
A review
The Problems Facing the Use of Tissue Culture
Technique in Date Palm (*Phoenix dactylifera* L.)

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Abstract :

Tissue culture is a recent technique mainly used for bulk rapid propagation of several commercial plant species including date palm (*Phoenix dactylifera*). Normally, date palm is propagated *in vitro* by three methods: the first method is by embryogenesis in which vegetative embryos can continuously be formed from embryogenic callus. The embryogenesis method is characterized by its ability to produce many plants in shorter periods. The second procedure is organogenesis which provides date palm buds that eventually give plantlets without passing through the callus stage. However, since plantlets are produced directly from tissues of mother plant without passing through callus stage, they are typically identical to mother plant. The third method is *in vitro* propagation using young flowers in which vegetative embryos can be inducing from embryogenic callus. This technique depends on culturing of date palm young flowers on nutritional media with high auxin concentrations to induce callus formation. There are some major obstacles in practical application of date palm tissue culture in the laboratory such as: browning of cultured tissues, vitrification of tissues, bacterial and fungal contaminations, early rooting of tissue cultured buds, deterioration of embryonic callus and its inability to form embryos, callus formation on bases of rooting plantlets. The identified abnormalities and variations in date palm plants produced from tissue culture, such as failure of fruit set, multiple carpels, dwarfism of date palm trees, albinism of leaves, abnormal growth and development of leaves and fruit strands, terminal bud bending, dryness of apical bud and changes in fruit quality were also discussed. In conclusion, most of these abnormalities mentioned previously recover in most cases as the plants get old (10-year-old).

Key words: browning, callus, contamination, somaclonal variation, date palm, dwarfism, fruit set, *Phoenix dactylifera* , problems, tissue culture

Introduction:

Date palm (*Phoenix dactylifera* L.) is a major tree crop in arid regions of the Middle East and North Africa. It has an important impact on the economy of many countries in these regions. Date palm is traditionally propagated through offshoots. The development of propagation methods through tissue culture resulted in massive expansion of date palm plantations. While most trees generated from

tissue culture are normal and true-to-type, several typical abnormal phenotypes can be detected (Gurevich *et al.* 2005). Tissue culture as a recent developed propagation tool has made a tremendous positive impact on the agricultural sector. The tissue culture derived plants are much safer than the conventional offshoots since they are free of the major diseases and pests such as 'Bayoud' and Red Palm Weevil. Furthermore, since all contaminated cultures are discarded from the process, only healthy plants are released to the growers (Aaouine, 2003). Al-Maarie (1995) and Alkhateeb and Ali-Dinar (2002) pointed out several advantages of this technique on date palm.

Normally, date palm is propagated *in vitro* by three methods; namely: embryogenesis, organogenesis and using young flowers. Each method has its advantages and disadvantages (Alkhateeb and Ali-Dinar, 2002). In embryogenesis, vegetative embryos can be continuously formed from embryogenic callus. The technique depends on culturing proper plant parts on nutritional media with high auxin concentrations to induce callus formation. The callus can be increased abundantly by continuous transference on proper nutritional media for rapid multiplication of callus tissues. The embryogenesis method is characterized by its ability to produce many plants in shorter periods of time (Fig. 1) as mentioned by Alkhateeb *et al.* 2006.

Organogenesis provides date palm buds that eventually produce plantlets without passing through the callus stage. Relatively few plantlets can be produced with this procedure in longer period of time compared to embryogenesis. However, since plantlets are produced directly from tissues of mother plant without passing through callus stage, they are typically identical to mother plant as shown in Fig. 2 (Aaouine, 2000, Alkhateeb and Ali-Dinar, 2002, Omer *et al.* 1992). Third method is *In vitro* propagation using young flowers in which vegetative embryos can be inducing from embryogenic callus. The technique depends on culturing of date palm young flowers on nutritional media with high auxin concentrations to induce callus formation. The callus can be increased abundantly by continuous transference on proper nutritional media for rapid multiplication of callus tissues (Fig. 3) (Alkhateeb *et al.*, 2006). Morphological responses were found dependent on the age and physiological stage of the explant as well as the hormones concentration of medium.

1. Advantages of using tissue culture techniques

Tissue culture as a recent developed propagation tool has made a tremendous positive impact on the agricultural sector. The following points reflect some of the advatages of this technique (Al-Maarie, 1995):

- a. Production of many date palm plants from fewer ones, particularly, with high quality cultivars like 'Barhee' date palm.

- b. Production of disease free date palm plants.
- c. Tissue cultured date palm plants are mostly with uniform growth, identical to mother plants and normally fruit after 4 years from planting.
- d. Tissue cultured date palm plants are easy to handle during transportation.
- e. Tissue cultured date palm plants have rapid growth and almost 100% survival rate compared to vegetative offshoots due to the presence of a strong root system on them.
- f. The technique can be used for genetical improvement of date palm plants e.g. development of tolerant date palm plants to environmental stresses, diseases etc... through cell and protoplast culture, *in vitro* mutagenesis, *in vitro* selection of cells with required genetical characteristics and gene transfer.

2. Problems often encountered by using tissue culture techniques

Several technical problems may be encountered during the different stages of tissue culture at the laboratory and field levels as following:

2.1 At laboratory level

2.1.1 Browning of cultured tissues

This phenomenon results from physiological changes within the cultured tissues that lead to gradual browning and eventual death of tissues (Fig. 4). The browning appears due to the oxidation of phenols within the tissues (Alkhateeb and Ali-Dinar, 2002). Tissue browning is a problem frequently observed during *in vitro* establishment of explants from woody plants (Block and Lankes, 1996). The problem of phenolic browning was minimized to a great extent by leaching of phenolic compounds due to agitation in antioxidants solution and by proper drying of explants prior to inoculation (Meghwal *et al.* 2001). Adopting certain measures, namely: culturing of plant parts during winter and spring seasons, incubation of tissues in the dark especially in the first three months, and adding charcoal to the medium can reduce this phenomenon.

2.1.2 Vitrification of tissues

It appears due to accumulation of water within the cultured tissues which reduces their growth and leads to death. This physiological condition generally result from several factors; use of liquid media, use of high concentrations of plant hormones and/ or ammonium, presence of high humidity levels and gases, particularly, ethylene inside culture tubes. Certain measures can be used to reduce this phenomenon, such as use of solid media, reduction of hormonal and ammonium concentrations, increase agar concentration and use of tube covers that allow proper leakage of gases (Alkhateeb *et al.* 2006).

2.1.3 Bacterial and fungal contaminations

Bacterial and fungal contaminations (Fig. 5) are major problems during culture stages, particularly inside the culture tubes, and often appear after 2-4 months from the start of culturing (Oda *et al.* 2003). To ensure the efficiency of bacterial control using antibiotics, the following are recommended: (1) cleaning contaminated tissues by washing in distilled water, dipping in antibiotic solution before culturing in the physiological medium; (2) sterilizing tools (scalpels and forceps at 180 deg C/2 h); and (3) using young date palm tissues in culture (Benjama *et al.* 2001).

Adopting certain measures, namely: culturing of plant parts during winter and spring seasons, addition of antibiotics to media also can be used to reduce bacterial contamination (Alkhateeb and Ali-Dinar, 2002). Zacchini and Agazio (2004) reported that mercury chloride and sodium hypochloride in the sterilization step and antibiotics in culture media allowed to overcome the heavy pathogen contamination in shoot culture of Nebbiara olive.

2.1.4 Early rooting of tissue cultured buds

Early rooting of buds generally reduces their ability to multiply during tissue culture stages. Rooting reduces the ability of buds to multiply by diverting most tissue nutrients to root formation rather than to bud shoot formation (Fig. 6). The rooting conditions normally result from the presence of high auxin concentrations that encourage rooting. Furthermore, low concentrations of minerals in culture media and incubation of culture in darkness also lead to early rooting of buds. Use of low concentrations of auxins, particularly NAA can reduce the phenomenon (Alkhateeb *et al.* 2006).

2.1.5 Deterioration of embryonic callus and its inability to form embryos

The embryonic callus normally losses its ability to form vegetative embryos after continuous transference of callus in the same media for longer periods of time (Fig. 7). The callus changes from nodular form of embryos to soft tissues that turn to brown color due to accumulation of phenolic compounds. Use of low concentrations of hormones in media or less frequency of callus transference can reduce occurrence of phenomenon (Alkhateeb *et al.* 2006).

2.1.6 Callus formation on bases of rooting plantlets

The occurrence of callus on bases of *in vitro* plantlets during rooting stage hampers and slows root formation (Fig. 8). Under these conditions, developed roots normally have weak connection to plantlet base and formed plants have low chances of success. Using low concentration of auxins can reduce the phenomenon (Alkhateeb and Ali-Dinar, 2002).

2.2 At field level

Under certain conditions micropropagation techniques often lead to somaclonal variation between date palm plants. Most somaclonal variations in tissue culture

appear on plants that come from callus tissues (Skirvin *et al.* 1994; Al-Wasel, 2000 and Ramage *et al.* 2004). Al-Wasel (2005) in a survey study on some tissue culture-derived date palm cultivars (Medjool, Barhee, Sukkary, Toory, Deglat-Noor, Khalas, and Nabtat-Saif), somaclonal variations were observed. Dwarfism, slow growth, morphological abnormality, terminal bud bending, fruit set failure (shees), and supernumerary carpels were the most common phenomena. The type and percentage of variations differ among cultivars. The fruit set failure and dwarfism highly occurred and caused an economical loss. The Occurrence of fruit set failure varied between 20-100%, and number of carpels was between 4-9 carpels, except cvs. Medjool and Toory which were highly produced fruits. Whereas dwarfism was between 3-25% depending on cultivars. Morphological abnormality and terminal bud bending were the highest in Sukkary (10-50%). Albino and leaf variegation were very low (1-3%).

Somaclonal variations in date palm plants can be permanent (genetic stable variations) or temporary (epigenetic variation). While the genetic variations in plants are fixed and difficult to be changed, epigenetic variations are unstable and mostly result from physiological changes. Plants with epigenetic variation normally recover with time once the causes of these physiological changes are removed (Skirvin *et al.* 1994). Several factors may contribute to the occurrence of somaclonal variations in tissue cultured date palm, namely: growth regulators, type of explants used in micropropagation process, genotypical nature of plants, length of duration cultured tissues are kept and frequency of subculturing and proliferation rate of cultured tissues (Alkhateeb *et al.* 2006). Types of somaclonal variations in date palm are following :

2.2.1 Failure of fruit set

Off-types are quite common among tissue culture-produced date palm trees which are often characterized with a low fruit setting capacity. Most flowers in such trees turn into parthenocarpic fruitlets having three carpels. In severe cases, supernumerary carpels are formed. Other flower abnormalities include distortions of carpels and stigmas. The abnormalities in most cases one is alleviated in older trees, with approx equal to 50% of trees reverting to normal within 10 years from transplanting. Many flowers on the abnormal trees have impaired pollen tube elongation, with growth being limited to the stigma or to regions near its point of attachment to the carpel. Directional growth of pollen tubes ceases and tubes grow in different directions or stop growing completely (Cohen *et al.* 2004). This phenomenon locally known as 'sheiss' has been lately noticed on tissue culture 'Barhee' date palm at 'Alqassim' area (Fig. 9). Although the phenomenon has also been noticed in other date palm cultivars, its occurrence in 'Barhee' is more serious often reaching 59-86% (Ali-Dinar and Alkhateeb 2005). Over the past few years, several researchers investigated the phenomenon to identify the nature and possible causes of the problem (Ali-Dinar and Alkhateeb 2005). Ali-Dinar and Alkhateeb (2005) reported that the failure of normal fruiting in young tissue culture of date

palm trees cv. Barhee was probably due to many interrelated events that lead to a slow growth of pollen tube at early stages of fruit growth and which may possibly be accentuated by the relatively high abscissic acid (ABA) contents during this period.

2.2.2 Multiple carpels

Normally date palm female flower has 3 carpels. After successful pollination and fertilization only one carpel develops to fruit while the other two shrink and die (Alkhateeb and Ali-Dinar, 2002). Often, in some micropropagated date palm cultivars, female flowers possess more than 3 carpels (Fig. 10).

2.2.3 Dwarfism of date palm trees

Abnormal growth of trees has been noticed with certain micropropagated date palm cultivars, namely: Barhee, Sukary, Majdool, Deglet Nour and Khalas. The trees acquire stunt growth habit with differences between cultivars (Fig. 11). For example, Al-Wasel (2001) found that in Barhee 12-18% trees were dwarf with packed leaves around their trunk, whereas Khalas cultivar exhibited 18-24.3%. The dwarf somaclonal variant arises from genetic changes that occur during the tissue culture process. Early identification of this problem is difficult in many plant species including date palm. Propagators must wait until plants are *ex vitro* in order to visualise the dwarfism phenotype of *Musa spp.* (Ramage *et al.* 2004).

2.2.4 Abnormal growth and development of leaves and fruit strands

The abnormal growth of leaves has been observed on certain micropropagated 'Sukhary' and 'Barhee' date palm trees (Fig. 12), while the twisted growth of fruit strands has been noticed on 'Barhee' trees (Alkhateeb *et al.* 2006).

2.2.5 Dryness of apical bud:

In the beginning the leaves around apical bud became very dry and consequently plants death (Fig. 13).

2.2.6 Terminal bud bending:

It is well known that date palm trees have one apical bud and if they lose it they will not grow. This phenomenon was recorded in tissue culture date palm trees cv 'Shaishy' (Fig. 14).

2.2.7 Albinism (variegation) of leaves

Certain leaves in some tissue culture date palm trees lose the ability to form chlorophyll and turn from green to variegated color (Fig. 15).

2.2.8 Changes on fruit quality

These changes were reported on fruit sugars and amino acids of micropropagated 'Deglet Noor' (Booij *et al.* 1993) and 'Barhee' (Alkhateeb *et al.* 2006).

3. Conclusion

Al Kaabi *et al.* (2005) were compared two tissue culture methods, involving organogenesis or embryogenesis, when applied to ten United Arab Emirates date palm varieties (*Phoenix dactylifera* L.). The frequency of somaclonal variation in the resultant plants was compared and related to the levels of variation at the DNA level, estimated by AFLP analysis (Amplified Fragment Length Polymorphism). The incidence of somaclonal variation in plants regenerated through organogenesis tissue culture was low whilst a survey of embryogenesis-derived trees in the field identified a relatively high level of abnormalities. The experimental conditions for the generation of reproducible AFLP markers were optimised using *EcoRI* and *MseI* primers. All ten date palm varieties were able to be distinguished by AFLP fingerprinting. Variability amongst 40 plants produced by organogenesis and embryogenesis, based on numbers of plants which showed aberrant patterns, was found to be 5 % and 12.8 %, respectively. However, based on the total numbers of variant DNA fragments, the embryogenesis plants showed a much higher level of variability (0.6 %) than that shown by the organogenesis plants (0.038 %).

In conclusion, most of these abnormalities mentioned previously recover in most cases as the plants get old (10-year-old). Furthermore, all these abnormalities were also observed in date palm propagated by offshoots which is the normal method of propagation for date palms (Alkhateeb *et al.* 2006). This was supported by the finding of Ali-Dinar and Alkhateeb (2005) and Djerbi (2000). They indicated that the plant showed a substantial improvement in vegetative growth and set normal fruit as the plant get older. They concluded that this may probably be to the relatively longer juvenility period of these plants induced by unstable interrelated factors and seemed that tree age plays a central vital role in these events. Ali-Dinar and Alkhateeb (2005) studied microscopically the reproductive process from anthesis to early stages of seed development in collected samples of pistils, ovaries and young fruit at 2-day intervals during the first 3 weeks after anthesis and pollination and at weekly intervals thereafter for normal and abnormal (sheiss) in Barhee date palm cultivar. They indicated that events of the reproductive process showed that pollens grew normally on the stigmatic surface and the pollen tubes were clearly progressing within the style 2-4 days after pollination in tissue culture and vegetative offshoots Barhee date palm Fig. (16 and 17). Six to 10 days after pollination, pollen tubes of vegetative offshoots and tissue culture trees (with few incidences of abnormal fruiting) had already entered the ovary while those of young tissue culture trees (with high percent of abnormal fruiting) were slowly progressing within the style. Ovule fertilization as estimated by the initial endosperm division was observed 2 weeks after pollination in

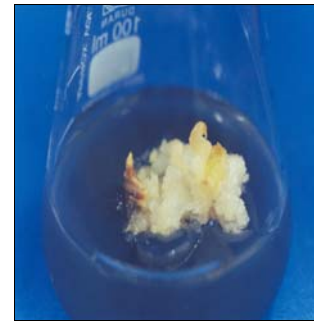
vegetative offshoots and old tissue culture trees. However, inner and outer integument in ovary of young tissue culture trees became less intact and separated from each other reflecting a possible subsequent failure of normal fruit setting due to failure of fertilization process. Fruit development was quite normal in offshoots and old tissue culture trees 6 weeks after pollination. Differences in pollen tube growth and the fertilization process between Barhee date palm trees may reflect possible physical or hormonal related factors that prevent normal progress of the reproductive process in young tissue culture trees.



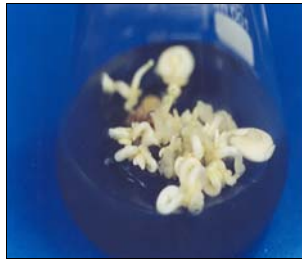
Initiation culture



Growing tissue



Callus formation



Embryogenic callus



Embryo germination



Acclimatization



Flowering



Fruiting

Fig. 1: Embryogenesis stages

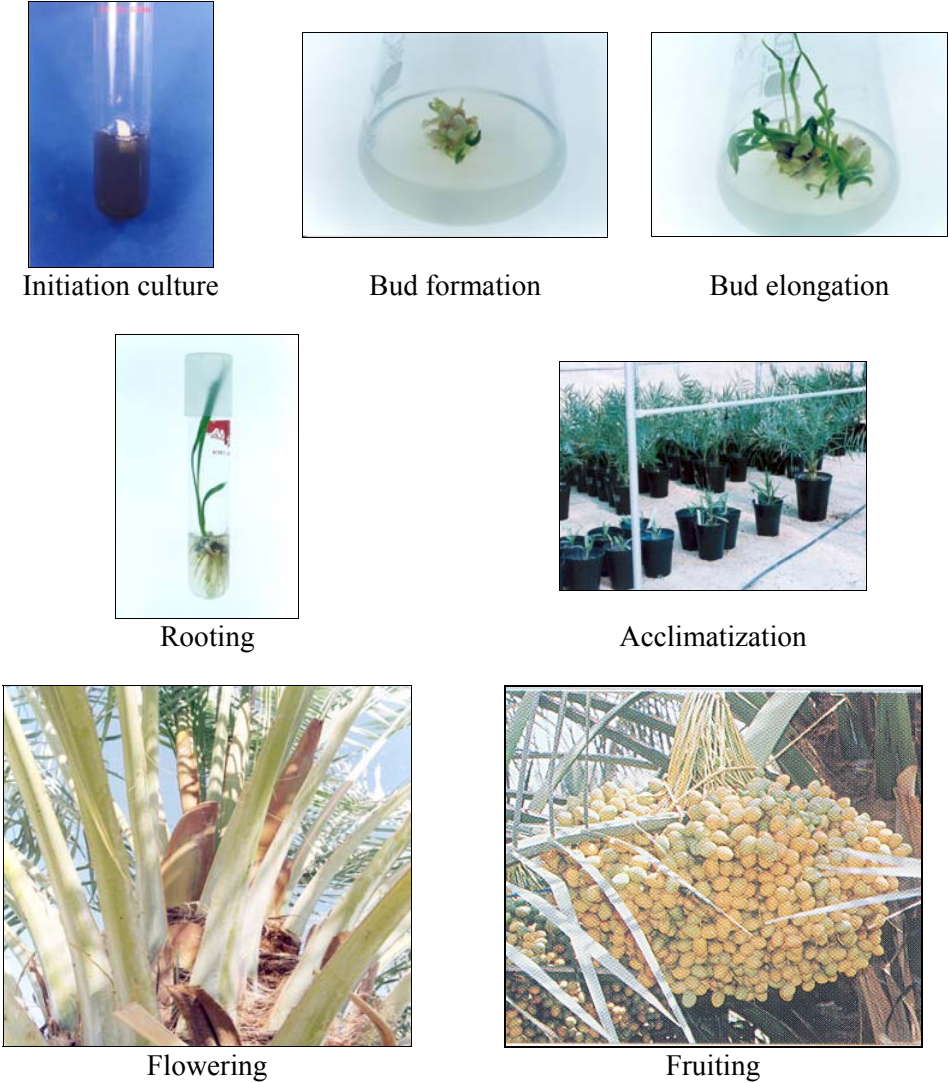


Fig. 2: Organogenesis st

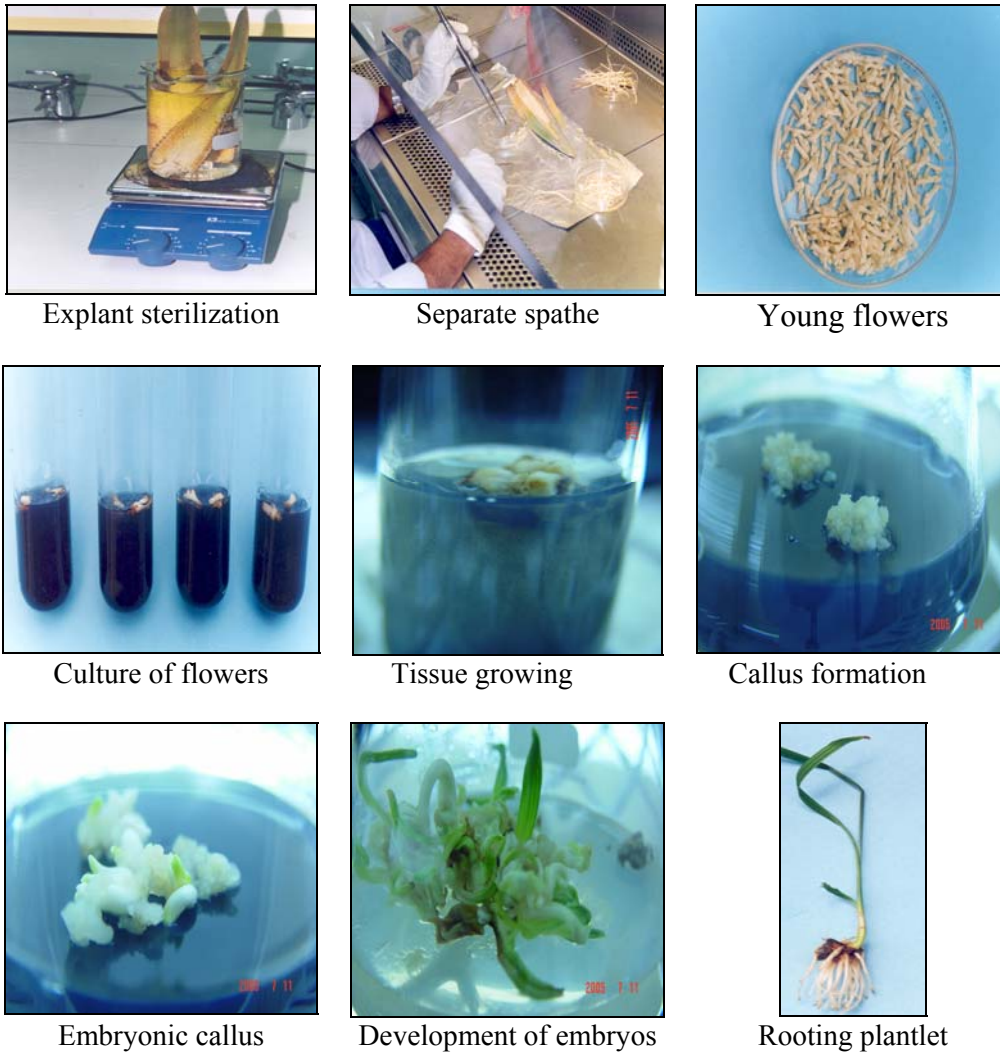


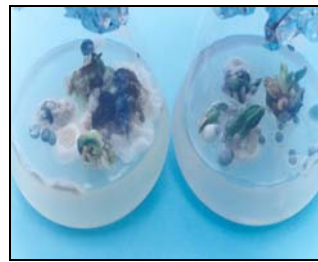
Fig. 3: Tissue culture by flowers



Fig. 4: Browning of cultured tissues



Bacterial contamination



Fungal contamination

Fig. 5: Bacterial and fungal contaminations



i. Bud without roots



ii. Bud with few early rooting



iii. Bud with ample early rooting

Fig. 6: Development of early rooting of tissue culture buds

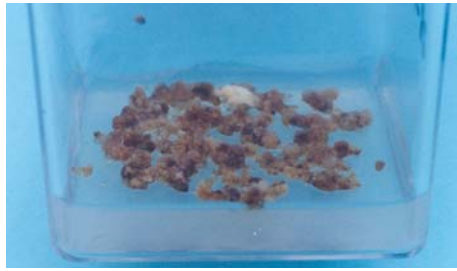


Fig. 7: Deterioration and browning of embryonic callus



Fig. 8: Callus formation on bases of rooting plantlets

Problems at the laboratory



Fig. 9: Failure of fruit set 'Sheiss' in Barhee date palm

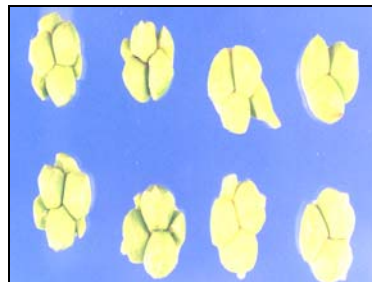


Fig. 10: Multiple carpels in female flowers



Fig. 11: 4-year-old dwarf tissue culture 'Khalas' tree.



Fig. 12: Abnormal growth of leaves in 'Barhee' tissue culture date palm trees.



Fig. 13: Dryness of apical bud



Fig. 14: Terminal bud bending



Fig. 15: Albinism (variegation) of tissue cultured date palm leaves and leaflets

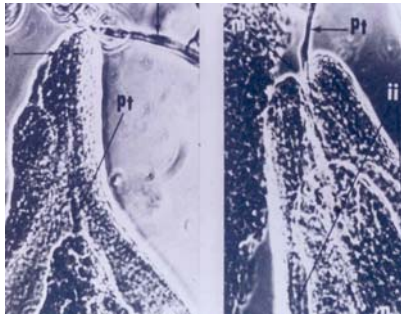
Types of somaclonal variations of date palm in field



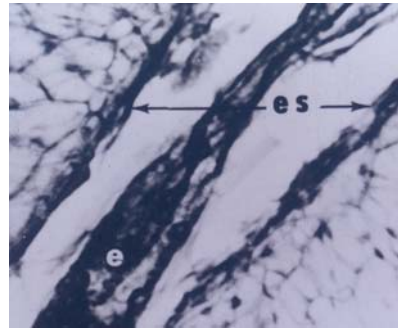
2- Pollen grain growth on stigma of 'Barhee' 2 days after pollination. pg: pollen grain, pt: pollen tube



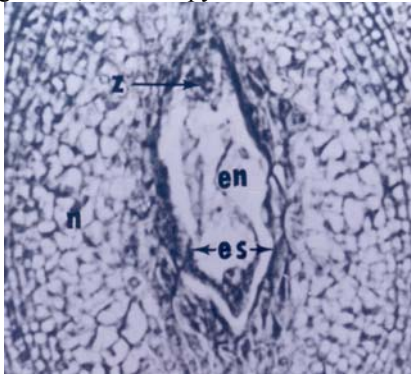
1- Normal and unnormal (sheiss) 'Barhee' fruits



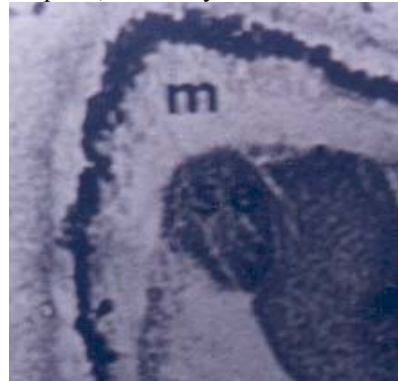
3- Pollen tube in 'Barhee' ovule 8-12 days after pollination pt: pollen tube, ii: inner integument, m: micropyle



4- Nuclear endosperm in 'Barhee' ovule 2 weeks after pollination. e: nuclear endosperm, es: embryo sac



5- Zygote 'sexual embryo' in 'Barhee' ovule 28 days after pollination. z: zygote, en: endosperm, es: embryo sac, n: nucellus



6- Enlarging sexual embryo 38 days after pollination. m: micropyle

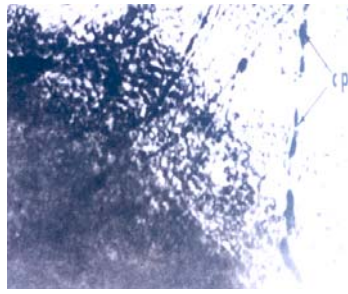
Fig. 16: The development of normal fruit.



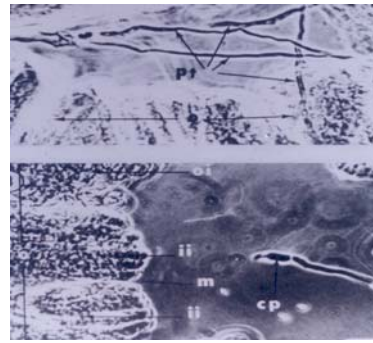
2- Pollen grain growth on stigma of 'Barhee' 2 days after pollination. pg: pollen grain, pt: pollen tube



1- Normal and unnormal (sheiss) 'Barhee' fruits



3- Callose plugs in pollen tube of 'Barhee' style 4 days after pollination. cp: callose plugs



4- Pollen tubes in 'Barhee' ovary 6-10 days after pollination. pt: pollen tube, ii: inner integument, m: micropyle, cp: callose plugs



5- Separated integuments of 'Barhee' abnormal fruit 2 weeks after pollination. m: micropyle, ii: inner integument, n: nucellus

Fig. 17: The development of abnormal 'sheiss' fruit.

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مقال استعراضى

المشاكل التي تجابه تقنية الإكثار النسيجي لنخيل التمر معمليا وحقليا

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الاحساء ، المملكة العربية السعودية

الملخص :

من المعروف أن تقنية زراعة الأنسجة هي تقنية حديثة استخدمت على نطاق واسع للإكثار السريع للعديد من النباتات ومن بين هذه النباتات نخيل التمر وعادة يتم إكثار نخيل التمر عن طريق تكوين الأجنة الخضرية وتتم عن طريق الحصول على الأجنة الخضرية بدءاً من تكوين الكالس الجنيني ، الذي يعطي مصدر مستمر للأجنة ، ويمكن إكثاره عن طريق تكرار زراعته بغرض تكوين العقد الجنينية والتي تتطور فيما بعد إلى أجنة خضرية. وتتميز هذه الطريقة بإنتاجها الغزير من النباتات في فترة قصيرة ، ولكن يعاب عليها أنه قد ينتج عنها نباتات غير مطابقة للنبات الأم بسبب مرورها بمرحلة الكالس والذي عادة إذا أستعمل لفترة طويلة يكون عرضة في بعض الأحيان لحدوث طفرات وراثية. أما الطريقة الثانية فهي طريقة التبرعم الخضري وتعتمد هذه التقنية بالدرجة الأولى على زراعة الأنسجة النباتية المأخوذة من قواعد الأوراق الغضة أو الفتية الموجودة قرب القمة النامية على بيئة غذائية تساعد على تكوين البراعم. وتحتاج هذه الطريقة إلى ضعف المدة اللازمة لإنتاج النباتات بطريقة الأجنة الخضرية ، كما أن عدد النباتات المتكونة قليل مقارنة مع طريقة الأجنة الخضرية إلا أن هذه الطريقة تتميز بكون البراعم التي يتم إكثارها تنشأ مباشرة من نسيج الأم دون المرور بمرحلة الكالس ، وبالتالي تكون النباتات الناتجة مطابقة للنبات الأم . أما الطريقة الثالثة فهي عن طريق زراعة الأزهار الفتية وذلك عند المراحل الأولى في التكوين وعلى العموم تعتبر هذه الطريقة في مراحلها الأولى وتحتاج إلى المزيد من الأبحاث. تواجه الزراعة النسيجية للنخيل عدة صعوبات سواء في المختبر أو في الحقل. فمن المشاكل التي تواجه العاملين في المختبر الآتي: التلوث البكتيري والفطري و التلون السبني والشفافية والتجذير المبكر للبراعم وتدهور الكالس الجنيني وفقدان قدرته على

تكوين الأجنة وأخيرا تكون الكالس على قواعد النباتات المجذرة. أما في الحقل فهي كالأتي: فشل عقد الثمار و تعدد الكرابل والتقرم وفقد الكلوروفيل و تشوهات في الأوراق وموت القمة النامية وتوقف القمة النامية عن النمو و أخيرا تغيير في المحتوى الكيميائي للثمار. وعلى الرغم من الصعوبات والظواهر السالفة الذكر التي قد تواجه زراعة الأنسجة إلا أنه من الملاحظ أنه يمكن التغلب عليها خاصة في المعمل أما في الحقل فهذا يحدث أيضا عند زراعة الفسائل العادية وغالبا ما تزول بمرور الوقت وتقدم العمر وتعود النخلة إلى حالتها الطبيعية في الإنتاج.

الكلمات الدالة: التلون البني، الكالس، التلوث، نخيل التمر، التقرم، فقد الكلوروفيل، تشوهات في الأوراق، الزراعه النسيجية .