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Callus induction and rhizogenesis in date palm (*Phoenix dactylifera* L.) cv. Ajwa

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Abstract

An efficient protocol for callus induction and rhizogenesis has been developed for date palm (*Phoenix dactylifera* L.) cv. Ajwa using various plant growth regulators (PGR) in modified MS medium. *In vitro* callogenesis study was conducted using young leaves of seedling. Fourteen weeks were required for embryogenic callus formation and rhizogenesis under continuous absolute dark condition. The explants cultured in modified MS medium supplemented with 5 mg/l 2, 4-D + 2 mg/l 2ip showed maximum 70% production of callus. Fifty percent explants produced callus in modified MS medium when supplemented with 5 mg/l 2, 4-D + 5 mg/l NAA. Forty percent of the explants produced callus in the same medium containing only 5 mg/l 2, 4-D. After eight weeks, embryogenic callus proliferation and rhizogenesis occurred when supplemented with low concentrations (0.1-1.0 mg/l) of 2, 4-D, 2ip and NAA in modified MS medium.

Keywords: Date palm, seedling, rhizogenesis, young leaves, growth regulators, activated charcoal, biotin.

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) cv. Ajwa is a monocotyledonous, dioecious plant and is considered the most important fruit tree in many Arab countries, such as Saudi Arabia, Egypt and Iraq. In addition to its value in fruit production, it is also one of the main trees used for ornamental and landscape purposes (Badawy *et al.*, 2005). It is considered as one of the most important cash crops in the Middle East as about 90% of the total world production is produced in this region. Fruits contain a variety of vitamins and minerals which have different functions that help maintain a healthy body to metabolize carbohydrates and maintain blood glucose levels, fatty acids for energy, and they help to make hemoglobin, the red and white blood cells (El-Sohaimy and Hafez 2010).

Propagation by offshoots is more widely used because the fruit will be of the same quality as the mother plant and ensures uniformity of the product (Al-Khayri, 2005). But these plants accumulate several bacterial, fungal, viral and mycoplasmal diseases from air, soil and insect-vectors, which results in a decline of their productivity (Anonymous 1969). Conventional propagation is by offshoots making them slow to establish new plantations. In addition, seed propagated palms do not bear true to type due to heterozygosity and require up to seven years fruiting. The need for rapid and efficient vegetative propagation systems for elite genotypes has therefore become urgent (Al-Khayri, 2005). Seed propagation also has some limitations as well, seed dormancy, low rate of germination and progeny variation (Chand and Singh, 2004), which consist of approximately 50 : 50 male and female trees (Carpenter and Ream, 1976).

To overcome these problems and fulfill the demand for planting material, it is necessary to develop the method of date palm propagation with the use of plant tissue culture (Mujib *et al.*, 2004). This technique also provides a rapid system for production of large number of genetically uniform and disease free plantlets for agriculture and forestry. Although there have been previous reports on date palm micropropagation through the organogenesis and somatic embryogenesis (Zaid and Tisserat, 1984; Daguin and Letouze, 1988), calli were usually initiated from the cut ends of the explants. There are many reports for date palm tissue culture concerning with the induction of callus and subsequent regeneration (Jahromi, 2014). Callogenesis is a prerequisite for the initiation of somatic embryogenesis, and requires the presence of auxin in the medium. Immature leaf explants were cultivated in medium supplemented with either 1 or 54 l M 1-naphtaleneacetic acid in order to induce either rhizogenesis (Gueye *et al.*, 2009). The low concentrations of cytokinin induced callus in date palm *in vitro* leaves culture and callus induction increased in the darkness (Baharan *et al.*, 2015). The objective of this study was to optimize media composition and to investigate the role of different concentrations and combinations of 2, 4-D, NAA and 2ip for *in vitro* callus induction and rhizogenesis of cv. 'Ajwa' date palms.

MATERIALS AND METHODS

This study was conducted at the Biotechnology Laboratory, Bangladesh Sugarcane Research Institute (BSRI), Ishurdi, Pabna.

Plant Material

The young leaves of 3-4 months old seedlings of cv. 'Ajwa' date palm (*Phoenix dactylifera* L.) were used as explants. Seeds of date palm were collected from Saudi Arabia, and were sown in polybags containing 50% sand, 40% loam soil, 5% cowdung and 5% bagasse. Water was applied as and when necessary. Seeds were germinated after one month.

Cleaning of Explants

To remove the attached soil and other debris, the seedlings were washed with tap water, and outer large leaves and fibers were carefully removed with a sharp knife. About 6-7 cm long leaf base was cut from the unexpanded leaf.

Surface Sterilization

6-7 cm long leaf base was washed with running tap water for 10-15 min. Then they were cleansed with 5% savlon and 70% ethyl alcohol and rinsed with sterile double distilled water. Then they were cleansed with liquid detergent Tween 20 (1% v/v) for 5 - 10 min and rinsed with sterile double distilled water. Finally, they were surface sterilized with 0.1% mercuric chloride (w/v) solution for 20 min and again washed well in sterile distilled water for three - four times to remove all traces of mercuric chloride (Badawy *et al.*, 2005).

Explant Preparation

After proper surface sterilization, the young leaves were cut into 1-2 cm long, with a diameter of approximately 0.5-1.0 cm pieces under the laminar flow hood.

Culture Media

The explants were cultured on modified MS semisolid media (MS-1 to MS-5) for morphogenesis. MS-1 contained all the components of MS medium + 0.05% activated charcoal (AC), MS-2 contained MS + 10% CW + 0.05% AC, MS-3 contained MS+ 170 mg/l NaH_2PO_4 + 10% CW + 0.05% AC , MS-4 contained MS + 100 mg/l Citrate + 170 mg/l NaH_2PO_4 + 10% CW + 0.05% AC and MS-5 contained MS medium + 2 mg/l biotin + 100 mg/l citrate + 170 mg/l NaH_2PO_4 + 10 % CW + 0.05% AC.

Callus Induction

Young leaves (0.5-1 cm) were placed on modified MS basal media (viz. MS-1, MS-2, MS-3, MS-4 and MS-5) solidified with 3% agar and supplemented with various concentrations of 2, 4-D (0, 1, 2, 3, 4, 5, 10, 20, 50 and 100 mg/l), in combination with 2, 4-D + NAA (0+0, 1+1, 2+2, 3+2, 3+3, 5+5, 10+10, 20+10 and 50+10 mg/l) and 2, 4-D + 2ip (0+0, 1+1, 3+1, 5+1, 5+2, 5+3, 10+1, 10+2, 10+3 and 20+3 mg/l), 0.5 g/l of activated charcoal was added to each media in order to remove the phenolic compounds. The primary callus obtained from the explants was subcultured in the same medium without PGRs was also tested for the induction of embryogenic cells. Cultures were kept in darkness at 28 ± 2ºC and subcultured after every three - four weeks for 14 weeks under the same culture conditions until maturation. Once embryogenic culture mass increased they were maintained and proliferated by subculturing and incubated under the same conditions as for initiation.

Culture Conditions

The pH of all the media was adjusted to 5.6 – 5.8 before autoclaving. The media were sterilized in an autoclave for 20 min. at 121ºC. Lighting was provided using white cool fluorescent tubes of 40 µmol/m^2/s light intensity.

For all the above studies, modified MS (MS-5) contained 3% (w/v) sucrose, 10% coconut milk, NaH_2PO_4(170 mg/l), citrate (100 mg/l), biotin (2 mg/l) and were solidified with 0.7% agar.

Data Analysis

Data were recorded on the percentage of calli and producing root (%). Values presented on tables and figures are means of three replicates from each experiment and at least twenty cultures were employed per treatment.

RESULTS AND DISCUSSION

Effect of 2, 4-D on callus induction

The data presented in Table 1 indicate that the highest percentage (40%) of explants produced callus in MS-5 medium containing 5 mg/l 2, 4-D, whereas the lowest percentage (5%) callus produced in MS-1. Profuse callus was developed at 5 mg/l 2, 4-D within eight weeks (Fig. 1A) and somatic embryo formed within fourteen weeks (Fig.1B) of culture. It was observed that callus initiation started at 2 mg/l 2, 4-D in MS-4 and reached to the peak (40%) at 5 mg/l 2,4-D in MS-5. After that it was gradually decreased up to 20 mg/l. No callus was initiated in the MS modified media fortified with 0, 1, 50, and 100 mg/l. 2, 4-D (El-Hadrami and Baaziz, 1995). After eight weeks, callus was maintained in all the modified MS media without AC at low concentration of 2.4-D (1.0 mg/l) and kept in dark condition for proliferation of embryogenic callus. Roots were formed from the embryogenic callus (Ahmed *et al.,* 2009, Zaid and Wet, 2002, Gueye *et al.,* 2009). However, many researchers observed 2, 4-D as the best auxin for callus induction as common in monocot and even in dicot (Sane, 2006). A similar promoting effect of 2, 4-D on callusing was earlier reported in date palm cultivars and other plants (Hassan and Roy, 2005).

Effect of 2, 4-D and NAA on callus induction

Combined **e**ffects of different concentrations of 2, 4-D and NAA are presented in Table 2. The highest percentage (50%) of explants produced callus in MS-5 medium containing 5 mg/l 2, 4-D + 5 mg/l NAA (Fig. 2A) and somatic embryo formed within six weeks (Fig. 2B) of culture. It was observed that callus initiation started at 2 mg/l 2,4-D + 2 mg/l NAA in MS-3, MS-4 and MS-5 reached to the peak (50%) at 5 mg/l2,4-D + 5 mg/l NAA in MS-5. After that it was gradually decreased up to 10 mg/l 2,4-D + 10 mg/l NAA. No callus was initiated in the MS modified medium fortified with the concentration of 2,4-D + NAA (0+0, 1+1, 20+10 and 50+10 mg/l) (Table 2). Moreover, the maximum callus produced in the MS-5 medium containing 5 mg/l 2,4-D + 5 mg/l NAA (AI-Khalifah, 2006).After eight weeks at low concentration of 1 mg/l 2,4-D + 1 mg/l NAA were added in MS-5 and without AC and kept in dark condition for proliferation of embryogenic callus. Roots were formed from the embryogenic callus (Bekheet *et al.,* 2008).

Table 1. Effect of different concentrations of 2, 4-D alone in different modified media on callus initiation from young leaves

Culture Medium	Percentage of callus initiated (Degree of callusing)								
	Concentration of 2,4-D (mg/l)								
	0	**1**	**2**	**3**	**4**	**5**	**10**	**20**	**50**
MS-1	-	-	-	-	-	5* (+)	-	-	-
MS-2	-	-	-	-	10 (+)	15 (+)	10 (++)	-	-
MS-3	-	-	-	10 (+)	15 (++)	25 (++)	15 (++)	10 (+)	-
MS-4	-	-	10 (+)	15 (++)	25 (++)	30 (++)	10 (++)	-	-
MS-5	-	-	20 (+)	25 (++)	30 (+)	40 (+++)	20 (++)	10 (+)	-

*Values are means of 3 replications, 20 explants used per replication. Degree of callusing: -; no response, +; slight callusing, ++; medium callusing, +++; profuse callusing

A **B**

Fig. 1. Callus development of date palm, A-Profuse callus induced from young leaf5 mg/l 2,4-D medium containing within eight weeks in dark condition. B- Rhizogenesis occurred from profuse callus in MS-5 medium containing with low concentration 1 mg/l 2,4-D for six weeks in dark condition.

Combined effect of 2, 4-D and 2ip on callus induction

The data presented in Table 3 also show that the highest percentage (70%) of explants produced callus in MS-5 medium containing 5 mg/l 2,4-D + 2 mg/l 2ip (Fig. 3A). Profuse callus was developed at 5 mg/l 2, 4-D + 2 mg/l 2ip within eight weeks in dark condition and callus formed somatic embryo within six weeks (Fig. 3B). It was observed that callus initiation started at 3 mg/l 2,4-D

+ 1 mg/l 2ip in MS-4 and MS-5 reached to the peak (70%) at 5 mg/l 2,4-D + 2 mg/l 2ip in MS-5. After that it was gradually declined up to 10 mg/l 2, 4-D + 3 mg/l 2ip. No callus was initiated in the MS modified media fortified with the combined conc. of 2,4-D + 2ip (0+0, 1+1 and 20+3 mg/l). Moreover, the maximum callus was formed in the concentration of 5 mg/l 2, 4-D + 2 mg/l 2ip (Bekheet *et al.*, 2008). After eight weeks at low concentration of 0.5 mg/l 2,4-D + 0.1 mg/l 2ip were added in modified MS medium and keept in dark for proliferation of embryogenic callus. Roots were formed from embryogenic callus (Gueye *et al.*, 2009). From all of the data, MS-5 medium showed best performance in callus induction, it is may be due to presence of biotin @ 2 mg/l (Al-Khayri 2001). Biotin has important role in callus proliferation and an important cofactor in carbohydrate metabolism (Begley *et al*., 1999).

Table 2. Effect of different combinations and concentrations of 2, 4-D and NAA in different modified MS media on callus initiation from young leaves

Culture Medium	Percentage of callus initiated (Degree of callusing)								
	Conc. of 2,4-D + NAA (mg/l)								
	0	**1+1**	**2+2**	**3+2**	**3+3**	**5+5**	**10+10**	**20+10**	**50+10**
MS-1	-	-	-	-	-	10* (+)	-	-	-
MS-2	-	-	-	-	10 (+)	15 (+)	5 (+)	-	-
MS-3	-	-	5 (+)	10 (+)	15 (++)	20 (++)	10 (++)	-	-
MS-4	-	-	5 (+)	15 (++)	20 (++)	25 (++)	10 (++)	-	-
MS-5	-	-	10 (+)	20 (++)	30 (++)	50 (+++)	10 (++)	-	-

∗Values are means of 3 replications, 20 explants were used per replication. Degree of callusing: -; no response, +; slight callusing, ++; medium callusing, +++; profuse callusing.

A **B**

Fig 2. Callus development of date palm, A-Profuse callus induced from young leaf 5 mg/l 2, 4-D + 5 mg/l NAA medium containing within eight weeks in dark condition. B- Rhizogenesis occurred from profuse callus in MS-5 medium containing with low concentration 1mg/l 2, 4-D + 1mg/l NAA for six weeks in dark condition.

Table 3. Effect of different combinations and concentrations of 2, 4-D and 2ip in different modified MS media on callus initiation from young leaves

Culture Medium	Percentage of callus initiated (Degree of callusing)								
	Conc. of 2,4-D + 2ip (mg/l)								
	0+0	**1+1**	**3+1**	**5+1**	**5+2**	**5+3**	**10+1**	**10+2**	**10+3**
MS-1	-	-	-	-	10* (+)	-	-	-	-
MS-2	-	-	-	-	20 (+)	-	10 (+)	-	-
MS-3	-	-	-	20 (+)	30 (++)	10 (+)	25 (++)	15 (+)	-
MS-4	-	-	10 (+)	15 (++)	35 (++)	5 (++)	30 (++)	10 (+)	-
MS-5	-	-	25 (+)	50 (++)	70 (+++)	35 (++)	20 (++)	35 (++)	10 (+)

∗Values are means of 3 replications, 20 explants used per replication. Degree of callusing: -; no response, +; slight callusing, ++; medium callusing, +++; profuse callusing

A **B**

Fig 3. Callus development of date palm, A-Profuse callus formed from young leaf in MS-5 medium containing 5 mg/l 2,4-D + 2 mg/l 2ip within eight weeks in dark condition, B- Rhizogenesis occurred from profuse callus in MS-5 medium containing low concentration of 0.5 mg/l 2,4-D + 0.1 mg/l 2ip within six weeks.

CONCLUSION

Efficient protocol for callus induction and rhizogenesis of date palm (*Phoenix dactylifera* L.) seedling from juvenile leaves required fourteen weeks in continuous dark condition was established. Callusing was high on modified MS-5 medium supplemented with 5 mg/l 2,4-D, combined effect of 5 mg/l 2,4-D + 5 mg/l NAA, and 5 mg/l 2,4-D + 2 mg/l 2ip. This protocol will be useful for morphogenesis which would be very effective for micropropagation of the plantlets and finally enable meeting the high demand for the 'Ajwa' date palm in Bangladesh.

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REFERENCES

Al-Khayri, J.M., 2005. Date Palm *Phoenix dactylifera* L. In: Protocols of Somatic Embryogenesis in Woody Plants, Jain, S.M. and P.K. Gupta (Eds.). Springer, Berlin, pp: 309-318.

AI-Khalifah, N. S. 2006. Micropropagation and DNA Fingerprinting of Date Palm Trees of Saudi Arabia, *Aariena* Pub. 1: 5-15.

Al-Khayri, J. M. 2001. Optimization of biotin and thiamine requirements for somatic embryogenesis of date palm (*Phoenix dactylifera* L.) *in vitro*. Cell Dev. Biol. Plant. 37: 453- 456.

Anonymous. 1969. The wealth of India: a dictionary of Indian raw materials and industrial products. Coun. Sci. Ind. Res. 3**:** 303-305.

Badawy, E.M. Afaf M.A. Habib, A. El-Bana and Gehan M. Yosry. 2005. Propagation of date palm (*Phoenix dactylifera*) plants by using tissue culture technique. Arab J. Biotech. 8**:** 343-354.

Begley, T.P., J.Xi,C. Kinsland, S. Taylor, and F. McLafferty. 1999. The enzymology of sulfur activation during thiamin and biotin biosynthesis. Curr. Opinion Chem. Biol. 3: 623-629.

Bekheet, S.A., H. S. Taha, M. S. Hanafy and M. E. Solliman. 2008. Morphogenesis of sexual embryos of date palm cultured *in vitro* and early identification of sex type. App. Sci. Res. 4:345-352.

Carpenter, J. B. and C.L. Ream. 1976. Date palm breeding, a review. Date Grow Inst. Rep. 53: 23-33.

Chand, S. and A.J. Singh. 2004. *In vitro* propagation of *Bombexceiba*L. (Silkcotton). *In vitro* Cell Dev. Biol. Plant. 40**:** 167-170.

Daguin, F. and R. Letouze. 1988. Regeneration of date palm *(Phoenix dactylifera* L.) by somatic embryogenesis improved effectiveness by dipping in a stirred liquid medium. Fruits.43: 91-194.

ElaheBaharan, Payam Pour Mohammadi, EhsanShahbazi and Seyedeh Zahra Hosseini. 2015. Effects of some plant growth regulators and light on callus induction and explants browning in date palm (*Phoenix dactylifera* L.) *in vitro* leaves culture, Iran. J. Plant Phy. 5(4): 1473-1481.

El-Hadrami, I. and M. Baaziz. 1995. Somatic embryogenesis and plant regeneration from shoot-tip explants in *Phoenix dactylifera* L. Biol. Plant. 37: 205-211.

El-Sohaimy, S.A. and E.E. Hafez. 2010, Biochemical and nutritional characterizations of date palm fruits (*Phoenix dactylifera* L.). J. App. Sci.6(8): 1060-1067.

Gueye, B., H. S. Ahmed, F. Morcillo, A. Borgel, D. Sane, J. L. Hilbert, J. L. Verdeil, and A. S. Blervacq. 2009. Callogenesis and rhizogenesis in date palm leaf segments: are there similarities between the two auxin-induced pathways?, Plant Cell Tiss. Organ Cult. 98: 47–58.

Hassan, A.K.M.S. and S. K. Roy. 2005. Micropropagation of *Gloriosasuperba*L. through high frequency shoot proliferation, Plant Tissue Cult. 15: 67-74.

Jahromi, A. A. 2014. Report of the survey studied on somaclonal variations in *in-vitro* propagated date palm plants. Indian J. Fun. Appl. Life Sci. 4 (S3):108-113. www.cibtech.org/sp.ed/jls/2014/03/jls.htm

Mujib, A.,M.J. Cho, S. Predieriand S.Banerjee. 2004. *In Vitro* Application in CropImprovement.Oxford IBHPubli. Co Pvt. Ltd.New Delhi.

Othmani Ahmed, C. Bayoudh, N. Drira, M. Marrakchi, and M. Trifi. 2009. Somatic embryogenesis and plant regeneration in date palm *Phoenix dactylifera* L., cv. Boufeggous is significantly improved by fine chopping and partial desiccation of embryogenic callus. Plant Cell Tiss. Org. Cult. 97: 71–79.

Sane, D., F. Aberlenc-bertossi, Y. K. Gassama-dia, M. Sagna, M. F. Trouslot, Y. Duval and Borgel. 2006. Histocytological analysis of callogenesis and somatic embryogenesis from Cell Suspensions of date palm (*Phoenix dactylifera*). Anna Bot. 98**:** 301–308.

Zaid A.and D. P. F. Wet. 2002. Date palm propagation. In: Date palm cultivation. FAO Plant Production and

Protection Paper no. 156. *Food and Agriculture Organization of the United Nations*, Rome, Pp: 73–105.

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