

Effect of Basal Medium, Explants Size and Density on the *In Vitro* Proliferation and Growth of Date Palm (*Phoenix dactylifera* L.) Cultivar '16-bis'

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Abstract

The effect of basal medium, explant size and density on shoot multiplication, growth, rooting and acclimatization of date palm cv. '16-bis' was evaluated. Bud clusters of different sizes (2, 3, 4 and 5 buds per cluster) were cultured at density of 1, 2, 3 and 4 clusters on Murashige and Skoog medium (MS), woody plant medium (WPM) and Nitsch medium (NM) supplemented with 0.5 mg/L 2-naphthoxyacetic acid and 0.5 mg/L kinetin for three months (multiplication phase). Separated shoots of different sizes (<3 cm; 3 to 4.5 cm and 4.5 to 6 cm) were cultured at density of 1, 2, 3 and 4 shoots on hormone free MS medium, WPM or NM for three months (Elongation-rooting phase). The proliferation and development of shoots were affected by the basal medium, explant size and density. The optimal shoot proliferation (18.1) was observed when 4 buds clusters were cultured at the density of 2 clusters per jar in MS medium. Separated shoots of 4.5 to 6 cm length exhibited the optimal *in vitro* development in terms of leaf length and greening, and root number and length when cultured on MS medium. In addition, these shoots reached the highest acclimation frequency with 80%. Our results would be utilized for an efficient propagation of plantlets of cv. '16-bis', a selected date palm cultivar resistant to the bayoud disease.

Keywords: acclimatization, organogenesis, *Phoenix dactylifera* L., regeneration, shoot multiplication

Introduction

Date palm (*Phoenix dactylifera* L.) is one of the most important fruit crops in the Middle East and North Africa. In Morocco, it is the main component of oasis ecosystems, where it provides food, shelter, fiber and other products. However, its population has been reduced significantly because of a very serious disease called bayoud (Sedra, 2011).

The bayoud disease caused by a filamentous imperfect fungus (*Fusarium oxysporum* f. sp. *albedinis*) (Quenzar *et al.*, 2001) is by far the most dangerous disease affecting date palm plantations (Sedra and Lazrak, 2011). In Morocco, the majority of the date palm groves are affected by this fungus (Quenzar *et al.*, 2001). Up to now, the only feasible method to combat bayoud disease and rehabilitate Moroccan palm groves is the selection, multiplication then the diffusion of resistant cultivars (Sedra, 2011).

The use of *in vitro* culture techniques would multiply selected genotype on a large-scale and in short period (Jain, 2012). Somatic embryogenesis is commonly used for the rapid propagation of date palm cultivars. However, undesirable genetic variability was observed in the regenerated plants (Khierallah and Bader, 2007). Regeneration through direct organogenesis has the advantage of using low concentrations of plant growth regulators and avoiding the callus phase, which minimize the risk of somaclonal

variation among regenerants. However, the success of this technique is highly genotypic dependent (Jain, 2012).

In this study, we aimed to evaluate the effect of basal medium, explant size and density on shoots proliferation and growth of the Moroccan date palm cultivar resistant to bayoud '16-bis', in order to optimize conditions for an efficient micropropagation and regeneration through organogenesis.

Materials and methods

Initiation and maintenance of organogenic cultures

Organogenic cultures of date palm cv. '16-bis' were initially established from shoot-tip explants according to the protocol of Beauchesne *et al.* (1986). Organogenic cultures were then cultured on three different basal media: MS (Murashige and Skoog, 1962), WPM (Lloyd and McCown, 1980) and NM (Nitsch and Nitsch, 1969). All media were supplemented with 30 g/L sucrose, 1 g/L polyvinyl-pyrrolidone and gelled with 6 g/L bactoagar.

Experiment 1: Effect of explants size on shoot proliferation

In the first experiment, the effect of four explant sizes on shoot proliferation was evaluated. Buds (< 1 cm length) from the organogenic cultures were separated into clusters of 2, 3, 4 and 5 buds (Fig. 1a). The clusters were cultured for three months at density of 1 per jar onto MS,

WPM and NM media for multiplication. All multiplication media were supplemented with 30 g/L sucrose, 1 g/L polyvinyl-pyrrolidone, 0.5 mg/L 2-naphthoxyacetic acid, 0.5 mg/L kinetin and gelled with 6 g/L bactoagar.

Experiment 2: Effect of explants density on shoot proliferation

In the second experiment, the effect of four densities of explants on shoot proliferation was studied. Clusters comprised 4 buds each were cultured onto the same multiplication media of the first experiment for three months at density of 1, 2, 3 and 4 clusters per jar.

Experiment 3: Effect of explants size on shoot length and rooting

In the third experiment, the effect of three shoot size (< 3 cm; 3 to 4.5 cm and 4.5 to 6 cm; Fig. 1b) on shoot length and rooting was tested. Shoots (with one root < 1 cm length; Fig. 1b) were isolated from the mother cluster and cultured individually for three months onto elonga-

tion-rooting media; i.e. hormone-free MS, WPM or NM. Media were supplemented with 30 g/L sucrose, 1 g/L polyvinyl-pyrrolidone and gelled with 6 g/L bactoagar.

Experiment 4: Effect of explants density on shoot length and rooting

In this experiment, shoots of 4.5 to 6 cm length were separated from the mother clusters and cultured at density of 1, 2, 3 and 4 shoots per jar. The elongation-rooting media were the same as experiment 3. Shoots were cultured in these media for three months.

Plantlets acclimatization

Plantlets with three leaves and good root system were removed from the culture media. The root system was carefully washed under tap water to remove residual media then soaked in a fungicide solution (1 g/L Pelt 44 PM) for 15 min. Acclimatization of the plantlets was done in a mixture of peat and gravel (1:1, v:v) in a tunnel covered with polyethylene and placed in a glasshouse. After two



Fig. 1. General aspects of shoot multiplication from clusters of different sizes. a Clusters of 2, 3, 4 and 5 buds used in this study; b Shoots of different sizes used during the elongation-rooting phase; c Shoot multiplication after 3 months of culture. This cluster was initially comprised 2 buds. Note the difference in growth between the initial buds and the newly formed ones; d Example of shoot multiplication after 3 months of culture when clusters comprised initially 4 buds and cultured at the density of 2 per jar; e Example of shoot multiplication after 3 months of culture when clusters comprised initially 4 buds and cultured at the density of 4 per jar

weeks, the polyethylene was opened gradually. The polyethylene was removed completely after four weeks. Inside the polyethylene tunnel relative humidity was 98% while it was 70% in the glasshouse.

Culture conditions

All culture media were adjusted to pH 5.7 then 25 ml aliquots of culture medium were dispensed into jars. Media were autoclaved at 121°C for 25 min. All cultures were incubated under a 16 h light/8 h dark photoperiod at 25°C and transferred onto fresh medium every month. Plantlets transferred to the glasshouse for acclimatization were kept at 27°C under natural light.

Parameters recorded and statistical analysis

In experiments 1 and 2, the average number of shoots per explant and average shoot length were calculated after three months of culture. In experiments 3 and 4, we recorded the average length of shoots, leaf greening (leaf greening was visually estimated and noted +, ++ and +++ for low, moderate and high intensity, respectively), average number of roots and length of roots after three months. Concerning the plants acclimatization, the survival rate was calculated after three months of culture in the glasshouse. In all experiments, each jar was considered as one replicate and the data represent the mean of ten replicates.

Analysis of variables was carried out using a completely randomized design at 5% significant level of SPSS version 16.0. Differences between means were separated with Student-Newman-Keuls test. Prior to analysis, percentage data were arcsine transformed.

Tab. 1. Effect of basal medium and initial number of buds per cluster on shoot multiplication and length of date palm cv. '16-bis' after 3 months of culture

Culture medium	MS				WPM				NM			
	2 buds	3 buds	4 buds	5 buds	2 buds	3 buds	4 buds	5 buds	2 buds	3 buds	4 buds	5 buds
Initial number of buds per cluster												
Average number of shoots after 3 months	12.2 ± 1.8 abc	17.4 ± 2.2 e	21.5 ± 2.5 f	20.4 ± 3.5 f	9.3 ± 2.7 a	12.2 ± 1.8 abc	14.7 ± 1.7 cde	14.5 ± 3.6 cde	10.4 ± 1.7 ab	13.3 ± 3.0 bcd	16.3 ± 4.7 de	17.1 ± 3.8 e
Average length of shoots (cm)	5.1 ± 0.8 d	4.3 ± 1.1 cd	3.1 ± 1.6 abc	3.2 ± 1.3 abc	4.1 ± 0.9 bcd	3.5 ± 1.5 abc	2.3 ± 0.9 a	2.2 ± 0.4 a	3.1 ± 0.9 ab	3.2 ± 1.0 abc	2.7 ± 0.4 ab	2.6 ± 0.9 ab

Note: Mean values with the same letters are not significantly different ($p > 0.05$)

Tab. 2. Effect of basal medium and number of clusters per jar (explant density) on shoot multiplication and length of date palm cv. '16-bis' after 3 months of culture

Culture medium	MS				WPM				NM			
	1 cluster	2 cluster	3 cluster	4 cluster	1 cluster	2 cluster	3 cluster	4 cluster	1 cluster	2 cluster	3 cluster	4 cluster
Average number of shoots per cluster after 3 months	21.5 ± 2.5 f	18.1 ± 2.7 e	12.5 ± 2.6 c	10.7 ± 1.6 bc	14.7 ± 1.7 d	9.4 ± 1.1 ab	7.3 ± 1.4 a	7.4 ± 1.7 a	16.3 ± 4.7 de	12.2 ± 1.3 c	8.3 ± 2.0 ab	7.7 ± 1.4 a
Average number of shoots per jar after 3 months	21.5 ± 2.5 bc	36.2 ± 5.5 f	37.5 ± 7.9 f	42.8 ± 6.5 g	14.7 ± 1.7 a	18.8 ± 2.3 abc	21.9 ± 4.4 bc	29.6 ± 6.8 de	16.3 ± 4.7 ab	24.4 ± 2.6 cd	24.9 ± 6.0 cd	30.8 ± 5.9 e
Average length of shoots (cm)	3.1 ± 1.6 a	2.6 ± 1.4 a	2.3 ± 1.7 a	2.5 ± 1.0 a	2.3 ± 0.9 a	2.5 ± 1.7 a	2.7 ± 1.4 a	2.5 ± 1.3 a	2.7 ± 0.4 a	2.3 ± 1.0 a	2.3 ± 1.0 a	2.4 ± 0.9 a

Note: Mean values with the same letters are not significantly different ($p > 0.05$)

Results

Effect of explants size on shoot proliferation

The explant size had a significant effect on shoot proliferation. When clusters of two buds were cultured onto proliferation medium, we observed buds elongation and greening. The formation of new buds was observed at the base of the elongated buds after the first transfer onto fresh medium (i.e. after one month of culture). After three months of culture, the number of newly formed buds was low: 12.2, 9.3 and 10.4 in MS, WPM and NM, respectively (Fig. 1c).

Four buds per cluster was the most suitable size for high shoot proliferation with averages of 21.5, 14.7 and 16.3 morphologically superior shoots per explant in MS, WPM and NM, respectively (Tab. 1). Clusters of 5 buds have also displayed high shoot proliferation. However, a pronounced tissue browning was observed.

Effect of explants density on shoot proliferation

The highest number of shoots per cluster was obtained when the density used was one cluster per jar (21.5, 14.7 and 16.3 shoots per explant in MS, WPM and NM, respectively). However, taking into consideration the number of shoots regenerated per jar (Tab. 2) and their morphology, we strongly suggest the use of 2 clusters per jar of MS medium.

When 3 and 4 clusters were cultured per jar, the average number of shoots regenerated per jar could be increased (Tab. 2). However, the shoots developed at the densities of 1 and 2 clusters per jar were morphologically superior. They were healthy and more uniform (Fig. 1d) while those

formed at the densities of 3 and 4 clusters per jar (Fig. 1e) showed morphological abnormalities (i.e. hyperhydricity and tissue browning; data not shown). Thus, a density of 2 clusters per jar is optimal in terms of cost and to avoid morphological abnormalities.

Effect of explants size on shoot length and rooting

The pattern of shoot growth and development was influenced by the explant size and the basal medium. Our findings showed that the optimum growth and development of shoots in terms of length and rooting was obtained in shoots from 4.5 to 6 cm length: In MS medium, the average shoot length was 14.2 cm, the average number of roots per shoot was 4.2 and their average length was 5 cm. In WPM and NM media, we observed lower averages: 11.8 and 10.7 cm shoot length, respectively; 3.4 roots per shoot in both the basal media; 3.5 and 3.4 cm root length in WPM and NM, respectively. Concerning the other sizes, shoots lower than 3 cm showed slow growth. Intermediate results were observed within shoots from 3 to 4.5 cm length (Tab. 3). Regardless of the initial shoot length, we observed that shoot greening intensity was higher in MS medium. This indicates that those plantlets have good photosynthetic capacity and consequently, better chance of survival during acclimatization (Mazri and Meziani, 2013).

Effect of explants density on shoot length, rooting and acclimatization

Increasing shoots density in the culture medium decreased shoot length, and root number and length in all the culture media. The highest length of shoots, the highest number of roots per shoot and their highest length were observed at the density of one shoot per jar. Nevertheless, the density of 2 shoots per jar also showed good development. For example, average values of 13.7 cm for shoot length, 4.3 for average number of roots per shoot and 4.3 cm for root length were noted at the density of 2 shoots per jar in MS medium. Shoots cultured at the densities of 3 and 4 per jar showed lower values (Tab. 4).

Shoots at the three leaves stage were transferred to the glasshouse. After three months under glasshouse conditions, more than 60% of plantlets survived (Tab. 4). The highest survival rate (80%) was observed in shoots that have been cultured onto MS medium at the densities of 1 and 2 shoots per jar. Thus, to save cost, we suggest the density of 2 shoots per jar during the elongation-rooting phase. The plantlets that survived grew vigorously and did not show any morphological abnormalities (Fig. 2).

Discussion

Plant growth and development under *in vitro* conditions depend upon different factors such as the genotype,

Tab. 3. Effect of basal medium and initial shoot length (cm) on shoot growth and development of date palm cv. '16-bis' after 3 months of culture

Culture medium	MS			WPM			NM		
	< 3cm	3 – 4.5 cm	4.5 – 6 cm	< 3cm	3 – 4.5 cm	4.5 – 6 cm	< 3cm	3 – 4.5 cm	4.5 – 6 cm
Initial shoot length (cm)	< 3cm	3 – 4.5 cm	4.5 – 6 cm	< 3cm	3 – 4.5 cm	4.5 – 6 cm	< 3cm	3 – 4.5 cm	4.5 – 6 cm
Shoot length after 3 months (cm)	10.4 ± 2.3 ab	11.7 ± 2.2 b	14.2 ± 2.0 c	9.2 ± 1.9 a	11.0 ± 0.8 ab	11.8 ± 1.3 b	9.3 ± 1.7 a	9.7 ± 1.4 ab	10.7 ± 1.8 ab
Shoot greening	+++	+++	+++	++	++	++	++	++	++
Root number	3.3 ± 1.3 ab	3.3 ± 0.6 ab	4.2 ± 1.0 b	3.2 ± 0.6 ab	3.2 ± 0.6 ab	3.4 ± 0.5 ab	3.0 ± 0.8 a	3.2 ± 0.6 ab	3.4 ± 0.8 ab
Root length (cm)	3.2 ± 0.6 ab	4.3 ± 0.4 ab	5.0 ± 0.4 c	2.5 ± 0.8 a	2.6 ± 1.5 a	3.5 ± 1.5 ab	2.4 ± 0.8 a	3.1 ± 1.4 ab	3.4 ± 1.2 ab

Note: Mean values with the same letters are not significantly different ($p > 0.05$). Intensity of greening: + low, ++ moderate, +++ high

Tab. 4. Effect of basal medium and number of shoots per jar (shoot density) on shoot growth, development and acclimatization of date palm cv. '16-bis' after 3 months of culture

Culture medium	MS				WPM				NM			
	1 Shoot	2 Shoots	3 Shoots	4 Shoots	1 Shoot	2 Shoots	3 Shoots	4 Shoots	1 Shoot	2 Shoots	3 Shoots	4 Shoots
Shoot length after 3 months (cm)	14.2 ± 2.0 d	13.7 ± 1.7 d	13.3 ± 1.3 d	11.4 ± 1.7 bc	11.8 ± 1.3 c	10.8 ± 1.1 bc	10.6 ± 1.9 bc	9.5 ± 1.3 ab	10.7 ± 1.8 bc	10.3 ± 1.4 abc	9.5 ± 1.0 ab	8.6 ± 0.5 a
Shoot greening	+++	+++	+++	+++	++	++	++	++	++	++	++	++
Root number	4.2 ± 1.0 c	4.3 ± 1.0 c	3.8 ± 0.9 bc	3.2 ± 1.2 abc	3.4 ± 0.5 abc	3.3 ± 1.2 abc	2.4 ± 0.5 a	2.3 ± 0.4 a	3.4 ± 0.8 abc	3.2 ± 0.9 abc	3.1 ± 0.7 abc	2.9 ± 0.8 ab
Root length (cm)	5.0 ± 0.4 d	4.3 ± 0.9 cd	3.7 ± 1.1 bc	2.6 ± 1.2 ab	3.5 ± 1.5 abc	2.5 ± 0.7 ab	2.6 ± 1.0 ab	2.2 ± 0.6 a	3.4 ± 1.2 abc	3.1 ± 1.3 abc	2.5 ± 0.7 ab	2.2 ± 0.4 a
Plantlet acclimatization (%)	80.0 ± 13.3 c	80.0 ± 9.4 c	68.0 ± 15.7 ab	62.0 ± 17.5 a	74.0 ± 16.4 b	72.0 ± 13.9 b	60.0 ± 13.3 a	60.0 ± 9.4 a	76.0 ± 12.6 bc	74.0 ± 9.6 b	62.0 ± 17.5 a	60.0 ± 13.3 a

Note: Mean values with the same letters are not significantly different ($p > 0.05$). Intensity of greening: + low, ++ moderate, +++ high



Fig. 2. Acclimatized plantlets under glasshouse conditions at 90 days after acclimatization

explant type, plant growth regulators and the basal medium (Neumann *et al.*, 2009). It has been demonstrated that the basal medium plays a crucial role in the *in vitro* multiplication and regeneration of many species, such as Easter lily (Ramsay *et al.*, 2003), *Populus alba* × *P. berolinensis* (Wang *et al.*, 2008), and *Aquilaria birta* (Hassan *et al.*, 2011). In fact, plant micropropagation is greatly influenced by the nature of the culture medium used (George *et al.*, 2008). In this study, the effect of three basal media on shoot proliferation, growth and development in date palm cv. '16-bis' was evaluated. The highest number of shoots per cluster, the optimum growth and development of shoots and the highest survival rate of plantlet acclimatization were obtained in MS medium. In addition, shoots that have been cultured onto MS medium were morphologically superior to those cultured onto WPM and NM media. Furthermore, shoots grown on MS medium showed higher level of greening which is important to have good photosynthetic capacity and better chance of survival during acclimatization (Mazri and Meziani, 2013). Shoot greening was influenced by the concentration of nitrogen in the culture medium (Mazri, 2012; Mazri and Meziani, 2013) which is higher in MS medium. MS medium was also used for shoot proliferation of many date palm cultivars such as cv. 'Maktoom' (Khierallah and Bader, 2007), cv. 'Barhee' (Fki *et al.*, 2011) and cv. 'Khanezi' (Al-Khateeb, 2006; 2008).

Regardless of the basal medium, our findings indicated that the explant size and density also influence bud shoot proliferation and growth in date palm culture. To the best of our knowledge, the effect of explant size and density has not yet been reported in date palm micropropagation. Our results demonstrate that 4 buds per cluster was the optimum size for bud shoot proliferation. The size of 2

buds per cluster led to an undesirable early elongation of the existing buds and hampered the formation of new buds. These findings are in contrast with previous results reported by Hamad and Taha (2009) on *Ananas comosus* L. Merr. Increasing the number of buds per cluster to 3 or 4 improved shoot proliferation and avoid the precocious elongation, whereas the size of 5 buds per cluster caused pronounced browning in cultured explants and decreased the number of healthy looking shoots produced, which is consistent with Abahmane (2011) who suggested the use of explant of small size to prevent browning of date palm *in vitro*-cultured tissues. Explant density also influenced bud shoot proliferation. Increasing explants density reduced the proliferation rate per cluster. This result is in good agreement with previous results of *Populus alba* × *P. grandidentata* (Chun *et al.*, 1986). However, Kane and Philman (1992) reported that explant density had no effect on regeneration rate per explant in *Pontederia cordata* L., which shows difference among species. In our case, increasing explant density showed tissue browning and hyperhydricity. Growth and development of isolated shoots was also influenced by the explant size and density. Shoots from 4.5 to 6 cm length exhibited rapid growth and development, especially in MS medium. Increasing shoot density decelerated their growth and development. This result showed that shoot length, and roots number and length were also influenced by differences in basal medium, shoot size and density.

Plantlets acclimatization can be considered as a major phase for successful establishment of micropropagated plantlets (Kumar and Rao, 2012). In this study, the survival rate of plantlets with no morphological abnormalities was in the range of 60% to 80%. In a previous work, higher survival rate was obtained in date palm cv. 'Najda'

under the same acclimatization protocol (Mazri and Meziani, 2013); this could highlight the genotype effect on plantlets acclimatization.

In conclusion, our results will be beneficial to optimize the conditions of the *in vitro* plantlet production of date palm cv. '16-bis'. This will be useful for the large-scale propagation of this cultivar resistant to *Fusarium oxysporum* f. sp. *albedinis*. As far as we know, this is the first report about cv. '16-bis' micropropagation.

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