplification products of 8 genotypes with these 2 ISSR primers yielded a total of 13 scorable bands, out of which 3 were found to be polymorphic (Figure 4). The size of the amplification product ranged from 300 to 4000 bp. Primer HB-9 showed the highest percentage value of polymorphism of 40%, compared to HB-10 with 12.5%. Similarly, Hussein et al. (2005) reported polymorphism at a low level (28.6%) using 10 ISSR primers among 14 date palm accessions. The present result of ISSR showed very low polymorphism (23%). This does not mean that ISSR is inefficient to detect polymorphism. There is a need to screen a larger number of primers to evaluate the present set of genotypes.

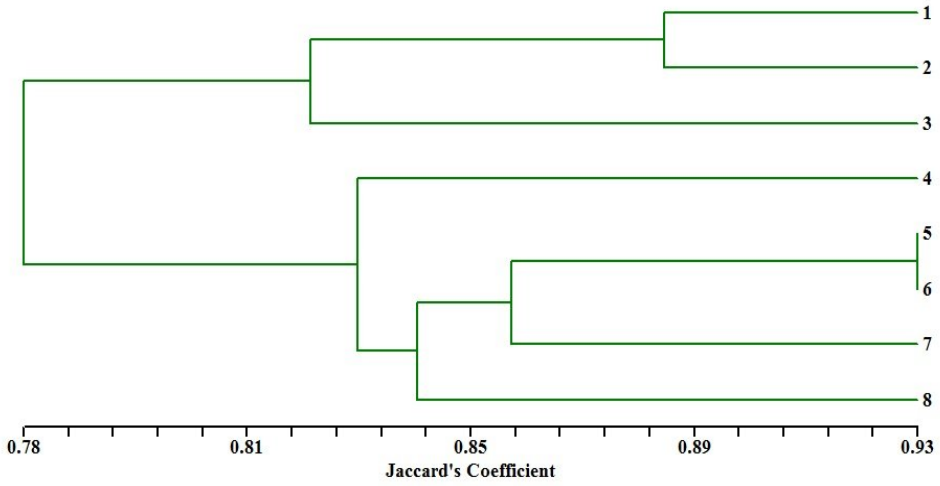

Figure 3. Dendrogram depicting the genetic relationship among 8 date palm genotypes consisting of 1-4are females (cvs. Ghanshyam, Early maturing, Late maturing and Seasonal) and 5-8 are males (M1, M2, M3 and M4) based on RAPD data.

Table 3. Details of amplification obtained with different ISSR primers.

Sr. No.	Name of primer	Sequence (5'-3')	No. of total bands	No. of polymorphic bands	No. of monomorphic bands	Polymorphism percent (P %)	Total No. of bands amplified
1	HB-9	$(GT)_6GG$	5	2	3	40	29
2	HB-10	$(GA)_6CC$	8	1	7	12.5	61
Total			13	3	10	23.07	90

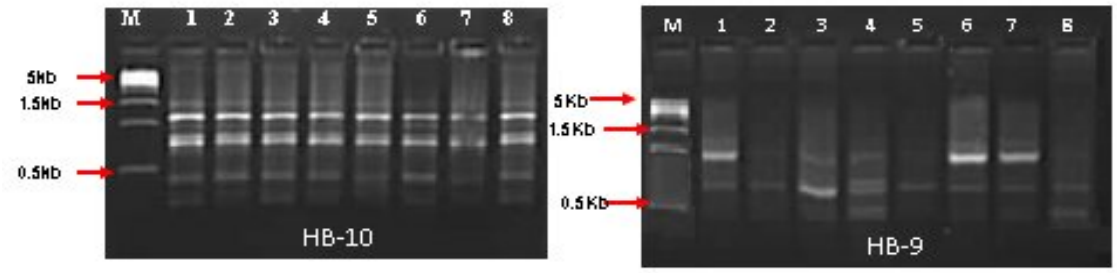

Figure 4. ISSR amplification pattern of 8 date palm genotypes obtained with two random primers. M is the DNA marker, lanes 1-4 are females (cvs. Ghanshyam, Early maturing, Late maturing and Seasonal) and lanes 5-8 are the males (M1, M2, M3 and M4).

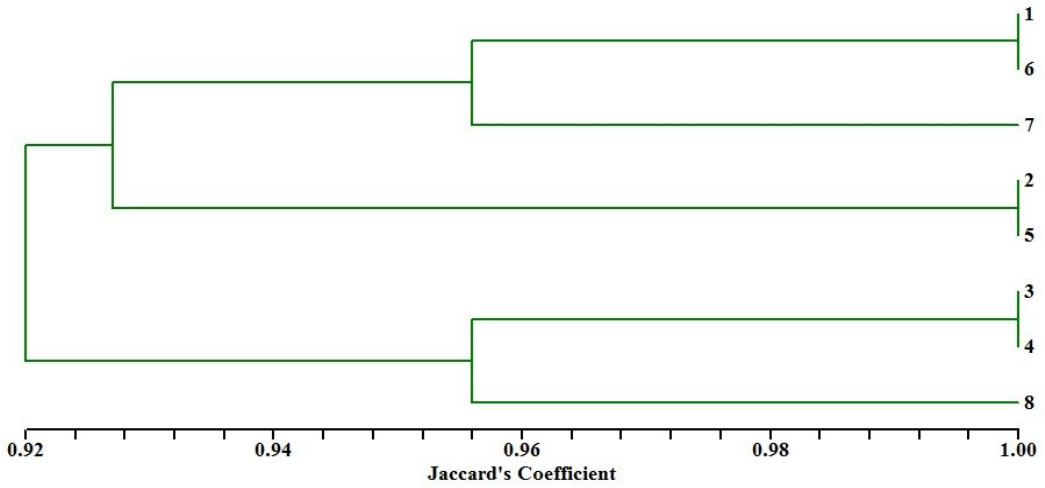

Figure 5. Dendrogram depicting the genetic relationship among 8 date palm genotypes consisting of 1-4 are females (cvs. Ghanshyam, Early maturing, Late maturing and Seasonal) and 5-8 are males (M1, M2, M3 and M4) based on ISSR data.

The primer HB-9 showed a total of 5 scorable bands, out of which 2 were polymorphic. A band size of ~400, 650 and 900bp showed 3 monomorphic bands, while a band size of ~670bp was present in only Late maturing and Seasonal which have large fruit size and Medium sweet, while absent in other 6 genotypes. A total of 7 bands were amplified by primer HB-10, out of which 6 were monomorphic ranging in size from ~400 bp to 1.5 kb and 1 polymorphic band which was absent in Ghanshyam, Male-3 and Male-4, while present in other genotypes.

A dendrogram (Figure 5) based on UPGMA analysis grouped the 8 genotypes into 2 main clusters, with Jaccard's similarity coefficient ranging from 0.92 to 1.00. The dendrogram showed 2 main clusters (A and B) with 5 and 3 genotypes respectively. Cluster A has 2 subclusters (A1 and A2). Subcluster A1 has Early maturing, Male-2 and Male-3 genotypes in which Early maturing and Male-2 with nearly similar ages (15 and 16 years), while Male-3 genotype was from a different location. Cluster B was further divided into 2 subclusters. Subcluster B1 consisted of Late

maturing and Seasonal (female) which were most similar, while subcluster B2 including Male-4 (a good male genotype) were different from Late maturing and Seasonal genotypes.

Combined analysis using RAPD and ISSR data

The combined analysis of RAPD and ISSR with similarity coefficient ranging from 0.77 to 0.91, showed polymorphism of 37.62% using 15 selected primers among 8 date palm genotypes. These results are consistent with Hussein et al. (2005) working with 14 date palm accessions, collected from different locations in Egypt, using a combination of RAPD and ISSR markers. They found low levels of intervarietal polymorphism and higher genetic similarities ranging from 91.4% to 99.6%. These results suggest a narrow genetic background in collected germplasm. Contrary to our results, Haider et al. (2012) observed higher polymorphism in 23 date palm cultivars from Syria representing 18 female and 5 male cultivars. The average polymorphism detected by the RAPD assay (58.5%) was higher than that observed for ISSR (50.6%). These results might be due to more genotypes and primers used in the study.

The amplification data obtained from RAPD and ISSR primers were used for a similarity matrix. This combined dendrogram (Figure 6) was similar to the RAPD dendrogram. In RAPD and ISSR combined marker data, the UPGMA dendrogram obtained had almost the same sample distribution pattern. On the other hand, the dendrogram from ISSR, with a high similarity matrix value, suggests that RAPD was more efficient than ISSR in date palm.

Principal co-ordinate analysis

PCA was performed to visualize the dispersion of genotypes in relation to the first 3 principle axes of variation (Figure 7). The genotype Male-1 (5) and Male-4 (8) genotypes were grouped together as good males while other genotypes were randomly distributed. Genotype no. 4 (female Seasonal), rain water sensitive, was in the central position. Furthermore, females no. 1, 2 and 3 (Early maturing, Ghanshyam and Late maturing) were on the same axis, while male samples no. 6 and 7 (Male-2 and Male-3) were on the other axis. Hamza et al. (2012) found grouping of date palm cultivars according to their fruit characteristics. PCA results showed the grouping of soft or semi-soft fruits and early or mid-season maturities. From PCA analysis, we conclude that Male-1 and Male-4, which are of same age (4 years) and the same location (Location-1) and taken from offshoots were grouped together. These results may further be confirmed with a larger number of genotypes and primers.

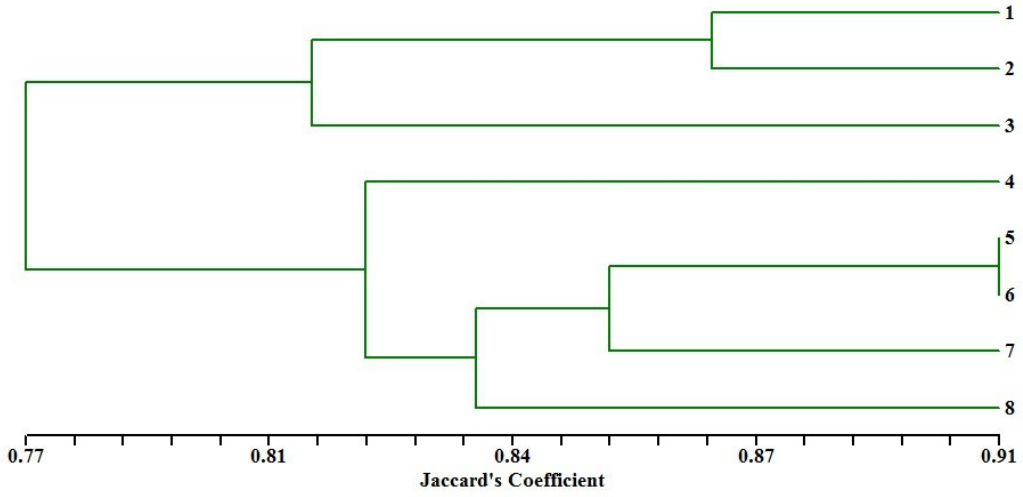

Figure 6. Dendrogram depicting the genetic relationship among 8 date palm genotypes consisting of 1-4 are females (cvs. Ghanshyam, Early maturing, Late maturing and Seasonal) and 5-8 are males (M1, M2, M3 and M4) based on pooled RAPD and ISSR data.

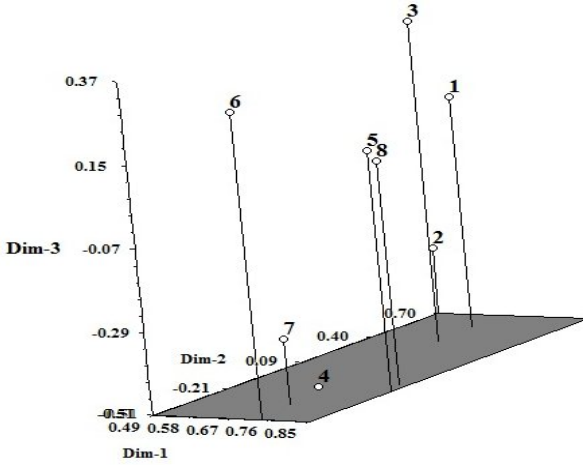

Figure 7. Principle coordinate analysis of 8 date palm genotypes, 1-4 are females (cvs. Ghanshyam, Early maturing, Late maturing and Seasonal) and 5-8 are males (M1, M2, M3 and M4).

Conclusion

The date palm is known as *kulpvriksh* in the Kutch region, due to its great potential to generate income in extreme environmental conditions and unproductive lands. Characterization of germplasm is a prerequisite for crop improvement and systematic study. The current study implies that RAPD and ISSR markers are effective tools to discriminate various date palm genotypes. Development of other date palm molecular markers like microsatellite (SSR) would facilitate distinguishing various genotypes based on particular traits. Molecular markers are indispensible for modern breeding to achieve date palm genetic improvement considering the lengthy and dioecious nature of date palm.

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REGULAR ARTICLE

Genotyping and identification of six date palm (*Phoenix dactylifera* L.) cultivars of the Gaza Strip by random amplification of polymorphic DNA

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Abstract

Date palm (*Phoenix dactylifera* L.) is one of the major fruit crops in the Gaza Strip. The main cultivars are Hayani, Bentaisha, Barhi, Zahedi, Ameri and Halawy. Because of the difficulty associated with morphological identification of date palm cultivars, development of cultivar specific genetic markers have been extensively studied. The purpose of this study was to optimize and apply a reliable molecular marker protocol for genotyping and identification of these cultivars. Random amplification of polymorphic DNA (RAPD) was used to study the genetic diversity among the six cultivars using 42 primers. Genetic similarity matrices were constructed for the six cultivars using the Nei and Li formula and clustered with the UPGMA to determine the relationships between the six cultivars. Nine primers were informative and reproducible, and gave a number of polymorphic bands (on average, 7.2 bands per primer). The overall level of polymorphism was 49%. Genetic similarity among the six cultivars ranged from 76.3 to 93.5%. Some primers gave reproducible cultivar-specific bands that may be reliable to identify that cultivar. Our study introduces for the first time a molecular marker approach, capable of distinguishing and studying the genetic diversity among the six date palm cultivars in the Gaza Strip.

Key words: Date palm, Cultivars, Gaza Strip, Molecular identification, PCR, *Phoenix dactylifera*, RAPD

Introduction

Date palm (*Phoenix dactylifera* L.), has been economically, spiritually and ornamentally associated with eastern Mediterranean life for at least the last 6,000 years (Popenoe, 1913; Barreveld, 1993; El Hadrami and Al-Khayri, 2012). The date palm can tolerate long periods of drought, and thus is widely grown and its fruits consumed in the dry regions of the Middle East, North Africa and the Indian subcontinent. The most important date-producing countries are Egypt, Saudi Arabia, Iran, United Arab Emirates, Algeria, Iraq, Pakistan, Oman, Tunisia and Libya (FAO, 2013). Date palm reportedly has more than 3,000 varieties around the world (Zaid, 2002). The overall production of dates has increased in the last 10 years worldwide to reach 7.5 million metric tons in 2011 (FAO, 2013). In the Palestinian territories, there has been an increase in date production to 4,688 metric tons in 2011 (FAO, 2013).

In the Gaza Strip, most of the date palms are planted in the coastal area and in many cases the plantations utilize mixed farming systems. The main date palm cultivars in the Gaza Strip are Hayani and to a lesser extent Bentaisha. In 1999 other cvs. such as Barhi, Zahedi, Ameri and Halawy were introduced (Banna, 2007).

Date palm cultivars are very difficult to distinguish by morphology. They are distinguished by the characters of the fruits, which are produced only after 4-5 years (Sedra et al., 1998). Therefore, molecular techniques may be a more reliable approach for cultivar discrimination. Genotyping using DNA-based markers has been used in the assessment of genetic diversity in plant species for different purposes (Agarwal et al., 2008; Appleby et al., 2009). It provides useful information regarding genetic diversity and the relationship between cultivars, while unaffected by environmental factors and the developmental stage of the plant. Among the various kinds of genotyping techniques characterized so far, restriction fragment length polymorphism (RFLP) was the first and until recently the most commonly used for estimation of the genetic

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diversity in plant species (Barnes, 1991). However, it is a time-consuming and labor-intensive assay**.** Polymerase chain reaction (PCR)-based techniques, which include random amplification of polymorphic DNA (RAPD), simple sequence repeats (SSR) and amplified fragment length polymorphism (AFLP) are playing an increasingly important role in DNA fingerprinting and pedigree analysis (Al-Khalifah and Askari, 2003; Agarwal et al., 2008; Al-Khalifah et al., 2012; Khanam et al., 2012; Zhao et al., 2013).

The arbitrarily designed RAPD short primers (usually 10-mer) may be used to reproducibly amplify segments of genomic DNA from a wide variety of species (Powell et al., 1995). Polymorphism among the amplification products is frequently detected and is useful as a genetic marker. RAPD assay may in some instances detect single base changes in genomic DNA (Atienzar et al., 2002)**.** However**,** distinguishable RAPD fingerprints among different varieties are obtainable only if suitable primers are used and PCR conditions are optimized (Ramos et al., 2008). On the other hand, the RAPD technique is relatively cheap, easily performed and interpreted, and possesses high discriminatory power and typing capacity (Buckingham, 2012).

So far, the discrimination between date palm cultivars, based on morphology, has created obstacles in proper date palm agriculture, and that may lead to serious problems in terms of time and cost. Therefore, in this study, we have optimized a molecular method capable of distinguishing among the six different Gaza Strip date palm cultivars using RAPD technique. The genetic diversity between these cultivars was also studied.

Table 1. A summary of the morphological characteristics of six date palm cultivars present in the Gaza Strip.

Fruit Charact-eristics	Date palm cultivar name					
	Hayani	Bentaisha	Halawy	Zahedi	Barhi	Ameri
Morphology						
Color	Dark red	Dark red	Yellow	Yellow	Yellow	Reddish yellow
Shape	Oblong, 4-7cm	Globular, 2-3 cm	Oblong, small to medium size	Ovate, medium in size	Ovate, 3-4 cm	Oblong, 4-5 cm
Weight	12-20 g	15-20 g	7-10 g	7-10 g	15-20 g	15-23 g
Consistency	High moisture content, (about 72%)	A high moisture content (about 89%)	Low-moderate moisture content	Dry at consumption	High moisture (about 75%)	Moderate moisture (about 62%) and high fiber content
Other features	The main cultivar in Gaza strip. Collected either red or black (rutab).	Less common cultivar than Hayani. Collected either red or black (rutab). Sweeter taste than Hayani.	Distinctive rich flavor and extremely sweet taste. Consumed fresh fruit at the Khalal stage.	Palm is fast growing and heavy bearing. Sugary fruits. Consumed as dry dates (tamar).	High quality and heavy yield. Excellent flavor with little astringency at Khalal stage. Marketed and consumed only as fresh fruit on strands at the Khalal stage.	Eaten dried.

Materials and Methods

Plant material source and preparation

Young leaves were collected from 9-year-old well characterized female date palm trees. Four samples, each from a different tree, were collected from each cultivar (Ameri, Halawy, Zahedi, Barhi, Bentaisha and Hayani). The tested date palms were selected, following consultation with the Palestinian Agricultural relief Committee (PARC). The PARC imported the four yellow cvs. (Halawy, Zahedi, Barhi and Ameri) in 1999 and distributed them to local farmers. Therefore, they provided information about the location of cultivars, age of the palm and evidence of fruit production. Leaf samples were collected from three areas of the Gaza Strip (Rafah, Deir al-Balah and Az-Zawayda). Table 1 summarizes some of the fruit morphological characteristics of the six cultivars. washed with 500 µl 70% ethanol. The pellet was dissolved in 100µl TE buffer and kept on ice for one hour, then mixed gently.

DNA extraction

DNA was extracted from 100 mg new tender white to pale yellow leaves to increase the DNA yield, and to avoid high levels of endogenous phenolics, polysaccharides, or other substances present in green leaves that may interfere with DNA extraction. In order to assure the cultivar type, 9-year-old trees were selected provided that they had already borne fruit.

The leaves were frozen and ground under liquid nitrogen to a fine powder using a mortar and pestle. The liquid nitrogen was allowed to evaporate and the powder was either stored at -70 ºC or immediately subjected to DNA extraction procedures using two methods. The first method involved the commercially purchased DNeasy plant mini kit (Qiagen, Germany), according to the manufacturer recommendations. The second protocol followed a previously published manual protocol (Dellaporta et al., 1983) with reduced plant material weight (100 mg instead of 1 g) using 10 ml falcon tubes and Sorvall centrifuge (Thermo Scientific). Ground plant tissue was lysed for 10 min at 65ºC with 1.5 ml extraction buffer (100 mM Tris-HCl pH 8; 50 mM EDTA pH 8; 500 mM NaCl; 10 mM 2-mercaptoethanole) and 100 µl 20% SDS. The lysate was incubated with 500 µl of 5 M potassium acetate for 20 min on ice followed by centrifugation at 25000 xg, 4ºC for 20 min. The supernatant was poured through a miracloth filter to a new tube containing 1 ml isopropanol and 1 ml 5 M ammonium acetate and incubated at -20ºC for 20 min. The nucleic acid was pelleted by centrifuge for 15 min. at 20000 xg; the supernatant was gently discarded and the pellet was dried for 5 min. The pellet was resuspended in 700 µl TE buffer (50 mM Tris 10 mM EDTA, pH 8) and contaminating RNA was digested with 4µl of 100 mg/ml RNase A for 1 hour at 37ºC. Insoluble debris was pelleted with 75 µl of 3 M sodium acetate and centrifugation for 15 min 25000 xg. The supernatant was then transferred to a clean tube containing 500 µl isopropanol and incubated at room temperature for 5 min. The DNA was pelleted at 20000 xg for 15 min, and the pellet

After purification, the DNA concentration was spectrophotometrically determined at absorbance of 260 nm, and its integrity was determined with 1% agarose mini-gel electrophoresis at 7 V/cm.

RAPD-PCR

A list of primers analyzed in the study is provided in Table 2 (Operon Technologies Inc., USA). The primers included 42 sequences selected from previously published reports based on reproducibility (Corniquel and Mercier, 1994; Aitchitt et al., 1998; Mokhtar et al., 1998; Ouenzar et al., 1998; Sedra et al., 1998; Mokhtar et al., 2000; Soliman et al., 2003; Saker et al., 2006; Eissa et al., 2009). The PCR reactions were performed in a final volume of 25 µl containing 30 ng total genomic DNA, 200 µM of each dNTP, 50 pmole primer and 1.25 units of Taq DNA polymerase (Promega,USA). The PCR cycling parameters consisted of an initial denaturation for 5 min at 94°C followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 35ºC for 1 min and extension at 72ºC for 2 min and a final delay phase at 72°C for 5 min. The quality of PCR was assessed by running a water negative control in each experiment. The amplified PCR fragments were separated on 1.4% agarose gel in 1x TAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.3 at 25°C), stained with ethidium bromide (0.5 µg/ml) and visualized by UV trans-illumination.

Data analysis

The results of RAPD were scored based upon the absence (-) or presence (+) of bands and the number of bands per primer. The scored data was transformed into numerical values based on the similarity and genetic distance. For each primer the scoring system was followed on the basis of the number of bands in a single cultivar and absent in the others; as well as the number of common bands in all cultivars.

Table 2. Sequences of 42 10-mer random primers analyzed with RAPD technique.

Primer No.	*Sequence 5' to 3'	Primer No.	Sequence 5' to 3'
1	CAGGCCCTTC	22	CTGCTGGGAC
2	AGGGGTCTTG	23	GTAGACCCGT
3	GTGATCGCAG	24	CCTTGACGCA
4	CAATCGCCGT	25	TTCCCCCGCT
5	TCGGCGATAG	26	TCCGCTCTGG
6	CAGCACCCAC	27	GGAGGGTGTT
7	TCTGTGCTGG	28	TTTGCCCGGA
8	TTCCGAACCC	29	GTGTGCCCCA
9	AGCCAGCGAA	30	CACCGTATCC
10	AGGTGACCGT	31	GAGAGCCAAC
11	CAAACGTCGG	32	CCCAAGGTCC
12	GTTGCGATCC	33	AAGACCCCTC
13	GTTTCGCTCC	34	GTTGGTGGCT
14	TGATCCCTGG	35	GGGAACGTGT
15	CATCCCCCTG	36	CTCAGTCGCA
16	GGACTGGAGT	37	ACATGCCGTG
17	TGCGCCCTTC	38	AGGCCCCTGT
18	TGCTCTGCCC	39	ATGCCCCTGT
19	GGTGACGCAG	40	AAAGCTGCGG
20	GTCCACACGG	41	GGGTTGTTGG
21	TGGGGGACTC	42	GTGGGTGTTG

* The primers sequences were obtained from (Corniquel and Mercier, 1994; Aitchitt et al., 1998; Mokhtar et al., 1998; Ouenzar et al., 1998; Sedra et al., 1998; Mokhtar et al., 2000; Soliman et al., 2003; Saker et al., 2006; Eissa et al., 2009).

The NL coefficient calculations were made to determine the similarity value between two cultivars using a single primer (Nei and Li 1979) using the following formula: NL = 2a/(b + c)); where, (a) number of similar bands in both cultivars, (b) total number of bands in the first cultivar and (c) total number of bands in the second cultivar. The similarity values for each primer were used for construction of a binary matrix for the six cultivars. The average of similarity values between each two cultivars using all primers was calculated and included in a single binary matrix. This matrix was also used for construction of a dendrogram according to UPGMA method (Michener and Sokal, 1957) using the online dendrogram construction utility, DendroUPGMA (http:// genomes.urv.cat/UPGMA) (Garcia-Vallvé et al., 1999).

Results and Discussion

Validation of DNA extraction and RAPD protocols

The quality and quantities of DNA may be important for the success of highly sensitive RAPD application. Extraction of the plant DNA using commercially ready-made reagent recipes is usually more convenient than methods with manual preparations, because it saves time, requires minimal preparation of buffers and reagents, and is less prone to error. However, commercial kits are more expensive. In this work a manual protocol for DNA extraction was successfully adapted with few modifications (Dellaporta et al., 1983).

The scale and purity of DNA extracted by the "Dellaporta protocol" and the commercial kit was satisfactory (concentration: 35-125 ng/µl and 25-65 ng/µl respectively). No degradation of DNA was observed (Figure 1), although the purity of manually extracted DNA was slightly reduced (260/280 OD ratio = 1.5 compared to 1.6-2 in case of the commercial kit). This was not critical for the quality of PCR reactions and did not compromise the number and quality of bands obtained in this study. Therefore we used the manual protocol in the rest of the study.

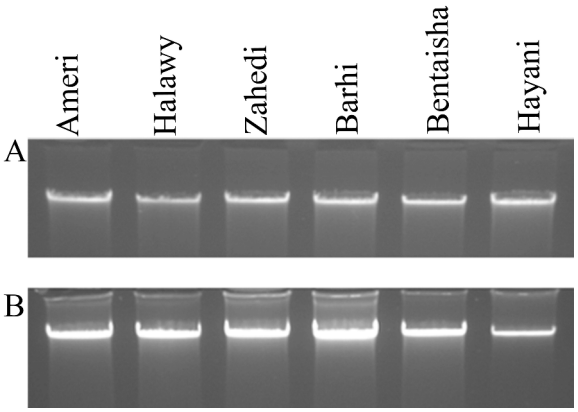

Figure 1. Agarose gel electrophoresis of total DNA. A representative 1% agarose gel showing DNA extracted from the six cultivars using the DNeasy plant mini kit (A) and the "Dellaporta protocol" (B).

For PCR reaction optimization, the GoTaq DNA polymerase kit (separate Taq DNA polymerase, buffer, MgCl2 and dNTPS) was used, rather than a ready-made master mix, which allowed adjustment of reactants amounts and concentration. The results of this work showed that the optimum PCR reaction composition for RAPD test are 1.25U Taq DNA polymerase, 200 µM each dNTPs, 1.5 mM $MgCl_2$, 50 pm of each primer and 30ng DNA.

The results showed that RAPD is sensitive to changes in the PCR kit components. This agrees with many previous works which discussed the RAPD reproducibility and sensitivity when changing the PCR reaction components (Sharma et al., 1995; Skorić et al., 2012). Therefore, the optimal RAPD conditions (concentration of the primer, MgCL2, Taq DNA polymerase and dNTPs;

the amount of DNA and amplification conditions) were maintained for fingerprinting and identification of date palm in the study.

RAPD fingerprinting of date palm cultivars

The RAPD principle is based on amplifying fragments of the target DNA with unknown sequences. Therefore, a large number of short primers with randomly selected sequences are used to find a few suitable ones. Forty-two random primers were examined in our study, 9 primers showed a number of polymorphic and reproducible bands in all six date palm cultivars (Table 3). Primers that generated either none or weak amplification patterns were discontinued. The reproducibility of the primers with identifiable polymorphic bands was confirmed with another three different samples of each date palm cultivar, collected from different regions of Gaza Strip. The 9 reproducible primers gave a total of 65 bands, including 32 polymorphic and 33 monomorphic bands (49% polymorphism). In comparison, the level of polymorphism obtained by similar studies was 58.5% (Haider et al., 2012) and 66% (Sedra et al., 1998). The number of bands ranged from 3 to 8 for each cultivar (on average 7.2 bands per primer), and the size of bands was 280-2000 bp.

The bands were recorded as present (+) or absent (-), and the genetic diversity of the six date palm cultivars was calculated using the similarity coefficient, and the UPGMA method was used to construct a dendrogram (Table 4 and Figure 2). The similarity values obtained from the nine primers ranged from 76.3 to 93.5% with a mean of 84.9% (Table 5). The highest similarity value (93.5%) was between Bentaisha and Hayani cvs.; both have red fruits while the other four cultivars have yellow fruits (Table 1), while the lowest similarity value (76.3%) was recorded between cvs. Hayani and Zahedi. The Ameri and Hayani cvs., which have similar fruit shape and size, although slightly different in color, also showed high similarity (91.7%). Barhi and Halawy cvs., which have similar sweet and least acidic fruits (Pundir and Porwal, 1998), have also demonstrated a high similarity value (91.8%).

The agarose gel of some primers gave reproducible cultivar-specific bands (Figure 3). Primers 6 and 33 repeatedly gave a band of about 1200 bp and 1000 bp, respectively, with Zahedi. Therefore, these primers might be reliable to identify the Zahedi cv. Primers 7 and 26 gave a band of at about 450 bp and 700 bp, respectively, with the four yellow fruit cultivars, which was absent in the two red fruit cvs. Hayani and Bentaisha. Thus they may be useful for distinguishing these two groups according to the fruit color. Similarly, primer 39 gave a specific band at about 630 bp with Ameri cv.

The RAPD primer No. 6 gave the highest polymorphism among the six date palm cultivars (9 polymorphic out of 11 bands). However, a single RAPD primer usually fails to distinguish among the six cultivars alone. Therefore, a combination of more than one primer must be used to identify the six cultivars.

Table 3. Polymorphism of nine RAPD primers applied on six date palm cultivars.

Primer ID	No. of polymorphic bands*	No. of monomorphic bands	Total No. of bands per primer	Polymorphism %
6	9	2	11	81.8
7	3	3	6	50
15	3	3	6	50
16	3	3	6	50
26	4	5	9	44.4
29	4	4	8	50
33	1	5	6	20
35	1	3	4	25
39	4	5	9	44.4
Total	32	33	65	49 %

* Polymorphic bands are those present only in a fraction of cultivars. Monomorphic bands are those present in all cultivars.

Table 4. RAPD profile for the six date palm cultivars obtained by nine primers.

Primer ID	Band size (bp)	Date palm cultivars					
		Ameri	Halawy	Zahedi	Barhi	Bentaisha	Hayani
6	2000	-	-	-	-	-	+
	1700	-	-	-	-	+	+
	1600	-	-	-	+	-	-
	1200	-	-	+	-	-	-
	830	+	-	+	+	+	+
	780	+	+	+	+	+	+
	720	+	-	-	-	+	+
	600	+	-	-	-	+	+
	570	-	+	+	-	-	-
	380	-	+	+	+	-	-
	350	+	+	+	+	+	+
7	1500	+	+	+	+	+	+
	820	+	+	+	+	+	+
	600	+	+	+	+	-	+
	490	+	+	+	+	-	-
	380	+	+	+	+	-	+
	320	+	+	+	+	+	+
15	2000	+	+	+	+	+	+
	1000	+	+	+	+	+	+
	750	-	+	-	+	+	+
	730	+	-	+	-	-	-
	600	-	+	-	+	-	-
	520	+	+	+	+	+	+
16	1400	-	+	+	+	+	-
	1100	+	-	-	-	-	+
	700	-	+	+	-	-	-
	540	+	+	+	+	+	+
	450	+	+	+	+	+	+
	320	+	+	+	+	+	+
26	1050	-	+	-	-	-	-
	950	+	+	+	+	+	+
	900	+	+	-	-	+	+
	800	+	+	+	+	+	+
	770	+	+	+	+	+	+
	700	+	+	+	+	-	-
	630	+	+	+	+	+	+
	480	+	-	+	-	+	+
	390	+	+	+	+	+	+
29	1600	-	+	-	+	-	-
	1200	+	+	+	+	+	+
	1100	-	-	+	-	+	+
	1050	+	+	+	+	+	+
	950	+	+	+	-	+	+
	750	+	+	+	+	+	+
	460	+	+	+	+	+	+
	280	+	-	-	-	-	+
33	1450	+	+	+	+	+	+
	1000	-	-	+	-	-	-
	750	+	+	+	+	+	+
	700	+	+	+	+	+	+
	450	+	+	+	+	+	+
	300	+	+	+	+	+	+
35	1050	+	+	+	+	+	+
	680	+	+	+	+	+	+
	500	+	+	+	+	+	+
	300	-	+	-	+	-	-

Table 4. Contd..

Primer ID	Band size (bp)	Date palm cultivars					
		Ameri	Halawy	Zahedi	Barhi	Bentaisha	Hayani
39	1450	+	+	+	+	+	+
	1300	+	+	+	+	+	+
	1300	+	+	+	+	+	+
	700	-	+	-	+	-	-
	650	-	-	+	+	-	-
	630	+	-	-	-	-	-
	580	+	+	-	+	+	+
	450	+	+	+	+	+	+
	300	+	+	+	+	+	+

The size of amplification fragments was estimated by comparison to 100 bp ladder as a

(+) indicates the presence of an amplified DNA fragment and (–) absence of that fragment.

Table 5. Similarity matrix based on the Nei and Li coefficients of the six date palm cultivars obtained from 9 RAPD markers.

Cultivar	Ameri	Halawy	Zahedi	Barhi	Bentaisha	Hayani
Ameri	1	0.816	0.845	0.917	0.870	0.917
Halawy		1	0.848	0.918	0.808	0.796
Zahedi			1	0.845	0.860	0.763
Barhi				1	0.804	0.792
Bentaisha					1	0.935
Hayani						1

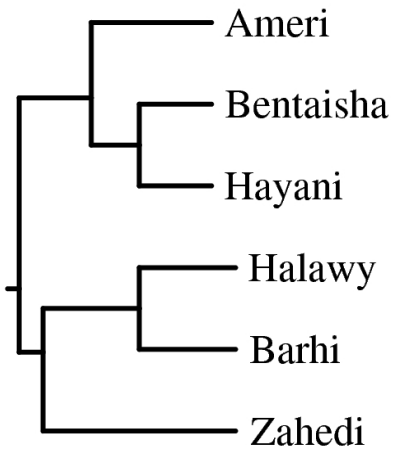

Figure 2. Phylogenetic dendrogram of six date palm cultivars using nine RAPD primers. The dendrogram was constructed according to the UPGMA method, using the online dendrogram construction utility, DendroUPGMA. Each arm of the dendrogram corresponds to the scaled genetic distance.

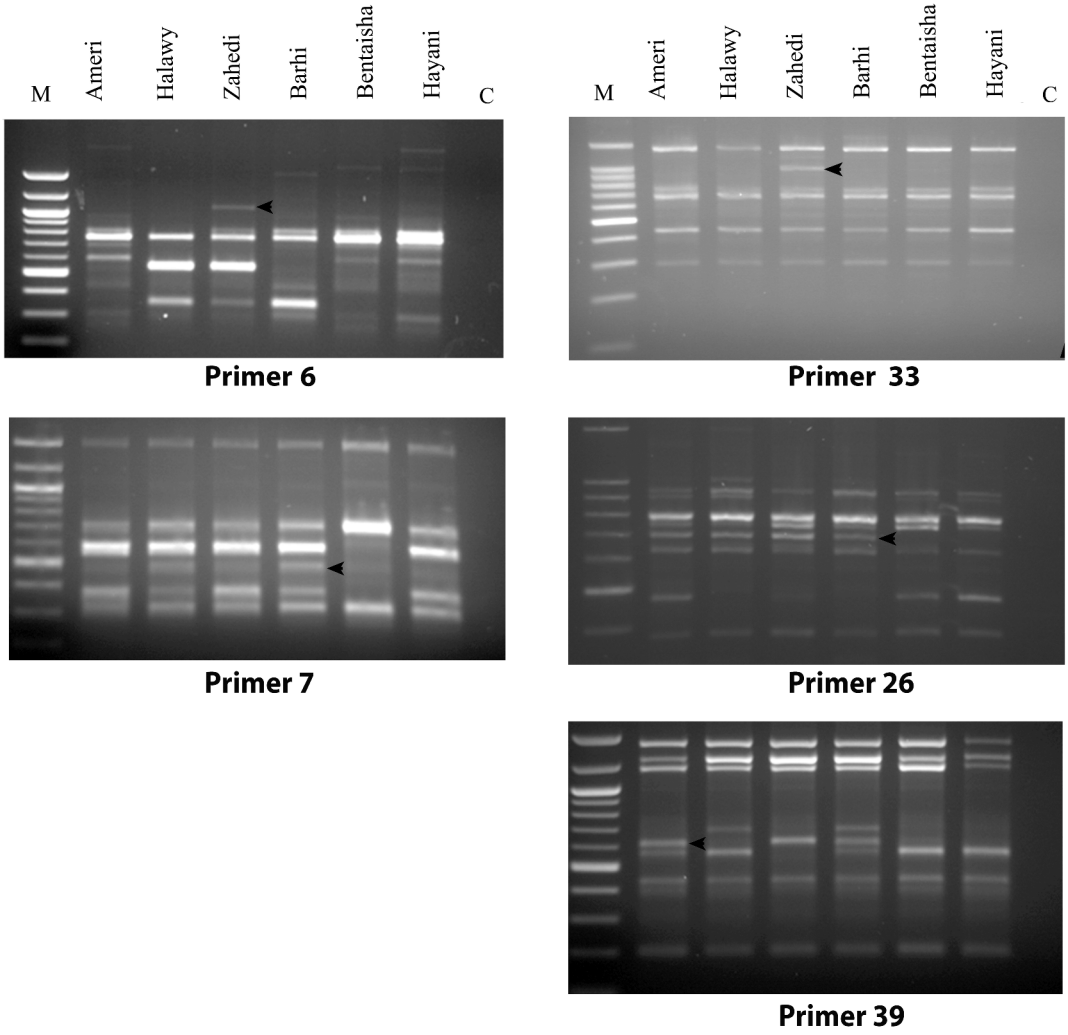

Figure 3. Agarose gel electrophoresis of RAPD experiments showing amplification with primers that gave specific marker bands with certain cultivars. These bands are indicated by black arrows. The name of cultivar is indicated above each lane and the primer number is indicated below each plate. **M** is a 100 bp DNA ladder and **C** is a negative PCR-control. The text and Table 4 give approximate size of the bands.

Conclusion

This is the first study to address genetic characterization of six date palm cultivars of the Gaza Strip. Among them, two cvs. (Hayani and Bentaisha) have been grown for a very long time while the other four were imported from other Palestinian areas in 1999 (Ameri, Halawy, Zahedi and Barhi). A RAPD protocol was optimized and found capable of differentiating all the six cultivars using 9 primers, which may be applied at an early stage of the tree growth, thus eliminating the need to wait for the fruit stage for cultivar identification.

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