Multiplication and germination of somatic embryos obtained from cell suspensions of date palm (*Phoenix dactylifera*)

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ABSTRACT

Establishment and development of embryogenic suspension cultures in date palm (*Phoenix dactylifera* L.) cultivars, namely Bouskri (BSK) was implemented using liquid medium with BAP (0.3mg/l) and 2, 4-D (0.1mg/l). Detailed morphological observations have revealed that the cells destined to become somatic embryos divided into spherical proembryos (globular stage) within 7-15 days, with subsequent conversion globular stage to elongation stage after 17 days and cotyledonary stage after 27 days of suspension cultures.

Effects of activated charcoal and glutamine in production of somatic embryos were studied. Activated charcoal (AC) used at 0.15%, improved growth rate somatic embryos and decreased tissue and medium browning, charcoal has significantly reduced phenolics and peroxidase activity in comparison with medium without charcoal. Addition of gultamine (100 mg/l) to the somatic embryo culture medium led to decreased peroxidase activity and increased proteins content in comparison with medium without glutamine.

Somatic embryos were conducted on MS liquid medium diluted a half without plant growth regulator and transferred after on MS solid medium led to improvement of the germination rate to (32%).

Key words: Date palm; somatic embryogenesis; suspension culture; activated charcoal; glutamine; phenolics; peroxidases.

1-INTRODUCTION:

Somatic embryos have been used as a model system to understand the mechanisms regulating plant embryogenesis, being an alternative for the propagation of plants with high rates of multiplication, with relevance in tree improvement programs (Anjaneyulu *et al.,* 2004). In the case of date palm, a regeneration protocol *via* somatic embryogenesis in liquid medium has been established (Fki *et al.,* 2003; Zouine and El Hadrami 2007; Abohatem *et al.,* 2011). This protocol allows the production of a large number of individual somatic embryos (SE) of uniform physiological and growth characteristics and with synchronized development (Abohatem *et al.,* 2011).

During the somatic embryogenesis in date palm (*P. dactylifera*), AC had been employed in every stage. For callogenesis and embryogenesis 0.15 g/l was employed. For embryo maturation and germination 0.25 g/l were used along with MS medium (Zouine *et al.,* 2005). AC induced somatic embryogenesis was also reported in *Phoenix dactylifera* L. by Fki *et al*. (2003), where the presence of 0.3 g/l AC in liquid medium resulted in the differentiation of large number of SEs.

Amino acid enrichment of the culture medium increased the number of regenerated embryos as well as storage protein accumulation and their conversion rate into vitro plantlets in the case of alfalfa (Lai *et al.,* 1992; Stuart *et al.,* 1985). Glutamine alone or in combination with casein hydrolysate and inorganic forms of nitrogen, has generally been used in the different phases of somatic embryogenesis (Fki, 1998; Morcillo *et al.,* 1999; Garin *et al.,* 2000).

The objectives of this study were (i) to describe morphological characteristics of somatic embryogenesis at different development stages from the cell suspension culture (ii) to compare the effect of activated charcoal and glutamine on embryogenic cells in suspension cultures on the basis of their phenolic contents, peroxidase

activities and protein contents and (iii) to improve germination rate of date palm somatic embryos

2-MATERIAL AND METHODS

2-1. Establishment of cell suspension:

To establish the cell suspension, the method described by Fki *et al.* (2003), and Zouine and El Hadrami, (2007) has been used in this study. Five hundred milligrams of embryogenic callus (granular aspect with globular embryos) were cut with sterile scalpel into small pieces (fine parts) as possible and then transferred in 50 ml of liquid medium in 250 ml Erlenmeyer. The content of Erlenmeyer is passed through sieves with a 500 µm mesh size and the filtrate is incubated on a rotary shaker (100 rpm) at 25 ± 2ºC under a 16/8-h (light/dark) photoperiod. The liquid medium is that MS/2 supplemented with 2,4-D (0.1 mg/ l), BAP (0.3 mg /l) (Abohatem *et al*. 2011).

Two factors (Activated charcoal and Glutamine) were tested in the culture medium which supplemented with 150 mg/l AC and without AC or supplemented with 100mg/l Glutamine and without G.

2.2. Development of somatic embryos in cell suspension culture:

The development and division of embryogenic cell during 15 days from cell suspension culture was observed under a microscope. Morphological characteristics of somatic embryogenesis at different developmental stages (globular, elongation and cotyledonary) were recorded from the cell suspension culture during the induction of somatic embryogenesis.

2.3. Maturation and germination of somatic embryos:

Maturation and germination of somatic embryos are conducted on MS liquid medium diluted a half without plant growth regulator for two weeks. After that somatic embryos are transferred and cultured on MS solid medium supplemented with NAA (0.1 mg/ l).

2.4. Extraction and analysis of phenolics:

Phenolics compounds were extracted and analysed as described by El Hadrami (1995). Fresh somatic embryogenesis tissues (250 mg) was homogenized with 2ml methanol (80%) at 4ºC and centrifuged three times at 7000g for 3 min, supernatants were recuperated each time. 100 µl of the supernatant was added to Folin-Ciocalteu reagent (250 µl) and Sodium carbonate (20%). The mixture was incubated at 40ºC for 30 min and the blue colour was determined at 760 nm.

2.5. Extraction and analysis of proteins:

Total soluble proteins were extracted according to the method described by Lecouteux (1993). Fresh somatic embryogenesis tissues (250 mg) was homogeneneised with 2ml Tris maleate buffer (0.1M, PH 6.5) and centrifuged for 6min at 7000g. The supernatant was used as the crude proteins extract. The total proteins were measured by spectrophotometer at 595 nm according to the method described by Bradford.

2.6. Peroxidase extraction, activity assays and elctrophoresis:

Somatic embryos tissues (250 mg FW) were homogenized in 1 ml of Tris maleate buffer pH 6.5 (0.1M). After centrifugation at 10 000 *g* for 10 min, the supernatant was used for enzymatic activities determination. Peroxidase (POX) activity was assayed as described by Baaziz et al. (1994).

To separate peroxidase isoenzyme, polyacrylamide gel electrophoresis of soluble proteins was carried out according to Baaziz (1989). For POX staining, the gel was incubated for 15 min in 100 ml of 0.1M sodium acetate buffer pH 5 containing 0.1g of benzidine and 0.1 ml 10% hydrogen peroxide. Gels were incubated with the substrates for 30 min in dark until dark bands appeared.

2.7. Statistical analysis:

Results were analyzed by variance analysis (ANOVA), followed by SNK test at P = 0.05 level to compare means (SPSS, 1996). The number of repetitions is three replicates with two independent experiments.

3. RESULTS AND DISCUSSION

3.1. Development of somatic embryos:

Microscopic observation was carried out to describe the development of embryogenic cell during 15 days from cell suspension culture (Fig.1A-B). During the first week, single cells of suspension cultures divided and formed small clumps of cells (Fig. 1C) whereas after one week, cells were dividing actively and formed large clumps of cells (Fig. 1D).

Similar result was observed in oil palm by Roowi *et al*. (2010) who showed that cell suspensions contained cellular aggregates composing of round cells with dense cytoplasm that were small in diameter (10–20 µM). Kramut and Te-chato (2010) described two types of cell aggregate, 5 to 10 cells and more than 10 cells. For aggregate consisting of more than 10 cells, those cell showed dense cytoplasm whereas 5 to 10 cell aggregate consisted of large vacuolar cells.

The morphological observations of cell suspension cultures have revealed that the cells destined to become somatic

embryos divided into spherical proembryos (globular stage) within 7-15 days (Fig. 1E), with subsequent conversion globular stage to elongated embryos after 17 days (Fig. 1F), cotyledonary embryos (Fig. 1G) were formed in the next 27 days and somatic embryos were formed in the next 35 days (Fig. 1H). To our knowledge, this is the first time description of the time course of date palm embryo formation in a suspension culture.

3.2. Effect of activated charcoal on phenolics, proteins content and peroxidases activities of date palm somatic embryos:

To limit tissue browning that cause an appreciable loss of culture viability, activated charcoal are commonly used in palm tissue culture to trap phenols and oxidized phenols. Activated charcoal used only at 150 mg/l improved growth rate somatic embryos and decreased tissue and medium browning (Fig.2).

The biochemical analysis showed that charcoal was significantly reduced phenolics (0.12 mg/g FW) in comparison with medium without charcoal (0.33 mg/g FW). It reduced peroxidase activity from 69.8 UE/g FW to 35.6 UE/ g FW. In addition, charcoal decreased proteins content (68.9 µg/g FW) in comparison with medium without charcoal (77.84 µg /g FW) (Table 1).

Until the actual time, there was a little information about biochemical parameters implicated in tissue browning in palms and particularly in date palm. The viability of somatic embryo is considered to be one of the most difficult to maintain. Previous works showed that this phenomenon is in relation with the high accumulation of caffeoylshikimic acids in the somatic embryo.

Activated charcoal was found to be the best antibrowning factor particularly during the first months of suspension culture. This result is in good agreement with the already known facts concerning the role of this compound to trap phenols and oxidezed polyphenols in palm tissue culture (Touchet *et al*. 1991; Teixeira *et al.,* 1993, 1994; Verdeil *et al.,* 1994; El Hadrami and Baaziz 1995; El Hadrami *et al.,* 1995; Othmani *et al.,* 2009).

3.3. Effect of gultamine on phenolics, proteins content and peroxidases activities of date palm somatic embryos:

The application of gultamine (100mg/l) to the somatic embryo culture medium led to decreased peroxidase activity (35.6 U E / g FW) in comparison with medium without glutamine (56.8 U E / g FW) and increased proteins content from 75.6 µg / g FW to 87.84 µg / g FW (Table 2).

Zouine and El Hadrami (2007) found that the use of 2, 4-D (0.1 mg/ l) in combination with glutamine (6.7 ×10 - 4M) gives a significant ($P < 0.05$) enhancement in soluble protein and sugars in embryogenic cultures of date palm. In Oil Palm somatic embryos, the utilisation of glutamine alone or in combination with arginine was found to be a factor of enhancement of protein accumulation (Morcillo *et al.,* 1999).

3.4. Effect of Glutamine and activated charcoal on peroxidase isoforms:

When separated by polyacrylamide gel electrophoresis, peroxidase extracts from somatic embryos of the date palm cultivar BSK, showed a major migration zone with Rf value interval 0.10-0.16 (Fig. 3), where the stain intensity increased with the high volume of embryo extract (60 µl).

Extracts with activated charcoal and glutamine were characterized by a peroxidase isoform of relatively high migration speed (Rf = 0.16). This latter diappeared in peroxidase extacts prepared from embryos cultivated on media without activated charcoal and glutamine. This result confirms that AC and G modulate browning by their action on qualitative aspect of peroxidases. They induce the formation of an enzyme isoform, which migrates at Rf = 0.16 on 11% polyacrylamide gels. This isoform correlated with embryo maturation.

3.5. Somatic embryos germination:

When the somatic embryos were conducted on MS liquid medium diluted a half without plant growth regulator and transferred after on MS solid medium supplemented with NAA (0.1 mg/l) an improvement of the germination rate (32%) was obtained. Only 8% of germination was reached when embryo were transferred directly on MS solid medium (Fig.4). Similar result was obtained by Fki et *al.* (2003), where they showed a 25% germination rate of date palm somatic embryos on modified MS medium deprived of plant growth regulator.

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Tables

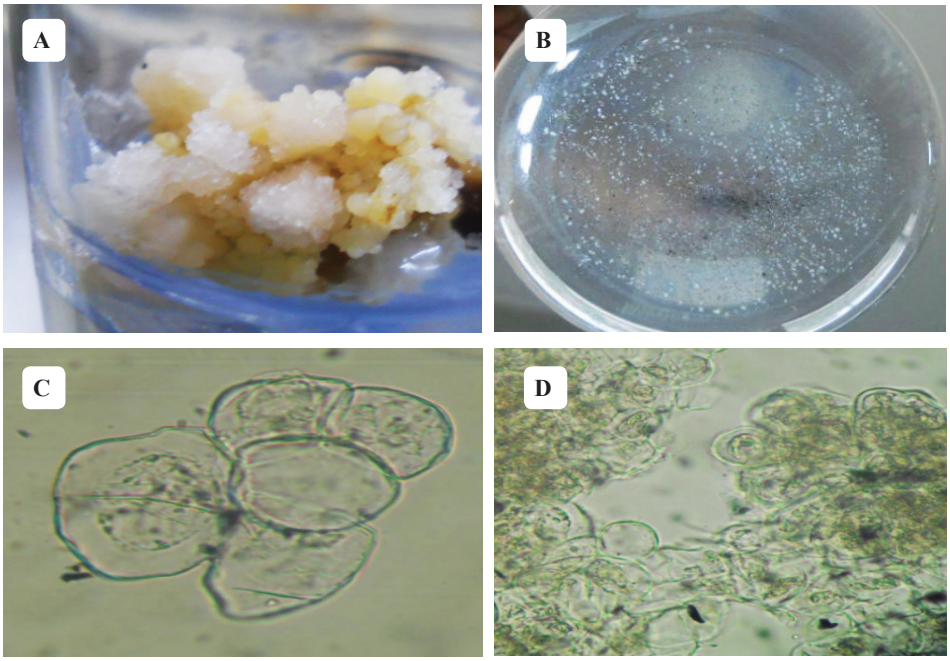
Table 1: Effect of activated charcoal on phenolics, proteins content and peroxidases activities of date palm somatic embryos

Medium	Total protein µg / g FW	Phenols mg / g FW	Peroxidase activity UE / g FW
With charcoal	87.84 ± 3.6	0.12 ± 0.05	35.6±23.2
Without charcoal	77.43 ± 2.8	0.33 ± 0.04	69.8 ± 26.8

Table 2: Effect of glutamine on phenolics, proteins content and peroxidases activities of date palm somatic embryos.

Medium	Total protein µg / g FW	Phenols mg / g FW	Peroxidase activity UE / g FW
With gultamine	87.84 ± 3.6	0.12 ± 0.05	35.6±23.2
Without gultamine	75.6± 4	0.15 ± 0.03	56.8 ± 33.7

Figures



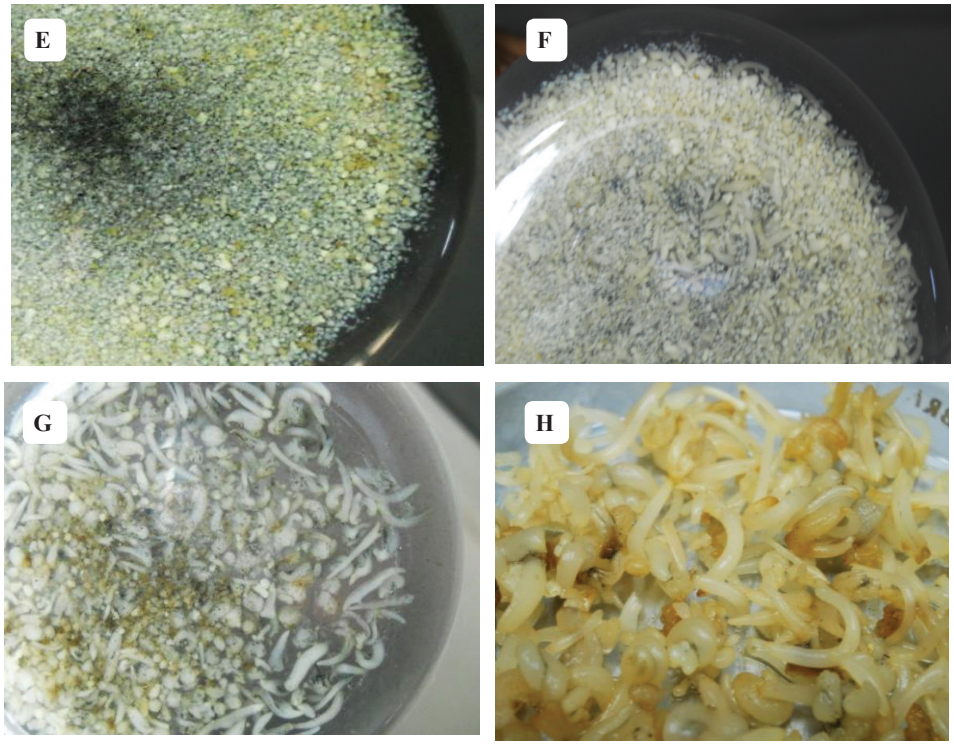


Fig. 1: The morphology of date palm cell suspension culture during the induction of somatic embryogenesis. (A) embryogenic callus, (B) cell suspension after 7 days of culture, (C) small clumps of cells during the first week under ×100 enlargement with light microscope, (D) large clumps of cells during the second week under ×100 enlargement with light microscope, (E) embryogenic cell in globular stage after 14 days of culture, (F) conversion of globular stage to elongated embryos after 21 days of culture, (G) cotyledonary embryos after 32 days of culture, (H) somatic embryo after 40 day of culture. Scale bar: 0.1mm.

Fig. 2: Effect of activated charcoal on phenolics and tissue browning in date palm somatic embryos

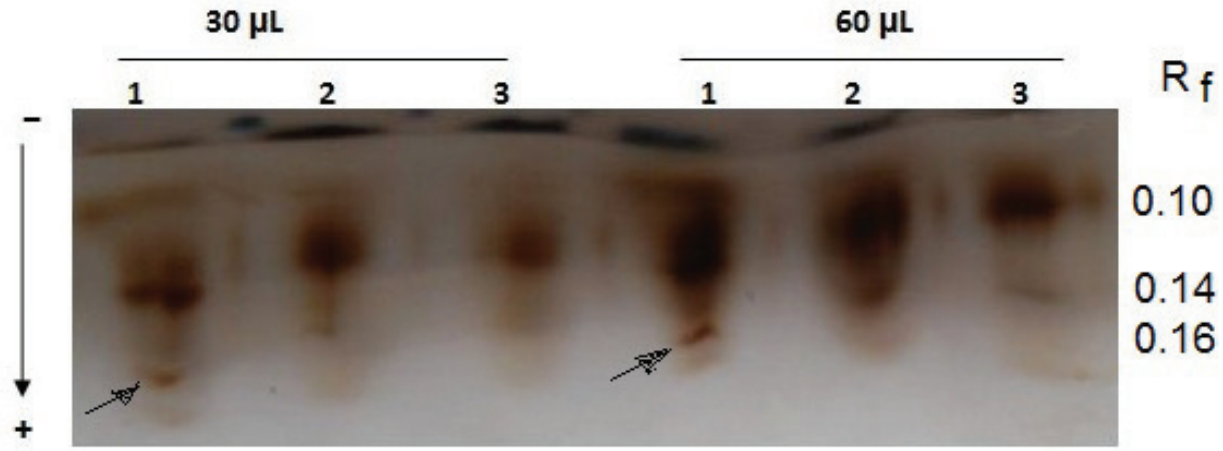


Fig.3: Zymogram of peroxidases extracted from somatic embryos of date palm (cultivar BSK), separated by polyacrylamide gel electrophoresis (11% gels) and revealed with benzidine, as substrate. Extract samples (30 µl and 60 µl) are loaded for cultures 'with AC and G' (1), 'without G' (2) and 'without AC' (3). Arrow indicates peroxidase isoform of Rf 0.16.

Fig. 4: Germination of somatic embryo