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Induction of direct somatic embryogenesis and callogenesis in date palm (*Phoenix dactylifera* L.) using leaf explants

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Abstract

An efficient method for direct somatic embryogenesis and callogenesis from date palm (*Phoenix dactylifera* L. cv. Estamaran) was established using leaf explants. The effect of pretreating leaf explants with three antioxidant combinations on the browning was studied. The results showed that both the pre-treatments were effective against the browning of explants. Surface sterilization of explants, without pre-treatment, enhanced the browning. After recognizing the importance of antioxidants, we cultured the explants on an MS medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) at 0.1, 0.5, 1, 2, and 5 mg/l, thiadiazuron (TDZ) at 0 and 5 mg/l and 6-benzyladeninepurine (BAP) at 0, 5, and 10 mg/l. Callogenesis and direct embryogenesis on the surface of the leaf explants were observed. Only on the medium containing 5 mg/l 2,4-D, 10 mg/l BAP, and 5 mg/l TDZ globular embryos were formed directly on the leaf explants. The percentage of callus formation increased with an increase in the 2,4-D concentration; however, high concentrations of cytokinin caused a reduction of callogenesis. A medium supplemented with 5 mg/l 2,4-D, 5 mg/l TDZ, and 5 mg/l BAP, and 2 mg/l 2,4-D, and 5 mg/l BAP produced the highest number of calli per replication (2.7) on the leaf surfaces and leaf margins. Along with the advantages of direct somatic embryogenesis, this protocol facilitated the prospect of micropropagation without resorting to the intermediary callus stage, an important aspect to commercial production, and without sacrificing the plant by excising the shoot tips, which are commonly used for date palm tissue cultures.

Key words: date palm, leaf explants, direct somatic embryogenesis, callogenesis

Introduction

Date palm (*Phoenix dactylifera* L.) belongs to the monocot family Arecacea and is classified as a dioecious tall evergreen (Jain, 2006). Vegetative propagation of the date palm is usually performed by offshoots, which often results in the transmission of pests, disease, pathogens, and insects, which decrease productivity. In addition, only a limited number of offshoots develop at an early period of the mother's palm life (Gueye et al., 2009). Date palms grow readily from seeds that may require 6–10 years to fruit, but half of the seedlings are males and the rest of the resultant females are of inferior quality because of the genetic segregation (Al-Khalifa, 2000). To overcome the propagation restrictions, micropropagation is viewed as a successful technique for date palm propagation (Bhattacharjee, 2006; Mujib et al., 2004).

The *in vitro* culture of date palm is mainly achieved through indirect somatic embryogenesis, where callogenesis is a prerequisite (Gueye et al., 2009). It should be stressed that the process of *in vitro* propagation may cause genetic and epigenetic alterations inducing somaclonal variations, particularly when a callus phase is required (Minocha, 1987). In contrast, micropropagation through direct embryogenesis, which lacks the callus phase, has the advantage of producing genetically identical plants. Various explant tissues of date palm have been examined including shoot tips (Al-Khayri, 2001; El-Bellaj et al., 2000), leaf primordials (Hegazy et al., 2009; Ibrahim and Hegazy, 2001), axillary buds (Sharma et al., 1984; Zaid and Tisserat, 1983), roots (Zayed et al., 2016), and inflorescences (Drira and Benbadis, 1985; Hegazy et al., 2009; Zouine and Hadrami, 2007). The

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most frequently used explants are apical shoot tips and floral buds, as they have been the most responsive to the *in vitro* culture. Date palm leaves are less frequently used than shoot tips in *in vitro* micropropagation. For callogenesis and rhizogenesis, young leaves from embryogenic suspension cultures (Fki et al., 2003; Othmani et al., 2009), and leaf segments from the seedling plantlet cultures were used (Gueye et al., 2009). Although a few reports on direct embryogenesis (Hegazy, 2008; Hegazy and Aboshama, 2010; Sidky and Zaid, 2012) from shoot tip explants in date palm have been published, no reports on the direct embryogenesis from adult leaf explants have been published thus far.

The use of leaf explants has the advantage of being a system where phytohormones can easily be manipulated to direct pluripotent cells to a particular cell fate (Imin et al., 2005; Nolan et al., 2003; Thomas et al., 2004). Furthermore, the ability of leaf explants to produce endogenous auxins might contribute to a more effective adventitious organ formation from the leaf than that of stem or meristem explants, which lack this ability (Vesperinas et al., 1998). In this regard, plant growth regulator treatments were assessed in terms of a new protocol derived from the past research to determine the most appropriate plant growth regulator concentrations and culture conditions for the induction of direct embryogenesis. The overall objective of this research was to study the induction of the direct embryogenesis of the high-quality date palm cultivar Estamaran, a semidry cultivar grown in the Middle East. This has been the first report of direct embryogenesis from leaves, the most convenient and readily available all-year-long explant source.

Material and methods

Plant material and sterilization

The inner and youngest leaves, next to the apex from the offshoots, were sampled from adult date palm plants (cv. Estamaran) growing at the Khuzestan Agricultural Sciences and Natural Resources University, Iran. Using a hatchet knife, we removed primary leaflets and cut them into 5–6 cm segments. Then, the samples were cut into 1.5–2 cm explants by using a sharp sterilized blade after the elimination of the narrow part of the top and bottom of each explant. The explants were surface sterilized by soaking in a sodium hypochlorite (5.25% NaClO) solution for 10 min, rinsed three times with sterile distilled water at 10 min each, and cultured on the MS medium (Murashige and Skoog, 1962) containing 30 g/l sucrose, 8 g/l agar agar, 1 g/l activated charcoal, and 0.5 mg/l 2,4-D. The cultures were incubated at 25 ± 1 °C and 16 h photoperiod provided by fluorescent light (50 μmol $m^{-2}s^{-1}$ light intensity). The pH values of all the media were adjusted to 5.7 by using HCl (1 N) and NaOH (1 N) before adding the agar, and distributed in 250 ml culture bottles (25 ml medium per bottle), and then autoclaved for 15 min at 121 °C and 1.1 kg/cm^2. The first and the second subcultures were under similar conditions and media, but with 2 g/l activated charcoal (AC) in the first subculture and 3 g/l AC in the second subculture.

Antioxidant pretreatment

For the prevention of the browning of explants, antioxidant solutions (75 mg/l citric acid and 75 mg/l ascorbic acid) were tested using three methods: 1) before surface sterilization (Hussain et al., 1995), 2) after surface sterilization (Hegazy and Aboshama, 2010) (filter sterilized using a 0.2 μm filter), and 3) without antioxidant treatment (only surface sterilization was applied). In treatments 1 and 2, the explants were transferred to beakers containing an antioxidant before or after explant sterilization and then cultured on the MS medium containing 30 g/l sucrose, 8 g/l agar agar, 1 g/l activated charcoal, and 0.5 mg/l 2,4-D (stimulation period).

Plant growth regulator treatment

After a three-month stimulation period (MS medium containing 30 g/l sucrose, 8 g/l agar agar, 1 g/l activated charcoal, and 0.5 mg/l 2,4-D), the explants were transferred to the induction MS medium containing 30 g/l sucrose, 8 g/l agar agar, and 3 g/l activated charcoal and supplemented with 30 plant growth regulator (PGR) treatments (see Table 1) including 2,4-D (0.1, 0.5, 1, 2, and 5 mg/l), TDZ (0 and 5 mg/l), and BAP (0, 5, and 10 mg/l). The cultures were maintained in the dark at 25 ± 1 °C (Abul-Soad and Mahdi, 2010) and subcultured every 4 weeks for 3 months under the same culture conditions.

After callogenesis and embryogenesis, the callus and embryogenic sectors were transferred onto a fresh MS medium without the plant growth regulators containing

Table 1. Treatment schemes of leaf explants of *Phoenix dactylifera* using plant growth regulators [mg/l]

Treatment number	2,4-D [mg/l]	TDZ [mg/l]	BAP [mg/l]	Treatment number	2,4-D [mg/l]	TDZ [mg/l]	BAP [mg/l]	Treatment number	2,4-D [mg/l]	TDZ [mg/l]	BAP [mg/l]
1	0.1	0	0	11	0.5	5	5	21	2	0	10
2	0.1	0	5	12	0.5	5	10	22	2	5	0
3	0.1	0	10	13	1	0	0	23	2	5	5
4	0.1	5	0	14	1	0	5	24	2	5	10
5	0.1	5	5	15	1	0	10	25	5	0	0
6	0.1	5	10	16	1	5	0	26	5	0	5
7	0.5	0	0	17	1	5	5	27	5	0	10
8	0.5	0	5	18	1	5	10	28	5	5	0
9	0.5	0	10	19	2	0	0	29	5	5	5
10	0.5	5	0	20	2	0	5	30	5	5	10

Table 2. Mean square (MS) values of the ANOVA for the effects of an antioxidant pretreatment on the leaf explant browning of date palm

Source of variation	d.f.	Mean square
Antioxidant	2	4.000 *
Error	6	0.667
C.V%		49

* Level of significance: 0.05

30 g/l sucrose, 8 g/l agar agar, and 3 g/l activated charcoal for 3 months with a four-week interval subculture in the dark.

Statistical analysis

The experiments were carried out in a completely randomized design with four replications for the antioxidant pretreatment and plant growth regulator treatments. Each replication consisted of one Petri dish containing three leaf explants. The analysis of variance (ANOVA) and multiple comparisons with Duncan's multiple range test were carried out using the SPSS statistical software (version 13.0).

Results and discussion

Effect of pre-treatment with antioxidants on explant browning

Antioxidant pre-treatments (75 mg/l citric acid and 75 mg/l ascorbic acid) were used before (Hussain et al., 1995) and after surface sterilization (Hegazy and Abo-

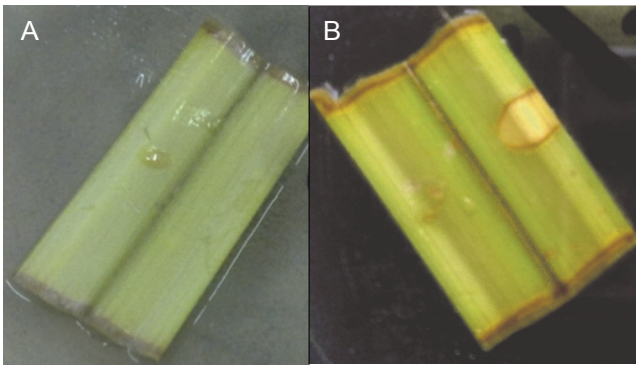

Fig. 1. Effect of pre-treatment of *Phoenix dactylifera* (cv. Estamaran) explants on browning: A) pre-treated explants and B) non-treated explants

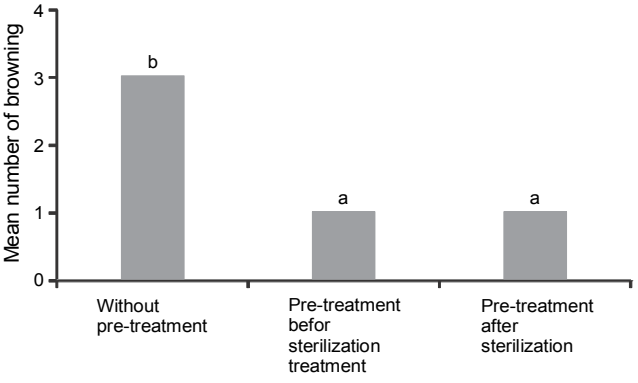

Fig. 2. Effect of antioxidant pretreatment on the leaf explant browning of date palm. Means followed by the same letter are not significantly different as indicated by Duncan's multiple range test at $P = 0.05$

shama, 2010). The effects of antioxidants were significant for explant browning (Table 2). It was found that

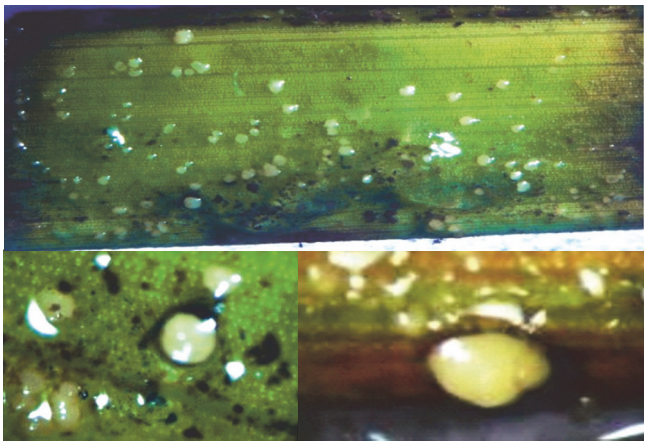

Fig. 3. Direct embryogenesis from the leaf explants of *Phoenix dactylifera* (cv. Estamaran).

both the pre-treatment methods tested were effective against explant browning (Fig. 1A). Importantly, surface sterilization without pre-treatment caused enhancement in explant browning, as shown in Figure 1B. The means of explant browning are presented in Figure 2; they were calculated after 12 days of culture, which involved the computation of the explant response to the total number of explants. According to Zaid (1987), the browning of the date palm tissue and the adjacent medium is assumed to be due to the oxidation of phenolic compounds and the formation of quinines, which are toxic to the tissues. Browning caused by the oxidation of phenolic has also been reported in cultured apple (Standardi and Romani, 1990) and oaks sp. (Bellarosa, 1988).

Plant growth regulators' effect on callogenesis and direct embryogenesis

Callogenesis and direct somatic embryogenesis from leaf explants were the two responses recorded in this experiment. The explants were stimulated and treated with auxin and light in the first 3 months of culture and then with plant growth-regulator treatments and kept in the dark. Both callogenesis and direct embryogenesis on the surface of the leaf explants were observed. Globular embryos appeared only in the treatment with 5 mg/l 2,4-D, 5 mg/l TDZ, and 10 mg/l BAP in the induction medium (Fig. 3). After embryogenesis, sectors with embryos were transferred on the MS medium without plant growth regulators; consequently, the number of embryos increased, but these embryos did not differentiate into elongated embryos. The first signs of callogenesis were recorded after 3 months of culture on the MS medium

Table 3. ANOVA results for the effects of plant growth regulators on callogenesis induction and browning in adult leaf explants of date palm

Source of variation	d.f.	Mean square
Callogenesis	29	1.95*
Error	60	0.62
C.V%		71

containing 30 g/l sucrose, 8 g/l agar agar, 1 g/l activated charcoal, and 0.5 mg/l 2,4-D, on the margins of the leaf explants as serrated swelling. Callus development was continued for another 3 months, but the callus did not increase in mass during this period. According to the data presented in Table 3 and Figure 4, plant growth regulators (2,4-D, TDZ, and BAP) affected the date palm callogenesis ($P \leq 0.01$). A medium supplemented with 5 mg/l 2,4-D, 5 mg/l TDZ, and 5 mg/l BAP, and 2 mg/l 2,4-D and 5 mg/l BAP produced the highest number of calli per replication (2.7) on the leaf surfaces (Fig. 5A) and leaf margins (Fig. 5B).

Cytokinins are required for callus induction and cell division. For callus formation, an appropriate equilibrium between the auxin and cytokinin applications is required (Minocha, 1987). In the present experiments, the best result was obtained in treatments containing 5 mg/l of 2,4-D, 5 mg/l TDZ, and 5 mg/l BAP, and 2 mg/l 2,4-D and 5 mg/l BAP. In previous research, it was shown that the use of BA in the culture media together with 2,4-D improved the callogenesis rates in *Acacia raddiana* (Sane et al., 2000). High callogenesis rates were also obtained in the presence of the combinations of 2,4-D with BA in date palm (Ahmar and Amsekhsi cultivar), which corresponded with our results. It has also been observed that the application of high concentrations of cytokinin caused a reduction of callogenesis (Sane et al., 2012). Vescovi and coworkers (2012) also found that the use of high levels of BA (9 mg/l) induced the apoptosis of the cells in the cultures of *Arabidopsis thaliana*.

In a study of Othmani and coworkers (2009), secondary embryos developed from somatic embryos (from leaves) were transferred to the MS medium without any growth regulator, which resulted in a complete destruction of the embryos. Using a liquid basal MS medium with 1.9 mg/l of 2,4-D resulted in an induction of

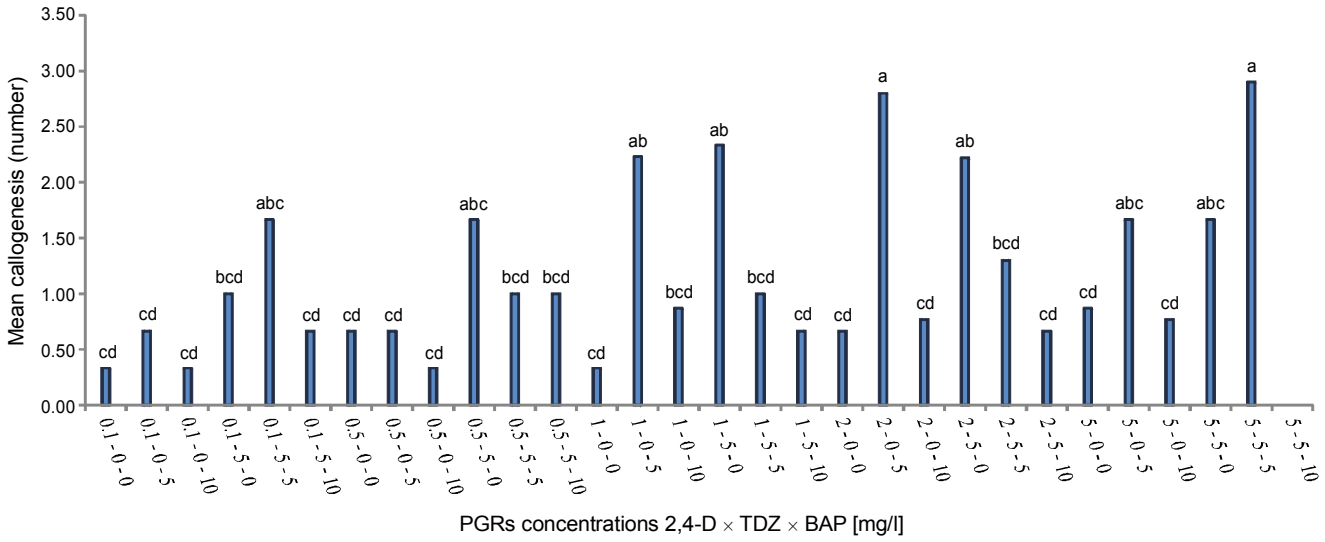

Fig. 4. Comparison of the effect of plant growth regulators (2,4-D × TDZ × BAP, on callogenesis in adult leaf explants of date palm cv. Estamaran. Means followed by the same letter are not significantly different as indicated by Duncan's Multiple Range Test at $P = 0.01$

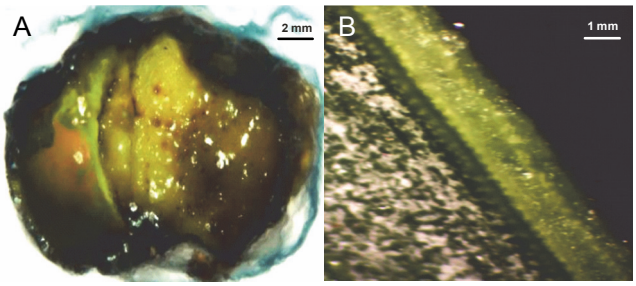

Fig. 5. Effect of plant growth regulators in the induction medium on the callogenesis in leaf explants of *Phoenix dactylifera* (cv. Estamaran). Callogenesis on the A) surface and B) edge of leaf explants in the treatment

cotyledonary embryo formation (Othmani et al., 2009). Al-Khayri and Al-Bahrany (2004) showed that the production of plantlets from somatic embryos in the MS medium without the use of growth regulators was possible. The results of the date palm tissue cultures of different varieties including cv. Amsekshi (Sane et al., 2006), cvs. Jihel and Bousthami Noir (Zouine and Hadrami, 2007), cv. Deglet Nour (Fki et al., 2003), and cv. Deglet Bay (Othmani et al., 2009) showed that 2,4-D was required for the induction of indirect somatic embryogenesis from leaf explants. Further, the TDZ treatment induced embryogenesis and enhanced the number of somatic embryos. TDZ has been suggested to either increase the levels of nucleosides or increase the accumulation or synthesis of purine cytokinins or to promote the conversion of adenine to adenosine (Capelle et al., 1983). TDZ increased the embryogenic response of the explants with normal growth and development; this phenomenon has already been observed in many other species. TDZ is very stable in the culture media and persistent in plant tissues (Mok and Mok, 1985). Victor and coworkers (1999) indicated that TDZ effectively induced somatic embryogenesis within a relatively short exposure time in peanut. A dual role for TDZ in the induction of somatic embryogenesis can be proposed: 1) a cytokinin-like activity that promotes cell division and differentiation, and 2) a minor auxin-like activity that seems to be crucial for the induction of embryogenic competence.

Conclusions

As a result of the research, a protocol for the callogenesis and direct embryogenesis of leaf explants of date palm has been developed. We found that the type of plant growth regulators and the antioxidant pre-treatment had a significant effect on callogenesis or direct embryogenesis. The rate of callusing increased with an increase in the auxin concentration. Lower levels of cytokinin (particularly BAP) induced callogenesis, but higher levels decreased the process.

This protocol provides a suitable technique for the callogenesis of date palm leaf explants and facilitates the vegetative propagation, conservation, and genetic en-

gineering of this species. To the best of our knowledge, this is the first paper describing direct somatic embryogenesis from the leaf explants of *Phoenix dactylifera*. Another advantage of this protocol via direct embryogenesis is that it opens up the prospects of genetic transformation. Moreover, the protocol can be applied for the mass multiplication of a selected parentage.

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