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Review Article

Prospects of *in vitro* conservation of date palm genetic diversity for sustainable production

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Abstract: 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. 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 become necessary for the development and improvement of date palm cultivars for sustainable crop production. The conservation, distribution, and utilization of natural and induced genetic diversity have become essential by the establishment of gene/germplasm bank both at the national and international levels. 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.

Keywords: Plant genetic diversity, sustainable agriculture, cryo-storage, somaclonal variation, gene bank.

مقترحات لحفظ التباين الوراثي لنخيل التمر للحصول على انتاج مستديم

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الملخص: يعد التباين الوراثي النباتي اساسياً لبرامج التحسين الوراثي للمحاصيل لتحقيق الزراعة المستدامة، وفقدها المتسارع هو نتيجة للنمو السكاني السريع والتصنيع و إزالة الغابات والكوارث الطبيعية. وفي المستقبل، قد يكون للتغير المناخي أثر سلبي على إنتاج نخيل التمر والمحاصيل الأخرى. يعد حفظ و توزيع والإستعمال الأمثل للتباينات / الموارد ا لوراثية النباتية يصبح ضرورياً لتطوير وتحسين أصناف نخيل التمر لتحقيق الإنتاج المستديم. يعد حفظ و توزيع والإستعمال الأمثل للتباينات الوراثية الطبيعية والمستحدثة اصبح اساسياً بتلأسيس بنوك الجينات و الأصول الوراثية على كل من المستوى الوطنى والدولي. ويشجع بنك الجينات الباحثين على التنقيب عن، و ملاحظة التباينات الوراثية للعشائر الطبيعية والأجناس المستأنسة في المزارع. تعد تقانة الحفظ في الأنبوب، الحفظ بالتجميد أو التخزين بالتجميد و التخزين بالتبريد نظم ممتازة لحفظ الموارد الوراثيةلاشجار الغابات و المحاصيل البستانية. ويعيب التخزين بالتبريد الحاجة إلي تجدبد المزرعة بتكرارية عالية وهو ما قد يؤدي إلى مخاطر التلوث والتباين الجسدي. التخزين بالتجميد يتميز بالقدرة على التخزين لفترات ممتدة بدون العيوب السابق ذكرها. لهذا السبب، تعد مزارع الأنبوب مناسبة كم في الأجنة الجسدية/ المعلقات الخلوية، كاللوس، ويجب أن يكون قادراً على استولاد نباتات بها أقل نسبة من التباين الجسدي. تعد تقنية الأجنة الجسدية هي الأكثر شيوعاً لنخيل التمر، والتي تعتمد على الطرز الوراثي وبيئة الزراعة المستخدمة للإكثار برغم احتمال حدوث تباينات جسدية فى النباتات المستولدة. تم حفظ الأجنة الجسدية لنخيل التمر بالتبريد ،لأول مرة، في تونس من خلال مشروع بحثي مع منظمة الأغذية والزراعة وهيئة الطاقة الدولية، وإن لم يتم استيلاد نباتات بعد. وفي آسيا، يعد المكتب الوطني للأصول الوراثية بالهند أكبر بنك موارد وراثية، ويحفظ بالأساس الأصول المحلية من بذور وأجزاء خضرية للمحاصيل كما يبتكر حاصلات جديدة.

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Introduction

The human population is rapidly increasing, especially in the developing countries and the demand for continuous supply of food is growing. Plant breeders are faced with the challenge to sustain the food production to meet the demand of ever-growing human population under the erratic climatic change. Several factors such as abiotic and biotic stresses, industrial pollution, deforestation, loss of genetic diversity, soil erosion, water shortages, improper facilities for the conservation and proper use of genetic resources, loss of arable land due to rapid industrialization, and poor infrastructure are responsible for having a negative impact on sustainable agriculture. The loss of genetic diversity is continuing unabated and as a result useful genetic material is regularly being lost, that will have great an adverse impact on sustaining food and agriculture. Moreover the climate change is gradually becoming another major hurdle for sustaining agricultural production. Thereby the conservation of local germplasm is utmost essential for proper utilization in future crop improvement. New tools would be required such as biotechnology, molecular biology, mutagenesis together with conventional plant breeding to assist plant breeders for the development of new cultivars and grow the on the available arable land under the climate change.

Date palm (*Phoenix dactylifera*) is highly heterozygous plant and considered as one of the most ancient cultivated plant since over 4000 years, and has gone through continuous selections by man and as a result the genetic diversity has narrowed down. Very few plant species have been so closely connected with the survival and well being of humans living in hot and arid environments. As well, date palm tree has a great socioeconomic importance and nutritional value in the Middle East and North Africa. In addition, date palm trees withstand adverse environmental changes such as drought, rains, flood, temperature fluctuations, and also help in preventing desertification. These drastic climatic changes could have adverse consequences by losing the genetic diversity. Date fruits are very important source of human nutrition as well as an export item for date palm growing countries. The most importantly the plant contributes in creation of 'microclimate' within the fragile oasis ecosystem and allows development of agriculture in drought and saline conditions; multiple cropping system such as grapes, tomato and so on. The genetic diversity of date palm represents different local varieties and cultivars grown under varied climatic conditions. The most popular Sudanese date palm varieties described as soft and dry types, and showed detectable genetic variation in fruit morphology (Figures 1 and 2; Elshibi, 2009). Moreover no conventional methods ***e.g.*** seed storage were not used to conserve genetic diversity due to high nature of heterozygosis for developing date palm breeding programs. With the development of *in vitro* culture techniques- organogenesis, somatic embryogenesis, embryo rescue–plant regeneration has already been accomplished, which is highly genotypic dependent; has made possible short- and long-term storage of *in vitro* cultures, large-scale multiplication of plants of elite genotypes.

In vitro propagation of date palm

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, 2007). In addition, half of this progeny will be composed of male trees which aren't distinguished before flowering stage. The female plants will 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.

The use of plant tissue culture techniques such as somatic embryogenesis and organogenesis is highly suitable for large-scale plant multiplication of vegetatively propagated crops. The success of this approach is very much genotypic dependent. *In vitro* techniques have successfully been applied for plant propagation in wide ranging crops including date palm (Jain, 2007). Micropropagation via organogenesis is widely used for rapid clonal propagation of elite genetic material of date palm. 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).

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, 2007). 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.

Figure 1. Genetic diversity is clearly visible in fruit morphology of best known Sudanese date palm cultivars (Elshibi, 2009).

Figure 2. In Sudan, farmers use a well-known trait 'orientation of spine along the base of the leaf' to distinguished between the Laggai and Khateeb cultivars- either alternate or opposite arrangement, with two spines or a single spine, respectively as well as by fruit shape and color. The spine character is unstable in other cultivars (Photography by S. Elshibi).

Conservation of plant genetic material

Both natural and induced plant genetic diversity conservation has become an important issue among plant breeders and agronomists for utilizing in improving food, feed, fibre, industrial crops worldwide, and its loss is a greater concern to plant scientists for present and future agriculture. The loss is mainly due to rapid industrialization, fire, deforestation, and even environmental pollution. Moreover, lack of availability or non-existence of desirable genotypes hampers plant breeders for developing new varieties. The most common method used for the conservation of plant genetic resources is by seeds and stored at low temperature. It is quite cost effective, safer, and easy to handle. The second approach is *in vitro* conservation of *in vitro* cultures in cold storage and cryo-storage (Bekheet et al., 2001, 2005, 2007). The success of this approach is very much dependent on plant regeneration and the maintenance of genetic fidelity of the stored genotypes. Nowadays international germplasm exchange has become more cumbersome and sometimes it is rather difficult to obtain elite genotype. Many countries don't like to share the germplasm because of patent and ownership problems, and have established National germplasm conservation or gene bank facilities. In India, National Bureau of Plant Genetic Resources (NBGR) was established in Delhi to conserve local and imported genetic material for proper utilization in developing new cultivars.

Community seed bank

The community seed bank is quite common at the village level for the preservation of local varieties and agriculture production in many developing countries. Farmers rely on informal seed systems based on local growers retention of seed from previous harvests, storage, treatment and exchange of this seeds within and between the communities. The informal seed sector is typically based on indigenous structures for information flow and exchange of seeds. Seed banks managed within this local seed system operate on a small scale at the community level with few resources. In date palm also the local high quality genetic material is conserved at the village or community level by preserving seeds. For more see http://www.bioversityinternational.org.

Seed gene banks

Seed banking is most widely used for the conservation of plant genetic resources. Initially the moisture content of seeds is lowered by drying them and stored at subzero temperatures in cold stores or deep freezers (Figure 3ab). Over 90 percent of global plant genetic material accession is stored by seed banking, e.g. all gene banks of CGIAR institutes. The main problem with this technique is inability of seeds to withstand desiccation at lower temperatures and that may hamper seed germination rate or survival of seeds may seriously effect and ultimately die. For more see http://www.bioversityinternational.org.

Figure 3a. Seed bank at CIMMYT.

Figure 3b. Seed bank at CATIE, Costa Rica.

Figure 4. Cryopreservation facilities at CATIE, Costa Rica.

Field gene banks

The genetic material is collected and planted in the field or orchard either in the same or different location. These gene banks have traditionally been used for perennial plants, including

- species producing little or no seeds;
- species that are preferably stored as clonal material
- species have a long life cycle to generate breeding and/or planting material.
- Species producing recalcitrant seeds

Crop species cocoa, rubber, coconut, coffee, sugarcane, banana, tuber crops, tropical and temperate fruits, vegetatively propagated crops, such as wild onion and garlic, and forage grasses are most common conserved in field banks. Date palm would be ideal to conserve in field gene bank. For more see http://www.bioversityinternational.org.

Bud bank or Vegetative banks

Seed banks are most widely used for genetic material conservation, and the role of bud bank or vegetative bank has received little attention (Klimesona and Klimes, 2007). The term bud bank was coined by Harper (1977). The bud bank consists of all buds that can potentially be used for vegetative propagation/regeneration (see more details Klimesona and Klimes, 2007). The advantage of bud bank is to exploit innate dormancy and induced dormancy, which is induced by drought or cold (Anderson et al., 2001). Another type of dormancy beneficial to bud banking is correlative inhibition which is represented by apical dominance. This mechanism prevents actively growing apical buds growth of axillary and adventitious buds situated below apical meristem. As a result buds remain available for vegetative regeneration until an injury breaks the apical dominance (Klimesona and Klimes, 2007).

***In vitro* storage bank**

In vitro storage, keeping plant tissues under strict sterile conditions are stored in petri dishes, glass tubes and vessels. By lowering the temperature above zero degree, plant tissue growth is drastically reduced and that minimize that frequent subcultures on the fresh culture media. These banks are unsuitable for long-term storage of plant material. For species with so-called 'recalcitrant' seeds or species that are vegetatively propagated, such as roots tubers and aroids, different conservation techniques are used at low temperatures. For more see http://www.bioversityinternational.org.

Cryo-storage bank

In these gene banks, the living tissues are stored at ultra low temperature -196°C in liquid nitrogen (Figure 4) for long-term storage as well as prevent tissue culture derived genetic variation. Plant species with recalcitrant seeds or vegetatively propagated plant species are conserved Nowadays a wide range of species can now be routinely cryopreserved: banana (*Musa* spp.) (Panis et al., 2001), cassava (*Manihot esculenta*), bramble fruits (*Rubus),* pear *Pyrus,* vegetables in the *Solanum* family, coffee (*Coffea arabica*), oil palm *(Elaeis guineensis)* and tea (*Camellia sinensis)*.

International germplasm banks

Several countries have established National germplasm banks for the conservation, utilization, and distribution of genetic material for research to develop new cultivars as well as to protect from their false claimers and bio-pirates, e.g. neem tree, basmati rice, and turmeric were claimed by some companies to have exclusive rights to use them, and the Indian Government challenged these companies, and finally won the case. High value genetic material is being protected by several Governments for preventing false claims of greedy companies. Date palm germplasm conservation needs to be established for preserving high quality date genetic sources and establish a genetic data base of available date palm genetic material that would facilitate in developing new cultivars in date palm growing countries.

Gene banks at various CGIAR institutes have been established and conserve different types of crop depending on the location (Table 1). Currently, CGIAR gene banks hold a total of 629,022 samples of crops and their wild relatives. There is no indication of well established date palm gene bank under the CGIAR and in any date palm growing

countries. Most likely date palm growers maintain their own high quality genetic material without sharing with other growers, and also for improving the date palm quality. In the future, date palm could be used as a bio-energy crop for the production of bio-ethanol, and would give extra income to growers. The selection of appropriate date palm genetic material would be crucial for producing date fruits for food, feed, and bio-energy.

Table 1. Gene banks of CGIAR institutes.

CGIAR gene bank	Full name	Website	Collections	# of sample collections
WARDA	Africa Rice Center	www.warda.org	Rice	14,751
CIAT	Intern. Ctr. Trop. Agriculture	www.ciat.cgiar.org	beans, cassava, forages	64,760
CIMMYT	Intern. Maize & wheat Imp. Centre	www.cimmyt.org	maize, rye, triticale, wheat	118,142
CIP	Intern. Potato Center	www.cipotato.org	potato, sweet potato, Andean roots and tubers	13,508
ICARDA	Intern. Center Agric. Res. in the Dry Areas	www.icarda.org	barley, chickpea, faba bean, forage, lentil, wheat	126,518
ICRISAT	Intern. Crops Res. Inst. for Semi-Arid Tropics	www. icrisat.org	chickpea, groundnut, pearl millet and others, pigeonpea, sorghum	110,476
IITA	Intern. Inst. Tropical Agriculture	www.iita.org	bambara groundnut, cassava, cowpea, soybean, yam	25,836
ILRI	Intern. Livestock Res. Institute	www.ilri.org	forages	17,032
IPGRI	Intern. Plant Genetic Resources Inst.	www.ipgri.org	banana and plantain	986
IRRI	Intern. Rice Res. Inst.	www.irri.org	rice	102,652
ICRAF	World Agroforestry Ctr.	www.worldagroforestrycentre.org	*Sesbania*	25

Doomsday Vault- a seed bank

Recently The Norwegian Government has built up an underground vault to store seeds to guard against major catastrophe like nuclear war, asteroid strikes, volcanic eruption, and severe climate change. It is a seed bank on a wild Arctic island 500 miles from the North Pole and stores all the known varieties of the world's crop. There are three underground chambers and each chamber has the capacity to store 1.5 million different seed samples. This facility is designed to have an almost "endless" lifetime.

National Bureau Plant Genetic Resources (NBPGR), Delhi, India

These facilities were created in 1976 to act as a nodal institute at the national level for acquisition and management of indigenous and exotic plant genetic resources for food and agriculture; and to carry out related research and human resource development, for sustainable growth of agriculture. The main aims of NBPGR are following-

- To plan, organize, conduct and coordinate exploration and collection of indigenous and exotic plant genetic resources

- To undertake introduction, exchange and quarantine of plant genetic resources
- To characterize, evaluate, document and conserve crop genetic resources and promote their use, in collaboration with other national organizations.
- To develop information network on plant genetic resources.
- To conduct research, undertake teaching and training, develop guidelines and create public awareness on plant genetic resources. They organize a short training course on *in vitro* conservation and cryopreservation.

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^{o}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.

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 (Mycock et al., 1995; Bekheet et al., 2007; Subaith 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. 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.

Conclusion

Date palm tree is very useful in North Africa and Middle East for providing nutrition to consumers, an important source of export item, capability to withstand adverse climate changes, provide a micro-climate in the desert for farmers to grow multiple crops, and could be used as a bio-energy crop for bio-ethanol production. Rapid industrialization, human population growth, erratic climatic changes, and deforestation- all these factors may lead to loss in date palm genetic diversity. Moreover, the genetic base of date palm has narrowed down due to continuous selection and gradually that may have negative impact on date palm genetic resources. So far, there is hardly known date palm gene bank to conserve, proper maintenance of gene bank record, utilization, and distribution of genetic material. The conservation, distribution and proper utilization of plant genetic diversity/resources become necessary for the development and improvement of plant cultivars for sustainable crop production by developing new varieties to face onslaught of adverse natural climatic changes and manmade disaster. Gene/germplasm banks should be established both at the national and international levels so that and facilitate easy accesses s of genetic material to plant breeders. Initially seed banks of date palm, maintained at low temperature, could be established and maintain records on the detailed information of each collected genetic material, and perhaps classify germplasm on specific traits beneficial to the industry. Also, develop a website on date palm gene bank to provide all detailed information on each stored genetic material. Mostly date palm is vegetatively propagated and seeds of elite selected germplasm may not be sufficient to supply to meet the demands of growers, researchers nationally and internationally. Seed bank is destroyed by terrorism, earthquake, natural disaster and manmade calamities as a result date palm germplasm utilization is hampered. Therefore, *in vitro* conservation and cryopreservation of genetic material is desirable for both short- and long-term storage. This approach is applicable only when availability of a reliable *in vitro* technique for plant regeneration. The most appropriate *in vitro* cultures suitable for plant regeneration somatic embryos/embryogenic cell suspension, callus, shoot tip culture, microspore, and others for short-and long-term storage. The genetic fidelity of regenerated plants needs to be maintained by preventing somaclonal variation. Cryo-storage is ideal for long-term storage of embryogenic cells, callus, shoot tip, control of different type of viruses to produce virus-free material, and prevent somaclonal variation. This method is being widely used for long-term storage in forest trees, banana, coffee and others. *In vitro* conservation and cryopreservation of date palm is feasible since date palm tissue culture is well established for plant regeneration via somatic embryogenesis and organogenesis. The use of molecular markers will be of great value to discriminate different genotypes, off-types or somaclones, and molecular marker-based data.

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