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EDITORIAL

Special issue on date palm papers presented at the "Arab Palm Conference 2011"

Historically, the cultivation of date palm, *Phoenix dactylifera* L., has been associated with the survival of humankind in arid regions; nonetheless this species continues to exhibit a critical need to improve the quality and diversification of products and by-products. Exploration of the potential use of date palm as a bioenergy crop and source of natural metabolites for pharmacy and health products remains scant. Broader commercial utilization of products from the entire date palm, including leaves and stems, would be of general benefit to date producers, especially to small farmers. Major issues currently challenging the date palm farming sector include global climate change, surface and ground water shortages, pests and diseases, storage and packaging innovations, and marketing strategies. Most of these issues can be efficiently addressed internationally in close collaboration with scientists and date palm growers to develop innovative approaches for date palm improvement.

The First Date Palm and Dates Conference (Arab Palm Conference 2011; http://www.arabpalm.org/) was organized to support date palm agriculture and foster research development. King Abdulaziz City for Science and Technology (KACST; http://www.kacst.edu.sa/), and the recently-established Date Palm and Dates League of the Arab World (DPDLAW) were the co-organizers. The conference was held under the patronage of His Excellency Dr. Fahd bin Abdul Rahman Balghonaim, the Minister of Agriculture at KACST Headquarters, Riyadh, Saudi Arabia on December 4-7, 2011. The goal of the conference was to address major challenges of contemporary date palm agriculture through scientific sessions focusing on date palm and date production, applications of innovative biotechnology, disease and pest management, secondary products and post-harvest techniques, as well as date processing, marketing and economics.

This special issue highlights some of the most important research studies presented at the Arab Palm Conference 2011, selected on the basis of a rigorous peer-review process; in addition it contains invited reviews on important topics relevant to the conference theme and also book reviews on recently published two date palm books. The Guest Editors wish to extend their gratitude to the contributors and reviewers of the manuscripts and to acknowledge the key role of the Editorial Board of the Emirates Journal of Food and Agriculture in the compilation of this issue.

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CONVENER NOTE

First Arab Palm Conference on the Development of Date Palm and Dates Sector in the Arab World

Introduction

The Arab world is the main producer and exporter of dates in the world. Date palms are grown in more than 40 countries, with annual world production of around 7.4 million tons and the Arab world produces approximately 5.4 million tons. In addition to providing fruits and other products, date palm trees also prevent soil erosion and damages caused by sandstorms. The Arab world has more than 84 million date palm trees. Some of the major date palm producing countries are Iraq, Saudi Arabia, Egypt, Tunisia, Morocco and Algeria and they face serious problems of poor yield and marketing constraints. Date palm yield has declined over a period of time and almost 28% of the production is lost due to pests and disease. Red palm weevil has become one of the major pests of date palm in many producing countries (Jaradat and Zaid, 2004). Also, inadequate facilities and lack of know-how in date processing and marketing have affected the economies of in these countries. Therefore, rehabilitation of date palm trees in the Arab countries needs collaborative efforts and investment in date palm projects.

Current Dates Production and Limitations

Date palm is an important fruit crop in the Kingdom of Saudi Arabia and it has more than 23 million date palm trees and over 320 varieties with annual earning of over 0.5 billion USD. The date palm plantations occupy 150,744 ha of land. Although the cultivation of date palms has developed considerably and great attention has been given to date production in Saudi Arabia, the level of date productivity is comparatively low compared with other date producing countries (Al-Obaid, 1996). This is due to many low-yielding cultivars, insufficient offshoots to establish new orchards, high price of new offshoots of good quality cultivars (Al-Sakran and Muneer, 2006), and water scarcity.

The other problems faced by the date palm farmers of Saudi Arabia are pests and a lack of marketing studies. Also, insufficient efforts are being made to adopt new technologies and modern machines to enhance the productivity of the date palm industry for export purposes. The most important pest that infests date palms in Saudi Arabia is the red palm weevil which has spread due to the lack of an efficient integrated pest management practices.

Arab Palm Conference 2011

The first date Arab palm conference, 4-7 December 2011, highlighted the importance of developing the date palm and dates sector in the Arab world, and to determine the role of this sector in supporting the national economies of the region. It was organized by King Abdulaziz City for Science and Technology (KACST in cooperation with the League of Arab Research Institutions, represented by the Date Palm and Dates Research League. KACST is an independent scientific organization and is serving both as the Saudi Arabian national science agency and national laboratories. The science agency function involves science and technology policy-making, data collection, funding of external research, and services, including the patent office and information technology. The Arab League for Palm and Dates sector is a Pan Arab organization dedicated to advancing research and development, growth and sustainability of the date palm and the date industry in the Arab world.

On the first day, the conference was inaugurated by the Minister of Agriculture, Saudi Arabia and the President of KACST, and opening of the Arab world date palm league (RABITAH) as well as inauguration of date palm exhibition. In all 71 oral presentations were made in nine different sessions during the conference. Two parallel sessions were conducted simultaneously. Session A dealt with date palm and dates production, agriculture biotechnology, diseases and pest management, and economics and value addition. Session B covered date palm and dates production, diseases and pest management, post-harvest and storage, and date palm and date processing. Two joint sessions were held: first on ‘Dates loss and opportunities of utilization’ and the second on ‘Developing investment in date palm and dates sector.’ In the poster session, 22 scientific posters were presented. KACST and The Date Palm and Dates League are publishing non-reviewed conference proceedings as a documentary publication containing full papers of all the presentations. Overall, the conference attracted 230 participants from 17 countries.

The conference reviewed many different topics related to date farming, including: tissue culture propagation, methods of improving production, best agricultural practices, integrated pest management, marketing, storage, and capacity building methods, promotion of the date palm and its products towards sustainable development and recent research milestones. During the conference, different issues were also reviewed such as production operations, packaging and marketing, genetic engineering and molecular biology and innovations that could serve the date palm and dates sector internationally.

This conference provided an excellent forum for meeting and networking among the various stakeholders including farmers, investors, scientists, policy-makers, private sector, and marketing managers. An international exhibition was also organized within the conference to feature international and local private sector and scientific organizations. The exhibition showcased the latest technologies, solutions and services for the sector like improved date palm productivity (agricultural operations), harvest and post-harvest operations, secondary products of palm and dates, technology and innovations for palm and dates processing, packaging, marketing and the role of the date and palm sector in supporting Arab national economies.

Executive Recommendations

There is an urgent need to establish a database for the cultivation, harvesting, marketing and manufacture of dates and date products. Improvement is also needed in the main processes of date cultivation such as propagation, offshoots, irrigation, fertilization, pest control, pruning, pollination, fruit thinning and harvesting techniques. In addition an increase in the research capacity in date palm genetics as well as reliable date palm tissue culture for clonal propagation of elite date palm cultivars, multiplication and supply throughout the year are also recommended. A milestone in molecular research on date palm has already been initiated by genome sequencing of Khalas cultivar by the researchers of Qatar Foundation at Weill Cornell Medical College; however more serious efforts are needed to develop this program for genetic improvement of dates in the future. http://qf-research-division.org/news2.php. Date fruit quality, application of programs and standard specifications for dates are highly desirable to improve the quality and economics. Therefore, it is strongly recommended to address major pests and diseases of date palm under the climate change, and germplasm conservation to set up date palm germplasm bank both *in vivo* and *in vitro*. There is also a need to support date manufacturing and marketing by establishing new date factories and improving the existing ones. The handling, storage, packaging, and transport of dates should be improved and is of a great importance to date palm sector.

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REVIEW ARTICLE

Socioeconomic and traditional importance of date palm

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Abstract

The date palm fruit is a drupe exhibiting a high diversity in texture, shape, color and chemical composition depending on the genotype, environment, season and cultural practices. The fruit typically characterize the variety. The socio-economic value of dates is particularly known from oases, where date palms grow and fruits were historically the medium of exchange between populations. The geographical distribution of date palm cultivars is not yet well studied and the international demand on some of them will have disastrous impacts on the sustainability of this crop in the long term. This impoverishes germplasm and narrows the diversity grown among oases. In addition, despite the presence of several reports on the chemical composition and the nutritional value of dates, many other potentialities of the fruits remain to be explored. Many claims report on the antibacterial, antifungal, antitumor, antiulcer and immuno-modulatory properties of dates. Recently, the antioxidant activity of some cultivars was investigated and attributed to phenolic compounds. Dates are very rich in phenolics, in quality and quantity, which opens many fields of investigation in terms of new potential uses. This study summarizes the recent progress in date research, providing an up-to-date overview of the worldwide production/commercialization and the traditional and medicinal uses. Other current and future applications of dates also are highlighted.

Key words: Date palm, *Phoenix dactylifera*, Dates, Chemical composition, Phenolics, Health benefits, Functional food, Nutraceuticals, Medicinal, Antioxidants

Introduction

Phoenix dactylifera L., date palm, is among the most important species in the Palm family (Arecaceae),which encompasses about 200 genera and more than 2,500 species (El Hadrami and El Hadrami, 2009; Jain and Johnson, 2011) and includes *P. canariensis* (Canary Islands date palm), *P. reclinata* (Senegal date palm) and *P. sylvestris* (Indian sugar palm). The species name was inspired by the finger like shape of the fruit and the genus from the legendary bird of Ancient Greece. It is a long-lived monocotyledonous species and one of the tallest domesticated trees. This perennial and dioecious species represents a cornerstone of the economy in many producing countries, especially in North Africa and the Middle East. Over 100 million trees are currently grown worldwide on an estimated area of 1 million ha. Date palm provides fruit, fuel, fiber and shade for other essential cover crops. The annual world production of dates has reached 6-8 million mt, representing a market exchange value of over 1 billion USD. The top three producing countries are Egypt, Iran and Saudi Arabia; the largest importer of dates is India (El Hadrami and El Hadrami, 2009; El Hadrami et al., 2011a).

Botanical description of the date palm

All species belonging to the genus *Phoenix* grow vertically to form an unbranched trunk driven by the activity of a single terminal shoot apex. To support such elevated vertical growth, the root system is highly developed and reaches deep for water resources. Date palm leaves are very developed and can reach several square meters in area. The leaves are erect, arranged in a spiral pattern on the trunk. Sheathing becomes denser at the top of the tree forming a crown with hundreds of leaves forming a terminal rosette. The leaves are pinnate with needle-sharp tips to defend the plant from grazing animals and reduce water loss.

Vegetative, floral or intermediate auxiliary buds can be found at the base of each leaf. During the juvenile life of the tree, these buds can form the so-called offshoots or suckers, which can develop

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into an adult palm and bear fruits at maturity. Date palm flowers also emerge from auxiliary buds within the terminal rosette and form branched clusters. Given that the date palm is a dioecious species, male and female flowers are found on separate palms and only female palms can bear fruit upon natural or artificial fertilization.

Flowers are typically small for such a large tree, white in color, and arranged on an abundantly-branched spadix surrounded by a large single spathe. The calyxes are three-toothed and cup-shaped, the same as the petals. The latter are twice as long in the female flowers. Date palm flowers consist in general of three ovaries, but only one is able to develop into a drupe. The stamens are six in number bearing linear dorsifixed anthers. Palm pollination is often wind-borne and artificial pollination is also a common practice to achieve higher yield. A mature fully-productive palm can bear several hundred kilograms of fruit each year. Dates are single-seeded fruits of a cylindrical, rounded or ovoid shape, with a fleshy sweet mesocarp covered with a thin epicarp, somewhat yellowish to reddish brown in color. The fruits are usually arranged on spikelets bearing a few dozen individual dates each. Spikelets are attached to a central stalk to form a bunch. The number of bunches per tree varies from 5-30 depending on the cultivar and environmental conditions.

Three major methods are currently practiced to propagate date palm (El Hadrami et al., 2011c). Historically, the most common method relies on the transplanting of offshoots exchanged or traded between growers and groves. Transplanted offshoots develop slowly. A well-maintained palm can produce up to three offshoots per year with a maximum of 10-40 during its lifetime. Under normal conditions, transplanted offshoots begin to bear fruit within 5-8 years with full maturity at around 30 years. Production often shows a decline after 100 years of cultivation, depending on the soil, microclimate and cultural practices and so on. The second method of propagation is by seed. Although it is the easiest, palm seedlings may take up to 10 years before beginning to flower and produce fruit. During the last few decades, a tremendous effort has been given to the development of alternative methods that are fast and reliable in generating large numbers of plants. This relies on the use of micropropagation and various tissue culture methods and biotechnologies (Jain, 2006; El Hadrami and El Hadrami, 2009; El Hadrami et al., 2011b).

Characteristics of important commercial cultivars

Date palm germplasm is claimed to encompass over 5,000 cultivars, some of which have been more or less characterized in detail (El Hadrami and El Hadrami, 2009). A majority of the cultivars are often susceptible to diseases and pests or yield poorly in comparison to elite varieties, hence leading to their exclusion from the groves. This trend had led to impoverished monoculture with a narrow genetic diversity, which threatens the perennial aspect of the crop. This is the case of Deglet Noor cv. in Tunisia and Algeria, where it represents over 65% of the total population, and Boufeggous-O-Moussa and Jihel cvs. in Morocco.

A comprehensive study from the 1920s, providing descriptions of the fruits of 1,500 cultivars, was published in 1973 (Popenoe, 1973). It also reports on the history and significance of the most important date cultivars according to country of origin.

Date fruit by-products

Besides the use of fresh fruits for human consumption, a number of by-products derived from dates also have various uses. These include jam, jelly, juice, syrup and fermented beverage. Cull dates from grading and sorting, as well from storage and conditioning are often utilized as animal feed. Several reports show that a number of bioactive compounds can be extracted from these by-products, thereby adding industrial value which could compensate for the economic loss from under-grading and/or deterioration. Various metabolites also are reported to be produced from dates or their by-products, such as citric acid, oxytetracycline and ethanol. In recent years, interest has been increasing to extract essential oils, polyphenols and dietary fiber from date seeds.

Due to the high production potential of dates worldwide, it may not always be possible to consume all the freshly-harvested fruit locally or export them. This has opened, in recent years, new opportunities to turn the surplus production into value-added products such as syrup and fermented juice. Al-Hooti et al. (2002) reported on a new technology developed on a laboratory scale to produce syrup from dates of Kuwaiti commercial cvs., Birhi and Safri, both very rich in sugars (88%). The study showed that the procedure developed, using pectinase/cellulase enzymes, recovers 68% of total soluble solids, as compared to regular procedures not relying on enzymes which recover only 35%.

Other studies have concentrated on finding ways to add value to some second-grade dates with a hard texture. Besbes et al. (2009) found that these dates are equal to first-grade dates in terms of their sugar, fiber and total phenolic contents. To add value to this type, the authors explored using them to produce jam, and conducted a series of tests of the composition and physical characteristics, such as the content of reducing sugars, firmness, moisture retention capacity, as well as customer acceptability in comparison with the made jam from first-grade fruits and consumed locally. Overall, the study revealed that jam from hard texture second-grade dates represents a commercially viable venture.

Socio-economic and traditional importance of dates

Date palm is socio-economically and traditionally important for local populations where the culture thrives (Jain, Al-Khayri and Johnson, 2011). Establishment of date palm groves helped nomad populations in the past to settle and organize communities and begin farming. These populations became a hub for marketing/trading commodities, animal and other products. Dates were and still are among the most important and appreciated products for trading at these oases-based communities. They are appreciated for their food and feed uses. Dry and soft dates are often eaten out-of-hand. They may also be press-packed, sliced or prepared in many ways to generate other products consumed locally and elsewhere. These include their use in pudding, bread, cakes, cookies, ice cream, candy bars and cereals. An entirely new industry has also been developed in recent years around date palm and dates. For example, factories with more or less sophisticated means of pitting, piercing, crushing and sieving dates provide a significant number of local jobs. Surplus production not consumed fresh often is transformed locally into paste, spread, powder (used locally as a sugar), jam, jelly, juice, syrup, vinegar and alcohol. Unused dates do not go to waste. They are often dehydrated, crashed and mixed with grains and straw to become a valuable feed for domestic animals.

Besides the fruit, young leaves and terminal buds are sometimes prepared as vegetables. Seeds also are often roasted, crushed and used in some countries as an extender to coffee or flour. Tapping date palm trees to extract the sugary sap is also a common practice in many countries in North and West Africa. The sweet sap is converted into sugar, molasses and alcoholic beverages. Date seeds are often softened by soaking in water to feed camels, sheep, goats and horses, or crushed dry and added to chicken feed.

Date seeds have other nutritional and value-added property potential (El Hadrami et al., 2011d). They contain over 60% carbohydrates, up to 10% fat and 5% protein and a substantial amount of dietary fiber (6-12%). They also represent a source of sterols, oestrone and some alkali-soluble polysaccharide. The seeds yield a yellow-green, moisturizing oil rich in essential fatty acids such as lauric (8%), myristic (4%), palmitic (25%), stearic (10%), oleic (45%), linoleic (10%) as well as traces of caprylic and capric acids, suitable for use in soap and cosmetic products. Date seeds can also be chemically processed to produce up to 65% oxalic acid or burned to produce charcoal for silversmiths. The seeds are also often strung into necklaces.

Date palm leaves are often used to make mats, screens, baskets, crates and fans or for religious purposes (e.g. Palm Sunday). Petioles are often appreciated as a source for cellulose pulp. Mature leaves are used in insulating board, packsaddles, ropes, coarse cloths and hats, given their richness in fiber. They also represent a source of fuel and raw material for making fishing implements and objects such as walking sticks and brooms.

A number of medicinal uses are directly or indirectly ascribed to the consumption of dates. The fruit is rich in tannins, making it a good astringent remedy for intestinal troubles. Formulations such as infusions, decoctions, syrups and pastes are often administered against colds, sore throat and bronchial cough. They are also taken to relieve fever, liver and abdominal aches, cystitis, gonorrhoea and edema. The roots are used to treat toothache and the pollen is appreciated for its estrogenic compound, estrone.

Historical importance of date production

Historically, date palm cultivation was practiced by ancient civilizations and is nowadays considered one of the oldest domesticated fruit-bearing trees. Remains of date palms were found in Jericho - the oldest site known to date to be the origin of agriculture. Date palm cultivation gained socioeconomic importance among tribes and countries due to its ecological plasticity and high adaptation to arid conditions where the annual precipitation rarely exceed 250 mm combined with hot summers up to 50°C and cold winters down to -10°C. Production of dates provides jobs for a manpower population estimated at 50 million people, 35% of which are located in the southern Mediterranean countries (El Hadrami and El Hadrami, 2009).

Another important fact for date palm cultivation relates to the microclimate the tree creates in the desert, fostering the growth of cover crops and vegetables to sustain local human populations and their animals. Dates are an excellent source for carbohydrates, proteins, fats, dietary fiber, essential minerals and vitamins (Al-Shahib and Marshall, 2003a,b). They are also used to produce syrup, jam, chutney, vinegar and fermented date juice. In addition, date palm trees provide shade to grow coffee, pomegranates, mango, citrus, cotton, maize, alfalfa, vegetables and cereals.

Other parts of the palms such as the leaves are often dried, dyed and plaited into mats, hats, trays or baskets. Combined with other material, this high-fiber foliage can also be used to produce ropes and twines. The woody midribs of the leaves are often used for roofing as well as to make dome-shaped traditional fishing cages. Sturdier trunks are used to make fishing crafts in some countries.

Current situation of date production

The date palm is currently grown for fruit production intended for local consumption, trade and export in 37 countries around the world. A few other countries grow the crop on small areas intended for local market consumption.

Geographical distribution of date palm

Phoenix dactylifera is a widely distributed species occurring in diverse geographic, soil and climatic areas (El Hadrami et al., 2011a). The vast majority of the trees are located in the Middle East and North Africa although the crop has been established in California, Arizona and Mexico in the Americas. The common requirement among all date palm growing areas is the high temperature (35° C) necessary for an optimal development of pollen and the low relative humidity for fruit setting and ripening. Such desert-adapted tree require large quantities of water drawn from deep in the soil through a well-established root system or from surface irrigation. Date palm grows in nearly rainless regions at 9-39° North latitude, which are represented by the Sahara and Southern fringe of the Near East (Arabia Peninsula, Southern Iraq and Jordan). Both wild and domesticated trees are morphologically and ecologically similar. Wild date fruits are small and inedible compared to those originating from domesticated trees. Cross-hybridization between the two types of trees occurs in some regions, making their distinction quite difficult to determine.

Worldwide date production

FAO estimates that the harvested area of date growing was 1.3 million ha in 2009 (FAO Statistics, 2010). This area had increased by 0.3 million ha over the previous decade. The largest area (833,351 ha) is located on the Asian continent, which includes the Middle Eastern producing countries, followed by Africa (416,695 ha), North Africa (392,200 ha). In the Americas and Europe the production covers only a few thousand hectares.

The worldwide production of dates was estimated in 2009 to 7.3 million mt, which represents an increase of over 1 million mt in a decade. The production mirrors the area of producing groves with production shared between Middle Eastern and North African countries (2.7 million mt).

According to FAO statistics, the world's largest producer over the last five years was Egypt with an average of 1.3 million mt followed by Iran (just over 1 million mt), Saudi Arabia (979,017 mt), United Arab Emirates (754,400 mt), Pakistan and Algeria (540,000 mt) each and Sudan (333,500 mt). Other significant producing countries are Oman, Libya, Tunisia, Morocco, Yemen, Mauritania, USA, Bahrain, Qatar, Spain and Kuwait.

In terms of annual yields, the USA leads with an estimated average of 67.093 hg/ha. Next is Africa especially the Northern parts, producing 64.974 hg/ha on average. In the Middle Eastern countries, although a large area of producing groves, yields are below the world average (53.798 hg/ha).

Advances in date palm breeding

Date palm breeding relies on the use of conventional methods as well as biotechnologies. From the 1950s to the 1970s conventional breeding programs were given special attention. However, the slow growth of the trees to generate backcrosses and recurrent selection among the progenies quickly became a burden. In addition, difficulties were encountered in selecting female trees, due to the fact that they are indistinguishable from the male trees until they mature, which may take 5-8 years, depending on the cultivar and growth conditions (El Hadrami and El Hadrami, 2009; El Hadrami et al., 2011a). The development of tissue culture in the 1970s and its extensive application for date palm production to the present has made a tremendous contribution to the breeding of this important crop. Currently, a number of micropropagation techniques and biotechnology approaches have made it possible to clonally produce large quantities of material intended to

replace dead or senescent trees in the groves and to establish new areas. These techniques have also provided conventional breeding with news ways to accelerate the breeding process and more quickly obtain progeny. There is still a long way is still to go before these biotechnologies will be able to help introgress new traits such as yield, quality, and tolerance/resistance to abiotic and biotic stresses. Recently, the entire genome of the date palm cv. Khalas was sequenced and made publically available (Al-Dous et al., 2009, 2011), and in the years to come this will help decipher the genetic secrets of this important monocotyledonous species.

Physico-chemical composition of date fruits

In recent years, reports on the physico-chemical composition of dates and their derived by-products have been on the rise (El Hadrami et al., 2011d). Elleuch et al. (2008) analyzed the color, moisture and oil-holding capacities and the rheological behavior of the dietary fibers extracted from dates flesh of two Tunisian cvs. Deglet Noor and Allig. The findings showed that on a dry matter basis, Deglet Noor cv. constituted 53% sucrose, 14% glucose, 13% fructose, 14% total dietary fiber, 2% protein and 2.5% ash, in comparison with Allig (14, 30, 29, 18, 3, and 2.5%, respectively). In both cultivars, the content of total dietary fibers, which is water insoluble, represents 10% of the dry matter.

Al-Shahib and Marshall (2003b) analyzed the fat content and fatty acid profiles of 14 cultivars of date seeds using gas chromatography-mass spectrometry. Quantitatively, the fat content range was 5-9% (w/w). In terms of quality, the authors reported the presence of 11 fatty acids; among them oleic acid was primary, representing up to 50% of the profile. Different results among samples were ascribed to both cultivar and cultural differences.

Studies of the saturated fatty acids in the flesh and seeds of dates revealed the presence of saturated fatty acids, including capric, lauric, myristic, palmitic, stearic, margaric, arachidic, heneicosanoic, behenic and tricosanoic acids (Al-Shahib and Marshall, 2003b). Unsaturated fatty acids included palmitoleic, oleic, linoleic and linolenic acids. A number of reports list the content of these fatty acids in several commonly grown varieties (Fayadh and Al-Showiman, 1990; Al-Showiman, 1990). Other studies (Saafi et al., 2008) have reported the physicochemical composition of the pulp and the pit and showed total sugars of 63.4% (51.6% reducing sugars and 11.8% sucrose) on a dry-weight basis, 3.9% protein and 0.3% oil in the pulp; versus 8.2% (6.6% and 1.5%), 5.3% and 8.3% for the pit. Chromatographic analysis revealed differential fatty acid profiles between pulp and pit. While linoleic acid was the major unsaturated fatty acid found in the pulp (32.8%), oleic acid was highly abundant in the pit (47.66%). The major saturated fatty acids were palmitic (20.6%) and lauric acid (17.4%) in the pulp and the pit, respectively. Other fatty acids encountered in these date by-products included myristic, stearic and linolenic acids.

Texture and firmness of the pericarp

As they ripen, edible dates go through four ripening stages termed *kimri, khalal, rutab* and *tamar* (Fayadh and Al-Showiman, 1990). These represent the immature astringent green, mature full colored, soft brown and hard raisin-like stages of development, respectively. In the first 4-5 weeks, the dates are fully green and become kimri; at this stage, average fruit size is 27.5 mm long x 17.8 mm in diameter and weighs 5.8 g on average, quickly increasing due to the accumulation of carbohydrates and moisture content. Acidity is quite high at this stage with an average protein level of 5.6%, fat 0.5%, and ash 3.7% (Al-Hooti et al., 1995).

In the khalal stage, the fruit color changes from green to a yellowish/reddish tone depending on the cultivar, over a period of 3 to 5 weeks. Sugar and moisture content decrease from the values recorded at the kimri stage along with a decrease in acidity. In this stage, the fruit averages 32.5 mm in length and 21 mm in diameter while the fruit weight increases to 8.7 g (Al-Hooti et al., 1995). The percentages of protein, fat and ash decrease to 2.7, 0.3 and 2.8%, respectively.

At the rutab stage, dates begin to soften (2-4 week period) due to an increased loss of moisture content and an increase of enzymatic activities of pectinases and polygalacturonases (Figure 1). The protein, fat and ash percentages in this stage decrease to 2.6, 0.3 and 2.6%, respectively. At the tamar stage, dates are drier and rather firm in texture while their color turns to a darker tone.

Freshly harvested dates are commonly consumed or stored for a short period of time before being eaten at the khalal and rutab stages. Keeping dates in storage past the rutab stage lowers their moisture content and they become tamar stage. This stage is the most appreciated by consumers and has a better storability and shelf life. A number of cultivars are not allowed to reach this stage and are commercially sold either as khalal or rutab fruit.

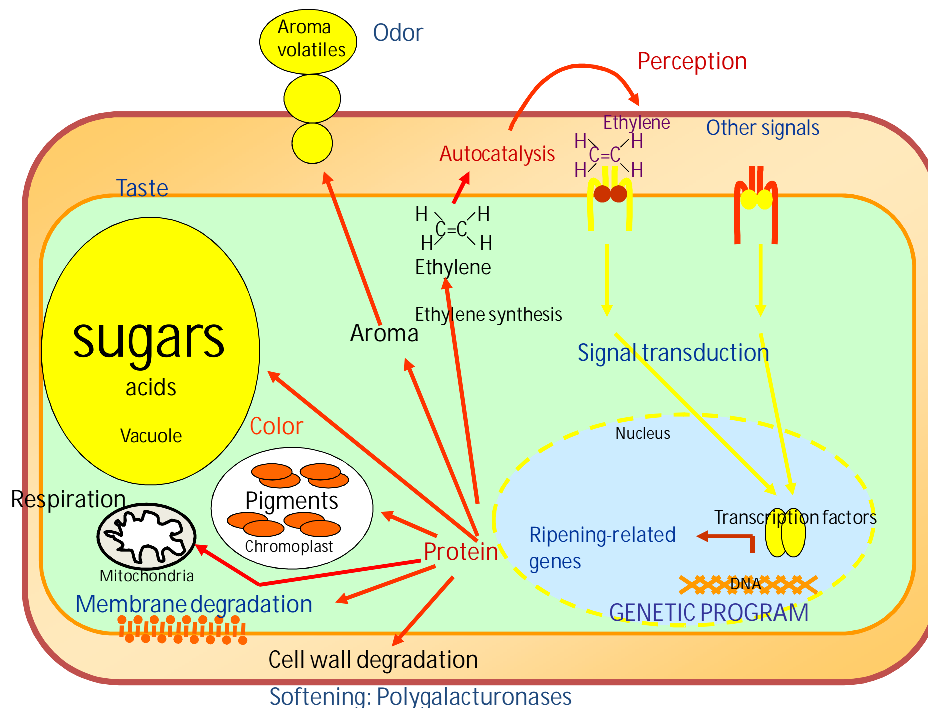

Figure 1. Illustration representing the series of metabolic changes that occur in dates during ripening process.

Firmness of the pericarp in dates mainly depends on the content of carbohydrates and fiber. There are relatively few reports on the content of dietary fiber in dates, especially during the various ripening stages. According to Spiller (1993), the dietary fiber content of widely retailed dried dates (rutab and tamar stages) was estimated at 4.4% with 3.2% insoluble and 1.2% soluble. Over the last few years a number of new methods have estimated these amounts to be higher.

Color and sweetness

As part of the various stages of ripening, dates undergo change in color from dark green to light green and yellow, light to red brown, and to dark brown. A number of experimental procedures were developed to guarantee the quality of traded date fruits in terms of ripeness and color. One in common use is the CIE Lab coordinates (L*a*b*), where L* measures the lightness ranging from black to white on a scale of 0 to 100; a* the greenness to redness on a scale of -100 to +100; and b* measuring the blueness (-100) to yellowness (+100). When the a* and b* values increase, the color becomes saturated and chromatic, while when they approach zero they indicate neutral colors e.g. white, grey or black. This method is also used to assess the quality of derived by-products such as syrup and juice.

Elleuch et al. (2008) used the CIE approach to compare Deglet Noor and Allig cvs. and showed that Deglet Noor is characterized by higher L*, a* and b* than Allig, suggesting that Deglet Noor is lighter, more yellow and red than Allig. Using the same technique, Al-Hooti et al. (1996) studied other cultivars from the United Arab Emirates, namely Bush bal, Gash, Gaffer, Gash Abash, Lulu and Shale and reported that the values of L* range between 17.5 and 23.1, indicating that these cultivars bear darker dates than the Deglet Noor or Allig cvs. studied by Elleuch et al. (2008).

The sweetness of dates depends on the ripening stage. At the kimri stage, dates are still green-yellow in color and very astringent. Their levels of reduced sugars are very low. At the khalal, rutab and tamar stages, total sugar content becomes 6-9 times higher than at the kimri stage. Reduced sugars at these stages are also high, 4-7 times greater than those recorded at the kimri stage (Ahmed et al., 1995).

Other date palm by-products are also known to be appreciated by local populations for their sweetness. A literature search indicated that little attention has been given to sap from date palm, as well as other palm trees such as the coconut and African oil palm. Palm sap is traditionally collected and consumed as a fresh juice or alcoholic beverage, especially in Asian countries, or to make palm sugar, appreciated in cakes, desserts, food coating, and drinks.

Metabolic and physiological changes during ripening

Date fruit shape and size, although genetically controlled, are also influenced by environmental components, especially the number of leaves supplying carbohydrates to each fruit branch, the tree water supply and the degree days to maturity.

A series of metabolic physiological changes occur in date fruits during growth, development and ripening. These changes are under the control of the genotype, branch size, temperature and heat units, light and water potential, carbohydrate supply and hormonal changes, especially gibberellin, auxin and ethylene levels. Although no clear studies have been conducted on dates, auxins may be involved in the stimulation of fruit development through the promotion of ethylene synthesis. Gibberellins, on the other hand, are believed to increase fruit stalk length within the branches, hence alleviating compaction and allowing dates to grow larger. As for ethylene, it is synthesized from methionine and the intermediate *S*-adenosylmethionine via the activity of two main enzymes; the 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and oxidase. The ACC oxidase depends of the level oxygen in storage and can be a rate-limiting factor for dates ripening. Dates continue to ripen after harvest, exhibiting a characteristic respiratory rise before ripening preceded by a spike in ethylene production, which accelerates the process.

The physiological changes that occur in dates during ripening begin with the recognition of external signals including the ethylene spike that occurs before the respiratory rise (Figure 2). Following complex signaling pathways, a number of transcriptional factors are activated inducing the transcription of ripening-related genes. Newly synthesized proteins are involved in many processes, including ethylene synthesis, which increases ethylene levels and accelerates ripening and the rise in mitochondrial respiration. Some of these proteins are involved in aroma and the creation of other volatiles responsible for the date-ripening odor. Others are involved in the synthesis of sugars and organic acids that improve taste. Among the proteins, there are also enzymes responsible for pigment degradation and color change during the kimri-khalal-rutab-tamar stages. The most important category of accumulated proteins is involved in membrane and cell-wall degradation (i.e., polygalacturonases, pectinesterases) ensuring softening of the fruit. El-Zoghbi (1994) showed that the percentage of pectin in dates decreases from the kimri stage (1.6g per 100g of dates) to 0.54% in the tamar stage, the fully ripe dry date. The study also pointed out that hemicelluloses, cellulose, lignin and total fiber contents decreased with the fruit transition from kimri to tamar stages. Pectinesterase activity in dates was also shown to increase with ripening reaching a maximum of 60.8 IU per 100g of tissue, thereby explaining the loss of pectin (El-Zoghbi, 1994). Similarly, cellulases activity was estimated to be 2-4 times higher in fruit during ripening (El-Zoghbi, 1994).

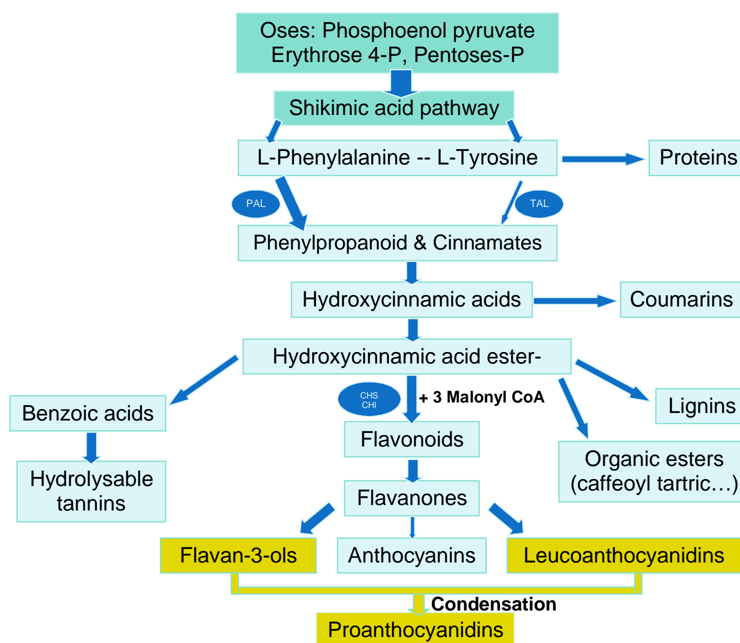

Figure 2. A simplified diagram representing the phenylpropanoid (shikimate) pathway in date palm.

Phenolic content and other secondary metabolites

In dates, three main families of phenolics (hydroxycinnamates; flavonols, flavan-3-ols, flavan-3,4-diols and proanthocyanidins) can be detected (El Hadrami et al., 1998; Daayf et al., 2003; El Hassni et al., 2004; J'Aiti et al., 2009). The major hydroxycinnamates found are derivatives of *p*-coumaric, chlorogenic, ferulic, and sinapic acids as well as their esters i.e. caffeoylshikimic acid derivatives (El Hadrami, 1995; El Hadrami et al., 1998; El Hassni et al., 2004; J'Aiti et al., 2009). Flavonoids such as luteolin, quercetin, and kaempferol, as well as more or less polymerized proanthocyanidins are also found in abundant quantities in fresh dates.

Saafi et al. (2009) examined the phenolic contents of dates derived from four commercial cultivars. The analysis revealed that the total phenolic ranged from 209.4 mg of gallic acid equivalents per 100 g fresh weight in cv. Kentichi to 447.7 mg equivalent in cv. Allig. Similar results were reported in other studies conducted using local cultivars grown in Oman and Bahrain (Al-Farsi et al., 2005, 2007; Allaith, 2008). Mansouri et al. (2005) and Biglari et al. (2008a) reported that total phenolic content ranged from 2.49 to 8.36 mg gallic acid equivalents per 100 g of fresh weight of Algerian and Iranian dates, respectively. Other studies conducted in the USA using Deglet Noor and Medjool cvs. showed a total phenolic content of 661 and 572 mg gallic acid equivalents per 100 g fresh weight.

A number of factors affect the phenolic content in dates and may justify the variation observed among studies. These include the cultivar, geographic origin, growing conditions, maturity of the tested dates, season, fertilizers, soil type, amount of sunlight received and conditions of storage, sampling and extraction.

Other chemical properties

Dates are rich in carotenoid and provatamin A. Boudries et al. (2007) analyzed the content of these two components in cvs. Deglet Noor, Hamraya and Tantebouchte, at khalal, rutab and tamar ripening stages. The major carotenoid pigments detected were lutein and β-carotene. Variation in total carotenoid content was detected among cultivars and ripening stages. Dates derived from the Deglet Noor cv. contained 61.7-167 µg per 100 g of fresh weight, while Tantebouchte and Hamraya cvs. ranged from 32.6-672, and 37.3-773, respectively. The study also showed that in general carotenoids disappear during ripening from the khalal to tamar stages.

Provitamin A value also varied with cultivar and ripening stage. In Deglet Noor cv. the values were 0.4-0.5 RE per 100 g of dates and remain unchanged during ripening. However, these values decrease during ripening from 11.7 to 1.6 RE and from 3.9 to 0.5 RE per 100 g of dates derived from cvs. Tantebouchte and Hamraya, respectively.

Dates possess a series of other characteristics due to their richness in dietary fibers. Rheological behavior, moisture- and oil-holding capacities are among the measurable parameters used to compare cultivars and ripening stages.

Nutritional value of dates

Dates represent an important nutritional element in the diet of local populations where the trees are grown. The fruit also becomes a part of the daily intake of residents in countries importing this fruit (El Hadrami and El Hadrami, 2009). Dates contain a high percentage of carbohydrate (total sugars, 44-88%), protein (2.3-5.6%), fat (0.2-9.3%), essential salts and minerals, vitamins and an elevated proportion of dietary fiber (6.4-11.5%) (El Hadrami and El Hadrami, 2009). They also contain oil in the flesh (0.2-0.5%) and the seed (7.7-9.7%). The seed represents 5.6-14.2% of the entire fruit weight.

A literature review over the last three decades showed an increasing number of reports on the chemical composition and nutritional value of dates (Yousif et al., 1976; Vandercook et al., 1977; Fayadh and Al-Showiman, 1990; Al-Hooti et al., 1995; Besbes et al., 2004) and date palm seeds (El-Shurafa et al., 1982; Al-Showiman, 1990; Devshony et al., 1992; Al-Hooti et al., 1998). These exhibit an increasing interest in utilization of the seed, often discarded or used as a feed for domestic animals, after technological or biological transformation of dates (Besbes et al., 2004).

Carbohydrates, proteins and fats

Dates are particularly rich in carbohydrates. Sugars, especially fructose, glucose, mannose, maltose, and other non-reducing sugars such as sucrose, represent over 80% of the dry matter. Glucose to fructose ratio varies between 1 and 2 depending on the cultivar and ripening stage. A small amount of the carbohydrates found in dates is represented by polysaccharides such as cellulose and starch (Shinwari, 1993). Elleuch et al. (2008) studied the sugar content of cvs. Deglet Noor and Allig and showed that sucrose was predominant in Deglet Noor, whereas in Allig, reduced sugars were more abundant with an equal proportion of fructose

and glucose. This difference was ascribed to the potential presence of high invertase activity in Allig cv. (Fayadh and Al-Showiman, 1990; Elleuch et al., 2008). Usually, the sugar content is lower in the kimri and khalal stages as compared to commercial dates at their full ripeness stage of tamar. Part of the sugar loss is also due to a well characterized non-enzymatic browning reaction, the Maillard reaction, which occurs during storage and involves sugars, free amino-acids and phenolics.

Analysis of the amino acid profile of dates and their seeds derived from the cvs. Deglet Noor and Allig showed the presence of 17 different amino acids, including glutamic acid that was foremost in the seeds, representing 17-18% of total amino acids. Other essential amino acids detected in the seeds included lysine, isoleucine, leucine, methionine, threonine, valine and phenylalanine. A number of proteins with molecular weights ranging from 22 kDa to 70 kDa were also abundant in the seed of both tested cultivars.

Fatty acids occur in both the flesh and seed of dates as a range of saturated and unsaturated acids. In seeds, at least 14 different fatty acids were detected, while in the flesh only eight occurred at very low concentration (Al-Shahib and Marshall, 2003b). Oleic acid represents one of the major unsaturated fatty acid in the seed with a content of 41.1-58.8%. Other detectable unsaturated fatty acids include palmitoleic, oleic, linoleic and linolenic acids. The seeds also contain 6-8% of a yellow-green, non-drying essential oil suitable for use in soap and cosmetic products. Seed oil is composed of 8% lauric; 4% myristic; 25% palmitic; 10% stearic; 45% oleic; 10% linoleic and traces of caprylic and capric acids (Morton, 1987).

Dietary fibers and vitamins

Fully mature dried dates have an average dietary fiber content of 4.4-6.5% (Spiller, 1993; Al-Shahib and Marshall, 2003a,b). Three quarters of this percentage represents insoluble fibers while the remaining proportion represents soluble ones. Depending on the method used for quantification, the content of these fibers can be more or less important (Yousif et al., 1982; Al-Showiman, 1998; Al-Shahib and Marshall, 2003a,b). Variation was also reported to be dependent upon the stage of ripeness (Al-Shahib and Marshall, 2003a,b). According to some studies, such as the one conducted by Al-Shahib and Marshall (2003a,b), six to seven dates (approximately 100g) consumed daily by an adult would provide 50-100% of the recommended daily intake. Dates were also reported to contain 0.5-3.9% pectin, thought to possess health benefits (Al-Shahib and Marshall, 2003a,b).

Dates are very rich in vitamins, especially β-carotene (vitamin A), thiamine (B_1), riboflavin (B_2), niacin, ascorbic acid (C) and folic acid (folacin) (Yousif et al., 1982; Considine, 1982; El Hadrami and El Hadrami, 2009). Some of these vitamins provide 10-50% of the daily recommended intake of an adult. Ripe fruits were reported to contain a substantial amount of carotenoids including lutein and various forms of β-carotene and minor carotenoids. The contents vary with the cultivar and stage of ripeness, with the total content of carotenoids decreasing towards the final ripening stages and in storage.

Essential minerals

Dates are reported to contain at least 15 essential minerals, including phosphorus, potassium, sodium, zinc, manganese, magnesium, copper, iron, fluorine and selenium (Al-Shahib and Marshall, 2003a,b). Depending on the mineral, content varies from 0.1 to 1000 mg per 100 g dry matter of dates. Variation also depends on the cultivar and ripening stage as well as the cultural practices during the growing season, and especially soil and plant fertility. Al-Hooti et al. (1995) reported on the mineral content of five cultivars of dates at various ripening stages. They found that the iron content decreased in four tested cultivars from kimri to tamar ripening stage, whereas it increased in cv. Lulu. On the other hand, the authors showed that the percentages of phosphorus, potassium, calcium, sodium, magnesium and zinc decreased in all five tested cultivars of dates from the kimri to tamar stage.

Fluorine in dates is proven to protect against tooth decay. Selenium is thought to play several roles including the prevention of cancer and the stimulation of the immune systems (Al-Showiman, 1998). Dates are also considered as a good supplement for correcting iron deficiencies and anemia.

Other nutritional properties

Dates are known for numerous other nutritional properties due to their richness in non-starch polysaccharides and lignin (Elleuch et al., 2008). Total insoluble and soluble non-starch polysaccharides estimated by Englyst et al. (1992) showed the presence of neutral sugars such as uronic and galacturonic acids. Other nutritional properties include a number of antioxidant molecules such as polyamines, phenolics, and

glutathione, known for their health enhancement attributes.

Dates represent an important source of the nutritional daily intake, especially for local populations and to the other few millions who consume dates on a regular basis. In addition, dates contain a number of phenolics such as phenolic acids, hydroxycinnamates and flavonoids including tannins. These compounds are known for their beneficial effects on human health as well as against oxidation-linked chronic or degenerative illnesses such as cancer and cardiovascular diseases. The antioxidant metabolites contained in dates seem to be linked to the role of these phytochemicals in maintaining specific cellular homeostasis, and contributing in a preventive manner to beneficial effects across diverse biological systems and cell types. Phenolics are well known for their inhibiting pathogens and parasites.

Health benefits of dates

Natural dietary antioxidants from fruits such as dates are believed to activate the enzymatic and non-enzymatic antioxidant systems (El Hadrami et al., 2005). Epidemiological evidence suggests that a diet rich in fruits and vegetables promotes a lower incidence of chronic diseases such as cancer, cardiovascular disorders and diabetes (Block et al., 1992; Serdula et al., 1996; Tapiero et al*.,* 2002; Duthie et al., 2003). Date consumption, therefore, can make a contribution to management of these degenerative oxidative diseases. Among all the dietary components of dates, phenolics account for most of the antioxidant properties, exhibiting a broad range of biological effects that can be classified into two main categories. One, the direct effect of phenolics as free radical quenchers, preventing nucleic acids, proteins and lipids oxidative damage (Götz et al., 1996; Rice-Evans et al., 1997; Offen et al., 1998; Morel and Barouki, 1999; Jakus, 2000; Droge, 2002). Two, the ability of phenolics to biochemically and physiologically or molecularly modulate cellular physiology.

In date palm growing areas, dates are used in the production of local beverages and alcohol and included in traditional medicines, tonics, aphrodisiacs and treatment of ulcers (Al-Qarawi et al., 2005). Date palm pollen grains have historically been used in Egypt to enhance/restore fertility, especially in women. Only a small number of pharmacological studies that have been conducted on dates. Al-Qarawi et al. (2003) showed that, depending on the type of extract used, fruit or pit extracts, a significant increase or decrease in gastrointestinal transit in mice was observed. Vayalil (2002) reported that date extract has strong antioxidant and antimutagenic properties. Date palm kernels are also included in many concoctions used to reduce skin wrinkles due to its anti-aging properties (Bauza, 2002), and to prevent irritant contact dermatitis due to natural fats.

In most Muslim countries, dates are consumed in quantity during the fasting month of Ramadan. This is believed to help protect the gastric mucosa from the damaging effect of gastric acid and prevent the development of peptic ulcers.

Anti-microbial properties of dates

In the current era, there is great reliance on processed food to feed burgeoning human populations. Modern methods of food production and processing have opened the door to invasive pathogens and pests, which cause yield and quality losses. Often fresh and transformed products still contain traces of pathogen toxins and other metabolites that can harm consumers in many ways causing temporary chronic diseases.

Being very rich in phenolics, dates are known to exhibit anti-viral, -bacterial and -fungal properties, making them a remedy for certain diseases and prevention of chronic inflammations. The fruit and its by-products are rich in dietary fibers, selenium, carotenoids, ascobate, and other essential antioxidant which may prevent the oxidative damages caused as a result of lymphocytes phagocytosis activity of invasive pathogens and pests.

Anti-tumoral and anti-ulcer properties of dates

Several studies have demonstrated the anti-carcinogenic properties of phenolics, several of which are abundantly present in dates (Mitscher et al. 1996; Yamada and Tomita, 1996; Uenobe et al. 1997). Phenolics are believed to interfere with the development of malignant tumors at various stages (Kuroda and Inoue, 1988). The anti-carcinogenic effect of phenolics has also been linked to their anti-mutagen activity (Mitscher et al., 1996; Yamada and Tomita, 1996; Uneobe et al., 1997) or their ability to inhibit the activity of enzymes involved in the formation of procarcinogens (i.e., cytochrome P450 class).

Phenolics were shown to be responsible for the decrease in carcinogenic potential resulting from mutagen exposure (Bravo, 1998). Phenolics such as caffeic and ferulic acids, highly present in dates, are known to react with nitrite and inhibit the in vivo formation of nitrosamine, hence inhibiting skin tumors (Kaul and Khanduja, 1998).

In addition to controlling oxidation-mediated disorders, phenolics have been ascribed the ability to reduce the impact of infectious diseases. Yoshida et al. (1990) examined the effects of quercetin, one of the flavonoid commonly found in dates, on cell growth of human malignant cells derived from the gastrointestinal tract and on cell cycle progression. Results showed that quercetin noticeably inhibited the growth of human gastric cancer cells with an IC_{50} value of 32-55 μM. The flavonoid was also able to suppress DNA synthesis by 14% as compared to the control while blocking cell progression from the G_1 to the S phase.

Al-Qarawi et al. (2005) used ethanol-induced gastric ulceration in rats as a model, to test the medicinal claim that dates are effective against gastric ulcers in humans. The study showed that both the aqueous and ethanolic extracts of dates derived from the fruit were effective in improving the severity of gastric ulceration and in extenuating the ethanol-induced increase in histamine and gastrin concentrations, and the decrease in mucin gastric levels. The basis of the gastroprotective action of date extracts may be multi-factorial, and may include an anti-oxidant action given the fact that ethanolic un-dialyzed extract was more effective than the other extracts used.

Immuno-modulatory properties of dates

Dates being rich in both phenolics and dietary fibers can play an important role in the modulation of the immune system and prevention of cardiovascular diseases. Lower incidences of cardiovascular disorders are expected in populations relying on a regular intake of dates. This is believed to occur through the inhibition of the oxidation of low-density lipoprotein (Frankel et al., 1993) and through the prevention of platelet aggregation. Phenolics contained in dates may also be able to reduce blood pressure and have anti-thrombotic and anti-inflammatory effects as shown for other fruits (Gerritsen et al. 1995; Muldoon and Kritchvesky, 1996). In addition, phenolics are known to inhibit α-amylase and α-glucosidase activities behind the postprandial increase in blood glucose level, often manifesting in type-II diabetes (Andlauer and Furst, 2003; McCue and Shetty, 2004).

The immune modulatory activities of phenolics derived from dates include anti-allergic (Noguchi et al., 1999) properties able to suppress the hypersensitive immune response. It also includes anti-inflammatory responses triggered by the suppression of the tumor necrosis factor-α-mediated pro-inflammatory pathways (Ma and Kinneer, 2002).

Functional and nutraceutical added-value of dates

In recent years there has been increasing interest in identifying food and by-products that can serve as functional foods and nutraceticals to enhance the well-being of individuals and reduce the risk of various diseases and disorders. Dates consumed fresh or in by-products represent a good source for many nutritional elements, making them a potential source of both functional food and nutraceuticals.

Dates as functional food

A number of studies in the last five years have been dedicated to the composition and functional characteristics of dates and their derived by-products (Al-Farsi et al., 2007; Biglari et al., 2008b; Al-Farsi and Lee, 2008). An examination of over 80 references by Al-Farsi and Lee (2008) on the content and nutritional value of date by-products revealed the presence of substantial amounts of dietary fiber, vitamins and total phenolics. This suggests that they are a good source for natural antioxidants, especially seed by-products, and could serve either as a functional food or incorporated as an ingredient in functional food. Ten essential minerals are also reported in dates, with selenium, copper, potassium, and magnesium being primary.

Dates in the era of nutraceuticals

Metabolic processes in humans generate oxidant by-products that over time cause extensive damages to DNA, protein and lipid. These damages contribute to various degenerative diseases and disorders linked to aging, such as cancer, cardiovascular disease, immune-system decline, brain dysfunction and cataracts. There are several other exogenous factors that contribute to an increase in oxidative damage. These include smoking, a diet rich in iron and/or copper (i.e., excessive meat uptake) and a poor diet that does not include sufficient fruits and vegetables.

Defences against oxidative damage include ascorbate, tocopherol and carotenoids; dates represent a good source for these antioxidants. In recent years, an increasing demand on the exploration of potential nutraceuticals derived from dates has been noted. Biglari et al. (2009) analyzed the total phenolics, especially flavonoids, in different cultivars of dates stored for six months and suggested that certain storage conditions could diminish the antioxidant activity of the fruits. Other studies have focused on adding value to various by-

products derived from dates such as the pits, jams, syrup, jellies and others (Elleuch et al., 2008).

Conclusion

Dates have been a part of human diet for over 6,000 years and are proven to contain high levels of carbohydrate, proteins, vitamins, dietary fibers, and essential minerals and antioxidants, while containing low levels of fat. The nutritional value of this fruit consumed fresh or in the form of many other derived by-products is important worldwide. An increasing number of recent studies have highlighted its potential use as functional food and nutraceuticals. In the next few years, more will be known about the physicochemical properties and nutritional values of this important fruit, hence improving its incorporation into the daily intake of millions of people thereby improving their diet and helping to prevent a variety of diseases. The current biotechnological advances deciphering the secret of this majestic tree have been evolving rapidly, with the latest being the sequencing of the entire genome, which hopefully will help accelerate breeding and finding ways to improve the nutritional value of dates.

Acknowledgments

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REVIEW ARTICLE

Date palm biotechnology: Current status and prospective - an overview

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Abstract

The date palm is one of the most ancient plants, grows in the regions of Middle East, North Africa, South Sahel, East and South Africa. Its sexually propagation hampers propagation of true-to-type genotypes due to heterozygosity. The vegetative propagation is carried out with the off shoots, produced from axillary buds situated at the base of the trunk during the juvenile life of palm tree. Offshoot production is slow; their numbers are limited, laborious and can't meet the rapidly growing demand of varieties. To speed up the date palm genetic improvement, *in vitro* culture techniques could be handy; however, genotype influence limits the effective use. Bioreactor is being used for large-scale production of somatic embryos. Somaclonal variation is common among *in vitro*-derived date palm plants. However, it could broaden genetic variability together with mutagenesis; molecular markers AFLP used to identify variability and to select useful variants. Dwarf date palm hybrid was developed by embryo rescue by interspecific hybridization of *Phoenix dactylifera* and *P. pusilla*. *In vitro* germplasm conservation is done by cryopreservation for long-term storage. Alternatively, *in vitro* shoot cultures and plantlets are stored at 4°C for short term-storage. Micro-calli is produced from date palm protoplasts; *Agrobacterium*-mediated transformation succeeded in GUS gene expression in callus. Date palm genomics can distinguish multiple varieties and a specific region of the genome linked to gender.

Key words: *Agrobacterium*-mediated transformation, Somaclonal variation, Genomics, Cryopreservation, Embryo rescue, Mutagenesis, Bioreactor

Introduction

The unique characteristics of date palm can be truly called 'tree of life' and is considered as one of the most ancient plant, and is distributed throughout the Middle east, North Africa, South Sahel, areas of East and South Africa, and even certain parts of Europe and USA. It makes a significant contribution towards the creation of equable microclimates within oasis ecosystems and thus enabling sustainable agricultural development in saline and drought affected areas. The rich fruit plays an important role in the nutrition of human population, and also several products are made that generate employment and thus influence socio economic aspect of people. Therefore it is widely acknowledged sustainability value in social, economic and ecological terms. Moreover, this crop has a great potential as a source of renewable energy, an alternate source to the fossil energy, by producing bio-fuel since its fruits high in carbohydrates, 44-88% total sugars.

Sexual propagation is widely used for date palm propagation. However this method can't be used commercially for propagating the cultivars of interest in a true-to-type manner. Interspecific hybridization between the date palm (*Phoenix dactylifera*) and the dwarf date palm (*P. pusilla*) has been successfully carried out, aimed at the development of short hybrid date palms (Sudhersan et al., 2009). Heterozygosity in date palm is related to the dioecious nature. Half of the date palm progeny is generally male and they don't produce fruits, and also large variation can occur in the progeny. There is no known method for sexing date palm at an early stage of tree development and that makes hard to eliminate non-productive male trees in the nursery before planting in the field. Another drawback of seed propagation is that the growth and maturation of seedlings is extremely slow. A date palm seedling may take 8-10 years or more before fruiting occurs. It is not surprising that little work has been done on date palm genetic improvement for developing new cultivars by traditional approaches. Therefore to speed up the date palm breeding programmes, particularly the areas where date palm is threatened by red weevil, devastating diseases like Bayoud and Brittle Leaf;

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as a source of bio-fuel, biotechnology would be of great help in overcoming these problems (Jain et al., 2011).

Problems facing date palm genetic improvement

The date palm cultivation encounters several constraints mainly due to its development under harsh desert conditions, *e.g.* water shortages, high temperature and irregular supply of amendments. Date palm also faces many biotic constraints, especially Bayoud disease caused by *Fusarium oxysporum* f. sp. *albedinis* (Figure 1) (Carpenter and Klotz, 1966; Djerbi, 1988).

Figure 1. Bayoud disease caused by *Fusarium oxysporum* f. sp. *albedinis*.

This disease is the most devastating to the date palm cultivation and was first described in southern Moroccan groves. Currently, it continues to spread across North African countries, especially in Morocco and Algeria where more than 12 million date palm trees have been destroyed so far. No effective means is known to control this disease and only a few cultivars with poor-quality fruits, unfortunately, are known to be resistant to Bayoud (El Hadrami et al., 1998). Therefore, proper date palm cultivation requires, disease resistant cultivars, pruning, pollination, fruit thinning, bunch removal and fruit harvesting, are highly essential for good quality fruit production. The cost of date production increases when the trees grow taller, due to the high labour cost in many producing countries. Mechanization is also expensive and unjustifiable in the case of small growers. Frequent climbing for fruit harvesting is highly dangerous in the case of taller old trees. Tree height is one of the major constraints to good quality date production. In order to reduce tree height and to develop dwarf date palms, a related dwarf palm species, *Phoenix pusilla*, was crossed with selected female date palm cultivars (Sudhersan et al., 2009).

Red palm weevil (RPW) (Figure 2) is a major pest in date palm growing countries in the Near East including the United Arab Emirates (UAE), Iran, Egypt and others (Jain et al., 2011). It appeared for the first time in the Middle East in 1985. It is a great cause of concern to the date palm growers in these countries. The control of RPW is mainly done by applying chemical insecticides through direct injection into the trunk of the date palm tree or by fumigation. Pheromone traps are also commonly used to control RPW, which still requires more refinement for more effectiveness to control this pest. Baculoviruses could be another way to control RPW, especially genetically engineered ones inserted with a set of genes dealing with neuro toxin, light-emission (fire fly gene), and heat tolerance. Another approach would be to express *Bacillus thuringiensis* (*Bt*) crystal insecticidal protein genes, to address problems related to insect pests (Sharma et al., 2002) and chitinase (to address problems related to basal stem rot).

Figure 2. Red palm weevil (*Rhynchophorus ferrugineus*).

Date palm propagation methods

Available techniques of rapid multiplication of date palm have contributed immensely to meet the increased demand of date palm fruits worldwide (Jain et al., 2011). Traditionally, date palm is propagated by both sexually through seeds and

vegetatively by off shoots that produced from axillary buds situated on the base of the trunk during the juvenile phase in date palm tree. It is quite slow for off shoots to develop and that hampers vegetative propagation of date palm trees. So far, there is no available technique to speed up in increasing the off shoot numbers as well as reduce the time in developing them. The use of off shoots preserve true-to-type character of multiplied genotypes. Moreover, sexual propagation of date palm is unsuitable for commercial production/propagation of true-to-type value-added genotypes. It is due to heterozygous nature of date palm seedlings and their dioecious nature (Jain, 2007a). In addition, half of this progeny is composed of male trees which aren't distinguished before flowering stage. The female plants produce variable fruits and generally of inferior quality (Eke et al., 2005). Furthermore, seed propagation method has another limitation that the growth and maturation of seedlings is extremely low, and therefore, date palm seedling may begin to fruit after 8-10 years of plantation. Although offshoot propagation is a true-to-type technique, it is not commercially practical for the following reasons:

- Offshoot production is limited to a relatively short vegetative phase of about 10 to 15 years;
- Only a limited number of offshoots are produced during this phase (20 to 30 offshoots, depending on variety);
- Some varieties produce more offshoots than others (some do not produce offshoots at all);
- Offshoot survival rate is low;
- The use of offshoots enhances the spread of date palm diseases and pests;
- Offshoot propagation is difficult, laborious, and therefore expensive.

In vitro propagation of date palm

The use of *in vitro* techniques such as somatic embryogenesis and organogenesis is highly suitable for large-scale plant multiplication of vegetatively propagated crops. The success of these techniques is highly genotypic dependent, however, have successfully been applied for plant propagation in wide ranging crops including date palm (Jain, 2007a). Micropropagation via direct organogenesis is widely used for rapid clonal propagation of elite genetic material of date palm (Khierallah and Bader, 2007). The performance of micropropagated date palm seems to be better than conventionally grown plants in terms of yield, early flowering time, and quite uniform in fruit quality and physical properties. Aaouine (2003) reported plant regeneration from 30 genotypes of date palm via direct shoot organogenesis. The major concern with this approach is somaclonal variation that is dependent on various factors including genotype, explants, plant growth regulators (Jain, 2001). Moreover, it is highly desirable to maintain genetic fidelity of regenerated plants, which can be studied by various molecular markers Micropropagation has an advantage of using low concentrations of plant growth regulators, consequently callus phase is avoided. Direct regeneration of vegetative buds minimizes the risk of somaclonal variation among regenerants. The duration of culture period is limited by frequent subcultures for maintaining and providing shoot cultures for plantlet production. However, the highest number of subcultures must be determined before starting the fresh cultures from the mother plants. This is done to prevent or reduce somaclonal variation. Currently, only a few laboratories use this technique to produce commercially *in vitro* date palm plants, mainly in Morocco, Saudi Arabia and United Arab Emirates. Micropropagation technique has been used commercially in selected date palm cultivars (Jain, 2006) described advantages and limitations of date palm micropropagation; major advantages are year round availability of plants, quality control, rapid production of plants of elite cultivars, and cold storage of elite genetic material.

Embryo rescue

Embryo rescue technique is carried out by the removal of a zygotic embryo from the seed and planting in a sterile nutrient culture medium. Embryo culture has several potential applications in agricultural crop improvement research programs. This technique has been used in several crops to produce new hybrids e.g. triticale; used for haploid production by making intergeneric and interspecific crosses, e.g. wheat and oat, *Hordeum vulgare* and *H. bulbosum*. It is used to save embryos that fail to develop naturally in interspecific or intergeneric hybridization where defective endosperms are common (Hodel, 1977). Embryo culture may also be used to reduce lengthy dormancy periods or with seeds difficult to germinate due to physical or physiological factors. Excised embryos cultured *in vitro*, under suitable basal nutrient culture media, usually germinate immediately. Embryo culture also can be useful in seedling developmental studies. Sudhersan et al. (2009) were successful in reducing the date palm height by embryo rescue of a cross between a dwarf palm species *Phoenix pusilla* and cultivated selected *P. dactylifera* cultivars (Sudhersan et al., 2009). This is the first

report on reducing the plant height in date palm by embryo rescue, and opens the way to genetically improve date palm in a short time.

Protoplasts

Date palm biotechnology is routinely being used in tissue, organ and cell culture for large-scale plant production and multiplication. Protoplast technique is yet to reach a stage of being used routinely in date palm genetic improvement, especially for somatic cell hybridization. The protoplasts are free of cell wall, consisting of cytoplasm bounded by the plasma membrane. The availability of commercial enzymes enables the production of large numbers of uniform protoplasts. Regeneration of fertile plants from isolated protoplasts was reported in tobacco (*Nicotiana tabacum*) for the first time by Nagata and Takebe (1971) and Takebe et al. (1971). The current status of protoplast plant regeneration has been reported for more than 400 plant species, (Davey et al*.,* 2005).

There are very few reports on date palm protoplast work. Chabane et al. (2007) reported callus formation from protoplasts in cvs. Deglet Noor and Takerboucht. Similarly, Rizkalla et al. (2007) succeeded in inducing callus from protoplasts in Barhee and Zaghloul cvs. So far, critical steps of plant regeneration from recalcitrant date palm protoplasts have been accomplished. For example callus formation was achieved in commercial cvs. Deglet Noor, Takerboucht, Barhee and Zaghloul. The use of feeder layer was the main factor for inducing cell divisions as well as subsequent microcallus and callus formation. However, plant regeneration from protoplast callus has yet to be accomplished before this technology can further be used for producing somatic hybrids. Another major application of protoplast technique is to genetic transformation of date palm by introducing useful genes, e.g. disease resistant, fruit quality, plant height and others. This approach would enable (1) the selection of resistant cultivars and cultivars with excellent fruit quality through field trials, (2) and then combining both traits in one cultivar through conventional crossbreeding or somatic hybridization. Also resistance genes can be taken from a cultivar or species with high resistance level to a particular disease through asymmetric somatic cell hybridization, partial genome transfer from donor to the recipient parent. By this approach, virus resistant plants have been produced by fusing protoplasts of *Solanum brevidens* and *S. tuberosum* (Valkonen et al., 1994); herbicide resistance in *Solanum nigrum* and *S. tuberosum*, and *S. nigrum* and *Lycopersicon esculentum* (Binding et al., 1982; Jain et al., 1988).

Somatic embryogenesis

Somatic embryogenesis has tremendous potential for rapid large-scale plant production. In date palm, this technology can be used for the large-scale propagation, thereby opening the way for the production of artificial seeds. Somatic embryogenesis of date palm has been quite successful in plant regeneration (Fki et al., 2003; Al-Khayri, 2005). The most frequently used explants of date palm are apical shoot tips and lateral buds for successful plant regeneration (Jain, 2007a). However, it should be noted that factors controlling callus induction are so numerous that's why other optimizations are still to be done to improve the quality of the embryogenic calli and to increase the frequency of callus induction from diverse explants. Both abnormal somatic embryo differentiation and somaclonal variation were associated with the utilization of high concentrations of 2,4-D. Reducing its concentration significantly had minimized the number of abnormal somatic embryos and somaclons (Fki, 2005). Smith and Aynsley (1995) studied field performance of tissue culture-derived date palm clonally produced by somatic embryogenesis, and the results demonstrated that these plants started bearing fruits within 4 years from field planting of small plants with leaf length 100 cm and 1.5 cm diameter at the base. The main advantages of somatic embryogenesis are ideal for cryopreservation, cost effective for large-scale propagation, and embryo production in a bioreactor (Table 1).

In addition, further studies are still to be done to find other biochemical and new molecular markers of embryogenesis in date palm. Most of the methods used to assess somaclonal variations have limitations: cytogenetically analysis cannot reveal alteration in specific genes, isozyme markers are subject to ontogenic variation, and molecular markers investigate only a small part of the genome. Hence, field performance analyses remain the most reliable strategy to assess genetic integrity in date palm. Studies related to the cryopreservation of date palm embryogenic cultures are scarce that's why developing innovative procedures will be beneficial for date palm genetic resources preservation and a fabulous support for commercial propagation laboratories. The preliminary studies revealed that embryogenic cultures constitute an adequate plant material for further experiments on mutation induction for useful mutants selection, transfer of genes and isolation of regenerable protoplasts.

Table 1. Advantages and disadvantages of somatic embryogenesis (Jain, 2007b).

Advantages	Disadvantages
Cost effective clonal propagation	Low number of field plantable plantlets
Both shoot and root meristem development in the same step of the process	Highly genotypic dependent
Quick and easy to scale-up in liquid cultures, e.g. bioreactors	Inability to produce somatic embryos from mature seeds in many plant species
Long-term storage via cryopreservation	Gradual fluctuation and eventual decline in embryogenic culture productivity
Establishment of gene bank	Somatic embryogenic cultures from seeds or seedlings have unproven genetic value
Production of somatic seeds by encapsulation of mature somatic embryos	Long life cycle may show genetic variability or new mutations at the later stage of development
Somatic seedlings may be rejuvenated	
Genetic transformation	
Automation of somatic embryo production	
Somatic seedlings are virus-free	
Mutation induction	

Finally, there are still a number of problems such as abnormal somatic embryos differentiation, endophytic bacteria proliferation in *in vitro* culture and somaclonal variation, needing further extensive research to be totally solved. Concerning the endophytic bacterial contamination, only juvenile explants could be used to establish clean *in vitro* tissue culture since antibiotics such as cefotaxim have only a bacteriostatic effect. Immaturity of vascular tissue in these explants may explain the absence of this kind of contaminants in such explants (Fki, 2005).

Genetic diversity conservation

Plant genetic diversity is highly essential for the genetic improvement of crops for sustainable agriculture and its gradual loss is as a consequence to rapid human population growth, industrialization, deforestation, and natural calamities (Jain, 2010a,b; 2011a,b). In the future, the impact of climate change may have an adverse impact on sustainable date palm productions as well other crops. The conservation, distribution and proper utilization of plant genetic diversity/resources have become necessary for the development and improvement of date palm cultivars for sustainable crop production by the establishment of gene/germplasm bank both nationally and internationally. The Gene bank should encourage researchers to survey and monitor the genetic diversity of natural populations and landraces on farmer's fields. *In vitro* conservation techniques, cryopreservation or cryo-storage and cold storage, are excellent system for genetic resources conservation of forest trees and horticultural crops. Cold-storage approach has disadvantage of frequent subculture and that may run into a risk of contamination and somaclonal variation. Cryo-storage has an advantage of long-term storage without going through frequent subcultures and somaclonal variation. For this, *in vitro* cultures are suitable, e.g. somatic embryos/ cell suspension, callus, and should be able to regenerate plants with minimal somaclonal variation. In date palm, the most common *in vitro* culture approach has been somatic embryogenesis, which is very much dependent on genotype and culture medium for plant multiplication, even though there is a risk of genetic variability among regenerated plants For the first time, cryo-storage of date palm somatic embryos was done in Tunisia, FAO/IAEA project, and plant regeneration is yet to be accomplished. In Asia, National Bureau of Plant Genetic Resources (NBPGRI, India) is the biggest germplasm bank, and conserves mainly local germplasm seed and vegetative propagated crops and introduces new crops as well.

***In vitro* conservation and cryopreservation of germplasm**

The purpose of date palm genetic material conservation is to protect from deforestation, man-made environmental pollution, and natural calamities such as hurricane, floods, drought, fire etc. In Grenada, hurricane Ivan and Emily in 2004 and 2005 damaged 90% nutmeg and other spice trees, and resulted in loss of agriculture production, elite germplasm, and exports. The basic requirement of *in vitro* conservation and cryopreservation of genetic resources is the reliable plant regeneration from *in vitro* explants and large-scale disease-free plant multiplication. In failing to plant regeneration, this

technique may is useless to storing *in vitro* cultures. Most common *in vitro* cultures are being used such as shoot tips, callus, cell suspension, microspore, and somatic embryos. At low temperature, 0-5°C, growth of stored shoot cultures is slowed down and that reduces the number of subcultures on the fresh culture media without influencing the genetic stability of cultures. It allows store cultures for several years as long as over 10 years depending on plant type. However, rooted shoots enhances storage time much longer, e.g. in strawberry shoot cultures that developed excellent roots could be stored for three years without change of culture medium under low light intensity and 4°C (S. M. Jain personnel communication). The growth rate can also be reduced by increasing sucrose concentration or addition of mannitol or sorbitol in the culture medium. Bekheet et al. (2001) were successful in the conservation of *in vitro* tissues including shoot buds and callus cultures of date palm var. Zaghloul by slow growth method for 12 months at 5°C in the darkness. *In vitro* conservation has many advantages: disease-free planting material, high plant multiplication rate, all year round plant supply to the growers, potential of producing low cost planting material, and maintain the genetic fidelity verified with molecular markers. The major disadvantages of *in vitro* conservation are: loss of genetic material by contamination due to bacteria, fungi, virus and mites; subcultures on the fresh culture medium; labour intensive; destruction of stored genetic material due to fire or earth quake; and power supply interruptions. Therefore, utmost precaution should be taken to use healthy plant tissues for storage, and also test for virus-free material especially for example in cassava, strawberry and so on before initiating *in vitro* cultures for storage.

Cryopreservation

Cryo-storage or cryopreservation is widely used for long-term storage of *in vitro* cultures of genetic material under ultra-low temperatures, usually at -196°C in the liquid nitrogen (Subaith et al., 2007; Bekheet et al., 2007). This method preserves contamination-free material and prevents somaclonal variation. Since date palm *in vitro* culture has been worked out for plant regeneration, several groups have been engaged in cryo-storage of date palm tissues such as shoot tips, nodular cultures, callus, and somatic embryogenic cultures (Bekheet et al., 2007). Cryoprotectant treatment is given before plunging the tissue in the liquid nitrogen for preventing ice crystal formation in the tissue in order to avoid any damage to the tissue that may adversely affect plant regeneration upon thawing of cryo-stored material. The common cryoprotects are polyehthylglycol (PEG), glucose, and dimethylsulfoxide (DMSO). In date palm, somatic embryo growth remains normal when treated with cryo-protectant mixture of glycerol and sucrose. The growth rate or germination rate of somatic embryos should remain normal after the cryopreservation and that would reflect any adverse impact of various treatments during the following the protocol.

Cryo-therapy for virus elimination

Cryopreservation has application for the elimination of viruses, which is also termed as cryo-therapy. Several viruses have been eliminated from various plants such as cucumber mosaic virus and banana streak virus from banana (Helliot et al., 2002), grape virus A (GVA) *in vit*ro-grown shoot tips of *Vitis vinifera* L. (Wang et al., 2003), potato leafroll virus (PLRV) and potato virus Y (PVY) from potato shoot tips (Wang et al., 2006). The cryopreservation method allows only the survival of small areas of cells located in the meristematic dome and at the base of the primordial (Helliot et al., 2002). Therefore, cryo-therapy would be an alternative efficient procedure to eliminate viruses to producing virus-free plant material and simultaneously long-term storage of genetic material.

Mutation breeding

The exploitation of genetic variability is essential for the development of new cultivars. Genetic variability can be induced by chemical and physical mutagens, T-DNA insertional mutagenesis, and tissue culture-derived variation or somaclonal variation. The most common physical mutagen used is gamma radiation. In this review, we will stick to physical mutagens only. Induced mutations are random changes in the nuclear DNA or cytoplasmic organ, resulting in chromosomal or genomic mutations that enable plant breeders to select useful mutants such as disease resistant, high yield etc. First of all, gamma irradiation breaks DNA into small fragments and secondly DNA starts repair mechanism. During this 2nd step, new variations develop or mutations occur. In date palm, there is hardly any work done on mutation induction, except that of FAO/IAEA Coordinated Research Project on development of Bayoud disease resistant date palm mutant varieties in North Africa (Jain, 2002, 2005, 2006). Mutation induction in date palm is feasible now due to a reliable plant regeneration system via somatic embryogenesis and organogenesis. Somatic embryogenesis system is more preferable approach due to single cell origin of somatic embryos and that

prevents or reduces the occurrence of chimeras. Moreover, mutant somatic embryos are germinated into direct plantlets in a single step, avoiding laborious rooting step. The irradiation of multicellular structures, e.g. seed, meristem tissue or offshoots, may result in chimeras in regenerated plants, and that would require a lot of extra work to dissociate chimeras by plant multiplication up to M1V4 generation (Jain, 2007a,b).

Mutant isolation

Mutant isolation can be done in two ways either in a single step or stepwise selection. In the first approach, irradiated cells are put under very high selection pressure for the isolation of mutant cell clumps/lines. The initial selection pressure should be as high as high LD_{75}. Remove isolated mutant cells and transfer them on the fresh culture medium with reduced selection pressure allowing them to recover from the initial selection pressure for about one week. The selected lines are put for shoot and root differentiation. Before selected mutant lines are put for shoot differentiation, they should be grown for 2 generations devoid of selection pressure and put them back again to the selection pressure. This step is done to make sure that the selected mutant lines are stable due to genetic changes rather than due to epigenetic changes. In the second approach, the selection pressure is reduced stepwise, from high to low concentration. All other steps are more or less similar to the first approach.

In vitro selection of mutants, normally type of the selection pressure varies, *e.g.* salt concentration, fungal toxin, polyethyl glycol (PEG), herbicide etc. For appropriate selection pressure, it is better to determine LD_{50} dose (Jain et al., 2010).

The third option is to select mutants at the whole plantlet level, *e.g.* by spraying herbicide or water withholding for drought tolerant selection, fungal toxin spraying or injection. In date palm, Bayoud disease resistant mutant plants were selected in the greenhouse by treating them with isolated toxin from *Fusarium oxysporum* f. sp. *albedinis* fungus causal agent (Jain, 2006). These plants are already in the field for the last four years. So far, they are doing just fine.

Somaclonal variation

Somaclonal variation is well suited to date palm genetic improvement by using selected somaclones with traits such as abiotic and biotic tolerance, high quality and other agronomic traits (Jain, 2001; El Hadrami and El Hadrami, 2009). It has a real advantage in widening the genetic basis of this species, relying more or less solely on vegetative propagation. Variation in the somaclones has often been associated with changes in chromosome numbers and/or structure, punctual mutations or DNA methylation or other epigenetic events (Jain et al., 1998; Brar and Jain, 1998). Somaclonal variation is undesirable from an industrial production stand point of view but may provide an enrichment of the genes pool. Its frequency depends, among others, on the genotype and the length of the proliferation process. Jain (2006) reported that rapid shoot proliferation can be achieved from various parts of the plant including shoot tips, stem cuttings, auxiliary buds and roots. He also pointed out that the selection of the genotype and the number of sub-culture cycles help limit the appearance of somaclones after the step of plant regeneration. Many off-type plants and abnormal dwarf phenotypes with low fruit sets as well as vitiated multi-carpel fruits (Fig.3) are observed among the *in vitro*-propagated date palm tree population. These phenotypes are not always detectable at seedling stages and often become apparent a few years after planting. However, the technological advances and the development of molecular markers have made it possible, in recent years, to early and accurately detect these variants and eliminate them for the mass production (Saker et al., 2000). These off-types and somaclones can be further investigated to enrich the genetic pool.

Figure 3. Somaclonal variation in multicarpel fruits of dates.
(Photos are provided by Dr. Nasser S. Alkhalifah, Riyadh, Saudi Arabia)

In vitro-selection represents useful biotechnology tools in date palm breeding for tolerance to biotic and abiotic stresses *i.e.*, drought, salinity, and diseases and pests (Jain et al., 2011). These techniques also offer an improvement of the value-added of the new genotypes with traits such as an increase in the number and/or size of fruits or their texture or taste, or a modification in flower structure (Witjaksono, 2003). By applying specific selective agents or providing particular conditions to *in vitro*-propagated tissues, somaclones with desired traits can be produced at a high frequency. Causes of somaclonal variation during the multiplication are diverse and tightly dependent upon the genotype, its level of ploidy, the growth conditions and duration of selection (Maluszynski and Kasha, 2002). Studies of the determinants of such a variation revealed that it can be due to changes at the gene level through genetic events such as duplication, translocation, mutation by insertion or deletion of transposable elements, or methylation. It can also occur at the chromosome level through instability, inversion, and transient or permanent ploidy changes (Kumar and Mathur, 2004). These phenomena often lead to irreversible pleiotropic and epigenetic events and the production of variants called chimera.

Genetic transformation

The global population growth rate is alarming and the situation demands to enhance food production to feed new mouths by developing new tools for plant breeders. Since date palm is more or less like a food crop and feeds people and serves as nutrition security, genetically engineered date palm would be able to generate disease and pest resistant plants by over expression of bio pesticide and antifungal. Growing of such palms will significantly reduce the hundreds of tons of pesticide applied yearly risking human health and degradation of the ecosystem. Genetic engineering would assist in reducing time scale in developing new cultivars; only when precisely single trait genes to be expressed without altering the remaining genetic makeup. However, genetically modified (GM) crops have yet to win the confidence of the consumer worldwide even though growing area of GM crops is expanding.

A large number of plant species have subsequently been genetically transformed, primarily using two different strategies for DNA delivery into totipotent cells; T-DNA delivery with *Agrobacterium tumefaciens* (Horsch et al., 1984) and direct gene transfer with particle bombardment. Generally, *Agrobacterium*-mediated transformation has several advantages over particle bombardment method e.g. integration of a well-defined DNA sequence, typically low copy number and preferential integration into actively transcribed chromosomal regions (Gheysen et al., 1998). Many approaches have been pursued in order to improve the efficiency of *Agrobacterium*-mediated transformation in recalcitrant monocot plant species, e.g. use of hypervirulent *Agrobacterium* strains, use of particular combinations of *Agrobacterium* and plasmids, optimization of co-culture media and conditions that increase the interaction of *Agrobacterium* with the plant cell (Cheng et al., 2004; Kumlehn et al., 2006). For date palm, *Agrobacterium*-mediated transformation used GUS (β-glucuronidase) as a reporter gene, which is easy to assay. So far, no conclusive report is available on the expression of economically-important genes in date palm to the present. The first report on successful infection of date palm embryogenic callus with *Agrobacterium,* and that led to the development of its gene transfer system (Saker et al., 2009). It involves callus production from shoot tip explants on callus induction medium (CIM) containing MS salts, B5 vitamins, 30 g/l sucrose, 10 mg/l 2, 4-D, 3 mg/l 2ip, 170 mg/l KH_2PO_4 and 3 g/l activated charcoal, followed by mass propagation of the proliferated microcalli on MS medium supplemented with 0.4 mg/l NAA and 0.1 mg/l 2ip. Factors influencing transient expression of the GUS gene were evaluated following the infection of embryogenic callus; results indicated that high bacterial density (OD_{600} 1-1.5) and prolonged infection (2 hrs) gave the highest percentage of GUS-expressing calli concluding date palm gene transfer achievable. Alternatively, direct gene transfer in date palm cells was optimized by particle bombardment method (Habashi et al., 2008; Saker, 2006, 2007). A construct harbouring a cholesterol oxidase gene, which renders plants resistance to insect attack, was introduced into embryogenic date palm callus using PDS1000/He particle bombardment system. Three calli out of 200 putative transformed microcalli gave positive GUS expression after bombarded with DNA-coated particles, gave positive GUS expression. The successful integration of GUS gene in GUS positive clones was verified by PCR. The reported system involves the establishment of embryogenic callus cultures from shoot tip explants, followed by shooting of the embryogenic callus with DNA coated particles under optimized physical conditions. The most effective physical factors

influencing gene delivery using a bio-listic gun were flight distance of micro-projectiles and their size and applied pressure, cell and tissue type dependent (Iida et al., 1990).

Molecular markers

Molecular markers are an increasingly important resource for all crops. DNA markers, especially those based on simple sequence repeats and single nucleotide polymorphisms, are playing an increasingly important role in plant variety identification, germplasm resource collection and breeding activities. In general, the molecular marker resources for date palm are somewhat limited. However, most of the available DNA marker types have been used on some material, mostly to cluster date palm varieties into related groups. The most profound effect on the development of the DNA marker resources for date palm is the newly available shotgun sequence. Mining this sequence database and the steady lowering of the costs of high throughput sequencing will increase rapidly the molecular marker resources and their application to date palm over the next few years

It is clear that the date palm genome is structured similarly to that of other characterized plants. Therefore all the tools that have been developed for using DNA markers are available. Preliminary studies have demonstrated that population structures and lineage relationships can be identified with the current crop of DNA markers. The availability of the complete genome sequence will facilitate the development of suitable marker among different marker types. The development of a series of sequenced tagged sites (probably based in SSRs) will supply resources needed for the screening of collections to reduce the number of samples kept in germplasm banks. They will also add impetus to identifying markers linked to the various disease-resistant genes. With the steady increase in the sequencing resources, SNPs will also become more useful but the relative costs of SNP and SSR analyses may well determine which of the two-marker systems becomes most widely used. It is undoubted that the collection of many high polymorphism information content SSR primer pairs and validated SNPs will provide the tools for phylogenetic analyses as well as germplasm conservation. However, once genomic regions associated with important characteristics such as disease resistance, taste and post-harvest stability, the sequencing of these regions and the identification of the actual bases for these characteristics can be incorporated into the breeding and improvement programs. The identification of off-types arising in tissue culture propagation and the complete genome sequencing of normal and off-type individuals will lead to the identification of both markers for assessing off-type individuals in the regenerated plants as well as the 'mutations' responsible for these off phenotypes. Therefore these molecular markers and the tools developed through their use will facilitate the improvements in available germplasm for increasing the area under date palm cultivation as well as for the overall improvement of the plant material available to growers.

Traditional and modern genetic improvement in date palm need extended time periods and considerable funds. Therefore, they can be assisted by molecular markers that give better and more efficient research strategies. Data based on molecular markers such Random Amplified Polymorphic DNA (RAPDs), have been developed to molecularly characterize date-palm genotypes of cultivars and to examine their phylogenetic relationships (Trifi et al., 2000). Earlier results showed the use of molecular markers as tools to evaluate genetic diversity and genotyping of date-palm cultivars (Jain et al., 2011). Based on statistical analysis, Sedra (2007c) reported certain informative molecular markers which are associated with specific phonological characters in date palm. Previous study of date-palm mitochondrial DNA gave evidence of two plasmid-like DNAs that seem to be linked to Bayoud disease resistance (Benslimane et al., 1996) but these markers cannot distinguish both cultivars studied (Trifi, 2001). Each marker corresponds to one part of date palm DNA and the genome has the size estimated to 1.7 pg and it is constituted of more than $١٠^{١٢}$ nucleic bases. These data seem to suggest that the higher the number of markers used the greater the probability to achieve more precise results. Trifi group, Tunisia used several hundred RAPD and inter-simple sequences repeats (ISSR) primers and identified several markers to distinguish partially or totally between resistant and susceptible cultivars of date palm. The difficulty and relatively weak efficiency were probably due to the nature of the genetic status of resistance.

Genomics

Genomics is carried out to study the whole genome of an organism, which is the sum total of DNA molecules harbouring all genes of an organism. This type of work is performed to study all the genes of a given cell, tissue and organism;

DNA (genome) as well as RNA (transcriptome), and protein (proteome) in the context of a regulatory network as well across taxa (evolution). The field includes intensive efforts to determine the entire DNA sequence of various organisms and to construct a genetic map, using large-scale sequencing technology, to generate massive, adequate and high-quality data, by using bioinformatics tools for assembly, annotation and in-depth analysis. A major branch of genomics is still focused on sequencing the genomes of various species, but the knowledge of full genomes has created the possibility for the field of transciptomics, proteomics, bioinformatics, function genomics, metagenomics and system biology.

A team from Weil Cornell Medical College in Qatar tried to sequence the entire date palm genome using Solexa (illumine) sequencer based on a shotgun method. They announced the finished draft map in 2009 and released the sequence data subsequently: (http://qatar-weill.cornell.edu/ research/datepalm Genome/index.html*).* According to their analyses, the genome assembly has a predicted genome size of ~550Mbp. The following are genome parameters of their draft sequence assembly:

- 45,000 scaffolds greater than 2kb
- Scaffold N50 is 4250bp
- 850,000 novel high quality SNPs between parental alleles
- GC content of the nuclear genome is 37%
- 302Mb of assembled sequence with 18.5Mb of ordered gaps
- Unique sequence is 292Mb at the 24-mer level

The date palm genomic project (DPGP) is being carried out at the King Abdulaziz City for Science and Technology (KACST) jointly with the Beijing Institute of Genomics, Chinese Academy of Science (BIG/CAS). The objectives are bioinformatics, genetics, biochemistry, transcriptomes and post-genomics. Data have been generated by using second-generation sequencers and sequence assembling has been working on most likely in a complex process where different types of data are integrated to ensure both quality and contiguity.

The first phase of the DPGP is focused on genomics and bioinformatics that pave the way for genetic and biochemical studies.

- The specific aims of the DPGP are: a working draft with sequence coverage; 10x from 454 and 50x from SOLiD; a complete map will be built with end-sequences from BACs and Fosmids; a genome diversity map built with shotgun sequencing of 30 cultivars; each with 30x of SOLiD reads; the date palm transcriptomes: full-length cDNA, over 30,000 unigenes; and expression profiles for leaves, roots, and flowers (~50 tissue samples).
- They have already preliminary data on genome sequencing and assembly, chloroplast genome sequencing and transcriptomics.

Conclusions and prospects

Date palm is life-line of people living in Sahara and sub-Sahara regions and also an important source of income in Near Eastern countries. Most of the date palm trees are very old, as old as 70-100 years and perhaps are becoming more vulnerable to various diseases and pests. One of the reasons could be due to global warming or global climatic changes. An increase in global temperature would bring new pests and disease and get rid of some existing types. Since date palm has a long life cycle, it could become more vulnerable to the global warming, and that is why it is highly desirable to pay more attention to the genetic improvement of date palm varieties that could with stand natural calamities without compromising the yield and quality. The use of chemical insecticide and pesticides is very common to control diseases and pests of date palm. These practices could become deadly health hazard to human health and that may also curtail their export market. Innovative techniques are needed to apply for the control of disease and pests, and that is where genetic modifications of organisms would be of highly effective. Genetic engineering of baculoviruses may be of great help in controlling the RPW by inserting a set of genes including neuro toxin (gene from scorpion or snake), light-emitting (fire-fly), and heat tolerance (bacterial gene). The engineered baculoviruses would multiply inside the insects and kill them instaneously. One could monitor the rate of viral multiplication inside the insect by light meter. Insertion of *Bt* gene in date palm won't be the right approach due to long life cycle of date palm and it would be rather difficult to predict the behaviour of transgene in the long run. Moreover, food safety regulations don't permit to insert *Bt* gene in food crops.

The progress of *in vitro* culture techniques has enabled date palm micro propagation more as a routine technique for large-scale plant production in many countries. The influence of genotype has handicapped micro propagation of different commercially valuable date palm varieties. This area needs serious attention by modifying the

culture medium well suited for several date palm cultivars. This type of work perhaps may require more empirical work in order to modify the composition of the culture medium. Now the question arises how well molecular approach would assist plant tissue culturists to modify the culture medium and growing conditions or the selection of appropriate explants or pre-conditioning of explants. To answer these questions, plenty of work is foreseen and in other words this area of research is 'virgin'.

The date palm shoot multiplication rate could be improved by using liquid culture system or 'bioreactor'. Few groups have started working on liquid culture for in vitro propagation of date palm. RITA bioreactor, based on temporary immersion system, should be tried in date palm shoot multiplication and somatic embryo production. Micro propagation via organogenesis or direct shoot formation is extensive labour-oriented. Somatic embryogenesis may reduce labour cost and also asset in developing automated somatic embryo production. However, genetic fidelity of micro propagated plants should be maintained with minimal somaclonal variation, otherwise there will be severe economic loses to the growers. Molecular marker analysis would be an ideal approach to identify genetic variability at the early stage of plant development. It would be difficult to identify point mutations or any genetic change at the early stage of plant development because it may not express phenotypically and may express at the later stage of plant development. This scenario occurred in oil palm tissue culture-derived plants in Malaysia and the oil palm industry lost millions of US dollars.

Haploid production in date palm has not yet been accomplished. Inflorescence culture will be one way to induce haploid somatic embryo production. Fki et al. (2003) induced callus from immature inflorescence of date palm var. Deglet Nour, and the calli originated from the proliferation of floral primordia showed embryogenic potential. The capacity of inflorescence to form callus was much higher than cultured leaves. They did not determine the ploidy level of callus and regenerated plants from inflorescence-derived callus. In the future, the success of this type of work would revolutionise date palm genetic improvement program as well as molecular genetics for useful gene identification.

Somatic embryogenic cell suspension is an excellent system for mutation induction and isolates useful mutants of date palm. Direct mutant somatic embryos can be produced and germinated into mutant somatic seedlings. These mutant seedlings can further be micro propagated for large-scale production. The utmost care should be taken while handling somatic embryogenic cultures, and in failing to do, the chances getting somaclonal variation becomes very high. This approach is an excellent example of combining mutagenesis and biotechnology for date palm improvement. Transgenic date palm is long way to go before consumers accept to consume them and consequently export market will also be lost. Therefore, transgenic approach to modify date palm should be followed with a great caution, even though it has a great potential to overcome several of its problems.

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REVIEW ARTICLE

In vitro mutagenesis for improving date palm (*Phoenix dactylifera* L.)

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Abstract

Genetic variability is needed for the improvement of crops, which can be either spontaneous or induced by mutagen treatments. The frequency rate of spontaneous mutations is very low and can't be used for plant breeding in developing new cultivars. Mutation breeding has been quite successful for producing new mutant cultivars with desirable traits in both seed and vegetative propagated crops (http://www-mvd.iaea.org). Somatic embryogenic cell cultures of date palm were irradiated with gamma radiation, and regenerated plants were transferred in the greenhouse and treated with Bayoud toxin, isolated from the causal fungus *Fusarium oxysporum* f. sp. *albedinis*. Several selected putative mutants tolerant to Bayoud disease were initially maintained in the greenhouse and finally transferred in the field for further evaluation. These plants have maintained tolerance to Bayoud disease under field conditions.

Key words: Bayoud, Gamma radiation, Mutatgen, Mutation breeding, Somatic embryogenesis

Introduction

The unique characteristic of date palm (*Phoenix dactylifera* L.) is a life-line to people living in Sahara and sub-Sahara regions. It is considered as one of the most ancient plants which was cultivated in Mesopotamia some 4,000 years ago (Omar and Hameed, 2006; Dakheel, 2003). Perhaps date palms are becoming more vulnerable to various diseases and pests. One of the reasons could be due to global warming or global climatic changes. An increase in global temperature would bring new pests and disease and get rid of some existing types. Since date palm has a long life cycle, it could become more vulnerable to global warming, and that is why it is highly desirable to pay more attention to the genetic improvement of date palm cultivars that could withstand natural calamities without compromising the yield and quality. Dakheel (2003) showed a unique date palm production system under harsh climatic conditions: high resilience and tolerance to environmental stresses-high temperature and radiation, low soil and atmospheric moisture, extended periods of drought, high salinity levels, and large diurnal and seasonal fluctuations.

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Date palm belongs to the monocot family Arecaceae and is an arborescent, dioecious tall evergreen and highly heterozygous plant (Jain, 2007). Date fruits are a most important source of human nutrition as well as an export item for many date palm-growing countries. The annual world production of dates in 2010 was approximately 7.9 million mt according to FAO (http://faostat.fao.org). The major bulk of date palm production, about 68% of the total world production, comes from Egypt, Saudi Arabia, Iran, United Arab Emirates, Pakistan, Algeria and Iraq. In Saudi Arabia, over 200 date palm cultivars are grown and produce 1,078,000 mt of dates, which is about 14% of the total world date production.

Date palm fruits are a source of nutrition to people living under harsh climatic conditions; and are a rich source of sweeteners, glucose, and fructose (Al-Eid, 2006). Date syrup is composed mainly of reducing sugars; glucose and fructose as major source of the sugar fraction. (Al-Ghamadi and Al-Kahatani, 1993a,b,c). Dates could be considered as an ideal food which provides a wide range of essential nutrients and potential health benefits.

Major Diseases and Pests

Date palm suffers from several diseases and insect pests leading to severe economic loss to the growers. There are about 25 diseases and disorders affecting date palm worldwide. Among them, 14 are caused by fungi (Karampour and Pejman, 2007). Date bunch fading disorder (DBF) is the most harmful phenomenon that damages both the

quality and quantity of date yield. In Iran, this disorder has caused wilting and drying of bunches and finally severe defoliation of date palm fruits during the last five years (Karampour and Pejman, 2007). In Egypt, 21 fungal species belonging to 15 genera were isolated from diseased date palm samples collected from different Egyptian localities (El-Deeb et al., 2007).

Bayoud disease is a serious threat to date palm plantations in North African Saharan and sub-Saharan regions; it is caused by a soil-borne fungus *Fusarium oxysporum f.* sp. *albedinis* (FOA). It was first observed in the Draa Valley, Morocco in 1870, and from there reached the Algerian Central oasis around 1889, and it rapidly advanced into new areas. The disease has destroyed more than 10 million palm trees in Morocco and nearly 3 million in Algeria. The rate of destruction by Bayoud disease is thus estimated at 5 percent per year (Oihabi, 2003). Presently, the disease is known to occur in Morocco, Algeria and Mauritania. Tunisia takes very drastic quarantine measures and strict surveillance to prevent the introduction of the disease from Algeria.

Quenzar et al. (2001) identified two circular plasmid-like DNAs (S and R plasmids) in the mitochondria of date palm. By employing a PCR-based approach, they showed that the presence of R plasmid and absence of S plasmid can be considered as a reliable molecular marker of Bayoud disease resistance. The study revealed that the presence of S plasmid and the absence of R plasmid are correlated to the susceptibility to Bayoud disease. This diagnostic molecular tool would ultimately become a simple, reliable, rapid and efficient approach to identify Bayoud resistant and susceptible genotypes from the large pool of date palm lines.

Red palm weevil (RPW) is a major pest in date palm growing countries in the Near East including the United Arab Emirates, Iran, Egypt and others (Oihabi, 2003). It appeared for the first time in the Middle East in 1985. It is a great cause of concern to the date palm growers in these countries. The control of RPW is mainly done by applying chemical insecticides through direct injection into the trunk of the tree or by fumigation. Pheromone traps are also commonly used to control RPW, which still requires more refinement for greater effectiveness to control this pest. Baculoviruses could be another way to control RPW, especially genetically engineered ones inserted with a set of genes dealing with neurotoxin, light-emission (firefly gene), and heat tolerance.

In Vitro **Culture of Date Palm**

The date palm is well known to propagate both sexually through seeds and vegetatively by offshoots that are produced from axillary buds situated on the base of the trunk during the juvenile life of the palm tree. Seed propagation of date palm is not appropriate for commercial production due to the heterozygous characteristics of seedlings, which is related to the dioecious nature of the date palm; half of the progeny are generally male and don't produce fruit, and also large phenotypic variation can occur in the progeny (Jain, 2006). Currently, there is no known method for sex determination of date palm at the early stage of tree development, making it rather difficult to discriminate between productive and non-productive male trees in the nursery before transplanting them to the field. Furthermore, the seed propagation method has another limitation in that the growth and maturation of seedlings is extremely low, and therefore, date palm seedling may not begin to fruit until 8-10 years of age. The ideal way would be to look for molecular markers for sex determination on the line of work done in papaya (Deputy et al., 2002).

Offshoot production is slow; their numbers are limited, laborious and can't meet the rapidly growing demand of cultivars. Normally offshoot numbers vary from 10-30, depending on the genotype, and are produced only within a certain period time in the mother palm's life (Jain, 2007). There are no field-based methods yet available for increasing the number of offshoots per plants. There are not many commercial tissue culture laboratories worldwide micropropagating date palm for large-scale plant production (for more information see: Al Kaabi and Zaid, 2003).

In vitro culture techniques such as somatic embryogenesis and organogenesis have been effectively used for large-scale plant multiplication of horticultural crops and forest trees (Jain and Ishii, 2003; Jain and Gupta, 2005; Jain and Hagmann, 2007). Plant multiplication via organogenesis is routinely followed in commercial laboratories worldwide especially in ornamental plant industries and also to some extent in fruits and cash crops like coffee, sugarcane, etc. The cost of plant production is generally high due to labor and energy costs that reduce the profit margin. Most of the commercial date palm micropropagation laboratories are operating in low labor cost countries. The performance of *in vitro* propagated plantlets seems to be better in terms of yield and early flowering. Al-Ghamadi and Al-Kahatani (1993a,b,c) made a detailed comparative analysis of fruit quality of micropropagated and conventionally

propagated plants and found no major variation affecting the fruit quality and properties. The results clearly indicate that *in vitro*-grown date palm are quite uniform in terms of fruit quality and physical properties. Smith and Aynsley (1995) reported the results of field performance of tissue culture-derived date palm clonally produced by somatic embryogenesis. These plants started bearing fruits within 4 years from field planting of small plants with a leaf length of 100 cm and 1.5 cm diameter at the base. Fruit from the tissue culture-derived plants of cv. Berhi was indistinguishable from the fruits of plants originated from suckers (offshoots). These results certainly justify the commercial scale of micropropagation procedures of somatic embryogenesis to provide rapid, cost-effective means of obtaining elite date palm planting material. However, this approach has a major bottleneck in that plant multiplication rate is highly genotype dependent, and may require modification of the culture medium depending on the genotype. For more information, see Jain (2006), who described advantages of date palm micropropagation and its limitations. Some of the major advantages of micropropagation are year round availability of plants, quality control, rapid production of plants of elite cultivars and cold storage of elite genetic material.

In vitro plant regeneration

Date palm tissue culture work has revolved around somatic embryogenesis (Fki et al., 2003; Al-Khayri, 2005) and organogenesis for plant regeneration (Khierallah and Bader, 2007; Al-Khayri, 2007). Aaouine (2003) reported plant regeneration from 30 genotypes of date palm via direct shoot organogenesis.. Furthermore, the initiation period for somatic embryogenesis induction is 4-6 months as compared to 8-10 months for organogenesis; and the total time from the induction phase to plant marketing is 40-44 months via somatic embryogenesis vs. 60 months via organogenesis (Aaouine, 2003).

Murashige and Skoog (1962) is the most commonly used culture medium for both somatic embryogenesis and organogenesis of date palm, which is also modified depending on the genotype or cultivar (Jain, 2006). Young offshoots from 2-3-year-old date palm cv. Maktoon were used for direct shoot induction after they were sterilized with commercial bleach and rinsed with sterile distilled water (Khierallah and Bader, 2007).

The somatic embryogenesis approach for date palm plant regeneration seems to be more effective for clonal propagation. Fki et al. (2003) improved somatic embryogenesis protocol of date palm cv. Deglet Noor for large-scale clonal propagation. Initially, embryogenic callus cultures were initiated from both leaf and inflorescence explants (Figure 1) on MS medium containing 0.5 and 10 mg/l 2, 4-D . These cultures were used to develop highly proliferating cell suspension cultures in liquid medium supplemented with 1 mg/l 2, 4-D. Somatic embryos were initiated from actively growing cell suspension, and finally somatic embryos were germinated and regenerated into plantlets. Abul-Soad et al. (2008) induced somatic embryogenesis of female inflorescence explants of date palm.

Figure 1. Date palm inflorescence for the induction of somatic embryogenesis.

The overall production of somatic embryos reached 10,000 units per liter per month. The partial desiccation of the mature somatic embryos significantly improved somatic embryo germination rate from 25 to 80%. The cutting back of cotyledon leaf was stimulatory to the germination rate. Furthermore, flow cytometry analysis showed no variation in ploidy level of somatic seedlings. Several research groups have modified the culture medium composition by adding vitamins, adenine sulfate, thiamine, glycine, glutamine, myo-inositol and activated charcoal (Al-Khayri, 2005). The role of vitamins in date palm tissue culture is not known. For more details see Jain (2006) and Al-Khayri 2005).

Advantages of Somatic embryogenesis

- Somatic embryos originate from a single cell and minimize or eliminate chimera depending on the plant species

- Somatic embryo cell suspension is ideal for mutation induction due to production of direct mutant somatic embryos
- Somatic embryos behave like a zygotic embryo in germination
- Single somatic embryo can be encapsulated to develop into a somatic seed that could germinate like a normal seed. This aspect still requires further research for use at a commercial scale
- Most suitable approach in woody species for plant regeneration
- Somatic embryos can be produced in a bioreactor which could be automated for large-scale production of somatic embryos
- Somatic embryos are suitable for long term storage by cryopreservation

Disadvantages of somatic embryogenesis

- Somatic embryogenesis is highly genotypic dependent and therefore culture medium modification may be needed for different genotype.
- Germination rate of somatic embryos is very poor in most of the crops
- Somatic embryogenic cultures can lose their property if they are not sub-cultured regularly on the fresh culture medium, and that raises the chance of getting genetic variability.

Mutation Induction

The exploitation of genetic variability is essential for the development of new cultivars. Genetic variability can be induced by chemical and physical mutagens, T-DNA insertional mutagenesis and tissue culture-derived variation or somaclonal variation. The most common physical mutagen used is gamma radiation. In this paper, we will focus on physical mutagens only. Induced mutations are random changes in the nuclear DNA or cytoplasmic organ, resulting in chromosomal or genomic mutations that enable plant breeders to select useful mutants such as disease resistance, high yield, etc. First of all, gamma irradiation breaks DNA into small fragments and secondly DNA starts a repair mechanism. During this second step, new variations develop or mutations occur. In date palm, there is hardly any work done on mutation induction, except that of FAO/IAEA Coordinated Research Project on development of Bayoud disease resistant date palm mutant cultivars in North Africa (Jain 2005, 2006). Mutation induction in date palm is feasible now due to a reliable plant regeneration system via somatic embryogenesis and organogenesis. The somatic embryogenesis system is the more preferable approach due to single cell origin of somatic embryos that prevents or reduces the occurrence of chimeras. Moreover, mutant somatic embryos are germinated into direct plantlets in a single step, avoiding the laborious rooting step. The irradiation of multicellular structures, e.g. seed, meristem tissue or offshoots, may result in chimeras in regenerated plants, and that would require a lot of extra work to dissociate chimeras by plant multiplication up to M1V4 generation (Jain, 2007).

Mutagen selection and treatment

Mutagens are mainly classified in two types: a) chemical and b) physical (ionizing radiations) (Jain, 2007). The main aim is to develop efficient methods for inducing the highest rate of gene mutations with the lowest chromosomal or physiological damage to the mutagen treated plant material. The mutagen selection is very much dependent on the plant material, e.g. organs (shoot tips, meristems, axillary buds) or cell suspension or protoplasts. *In vitro* mutagenesis, protocols for preparing cultures are similar to micropropagation for *in vitro* mutation breeding (Predrieri, 2001).

Mutant isolation can be done in two ways, either in a single step or stepwise selection. In the first approach, irradiated cells are put under very high selection pressure for the isolation of mutant cell clumps/lines. The initial selection pressure should be as high as high LD_{75}. Then isolated mutant cells are removed and transferred to fresh culture medium with reduced selection pressure allowing them to recover from the initial selection pressure for about 1 week. The selected lines are put for shoot and root differentiation. Before selected mutant lines are put for shoot differentiation, they should be grown for two generations devoid of selection pressure and then put them back again under the selection pressure. This step is done to make sure that the selected mutant lines are stable due to genetic rather than epigenetic changes. In the second approach, the selection pressure is reduced stepwise, from high to low concentration. All other steps are more or less similar to the first approach.

The other option is to select mutants at the whole plantlet level, e.g. by spraying herbicide or withholding water for drought tolerant selection, fungal toxin spraying or injection. In date palm, Bayoud disease resistant mutant plants were selected in the greenhouse by treating them with isolated toxin from *Fusarium oxysporum* f. sp. *albedinis* fungus causal agent (Jain, 2006). These

plants have already been in the field for four years and have not shown any sign of disease.

Chemical mutagen treatment

In this section, we will describe the ethyl ethane sulfonate (EMS) mutagen treatment, which is most commonly used for mutation induction. Similar steps can be used for other chemical mutagen with some minor modification in the protocol.

The doses flanking the LD_{50} value shall be selected for generating the mutant populations for evaluation. The quantity of callus and cells (from suspensions) and the number of cultures to be treated need to be judged from their growth rates and regeneration potential. Use high cell density for mutagen treatment and select growing cell clumps after plating of mutagen treated cell suspension on a solidified culture medium. Thus all dead cells are eliminated. The larger the surviving population of cells the better it is for regeneration of large number of plants, which is ideal for selection of mutant lines. Given the low differentiation levels during the *in vitro* phase, cultures need to be critically and regularly observed for variations. The response of the cultures (in terms of media color change, alterations in growth rate, somatic embryo germination rate, plantlet morphology, etc.) to the mutagenic treatments shall be recorded at every stage.

- Prepare a fine cell suspension, clump size 3-4 cells, in the liquid medium from a fragile callus. Determine the doubling time of cells, growth and stationary phase.
- Carefully add freshly prepared EMS (0.5-1.0%) solution in actively growing cell suspension in the growth phase stage
- Use high cell density 100,000 cells/ml
- Shake them while mutagen treatment is on, 100 rpm on a horizontal shaker, for 6 hrs. Treatment time can be modified for other crops.
- Transfer cells in centrifuge tubes and centrifuge at 5,000 or 6,000 rpm for 5 min
- Remove the pellet, add fresh culture medium, and centrifuge at 5000 or 6000 rpm and repeat this process 5-6 times for removing mutagens. Utmost care should be taken to dispose of mutagens while washing the cells.
- Spread uniformly mutagen treated cells on the upper surface of agar culture medium containing plant growth regulators, and allow them to stand for 96 hrs to recover from mutagen treatment shock. We can also put a sterile filter paper on the upper surface of the agar medium and transfer cells.
- Put mutagenized cell population under the selection pressure, e.g. fungal toxin for the selection of disease resistant lines. In case of strawberry, crude extract of a fungus *Phytophthora cactorum* which is a causal agent of fruit rot disease was used. Filter sterile crude fungal extract is directly added in the culture medium. Grow mutagenized cell population on this medium and select surviving clump of cells and transfer to the fresh culture medium containing crude extract.
- The surviving tissue should be transferred onto the fresh shoot regeneration medium for callus production or direct shoot formation depending on plant or genotype. Keep the selection pressure during shoot differentiation.
- Harden *in vitro* plantlets in the greenhouse under 70-90% relative humidity
- Perform initial screening of plantlets, 100-200 at one time, in the greenhouse by inoculating each plant with fungal crude extract
- Field testing in “hot spot” areas under field conditions.

Physical mutagen treatment

Cell suspension

- Prepare and use actively growing cell suspension, as described in section 3.1.
- Spread cells uniformly on filter paper and place them on a solidified culture medium in a Petri dish and seal it with parafilm. The advantage of using filter paper is that cells are uniformly spread and easy to transfer on the fresh culture medium.
- Irradiate cells with an optimal dose, LD50, of gamma rays or any other physical mutagen
- Transfer irradiated cells onto the fresh culture medium in order to recover from irradiation shock for 96 h.
- Follow steps as described in section 3.1.

Determination of radiosensitive dose

All plants show variation in radiosensitivity and therefore it is important to make a radiosensitive curve to determine LD_{50} dose for mutation induction. High radiation doses are detrimental to the plant genome and cause heavy damage to DNA. This leads to large number of mutations, which are mostly undesirable, cumbersome to identify any useful mutants, and handling of mutant population becomes more difficult. In some crop plants, low radiation dose promotes shoot growth, e.g. in citrus

30 Gy dose stimulates shoot growth (Jain, personal communication) and 10 Gy maintains the somatic embryogenesis nature of date palm for up to 3 years (Drira, personal communication). In date palm 20-30 Gy was used for mutation induction depending on the genotype used. For Deglet Noor date palm cv., LD_{50} of somatic embryogenic cell suspension cultures were 20 Gy and used for mutation induction. Actively growing cell suspension in the growth phase was transferred on the filter paper in the Petri dish. Cell clumps were uniformly spread on the filter paper and the dishes were sealed with Para film. Cells were irradiated with different gamma radiation doses and they were transferred for overnight to the fresh solid culture medium for recovery from the radiation treatment. The irradiated cells were transferred in the liquid medium, and distributed in 50 ml flasks containing 30 ml liquid medium. The cell viability test was made with FDA staining to determine the cell survival rate after the different radiation dose treatments. The number of surviving cells was calculated on the basis of cells per ml per radiation dose. After this step, in each flask, 100,000 cells per ml were added as starting material and the cell growth was determined after 1 week. The number of cells per ml per radiation dose treatment was counted with a haemocytometer. These results established the radiosensitive curve and determined LD_{50} radiation dose for mutation induction.

Post mutagenesis handling

The aspect of *in vitro* mutagenesis is crucial for handling mutated cell population and identification of mutants. The number of subcultures depends primarily on the crop or genotype LD_{50} dose, and other factors such as proliferation rate, number of plants to be field evaluated, etc. For example, *in vitro* selected banana putative mutant lines showing resistance to Fusarium wilt caused by *Fusarium oxysporum* f. sp. *cubense* (FOC), multiplied in large numbers by micropropagation up to M1V4 generation, in the range of 500-1000 shoots, which are further rooted to develop into well-developed plantlets for greenhouse evaluation before field trials. *In vitro* shoots can be directly rooted in the greenhouse under 70-90% humidity and avoid one step of *in vitro* rooting. The number of plants is very much dependent on the greenhouse facilities for handling large number of plants at one time.

Greenhouse weaning

The putative multiplied mutant plantlets may respond differentially to greenhouse hardening and field transfer. Considerable mortality can be expected at both these stages. Hence, only experienced staff should do the greenhouse hardening and subsequent field transfer. This step requires 70-90% relative humidity in the greenhouse or plastic house otherwise *in vitro* plants would wither and die. Normally *in vitro* culture conditions, the relative humidity is over 90%. Greenhouse-hardened plants may be field evaluated following standard procedures depending on the trait to be evaluated either in a single or multi-location trials.

Greenhouse screening

Well adapted *in vitro* mutant plantlets are subjected to the selection pressure for the selection of solid mutants before transfer to the field evaluation. The selection pressure could be applied 2-3 times to make sure that the selected mutant lines are solid mutants.

For example in banana, the selection of mutant plants against *Fusarium oxysporum* f. sp. *cubense* (FOC) is done by dipping roots of 500 plants into conidial suspension (500,000 conidia per ml) of the fungus for 20 min and transferring them to sterile perlite. The selected plants could well undergo one round of selection before transfer them to the “hot spot” in the field. In date palm, mutant plants were treated with *Fusarium* toxin and selected 10 mutant plants in the greenhouse, which later on transferred in the field (Figure 2). These mutants showed tolerance similar to the check resistant cv. Saver Layalet from Morocco.

Figure 2. Mutants plant are selected after infection with Bayoud toxin.

Field trials

The selected mutant plants are directly transferred to the “hot spot” in the field depending on the selected trait, e.g. salinity, drought, acidity, soil infected with fungal disease etc. The performance of the mutant plants is evaluated on the basis of survival rate and other agronomical

aspects including yield. In date palm, Bayoud tolerant 10 mutant plants were transferred for the field evaluation, and did not show any sign of disease (Figure 3).

Figure 3. *In vitro* derived plants are growing in Zagora a Bayoud prone area for further testing.

Conclusions

Somatic embryogenic cell suspension is an excellent system for mutation induction and isolates, providing useful mutants of date palm. Direct mutant somatic embryos can be produced and germinated into mutant somatic seedlings. These mutant seedlings can further be micropropagated for large-scale production. The utmost care should be taken while handling somatic embryogenic cultures, and, failing to do, the chances of getting somaclonal variation becomes very high. This approach is an excellent example of combining mutagenesis and biotechnology for date palm improvement. Transgenic date palms have a long way to go before consumers accept to consume them and consequently the export market will also be lost. Therefore, the transgenic approach to modify date palm should be followed with great caution, even though it has a great potential to overcome several problems. There is a complete lack of date palm molecular biological research to address several issues facing date palm genetic improvement. Molecular marker-assisted selection and breeding need serious attention by identifying trait specific genes from natural or induced genetic variability. Functional genomic date palm breeding would probably become a reality in time to come and that would speed up genetic improvement of date palm.

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REGULAR ARTICLE

Enhancement of date palm as a source of multiple products: Examples from other industrialized palms

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Abstract

Multipurpose palm species development would benefit from a broader consideration of the varied economic products palms can potentially provide. All economic palm species have a primary product which accounts for their exploitation or domestication and industrialization. A nearly-exclusive emphasis on the primary product has often obscured the potential value of secondary products. Responsible disposal of residues from harvesting and processing of primary palm products often has the potential of being transformed from a disposal expense and potential source of environmental pollutants into secondary products of value. Examples from other palms which may have applicability to date palm production include: 1) In the oil palm industry, empty palm oil fruit bunches are used as fuel to generate electrical energy and yield a fine ash with industrial uses. 2) In coconut plantation operations, pruned leaves and unwanted husks can be used as fertilizer for the plantation or burned to generate energy. 3) Replacement of ageing plantation trees provides an abundance of woody material requiring disposal, affording a periodic opportunity for innovative secondary product harvest. Technical research on the utilization of palm by-products is typically focused on an individual species, but the results often have broad potential adoption for other economic palms.

Key words: Biofuel, By products, Edible oil, Fruit bunch, Endosperm, Organic fertilizer, Palm, *Phoenix dactylifera,* Sap, Stem wood

Introduction

The palm family (Arecaceae) is comprised of 183 genera and over 2,400 species, and has a worldwide distribution between 44° north and south latitudes (Govaerts and Dransfield, 2005; Dransfield et al., 2008). Five major palm species are domesticated fully and are grown as economic species: areca or betel nut palm (*Areca catechu*), peach palm (*Bactris gasipaes*), coconut palm (*Cocos nucifera*), oil palm (*Elaeis guineensis*) and date palm (*Phoenix dactylifera*). The taxonomic relationship among the five species, which represent three of the five palm subfamilies, is presented in Table 1.

Although the five palm species are classified in different subtribes they are comparable, in terms of the variety of products they yield. The five share certain characteristics, for they are all pinnate-leafed, large, erect and slender to thick palms. Two produce basal suckers (date and peach palms) which can be used for propagation. The date palm's oasis habitat differs from the other four species which are tropical wet climate palms. All five were domesticated primarily for their fruits. Within the subsistence economies where domestication took place, every part of the five palm trees would have been evaluated carefully for any possible utility or product.

The purpose of this paper is to outline the multipurpose character of the areca, peach, coconut, oil and date palms. The information presented can serve as a stimulus to broader consideration of the date palm, to enhance current secondary products and to promote potential new uses.

Shared fruit characteristics

Fruit of palms in the Arecoideae subfamily (areca, peach, coconut and oil palms) are similar. Each has a well-developed exocarp, mesocarp, endocarp (shell) and endosperm (kernel). The terms *nut* and *seed* as applied to palms often create confusion. In the coconut, for example, both *nut* and *seed* may refer to either the entire fruit or to the fruit after the husk has been removed. Generally, use of more precise botanical terms is preferable.

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Table 1. Taxonomic Relationship of Five Major Economic Palm Species.

Family	Subfamily	Tribe	Subtribe	Genus and Species/Common Name
Arecaceae	Arecoideae	Areceae	Arecinae	*Areca catechu* (areca palm)
		Cocoseae	Attaleinae	*Cocos nucifera* (coconut palm)
			Bactrinidae	*Bactris gasipaes* (peach palm)
			Elaeidinae	*Elaeis guineensis* (oil palm)
	Coryphoideae	Cryosophileae	Phoeniceae	*Phoenix dactylifera* (date palm)

The arecanut mesocarp is a source of alkaloids. Coconut has a mesocarp fiber (coir), activated carbon and other products from the endocarp, and oil and other food products from the endosperm (meat). Peach palm has an oily starchy mesocarp which can be cooked and eaten or processed into starch. Peach palm also furnishes palm heart (apical meristem) as a commercial green vegetable. Major products from the oil palm derive from the mesocarp and endosperm, both yield excellent quality food oils.

Coconut and oil palms, once they reach sexual maturity, produce fruit continuously over the year, but with month-to-month fluctuations. Spreading of the harvest of these two tree crops over the entire calendar year is a major economic advantage.

Unique among the five palms, the date palm, in desert climates, has intangible value by creating shade and microclimatic conditions suitable for the growth of other crop plants. Also, the palms function in oases as windbreaks and to stabilize earthen irrigation works.

Once established, palm plantations can provide a sustainable and reliable supply of fruits and other products for decades. Modern commercial palm plantation development has focused almost exclusively on a single economic product; as a result, insufficient attention has been paid to secondary products from the fruits and other parts of the palms.

Individual Multipurpose Palms

An effective approach in this article is to examine and describe the areca, peach, coconut, oil and date palms in terms of their individual harvest and processing practices, focusing on products. Summary descriptions of the individual palms provide a background for a general discussion of enhanced utilization of present and future date palm products.

Areca catechu

The areca palm was domesticated in South and Southeast Asia for its fruit. This solitary slender tropical palm is grown from seed in either pure stands on large estates or by small farmers in mixed systems. Commercial cultivation is limited to the Asian realm. The palm is quite attractive and is also grown ornamentally.

The fruit endosperm is the chief economic product, chewed for its mild narcotic effect derived from presence of the alkaloid arecoline. The endosperm also contains about 10% oil. Fruit bunches are produced seasonally, cut when mature and transported out of the field. At a processing site, fruits are removed from the bunches, husked and then dried or boiled, to reduce the tannin content, and cut into pieces for chewing. Boiling of the fruits yields a tannin-rich liquid. Betel chewing is almost exclusively limited to the areas where the palm was domesticated; like tobacco use, it is linked to the occurrence of mouth cancer.

Areca fruit husks represent about 70% of the entire fruit and research has shown that the material has potential use in making hard board, latex-bound fabric, pulp and paper and as a source of furfural, an industrial chemical compound. Husks can also be used directly as fertilizer.

Other tree products include leaves for thatch; the large leaf sheaths made into biodegradable plates, sandals; also suitable for plyboard and panels. Dead pruned leaves serve as mulch and fertilizer.

Areca palms have an economic life of about 40 years. Replacement plantings generate other products from the trees removed. The palm heart has a bitter taste, but is edible. Entire stems are employed in construction of rustic structures, and the attractive yellow wood cut into a variety of articles such as rules, shelves, etc. Residue can also be incorporated into the soil of plantations as fertilizer.

Detailed accounts of areca palm products and utilization are provided by Bavappa et al. (1982) and Bhat and Nair (1985).

Bactris gasipaes

This tropical Central and South American palm was domesticated by indigenous people for its fruit, which must be cooked before eating. Peach palm is a lesser-known commercial palm because its cultivation is restricted to the region of domestication. Traditionally a small farmer tree crop in mixed systems, the palm produces multiple

slender stems. Chiefly propagated by seed, the palm bears one or two fruit crops annually. Peach palms also are cultivated to produce seed to establish new plantings; the palm is also grown as a handsome ornamental, but has the disadvantage that most specimens have a spiny stem. Long known as a source of quality heart of palm, in recent decades the palm has been cultivated specifically for that product which is consumed locally and exported. Palm heart extraction destroys the stem and precludes fruit production.

Mature fruit bunches are cut from the trees and taken to a processing site. Removed from the bunches, the fruit are sold fresh or cooked. Fruit destined for flour production must be cooked within about a day of harvest. A traditional alcoholic beverage is prepared by cooking, mashing and fermenting the mesocarp pulp. The fruits are also a source of cooking oil and meal for animal rations; the male flowers are edible.

Harvesting palm hearts consists of felling the stem and extracting the apical meristem from the top of the stem, where it is surrounded by several protective sheaths. Harvested palm hearts are 60-80 cm in length, varying in diameter depending upon stem size. They must be transported quickly for processing to avoid moisture loss and fungal damage. At the factory, the hearts are peeled to expose the soft core, cut into short lengths and cooked in cans or jars. Peelings are suitable animal feed. Stem and leaf remains are left in the field as mulch and fertilizer for the remaining smaller stems of the cluster.

Other peach palm products include green leaves from felled stems for thatching and entire stems as framing for simple rural shelters. When older thicker stems are cut, the hard dark wood can be sawed into parquet flooring, made into furniture and carved into decorative or useful articles.

The economic life of a peach palm plantation for fruit production is estimated to be 50-75 years. Clearing and cutting of an old stand to replace it with new seedlings affords an opportunity for palm heart harvest and stem utilization. Growth of peach palm for palm heart production is done at a high planting density. Harvest begins after 1-2 years, before flowering, with natural regeneration from basal offshoots.

Detailed information regarding peach palm cultivation, for both fruit and palm heart, is contained in Villachica (1996), Mora-Urpí et al. (1997) and Mora-Urpí and Gainza (1999).

Cocos nucifera

The most widely cultivated of economic palms, and the best known, the coconut was domesticated in the southern Pacific and Indo-Atlantic regions for its remarkable fruit. The fruit has been described as a portable source of water, food and fuel (Gunn et al., 2011). A short to tall, solitary palm of moderate diameter, seed and tissue culture propagated palm; it flowers and fruits continuously. Coconuts are grown by small farmers in mixed cropping and grazing systems, and also as a large-scale monoculture. It is also a popular tropical ornamental.

A large number of primary products are derived from this remarkable tree, which aptly has been called the *tree of life*. The entire fruit provides an endosperm for copra which contains an excellent edible oil, along with fresh coconut meat; coconut water; the endocarp (shell) has numerous uses; and the mesocarp (husk) yields coir fiber. Palm toddy (sap) can be obtained from tapping the unopened inflorescence, although this practice is incompatible with fruit production. The apical meristem (heart) is an edible green vegetable, although harvest destroys the tree. The coconut stem can be sawed into lumber for construction and furniture, and a variety of useful objects.

The most valuable product from this palm is the endosperm oil, which has numerous food, cosmetic and industrial uses, and is a suitable biofuel. Coconut fruit harvest involves cutting individual fruits from the bunch while it is still on the tree. If mature fruits are harvested solely for copra (oil), they are typically husked in the field and only the nut transported to a copra processing or fresh coconut processing facility. Copra cake, after oil extraction, can be used in animal feed and as fertilizer. With integrated coconut processing, the entire fruit is taken to the factory where the husks are removed to extract the coir fiber. Coconut shells may be made into charcoal, activated carbon, or carved into buttons etc. Waste coconut water of mature fruits is unsuitable as a beverage but can be used in industrial fermentation. Green immature nuts are harvested when fresh coconut water is desired, which can be drunk directly from the nut or drained, processed, and preserved in containers.

Tapping the unopened coconut inflorescence yields a sap consumed as a sweet drink; sweet sap can be boiled down to palm sugar or left to ferment naturally into a palm wine. In turn, the wine can be distilled into spirits (arrack). All four of these products are commercialized in South and Southeast Asia.

Nonedible residues from any of the processing steps described can serve as fuel or transported back to the fields for mulch or buried as organic fertilizer. Pruned leaves and empty fruit bunches have similar uses.

Coconut palm leaves have utility as thatching material, and fresh young leaves can be woven into mats, strong baskets and coarse hats. Leaf midribs can serve as basket framing, to make animal cages and fish trips, or split to make hand brooms. Fences can be constructed from the petioles.

Coconut plantations have an economic life of 60-70 years. Clearing of old unproductive fields affords an opportunity for timber and leaf harvest, as well as palm hearts. Residues can be incorporated into the soil as fertilizer for the new plantings.

Grimwood (1975), Ohler (1984) and Killmann et al. (1996) are basic published sources of information on both commercial and subsistence products from coconut.

Elaeis guineensis

The oil palm is native to Tropical West Africa where it was domesticated as a subsistence crop by indigenous people for its fruit as a source of edible oil, and it continues to be a small farmer crop of West Africa. Another subsistence item is palm wine. The palm is often referred to as the African oil palm to distinguish it clearly from several palm species native to Tropical America which also are sources of palm oil. The African species is the world's major commercial oil palm species and has been introduced to Asia and the Americas where it is cultivated. Oil palm has the distinction of giving the highest yields per unit area of any oilseed crop plant. A solitary palm, it develops a moderately thick stem and is propagated by seed and tissue culture. Once mature, it continuously flowers and fruits.

Cultivation continues as a subsistence crop in Africa where the palm is a common feature of cleared forest areas, in association with food crops and livestock. In regions where it was introduced as an improved crop, such as Southeast Asia and Tropical America, the palm is grown in pure stands on large estates in association with processing factories.

The palm is a source of two similar oils, from the mesocarp and endosperm. Ripe fruit bunches are cut from the trees and transported to the factory. There, the fruit bunches are steam sterilized, fruits stripped from the bunches, mesocarp oil is extracted and seeds are separated to undergo a separate extraction process. Both oils are refined and have wide food and industrial uses. Subsistence farmers primarily extract the mesocarp oil using simple means, mixing the fruits with water and boiling them in a steel drum until the oil at the surface can be skimmed off.

Several secondary processing products are of value. Empty fruit bunches are burned as oil mill fuel and the ash collected to use as fertilizer, in soap making, mixed with concrete, etc. Palm oil bunch ash is a commercial raw material sold internationally. Dried mill effluent and palm kernel cake can be added to animal rations at a low proportion. Solids from effluent are returned to field.

In Africa, tapping the male inflorescence, and sometimes the stem, yields sap which is collected in jars fastened to the tree. Once or twice a day the accumulated sap is transferred to larger containers and taken from the field. It ferments naturally into a mild palm wine. Although chiefly a subsistence beverage, on a small scale it is processed and bottled for commercial sale. The sap contains yeast and also is used in making leavened bread.

Secondary products are fresh leaves pruned for thatch, construction etc., especially by small-scale subsistence growers. Leaves can be added to animal feed. Pruned dead leaves serve as mulch and fertilizer.

An oil palm plantation has an economic life of only about 25 years, the least of any of the five palms. When old plantations are cleared for replanting, huge quantities of stem and leaf materials are generated. Often the residue is burned and the ashes spread on the field. Denser portions of stem can be used as an ingredient in particleboard, but economic viability is unclear. Oil palm has an edible heart, but apparently this food item is not utilized. Leaves, trunks, roots are recycled as fertilizer on site.

Poh et al. (1994), Killmann et al. (1996) and Corley and Tinker (2003) are key references on African oil palm.

Phoenix dactylifera

The date palm is believed to have been domesticated in the Mesopotamian region more than 6,000 years ago, and is among the oldest cultivated tree crops. From its homeland, the date palm was dispersed to North Africa, the Arabian Peninsula and to South Asia, and subsequently to southern Africa, the Americas and Australia, wherever suitable climatic conditions were found to enable commercial fruit production. The date palm develops a moderately thick stem, may reach a height of 20 m. and produces basal suckers in its

earlier years. It may be propagated by seed, offshoot separation and tissue culture. A single annual fruit crop is produced under normal conditions.

Date palm is second only to the coconut with regard to being a source of numerous useful products, but among the palms it holds the distinction of having the greatest number of named cultivars on the basis of fruit characteristics. Date fruits overwhelmingly represent the economic value of the tree; all other products combined are of comparatively minor importance.

Harvesting of ripe date fruits commonly consists of cutting the fruit bunches from the trees, stripping the fruits in the field and transporting them to a processing plant. In a very few elite cultivars of high value, ripe fruits are individually handpicked from the fruit bunch while it is still on the tree. Fruits are sold fresh or dried, and they store well under low temperature conditions. Many food products are prepared from the date fruit, including date paste for bakery products, preserves, condiments, juice, spread, syrup and sugar. Second grade fruits unsuitable for sale can be processed into wine and alcohol, the latter a biofuel resource; organic acids and protein. Date pits contain up to 10% of good quality oil. Waste products from fruit and pit processing can be fed to animals. Pollen is reputed to have medicinal properties.

Under modern plantation practices, field operations generate quantities of empty fruit bunches, cull fruits and pruned leaves. Commonly these residues are left in the field as mulch and fertilizer. However, in desert oases of small-scale agriculture, where woody material is scarce, the date palm provides leaves for shading, thatching and weaving into baskets, mats, rope, hats etc. Midribs and petioles have utility in construction and fencing. Stem wood can be split or sawed into construction material. All of these are also fuel sources. The entire date palm and date palm leaves have symbolic and ritual significance in major religions.

The productive economic life of a date palm is not known with precision. A rough estimate is about 75 years; however trees may be replaced when they reach excessive heights because of the difficulty of pollination, pruning and harvesting. When the palms are cleared for replanting, abundant quantities of leaves and stem wood create a disposal problem. Felled trees also represent a source of edible palm hearts. Currently, these materials are little utilized.

Comprehensive information on date palm growing and its numerous products can be found in Barneveld (1993), which updates earlier studies by Dowson (1962, 1982), and Zaid (2002).

Discussion

Because the harvest and most processing of primary and secondary palm products occur in two separate locations, field and factory, it is logical to discuss enhanced utilization in two sections.

Field

As with every tree crop, field harvest consists of taking from the plant its key economic product, commonly the fruit or fruit bunch, and transporting it to another location for processing. Field harvest may involve on-site primary processing to reduce bulk and weight, such as removal of fruits from the bunches and leaving the empty bunches in the field. Areca, peach and oil palm bunches are cut and transported away; coconuts are harvested individually without bunch cutting; date fruits commonly are stripped from the bunches in the field.

Date harvest typically involves leaving the empty date fruit bunches in the field. In coconut harvest, if coir mesocarp fiber is not to be extracted, the fruits may be husked after harvest and the husks left in the field. If the fiber is to be exploited, the entire fruit is taken to the factory. As part of the fruit harvest of all five palms, some green leaves may be pruned from the trees to afford easier access to the fruit bunches.

Factory

Palm fruit processing varies from simple to complex, the facilities also ranging from small household sites to modern factories. In all cases, the processing generates residue or waste products. These may be empty fruit bunches, shells, peelings, spent pulp or seeds.

Arecanut palm fruit processing is a relatively simple operation, consisting of detaching fruits from the bunches and boiling them whole to reduce tannins. Next the fruits are husked and the endosperm removed and cut up, dried, often mixed with a pigment and other ingredients, and packaged for sale. Some variation exists in the processing procedures employed. Arecanut husks have utility in hardboard and paper making, and as fuel.

Peach palm processing involves stripping fruits from the bunches which are sold fresh or boiled; fruits may also be canned. The only residue is the empty fruit bunch. Processing the peach palm mesocarp into flour generates waste made up of fruit peelings and endosperm. In processing palm

hearts, the incoming palm heart lengths are peeled to remove the inedible outer sheaths, cut into sections, cooked and preserved. Residues from flour and palm heart processing can serve as partial livestock rations. Empty fruit bunches are not utilized.

Oil palm fruit bunches arriving at the factory are steamed, the fruits stripped off and the mesocarp and endocarp oil separately extracted. Empty fruit bunches are employed as fuel and the ash collected for industrial uses. Livestock can consume press cake as part of their rations and other waste products are returned to the field as fertilizer.

Coconut processing may be simple, involving removal of the endosperm from the split endocarps if the product is dried copra for later oil extraction. This is the practice when coconut production is insufficient to support a local processing facility. Alternatively, the endosperm may be removed and immediately processed into various fresh coconut products (e.g. shredded coconut, coconut milk) or oil. In some integrated coconut processing operations, the husk fiber is also an important product. Husks must be retted in water for a period of time to loosen the coarse fiber, after which it is separated, dried and spun into numerous coir products.

The coconut shell (endocarp) is a valuable product. As a direct fuel it has high caloric value, and can be made into excellent charcoal as well as activated carbon. Splitting mature coconuts to extract the endosperm releases coconut water, which is commonly allowed to drain away. However, the liquid contains some carbohydrates and amino acids. If the coconut water is captured, it has use as a raw material for fermentation processes, and can be added to cattle feed before fermentation occurs.

Considering these palm species together, they have the most in common in terms of some residues being used as fuel, often mixed with other combustible material, to generate electrical energy. Depending upon physical and chemical characteristics, waste products of the five palms also serve as an additive in animal rations, or are returned to the plantation fields as fertilizer. Because conveyances bringing fruits to the factory return empty, transporting the biomass back to the field is efficient. Utilization of crop and processing waste products is important because rather than creating environmental pollutants through their disposal, they can become low-value products themselves.

Significant research has been carried out in recent years on secondary product use of the palms discussed in this paper. However, there has been inadequate evaluation of the results across major economic palm species. Three selected examples stimulated by this study, applicable to date palm, can be cited. One, empty fruit bunches of the date palm appear to be little used; yet, evidence from the oil palm suggests that the ash derived from burning them has exceptional qualities with potential industrial applications. Two, the palm heart (apical meristem) of the date palm is edible; yet this food item is scarcely mentioned in the literature. In the Philippines, when coconut plantations are replaced, the palm hearts are extracted and processed into a preserved product sold domestically and internationally. Three, date palm leaves and stems have shown potential in the manufacture of paper, panel board, particle board, etc. A concerted effort to valorise these raw materials should be undertaken.

Conclusion

The date palm has been a multipurpose species since it was first domesticated some 6,000 years ago. In modern times, significant progress has been made in the development of direct and derived date fruit products and the utilization of by-products from packing and processing; however, comparatively minor attention has been given to date palm products other than the fruits. By highlighting the multipurpose character of five major economic palm species, this paper attempts to broaden the thinking about how other nonfruit and fruit products can be developed.

General economic benefits can be derived from broader utilization of all parts of the date palm. Additional socio-economic benefits will also accrue to date farmers in terms of expanding the number of saleable products they produce, and diversifying their income sources.

One key recommendation is that the excellent study *Date Palm Products* (Barreveld, 1993) be updated in light of the numerous published studies over the past two decades. The revision should also place greater emphasis on nonfruit products and include relevant research on comparable palm species.

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Effect of X-irradiation on date palm seed germination and seedling growth

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Abstract

This study was conducted to examine the effect of low doses of X-rays on date palm (*Phoenix dactylifera* L., cv. Khalas) seed germination and seedling growth. A significant reduction in seed germination was observed in response to X-irradiation as compared to the non-irradiated control. A significant reduction in seed germination percentage (82.85%) began at 0.25 Gy of X-rays and persisted to decline to 61.42% at 15 Gy. Based on accumulative growth percent increase (AGPI), a significant enhancement of root growth was observed in response to increasing X-rays doses. The mean root length increase ranged between 3.7% at 0.05 Gy to 8.05% at 15 Gy after 1 week of X-rays exposure. Similarly after 2 weeks of exposure, a significant increase in root growth started at 0.1 Gy and root growth reaching the highest value (10.94%) at 15 Gy, while root growth reached 15.19% in week 4 at the same dose. A significant increase in leaf length of 0.05-0.25 Gy was observed but at higher doses reduced growth occurred. This study provides an insight into the potential use of X-rays in manipulating growth parameters of date palm and enhances understanding of the physiological responses inflicted by irradiation stress.

Key words: Date palm, Leaf, Radiation, Root, Seed germination, Growth, X-ray

Introduction

The date palm (*Phoenix dactylifera* L.) is one of the most economically important tree species in the arid regions (Alshuaibi, 2011; Al-Abbad et al., 2011). Propagation is normally carried out by offshoots; however, tissue culture techniques are currently used for commercial mass propagation of date palm (Al-Khayri, 2007; Zaid et al., 2011). There are more than 3,000 named cultivars worldwide concentrated in areas between latitudes 10° and 30° North, mostly in the Middle East and North Africa with the greatest production in Iraq, Saudi Arabia, Iran, Pakistan, and Egypt (Johnson, 2011). Worldwide date production has increased exponentially over the last three decades with an annual expansion of about 7%. The total number of date palms in the world has been estimated to be approximately 100 million trees with fruit production of 2.6 million metric tons (Rajmohan, 2011). Date palm production is challenged by numerous diseases and pests (Zaid et al., 2002) especially because of the incompatibility of traditional breeding methodologies with the long generation cycle and the dioecious, heterozygous nature of date palm (El-Hadrami et al., 2011). Modern biotechnological approaches, including radiation technology, are gaining interest to augment the efforts of date palm genetic improvement (Jain, 2011).

The application of nuclear technology for genetic improvement of date palm was demonstrated through inducing mutations by exposing callus to gamma radiation and subsequent *in vitro* selection of plants resistant to Bayoud disease (Jain, 2005; 2007). Studies related to the effect of radiation on date palm physiological and molecular process are limited. However, some physiological responses of date palm seedlings to static magnetic field (SMF) and alternating magnetic field (AMF) were recently elucidated (see review by Dhawi and Al-Khayri, 2011). Magnetic field inflicted modifications in proline content (Dhawi and Khayri, 2008a); chlorophyll (Dhawi and Al-Khayri, 2008b); DNA (Dhawi and Al-Khayri, 2009a); ion content including Mg, Ca, Na, P, K, Fe, Mn and Zn (Dhawi and Al-Khayri, 2009b; Dhawi et al., 2009); and water content as well as growth expressed in fresh weight (Dhawi and Khayri, 2009c). These parameters were also found to be modified by X-rays irradiation of date palm

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seedlings (Al-Enezi and Al-Khayri, 2012a,b). Other studies examined the effects of X-rays radiation on several physiological and biochemical processes in different plant species including cotton, *Gossypium barbadense* (Younis et al., 1962); barley, *Hordeum disticum* (Joshi and Ledoux, 1970); beans, *Vicia faba* (Roy, 1974); wheat, *Triticum aestivum* (Erickson et al., 1979); okra, *Abelmoschus esculentus* (Rao and Rao, 1978); corn, *Zea mays* (Romanova et al., 2000); lotus, *Nelumbo nucifera* (Arunyanart and Soontronyatara, 2002); rocketsalad, *Eruca vesicaria* (Atsushi and Miho, 2006); slender goldenweed, *Haplopappus gracilis* (Tage, 2006); tangerines, *Citrus reticulala* (Palou et al., 2007); as well as green tea, *Camellia sinensis*; sage, *Salvia officinalis*; cinnamon, *Cinnamomum verum* and ginger, *Zingiber officinale* (Al-Nimer and Abdual-lateef, 2009).

Exposure to ionizing radiations, such as X-rays and gamma rays, is suggested to inflict physicochemical stresses thus inducing growth and physiological modifications (Levitt, 1972; Ahloowalia and Maluszynski, 2001). The effects of ionizing radiation are largely damaging and at high doses the effect is detrimental (Ehrenberg, 1955). However, some reports provide evidence of a stimulating effect on growth when seeds or seedlings are exposed to low-dose ionizing radiation (Breslavets 1946; Younis et al., 1962; Zaka et al., 2004; Mortazavi et al., 2006). Growth stimulation in plants by very low doses of ionizing radiation became a widely recognized phenomenon known as *hormesis* (Sheppard and Regitnig, 1987; Sheppard and Chubey, 1990; Macklis and Bresford, 1991; Luckey, 2003). Moreover, the sensitivity to radiation is dependent upon several factors including radiation form and dose as well as plant physiological status, age and genotype (Yoshida et al., 1999; Din et al., 2004; Esnault et al., 2010; Borzouei et al., 2010; Tabasum et al., 2011).

Whereas previous date palm studies investigated the effect of exposure to X-rays at the seedling stage, the current investigation examined the influence of low doses of X-rays irradiation on seed germination and growth of both roots and leaves of the emerging seedlings. Based on the available literature, this is the first study addressing the effect of radiation on seed germination of palms in general.

Materials and Methods

Plant material

Seeds were collected from female date palm trees of cv. Khalas, a mid-season cultivar grown in the Al-Hassa oasis, Saudi Arabia. Seeds were surface sterilized with 1% sodium hypochlorite for 5 min and soaked in water for 24 h prior to irradiation.

Irradiation treatments

Soaked date palm seeds were placed in 9 cm Petri dishes (10 seeds per dish) containing 10 ml distilled water. Using a therapeutic medical X-rays device (Clinac 23EX Linear Accelerator, Varian Medical Systems, USA), seed samples were exposed to different X-rays doses (0, 0.05, 0.1, 0.5, 1, 2.5, 5, 7.5, 12.5, and 15 Gy) using 7 Petri dishes (70 seeds) per treatment.

Growth conditions

Following irradiation the seeds were germinated on moist filter paper at 37 °C. After 2 weeks, the germinated seeds were counted and the seedlings were planted individually in a 20-cm plastic pot containing potting mix (1 soil: 1 peat moss: 1 vermiculite). They were maintained in the greenhouse under natural light at 30-40 °C with a relative humidity of approximately 50% and watered as needed to ensure adequate moisture.

Determination of seed germination percentage

Germination percentage of seeds exposed to X-ray was calculated by using the equation:

Germination percentage = Number of germinating seeds / Total number of seeds × 100

Determination of the growth rate

Growth was calculated by measuring the root length of seedlings and leaves every week for a period of 4 weeks. Growth was calculated as accumulative growth percent increase (AGPI) according to the following equation:

Accumulative growth percent increase (AGPI) = (Final length - Initial length) / (Final length) x 100.

Seedling growth was assessed based on the differences in length between weeks 1 and 2 (AGPI 1), week 2 and 3 (AGPI 2), and week 3 and 4 (AGPI 3).

Statistical analysis

The experiment was randomly designed as a single factor (X-rays dose) with 11 levels and 7 replications (plates), N=77 Data were subjected to analysis of variance (ANOVA) and the means were separated, where appropriate, using the least significant difference (LSD) at 5% Standard deviation for each treatment was also calculated.

Results and Discussion

Seed moisture at the time of irradiation plays an important role in the expression of radiation effects (Ohba, 1961; Bhattacharya and Joshi, 1977). Seeds are normally dry and require absorption of water

before cellular metabolism and hydrolytic enzymes are activated (Bewley, 1997). To ensure proper moisture to support metabolic activities, the date palm seeds were soaked in water prior to irradiation. This procedure was recommended by Younis et al. (1962) working with cotton seeds. The date palm seeds were also kept in water during irradiation as a precaution to prevent desiccation.

Several studies conducted on different plants types have proven the ability of X-radiation to cause important physiological and morphological changes such as changes in the constituents of medicinal plants (Al-Nimer and Abdual-lateef, 2009), chromosomal aberrations (Moutschen et al., 1987), morphology of axillary buds (Langenauer et al., 1972), plastids variations (Palta and Mehra, 1968), gametophyte generation (Palta and Mehra, 1973) and mutations (Lesley and Lesley, 1965). This is due to high doses of X-radiation which form free radicals, known for their adverse effect on major macromolecules including DNA and proteins (Jade et al., 2010; Legue and Chanal, 2010). Moreover, X-rays were observed to affect root growth (Scandalios, 1964), percentage of seed germination (Sheppard and Chubey, 1990) and leaf growth (Sheppard et al., 1987).

According to the analysis of variance (Table 1), seed germination percentage was affected by X-rays in a significant one-way. The results show that the germination percentage of treated seeds significantly decreased by X-irradiation as compared to the control. The significant reduction in seed germination percentage (82.85%) began at 0.25 Gy of X-rays and continued to decline to up to 61.42% at 15 Gy (Figure 1). Similarly, Dhakshanamoorthy et al. (2010) found that germination of Barbados nut, *Jatropha curcas* L. decreased with the increase of gamma rays at doses ranging from 5 to 25 Kr. Similarly, Hameed et al. (2008) found that the percentage seed germination of chickpea *Cicer arietinum* was inversely related to the irradiation doses of gamma rays tested at 100-1000 Gy. Reduction of seed germination percentage was observed in other different plant including barley, *Hordeum vulgare* (Joshi and Ledoux, 1970); petunia, *Petunia hybrida* (Gilissen, 1978); jack pine, *Pinus banksiana* (Rudolph, 2003); Scots pine, *Pinus silvestris* (Zelles, 2003) and wheat, *Triticum aestivum* (Floris and Anguillesi, 2003). Unlike date palm seeds, germination of broccoli (*Brassica oleracea* L.) seeds was improved at 2 Gy of X-rays but higher doses, 8 and 16 Gy, caused reduction of the germination rate (Sheppard and Chubey, 1990). In contrast, Borzouei et al. (2010) working with wheat (*Triticum aestivum* L.) showed that mean germination time, root and shoot length, and seedling dry weight decreased with increasing radiation doses; whereas, final germination percentage was not significantly affected by gamma radiation doses ranging from 100 to 400 Gy. In a study of hard wheat (*Triticum durum* Desf.), low doses of gamma rays, 5 to 30 Gy, did not affect seed germination, shoot and epicotyl growth (Melki and Marouani, 2009).

Table 1. Analysis of variance for seed germination percentage of date palm after 4 weeks under the influence of different levels of X-rays; P values less than 0.05 are significant.

Factor	df	M S	F	p-value
X- Ray	10	906.0	9.689	0.0001
Error	66	93.5		

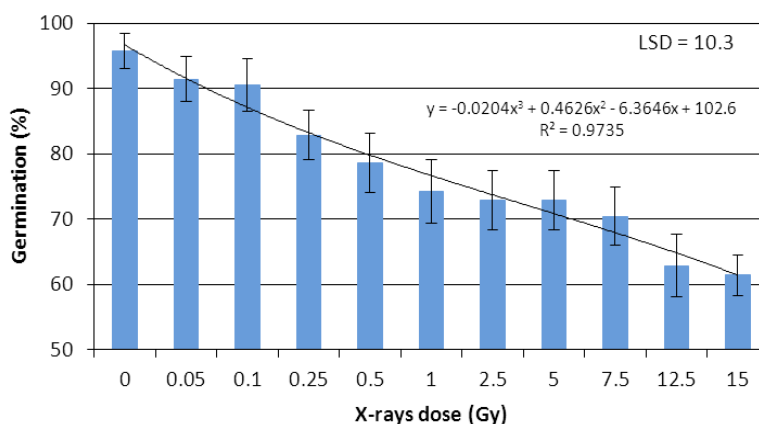

Figure 1. Effect of seed X-rays irradiation on the germination percentage of date palm seeds.

Table 2. Analysis of variance for the roots length of date palm plant under the influence of different levels of X-rays after 1, 2 and 3 weeks; P values less than 0.05 are significant.

Factor	df	MS	F	p-value
Accumulative root length growth increase after 1 week				
X-Ray	10	13.658	70.579	0.0001
Error	66	0.194		
Accumulative root length growth increase after 2 weeks				
X-Ray	10	16.218	16.885	0.0001
Error	66	0.961		
Accumulative root length growth increase after 3 weeks				
X-Ray	10	32.393	18.124	0.0001
Error	66	1.787		

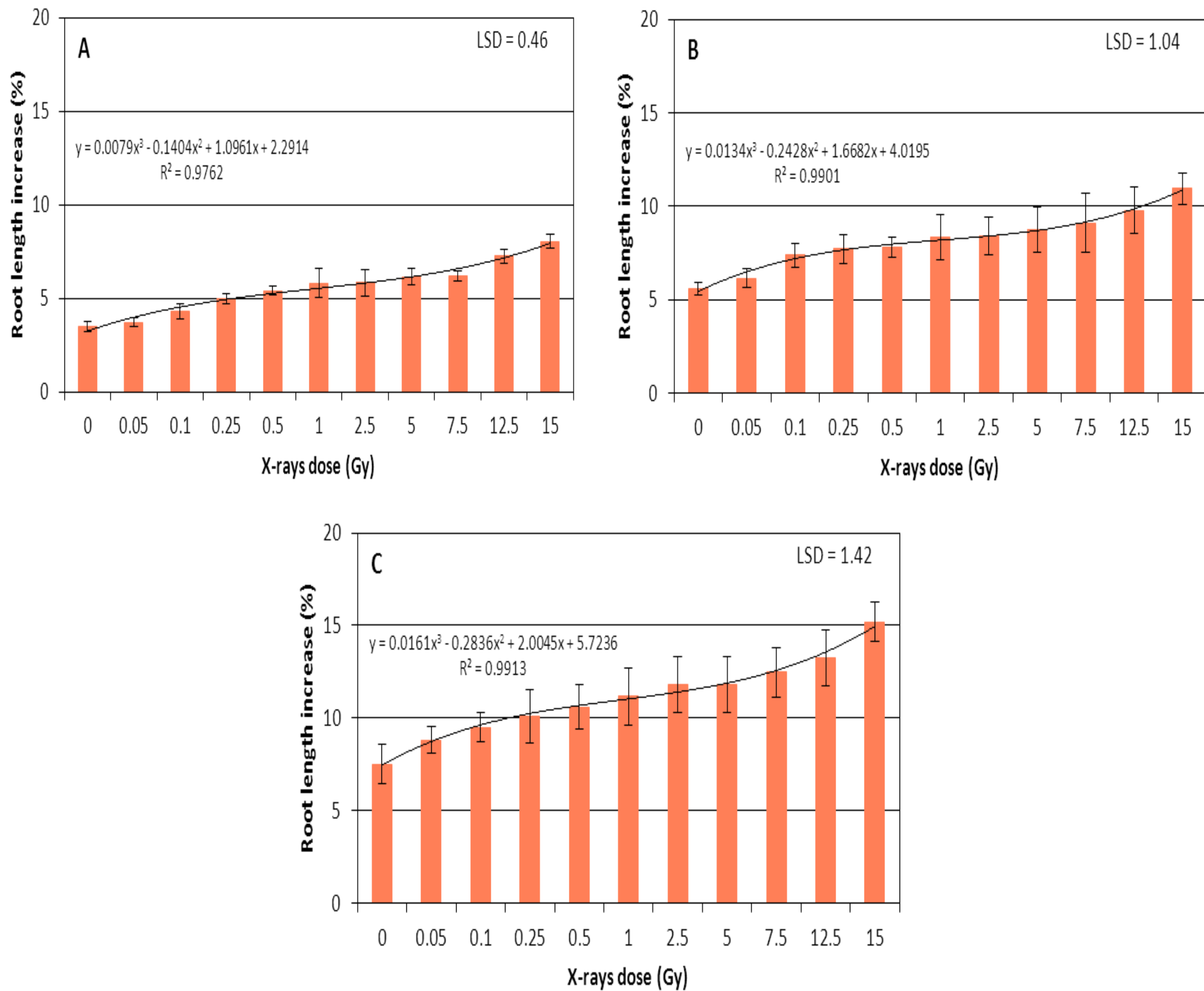

Figure 2. Accumulative growth percent increase (AGPI) of roots of date palm seedlings under the influence of different levels of X-rays measured after 1 week (A), 2 weeks (B), and 3 weeks (C).Table 3: Analysis of variance for leaves

growth of date palm under the influence of different levels of X-rays after 1, 2 and 3 weeks; P values less than 0.05 are significant.

Factors	df	MS	F	p-value
Accumulative leaf length growth increase after 1 week				
X-Ray	10	138.419	104.447	0.001
Error	66	1.325		
Accumulative leaf length growth increase after 2 weeks				
X-Ray	10	159.027	155.158	0.001
Error	66	1.025		
Accumulative leaf length growth increase after 3 weeks				
X-Ray	10	148.18	126.121	0.001
Error	66	1.175		

The analysis of variance for the cumulative roots growth during the 4 weeks indicates a significant increase under the influence of X-rays in one direction, a level of exposure at level of 5% (Table 2). Root growth increased significantly when exposed to different doses of X-rays, and this appears in the first week after exposure and the mean length of roots ranged between 3.7% at the dose of 0.05 Gy to 8.05% at 15 Gy (Figure 2). The significant increase began at 0.1 Gy of X-rays exposures. On the other hand, the high doses of 2.5-15 Gy of X-rays exposures showed apparent differences in the root length among them, with the exception of the doses ranging from 5-7.5 Gy compared to doses of less than 0.05-5 Gy which showed significant statistical differences. Similarly in the second week of exposure, the significant increase in root length began at dose 0.1 Gy and root growth reached the maximum value at 15 Gy (10.94%), while root growth reached 15.19% in the third week at the same dose. In chickpea, 15 Gy of gamma rays induced a significant improvement in root length, nearly 20% more than the control (Melki and Sallami, 2008). Similarly, Melki and Marouani (2009) obtained improvements of 18% to 32% in root number and root length of wheat (*Triticum durum* Desf.) seeds irradiated with 20-Gy gamma dose and concluded that the improvement of root growth might be useful in case of drought.

The results of analysis of variance ANOVA also showed the one-way significant interaction which had an effect on the proportion of the cumulative leaf growth of date palm seedlings exposed to X-ray at 5% level of significant (Table 3). One week after exposure to X-rays, leaf growth of seedlings was enhanced at 0.05 Gy reaching 24.56% more than the control. However, the maximum leaf length occurred at a dose of 0.25 Gy reaching a 30.7% increase as compared to the control. In contrast, at doses ranging from 0.5-15 Gy, significant decreases in leaf length was observed (Figure 3). After 2 weeks of exposure to X-ray radiation, total leaf length under the influence of doses of 0.05-0.5 Gy significantly increased as compared to the control experiment, while the average leaf length showed a significant reduction at doses of 2.5-15 Gy compared to experience control. After 3 weeks, a similar response was observed which clearly showed a significant increase of leaf length at 0.05-0.5 Gy as compared to the control. Whereas, a significant reduction in the leaf length occurred at 5-15 Gy. Although the X-rays led to a significant increase in leaf growth, this increase was only at the low doses, reaching maximum at 0.25 Gy, while high doses led to a significant decrease in the leaf growth. These results are consistent with previous studies which revealed increase leaf growth at low doses of X-rays exposures in other plants such as cotton (Younis et al., 1962) and okra, *Abelmoschus esculentus* (Rao and Rao, 1978). In a related study, Dhakshanamoorthy et al. (2010) found that a stimulatory effect on shoot length, seedling length and vigor index, plant height, petiole length and yield characters in Barbados nut, *Jatropha curcas* plants when seeds were exposed to 5 Kr of gamma radiation while at 25 Kr an inhibitory effect was observed.

Overall, date palm seed germination and root growth were significantly reduced with increasing doses of X-rays radiation; however, leaf growth was enhanced to an optimum level at 0.25 Gy, beyond which growth decreased. In a previous report, a significant increase in DNA content and ion content in date palm seedlings with as low dose as 0.05 Gy was noted, reaching the highest level at 0.25 Gy. This was accompanied by significant reductions of

photosynthetic pigments content (chlorophyll a and carotenoids) starting at 0.05 Gy and chlorophyll b reduction at 0.1 Gy (Al-Enezi and Al-Khayri, 2012a). In another study, a direct relationship between the X-rays dose and proline accumulation along with increased fresh weight and water content were observed in irradiated date palm seedlings (Al-Enezi and Al-Khayri, 2012b).

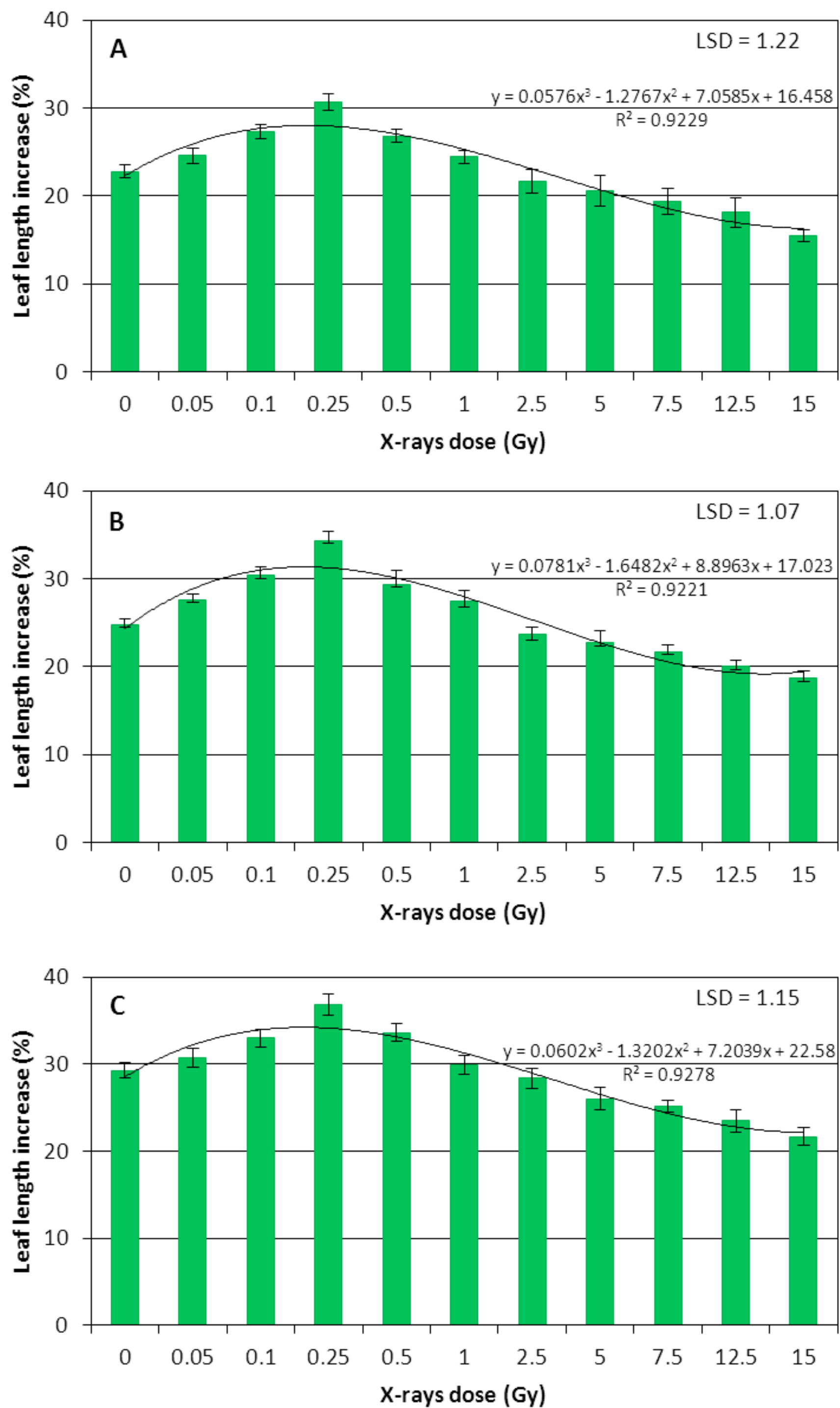

Figure 3. Accumulative growth percent increase (AGPI) of leaves of date palm seedlings under the influence of different levels of X-rays measured after 1 week (A), 2 weeks (B), and 3 weeks (C).

The observed modifications of growth in response to irradiation may be due to changes in membrane permeability, transpiration and the opening of stomata (Roy, 1974). Ionizing radiations can also cause changes in the activity of endogenous plant growth regulators and may in turn influence growth (Maherchandani, 1975). Changes of membrane permeability may reduce the absorption of some nutrients, especially calcium, which plays a major role in various growth and development processes (Sanders et al., 2002; Sze et al., 2000). Reduction in calcium leads to the appearance of yellowed leaves, reduced plant growth, breakdown of cell walls, and an increase of plant sensitivity to pathogens (Medveddev, 2005; White and Broadley, 2003). Ion content increased in response to low doses of X-rays in date palm seedlings; however, doses above 0.25 Gy caused reduction of calcium ions as well as sodium, potassium, phosphorus and magnesium ions in leaf tissue (Al-Enezi and Al-Khayri, 2012a). The current study has shown that treatment with doses above 0.25 Gy also inflected reduction in leaf growth.

In conclusion, the current study has shown that X-rays radiation exerts an inhibitory influence on germination of date palm seeds. Root growth was stimulated in response to increasing doses; however, leaf growth reached a peak at 0.25 Gy, above which growth started to decline. This suggests that root growth is more sensitive to irradiation than leaf growth. Information gained from this study may facilitate the development of strategies for the utilization of X-rays in date palm mutagenesis and physiological studies. Because of genetic variability controlling the sensitivity to irradiation, future studies with various cultivars are essential to further enhance our understanding of date palm responses to irradiation stress. Moreover, investigations involving higher doses are necessary to determine the lethal dose and the induction of mutagenesis to assist genetic improvement of date palm.

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REGULAR ARTICLE

A new concept for production and scaling up of bioactive compounds from Egyptian date palm (Zaghlool) cultivar using bioreactor

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Abstract

A promising and successful protocol for enhancement and production of total phenolic and peroxides compounds from Egyptian date palm cultivar Zaghlool cells in stirred tank reactor was established. The influence of cell culture cultivation in combination with *Aspergillus niger* extract, and methyl-jasmonate elicitors incorporation in the feeding medium on cell growth patterns and production of active compounds was investigated. The maximum value of cell growth parameters and the highest content of bioactive compounds were obtained from elicitation of modified MS-medium with *Aspergillus niger* extract at 0.1% in combination with methyl-jasmonate (100 µM), as compared with other concentrations after 10 days of cultivation. The chemical analyses of phenolic and peroxidase compounds were spectrophotometrically performed.

Key words: Date palm, Elicitors, Phenolic and Peroxidase compounds, Bioreactor

Introduction

Date palm (*Phoenix dactylifera* L.) is a dioecious fruit tree native to the hot arid regions of the world, mainly grown in the Middle East and North Africa. Since ancient time this majestic plant has been recognized as the *tree of life* because of its integration into human settlement, wellbeing, and food security in hot and barren parts of the world, where only a few plant species can flourish (Al-Khayri, 2007). Date palm trees provide the most sustainable agro-ecosystems in harsh dry environments, providing raw materials for housing, furnishings, and many handcrafts. In addition the palm supplies nutritious delicious fruits that can be consumed fresh, dried, or processed, and are a source of sugars, minerals, and vitamins. Economically, date palm provides a major source of income for local farmers and associated industries in communities where it is grown. Biotechnology is a set of rapidly emerging and far-reaching new technologies with great promise in areas of sustainable food production, nutrition security, health care and environmental sustainability. The objective of biotechnology is to use its tools to help convert a country's diverse biological resources into useful products and processes that are accessible to its people for economic development and employment generation (Jain et al., 2011). Bioreactors have several advantages for the mass cultivation of plant cells: 1) better control for scale-up of cell suspension cultures under defined parameters for the production of bioactive compounds; 2) constant regulation of conditions at various stages of bioreactor operation; 3) easy and efficient handling of culture such as inoculation or harvest; 4) enhanced nutrient uptake by submerged culture conditions which stimulate the multiplication rate and a higher yield of bioactive compound and 5) large numbers of plantlets are easily produced and can be scaled-up (Fulzele and Heble, 1994; Othmani et al., 2011).

Phenolics are intermediates of phenylpropanoid metabolism (Cvikrová et al., 1996) and precursors of lignin (Lewis and Yamamoto, 1990) and phenylpropanoid phytoalexins (Kessmann et al., 1990). Their deposition in cell walls is an important defense-mechanism response to pathogen infection (Bolwell, et al., 1985). Plant cells cultivated *in vitro* synthesize phenolic compounds; however, in some cases changes in the quality and quantity of the substances are recommended (Zagoskina and Zamprometov, 1983). This is probably due to the specificity of tissue culture as an artificial biological system in which the basic function of phenols is to interfere with cell proliferation (Ozyigit et al., 2007). Longenbeck (1983) reported that a substitution pattern of phenols was affected

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according to the rate of IAA degradation. Also, he reported that phenols were found to react with hydrogen peroxide produced during IAA degradation, thereby protecting cellular constituents from their toxic effect.

Peroxidase is an enzyme known to play a very crucial role in scavenging free radicals within plant systems (Regalado et al., 2004) in addition to their involvement in various metabolic activities. Outside plant systems, this enzyme has several commercial applications, the major one being its use as an important component in chemical diagnostics and laboratory experiments (Ayala, 2000). A wide range of chemicals can be modified using peroxidase and hence it has various applications in waste water treatment to remove phenolics, and in the synthesis of various aromatic compounds. On the other hand**,** Booij et al. (1993) reported that the changes in soluble peroxidase correlated well with budding, and that the modification of peroxidase activities and expression of iso-peroxidase always preceded the morphological appearance of buds. Thus, evaluation and determination of peroxidase during subculture can lead to a better understanding of the physiological processes, as well as establish optimum conditions for the culture of date palm.

Elicitors are molecules that stimulate defense or stress–induced responses in plants (Radman et al., 2003). However, the broader definition of elicitors includes both substances of pathogenic origin and compounds released from plants by the action of pathogens (endogenous elicitors). Natural elicitors can be divided into two types: biotic and abiotic. The biotic elicitors have a biological origin, derived from the pathogen or from the plant itself, while abiotic elicitors are not of biological origin and are the result of physical factors and chemical compounds (Kumar and Shekhawat, 2009).

Zhao et al. (2000) reported that using combined biotic elicitor treatment of *Aspergillus niger* mycelium and tetramethyl ammonium bromide with *Catharanthus roseus*, cell cultures enhanced the accumulation of ajmalicine content, as compared with the control medium.

On other hand**,** Fritz et al. (2010) reported that jasmonic acid (JA) and methyl-jasmonate (MJ), are plant hormones involved in chemical and physiological defense responses. Moreover**,** Balbi and Devoto (2008) stated that JA and MJ are oxylipins (oxygenated fatty acids) that originate from linolenic acid released from chloroplast membranes by lipase enzymes and subsequently oxygenated by lipoxygenases (LOXs) to hydroperoxide derivatives. Elicitation or stress stimulus leads to a rapid release of α-linolenic acid from the lipid pool of the plant cell through which an intracellular signal cascade elicits secondary metabolite production important for plant defense (Memelink et al., 2001). α-Linolenic acid is converted by a lipoxygenase, an allene oxide synthase (AOS) and an allene oxide cyclase (AOC) into the intermediate 12-oxo-phytodienoic acid. This compound is converted into JA through the action of a reeducate and three rounds of β-oxidation (Mueller, 1997; Menke et al., 1999).

The main objective of this investigation focused on the effect of different concentrations among of *Aspergillus niger* (AN) and MJ as biotic elicitors on primordial leaf cell growth parameters; and to determine the accumulation rate of phenolic and peroxidase compounds in suspension cultures of Egyptian date palm (Zaghlool) cultivar using a bioreactor.

Material and Methods

Plant materials

Female date palm (*Phoenix dactylifera* L.) offshoots of Egyptian date palm cultivar (Zaghlool) were obtained from Rashed in North Egypt; the offshoots were separated during the fruiting stage from the mother plants. The measurements and parameters of the offshoots were 120-150 cm in height, 35 cm in diameter and 45-50 kg. in weight. These offshoots were used as mother plant materials for initiation of *in vitro* cultures.

Sterilization

Sterilization of the obtained shoot tip was carried out according the method described by Taha et al. (2010).

Nutrient media and callus production

Explants of sterilized primary basal leaves excised from the base of shoot tips were cultured on solidified Murashige and Skoog (1962) nutrient medium (MS) supplemented with 1.7 g/l phytagel and 30 g/l sucrose. The MS-nutrient medium was fortified by 170 mg/l NaH_2Po_4 $2H_2O$ + 200 mg/l H_2PO_4 + 1 mg/l thiamine HCI and 3 g/l activated charcoal and augmented with 3 mg/l 2,4-D + 3 mg/l 2iP and 5 mg/l BA. This is the best medium for callus production according to Taha et al. (2010). The pH of the culture medium was adjusted to 5.7 with 0.1 M NaOH or 0.1 M HCl before adding phytagel. The culture medium was dispensed into 150 ml jars, each containing 40 ml and autoclaved at 121°C ± 1 for 20 min. Cultures were incubated in darkness in a growth chamber at a constant temperature of 28°C ± 1 then incubated under light condition (2000 Lux) from cool white

fluorescent lamps, and subcultured every 6 weeks on fresh new medium. After three subcultures, white calli were initiated and observed.

Cell production

Calli from the previous experiment were retained and re-suspended in an agitated liquid MS medium containing 1 mg/l 2,4-D + 1 mg/l 2iP + 3mg/l NAA according to the best results obtained by Taha et al. (2010).

Establishment of bioreactor experiments

A 2-L turbine stirred tank bioreactor (STB) of the National Research Centre (NRC) was used with a working volume of 1.5-1.7 L (B. Braun, Biotech, International, Germany). The culture was aerated through a stainless steel sparger. The flow rate was set up according to the type of experiment and maintained at the normal level with a mass flow control system until the end of the culture period. Two six-bladed turbine impellers (D=45 mm) were used for mixing at a rotation speed of 120 rpm. The temperature was maintained at 26°C with a thermostatic outlet spongy sheet surrounding the vessel. Aeration was performed by filtered sterile air at the rate of 0.5 l/min. Dissolved oxygen concentrations were measured with a sterilizable oxygen electrode (Ingold). Dissolved oxygen concentration was monitored with a sterilizable pO2 electrode to maintain different levels of dissolved oxygen concentrations in the bioreactor broth, with the inlet air dosed by a mass flow controller connected with software and pO2 electrode. The bioreactor was inoculated with one part of suspension culture and five parts of medium, and the cell cultures were kept at 25°C. The MS-nutrient medium containing cell lines were introduced into a glass tank bioreactor under sterilized air condition. The parameters affecting either mass cell culture and/or enhancement of phenolic and peroxides compounds in lyophilized suspension culture of Zaghlool date palm cultivar were investigated, as follows:

- Effect of controlled pH medium at the degree of 5.7 using either 0.2 N NaOH or 0.2 N HCl with ADI 1030 Bio-controllers (Applikon) equipped with a sterilizable pH–electrode (Ingold) and peristaltic pumps for alkali and acid addition.
- Effect of uncontrolled of pH medium.
- Each experiment was done for 2 weeks after inoculation. At the end of 15 days of inoculation, the cells obtained were harvested and chemically analyzed for an accumulation of phenolic and peroxidease compounds. The percentage of these target compounds were recorded as the control treatment.
- Effect of two type of biotic elicitors at different concentrations on enhancement of cell growth parameters, phenolic and peroxidase compounds production.
- Effect of *Aspergillus niger* extract: An extract of the fungus, *A. nige*r was obtained from The Department of Plant Pathology of the National Research Centre. Preparation of the fungus elicitor was carried out according to the method described by Taha (2002). In this experiment, 0, 0.1, 0.2 and 0.3% of suspended *A. niger* extract (P.C.V) were added to the culture media.
- Effect of methyl-jasmonate**.** In this experiment, 0, 50, 100 and 200 µM of methy-jasmonate were used.

Measurement of cell growth parameters

The fresh weight and dry weight (w/v) were determined using a sampling unit of suspension culture (2 ml) every 2 days for 2 weeks.

Total phenolics

Extraction and determination of total phenolics in different date palm cell lines were determined using Folin–Ciocalteau reagents (Singleton and Rossi, 1965). Date palm cell line extracts of (40 µl) or gallic acid standard were mixed with 1.8 ml of Folin–Ciocalteu reagent (prediluted 10-fold with distilled water) and maintained at room temperature for 5 min, then 1.2 ml of sodium bicarbonate (7.5%) was added to the mixture. After 1 hour at room temperature, the absorbance was measured at 765 nm. Results were expressed as ng gallic acid equivalents (GAE/g-dW) sample (Shui and Leong, 2006).

Peroxides

The peroxides activity in lyophilized cell cultures (cell and liquid medium) was monitored for total peroxides according to the method described by Wititsuwannakul et al. (1997).

Data analysis

All experiments were designed in a completely randomized design. The results obtained were statistically analyzed using standard error (SE) according to the method described by Snedecor and Cochran (1980).

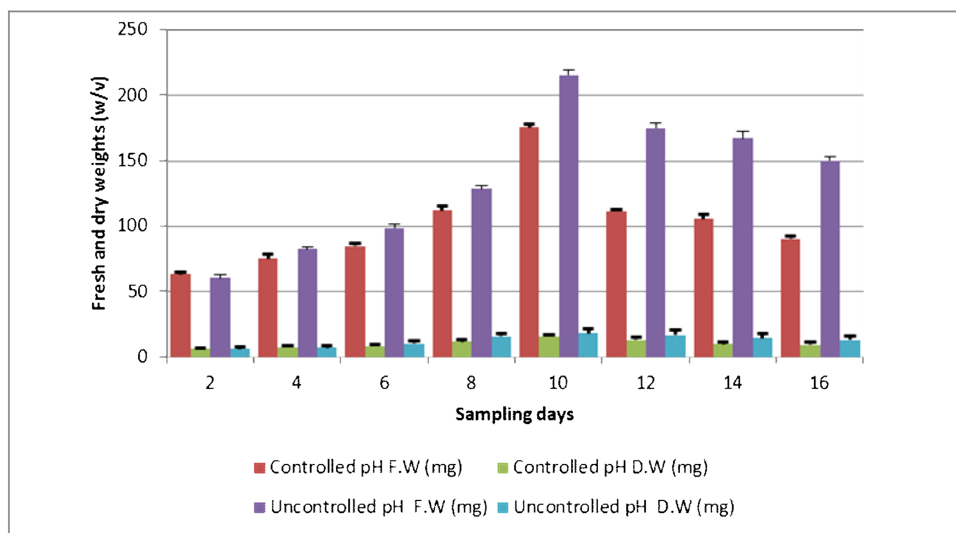

Figure 1. Effect of controlled and uncontrolled pH MS agitated liquid medium containing 1 mg/l 2,4-D + 1 mg/l 2iP + 3 mg/l NAA on cell growth parameters (fresh and dry weights) of Zaghlool date palm cultures, cultured in STB and incubated under 16/8 of daylight condition at 26±1ºC.

Results

Bioreactor experiments: Effect of different conditions on date palm cell growth parameters

1-Effect of controlled and uncontrolled pH medium

The effects of controlled and uncontrolled pH MS-liquid medium containing 1 mg/l 2-4-D, 1 mg/l 2iP and 3 mg/l NAA on Zaghlool cell growth parameters (fresh and dry weights) are presented in Figure 1. The scheduled time of measurement of different cell growth parameters i.e., fresh and dry weights was at intervals of 2 days for 16 days of culturing period. Culturing of date palm cells was carried out in 2-L turbine stirred tank bioreactor (STB) with a working volume of 1.5-1.7 liters. The highest values of fresh weight for controlled pH MS-medium 175.6 and 112.3 (w/v) were recorded in days 10 and 8, respectively. However those recorded 215.8 and 175.3 (w/v) in days 10 and 12, respectively, for uncontrolled pH MS-medium.

Regarding dry weights, the highest values recorded of 18.37 and 15.86 (w/v) were from uncontrolled and controlled pH of MS media, respectively.

Effect of *Aspergillus niger* extract

The effect of elicitation of uncontrolled MS medium with different concentrations of AN as a biotic elicitor on the optimization of different conditions affecting maximization of date palm cell growth parameters was investigated. Filtered and sterilized mycelium of AN as the biotic elector at different concentrations of 0, 0.1, 0.2, 0.3% was used. Sterilized filtrate of AN was used for enhancement of both cell growth parameters as well as the accumulation rate of total phenolic and peroxidase compounds in a suspension culture of Zaghlool date palm. Data presented in Table 1 clearly show that the highest value of fresh (245.4; 235.12; 223.17 and 197.8 w/v) and dry (24.12; 22.17; 20.15 and 18.2 w/v) weights were recorded with 0.1, 0.2, 0.0 and 0.3% of AN, respectively. The best results of fresh and dry weights were recorded at day 10 of cultivation, as compared with other schedule times. Furthermore, the optimum concentration of AN for achievement of different cell growth parameters was 0.1% compared with other concentrations.

Effect of methyl-jasmonate

MJ at different concentrations i.e., 0.50, 10 and 200 µM were used for scaling-up and production of mass cell cultures, as well as attainment of active compounds in suspension culture of Zaghlool date palm cultivar. Furthermore, MJ at different concentrations was incorporated with uncontrolled MS-medium containing 0.1% AN as shown in Table 2. The highest values of fresh (364.12; 331.45; 275.5; 249.75) and dry weights (39.28; 35.37; 31.05; 28.15) were recorded within fortified modified MS medium with 0, 50, 100 and 200 µM of MJ, for fresh and dry weights of cell cultures, respectively. In general these experiments clearly indicated that supplementation of uncontrolled MS liquid medium with 0.1% of AN and 100 µM of MJ enhanced mass cell production from Zaghlool

date palm cultivar using STB (Fig. 2) for 10 days, as compared with other concentrations and cultivation times.

Bioactive compounds determination

The total phenolic ng GAE/g dw and peroxidease (u/mg) compounds of lyophilized Zaghlool date palm cell cultures were determined in the previous experiments. Illustrated data in Figure 3 clearly show the effect of modified liquid MS-medium with either controlled or uncontrolled pH media or elicitation of culture medium with 0.1, 0.2 or 0.3% of AN or with 50, 100 or 200 µM of MJ, as biotic electors on attainment of total phenolic and peroxides compounds production in different cell lines of Zaghlool date palm cultivar. The highest values of total phenolic (25.35 ng GAE/g dw) and peroxides (5432 u/g) compounds were recorded with uncontrolled pH of MS-medium augmented with 0.1% of AN and 100 µM of MJ, compared to other supplementation and concentrations.

This study clearly indicates that combining different elicitors derived from the secondary metabolites process, especially total phenolic and peroxides compounds in date palm cell cultures, along with AN and MJ, play a critical role in elicitation of date palm cell cultures. Moreover, scaling-up and production of mass cell cultures and active compounds from date palm cell cultures using a serried tank bioreactor 2L was established.

Discussion

The scaling-up of mass cell cultures and maximization of bioactive products through bioreactors must be followed up. Bioreactors have two advantages over flasks for culturing plant cells. First, better control can be exerted on the system (i.e., pH and dissolved gas concentrations). Second, most bioreactors are scalable and hence better able to reproduce on a larger scale those conditions which were observed on a smaller scale to be the most desirable for culture performance. With the aim of implementing an industrial scale process, the behavior of cell culture in bioreactors has been receiving significant investigative attention (Zhong, 2001; Huang et al. 2002). However, understanding how to improve cell cultures through rational modification of the reactor environment remains a challenge.

One of the methods frequently used to increase the productivity of plant cell culture is use of so-called elicitors (Singh, 1996). Elicitors can be any type of compound that improves the production of phytoalexins (Muller, 1956; Kue, 1972). Phytoalexins are antibiotically active compounds, used by plants to resist microbial attacks (Darvill and Albersheim, 1984). Many secondary metabolites belong to the group of phytoalexins; therefore, if the correct elicitor can be found, it is possible to enhance the production of the desired secondary metabolite (Eilert, 1987). In addition, Wijnsma et al. (1985) reported that, the anthraquinons in *Cinchona ledgeriana* cell cultures were increased when the cells were treated with 0.5 mg/ml of AN as elicitor. JA activates stress response in cells in two ways: (1) JA produced at the wound site, serves as a mobile signal to activate responses in systemic tissues; (2) wound-induced production of a mobile signal other than JA that activates synthesis of the hormone in systemic tissues (Abraham and Howe, 2009). It can be concluded that setup of Zaghlool date palm cell culture in STB for 10 days in MS-liquid modified with uncontrolled pH medium achieved a maximum of different cell growth parameters compared them with controlled pH medium.

Table 1. Effect of elicitation of uncontrolled pH liquid MS-medium with different concentrations of Aspergillus niger extract on enhancement of fresh and dry weights of Zaghlool date palm suspension cultures. Cultures were carried out using serried tank bioreactor 2L for 2 weeks at 26 ºC under 16/8 daylight condition.

Days duration	MS medium* supplemented with different concentrations of Aspergillus niger (%).							
	0		0.1		0.2		0.3	
	FW	DW	FW	DW	FW	DW	FW	DW
0	61.84±3.15	8.66±1.52	95.38±3.25	9.16±1.69	64.25±2.95	8.74±1.53	59.4±2.63	8.42±1.42
5	100.5±5.22	10.82±1.79	125.63±5.93	12.63±2.48	119.6±4.87	17.3±2.23	107.6±3.52	11.2±2.15
10	223.17±7.5	20.15±2.24	245.43±8.25	24.12±3.54	235.12±7.96	22.17±2.56	197.8±5.18	18.2±2.36
15	185.3±6.13	17.42±2.15	215.37±7.61	21.7±3.15	205.14±6.85	19.63±3.15	191.6±6.45	15.87±2.05

*The pH of MS- medium was set up as uncontrolled. Each treatment was the average of 3 replicates ± Standard Error FW: Fresh weight (w/v); DW: Dry weight (w/v).

Table 2. Effect of incorporated of uncontrolled pH liquid MS-medium containing 0.1% of Aspergillus niger extract with different concentrations of methyl-jasmonate on enhancement of fresh and dry weight of Zaghlool date palm suspension cultures. Cultures was carried out using serried tank bioreactor 2L for 2 weeks at 26ºC.

Days duration	MS medium* supplemented with 0.1% of Aspergillus niger (%) and different concentrations of Methyl-Jasmonate (µM).							
	0		50		100		200	
	FW	DW	FW	DW	FW	DW	FW	DW
0	66.25±3.42	8.73±1.55	65.18±3.53	8.07±1.15	64.35±2.95	8.12±1.76	65.25±2.25	7.95±1.25
5	100.63±5.43	11.32±1.93	112.13±4.65	13.25±2.15	135.12±5.25	15.17±2.09	125.15±3.98	15.33±2.95
10	249.75±7.95	28.15±3.05	275.25±6.33	31.05±3.17	364.12±8.14	39.28±4.56	331.1±5.22	35.37±2.85
15	235.13±6.25	25.45±2.89	264.85±5.89	28.19±3.85	325.13±8.09	35.62±3.97	305.52±4.87	32.49±2.97

Each treatment was the average of 3 replicates ± Standard Error. *The pH of MS- medium was setup as uncontrolled. FW: Fresh weight (w/v); DW: Dry weight (w/v).

Relative to the results we obtained, Zabetakisa et al. (1999) mentioned that elicitation through the use of MJ increased the tropane alkaloid from *Datura stramonium* as compared with a fungal elicitor and oligoalacturonide. Also, Taha (2003) established an efficient protocol for enhancement of total alkaloids production from suspension cultures of *Atropa belladonna* using various concentrations of AN extract. He reported that the optimum augmentation of liquid MS-medium was 1 mg/l of NAA and BA and extract of AN at a concentration of 10% (~ 0.5 mg/ ml), gave the highest value for cell growth and total alkaloid accumulation in the different type of cell cultures after 10 days of cultivation.

In general, the results obtained may be due to enhancement, achievement and production of total phenolic and peroxides from cell cultures of Zaghlool date palm cultivar using advanced techniques of scaling up through bioreactors such as STB.

The highest values of total phenolic compound accumulation (25.37 ng GAE/g dw) of lyophilized date palm cell cultures was recorded with elicitation of uncontrolled MS medium with 0.1% of AN and 100 µM. The results obtained are in agreement with those of Taha et al. (2011) who reported that the maximum value of cell growth parameters and highest content of inulinase activity (0.395 u/ml) resulted from elicitation of augmented MS-medium with AN extract at the level of 0.2% in combination with MJ (150 µM), as compared with other concentrations after 2 weeks of cultivation. In addition the results obtained agree with those obtained by Del Río et al. (2003) who reported that the highest phenol levels were detected after 120 days in leaves, stems and roots (in that order) of *Olea europaea*. This is because leaves are the principal producers of phenolic compounds, the pathway of shikimic acid, which acts as the precursor of phenolic compounds, beginning in their photosynthetic cells. Several studies in different plant species have shown that various phenolic compounds are synthesized and accumulate in different leaf tissue (Del Río et al., 2000; Botía et al. 2001). The present study revealed that the phenolic content increased quantitatively with the increase in age of suspension to week 3 of cultivation may be due to the hyperactivity of oxidative enzymes (Cochrane, 1994). An increase in production of phenolic compounds has been associated with a decrease in growth and a decline in protein synthesis.

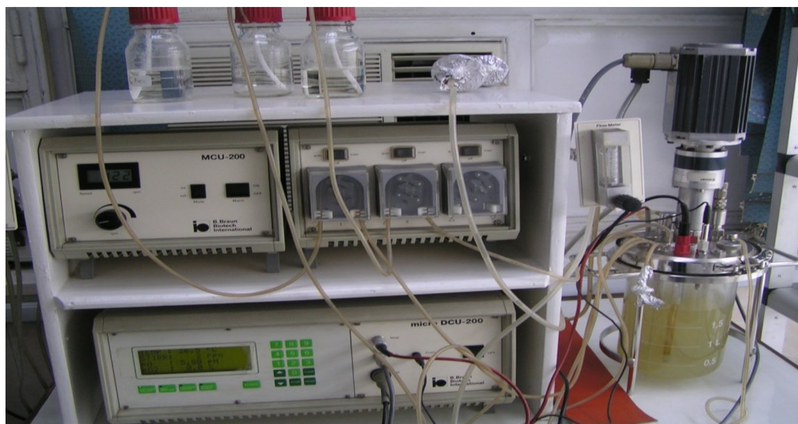

Figure 2. B-Braun Biotechnology International stirred tank bioreactor (2L).

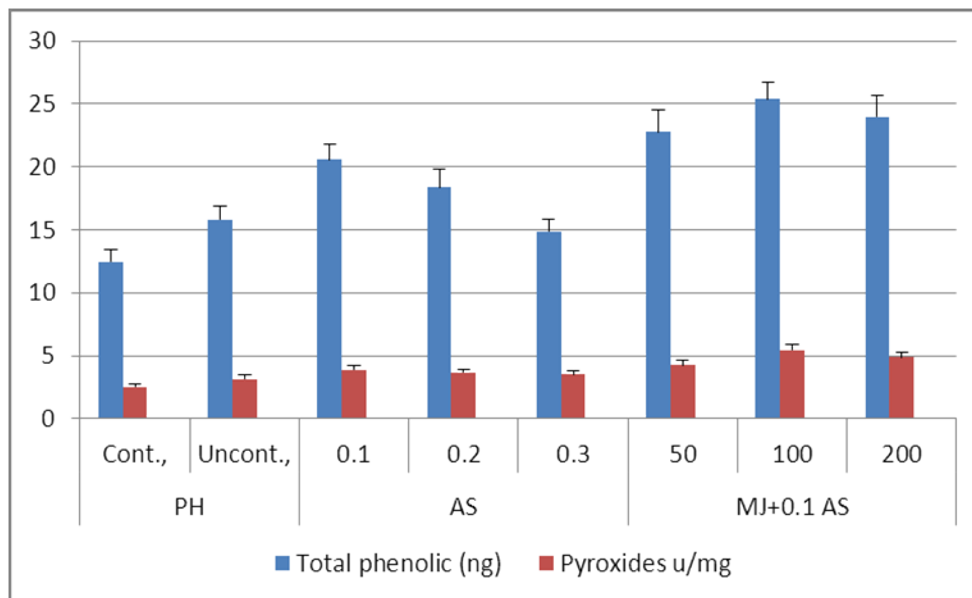

Figure 3. Effect of controlled and uncontrolled pH of MS-medium , or elicitation of uncontrolled pH liquid MS-medium with either Aspergillus niger extract (As %) or methyl-jasmonate (µM) incorporated with 0.1% of AS on accumulation of total phenolic (ng GAE/g dw) and peroxides (u/mg) of date palm lyophilized cell cultures. Cultures were carried out using STB 2L for 2 weeks at 26ºC and incubated under 16/8 of daylight condition.

Regarding, plant peroxidases, Obinger et al. (1996) reported that phenol oxidizing enzymes are widely used as markers in the plant kingdom, because of their high polymorphism. In agreement with our results, Azeqour et al. (2002) mentioned that date palm leaves contain highly active peroxidases. However since plant peroxidases are involved in many functions such as growth, vegetative development, resistance to biotic and abiotic stresses (Gonzalez-Verdejo et al., 2006; Mc Innis, 2006), the exact role of these enzymes is not yet understood in date palm. Plant cell and organ cultures grown *in vitro* usually exhibit changes in physiological and biochemical responses upon exposure to biotic and abiotic elicitors (Sircar and Mitra, 2008). Elicitation is a process of induced or enhanced synthesis of secondary metabolites by the plant cells to ensure their survival, persistence and competitiveness.

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REGULAR ARTICLE

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

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Abstract

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

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

Introduction

The date palm (*Phoenix dactylifera* L.) is a staple food crop with high nutritional value and yield and can be cultivated under unfavorable soil and water conditions. Most of date palm cultivation is in North Africa, the Middle East and similar regions in the world (Botes and Zaid, 2002). It is traditionally propagated by offshoots which are limited in number, particularly in some elite cultivars which restricts agricultural expansion and land reclamation. To overcome this problem, tissue culture techniques have been utilized for mass propagation of elite date palm cultivars. Tissue culture of the date palm was initiated four decades ago and depended mainly on offshoot tip explants (Reuveni et al., 1972). Explants derived from offshoots and use of a high-auxin medium caused many technical problems such as endogenous bacterial contamination, browning, somaclonal variation and long-term duration of production. Micropropagation by shoot tips of offshoots takes about 3 years, if the protocol is well-known (Abul-Soad et al., 2004a). A viable alternative explant is needed to overcome the above mentioned problems. Most of the commercial laboratories worldwide are using somatic embryogenesis from shoot tip explants (Tisserat, 1979; Al-Khayri, 2001; Abul-Soad et al., 2002b; Abul-Soad, 2003).

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

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

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

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

Material and Methods

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

Plant materials

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

Surface sterilization

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

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

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

zThe explants cultured onto 100 mg l-1 2,4-D for 6 weeks then transferred onto 10 mg l-1 2,4-D up to 24 weeks. yThe minimum and maximum spikelets length within a single inflorescence.

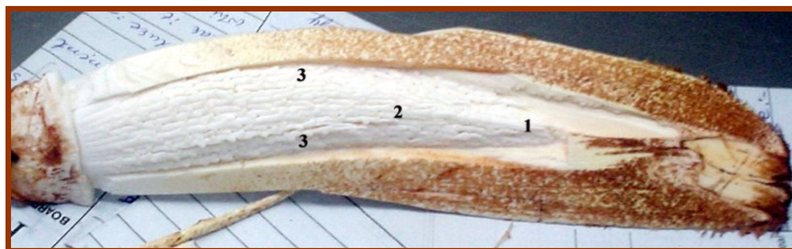

Figure 1. Initial spikelet explants of Kashoo-Wari cv. female inflorescence of a pyramidal structure, No. 1 is the longest spikelets (50 mm), No. 2 is the middle (45 mm) and No. 3 is the shortest (40 mm).

Media composition and treatments

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

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

Incubation conditions

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

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

Results and Discussion

The explant length

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

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

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

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

Spikelet lengths differed due to certain other factors such as the location of the immature spathe on the palm crown, which was constant for all four trees of Aseel cv. used in this study. In the case of Dedhi-2 cv. and Dedhi-3 cv. which were excised on the same day (6 Feb.), the location from which the spathe was excised was different. Consequently the inflorescence length differed and measuring 22-26 mm and 19-21 mm, respectively. This result confirms the significance of location in the palm crown when excising immature spathes. Spathes always emerge in three groups appearing one after the other. The earliest set of spathes emerge during the middle of the flowering season, followed by an outer set and finally the upper set near the central apical meristem (heart) of the date palm tree (Abul-Soad, 2003; Abul-Soad et al., 2004b).

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

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

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

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

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

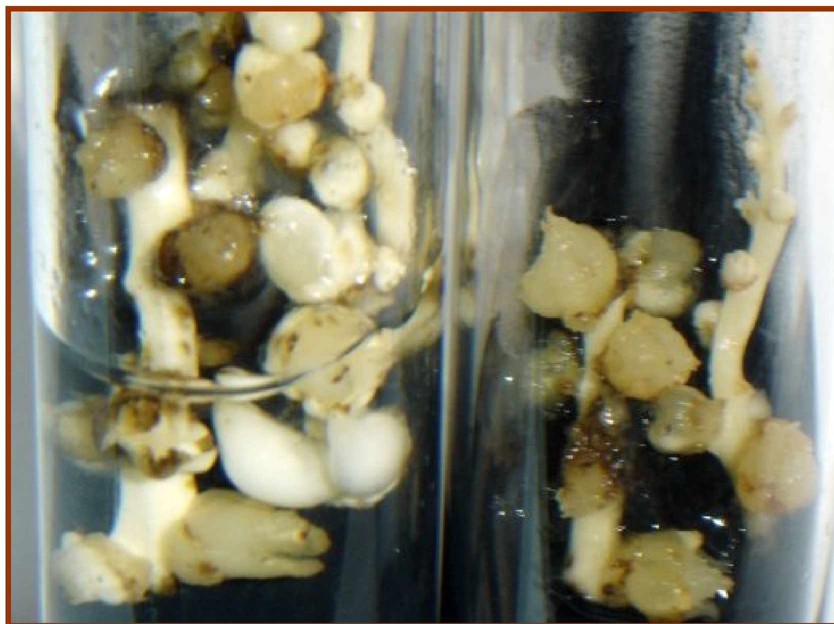

Figure 2. The vitrified and swollen florets on spikelet explants on the high-auxin medium (100 mg l^{-1} 2,4-D) after 24 weeks.

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

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

The phenomenon of burned terminal parts of spikelet explants was recorded for the majority of the cvs. studied after 6 weeks in culture (Figure 3). This effect could be the result of quick degradation of the tissues on unsuitable medium composition and/or depletion of endogenous hormones within the explant by the larger florets located in the middle of spikelet explant. It has been reported that some of florets of the intact spikelet explants react to the medium formula used, but not for all the florets; it occurs mostly with those found in the middle (Abul-Soad et al., 2004b; Abul-Soad, 2011). Using 100 mg l^{-1} 2,4-D medium may be causing burning of the terminal parts of the spikelet explants such that they become dark brown in color. However, in the responding explants (induced pro-embryos), fewer brownish tips were observed. Moreover, the explants of some cultivars were more sensitive to the phenomenon of burned terminal parts. The competence among the florets lying on the spikelet stalk explant may be caused by depletion of the endogenous hormone and subsequent browning and drying of terminal parts bearing the lying florets on the stalk occurred. This impact could be twice as significant in the case of using high concentration of 2,4-D, and the susceptibility of the cultivar to burned terminal parts of spikelet explants. Sensitivity was found to be cultivar dependent on the same nutrient medium composition.

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

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

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

The initial 2,4-D period

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

The overwhelming majority of Batch 1 and III explants produced swollen florets which appeared vitrified (Figure 2). It is noteworthy that such a shape was dominant over all the explants cultured onto the high-auxin medium, where no growth progressed after 24 weeks from the initial culture (Table 2).

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

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

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

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

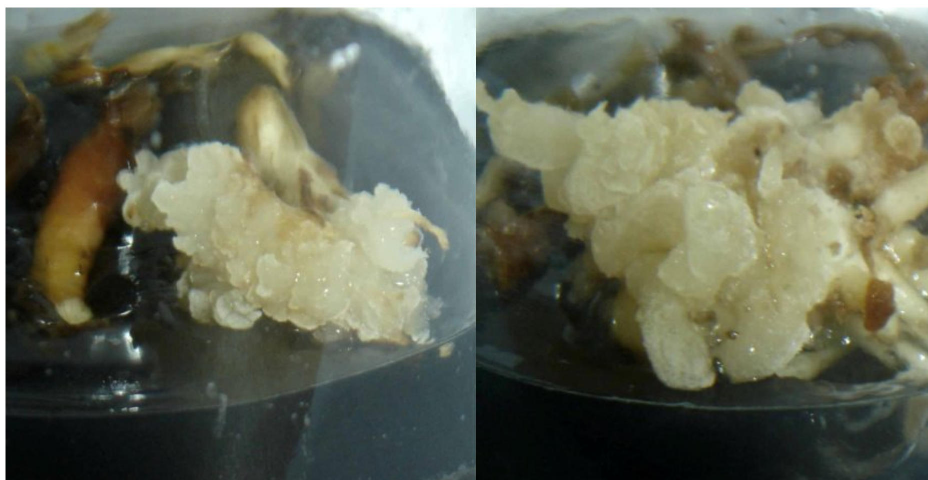

Figure 4. Unfriable callus (at left photo) and mixed with roots (at right photo) formed from the florets of spikelet explants of Kashoo-Wari cv. after 3 subcultures.

The length vs. 2,4-D initial period

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

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

The predominant response was the swollen florets some of which induced pro-embryos. Longer explants of late excised spathes such as Aseel-4 and Khormo cvs., possessed florets of about 5 mm in diameter. Very few of the swollen florets of Kashoo-Wari cv. of Batch II which produced pro-embryos developed into green shoots when shifted to the light conditions (2,000 l m^{-2}). Other explants, because of the media composition of 2,4-D, turned brown and died at the end (42 weeks). However, very few small explants of Aseel-2 cv. and Dedhi-1cv. produced only a small amount of unfriable white callus from the tiny florets. The callus produced was not from the stalks bearing these florets. The most appropriate age/length of the inflorescence in terms of spikelet length was 15-50 mm; most of the organs induced on Batch II were in this range.

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

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

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

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

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

Conclusions

The immature spathe could be useful in date palm tissue culture if excised a couple of weeks before the brown tip of the first spathe appears in the crown and for a week thereafter. During this time period, the length of the immature spathe varies and subsequently the spikelet explants within it varies. The length of spikelet explants varied among different cultivars when excised from a particular location on the crown on the same day. In addition, spikelet explants were variable within a cultivar according to the time of excision, location on the crown and within a single spathe.

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

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REGULAR ARTICLE

Determination of the date palm cell suspension growth curve, optimum plating efficiency, and influence of liquid medium on somatic embryogenesis

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Abstract

Understanding the behavior of date palm (*Phoenix dactylifera* L.) cell suspension growth and differentiation would foster effective utilization for mass micropropagation and various in vitro investigations. The objectives of this study were to define the growth curve, identify the optimum plating density, and examine the efficiency of somatic embryogenesis of date palm suspension culture on solid and liquid media. Cell suspensions were established from shoot tip-induced callus of cv. Barhee inoculated in MS medium containing 10 mg l^{-1} naphthaleneacetic acid and 1.5 mg l^{-1} 2-isopentenyladenine. Various growth phases including lag, exponential, linear, progressive deceleration, and stationary, along with their specific onsets and durations, were identified based on packed cell volume method. The growth pattern characterizing the exponential phase in date palm cell suspensions commenced 4 weeks after culture initiation which makes this period the most suitable for sub-culturing, assessment of the effects tissue culture factors, and in vitro selection. The effect of cell plating density on cell growth after transferring to solidified medium was also determined. The highest plating efficiency, 14.6%, was obtained at cell density of 10,000 cells ml^{-1}. To stimulate somatic embryogenesis, cell suspension masses were transferred to agar or liquid media devoid of phytohormones. Plant regeneration was marked by the development of globular somatic embryos which progressively matured and germinated. Culturing suspension mass in liquid medium expedited regeneration and resulted in 3.5-fold more somatic embryos than agar medium.

Key words: Cell suspension, Growth curve, Micropropagation, *Phoenix dactylifera*, Plating efficiency, Somatic embryos, Tissue culture

Introduction

Date palm (*Phoenix dactylifera* L.), belongs to the monocotledonous family Arecaceae, and is an economically important tree species predominantly concentrated in arid regions of the Middle East and North Africa (Zaid, 2002). Propagation by seeds produces female trees with inferior fruit quality. Offshoots are preferred for propagation because they produce genetically identical trees. However, offshoots availability is limited. Alternatively, micropropagation provides an efficient means for mass propagation of date palm. Breeding of date palm is hindered by the long generation time and high heterogeneity (El Hadrami and El Hadrami, 2009). This makes biotechnological approaches, such as genetic transformation (Saker et al., 2007a; Habashi et al., 2008; Mousavi et al., 2009; Saker et al., 2009) and in vitro selection (El Hadrami et al., 2005; Al Mansoori et al., 2007; Jain, 2010) indispensable for date palm genetic improvement.

Research in date palm tissue culture has received increasing interest resulting in plant regeneration protocols for numerous commercial date palm cultivars. Several reviews have described research progress of date palm micropropagation (Tisserat, 1991; Omar et al., 1992; Benbadis, 1992; Bhaskaran and Smith, 1995; Al-Khayri, 2005; Al-Khayri, 2007; Singh and Shekhawat, 2009; Abahmane, 2011; Abul-Soad, 2011; Fki et al., 2011; Othmani et al., 2011). The literature indicated that plant regeneration of date palm was achieved via organogenesis and somatic embryogenesis depending upon the genotype and the composition of the culture medium. Date palm somatic embryogenesis was improved using biotin (Al-Khayri, 2001), abscisic acid (ABA) supplement (Sghaier et al., 2009; Sghaier-Hammami et al., 2010), coconut water additive (Al-Khayri, 2010), and suitable basal salt formulation (Al-Khayri, 2011). Moreover, the stimulation of direct somatic

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embryo regeneration from shoot tip explants using N-phenyl N'-1,2,3-thidiazol-5-ylurea (TDZ) was achieved by Sidky and Zaid (2011).

Prompted by successes in numerous plant species; for examples, soybean (Hayashi and Woshida, 1988), orchardgrass (Britain-Loucas et al., 1998), and peach (Schiavone and Wisniewski, 1990), liquid media were eventually utilized in date palm tissue culture. Several researchers successfully obtained in vitro plant regeneration using suspension cultures established from date palm embryogenic callus (Sharma et al., 1986; Bhaskaran and Smith, 1992; Veramendi and Navarro, 1996; Taha et al., 2001; Fki et al., 2003; Zouine and El Hadrami, 2004; Zouine et al., 2005; Sané et al., 2006; Saker et al., 2007b; Zouine and El Hadrami, 2007; Badawy et al., 2009; Othmani et al., 2009a). These studies proved that suspension culture is a prolific source of somatic embryos suitable for mass propagation of several date palm cultivars. In addition, Othmani et al. (2009b) demonstrated the applicability of embryogenic suspension culture for high output of date palm somatic embryos using temporary immersion bioreactor (TIB) system.

Other researchers utilized date palm suspension cultures for in vitro physiological studies related to abiotic stress responses; for example, changes of proline and ions accumulation in response to salinity (Al-Khayri, 2002) and PEG-simulated drought (Al-Khayri and Al-Bahrany, 2004). Moreover, liquid cultures facilitated anatomical and histological studies related to the development of date palm somatic embryos (Bhaskaran and Smith, 1992; Sané et al., 2006; Sghaier et al., 2008). Additionally, suspension culture proved useful in conducting biochemical studies involving the role of protein accumulation in date palm somatic embryogenesis (Zouine and El Hadrami, 2004; Sghaier et al., 2009; Sghaier-Hammami et al., 2010).

Efficient manipulation of cell cultures, however, requires understanding the growth behavior of a given culture system especially identifying the onset and the duration of various culture growth stage phases: lag, exponential or log, linear, progressive deceleration, and stationary. This information can be used to establish a growth curve. Growth curves are essential to assess culture performance and metabolic activities at various growth phases. Furthermore, they provide guidelines for determining the optimal time for sub-culturing of cell suspension cultures.

Cell suspension offers a tremendous opportunity for date palm genetic improvement based on mutagenesis and in vitro selection studies (Jain, 2005, 2007). The success of this technique relies on using actively dividing cells found in the exponential phase, i.e. the most rapid growth. This emphasizes the importance of distinguishing growth phases for mutagenesis and in vitro selection studies. A common approach of in vitro selection involves plating cell suspensions on a solidified medium containing selection agents. The cell density is a determining factor in the growth efficiency of the plated cells, i.e. the plating efficiency (PE). For this reason, it is imperative to identify the optimum plating efficiency to maximize the effectiveness of in vitro selection of date palm biotypes.

The literature lacks information distinguishing the growth phases of date palm cell suspension. Likewise, no data were reported on the effects of the plating density. Moreover, previous studies demonstrated cultivar-dependency in date palm somatic embryogenesis improvement using liquid medium. The objectives of the present study were: to develop a growth curve distinguishing various culture phases of date palm cell suspension, to identify the optimum plating efficiency, and to assess somatic embryogenesis in liquid and agar media.

Materials and Methods

Explant preparation

Date palm cv. Barhee offshoots, 3-4 years old, were removed from mother trees, the outer leaves were removed exposing the shoot tip regions that were excised and immediately placed in a chilled antioxidant solution consisting of ascorbic acid and citric acid, 150 mg l^{-1} each, to prevent browning. The shoot tip tissue, about 8 cm long, was surface sterilized in 70% ethanol for 1 min followed by 15 min in 1.6% w/v sodium hypochlorite (30% v/v Clorox, commercial bleach) containing 3 drops of Tween 20 (Sigma Chem Co, St. Louis, MO) per 100 ml Clorox solution. The tissue was then rinsed with sterile distilled water four times and placed again in sterile antioxidant solution in preparation for explant excision. The tissue surrounding the shoot tips was removed until the leaf primordia were exposed and detached at the base. The shoot tip terminal, about 1 cm long, was sectioned longitudinally into four sections. Each offshoot yielded 10 explants, 6 smallest leaf primordia plus 4 terminal tip sections, which were used for culture initiation. The culture conditions used throughout the study, except where specified, consisted of a 16–h photoperiod of cool-white florescent light (40 µmol m^{-2} s^{-1}) and 23 ± 2°C.

Culture medium

The explants were cultured on a medium consisting of MS salts (Murashige and Skoog, 1962) supplemented with (per liter) 170 mg NaH_2PO_4, 125 mg *myo*-inositol, 200 mg glutamine, 5 mg thiamine-HCl, 1 mg nicotinic acid, 1 mg pyridoxine-HCl, 30 g sucrose, and 7 g agar (purified Agar-agar/Gum agar, Sigma). This basal medium was used throughout the system with modifications made according to each stage. The callus initiation medium contained (per liter) 100 mg 2,4-dichlorophenoxyacetic acid (2,4-D) (452.5 μ*M*), 3 mg 2-isopentenyladenine (2iP) (14.7 μ*M*), and 1.5 g activated charcoal (acid-washed, neutralized, Sigma). These cultures were maintained in the dark for 12 weeks during which they were transferred at 3-week intervals. At the end of this period, resultant callus was separated and transferred to callus proliferation medium that contained (per liter) 10 mg naphthaleneacetic acid (NAA) (53.7 μ*M*), 30 mg 2iP (147 μ*M*), and 1.5 g activated charcoal. These cultures were maintained for an additional 3 weeks. To proliferate embryogenic callus, the callus was transferred to a medium containing (per liter) 10 mg NAA (53.7 μ*M*), 6 mg 2iP (29.6 μ*M*), and 1.5 g activated charcoal. These cultures were maintained for 9 weeks during which they were transferred at 3-week intervals. Embryogenic callus was maintained for 12 weeks on a medium containing (per liter) 10 mg NAA (53.7 μ*M*) and 1.5 mg 2iP (7.4 μ*M*).

Cell suspension establishment

Date palm suspension cultures were initiated by inoculating scalpel-macerated embryogenic callus (1 g per flask) in 150-ml Erlenmeyer flasks containing 50 ml liquid medium. The medium consisted of MS salts with the same supplements described for callus culture medium but without agar and activated charcoal. The suspension cultures were incubated on a rotary shaker set at 150 rpm under a16–h photoperiod of cool-white florescent light (40 μmol m^{-2} s^{-1}) and 23 ± 2°C. After 3 days the suspensions were filtered through a 500 μm-stainless steel sieve and the cell filtrates were brought to a final volume of 50 ml by adding a fresh liquid medium. The cell filtrates were placed in culture flasks and maintained under the same culture conditions for use in the current study. At 2-week intervals, the cell suspension was re-sieved and half of the medium was replaced with fresh medium.

Suspension growth determination

The packed cell volume (PCV) method was used to monitor cell growth. To determine the PCV, 5 ml of cell suspension was placed in a sterile graduated centrifuge tube and centrifuged at 2000 g for 5 min. The packed cell volume was recorded as percentage cell mass of the total centrifuged volume and then the samples were returned to the original cultures. Measurements were taking weekly for 12 weeks. The PCV values were plotted in relation to time, to construct a growth curve reflecting various phases of cellular growth.

Plating efficiency

Concentrated cell suspension was diluted to give initial cell density of 100, 500, 1000, 5000, 10000, 50000, and 100000 cells ml^{-1} with the aid of a hemocytometer. Samples of cell suspensions were mixed with melted agar medium after setting the autoclaved medium to cool down to 30-35°C. The medium used was identical to the suspension medium but contained 7 g l^{-1} agar and dispensed in 15x100 mm Petri dishes, at 20 ml per dish. The cell suspension and molten medium were mixed and evenly distributed in the plate. These were allowed to solidify forming a fixed thin layer of cell. To assess the recovery potential of cell suspension to form cell colonies in relation to the initial cell concentration, colonies formed were counted using an illuminated colony counter. The plating efficiency was determined using the following equation: PE = (final number of colonies per plate / initial number of cellular units per plate) x 100.

Somatic embryogenesis efficiency in liquid and solid media

Cell suspension cultures were induced to undergo somatic embryogenesis by transferring them to a hormone-free medium, either in a liquid or solidified state. The medium consisted of MS salts supplemented with the same additives described above. The liquid cultures were incubated on a rotary shaker at 150 rpm. The cultures were incubated at a 16–h photoperiod of cool-white florescent light (40 μmol m^{-2} s^{-1}) and 23 ± 2°C. The numbers of resultant embryos were counted at bi-weekly intervals for 14 weeks.

Experimental design and statistical analysis

To determine the effect of time on cell suspension growth expressed in PCV, a one-factor randomly designed experiment with the main factor time at 12 levels using 5 replications. To assess PE, plates were inoculated at 7 levels of initial cell concentration in a one-factor randomly designed

experiment with 5 replications. To evaluate the formation of somatic embryos in relation to time and medium solidification status, a two-factor randomly designed experiment was conducted with the main factors being the solidification status at 2 levels and time at 7 levels using 5 replications. The data were subjected to analysis of variance (ANOVA) and the means were separated, where appropriate, with a least significant difference (LSD) at 5% significance level.

Results

Suspension growth determination

The present study resulted in the determination of the growth pattern of date palm in vitro cell suspension which was useful to construct a growth curve identifying the onset and duration of the various growth phases. Based on their characteristic growth pattern, these phases were divided into lag phase, exponential or log phase, linear phase, progressive deceleration phase, and stationary phase.

According to ANOVA (Table 1), growth of date palm cell suspension expressed in PCV, was significantly affected by time (i.e. culture duration). Initially, cell growth progressed slowly as the cells acclimatize to the new environment, but as time passed, growth proceeded relatively rapidly then reached a steady state. The slow growth period, associated with the lag phase, extended from the time of initiating the suspension cultures until week 3 of culture (Figure 1). The exponential phase, where the highest growth rate occurred, was observed within 4-5 weeks of culture initiation. Following this phase, a linear growth pattern commenced and lasted until week 7. Progressive growth deceleration occurred beginning at week 8 followed by a stationary phase pattern starting with week 10. Maximum growth volume (12.1%) was reached 8 weeks after suspension culture initiation.

Table 1. Analysis of variance of the effect of time on growth of date palm cell suspension expressed in packed cell volume (pcv).

Source	df	SS	MS	*F* value	*P* > *F*
Time	11	838.920	76.265	82.264	0.0001
Error	48	44.500	0.927		

P values less than 0.05 are significant. SS, sum of squares. MS, mean of squares.

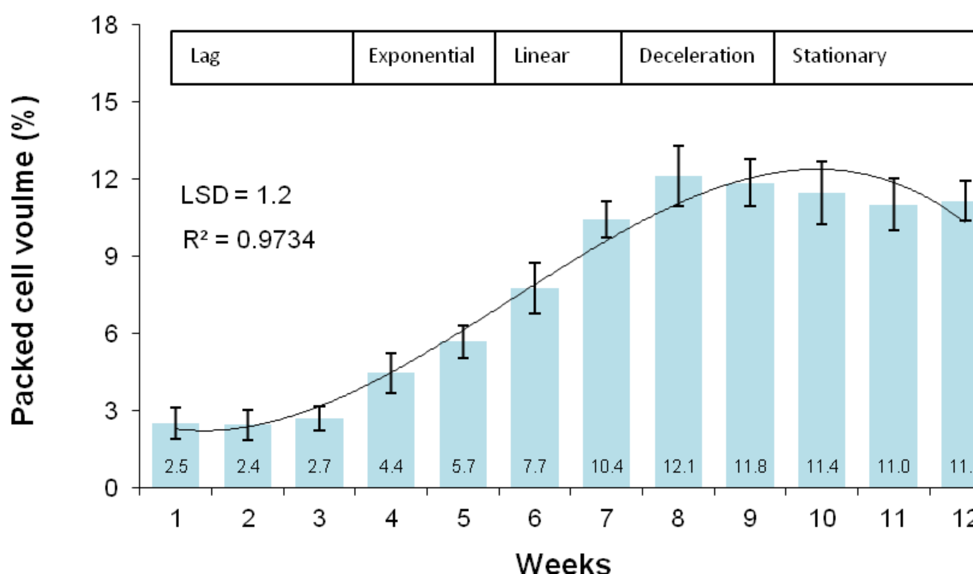

Figure 1. The growth curve of date palm cell suspension culture showing PCV in relation to time as it pertains to each of the growth phases (lag phase, exponential or log phase, linear phase, progressive deceleration phase, and stationary phase).

Table 2. Analysis of variance of the effect of initial cell concentration on re-growth of date palm cell suspension after plating expressed in terms of colony count and plating efficiency.

Source	df	SS	MS	*F* value	*P* > *F*
Colony count					
Cell density	6	54223.486	9037.248	451.540	0.0001
Error	28	560.400	20.014		
Plating efficiency					
Cell density	6	748.686	124.781	23.292	0.0001
Error	28	150.000	5.357		

P values less than 0.05 are significant. SS, sum of squares. MS. mean of squares.

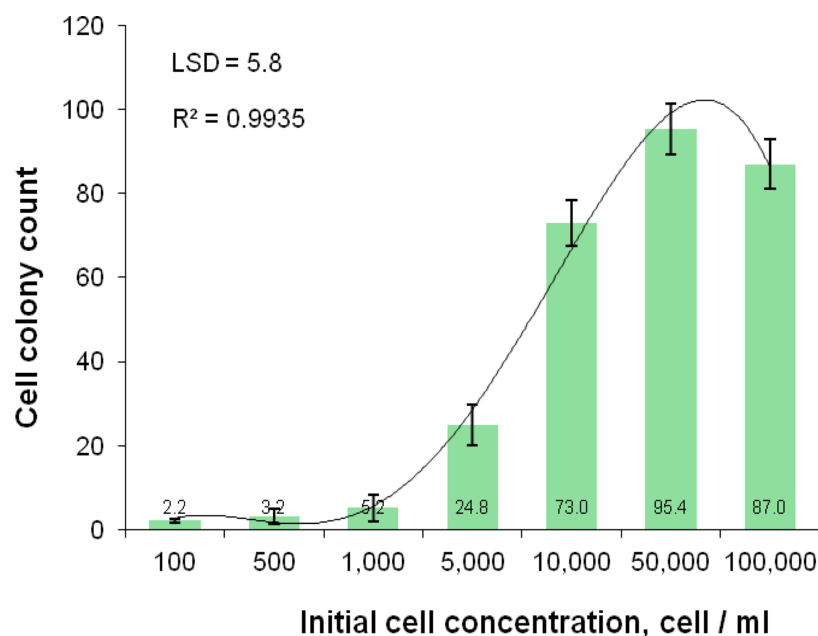

Figure 2. The growth of date palm cell suspension, expressed in number of colonies, recovered after plating on a semi-solid medium at various cell densities.

Cell colony count and plating efficiency

The cell suspension consisted mainly of single cells and small cell aggregates. Plated cells, that were capable of dividing, formed cell colonies that became visible after 3 weeks of plating. Colony count was significantly influenced by the cell density, as revealed by ANOVA (Table 2). Accordingly, when cell count was used to calculate the percentage of cell growth, to express growth in terms of PE, a significant effect due to cell density was also observed (Table 2).

Generally, cell colony count was directly proportional to the initial cell concentration (Figure 2). At low density, 100 to 1000 cells ml^{-1}, cell growth was negligible. The minimum cell density required to obtain detectible cell colony formation was 5,000 cell ml^{-1}. Whereas, the maximum count, 95.4 colonies, was observed at 50,000 cells ml^{-1}. Cell density above this level, appear to impose constrains on growth of date palm plated cells. However, the effects of density ranging from 50,000 to 100,000 cells ml^{-1} were not significantly different (Figure 2).

In terms of PE, which takes into account the percentage of cells that formed colonies in relation to the initial cell population, 10,000 cells ml^{-1} resulted in the optimum PE, 14.6% (Figure 3). Increasing the concentration to 50,000 cells ml^{-1} started to decrease the PE; although, this concentration was associated with the highest colony count. Statistically, however, cell density ranging from 10,000 to 50,000 cells ml^{-1} had similar effect on plating efficiency. At a higher cell concentration, 100,000 cells ml^{-1}, a significant decrease in PE was noted in comparison to the optimum cell densities.

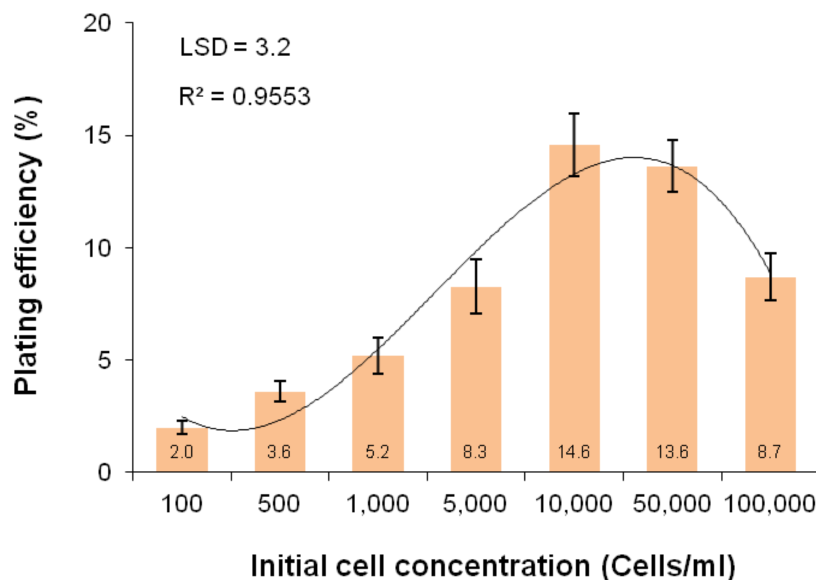

Figure 3. The growth of date palm cell suspension, expressed in plating efficiency, recovered after plating on a semi-solid medium at various cell densities.

Somatic embryogenesis efficiency in liquid and solid media

The culture conditions provided by the liquid medium significantly surpassed that of the solidified medium in supporting somatic embryogenesis of date palm, as indicated by ANOVA (Table 3). Liquid medium did not only increase the number of resultant somatic embryos but also expedited the formation of somatic embryos. This effect was indicated by the significant two-way interaction between medium solidification status (liquid or solid) and the time required for embryo acquisition.

Initially, the cell suspension proliferated callus prior to somatic embryo formation was observed. Within 4 weeks of culturing somatic embryo formation was obvious in the liquid culture medium, although a small percentage of the cultures commenced embryo formation prior to that, within the first 2-3 weeks after introducing to hormone-free medium. At this time the agar solidified cultures exhibited negligible development and it was not until week 6 that somatic embryos developed. At week 8, liquid cultures produced somatic embryos almost 3.5 times as many as that of the agar cultures, respectively, 43 and 12 embryos per culture (Figure 4). In the following weeks, the liquid medium maintained its significant superiority over the solidified medium reaching the end of the process of somatic embryo formation at week 12. Conversely, agar medium cultures continued to produce more embryos in week 14; however this increase was not significant. At the end of the experiment, liquid cultures produced 3.5 times the somatic embryos as compared to agar cultures, respectively, 69 and 20 embryos per culture. The liquid cultures exhibited expedited regeneration process whereby 62% and 85% of the somatic embryos were produced within 8 and 10 weeks, respectively.

Table 3. Analysis of variance of the effect of medium physical status (solid and liquid) on somatic embryogenesis in date palm expressed in term of embryo number produced over time.

Source	df	SS	MS	*F* value	$P > F$
Medium status	1	666.514	666.514	65.253	0.0001
Time	6	3075.143	512.524	50.177	0.0001
Medium status X Time	6	226.286	37.714	3.692	0.0004
Error	56	572.000	10.214		

P values less than 0.05 are significant. SS, sum of squares. MS. mean of squares.

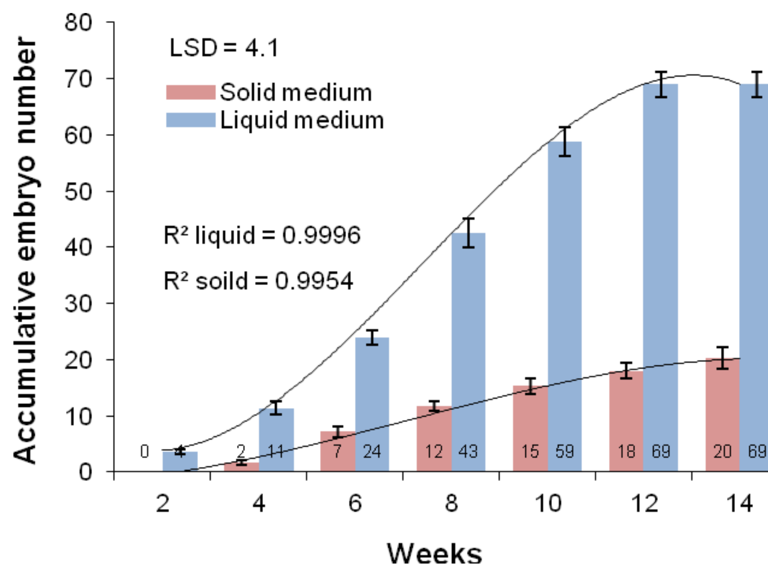

Figure 4. Progress of somatic embryogenesis in date palm cell suspension cultured either in liquid or agar medium in relation to time.

Discussion

Suspension culture establishment

For establishing date palm suspension cultures, different researchers utilized variable sieve pore sizes for filtration. Filter pore size arbitrarily ranged from 380 µm (Othmani et al., 2009a) to 500 µm (Fki et al., 2003; Zouine et al., 2005; Saker et al., 2007b). Larger size, 2000 and 1000 µm filter combination, was used by Sané et al. (2006) for the filtration of date palm embryogenic mass. In the current study 500 µm pore size appear to be effective for the establishment of date palm suspension. The optimum filtration pore size for establishing suspension culture merits further investigations related to the influence of cultivar and culture stage. According to a study by Badawy et al. (2009), the optimum filtration pore size during the maturation stage of date palm somatic embryos was 500 µm mesh filter diameter. This resulted in increased globulrization and higher numbers of somatic embryos when 500 µm mesh diameter was used as compared to 100 µm and 200 µM mesh filter diameter.

Suspension growth determination

There are general technical difficulties in defining the growth of plant cell in vitro cultures which pertains to maintaining aseptic conditions while obtaining consecutive samples from culture vessels. In vitro cell growth can be measured in relation to the number of cells, the cell mass, the volume of cells, or as a function of biochemical processes such as sugar dissimilation (Schripsema et al., 1990). Various methods are employed to measure in vitro cell growth, including cell or colony counting, dry weight, and fresh weight, and packed cell volume (Dixon, 1985). Different growth curves are obtained depending upon the methods used (Majerus and Pareilleux, 1986; Yamamoto and Yamada, 1986).

Since cell stretching and volume increase of cells comes after maximum cell division is reached, the maximum fresh weight is reached some time after the maximum dry weight is obtained. Growth patterns of cell number usually parallel that of the dry weight, while the packed cell volume usually parallels that of the fresh weight. Growth curves obtained with different methods vary and may be influenced by the genotype, cell line, and various components of growth medium (Zilkah and Gressel, 1977; Schripsema et al., 1990).

PCV procedure is commonly used to monitor growth of plant cell cultures (Santos-Diaz and Ochoa-Alejo, 1994; Falco et al., 1996). In the present study, this method was used to develop a growth curve for date palm suspension culture

identifying the onset and duration of the various growth phases including lag phase, exponential or log phase, linear phase, progressive deceleration phase, and stationary phase.

Cell colony count and plating efficiency of cell suspension cultures

A major requirement for obtaining growth from in vitro cell cultures after transferring them to agar medium is using suitable initial density of cells for inoculation in the agar culture plates. It is pertinent to use the proper plating densities because lower plating densities reduces the nutrient deficiency effect which can limit growth; whereas, higher densities elicits nutritional competition and limitation to growth. Moreover, accumulation of growth inhibitors like phenolic compounds at higher densities may adversely affect cell growth (Marchant et al., 1997; Yu et al., 2000; Aziz et al., 2006). Usually a high initial cell number, above 1000 cells per ml, is required for plating to obtain development of colonies and subsequent growth to microcalli of appreciable sizes. Using proper initial cell density would facilitate colony isolation and characterization in various in vitro physiological and genetic studies. The optimum cell density that gives the highest percentage of colony growth may differ among plant species. Plating densities of 1,000-100,000 cells per ml fall within the optimum PE range depending on the plant species (Bellincampi et al., 1985).

The current study showed that initial cell density of 10,000 cells ml^{-1} resulted in maximum PE, 14.6%, in date palm. Although this number is acceptable PE may be enhanced by various manipulations. For example, colony formation may be increased by the raft nurse or feeder layer technique (Bellincampi et al., 1985). Nurse culture proved advantageous in date palm protoplast culture (Chabane et al., 2007) and may be beneficial for cell growth as well. Improvement of the PE may also be achieved using conditioning medium (Bellincampi et al., 1985; Astarita and Guerra, 2000). These approaches are worth testing in future studies aimed at improving the PE of date palm suspension cultures. Moreover, the number of microcalli developed from date palm protoplast culture was found to be cultivar dependent (Chabane et al., 2007). Date palm plated cell suspensions are expected to behave similarly; however, more research is required to assess genotypic differences.

Somatic embryogenesis efficiency in liquid and solidified media

Cell suspension cultures offer several distinct advantages over solidified-medium cultures. In liquid medium, calus clusters and regenerated embryos usually disassociate and float freely in the medium; consequently, they are totally submerged and evenly exposed to the medium ingredients and precursors. Ample aeration is achieved by gently agitating the culture vessels on an orbital shaker. These conditions provide an excellent growth environment that allows for more precise manipulation of medium components and control of cells and embryos development leading to plantlets regeneration (Ammirato, 1984). Availability of an efficient cell suspension system offers the necessary tools for large scale cloning of elite cultivars, production of artificial seeds (Onishi et al., 1994; Bekheet et al., 2002), and in vitro selection (Larkin and Scoweroft, 1981). Cell suspension cultures are also convenient for mass production of fine chemicals in bioreactors and offer a simplified model system for the study of plant responses (Schripsema et al., 1995; Mustafa et al., 2011). Studies with different plant species have demonstrated the advantages of liquid media. For instance, Jayasankar et al. (1999) noted a twofold increase of grapevine, *Vitis vinifera*, somatic embryo regeneration when liquid medium was used.

The current study has also shown that date palm somatic embryogenesis efficiency using liquid medium was far superior to solid medium. This confirms previous findings reported in different date palm cultivars. Fki et al. (2003) reported 20-fold increase in cv. Deglet Nour when embryogenic suspensions were used instead of solid cultures. The highest rate obtained in liquid was 200 embryos per month per 100 mg callus, compared to only 10 embryos with agar medium. Embryogenic callus of cvs. Bousthami noir and Jihel placed in liquid medium yielded 72 embryos per 100 ml of culture medium within 2 months, while those placed on solid medium yielded 16 embryos after 4 months (Zouine et al., 2005). This expediting action of liquid medium was also observed in the current study with cv. Barhee. Saker et al. (2007b) reported 2 to 10 times greater numbers of somatic embryos of cv. Sewi cultured in different liquid media as compared to the corresponding solidified media. The highest number recorded was 129 embryos per flask inoculated with 0.5 g callus. Working with date palm cv. Sakkoty, the highest number of somatic embryos obtained by Badawy et al. (2009) was 48 embryos formed from liquid cultures inoculated with 0.2 g callus. In comparison, cv. Barhee tested in the present study produced 69 embryos per flask inoculated with 1 g

callus as compared to 20 embryos on agar medium; i.e. 3.5-fold increase. It is worthy to note that this increase was detected as early as week 6 of culturing on the regeneration medium and retained throughout the culture duration. This implies that data can be accurately collected to assess the effects of various tissue culture parameters on date palm cell suspension within 6 weeks, without the need to continue observations until week 14, thus reducing the experiment duration.

In conclusion, the current investigation has resulted in an efficient protocol for date palm cell suspension culture suitable for monitoring cell growth and differentiation. A growth curve specifying various culture stages of date palm suspension cultures was developed to assess future studies designed to observe changes in cell growth and somatic embryogenesis in response to modifications in culture factors. The growth curve will facilitate identification of the best time for sub-culturing. Based on testing colony growth at various cell densities, the optimal plating efficiency for date palm was determined. This parameter is essential for future in vitro studies related to somaclonal variation selection and protoplast culture. The study has also confirmed that liquid medium was more supportive of somatic embryogenesis than solidified culture medium, not only by giving higher embryo numbers but also by expediting embryo regeneration.

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REGULAR ARTICLE

Molecular and morphological identification of some elite varieties of date palms grown in Saudi Arabia

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Abstract

Date palm (*Phoenix dactylifera* L.), is a highly out breeding, dioecious plant species of enormous genetic diversity. Genotype identification of date palm is an intricate empirical exercise based on morphological characters. In date palms most of the female cultivars are recognised by their fruit characteristics such as size, shape, colour and taste. Morphological characters of the tree are also taken into consideration for cultivar identification. However some date palms have similar or narrow distinguishing morphological characters that complicate cultivar identification and require genetic evidence to prove phylogenetic relationships. RAPD analysis has been successfully applied for cultivar identification of date palms. The objectives of the present study were to characterize some elite cultivars of date palms using morphological characters of fruits and to correlate the results with RAPD markers. A total of 14 well-known cultivars of date palm (Barhy, Deglet Noor, Hilaliah, Hilwa, Khalas, Makhtomi, Moneifi, Nabtet Ali, Omal Khashab, Rothana, Sabbaka, Shagra, Sukkary, Wannanah) were selected from Saudi Arabia. Analysis of the morphological data of fruits revealed a high level of diversity in length-width ratio, colour, shape of the fruit, fruit-base and in the percentage of area covered by the fruit cap. The length-width ratio of these 14 cultivars ranged from 1.1 to 2.62, indicating a great variation in their shape. Correlation of morphologic characters with genomic similarity using RAPD markers showed that the fruit shape is one of the characteristics most influenced by genetic variation. Wherever there was insignificant length-width ratio among cultivars, more genomic similarity was observed. Genetic variations at the molecular level have resulted in the production of many elite date palm cultivars which are highly variable in fruit size, shape, colour, texture, sugar and protein content. The methodology followed in this study can be extended to other cultivars, which may ultimately result in the compilation of an authentic manual describing the diagnostic characters of date palm cultivars with their existing synonyms. The RAPD analysis will help to resolve the ambiguity regarding the identity of narrowly-distinguishable cultivars and to assess genetic diversity for the conservation of date palm germplasm in Saudi Arabia.

Key words: Morphology, Fruit shape, Length-width ratio, Genetic diversity, RAPD

Introduction

Date palm (*Phoenix dactylifera* L.) cultivation is the main source of agricultural income in many countries of arid regions of West Asia and North Africa. With its ability to accumulate exceptionally high levels of metabolites under extreme arid conditions, it is a unique physiological entity (Al-Khalifah et al., 2006). Being a key species, adapted to the harsh environmental conditions of arid zones, date palms are regarded as one of the important components of biodiversity in the inhospitable desert areas. *Phoenix dactylifera* L. is interfertile with its allied species (Muirhead, 1961) and successfully pollinated with *P. rectinata* and *P. atlantica* in Africa. In India and Pakistan it is pollinated with *P. sylvestris* and in Spain with *P. canariensis* (Oudejans, 1979; Benbades, 1992). This highly out breeding behavior has brought about immense genetic diversity in this species. Zaid and de Wet (1999) reported the occurrence of 3,000 cultivars around the world. There are about 450 cultivars in Saudi Arabia (Bashah, 1996), 400 in Iran (FAO, 1996), 600 in Iraq (FAO, 2008), 250 in Tunisia (Kearney, 1906), 244 in Morocco (Saaidi, 1979) as well as many cultivars in other date-growing countries (Zaid and de Wet, 1999).

Most of the cultivar identification studies are of an enumerative type based on local names which vary from place to place. These cultivars are location specific, known by different names at different places, or one name is assigned to different cultivars at different places. This has created much ambiguity in listing the cultivars based on local names. A scientific approach of characterizing cultivars and assigning ~~a~~ more

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acceptable legitimate name to the cultivars has seldom been attempted for this species, especially in Saudi Arabia.

Genotype identification of date palm is commonly based on morphological characters (Sedra et al., 1998). In date palm most of the female cultivars are recognised by their fruit characteristics such as size, shape, colour and taste along with the morphological characters of the tree for cultivar identification. During the ripening process, the date fruits pass through four distinct stages of maturity, i.e. *kemri, beser (khalal), rutab* and *tamar* (Al-Ghamdi, 1993). When the fruits are young they are green in colour (varying in different cultivars) and are termed *kemri*. The beginning of ripening marks the *beser* stage, the half ripened stage is called *rutab* and fully ripened, soft textured stage is called *tamar*. Colour variations during the ripening of fruits are important morphological markers for the cultivar identification.

Some date palm cultivars have similar or narrow distinguishing morphological characters that complicate cultivar identification and require evidence to prove phylogenetic relationships at the interspecific level. RAPD analysis is a comparatively simple, quick and inexpensive procedure for generating genomic markers (Welsh and Mc Clelland, 1990; Williams et al., 1990). This technique has been successfully applied for cultivar identification of date palms (Saker et al., 2000; Al-Khalifah and Askari, 2003; Askari et al., 2003; Al-Khalifah, 2006). The objectives of this study were to characterize some elite cultivars of date palm using morphological characters of fruits and to correlate these results with RAPD markers.

Materials and methods

A total of 14 well-known cultivars of date palm (Barhy, Deglet Noor, Hilaliah, Hilwa, Khalas, Makhtomi, Moneifi, Nabtet Ali, Omal Khashab, Rothana, Sabbaka, Shagra, Sukkary, Wannanah) trees were tagged in two orchards of the Al-Qassim area, Saudi Arabia. Colour variations during the three fruit ripening stages (*beser, rutab* and *tamar*) were recorded directly from the tagged trees. One hundred fruits from each cultivar were collected during *rutab* stage and their length and width measured using Vernier Calipers. Shape and colour of the fruits was documented using a digital camera. The base and apex of the fruits were also noted carefully and the diameter of the fruit cap (persistent calyx) was measured using a millimeter scale. Based on these data the total area of the fruit base covered by the fruit cap was calculated.

For RAPD analysis young sprouting leaves from each cultivar were collected. Total genomic DNA was extracted using the protocol of Dellaporta et al. (1983). After determining the quality and quantity of extracted DNAs with a UV Spectrophotometer, the stock DNA samples were diluted in distilled water to make a working solution of 10 ηg/μL.

A Polymerase Chain Reaction (PCR) was performed as described by Al-Khalifah and Askari (2003) using 130 random 10-mer RAPD primers (OPERON Tech., USA) of A to G series. The PCR products of each primer were separated by electrophoresis according to their molecular weight on 1.4% (w/w) agarose gels. The profiles of each primer were then documented by Gel Documentation System of Bio-Rad (Hercules, Calif.). The length of the amplified RAPD fragments was estimated by running the Kilo Base DNA marker (Amersham Pharmacia Biotech.) in the gel as a standard size marker. Amplification profiles of all the cultivars were compared with each other using the Diversity Data Base software package (Bio-Rad).

Results and Discussion

Analysis of the morphological data of fruits showed a high level of diversity in length-width ratio, colour, shape of the fruit, fruit-base and in the percentage of area covered by the fruit cap (Table 1). Fruit shape varied from globular, elliptic, ovate, oblong, to linear oblong as in Deglet Noor (Figure 1,2). Many intermediary forms or combination of one or two forms were also observed. The length-width ratio of these 14 cultivars ranged from 1.1 to 2.62, indicating a great variation in their shape. Even within cultivars having the same or insignificantly different length-width ratio, there was variation in shape, mainly due to the position of the widest portion, i.e. widest near the base in Shagra and Wannanah and widest near the middle as in Moneifi. Fruit base varied from truncate to cordate or sometimes oblique. During the *kemri* stage all cultivars had green coloured fruits which turned to yellow or red or various degrees of a combination of red and yellow in the *beser* stage. During the *rutab* stage the ripening process usually starts from the tip of the fruit which brought different colorations to the fruits (Table 1). *Tamar* is the harvesting stage in which they showed colour variation from amber, golden brown, reddish brown to chocolate brown. The size of the fruit cap and percentage of the fruit-base covered by the fruit cap are important morphological markers to distinguish cultivars. This marker showed variations of 25-90% coverage in different cultivars (Table 1).

Table 1. Comparative fruit morphology of 14 date palm cultivars.

Cultivar	Shape	Colour variation during ripening			Length-width ratio	Fruit Cap (%)	Base
		Beser	*Rutab*	*Tamar*			
Barhy	Globular-Broadly elliptic	Lemon yellow	Amber	Golden brown	1.21	40	Truncate
Deglet Noor	Linear-oblong	Lemon yellow	Reddish brown	Amber	2.62	90	Truncate
Hilaliah	Globular	Yellow with rose tinge	Amber	Reddish brown	1.1	90	Truncate
Hilwa	Oblong	Scarlet red	Dark red	Chocolate brown	1.5	50	Shallowly cordate
Khalas	Ovate	Light yellow	Amber	Amber	1.46	30	Oblique
Makhtomi	Ovate-oblong	Greenish-yellow	Amber	Amber	1.46	60	Shallowly cordate
Moneifi	Elliptic	Yellow	Amber	Reddish brown	1.51	50	Truncate
Nabtet Ali	Elliptic oblong	Light yellow	Reddish brown	Reddish brown	1.44	25	Shallowly cordate
Omal Khashab	Oblong	Reddish-yellow	Amber	Amber	1.84	60	Truncate
Rothana	Elliptic-oblong	Lemon yellow	Amber	Reddish brown	1.4	30	Deeply cordate
Sabbaka	Oblong	Light yellow	Reddish brown	Light brown	1.5	50	Shallowly cordate
Shagra	Ovate-oblong	Yellow with red dots	Brown	Reddish-brown	1.32	33	Cordate
Sukkary	Ovate	Reddish yellow	Reddish brown	Reddish brown	1.43	60	Cordate
Wannanah	Ovate	Yellow with red dots	Chocolate brown	Chocolate brown	1.44	30	Oblique

Random Amplified Fragment DNA (RAPD) markers were also produced for the identification of these cultivars. Out of 130 primers screened for reproducible and polymorphic DNA amplification patterns, 42 were selected for DNA fingerprinting. The DNA profiles produced by 14 cultivars with OPERON A06 primer are presented in Figs. 1,2 along with their fruit morphology. The analysis of pair-wise genetic distance and similarity matrix based on the Nei and Li (1979) similarity coefficient showed an average of more than 50% similarity among the cultivars (Table 2, from Al-Khalifah, 2006). Cluster analysis using the unweighted pair group method of arithmetic means (UPGMA) and the dendrogram (Fig. 3 from Al-Khalifah, 2006) showed maximum similarity between Makhtomi and Nabtet Ali (0.70), followed by Barhy and Hilaliah (0.65). Out of the 19 cultivars screened by Al-Khalifah (2006), 12 formed couples and the rest showed various percentages of similarity to either one couple or to more than one couple.

A correlation of morphologic characters with genomic similarity showed that fruit shape is one of the characteristics most influenced by genetic variation. Wherever there was an insignificant length-width ratio between cultivars, more genomic similarity was observed. In the case of Makhtomi and Nabtet Ali, where the maximum genomic similarity was observed, their length-width ratios only differed by 1.46 and 1.44, respectively. The second genomically similar couplet (Hilaliah-Barhy) also showed a very narrow variation in their length-width ratio (1.1-1.2). The other pairs that followed the same rule were Khalas-Makhtomi (1.46-1.46), Sabakka-Rothana (1.5-1.4), and Shagra-Wannanah (1.32-1.44). But there was an exception exhibited by the Nabtet Ali-Wannanah pair, where the length-width ratio was similar (1.44) but the genomic similarity was the least (44.1%). But in this case, irrespective of their similar length-width ratio they were very distinct in their fruit morphology, i.e. their shape (elliptic-oblong and ovate), colour and fruit base.

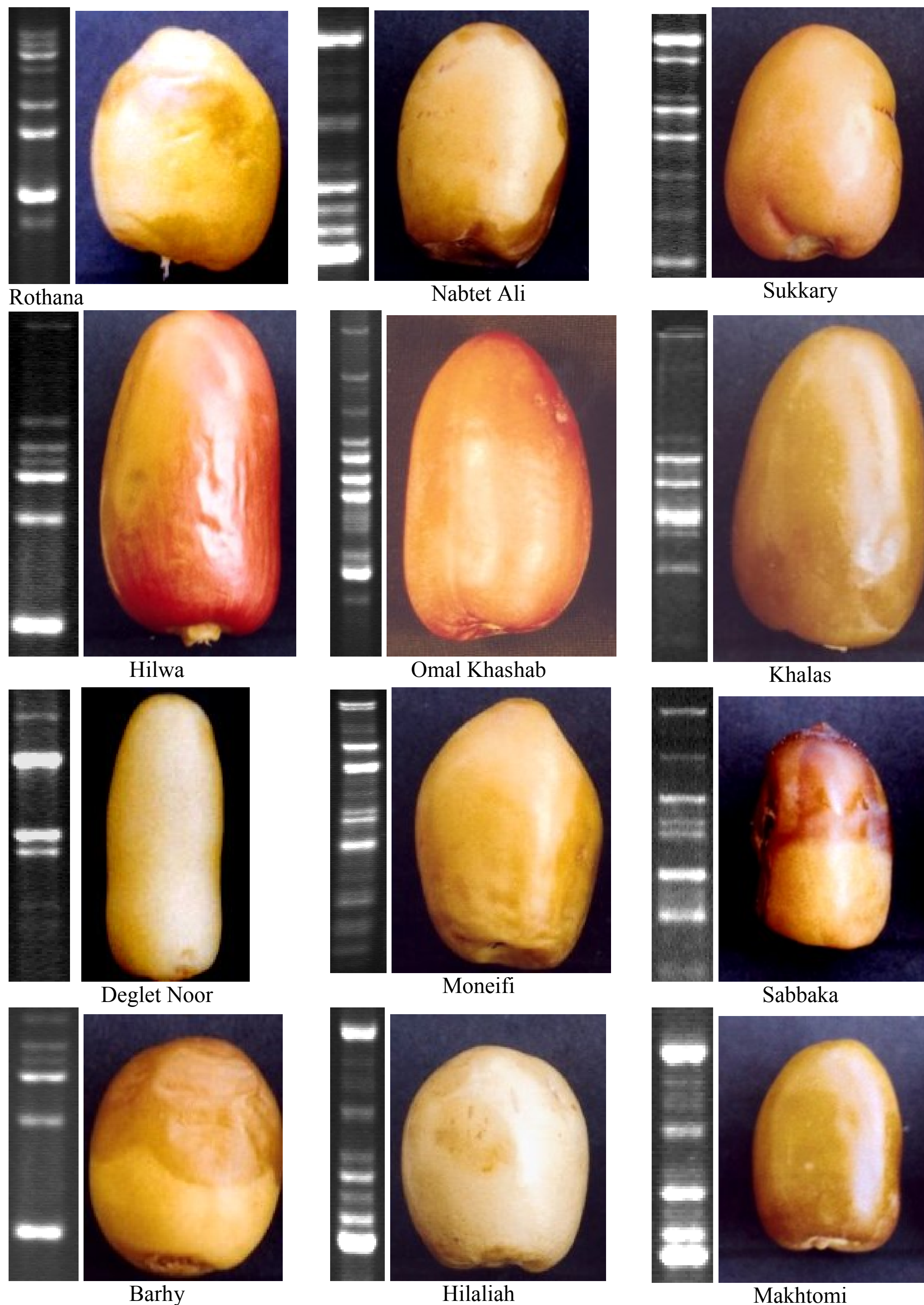

Figure 1. Fruit morphology and DNA profiles of 12 cultivars produced by A-06 OPERON primer.

Results generated by the present data using different RAPD primers suggest genetic diversity among date palm cultivars. Molecular phylogeny of 13 date palm cultivars studied by Al-Khalifah and Askari (2003) and 7 cultivars by Askari et al. (2003) also showed the same tendency of genetic diversity. These genetic variations at the molecular level have resulted in the production of many elite cultivars which are highly variable in fruit size, shape, colour, texture, sugar and protein content.

Table 2. Similarity matrix based on the Nei and Li coefficients of 19 date palm cultivars obtained from RAPD markers (Al-Khalifah, 2006).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Nabtat Ali	1	100.0																		
Maktoomi	2	70.2	100.0																	
Moneifi	3	60.6	67.4	100.0																
Mowakil	4	60.5	63.1	57.0	100.0															
Omal Khashab	5	60.0	59.3	57.6	70.2	100.0														
Bayadh	6	57.5	60.6	60.0	70.3	59.9	100.0													
Khalas	7	56.6	61.6	58.0	73.0	67.3	71.3	100.0												
Rothana	8	54.4	51.6	48.0	55.1	55.4	52.1	51.9	100.0											
Hilaliah	9	53.7	56.4	47.6	64.9	56.0	57.1	58.7	50.9	100.0										
Madjhool	10	52.4	54.5	49.3	63.1	55.0	57.2	61.5	50.1	56.8	100.0									
Sukarry	11	51.9	58.1	54.6	66.0	57.2	64.7	59.3	57.9	57.6	52.7	100.0								
Kwairiah	12	51.7	56.8	50.7	55.7	54.0	53.6	53.9	56.3	58.7	51.3	55.1	100.0							
Roshodyia	13	50.5	51.9	57.8	53.9	56.8	49.6	54.5	54.0	49.5	46.5	50.5	63.1	100.0						
Hilwa	14	50.1	49.4	50.6	58.7	59.0	55.0	60.8	52.5	55.3	61.6	50.6	51.6	55.8	100.0					
Barhy	15	50.0	54.3	52.4	57.5	57.8	61.2	63.8	50.9	64.8	53.8	54.4	59.8	53.0	59.0	100.0				
Sabaka	16	46.8	48.0	53.9	52.2	48.8	56.2	48.9	58.8	47.4	44.6	58.9	44.7	48.2	43.2	48.9	100.0			
Shagra	17	46.4	48.2	44.6	62.7	54.8	50.0	54.9	44.8	55.2	64.4	48.4	56.8	48.7	54.9	52.6	46.2	100.0		
Deglet Noor	18	44.6	50.4	50.6	51.9	59.3	52.3	54.0	42.2	51.3	59.8	47.3	52.4	51.6	52.1	54.4	42.8	56.9	100.0	
Wanana	19	44.1	47.5	48.2	59.2	47.3	62.6	57.3	49.1	54.5	54.8	58.6	62.4	55.6	56.2	56.4	48.9	51.0	54.1	100.0

The methodology followed in this study also can be extended to other cultivars, which may ultimately result in the compilation of an authentic manual describing the diagnostic characters of date palm cultivars, with the existing synonyms. The addition of tree characteristics, protein and sugar content of each cultivar to these data in future will make an ideal manual that can be used as a reference to identify the presently-known date palm cultivars. The RAPD analysis will help to resolve the ambiguity regarding the identity of narrowly-distinguishable cultivars and to assess genetic diversity for the conservation of date palm germplasm in Saudi Arabia and other countries.

Figure 2. Fruit morphology and DNA profiles of two morphologically similar cultivars produced by A-06 OPERON primer.

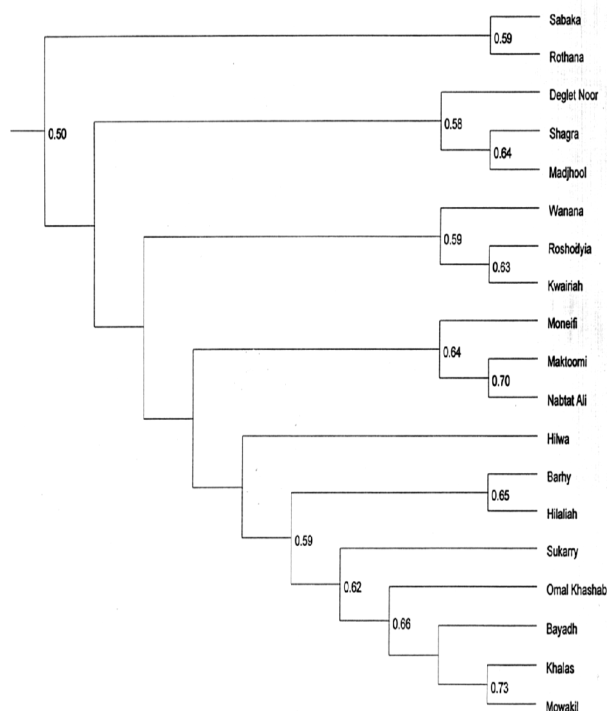

Figure 3. A dendrogram of phylogenetic relationships among 19 cultivars of date palm based on the RAPD analysis using 42 primers (Al-Khalifah, 2006).

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REGULAR ARTICLE

Potential applications of gene silencing or RNA interference (RNAi) to control disease and insect pests of date palm

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Abstract

Gene silencing or RNA interference (RNAi), a recently-discovered regulatory and defense mechanism in plants, animals and other organisms, has great potential to control plant pests. A gene essential for survival or development of the plant pest is targeted, and an inverted repeat construct of the gene is transformed into susceptible host plants. Plant transcription produces a double-stranded RNA (dsRNA), which the plant recognizes as a foreign molecule. Dicer, the plant's protective ribonuclease enzyme, hydrolyzes the dsRNA to small interfering RNAs (siRNAs). The feeding pest ingests the siRNAs, causing the pest's RNAi mechanism to hydrolyze the messenger RNA of its own essential gene. This "silences" that essential gene in the pest, which either dies or is debilitated, and the transgenic plant is resistant to that pest. RNAi, having been shown to provide resistance against insects (Diabrotica, Helicoverpa), bacteria (*Agrobacterium, Staphylococcus*), nematodes (Heterodera, Meloidogyne) and parasitic plants (*Orobanche, Striga, Triphysaria*), should provide effective, durable resistance to red palm weevil (*Rhynchophorus ferrugineus*), Bayoud disease (*Fusarium oxysporum* f. sp. albedinis), Al-Wijam, and other serious pests of date palm.

Key words: Disease, Gene silencing, Insect, RNAi

Introduction

Date palm (*Phoenix dactylifera*) has been a cultivated tree crop for at least 5,000 years (Johnson, 2011). It is a very important plant throughout the world, and is perhaps the most important plant in Saudi Arabia and throughout the Middle East. It has high socioeconomic importance, due not only to its food value, but also its capacity to provide many other products such as shelter, fiber, clothing, aesthetic beauty and furniture (Mousavi et al., 2009). It has high natural tolerance to very adverse growing conditions, including drought, salinity and high temperatures (Bakheet et al., 2008). In 2007 nearly 1.1 million ha of date palm were harvested, yielding 6.91 million tonnes. The major producers were Egypt (19%), Iran (15%) and Saudi Arabia (14%) (http://faostat.fao.org 2007).

Each year plant pests cause serious economic losses throughout the world in palm species, especially in date and coconut palms. In date palm up to 30% of production can be lost to pests and diseases (El-Juhany and Loutfy, 2010), including the red palm weevil, (RPW *Rhynchophorus ferrugineus*), the Bayoud disease (*Fusarium oxysporum* f. sp. *albedinis*) (Quenzar et al., 2001; Zaid et al., 2002) and phytoplasma diseases (Nixon, 1954; Alhudaib et al., 2007; Harrison and Elliott. 2009). Depending on the level of infestation, the RPW can cause losses up to $130 million annually in the Middle East countries alone, and additional millions of dollars of losses on coconut and other palm species (Faleiro, 2006; El-Sabea et al., 2009). Because date palm is a long-lived plant and because it is genetically heterogeneous and difficult to propagate, it is essential to develop durable resistance against these important pests and to incorporate it into horticulturally desirable cultivars.

Recent advances in biotechnology, both in date palm and in new disease resistance strategies, provide encouraging opportunities for developing pest resistant date palms. Tissue culture from somatic embryos of date palm has been advanced significantly, and large scale micro-propagation (Al-Khayri, 2010) is now possible. In addition, transformation of embryonic date palm callus cells has been achieved (Mousavi et al., 2009; Saker et al., 2007) using biolistics (particle bombardment). Successful transformation was demonstrated both by β-glucuronidase (GUS) expression (histochemical staining) and direct detection by polymerase chain reaction (PCR). However,

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transgenic plants have not yet been regenerated. Even greater success has been achieved in the oil palm (*Elaeis guineensis*), where stable transformation has been achieved both by biolistics and by *Agrobacterium tumefaciens* (Ismail et al., 2010), and transgenic plants have been regenerated.

RNAi is a recently discovered mechanism for regulating gene expression. It functions in diverse organisms including plants and fungi (Van West et al., 1999; Hammond and Keller. 2005; McDonald et al., 2005; Eamens et al., 2008), bacteria (Escobar et al., 2001; Hannon, 2003; Katiyar-Agarwal et al., 2006; Yanagihara et al., 2006), insects (Baum et al., 2007; Mao et al., 2007) nematodes, (Fire et al., 1998; Huang et al., 2006; Sindhul et al., 2009), hydra (Matzke, and Matzke, 2004), humans (Hannon, 2003), parasitic plants (Tomilov et al., 2008; Aly et al., 2009), and plant viruses (Ahlquist, 2002; Baulcombe, 2005; Dietzgen and Mitter, 2006). It holds great promise to control human, plant and animal diseases (Matzke and Matzke, 2004). RNAi is considered an ancient defense mechanism whereby the host organism recognizes as foreign a double-stranded RNA (dsRNA) molecule and hydrolyzes it with a ribonuclease named dicer. This hydrolysis produces small and specific RNA fragments of 21–28 nucleotides called small interfering RNAs (siRNAs). The siRNAs then combine with constituitive proteins to form the RNA-induced silencing complex (RISC). The RISC diffuses in the cell, and its resident siRNA hybridizes to the specific messenger RNAs (mRNAs) with sequences complementary to that of the siRNA. The new double-stranded region stimulates the hydrolysis of that mRNA by dicer to produce more siRNAs. This process is repeated each time the siRNA hybridizes to its complementary mRNA, effectively destroying and preventing that mRNA from being translated, thus "silencing" the expression of that specific gene (Eamens et al., 2008).

Stimulated by these discoveries, Venganza, Inc. developed a new technology for plant disease control called host-mediated silencing of pest genes (HMSPG). Venganza has filed a patent on HMSPG (www.venganzainc.com), and has used HMSPG to develop plants resistant to several fungal pathogens in addition to the oomycete *Phytophthora* species shown in the figures below. The molecular approach is to target an essential gene of the pest by producing a DNA construct containing an inverted repeat of that essential gene. The susceptible host plant is transformed with the inverted repeat construct, and transcription in the plant produces a dsRNA with the sequence of the targeted and essential pest gene. The plant recognizes the dsRNA as a foreign molecule, and the plant's protective dicer enzyme hydrolyzes the dsRNA into siRNAs. When the pest attacks the transgenic plant, it ingests those siRNAs, which then cause the RNAi mechanism within the pest to hydrolyze the mRNA of the pests' own essential gene. Silencing the pest gene stops the infection because the pest dies or is no longer pathogenic (depending on the choice of the gene), and the transgenic plant is now resistant to that disease. Venganza first demonstrated HMSPG in tobacco using the cutinase gene from *P. nicotianae*, because cutinase is essential for pathogenicity in Phytophthora (Munoz and Bailey, 1998). Interestingly, this essential gene sequence from *P. nicotianae* also was effective in conferring resistance against several related pathogenic fungi.

Materials and Methods

The materials and methods used in the following research are described in the Niblett patent application (Niblett, 2006), in the individual references cited, and in "Molecular Cloning: A Laboratory Manual" (Sambrook et al., 1989).

Results and Discussion

As shown in Figure 1, plant A, typical of those transformed with the cutinase gene construct (pVZA100), is resistant to *P. nicotianae*, whereas the untransformed or wild type plant B is susceptible. This resistance was effective against both Races 0 and 1 of *P. nicotianae*. Similar results have been obtained with other fungal pathogens on dicot and monocot hosts.

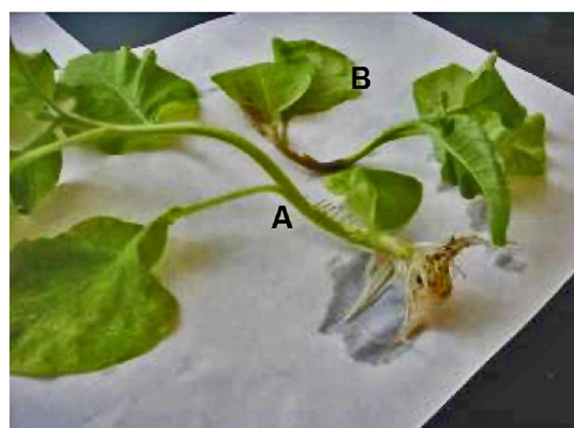

Figure 1. Resistance to P. nicotianae conferred to tobacco by transformation with pVZA100 (plant A), and a susceptible untransformed control plant (B).

Figure 2 demonstrates that the molecular mechanism of the resistance is RNAi. Panel A

shows the effect of pVZA100 transformation on *P. nicotianae*. Lane 1 is a 35 nt marker and Lane 2 shows the intact 620 nt cutinase mRNA from a wild type culture. RNAs from four transformed cultures (A-D; lanes 3-6) contain 21-25 nt siRNAs that hybridized to the cutinase probe, demonstrating that the cutinase mRNA has been hydrolyzed in the transformed cultures. Furthermore, the *P. nicotianae* isolates containing the intact mRNA were pathogenic, whereas those transformed with pVZA100 and containing the siRNAs were nonpathogenic. Cultures of *P. nicotianae* re-isolated from resistant transgenic tobacco plants showed the same siRNA profiles as those in Figure 2 A-D. The nonpathogenic cultures of *P. nicotianae* that had been transformed with pVZA100 or re-isolated from resistant transgenic tobacco were transferred monthly on growth media for three years and never regained pathogenicity, indicating that the RNAi is long-lasting, if not permanent.

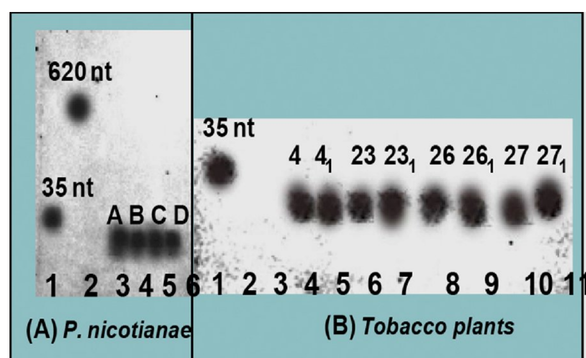

Figure 2. Hybridization of a of cutinase probe to PAGE-separated RNA extracted from transgenic and wild-type cultures of P. nicotianae and tobacco plants.

Panel B of Figure 2 shows direct evidence for RNAi activity in tobacco plants transformed with pVZA100. Lane 1 is the 35 nt size marker. RNAs from wild type and pCAMBIA1201 control transformed plants (lanes 2 and 3) showed no hybridization because cutinase is a fungal gene, not present in plants. However, the four tobacco lines transformed with pVZA100 (lines 4, 23, 26 and 27, and their T1 seed progeny; 4_1 etc.) all contained the cutinase siRNAs, and were resistant to *P. nicotianae*, as in Figure 1. These lines were randomly selected from about 50 individual transformation events.

Figure 3 shows that the tobacco plants transformed with pVZA100 also were resistant to the blue mold disease caused by *Peronospora tabacina*. *Peronospora* and *Phytophthora* are related taxonomically as members of the same Order (Peronosporales), but they are in different families (*Peronospora* = Peronosporaceae and *Phytophthora* = Pythiaceae). Therefore the pVZA100 construct also confers broad resistance to a distantly related fungal pathogen. Figures 4 and 5 provide additional evidence that HMSPG confers a broad type of resistance. In Figure 4 the cutinase gene from *P. nicotianae* (pVZA100) provides resistance against *P. sojae*, in soybean. This resistance was effective against all seven races of *P. sojae* tested.

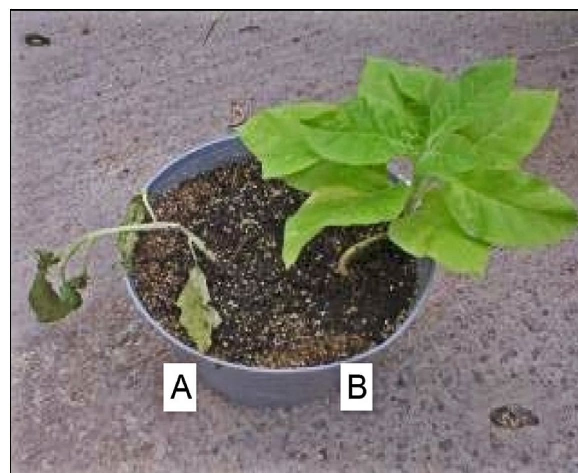

Figure 3. Resistance to tobacco Blue Mold (Peronospora tabacina) conferred by transformation with pVZA100 (plant B). Plant A is an untransformed control plant.

Figure 4. Resistance to P. sojae in soybean plants by transformation with pZA 100.

In Figure 5, pVZA100 (Row 2) confers resistance against *P. infestans* in potato as compared with Row 1, which contains wild type plants and those transformed with pCAMBIA1201 alone). Rows 3 and 4 show resistance in potato plants transformed with pVZA300 and pVZA400,

which contain the elicitin and ribosomal RNA (rRNA) genes, respectively, from *P. infestans.* Note that the level of resistance conferred against *P. infestans* by pVZA100 is not as high as with pVZA300 and pVZA400. This may reflect the lower sequence identity (82%) between the cutinase gene of *P. nicotianae* and that of *P. infestans.* The resistance conferred by pVZA100, 300 and 400 was effective against both mating types A1 and A2 of *P. infestans.* The pVZA300 construct used here contains a gene from both *P. infestans* and a plant insect pest, indicating that a construct containing two genes remains functional and is effective against its target gene in *P. infestans*. We have recently used "gene stacking" on a single construct to confer resistance to three different fungal pathogens.

Figure 5. Resistance to late blight (P. infestans mating type A2) conferred to potato by transformation with pVZA100 (Row 2), pVZA300 (Row 3) or pVZA400 (Row 4), compared to wild type plants, and those transformed with pCAMBIA 1201 (Row 1).

HMSPG and similar strategies are widely applicable to a broad spectrum of plant pests. For example, inverted repeats of genes from multiple plant viruses were used to obtain resistance to four different viruses (Dietzgen and Mitter, 2006). HMSPG also is effective against insects, nematodes, parasitic plants, and bacteria, which is the capability we propose to implement here against the pests of palm. Corn plants transformed with a construct containing an inverted repeat from a vacuolar ATPase gene from Western corn rootworm showed significant reduction in damage from this insect (Baum et al., 2007), and cotton plants were protected from the cotton bollworm when transformed with an inverted repeat from a bollworm cytochrome P450 gene (Mao et al., 2007).

Inverted repeats of nematode parasitism genes also have been effective in controlling both root knot (Huang et al., 2006) and cyst nematodes (Sindhul et al., 2009). Working with the parasitic plant *Triphysaria versicolor*, a species of broomrape, (Tomilov et al., 2008) demonstrated that lettuce plants containing an inverted repeat of the GUS gene could silence an active GUS gene in the *T. versicolor* when it fed on the lettuce. Furthermore, feeding that same “silenced” *T. versicolor* on lettuce expressing the GUS gene, silenced the GUS gene in lettuce. This demonstrated that the “silencing principle” (siRNA) moves back and forth between lettuce and *T. versicolor.* Using *Orobanche aegyptiaca,* another species of broomrape, Aly et al. (2009) demonstrated that tomato plants transformed with an inverted repeat of the mannose 6-phosphate reductase gene of *O. aegyptiaca* showed a 58% greater mortality of the broomrape tubercles that developed on the transgenic tomatoes.

With bacteria, siRNAs have been effective *in vivo* and *in vitro* against the coagulase enzyme of the human pathogen *Staphylococcus aureus* (Yanagihara et al., 2006), and the crown gall disease of plants caused by *Agrobacterium tumefaciens* was controlled in tobacco, Arabidopsis and tomato plants transformed with inverted repeats of the *A. tumefaciens* genes *iaaM* and *ipt*, which encode precursors for auxin and cytokinin biosynthesis (Escobar et al., 2001).

Because HMSPG is effective against bacteria there is reason to be optimistic that it will be effective against phytoplasmas. Because phytoplasmas cannot be cultured we performed a preliminary experiment to test the efficacy of HMSPG against *Xanthomonas campestris* pv. *campestris* (Xcc), a serious bacterial pathogen of cabbage and other vegetables. We used the *in vitro* incubation assay that we developed for fungi (Bailey and Niblett, 2010) to identify candidate genes for HMSPG. With fungi, spores or mycelium are incubated in the dsRNAs and siRNAs, and viability is measured by colony formation or infectivity. Here we prepared dsRNAs from the Xcc 23S rRNA and enolase as candidate genes. The bacteria were incubated directly in the dsRNAs or in siRNAs prepared from the dsRNAs by digestion with ribonuclease III and then plated on YDC medium to measure viability by colony formation. The control dsRNA and siRNAs were prepared from the β-glucuronidase (GUS) gene of *Escherichia coli.* Our data (not shown) indicates

that Xcc dsRNAs and siRNAs reduced colony formation by 24 to 55%, as compared to about 10% reduction in the controls treated with GUS ds- or siRNAs. Similar reductions in viability were obtained with candidate fungal genes that subsequently provided strong resistance in transgenic plants. Therefore, as demonstrated with fungi, both the 23S rRNA and enolase genes have high potential for conferring resistance to Xcc *in planta*, and likely to other bacterial and phytoplasma pathogens.

Advantages of RNAi and HMSPG

A major concern voiced against transgenic plants is the possible expression of a protein that might cause an allergic response in consumers. Therefore, a major asset of our RNAi strategy is that no protein is expressed. We further ensure this in our construct design by avoiding both 5' terminal and internal ATG initiation codons and by inserting one or more stop codons in all six possible reading frames. Potential off-target effects on host plant genes or other species are minimized by designing constructs to produce siRNAs with a maximum of 15 contiguous base pairs of identity to known coding sequences. This is a conservative strategy, given that an upper limit of 18 contiguous base pairs is generally considered adequate to avoid off-target effects (Xu et al., 2006).

Using conventional breeding techniques it may be difficult or impossible to achieve disease resistance in an important crop species when genes for resistance to a particular pest do not exist or that crop is difficult or very time-consuming to breed for resistance because of sterility, ploidy differences or incompatibilities (e.g. date palm, bananas, potatoes, etc.). Also, when plant resistance genes are identified and transferred into desirable varieties, that resistance may not be durable because of the presence of diverse genotypes of the pest, or because the pest may mutate and rapidly "defeat" that resistance gene.

HMSPG has now been demonstrated to be effective against all fungi for which it has been tested. Evidence for Oomycetes is presented above. From ongoing projects we have evidence in transgenic monocots and dicots for resistance to a Basidiomycete and three species of Ascomycetes, while others have shown RNAi activity in the ascomycetes *Fusarium graminearum* (*Gibberella zeae*) and *Aspergillus flavus* (McDonald et al. (2005), Nowara et al. (2010) and Yin et al. (2011) have recently shown it to be effective against the obligate parasites *Blumeria* and *Puccinia*, respectively. As noted above, HMSPG also is effective against insect, bacterial and nematode pests.

Durability of RNAi-Derived Resistance or HMSPG

Venganza and others have demonstrated that sequence identity to the target gene must be about 70-80% to provide high level resistance (Fig. 5). Therefore, it would require mutations altering 20-30% of an essential gene sequence for a pest to overcome or develop resistance to HMSPG. Such mutation in an essential gene would likely be lethal to the pest. Furthermore, even if a pest does mutate sufficiently to overcome the action of a single siRNA, the many other siRNAs also produced from that same essential gene construct (Ho et al., 2006) will be present to hydrolyze the mRNA transcript of that essential gene at many additional sites, and only a single hydrolysis is necessary to cause the desired silencing of that essential gene. Hence, this form of resistance is recalcitrant to mutation and should provide durable resistance to all mating types, races, strains, biovars and pathovars of the important pests of date palm that we have described. Our recent discovery of near identity among the sequences of essential genes in fungal pathogens from the US and Africa strongly supports this concept.

Because of their interest in HMSPG, the National Agricultural Research Organization of Uganda negotiated a contract with Venganza, Inc. in 2009 to identify candidate genes potentially effective for the control of *Fusarium oxysporum cubense* (FOC) and *Mycosphaerella fijiensis* (MF), which cause Fusarium wilt and black sigatoka, respectively, in bananas. Using our *in vitro* assay we identified candidate genes for conferring resistance to these pathogens. Genes known to be essential for fungal survival were selected. Because the FOC genome has not been sequenced, the sequenced genome of the closely related *Fusarium graminearum* (*Gibberella zeae* = GZ) was used to identify sequences for PCR oligonucleotide primers to amplify gene segments from genomic DNA of FOC and MF, which like GZ are both ascomysetes. The well-annotated genomic sequence of *Neurospora crassa*, another ascomycete, and accessible through the National Center for Biotechnology Information (NCBI), also was used for comparison, as were the expressed sequence tags (ESTs) for FOC and MF available through NCBI. PCR primers were designed for 14 FOC and 12 MF candidate genes, and the amplicons obtained in all 26 cases from genomic DNA of both Ugandan and Florida isolates of FOC and MF. The

dsRNAs transcribed from the gene segments were effective against both Ugandan and Florida isolates of FOC and MF. Sequencing of the cloned amplicons from both Ugandan and Florida isolates of FOC and MF revealed that the nucleotide sequences of the same essential genes were essentially identical, demonstrating the highest possible level of conservation among these essential genes, and therefore the broad applicability of HMSPG to disease control on both continents, and probably worldwide.

Conclusions

HMSPG now provides to agriculture and plant breeders an entirely new and unique source of genes for pest resistance - the essential genes of the pests themselves. Using HMSPG to develop resistant plant varieties is potentially much more rapid than conventional breeding. HMSPG inserts specific genes for resistance into proven and accepted plant varieties with no phenotypic changes. Several genes may be stacked on a single or on multiple constructs, thereby conferring durable resistance to several pests with a single transformation event. This benefits plant breeders because transgenes are inherited as single dominant genes. Where sequences of essential genes are not available for a particular pest, we have demonstrated that sequences of closely related pests can be used to prepare PCR primers and those amplicons sequenced to confirm the gene's identity. Therefore, we conclude that HMSPG should be tested immediately and could provide durable resistance to serious pests affecting date palm.

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REGULAR ARTICLE

Envision of an international consortium for palm research

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Abstract

An increasing number of insects and diseases are destroying palm trees of high economic and aesthetic value throughout the world. Global climate change presents another challenge for palm distribution. Efforts to reduce damage caused by these biotic and abiotic stresses are being made by scientists worldwide. Individual efforts may be duplicative and sometimes unsuccessful. Interdisciplinary approaches combining expertise of pathologists, entomologists, biotechnologists, and breeders should be more effective. We propose the formation of an International Consortium for Palm Research (ICPR) to foster innovative international research collaborations for palm improvement, productivity and utilization. Funding of this nonprofit Consortium is envisioned to include donations from potential beneficiaries in proportion to the potential benefits received. Potential donors might include agricultural ministries and public and private organizations in various affected countries as well as international organizations interested in palm development. The host of ICPR is envisioned to be the Kingdom of Saudi Arabia with an international board directing activities and awarding meritorious research proposals. Tangible evidence of scientific accomplishment, publications, ancillary funding, and international patent applications would be key criteria for receiving research awards. Current research priorities are highlighted including the extremely serious red palm weevil (*Rhynchophorus ferrugineus*) occurring from the Middle East to Asia and California, USA; *R. palmarum* vectoring the very serious red ring nematode (*Bursaphelenchus cocophilus* baujard) in the Caribbean and Central and South America; at least three species of *Fusarium*, one possibly airborne, occurring from Morocco to Florida, USA; *Phytophthora palmivora* destroying the oil palm industry in Colombia; Ganoderma causing serious losses from Malaysia to Florida, USA; and phytoplasmas including lethal yellowing and Al-Wijam.

Key words: agriculture; Arecaceae; biotechnology; collaboration; consortium; development; research; palm; pests

Introduction

Many species of palm trees provide food, shelter, fiber, income and aesthetic value to millions of the world's citizens. Despite these major economic and social contributions, palm production throughout the world is threatened by an increasing number of insect and disease pests as well as overall production inefficiency (El-Juhany, 2010). Moreover, abiotic factors such as water availability, soil composition, and temperature range are main determinants of agricultural productivity. Many palms are grown under adverse environmental conditions to which they have acclimatized. Current global climate change is perceived to negatively impact crops and thus threaten global food security (Schmidhuber and Tubiello, 2007). Global warming may encourage the appearance of new insects and diseases and the disappearance of some beneficial organisms. Plant breeders and agronomists are faced with the difficulty of predicting the impact of climate changes on global or regional or national agriculture and therefore new varieties must be developed and distributed regularly at the national and regional levels for sustainable crop production. In addition to developing new varieties that can be readily adapted in a short period on different locations with varying agro-climatic and growing conditions, and low available resources (Jain, 2010). Conservations of genetic resources along with the applications of conventional breeding in combination with the appropriate biotechnological techniques such as mutagenesis, genetic engineering, and molecular breeding are essential for such strategies. Expanding the utilization of palm species to innovative technologies, like the

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production of natural pharmaceuticals and the potential use in biofuel industry, would further the economic importance of these species.

In this communication, we propose the establishment of an International Consortium for Palm Research (ICPR) to foster research collaboration with the objective of supporting palm improvement and utilization on a global basis. Despite notable research progress, numerous problems still persist and threaten the existence of many palm species.

Consortium is a Latin word meaning partnership, association or society. It can be further defined as “an agreement, combination, or group (as of companies) formed to undertake an enterprise beyond the resources of any one member” (Merriam-Webster Online Dictionary, http://www.merriam-webster.com/). This organizational and funding approach is a common practice to support various aspects of agriculture worldwide, where the research needs for a commodity are greater than the resources of a single member. These groups may be formed under several different names including associations, foundations and growers groups for commodities as diverse as potatoes, grapes, wheat, hops, soybeans, corn, cotton, horseradish, citrus, etc. Each consortium collectively funds research specific to their commodity needs through a self-tax or check-off system. The growers contribute a certain amount or percentage per unit of production (pound, box, bushel, barrel, acre, hectare, etc.). The funds are collected and pooled, and then allocated to support meritorious competitive research projects deemed necessary to improve production practices and sustain yields for that particular commodity. We propose this consortium approach for solving problems in palm production on a global basis.

Background

The order Arecales contains only one family, the Arecaceae (syn. Palmae), which comprises 2,400 species in 190 genera. Recently a new phylogenetic classification of Arecaceae was published (Dransfield et al., 2005), and modern molecular techniques have contributed significantly to establishing genetic relationships among the palms (Anzizar et al., 1998; Baker et al., 1999; Asmussen et al., 2006). Moore and Uhl (1973, 2011) stated that the palms with the greatest importance in world commerce are the coconut (*Cocos nucifera*) and the African oil palm (*Elaeis guineensis*); both are prime sources of vegetable oil and fat.

The top coconut producing countries are Indonesia, Philippines, India, Brazil and Sri Lanka, respectively. Collectively, they produce 50.7 million tons annually, or 93% of the world’s production (FAOSTAT data, http://faostat.fao.org). Coconut has culinary, domestic and industrial uses, and nearly 12 million hectares are planted in 86 countries. About 96% of the crop is grown by 10 million resource-poor families, on holdings under 4 ha, and more than 80 million people depend directly on coconut and its processing for their livelihoods (http://www.fao.org/docs/eims/upload/216252/Infosheet_Coconut.pdf).

Oil palm provides a major source of oil for cooking, biodiesel fuel production and many industrial uses. It is cultivated in tropical areas of Asia, Africa and South America. With the global demand for edible vegetable oils increasing strongly in recent decades, palm oil production has expanded rapidly to meet that demand. Since the 1990s the area under cultivation increased about 43%. Seventeen countries produce palm oil, and about 4.5 million people earn a living from it. The five major producing countries are Indonesia, Malaysia, Thailand, Colombia and Nigeria, respectively. Collectively, they produce 47 million MT annually, or 94% of the 50.2 MMT produced, with Indonesia alone producing 51% of the total (http://www.greenpalm.org/en/about-palm-oil/where-is-palm-oil-;grown http:// www. indexmundi.com/ agriculture/? commodity=palm-oil&graph= production).

The date palm (*Phoenix dactylifera*) also is of major importance. It has been cultivated as a tree crop for at least 5,000 years (Johnson, 2011). A very important plant throughout the world, it is perhaps the most important plant in Saudi Arabia and throughout the Middle East. It has high socioeconomic importance, due not only to its food value, but also its capacity to provide many other products such as shelter, fiber, clothing, aesthetic beauty and furniture (Mousavi et al., 2009). It has high natural tolerance to very adverse growing conditions, including drought, salinity and high temperatures (Bakheet et al., 2008). In 2007 nearly 1.1 million ha of date palm were harvested, yielding 6.91 million tonnes. The major producers were Egypt (19%), Iran (15%) and Saudi Arabia (14%) (FAOSTAT data, http://faostat.fao.org).

In addition, many species of palms are used extensively as ornamentals in warm regions throughout the world, or indoors when a tropical effect is desired. The word palm is even used to indicate the tropical or verdant nature of municipalities such as Palm Beach, Palm Coast and

Palm Springs in the United States. Sales of palms for ornamental purposes provide millions of dollars annually to economies throughout the world. Annual sales are valued at $70 million in California and $127 million in Florida alone (Anonymous, 2010).

Breeding palms for pest resistance or for other desirable characteristics by traditional breeding techniques is very difficult and time consuming. It is hindered by life cycle longevity, the palm's highly heterogeneous genetic nature, and the difficulty to propagate uniform plants in large numbers. However, with recent scientific advances palm improvement can be accomplished through biotechnological approaches (Jain et al., 2011), such as genetic transformation (Saker et al., 2007; Habashi et al., 2008; Mousavi et al., 2009; Saker et al., 2009) and *in vitro* selection (El Hadrami et al., 2005; Al Mansoori and Alaa El-Deen, 2007; Jain, 2010). Combining these approaches with other emerging molecular techniques holds great promise to control insects (Whyard et al., 2009) and disease pests of palm (Niblett and Bailey 2012, this issue). Mutagenesis is another effective approach to combat palm diseases (Jain, 2005, 2007, 2010, 2011).

Consortium Structure and Function

Justification

Numerous researchers are working on palm improvement worldwide. Unfortunately, many are working in isolation, their research may be poorly funded, and their research may be inadvertently duplicated. To minimize these issues, to maximize research output and to give palm research new visibility we envision the creation of the International Consortium for Palm Research (ICPR).

Objective

To foster innovative international research collaborations for palm improvement, productivity and utilization.

General priorities

The consortium is perceived to engage in research towards: improving resistance to palm pests, diseases, and other biotic stresses, enhancing palm tolerance to abiotic stresses and global climate change, promoting conservation and utilization of genetic diversity of palm, and developing novel industrial applications for palm products (e.g. bio-energy, pharmaceuticals, etc.)

Multidisciplinary

This consortium advocates interdisciplinary research approach involving different science disciplines including biotechnology (e.g. bioinformatics, genetic engineering, genomics, molecular biology, and plant tissue culture), entomology, plant breeding and genetics, plant pathology, plant physiology, and food science and nutrition.

Membership

Membership is open to individuals and organizations with nominal fee. Members should be affiliated with industry or academia and have demonstrated significant research activities related to palms.

Location

We propose that the consortium headquarters be located at the Date Palm Research Center, King Faisal University, Al-Hassa Saudi Arabia. This location was selected because of their proven expertise in palm technology, the interest of the institution, and the proximity to major date palm agricultural areas.

Governance

The consortium will be managed by unsalaried Board of Directors (BoD) representing different geographical regions, commodities and expertise. They will normally serve three-year terms except for the initial BoD members who may be reassigned to ensure continuity and orderly succession. The BoD will make the daily management decisions, and their decisions will be made by majority vote and announced to the Membership. The BoD shall present proposed bylaws to the membership for ratification and adoption, and they shall solicit the involvement of the membership by electronic polls and for review of manuscripts and submitted grant proposals, and for other issues as they may occur.

Bylaws

The consortium bylaws will be drafted by the BoD in consolation with members and financial donors. Those of the West Chester University Research Consortium might be viewed as an example at: http://www.wcupa.edu/wcurc/bylaws.htm

Funding acquisition

Support funding will be solicited from growers and grower organizations involved with date, oil, coconut and ornamental palms. Equally important are the countries, states, municipalities and companies that depend on palm trees to provide the ambiance in which they do their business. These include such diverse agencies as the governments

of Saudi Arabia and Malaysia, Colombian Oil Palm Growers, the Walt Disney Company, cities of Palm Springs, Palm Beach, Miami, Miami Beach, California Date Growers Association, Florida, Arizona, Nevada and California Departments of Tourism, Florida and California Ornamental Growers and Nurserymens Associations, etc.– all contributing in proportion to the value that palms bring to their agency or institution.

Information resources

A full-time paid computer specialist will be responsible to maintain an active and up-to-date website, with a bulletin board for notices of general interest, a list of active members and their affiliations, a periodic electronic newsletter, and a literature collection of recent major palm publications, in addition to links to existing information resources. The consortium website is intended to be the first place an interested party would go to for information related to palms. Examples of linked resources include: International Palm Society website, Coconut group, Oil palm group, Date Palm Global Network, and Arab Palm League.

Funding of research proposals

The BoD will set annually the research priorities. Whenever funds are available a call for proposals will be sent to the membership. Proposals will be peer-reviewed by a selection panel comprised of experts selected by the BoD, who will maintain database of international experts. The high quality research proposals will qualify for funding, that would include appropriateness of the proposal, tangible evidence of scientific accomplishment, publications in international refereed journals, ancillary funding, and international patent applications. The project director would submit an annual progress report and further planning of the project. The further renewal of project funding would be done based on the annual progress. Within three months following completion of the research, a detailed report will be submitted to the BoD, along with any publications or patent applications. Ownership of patents would be mutually agreed upon according to predefined rules governing this process.

Examples of Research Priorities

Palms are affected by large number of pests, including insects, nematodes and diseases caused by fungi, bacteria and phytoplasma (Downer et al., 2009). Four of these major pests will be discussed as examples:

Red palm weevil

The red palm weevil (RPW), *Rhynchophorus ferrugineus*, a member of Coleoptera: Curculionidae, is a concealed tissue borer and lethal pest on over 20 species of palm, including date palm (Figure 1) and coconut palm (Abraham et al., 1998; Esteban-Duran et al., 1998). Gomez and Ferry (Gomez and Ferry, 2002) indicate it "has become the most important pest of the date palm in the world". Its aboriginal home is South and Southeast Asia, where it is a key pest of coconut (Nirula, 1956; Faleiro and Kumar, 2008). First reported on date palm in Iraq (Buxton, 1920), then from Rass-El-Khaima in the UAE in 1985, it reached the eastern region of the Kingdom of Saudi Arabia in 1985, and afterwards spread to numerous other areas in the Kingdom (Abuzuhairah, 1996). It was recorded in Iran in 1990 (Faghih, 1996), then in Egypt in 1992 (Cox, 1993). By 1994, it had been found in the south of Spain (Barranco et al., 1995) and in 1999 in Israel, Jordan and the Palestinian Authority Territories (Kehat, 1999).

The RPW has now spread to all the countries of the Gulf region in the Middle-East, infesting approximately 5% of the palms in the region with an annual infestation rate of about 1.9% (Abraham et al., 1998; Zaid et al., 2002). FAO has now identified it as a category-1 pest of date palm in the Middle-East (El-Sabea et al., 2009). Subsequently, the weevil moved from North Africa into Europe, where it was reported for the first time in Spain, Portugal and the Canary Islands (Faleiro, 2006a,b) and in 2009 in the Caribbean (Curacao, Dutch Antilles), potentially from date palms imported from Egypt for landscaping (Reijnaert, 2009). In August of 2010 the RPW was reported in Laguna Beach California (Anonymous, 2010), where it now poses a serious threat to California's $30 million dollar date crop industry, as well as ornamental palm tree sales valued at $70 million in California and $127 million in Florida.

The RPW can be controlled by insecticide applications, but it is expensive and pollutes the environment. Instead, an integrated pest management approach has been adopted. It includes prohibition of movement of infested plants, extensive monitoring of insect populations, the use of food-baited pheromone traps to reduce adult weevil populations and strategic insecticide applications. But this program is labor intensive and expensive to implement. The consortium is in support of using advanced technologies to tackle this insect problem. Technologies intended include biological control aspects, genetic engineering of baculovirus containing neuro-toxin gene and Bt gene for examples.

Figure 1. Red palm weevil infestation. Source: (El-Sabea et al., 2009).

Fusarium diseases

Bayoud disease (BD) of date palm is caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *albedinis* (FOA). It is a lethal root rot and vascular wilt disease of date first reported in 1870 in the Drâa valley of Zagora, Morocco. It has been spreading continuously eastward, and within one century had killed more than twelve million palms in Morocco and three million in Algeria (Zaid, 2002; Quenzar, 2001). Oases that formerly had 300-400 palms per hectare were reduced to 40-50 palms per hectare (Djerbi et al., 1986), and BD was destroying the most renowned varieties such as Medjool, Deglet Nour, and Bou Fegouss). BD also has reduced the production of desirable annual crops formerly supported under date palm culture, and has accelerated the desertification of the region, with farmers abandoning their land and moving to large urban centers.

FOA is a soil inhabiting fungus, persisting as hard-walled chlamydospores in dead tissues of diseased palms. Chlamydospores are released from disintegrating tissues into the soil, where they remain dormant and survive for more than eight years. Controlling BD is difficult, if not impossible, by current cultural methods once FOA becomes establishes in a plantation, because of the persistence of the fungus, the movement of soil and the flow of irrigation water. Individual infected plants can be eradicated and the soil fumigated with

methyl bromide or chloropicrin, but this is expensive and time-consuming. Some BD-resistant date palm selections have been reported (Djerbi et al., 1986), but introgression of resistance into desirable varieties by conventional breeding is difficult and time-consuming.

Fig. 2. Queen palm in Florida dying from Fusarium wilt.

In addition to FOA there are several additional Fusarium pathogens of palm. Elliott (Elliott, 2009) recently confirmed the spread of new palm diseases in Florida including two formae speciales of *Fusarium oxysporum*, with f. sp. *canariensis* (FOC) causing Fusarium wilt of Canary Island date palm (*Phoenix canariensis*) and a new forma specialis causing Fusarium wilt of queen palm (*Syagrus romanzoffiana*) (Figure 2) and Mexican fan palm (*Washingtonia robusta*). FOC also attacks Canary Island date palm in Australia, Italy, France, Japan, the Canary Islands and California, where it can kill 40-50 year old trees (Feather et al., 1989; Simone and Cashion, 1996; Smith et al., 2003). The California isolate caused sufficient concern to warrant a quarantine to protect the California date industry (Feather et al., 1989). Fusarium remains a threat to palm species worldwide. Resistant to Fusarium may be developed by mutation technology and genetic engineering in conjunction with genomic studies of palms and their pests and diseases. Radiation-induced mutation proved applicable to the development of date palm resistance to FOA (El-Hadrami et al., 2005; Jain, 2005, 2007, 2011).

Phytophthora palmivora

Bud rot disease or "pudricion del cogollo" is a major disease of African oil palm in Colombia (Fig. 3). This soil and air borne disease has killed thousands of trees and been known for more than 40 years in Central and South America. But the causal agent has only recently been identified as *Phytophthora palmivora* (Torres et al., 2010). It has spread widely in Colombia and threatens to make oil palm production unprofitable. Bud rot of coconut also is caused by *P. palmivora* (Ridings, 1972). It would be economically ruinous to the Malaysian oil palm industry if *P. palmivora* was introduced there. Two species of Ganoderma currently cause butt rot and serious losses in oil palm production in Malaysia (Zakaria et al., 2005).

Phytoplasmas

Phytoplasmas are bacteria without cell walls, and they cannot be cultured in microbiological media. There are several different phytoplasmas that affect palms. These diseases usually occur in tropical or subtropical climates and cause symptoms ranging from mild yellowing to death of the infected plants. Transmission from plant-to-plant requires an insect vector, usually a leafhopper or plant hopper.

Probably the best known and most destructive phytoplasma disease is lethal yellowing (LY) of coconut, which has killed millions of coconut palms in Florida and throughout the Caribbean (Fig. 4). It infects and kills many species of palm including *P. dactylifera*, and is vectored by a planthopper, *Haplaxius crudus* (previously *Myndus crudus*) (Torres et al., 2010). Texas *Phoenix* palm decline (TPPD) occurs on *Phoenix canariensis* in Texas and on *Phoenix sp*. and *Sabal palmetto* in Florida (Harrison and Elliott, 2009b). Al Wijam (AW), another phytoplasma disease, was first observed on date palm in Al Hassa, Saudi Arabia (Nixon, 1954), and it was recently characterized by Alhudaib et al. (Alhudaib et al., 2007). Phytoplasma are currently classified by comparing restriction fragment length polymorphism (RFLP) patterns for polymerase chain reaction (PCR) amplicons of their 16S rDNA and naming them after the major phytoplasma whose RFLP pattern they most closely resemble. Therefore, LY is *Candidatus Phytoplasma palmae* subgroup 16SrIV-A; TPPD is Candidatus Phytoplasma palmae subgroup 16SrIV-D; and AW is *Candidatus* Phytoplasma asteris group 16SrI (Alhudaib et al., 2007; Harrison and Elliott, 2009a,b).

Figure 3. Thousands of oil palms dying in Colombia from infection by *Phytophthora palmivora*.

Figure 4. Coconut palms in Jamaica dying from lethal yellowing phytoplasma.

Phytoplasma diseases cannot be controlled, but symptoms are diminished and tree life extended by injections of tetracycline. This is practical only for "specimen trees" in an expensive landscape (Harrison and Elliott, 2009a).

Genetic resistance is available in palm species to some or all of the serious pests of palm, but it has not been introgressed into those which are susceptible because of the difficulties in palm breeding. Current and emerging molecular techniques show promise for overcoming many of these impediments.

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BOOK REVIEW

Dates: Production, Processing, Food, and Medicinal Values

[A. Manickavasagan, M. Mohamed Essa and E. Sukumar (Eds.). 2012. Medicinal and Aromatic Plants – Industrial Profiles, Vol. 50. CRC Press, Boca Raton, Florida, USA. 415 pp. Illustrated. Hardcover. ISBN 9781439849453. USD 149.95.]

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World date palm (*Phoenix dactylifera*) cultivation has expanded rapidly in recent years, with fruit production more than doubling from 1991 to 2010, to reach 7.9 million metric tons. This expansion has been led predominantly by the countries of the Arabian Peninsula and North Africa, and bolstered by a steady stream of technical conferences, research articles and books on the date palm.

This well-edited and attractively-printed new book represents the work of numerous contributors from a dozen date-growing countries; more than one-half the contributors, as well as the editors, are based in Oman, and hence many Omani examples are cited. In organization, the book is made up of 29 chapters which are divided into four sections as indicated in the subtitle. Part one on production is the longest. Chapters on mechanization of pollination and fruit harvesting are particularly welcome. Also included are discussions of water management, salinity problems, fertilizer application, pest management, date marketing, tissue culture techniques, as well as an account of a date palm genome project in Saudi Arabia. Publication of the latter chapter is perplexing since the same material appeared a year ago in another date palm book (Jain, S. M., J. M. Al-Khayri and D. V. Johnson, Eds., 2011. Date Palm Biotechnology. Springer, Dordrecht, Netherlands).

Fruit processing is broadly addressed in Part two. Detailed reviews are presented on drying fruits using the sun, solar tunnels, hot air and microwave, and the associated physical and chemical changes which occur. Other chapters deal with computer-assisted fruit assessment, a comprehensive review of alternative fumigants as methyl bromide use is phased out, the range of products derived from date fruits and seeds, and the potential of using processing waste for biofuel production.

The third part of the book focuses on date use as food and feed, and products derived from them, along with their physical, chemical and structural characteristics. Also examined is the use of dates as a fermentation substrate and as a sugar additive in food. Date fruits unfit for human consumption, along with fruit processing waste, have long been fed to animals at a low percentage of overall diet. Shredded date palm fronds can also be fed to animals during times of feed shortage. Such wider uses of products from the entire date palm merit greater research attention.

A chapter tracing the historical and religious role of the date palm introduces the last section of the book. Also discussed is the nutritional value of dates as a functional food, neutraceutical and antioxidant source. Medicinal use of the date and other species of *Phoenix*, are discussed in relation to Ayurveda and other traditional medicines of India, with claims of treating a host of diseases, despite acknowledgement of the lack of supporting scientific research. The final chapter reports on preliminary research in Oman on the effect of date consumption as an inhibitor of fibrillation of Aβ protein as a defence against Alzheimer's disease, which could lead to developing a new treatment therapy.

This book covers a wide range of topics and provides current reviews of many of the key issues confronting date cultivation today; as such it represents an important milestone in date palm science and I recommend it.

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