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CALLUS INDUCTION IN "SHAHANI" DATE-PALM EMBRYO

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ABSTRACT

Embryos excised from mature seeds of "Shahani" date palm (*Phoenix dactylifera* L.) were cultured on a modified Murashige and Skoog (MS) medium containing 30 g l^{-1} sucrose, 3 g l^{-1} activated charcoal, 8 g l^{-1} agar with or without 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylamino purine (6-BAP).

Bacterial infection, originating from the internal tissues of explants, could not be controlled by surface sterilization. Contaminants emerging at the basal cut surface of the explants, were isolated and identified as short-rod shaped Gram-negative bacteria. Among the antibiotics tested, inclusion of gentamicin or kanamycin at the concentration of 100 mg 1^{-1} in the medium proved to be effective and resulted in control of bacterial contamination up to 90%. Excised embryos germinated and developed into seedlings in treatments devoid of hormones and/or containing 10, 20, 30 mg l^{-1} 2,4-D plus 10 mg l^{-1} 6-BAP under dark condition. Excised embryos also germinated and developed into seedlings in treatments devoid of hormones and/or containing 10, 20 mg l^{-1} 2,4-D plus 10 mg l^{-1} 6-BAP in light condition. Excised embryos cultured on media containing 40, 50, 60, 70 mg l^{-1} 2,4-D plus 10 mg l^{-1} 6-BAP produced callus under both light conditions after 12 weeks. Maximum callus formation (60% of explants) occurred in medium containing 40 mg l^{-1} 2,4-D plus 10 mg l^{-1} 6-BAP under dark condition.

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پینه انگیزی در رویان خرمای شاهانی

یوسف‌علی سعادت و مرتضی خوشخوی
به ترتیب دانشجوی سابق کارشناسی ارشد و استاد بخش باغبانی دانشکده کشاورزی، دانشگاه شیراز، شیراز، ایران.

چکیده

رویان‌های جدا شده از بذرهای خرمای شاهانی بر روی محیط کشت تغییر یافته موراشیگی و اسکوگ دارای ۳۰ گرم در لیتر ساکاروز، ۳ گرم در لیتر ذغال فعال شده و ۸ گرم در لیتر آگار همراه با یا بدون اسید ۲،۴- دی کلرو فنوکسی استیک (2,4-D) و ٦-بنزیل آمینوپیورین (6-BAP) کشت شدند. آلودگی باکتریایی منشاء گرفته از بافتهای درونی ریز نمونه‌ها بوسیلهٔ گندزدایی سطحی کنترل نشد. با جداسازی و رنگ آمیزی آلوده گرها که از ناحیهٔ تماس ریزنمونه‌ها با محیط کشت پدیدار می‌شدند مشخص گردید که آنها از نوع باکتری‌های گرم منفی و با شکل ظاهری میله‌ای کوتاه می‌باشند. از میان پادزیوهای (آنتی بیوتیک‌های) بکار رفته در این پژوهش افزودن جنتامایسین یا کانامیسین با غلظت ۱۰۰ میلیگرم در لیتر به محیط کشت، موجب از میان رفتن آلودگی باکتریایی در حدود ۸۰ تا ۹۰ درصد گردید. رویان‌های جدا شده که بر روی محیط‌های بدون هورمون و یا دارای ۱۰، ۲۰ یا ۳۰ میلیگرم در لیتر 2,4-D با ۱۰ میلیگرم در لیتر 6-BAP کشت شده بودند در شرایط تاریکی تنژیده و به صورت گیاهک‌های کامل درآمدند. رویان‌های کشت شده بر روی محیط کشت بدون هورمون و یا دارای ۱۰ یا ۲۰ میلیگرم در لیتر 2,4-D با ۱۰ میلیگرم در لیتر 6-BAP در نور نیز تنژیده و بصورت گیاهک کامل درآمدند.

رویان‌های جدا شده که بر روی محیط کشت‌های دارای ۴۰، ۵۰، ٦۰ یا ۷۰ میلیگرم در لیتر 2,4-D با ۱۰ میلیگرم در لیتر 6-BAP کشت شدند، پس از ۱۲ هفته در شرایط نور و تاریکی بافت پینه تولید نمودند. حداکثر تشکیل بافت پینه (٦۰% ریزنمونه‌ها) در محیط کشت دارای ۴۰ میلیگرم در لیتر 2,4-D با ۱۰ میلیگرم در لیتر 6-BAP در شرایط تاریکی صورت گرفت.

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is an important crop growing in tropical regions of Iran. Vegetative propagation of date palm is achieved only by off-shoots that arise from lateral buds. Problems associated with propagation by off-shoots include, potential distribution of insects and diseases, transport, and maintenance (1). In addition, separation of the off-shoots from mother plants is difficult and laborious, hence expensive. Each date palm produces only 6 to 12 off-shoots during its life cycle and mostly during its juvenile stage of development. Therefore, this method of propagation is a very slow process of multiplying the date palm. The sole prospect of speeding up the process of vegetative propagation seems to be the use of tissue culture methods. The main advantages of using tissue culture for date palm are the greater plant production potential from a single palm, as well as production of a large number of palms all with a genetically uniform structure. Plant cell, organ and tissue culture procedures may also be a key to speed up the breeding programs of date palm and introduction of new hybrids or cultivars. Embryo culture may aid in early identification of crosses with hybrid vigor, assuming that rapid vegetative growth can be correlated with yield (11).

Eeuwens and Blake (5) reported that for rapid growth and callus initiation on explants derived from date and coconut palms, Y3 minerals formulated by Eeuwens (3) was superior to White's (19), B5 (7), and Murashige and Skoog (MS) (10) media. Sharma *et al.* (14) reported that out of all combinations of media and growth regulators used, medium S (15) containing 5 mg l^{-1} naphthalenacetic acid (NAA), 0.1 mg l^{-1} casein hydrolysate was the best for growth of date palm explants. The basal medium used for date palm tissue culture by other researchers (6, 12, 16 17, 18, 20) was generally a modified version of MS medium (10). Eeuwens and Blake (5) reported that cytokinin was important for the growth of shoot tissues of date palm and that 6-benzylamino purine (6-BAP) was the most promotive cytokinin. Eeuwens (4) examined the effects of a factorial combination of 2,4-D, 6-BAP and gibberellin (GA_3) on the growth of *Cocos* and *Phoenix* and showed that the positive interaction between 2,4-D (10^{-7} M) and 6-BAP (5×10^{-6} M) was highly significant ($P<0.01$). A low concentration of GA_3

(10^{-5} M) appeared to be slightly inhibitory.

For maximum callus induction, Tisserat (17) tested various qualitative and quantitative addition of auxin to nutrient media. Generally, agar media containing 0.1 or 1.0 mg l^{-1} P-chlorophenoxy acetic acid (P-CPA), NAA or 2,4-D or 100 mg l^{-1} 2,4-D, 3 mg l^{-1} 2-isopentyladenine (2iP) and 0.3% activated charcoal produced the highest callus fresh weight among the chemicals and environmental treatments tested. Culturing callus on a liquid medium usually produced lower growth rates compared to corresponding agar treatment. Gabr and Tisserat (6) reported that indoleacetic acid (IAA) and NAA were notably less effective than 2,4-D in callus production from cultured shoot tips of date palm, while NAA was found to be more effective than IAA. Reuveni and Lilien-Kipnis (13) obtained callus and root formation from cotyledonary sheath explants of date palm embryos *in vitro*. Ammar and Benbadis (1) were able to produce callus in cotyledonary sheath sections taken from zygotic embryos germinated *in vitro*. Renolds and Murashige (12) reported that germpore containing ovule sections, excised from date palm fruits 2-3 months after pollination, produced callus on a medium containing 100 mg l^{-1} 2,4-D, 1 mg l^{-1} 2iP and 0.3% activated charcoal. Sharma *et al.* (12) reported that when isolated embryos were cultured, seedlings were formed within 6 weeks. Callus cultures have successfully been established from the segments of these seedlings. Zaid and Tisserat (20) reported that when excised embryos of date palm were cultured on a basal medium containing 2,4-D and 2iP, callus production occurred. Callus produced varied in type and consistency between cultivars. NAA and IAA were less effective than 2,4-D for callus production from embryos. A dosage-response relationship between callus initiation and concentration of auxin has been reported (16).

One persistent problem associated with date palm tissue culture is the occurrence of bacterial contaminations. Leary *et al.* (9) reported that pure cultures of a gram-negative, rod-shaped bacterium were isolated from the internal tissues, including the meristem of apparently healthy off-shoots of date palm. Biochemical and physiological tests indicated that the bacterium was *Bacillus circulans*. The *B. circulans* isolated from the date palm tissue culture was found to be sensitive to tetracycline (30 mg l^{-1}), streptomycin (10 mg l^{-1}), neomycin (20 mg l^{-1}) and chloramphenicol (30 mg l^{-1}). The use of one or

more of these antibiotics in the medium controlled the destruction of the cultures by *B. circulans*.

So far there is no report available on tissue culture of "Shahani" date palm which is an important cultivar in Fars province of Iran. This paper reports the embryo culture, bacterial contamination control and nutritional and hormonal requirements for *in vitro* culture of this cultivar.

MATERIALS AND METHODS

1. Plant Material

Excised embryos were obtained from mature seeds of "Shahani" date palm. Seeds were soaked in tapwater for 48 hr prior to surface sterilization. A cutter was used to excise the middle parts of the seeds containing embryos. Surface sterilization carried out with 70% ethyl alcohol for 1 min and then with 20% chlorox (containing about 5.25% sodium hypochlorite) solution for 20 min. The middle parts of seeds were excised and placed on the top of two sheets of Whatman No. 4 filter paper in a disposable sterile petridish containing 6 ml sterilized distilled water and incubated at 30°C for 6 days under a dark condition. When the seeds had germinated and their radicles were 2–4 mm in length the embryos were excised aseptically using a scalpel and forceps under a laminar air flow cabinet. Excised intact embryos were planted on the surface of different media so that the radicle tips were immersed into agar and plumules remained on the surface of the media.

2. Nutrient Media

The inorganic salts of MS medium were supplemented with: thiamin-HCl 0.4, pyridoxin-HCl 0.5, nicotinic acid 0.5, glycine 2, meso-inositol 100, casein hydrolysate 500, neutralized activated charcoal 3000 and bacto agar 8000 (all in mg l^{-1}). The explants were cultured on media devoid of hormones and/or containing 10, 20, 30, 40, 50, 60, 70 mg l^{-1} 2,4-D plus 10 mg l^{-1} 6-BAP.

3. Elimination of the Bacterial Contamination

In spite of extreme care for sterilization, bacterial contamination was observed during the course of this experiment. The procedures mentioned below were employed to overcome this problem, following the methods described by Johnson and Case (8).

3. 1. Isolation of bacteria. A nutrient agar plate containing 0.5% glucose was aseptically inoculated with contaminated samples, and streaked the first sector of the petri plate. The next sectors were then streaked in the same manner. The plate was incubated in an inverted position at 35°C for 48 hr until discrete, isolated colonies were developed.

3. 2. Gram staining. A drop of distilled water was placed on a microscope slide and a small amount of culture medium containing the isolated bacterium was to the drop, mixed and spread to cover a Ca. 1-cm circle. The smear was heat-fixed by passing the slides through a flame 2 or 3 times.

For staining, the smear was covered with crystal violet, for 30 seconds and washed carefully with distilled water. The smear was covered with Gram's iodine for 30 seconds, decolorized with 95% ethyl alcohol, washed with distilled water, stained for 30 seconds with safiranin and washed with distilled water. The slide was blotted with paper towel and observed under microscope using an oil immersion objective.

3. 3. Selection and evaluation of antibiotics. Two Muller Hinton agar plates were labelled for each isolated colony. Each culture was aseptically swabbed on plates in three directions. By sterilized forceps, six different antibiotic disks were placed on the surface of each agar plate. The disks were gently tapped to attain better contact with the agar. The following antibiotic disks were used: ampicillin, cephalothin, erythromycin, gentamicin, kanamycin, cloxacillin, nalidixic acid, nitrofurantion, tetracycline and trimethoprim-sulfamethoxazole. Plates were incubated at 35°C for 24 hr. The zone of inhibition was measured and compared with standard tables (8).

4. Sterilization

Seeds were surface-sterilized by immersion in 70% ethyl alcohol for 1 min and then 20% chlorox (Containing about 5.25% sodium hypochlorite) solution, containing one drop Tween-20 emulsifier for 100 ml solution, for 20 min. They were rinsed 3 separate times with sterilized distilled water. The nutrient media were sterilized by autoclaving for 15 min at 120° C and 1.5 kg cm^{-2} steam pressure. After autoclaving, the nutrient media were transferred to a laminar air flow cabinet. When the nutrient media cooled down to about 30-40° C appropriate concentrations of antibiotics were added aseptically to nutrient media to eliminate bacterial contamination. Then, 25-35 ml of media were poured into 50- and 100- ml sterilized round glass bottles.

5. Culture Conditions

Cultures were kept under a 16-hr photoperiod of 900 lux light intensity emitted by a cool-white fluorescent lamp at 27±2°C. Continuous darkness was obtained by placing the cultures in a plastic box covered by black cloth. The initial cultures and subsequent subcultures were incubated for a period of 4-6 weeks. Data were recorded at a 4-6 weeks interval.

RESULTS

1. Germination Studies

Within the first week after *in vitro* culture of explants, enlargement of embryos was commonly observed and cotyledons of the embryos elongated and turned green. Germination was evaluated by emergence of the primary root and first foliar leaf, which usually occurred during the first 5-8 weeks in culture in treatments devoid of hormones as well as those containing 10, 20 or 30 mg l^{-1} 2,4-D plus 10 mg l^{-1} 6-BAP under dark condition. Germination also occurred in treatments devoid of hormones and/or containing 10 or 20 mg l^{-1} 2,4-D plus 10 mg l^{-1} 6-BAP under light condition. The embryo germination percentages among treatments were 20, 25 and 55% in media devoid of hormones or containing 10 or 20 mg l^{-1} 2,4-D plus 10 mg l^{-1} 6-BAP under light,

respectively (Table 1). However, embryo germination did not occure in media containing more than 20 mg l^{-1} 2,4-D under light condition. Under dark condition, embryo germination rates were 25, 22, 20 and 11% in treatments devoid of hormones and containing 10, 20 or 30 mg l^{-1} 2,4-D plus 10 mg l^{-1} 6-BAP, respectively (Table 1). Under light, embryos germinated 1 to 2 weeks later than those under dark condition but the growth of seedlings was greater in the light compared with dark condition.

Table 1. Embryo survival and germination percentages of "Shahani" date palm embryos on media containing various concentrations of 2,4-D and 6-BAP under light and dark conditions after being 8 weeks in culture.

Conc. of 2,4-D (mg l^{-1})	Conc. of 6-BAP (mg l^{-1})	Embryo survival (%)		Germination (%)	
		Dark	Light	Dark	Light
0	0	75	20	25	20
10	10	67	30	22	25
20	10	100	55	20	55
30	10	100	100	11	0
40	10	62	91	0	0
50	10	75	100	0	0
60	10	0	89	0	0
70	10	0	12	0	0

In medium devoid of hormones, embryo survival percentages were 20% under light and 75% under dark condition. Maximum embryo survival (100%) was observed in media containing 20 or 30 mg l^{-1} 2,4-D plus 10 mg l^{-1} 6-BAP under dark condition and in media containing 30 or 50 mg l^{-1} 2,4-D plus 10 mg l^{-1} 6-BAP under light condition.

Minimum embryo survival percentage was observed in media containing 60 or 70 mg l^{-1} 2,4-D plus 10 mg l^{-1} 6-BAP under dark (Table 1). The single slender primary roots were dominant during the first 8-weeks in culture.

2. Callus Initiation

Excised embryos cultured on media containing 40, 50, 60 or 70 mg l^{-1} 2,4-D plus 10 mg l^{-1} 6-BAP produced callus both under dark and light conditions after 12 weeks. Formation of callus was evident in 6-weeks old cultures as cessation of embryo enlargement and swelling of hypocotyls. Maximum callus initiation (60% of explants) occurred in the medium containing 40 mg l^{-1} 2,4-D plus 10 mg l^{-1} 6-BAP under dark condition (Fig. 1).

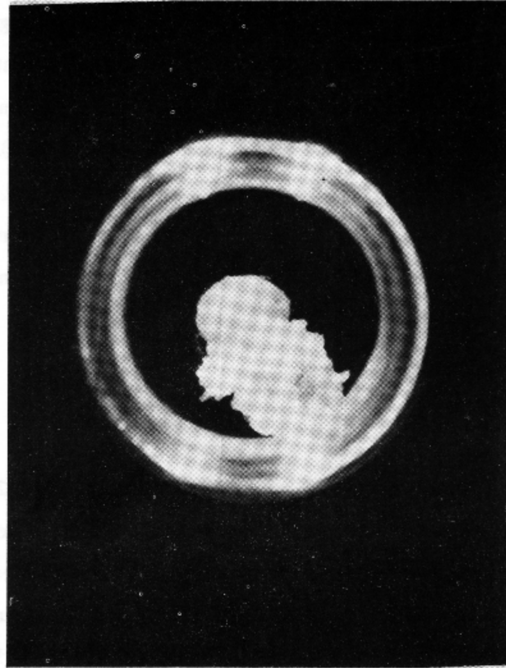

Fig. 1. Callus production by an embryo of "Shahani" date palm on a medium containing 40 mg l^{-1} 2,4-D and 10 mg l^{-1} 6-BAP after 20 weeks in culture under a dark condition.

The callus was friable and yellow-white in color. Callus formed on media containing 60 and 70 mg l^{-1} 2,4-D plus 10 mg l^{-1} 6-BAP was compact and had a brownish-yellow color. The lather treatments resulted in little callus formation under both light and dark conditions.

3. Elimination of Bacterial Contamination

In spite of surface sterilization of explants, bacterial contamination was observed as a persistent problem during this research work. By Grams's staining it was shown that the isolate from "Shahani" embryo was a short rod-shaped Gram-negative bacterium which rapidly grew on nutrient agar containing 0.5% glucose after 24 hr at 35°C.

In test with the antibiotics, the diameter of inhibition zone around the antibiotic disks was measured and compared with zone of interpretive standards (8). The results are summarized in Table 2. On the basis of results obtained, addition of 100 mg l^{-1} gentamicin or kanamycin to the nutrient media resulted in control of bacterial contamination up to 80-90%. Fifty mg l^{-1} gentamicin or kanamycin controlled bacterial contamination up to 50-55%.

DISCUSSION

In this study, embryos cultured on media containing 10 and 20 mg l^{-1} 2,4-D germinated and produced normal plants; whereas, Tisserat (16) reported that embryos cultured on media containing 10 mg l^{-1} 2,4-D produced callus. Moreover, in the present investigation, embryos which were cultured on media containing more than 70 mg l^{-1} 2,4-D failed to grow in culture and turned brown and died. This is not in accordance with Tisserat (16) who reported that embryos cultured on medium containing 100 mg l^{-1} 2,4-D produced callus. This different response may be due to either different plant genotypes or composition of culture media used for different cultivars. Maximum callus induction in this study was produced

Table 2. Effectiveness of antibiotic disks in suppressing growth of bacteria isolated from "Shahani" date palm embryos.

Antibiotics	Disk Potency g	Isolate No. 1		Isolate No.2	
		Inhibition zone diameter (mm)	Interpretation	Inhibition zone diameter (mm)	Interpretation
Ampicillin	10	0	resistant	0	resistant
Cephalothin	30	0	resistant	0	resistant
Chloramphenicol	30	20	sensitive	25	sensitive
Cloxacillin	5	0	resistant	0	resistant
Erythromycin	10	0	resistant	0	resistant
Gentamicin	10	15	sensitive	16	sensitive
Kanamycin	30	20	sensitive	20	sensitive
Nalidixic acid	30	23	sensitive	22	sensitive
Nitrofurantion	300	0	resistant	15	intermediate
Streptomycin	10	15	sensitive	10	resistant
Tetracycline	30	16	intermediate	18	intermediate
Trimethoprim-sulfamethoxazole	23.75	30	sensitive	26	sensitive

in dark condition. This is in agreement with findings of Sharma *et al.* (14), Gabr and Tisserat (6) and Zaid and Tisserat (20). Illumination has been shown to have stimulatory effect on the production of phenolic compounds (2). Hence, the callus production in dark may be attributed to the low level of phenolic compounds under these conditions.

The organisms isolated from the "Shahani" date palm embryos were bacteria which grew rapidly on nutrient agar medium containing 0.5% glucose. It was also unlikely that bacteria were airborne, since all operations were carried out under a laminar air-flow cabinet. Therefore, it may be assumed that bacteria had an endophytic origin. This is in accordance with Leary *et al.*(9) who isolated *Bacillus circulans* from the heart tissues,

vegetative bud meristem, shoot primordia, young branch bract and mature frond of a healthy date palm off-shoots. Their results showed that the *B. circulans* is of endophytic origin. *B. circulans* is a Gram-negative and rod-shaped bacterium as in the present investigation. By disk diffusion sensitivity test it was found that bacteria isolates were sensitive to gentamicin and chloramphenicol which is in agreement with the results obtained by Leary *et al.* (9). Plant materials which obtained from different regions in the present investigation were accompanied by various levels of bacterial contamination. For instance, seeds which were taken from Darab contained a higher level of contaminants than those obtained from Jahrom area. Moreover, two samples of seeds which were obtained from two different trees in the same orchard in Jahrom showed different levels of contamination. Therefore, it may be necessary to perform a preliminary experiment to determine the degree of contamination. Less costly media such as sucrose-agar with or without minerals can be used for this purpose.

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LITERATURE CITED

1. Ammar, S. and A. Benbadis. 1977. Multiplication végétative du palmier dattier (*Phoenix dactylifera* L.) par la culture de tissus de jeunes plantes issues de semis. C.R. Acad. Sci. Séries D. 284:1789-1792.
2. Durand-Cresswell, R. and C. Nitsch. 1977. Factors influencing the regeneration of *Eucalyptus grandis* by organ culture. Acta Hortic. 78:149-155.
3. Eeuwens, D.J. 1976. Mineral requirements for growth and callus initiation of tissue explants from mature coconut (*Cocos nucifera*) and date (*Phoenix dactylifera*) palms cultured *in vitro*. Physiol. Plant. 36:23-28.

4. Eeuwens, D.J. 1978. Effects of organic nutrients and hormones on growth and development of tissue explants from coconut (*Cocos nucifera*) and date (*Phoenix dactylifera*) palms cultured *in vitro*. Physiol. Plant. 42:173-178.

5. Eeuwens, D.J. and J. Blake. 1977. Culture of coconut and date palm tissue with a view to vegetative propagation. Acta Hortic. 78:273-286.

6. Gabr, M. and B. Tisserat. 1985. Propagating palms *in vitro* with special emphasis on the date palm (*Phoenix dactylifera* L.) Scientia Hortic. 25:255-262.

7. Gamborg, O.L., R.A. Miller and K. Ohima. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50:151-158.

8. Johnson, T.R. and C.L. Case (eds.) 1968. Laboratory Experiments in Microbiology. The Benjamin/Cummings Publishing Co. pp. 11-17.

9. Leary, J.V., N. Nelson, B. Tisserat and A. Allingham. 1986. Isolation of pathogenic *Bacillus circulans* from callus cultures and healthy off-shoots of date palm (*Phoenix dactylifera* L.). Appl. Environ. Microbiol. 52:1173-1176.

10. Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

11. Renolds. J.F. 1982. Vegetative propagation of palm trees. In: J. M. Bonga and D.J. Durzan (eds.). Tissue Culture in Forestry. Martinus Nijhoff/Dr. W. Junk Publishers, The Hauge, Netherlands. pp. 182-207.

12. Renolds. J.F. and T. Murashige. 1979. Asexual embryogenesis in callus cultures of palms. *In Vitro* 15:383-387.

13. Reuveni, O. and H. Lilien-Kipnis. 1974. Studies of the *in vitro* culture of date palm (*Phoenix dactylifera* L.) tissues and organs. Volcani Inst. Agric. Res. Pamphlet No. 145:1-40.

14. Sharma, D.R., R. Kumari and J.B. Chowdhury. 1980. *In vitro* culture of female date palm (*Phoenix dactylifera* L.) tissues. Euphytica 29:169-174.

15. Staritsky, G. 1970. Tissue culture of oil palm (*Elaeis guineensis* JACQ). as a tool for its vegetative propagation. Euphytica 19:288-292.

16. Tisserat, B. 1979. Propagation of date palm (*Phoenix dactylifera* L.) *in vitro*. J. Exp. Bot. 119:1275-1283.

17. Tisserat, B. 1982. Factors involved in the production of plantlets from date palm callus cultures. Euphytica 31:201–214.

18. Tisserat, B. 1988. Palm tissue culture. U.S. Dept. of Agric. Agric. Res. Service. No. 55, 60 p.

19. White, P.R. 1943. A Handbook of Plant Tissue Culture. J. Cattell, Lancaster, Pa., U.S.A.

20. Zaid, A. and B. Tisserat. 1984. Survey of the morphogenetic potential of excised palm embryos *in vitro*. Crop Res. (Hortic Res.) 24:1–9.