Research Article

A simplified Protocol to Induce Callogenesis in Protoplasts of Date Palm (*Phoenix dactylifera* L.) Cultivars

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Received: November 03, 2014; **Revised:** January 29, 2015; **Accepted:** February 24, 2015

Background: In Algeria, date palm is currently confronted to the Bayoud disease. Biotechnological tools such as protoplasts fusion can appear as an alternative to ensure rapid multiplication and improvement of this species.
Objectives: Callogenesis induction in protoplasts isolated from embryogenic callus of three date palm cultivars.
Materials and Methods: Some factors influencing the isolation and culture of protoplasts segregated from the calli of three date palm (*Phoenix dactylifera* L.) cultivars (Deglet Nour, Akerbouch and Degla Beida) were studied. Protoplasts of each cultivar were cultured on a semi-solid medium supplemented with various hormonal balances.
Results: Maceration with an enzymatic solution containing 1.5% cellulase and 1% macerozyme R10 in the presence of 0.5 M mannitol for more than 16 h with gentle agitation allows isolation of a great number of viable protoplasts. In addition, purification of protoplasts on a cushion of 21 or 25% sucrose was effective in cell debris removal and maximum recovery. The culture of isolated protoplasts on a semi-solidified Murashige and Skoog medium, with 0.3% agarose, 2 mg. L^{-1} 2,4-D and 0.5 mg.L^{-1} BAP allowed good viable protoplast maintenance as well as cell wall regeneration. After more than two months of culture, cell divisions were still occurring and microcalli became visible to the naked eye, containing a large number of cells.
Conclusions: The developed protocol can be useful for application of somatic hybridization to improve date palm cultivars.

Keywords: Date palm; Enzymatic solution; Hormonal balance; Microcallus; *Phoenix dactylifera*; Protoplasts

1. Background

The date palm (*Phoenix dactylifera* L.) is a perennial dioecious monocot, with a diploid genome (2n = 2x = 36) that belongs to Palmaceae or Arecaceae. Date palm is normally being reproduced via vegetative propagation by offshoots, which maintains the genetic identity of cultivars (1). However, number of plantlets are limited to the number of offshoots, *i.e*. 10-15 per tree (2). Therefore, slow rate of production via conventional means, especially where mother plants become affected by a disease leads to sever problem in establishing new orchards. Protoplast isolation and somatic hybridization followed by microcalli production and plantlet regeneration may expedite the procedure, allowing micropropagation with great extent.

The main cultivars of date palm, Deglet Nour (DN) and Medjhool, are currently impacted negatively by Bayoud disease caused by a telluric fungus; *Fusarium oxysporum* (f.sp *albedinis*) (3, 4). Few resistant cultivars to this fungus are available to hamper its devastating effect. However, many of which such as Akerbouch (5, 6) have dates of poor quality. Meanwhile, classical improvements through breeding require a long time (about 15-30 years) to produce resistant plant materials with good fruit quality (7, 8). In this regards *in vitro* culture appears as an alternative to ensure rapid multiplication and improvement of many plant species (3).

In this context, somatic hybridization by protoplast fusion may help to resolve the problem of Bayoud. This technique has been successfully used for the genetic improvement of many other species (1).

Somatic hybridization combines the nuclear and cytoplasmic genomes of related species, entirely or partially, at the interspecific and intergeneric levels, avoiding barriers of natural sexual incompatibility (9). However, this technique has not been applied to the improvement of the date palm (1).

In dicots, leaves are the common plant material for protoplast isolation (1, 10), while in monocots including date palm callus and embryogenic cell suspensions are known to be the preferred source for protoplasts isolation and culture (3, 7, 11-13).

The aim of this work was to establish an effective system to isolate protoplasts from embryogenic callus of three date palm cultivars, followed by the induction of cell divisions to obtain microcalli for regeneration of plantlets via somatic embryogenesis. Several factors such as cell wall degrading enzymes, time and type of maceration, and hormonal balance of protoplast culture were analyzed.

2. Materials and Methods

2.1. Plant Material

Protoplasts were isolated from embryogenic calli initiated from fragments of offshoot apical meristems of three date palm cultivars, namely Deglet Nour (DN), Akerbouch (Ak) and Degla Beida (DB). Explants were disinfected and cultured on a modified Murashige and Skoog (MS, 1962) with different plant hormones combinations, and concentrations of activated charcoal according to the protocol established (14-16). The cultures were maintained in darkness at 27ºC with regular subculture for two months on the same medium.

2.2. Protoplast Isolation

Nodular callus (# 0.6 g) was used to isolate protoplasts. The callus was cut into small pieces, mixed with 10 mL of enzymatic solution containing cellulose and pectinase (macerozyme R10) in a Petri dish of 9 cm in diameter, and placed in dark at 27ºC. MS medium supplemented with 204 mM KCl, 67 mM $CaCl_2$, pH 5.6 (Chabane *et al*., 2007) was filter-sterilized (0.2 µm). Several factors including enzyme and mannitol concentration, duration and mode of maceration and sucrose cushion were considered to optimize the protoplast isolation.

2.3. Enzyme Concentrations

Enzymatic solutions of three different concentrations of cellulase (1, 1.5 and 2%) and macerozyme R10 (0.2, 0.5 and 1%) were subjected to two modes of maceration (Stationary or agitation at 50 rpm). The concentration of the osmotic agent (mannitol) was 0.5 M. The mixture (callus + enzymatic solution) was incubated over a 16 h period in darkness at 27ºC.

2.4. Mannitol Concentration

Three concentrations of mannitol (0.4, 0.5 and 0.6 M) combined with two modes of maceration (stationary or agitation under 50 rpm) were tested in the presence of an enzymatic solution containing 1.5% cellulase and 1% macerozyme R10. The mixture was incubated over a 16 h period in darkness at 27ºC.

2.5. Duration and Mode of Maceration

To determine the best duration and method of enzymatic maceration, different durations (observations carried out after every 2 h over a 24 h period) combined with three modes of maceration (stationary, 50 or 100 rpm of agitation) were tested in the presence of 1.5% cellulase, 1% macerozyme R10, and 0.5 M of mannitol. The mixture was incubated in dark at 27ºC.

2.6. Protoplast Purification

After incubation, the mixture is filtered by passage through a grid with pores of about 350 µm to remove debris and large cell colonies. The filtrate was centrifuged at 65 ×g for 5 min. The supernatant was discarded and pellet containing the protoplasts was diluted in 1 mL of solution A, consisting of a basal medium without enzymatic solution supplemented with 0.5 M mannitol. The pellet was rinsed twice by centrifugation at 65 ×g for 5 min to remove all enzyme trace . To increase the viability of protoplasts and prevent their breakdown (because of the effect of salts), the protoplasts were rinsed once with a solution B (solution A without KCl). The protoplasts were suspended in 1 mL solution B, allowing their flotation on 2-3 mL of 21 or 25% sucrose solution. Protoplasts were recovered by centrifugation at 65 ×g for 5 min from interphase of two solutions. Recovered protoplasts were suspended in 1 mL of solution B and homogenized for counting.

2.7. Protoplast Culture

Only protoplasts of DN and Ak were cultivated. The isolation was carried out with an enzymatic solution containing 1.5% cellulase and 1% macerozyme R10 during 14 to 16 h in dark at 27ºC in the presence of 0.5 and 0.6 M mannitol, for DN and Ak, respectively. After purification on a layer of 21 to 25% sucrose,

protoplasts were cultivated at a density of 9.5×10^4 protoplasts $m.L^{-1}$ by spreading on 5 mL of MS medium solidified with 0.3% agarose and fortified with three different concentration and combination of hormones; 2,4-Dichlorophenoxyacetic acid (2,4-D) and 6-Benzylaminopurine (BAP) (2/0.5; 1/0.5, and 0.5/1 $mg.L^{-1}$) considered as hormonal balances (HB). The medium was also supplemented with 40 $g.L^{-1}$ sucrose, 72 $mg.L^{-1}$ glucose and 258.4 $mg.L^{-1}$ KH_2PO_4 (pH 5.7). The liquid medium containing the appropriate hormonal balance and mannitol instead of glucose and sucrose was added after 3, 4 and 6 weeks, with reduction of mannitol concentration from 0.25 to 0.125 and finally 0 M. All the cultures were maintained in complete darkness at 24±2ºC.

2.8. Observations and Data Analyses

Only viable protoplasts were counted. The viabil-

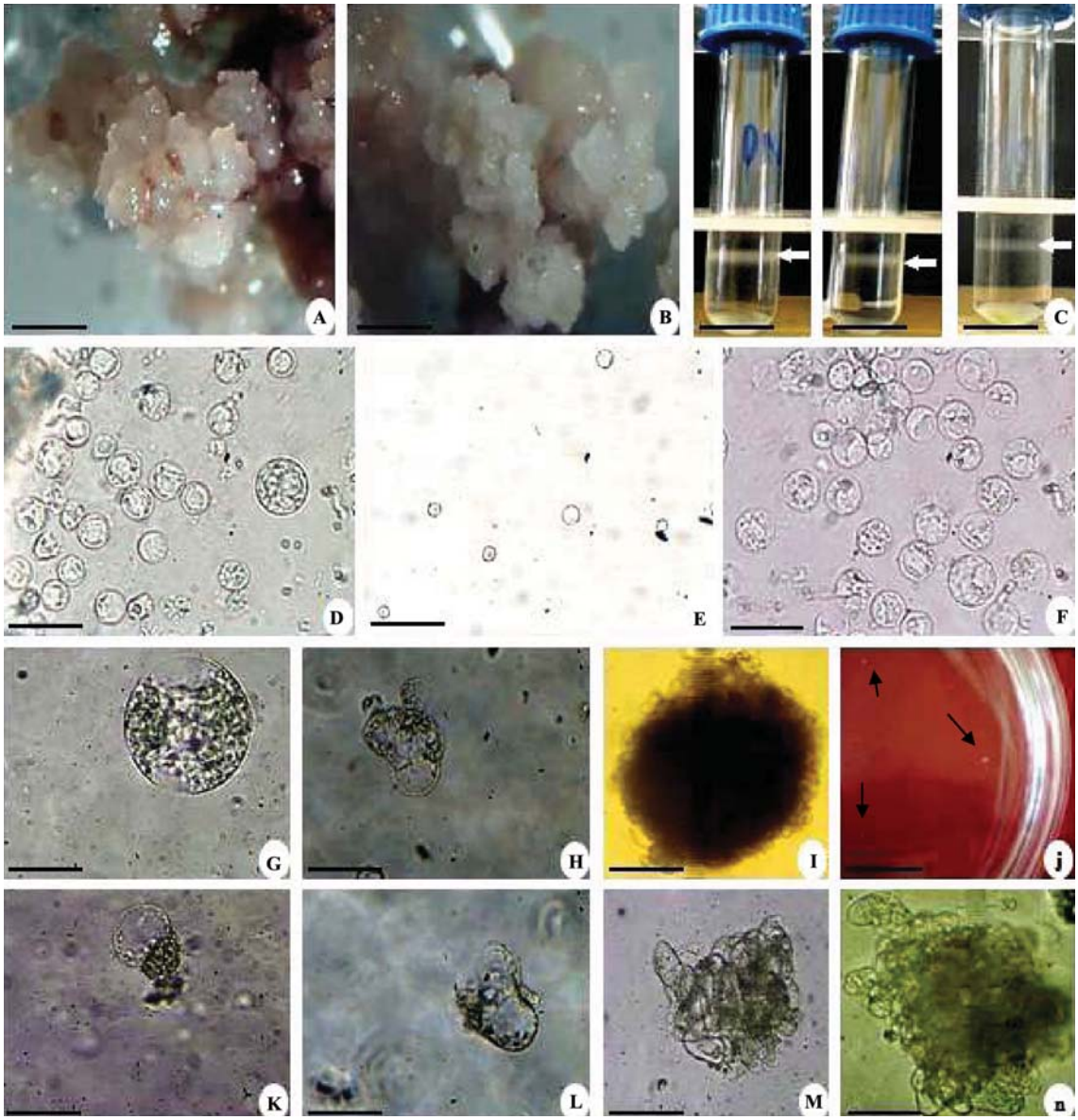

Figure 1. Culture of protoplasts isolated from calli of three date palm cultivars. A-B: Embryogenic callus of Deglet Nour and Akerbouch (G = ×45); C: Purification of protoplasts of 3 cultivars on a layer of 25% of sucrose (Bar = 1.4 cm); D: DegletNour-ES N°1-50 rpm (Bar = 27.5 µm); E: Akerbouch-ES N°2-Stat (Bar = 110.0 µm); F: DeglaBeida-ES N°1-Stat (Bar = 27.5 µm); G: DegletNour-HB N°1-24h (Bar = 12.8 µm); H: DegletNour-HB N°1-3days (Bar = 11.6 µm); I-J: Microcalli visible to the naked eye: DegletNour-HB N°1-74 days (Bar = 135.0 µm and 1.9 cm); K: Akerbouch-HBN°1-3days (Bar = 12.8 µm); L: Akerbouch-HBN°1-5days (Bar = 12.8 µm); M-N: Microcolony: Akerbouch-HBN°2-74 days (Bar = 86.1 and 38.8 µm).→ indicates layer purified protoplasts and Microcalli

ity of protoplasts was determined by treatment with 0.1% methylene blue solution. Protoplasts were counted using Malassez cell. At least three counts per treatment were made. The results were expressed as the number of viable protoplasts per grams of fresh material of callus. The protoplasts diameter was determined using an ocular micrometer. Protoplast viability, cell wall regeneration and cell divisions were estimated by following ten culture fields by observing under inverted microscope at magnification of 400. All experiments were repeated at least twice and averages were represented graphically and statistically analyzed. Data analysis (Anova/Manova) was carried out with "Statistics" program, version 5.

3. Results

3.1. Protoplast Isolation

Yield and viability of isolated protoplasts were found to be highly dependent on the genotype and conditions of isolation (enzymatic solution, duration and mode of maceration). The best yields, 4.7 and 2.5×10^5 protoplasts per g of fresh weight (f.w.) were obtained with enzymatic solution N°1 (ES N°1) containing 1.5% cellulase and 1% macerozyme R10 under stationary conditions or gentle agitation at 50 rpm for DN and DB, respectively (Figure 1D and F and Figure 2) and with 1% cellulase and 0.2% macerozyme R10 (ES N°2) without agitation for Ak, 1.4×10^5 protoplasts per g of f.w. (Figure 1E). Similarly, ES N°2 under stationary conditions allowed good viability of protoplasts in DN and DB, while a combination of 2% cellulase and 0.5% macerozyme R10 (ES N°3) without agitation is more beneficial for Ak (Figure 2). The viability results are 57, 70 and 62% respectively for the three cultivars.

The concentration of the osmotic agent (mannitol) significantly affected the number and viability of isolated protoplasts. A concentration of 0.6 M mannitol significantly improved the yield of isolated protoplasts (4.9×10^5 protoplasts per g f.w.) from callus of cultivar Ak mainly under 50 rpm of agitation, while 0.5 M of mannitol was the best concentration for DN and DB with 4.7 and 2.5×10^5 protoplasts per gram f.w., respectively. However, better viability, 78, 65 and 56% respectively, for DN, Ak and DB was obtained with low concentrations of mannitol (0.4 or 0.5 M) under stationary conditions or 50 rpm of agitation depending on the cultivar (Figure 3).

The yield of viable protoplasts increased with the duration in relation with the mode of maceration. The best yields, 4.7 and 4.2×10^5 protoplasts per g of f.w. were obtained after 16 h at 50 rpm agitation for DN and 18 h under stationary conditions for DB. Although 8 h without agitation is more beneficial for Ak (1.0×10^5 protoplasts per g f.w.), less than 8 h of maceration under 50 rpm of agitation produced the best rates of viability, 81, 83 and 60%, respectively for DN, Ak and DB, (Figure 4). In general, the stationary maceration was beneficial for having more viable protoplasts in DB, while a low agitation of 50 rpm improved the protoplasts isolation in other two cultivars (Figures 2, 3 and 4).

Protoplasts purification on a layer of 21 or 25% of sucrose was effective in removing cell debris and recovering the maximum number of released protoplasts. A concentration of 25% of sucrose was beneficial in the case of DN and DB, while 21% was sufficient for Ak (Figure 1C). The results in terms of recov-

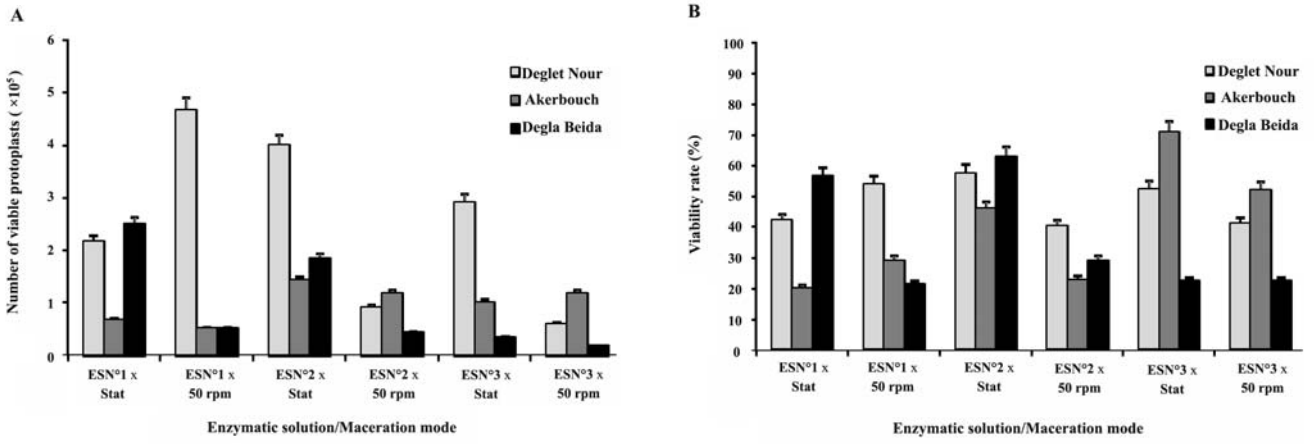

Figure 2. A: Effect of the interaction (Enzymatic solution × Maceration mode) on yield and B: viability of protoplasts isolated from calli of three date palm cultivars

ery percentage of viable and purified protoplasts were 85%, 87% and 95% for DN, Ak and DB, respectively (Figure 5).

3.2. Protoplast Culture

The viability of protoplasts before cultivation was about 72 and 47% for DN and Ak, respectively. Nevertheless, viability decreased with time of culture, hormonal balance and type of cultivar. The protoplasts of DN had good maintenance of viability and cell wall regeneration than protoplasts of Ak on different hormonal balances tested for all culture. After 15 days of culture, a combination of 2 mg.L^{-1} of 2,4-D and 0.5 mg.L^{-1} of BAP (HB N°1) allowed good maintenance of protoplasts viability for DN and Ak, 46.4 and 20%, respectively. A good cell wall regeneration shown by more cell elongation was noted, 43.6 and 18% for DN and Ak, respectively (Figure 6). This led to the best cell division rates, 17.5 and 2.8%, respectively (Figure 7). Equally, in presence of the HB N°1, protoplasts began to regenerate their walls after 24 h of culture, as evidenced by elongation and thickening of the cell periphery (Figure 1G and K). By contrast, protoplast elongation was not seen for two other hormonal balances for at least 48 h of culture, and the viability of these protoplasts decreased rapidly as a result of low cell wall regeneration and cell division (Figures 6 and 7). The first cell divisions were observed after 48 to 72 h on the HB N°1 (Figure 1H and L) and more than 4 days on the two other hormonal balances. The succession of cell divisions led to microcolonies, followed by appearance of small number of microcalli visible to the

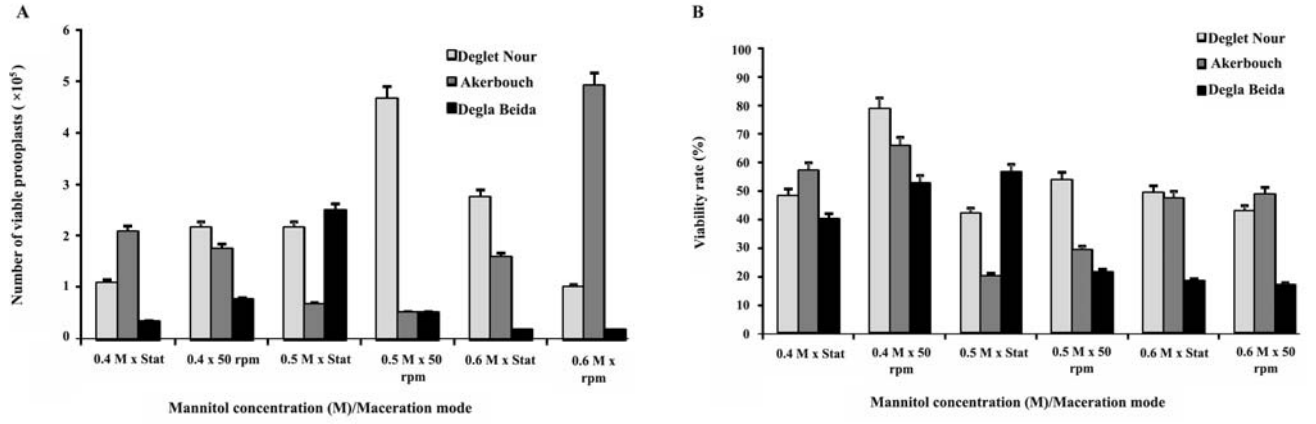

Figure 3. A: Effect of the interaction (Mannitol concentration × Maceration mode) on yield and B: viability of protoplasts isolated from calli of three date palm cultivars

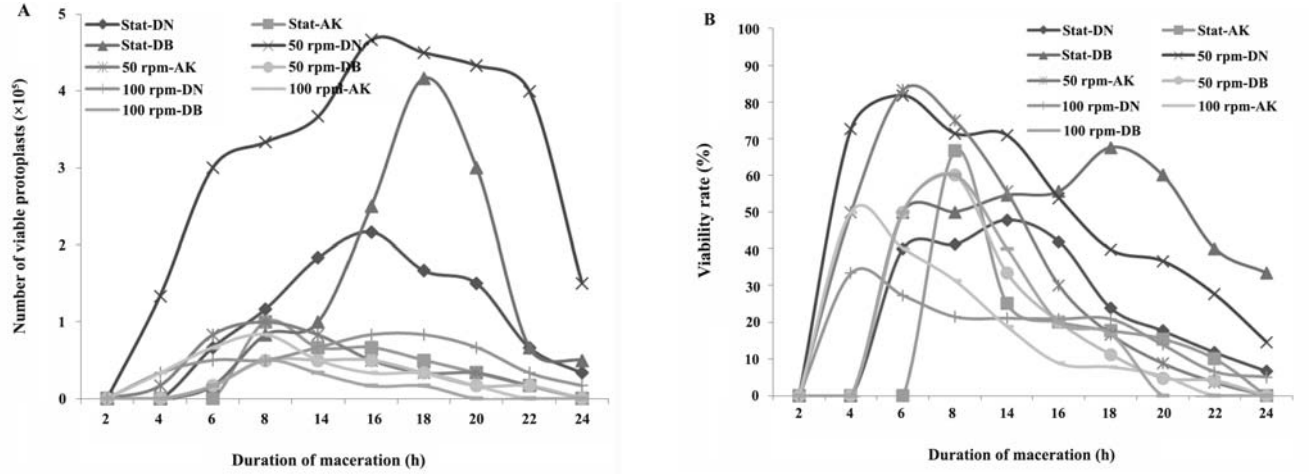

Figure 4. A: Effect of duration and mode of maceration on yield and B: viability of protoplasts isolated from calli of three date palm cultivars: DegletNour (DN), Akerbouch (Ak) and DeglaBeida (DB)

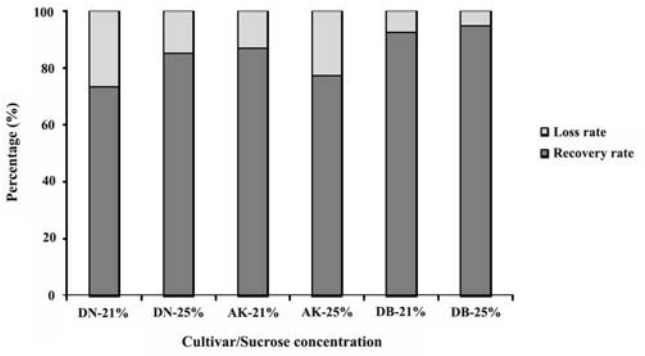

Figure 5. Effect of sucrose concentration (21 or 25%) in the layer of purification on recovery and the loss rate of protoplasts in three date palm cultivars: Deglet Nour (DN), Akerbouch (Ak) and Degla Beida (DB)

naked eye, including a large number of cells, after culture for more than 2 months (Figure 1 I, J, M and N).

4. Discussion

We obtained microcalli from protoplasts isolated from calli of two main cultivars (among the 940 listed) of date palm grown in Algeria (17). Our objective was to optimize a protoplast cultivation protocol to be used in improvement of date palm by somatic hybridization between DN (for the quality of its dates) and Ak (for its resistance to Bayoud).

4.1. Protoplasts Isolation

The isolation of protoplasts is strongly influenced by genotype, plant material, composition of the enzymatic solution (1, 18) and the isolation conditions (19-21). Chabane *et al*. (7) indicated that embryogenic calli were the appropriate material for protoplast isolation in date palm.

An effective enzymatic formula should be able to release the maximum number of protoplasts without compromising their viability (21). According to Gabr and Tisserat (22), a combination of 1% cellulase and 0.2% macerozyme R10 allows the release of 1.70×10^5 protoplasts per mL from calli of DN. In our case, a yield of 4×10^5 protoplasts per g of f.w. (equivalent to 2.4×10^5 protoplasts per mL) was obtained with the same enzymatic solution (ES Nº2) on calli of DN. However, the addition of pectolyase, Y23 significantly improved the isolation of protoplasts of date palm (7). This pectinase was found to be more useful than the macerozyme R10 to isolate protoplasts from callus of plum (23-25). However, the excess enzyme led to a decrease in the viability of isolated protoplasts (10, 18, 26), perhaps by enzymatic toxicity (27, 28) or due to a strong and rapid enzymatic activity often causing membrane damage (29, 30). Consequently, in the present study, the combination of 1.5% cellulase and 1% macerozyme R10 in the presence of 0.5 M mannitol yielded the highest amount of protoplasts.

The concentration of the osmotic agent strongly influences the number and viability of protoplasts released (31). With date palm, Chabane (3) used 0.5 M of mannitol, while, Gabr and Tisserat (22) and Rizkalla *et al*. (11) used about 0.4 M of sucrose. Good yields and viability are not always guaranteed with the same concentration of the osmotic agent (32). According to Zhu *et al*. (18), too little or high concentrations of mannitol reduce the yield of protoplasts of *Echinacea* callus. For our protocol, an optimal yield of protoplasts was obtained with 0.5 M mannitol.

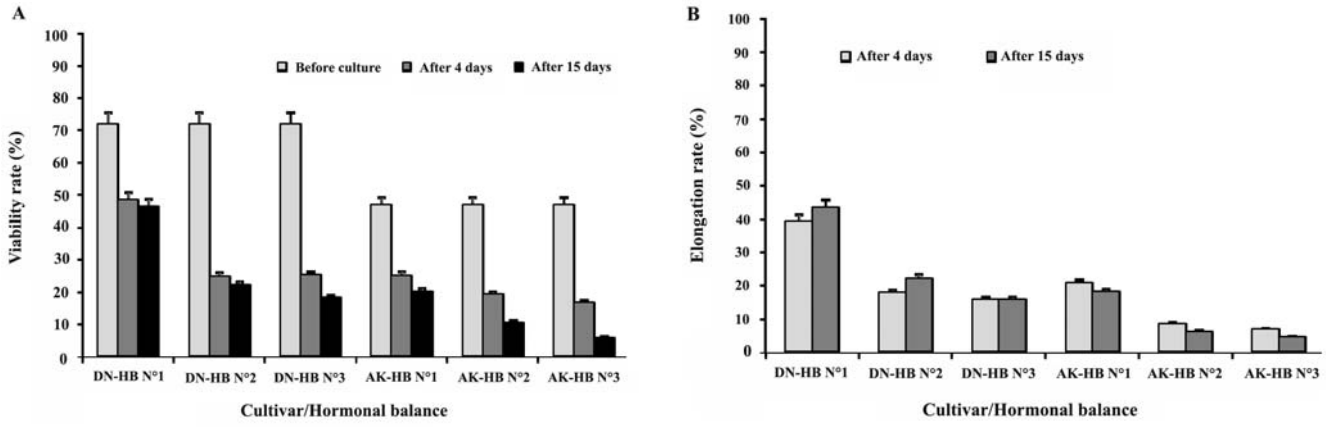

Figure 6. A: Effect of hormonal balance on viability and B: elongation of protoplasts of Deglet Nour (DN) and Akerbouch (Ak) during the first 15 days of culture. HB= Hormonal Balance

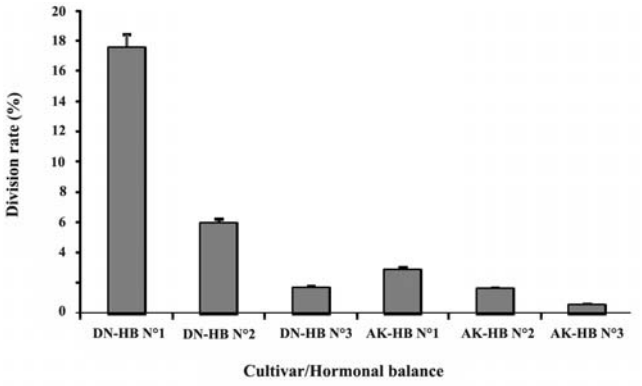

Figure 7. Effect of hormonal balance in the division of protoplasts of Deglet Nour (DN) and Akerbouch (Ak) after 15 days of culture. HB = Hormone Balance

A short maceration leads to a low yield of protoplasts, while a long duration decreases the viability (21, 33). With date palm, Gabr and Tisserat (22) indicated that a long incubation period may have an adverse effect on the quality of isolated protoplasts. Similarly, Chabane *et al*. (7) found that incubation for more than 12 h resulted in the reduction in yield and viability of isolated protoplasts. However, we found that an optimal yield of protoplasts in date palm was obtained with 16 h maceration.

The liberation of protoplasts from calli requires a certain agitation ranging from 20 to 60 rpm depending on the species (20) and disintegration of tissue into small pieces (1). Nevertheless, Sinha *et al*. (19) and George (34) suggested that the importance of agitation was only at the end of the incubation when it allows the release of protoplasts from digested tissues. In the case of date palm, the stationary maceration resulted in good yields (7, 22). For our protocol, an optimal yield of protoplasts was obtained with gentle agitation.

4.2. Protoplast Culture

The decrease in viability of protoplasts is related to their extreme sensitivity to osmotic shock and other physical disturbances during the culture-period (35). With date palm, Chabane (3) has shown that protoplasts of DN have greater viability than those of Takerboucht after 10 days of culture. According to Mattoo and Handa (36), auxins and cytokinins induce ethylene biosynthesis, implicated in cell death, even at low levels, while Beyl (37) and Gaba (38) indicated that 2,4-D induces the formation of ethylene only at high levels. Lysis of protoplasts may occur because of the accumulation of certain toxic products (ROS and RNS) of metabolism or proteolytic enzymes released by dead cells, and following high osmotic turgor pressure (31, 35, 39). For this reason, calcium is used to maintain the membrane integrity (1, 40, 41) and cellulose synthesis (34).

Cell wall regeneration is correlated with the loss of spherical form and subsequent cell elongation. cytokinins and auxins especially, promote cell elongation by promoting the synthesis of certain enzymes involved cell wall synthesis and an increase in membrane permeability, probably to water (34, 42-44). However, the effect of synthetic auxins, such as 2,4-D appears to be directly related to the level of endogenous auxin, *i.e*. IAA (45). Protoplasts cultured on a suitable medium initiate the synthesis of a new wall within a few minutes or hours after their introduction (9, 42, 46). With date palm, Chabane (3) noted the regeneration of the cell wall three days after spreading. In our case, the change in shape and cell elongation began 24 h after the protoplasts introduction.

The mitotic activity is influenced by the combination and concentration of growth regulators during culture (47). Auxins induce the cell cycle by activation of genes responsible for DNA replication, and cytokinins control the events of mitosis (37, 38, 42, 44, 45, 48, 49). Furthermore, the ratio of auxin to cytokinin is very important in the development of protoplasts. It is frequently greater than one (50). Our best results were obtained with the HB N°1, where the ratio was greater than 1.

The emergence of divisions varies from one cultivar to another. This difference may be due to either varying nutritional and hormonal requirements (51) or to a difference in the culture density (9). In case of date palm, Chabane (3) observed the first division after 7 days with DN and subsequent divisions after 15 days. However, the first divisions of callus protoplasts of several species were observed during the first days of culture (10, 50, 52- 54) and during the first ten days for the following divisions (50). Thereafter, the appearance of date palm microcalli appeared after more than two months of culture (3). However, no plant regeneration was obtained in our work.

5. Conclusions

We obtained good yields of microcalli from protoplasts isolated from calli of date palm cultivars DN and Ak. These two cultivars are known for the quality of their dates (DN) and resistance to Bayoud (Ak). The development of this protocol is a necessary first step towards application of somatic hybridization as a tool for new cultivar development with desirable fruit qual-

ity and resistance to Bayoud in date palms. Our optimal protoplast isolation protocol includes maceration with 1.5% cellulase and 1% macerozyme R10 in the presence of 0.5 M mannitol for more than 16 h with gentle agitation. In addition, purification of protoplasts on a cushion of 21 or 25% sucrose was effective in removing cell debris and recovering the maximum amount of released protoplasts. The culture of isolated protoplasts on MS semi-solidified medium, with 0.3% agarose and 2 $mg.L^{-1}$ of 2,4-D and 0.5 $mg.L^{-1}$ of BAP allowed good maintenance of protoplast viability with good regeneration of cell walls. Microcalli with large number of cells were obtained after a few months of culture.

Developing efficient protoplast culture systems and taking advantage of molecular data about date palm genome at the same time would speed up targeted breeding. Protoplast culture can help to accelerate identification of necessary genetic resources to create resistant varieties with high quality such as Khalas. Genome sequencing of Khalas on the other hand revealed of the presence of 41,660 genes, which are more than 96% of genes from more than 90% of sequenced genome (605.4 Mb of 671 Mb) (55). Moreover, the complete genome sequences of chloroplast and mitochondrial transcriptome are available (56, 57). These growing bod of knowledge would be beneficial in years to come.

Acknowledgements

The authors gratefully acknowledge funding support from ENSA and the Algerian Ministry of High Education and Research (MESRS), and they thank Dr. Mark Bernads (University of Western Ontario, Canada) for his precious contribution in the improvement of the text of this article.

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