

Evaluation of *In Vitro* Shoot Elongation and Rooting of Date Palm, and Determination of Physiological Characteristics of Regenerated Plantlets

Reda MEZIANI^{1*}, Mouaad Amine MAZRI², Mahassine ARHAZZAL^{1,3},
Ilham BELKOURA³, Chakib ALEM⁴, Fatima JAITI⁴

¹Institut National de la Recherche Agronomique, CRRA-Errachidia, UR Systèmes Oasiens, Laboratoire National de Culture des Tissus du Palmier Dattier, BP 2, Errachidia, Morocco; redameziani@yahoo.fr (*corresponding author)

²Institut National de la Recherche Agronomique, CRRA-Marrakech, UR Agro-Biotechnologie, Laboratoire de Biotechnologie Végétale, BP 533, Marrakech, Morocco; m.a.mazri@gmail.com

³Ecole Nationale d'Agriculture, Département des Sciences de Base, Laboratoire de Culture In Vitro, BP S/40, Meknes, Morocco; mahassine.ar@gmail.com; bilham@enameknes.ac.ma

⁴Université Moulay Ismail, Faculté des Sciences et Techniques, BP 509, Errachidia, Morocco; alem04@yahoo.fr; fatimajaiti@yahoo.fr

Abstract

The effects of various culture conditions on shoot elongation, rooting and plantlet acclimatization were tested. Adventitious shoots obtained through direct organogenesis of date palm (*Phoenix dactylifera* L.) cv. 'Mejhoul' were used as explants. The effects of culture medium texture, plant growth regulators, polyvinylpyrrolidone, adenine, myo-inositol, L-glutamine, and carbon source on *in vitro* plantlet quality and subsequent acclimatization were evaluated. The most effective culture medium was the semi-solid and half-strength Murashige and Skoog medium without plant growth regulators, supplemented with 30 g L⁻¹ sucrose. After 3 months of culture on this medium, the average shoot length was 13.6 cm, the average number of adventitious roots per shoot was 3.6, and the average root length was 3.85 cm. The survival rate of these plantlets in acclimatization was 90%. On the other hand, liquid medium, plant growth regulators, polyvinylpyrrolidone, adenine, myo-inositol and L-glutamine did not increase the survival rate during acclimatization. Along with these experiments, some physiological characteristics of the plantlets obtained *in vitro* were also determined. Chlorophyll content and fluorescence, foliar surface and stomatal conductance were measured after 3 months of culture in each medium. The ranges were as follows: Chlorophyll content, 11.7-31.8 CCI; chlorophyll fluorescence, 0.633-0.795; foliar surface, 7.35-13.29 cm²; and stomatal conductance, 10.3-36.0 mmol m⁻² s⁻¹. Interestingly, positive correlations between the physiological characteristics of the plantlets and their survival percentage in the glasshouse were revealed. The findings of this investigation will be valuable for large-scale and cost-saving production of date palm cv. 'Mejhoul' plants.

Keywords: chlorophyll content; chlorophyll fluorescence; foliar surface; morphological characteristics; organogenesis; *Phoenix dactylifera* L.; stomatal conductance

Abbreviations: 2iP, 6-(dimethylallylamino) purine; BAP, 6-benzylaminopurine; CCI, chlorophyll content index; CGS, commercial granulated sugar; GA₃, gibberellic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; MS, Murashige and Skoog; NAA, 1-naphthaleneacetic acid; NOA, 2-naphthoxyacetic acid; PGR, plant growth regulator; PVP, polyvinylpyrrolidone.

Introduction

Date palm (*Phoenix dactylifera* L.) belongs to the genus *Phoenix*, which consists of 14 species (Johnson, 2011). It is mainly grown in the MENA region but also distributed in other countries such as Spain, Australia and the USA (Shabani *et al.*, 2014). Date palm is one of the most commercially important fruit species in the Middle East and

North Africa. It is cultivated for its edible fruits and is known to play roles in creating equable microclimates within oasis ecosystems and generating employment (Jain, 2012). In Morocco, date palm is mainly grown in pre-Saharan arid regions with about 453 cultivars (Sedra, 2015).

'Mejhoul' cv. (also known as 'Medjool') is one of the best and the most sought after date palm varieties in the world (Sedra, 2015). In Morocco, cv. 'Mejhoul' is threatened by

Bayoud, a vascular disease caused by the pathogen *Fusarium oxysporum* f. sp. *albedinis*. Unfortunately, Bayoud has significantly decreased the population of cv. 'Mejhou' (Sedra, 2011). Nowadays, the only effective way to preserve this variety is by rapid and large-scale propagation through *in vitro* culture techniques, followed by plantation in Bayoud-free areas.

Date palm can be micropropagated through either somatic embryogenesis or organogenesis (Mazri and Meziani, 2015). In Morocco, organogenesis is the mainly used technique since it allows production of true-to-type plants (Sedra, 2005; Meziani et al., 2015). Over the last years, organogenesis has played a major role in the propagation of date palm genotypes of commercial interest.

The organogenesis pathway involves four steps: adventitious bud initiation, shoot bud multiplication, shoot elongation and rooting, and plantlet acclimatization (Mazri and Meziani, 2013). In previous papers, the effects of different culture conditions on adventitious bud initiation (Meziani et al., 2016) and shoot bud multiplication of date palm cv. 'Mejhou' (Meziani et al., 2015; 2016; Mazri et al., 2016) were reported. Studies on *in vitro* shoot elongation and rooting of date palm are scarce. Although there are some studies dealing with the effects of medium composition and growth conditions on *in vitro* shoot elongation and rooting in some date palm cultivars (Khierallah and Bader, 2007; Khan and Bi Bi, 2012; Mazri, 2013; Mazri and Meziani, 2013), it is well known that *in vitro* propagation of date palm depends on the genotype (Zaid et al., 2011).

Chlorophyll content, foliar surface and stomatal conductance are physiological characteristics that can affect plantlet quality, survival, growth and development after transplantation. Chlorophyll is a photoreceptor, an indicator of the photosynthetic potential of plants and a catalyst for the conversion of sunlight into chemical energy. It plays a major role in the photochemical synthesis of carbohydrates (Vernon and Seely, 1966; Oliveira et al., 2016). Plant foliar surface is a physiological index for evaluating some metabolic processes such as growth, transpiration, photosynthesis and respiration. It also constitutes a boundary between plant tissues and the environment and plays a major role in the defense against pathogen infections (Derridj et al., 1996; Lee et al., 2008; Camen et al., 2010). Stomatal conductance is an important factor in controlling leaf transpiration, regulating water losses by the plant and in the emission and uptake of acids (Burrows and Milthorpe, 1976; Cabrera et al., 1995; Filella et al., 2009). It is also an indicator of stomatal opening and photosynthesis activity (Jones, 1992; Johnson et al., 1979).

Herein, the effects of different groups of factors on shoot elongation and rooting, and plantlet acclimatization of date palm cv. 'Mejhou' were investigated. Accordingly, the effects of medium texture, plant growth regulators (PGRs), carbon sources, polyvinylpyrrolidone (PVP), L-glutamine, myo-inositol and adenine were tested. Furthermore, the chlorophyll content and fluorescence, foliar surface and stomatal conductance of the *in vitro* plants were measured and their correlations with acclimatization were determined.

Materials and Methods

Origin of plant material

In vitro young plantlets of date palm cv. 'Mejhou' were obtained according to Meziani et al. (2015; 2016). Briefly, shoot tips of date palm (*Phoenix dactylifera* L.) cv. 'Mejhou' were obtained from 3-year-old offshoots collected from a date palm orchard in Erfoud, Morocco. The shoot tips were disinfected and explants from meristematic tissues were extracted as described by Mazri et al. (2016). The explants were cultured on half-strength Murashige and Skoog (1962) medium (MS/2, consisting of ½ MS macro-elements, MS microelements and MS vitamins) supplemented with 14.2 µM indole-3-acetic acid (IAA), 13.4 µM 1-naphthaleneacetic acid (NAA), 0.5 µM 6-(dimethylallylamino) purine (2iP), 1.5 g L⁻¹ PVP (Sigma, Steinheim, Germany), 30 g L⁻¹ sucrose (Sigma, St. Louis, MO, USA) and 6 g L⁻¹ agar (Sigma, St. Louis, MO, USA) for 9 months under dark conditions at 25 ± 1 °C, with monthly subcultures. The formed shoot buds were cultured for 3 months (with 1-month subculture intervals) on MS/2 medium supplemented with 0.9 µM 2-naphthoxyacetic acid (NOA), 1.1 µM IAA, 1.8 µM kinetin, 1.9 µM 2iP, 1 g L⁻¹ PVP, 30 g L⁻¹ commercial granulated sugar (CGS; Cosumar, Casablanca, Morocco), and 6 g L⁻¹ agar, under a 16 h light photoperiod (13.5 µmol m⁻² s⁻¹ light intensity). Afterwards, young plantlets of about 4-5 cm length and one root were singled out and used for the subsequent experiments.

Effects of medium components on shoot elongation and rooting

In the first experiment, the effects of medium texture on shoot elongation and rooting were evaluated. Shoots were cultured on MS/2 medium supplemented with 30 g L⁻¹ CGS. The medium was either semi-solid (CM1) or liquid (CM2).

In the second experiment, the effects of various PGR combinations were tested (Table 1). Based on the results from the first experiment, indole-3-butyric acid (IBA), NAA, 6-benzylaminopurine (BAP) and kinetin were added to semi-solid MS/2 medium either alone or in combination at the concentration of 1 mg L⁻¹ each, while filter-sterilized gibberellic acid (GA₃) was added to the medium at 1 mg L⁻¹ after autoclaving. All culture media used in this experiment were supplemented with 30 g L⁻¹ CGS.

In the third experiment, the effects of PVP, adenine, myo-inositol and L-glutamine were evaluated at the concentrations of 1 g L⁻¹ each (Table 1); again, semi-solid MS/2 medium supplemented with 30 g L⁻¹ CGS was used.

Finally, the effects of various carbon sources on shoot elongation and rooting were evaluated. Semi-solid MS/2 medium was supplemented with sucrose, sorbitol or mannitol at 30 g L⁻¹ (all purchased from Sigma, St. Louis, MO, USA; Table 1).

Culture conditions

In all experiments, the culture medium used was MS/2. When semi-solid medium was required, 6 g L⁻¹ agar was added. The pH of all media was adjusted to 5.8 and 40 ml volumes were dispensed into jars (12 cm in height and 6.5 cm in diameter) before autoclaving at 121 °C for 25 min.

The cultures were kept at 25 °C under 16 h photoperiod ($40 \mu\text{mol m}^{-2} \text{s}^{-1}$). In all experiments, shoots were cultured for 3 months with transfer to fresh medium at 1-month intervals.

Physiological characteristics determination

Chlorophyll content determination

The chlorophyll content was measured after 3 months of culture on elongation-rooting media using a CCM-200 Chlorophyll Content Meter (Opti-Sciences, Tyngsboro, Massachusetts, USA). The measurements were performed on fully expanded leaves (one leaf per plantlet and 10 plantlets per treatment). The chlorophyll content was expressed as chlorophyll content index (CCI), which is the ratio of percent transmission at 931 nm to 653 nm through a leaf sample.

Chlorophyll fluorescence determination

The chlorophyll fluorescence, representing the photochemical capacity of Photosystem II, was measured after 3 months of culture on elongation-rooting media using a chlorophyll fluorometer (OS-30p, Opti- Science Inc., Hudson, NH, USA). Briefly, the plant leaves were dark adapted for 30 min (25 °C) then initial fluorescence (F_0), maximum fluorescence (F_m) and their ratio (F_v/F_m ; where F_v is the difference between F_m and F_0) were calculated. For each treatment, one leaf per plantlet and 10 plantlets were used.

Foliar surface determination

The foliar surface of one leaf per plantlet and 10 plantlets per treatment was measured using a digital planimeter (Ushikata X-PLAN 380 dIII, Japan) after 3 months of culture on elongation-rooting media.

Stomatal conductance measurement

Stomatal conductance was measured with an AP4 porometer (Delta-T Devices, Cambridge, UK) after 3 months of culture on elongation-rooting media, using one leaf per plantlet and 10 plantlets per treatment.

Plantlet acclimatization

The obtained plantlets were acclimatized as described by Mazri *et al.* (2017). Briefly, the plantlets were removed from the culture media and the root system was washed with tap water. The roots were immersed for 15 min in a solution of 1 g L^{-1} Pelt 44 PM systemic fungicide (Bayer Crop Science, Bayer Maghreb SA, Casablanca, Morocco) then the plantlets were transferred to plastic bags containing a mixture of peat and gravel (1:1, w/w). The plantlets were sprayed with a solution of 0.5 g L^{-1} Pelt 44 PM then covered with a polyethylene bag. The polyethylene was gradually removed to allow the plantlets to acclimatize to glasshouse conditions (27 °C, 70% relative humidity).

Table 1. Composition of culture media used in this study

Experiment	Culture medium	Agar	IBA	NAA	BAP	KIN	GA ₃	CGS	Sucrose	Sorbitol	Mannitol	PVP	Adenine	Myo-inositol	L-Glutamine
1	CM1	+	-	-	-	-	-	+	-	-	-	-	-	-	-
	CM2	-	-	-	-	-	-	+	-	-	-	-	-	-	-
	CM3	+	+	-	+	-	-	+	-	-	-	-	-	-	-
	CM4	+	+	-	-	+	-	+	-	-	-	-	-	-	-
	CM5	+	-	+	+	-	-	+	-	-	-	-	-	-	-
	CM6	+	-	+	-	+	-	+	-	-	-	-	-	-	-
2	CM7	+	+	+	+	-	-	+	-	-	-	-	-	-	-
	CM8	+	+	+	-	+	-	+	-	-	-	-	-	-	-
	CM9	+	+	-	+	+	-	+	-	-	-	-	-	-	-
	CM10	+	-	+	+	+	-	+	-	-	-	-	-	-	-
	CM11	+	+	+	+	+	-	+	-	-	-	-	-	-	-
	CM12	+	-	-	-	-	+	+	-	-	-	-	-	-	-
	CM13	+	-	-	-	-	-	+	-	-	-	+	+	+	+
	CM14	+	-	-	-	-	-	+	-	-	-	+	+	+	-
	CM15	+	-	-	-	-	-	+	-	-	-	+	+	-	+
	CM16	+	-	-	-	-	-	+	-	-	-	+	+	-	-
	CM17	+	-	-	-	-	-	+	-	-	-	+	-	+	+
	CM18	+	-	-	-	-	-	+	-	-	-	+	-	+	-
CM19	+	-	-	-	-	-	+	-	-	-	+	-	-	+	
3	CM20	+	-	-	-	-	-	+	-	-	-	+	-	-	-
	CM21	+	-	-	-	-	-	+	-	-	-	-	+	+	+
	CM22	+	-	-	-	-	-	+	-	-	-	-	+	+	-
	CM23	+	-	-	-	-	-	+	-	-	-	-	+	-	+
	CM24	+	-	-	-	-	-	+	-	-	-	-	+	-	-
	CM25	+	-	-	-	-	-	+	-	-	-	-	-	+	+
	CM26	+	-	-	-	-	-	+	-	-	-	-	-	+	-
	CM27	+	-	-	-	-	-	+	-	-	-	-	-	-	+
4	CM28	+	-	-	-	-	-	-	+	-	-	-	-	-	-
	CM29	+	-	-	-	-	-	-	-	+	-	-	-	-	-
	CM30	+	-	-	-	-	-	-	-	-	+	-	-	-	-

The basal medium consisted of half strength MS medium.

BAP, 6-benzylaminopurine; CGS, Commercial granulated sugar; GA₃, gibberellic acid; IBA, indole-3-butyric acid; KIN, kinetin; NAA, 1-naphthalene acetic acid; PVP, polyvinylpyrrolidone. (+): presence; (-): absence.

Concentrations: Agar (6 g L^{-1}); PGRs (1 mg L^{-1}); carbon sources (30 g L^{-1}); PVP, adenine, myo-inositol and L-glutamine (1 g L^{-1}).

Statistical analysis

In all experiments, four young plantlets per jar were used. Each jar was considered as one experimental unit and was repeated 10 times. Therefore, 40 young plantlets were used in each medium treatment. The experiments were established as a completely randomized design. After 3 months of culture, the following data were recorded: shoot length, root number and length, chlorophyll content and fluorescence, foliar surface, and stomatal conductance. The data are reported as means \pm standard deviations. Three months after *ex vitro* transfer, the survival rate of plantlets was calculated.

Data were subjected to analysis of variance. Significant differences between means were assessed with the Student-Newman-Keuls test at the 0.05 significance level using SPSS for windows (SPSS v.21, IBM, Chicago, IL, USA). Percentage data were analyzed after arcsine transformation and correlations between physiological characteristics and plantlet acclimatization were evaluated using Pearson's correlation coefficients.

Results

Effects of medium texture on shoot elongation, rooting and plantlet acclimatization

After 3 months of culture, there was a significant difference in shoot length between semi-solid (CM1) and liquid (CM2) media, while no significant difference was found in terms of root number and length. The average shoot length was 13.15 cm in CM1 medium while it was 16.55 cm in CM2 medium. The average number of roots per shoot was 3.3 (3.15 cm length) in CM1 medium and 3.7 (3.45 cm length) in CM2 medium. Interestingly, the shoots cultured on CM1 medium were thicker, greener and more vigorous than those cultured on CM2 medium. As regards

to the physiological characteristics, the statistical analysis revealed significant differences in terms of chlorophyll content, chlorophyll fluorescence, foliar surface and stomatal conductance between CM1 and CM2 medium (Table 2). In addition, three months after transfer to the glasshouse, plantlets derived from shoots cultured on semi-solid medium survived at a significantly higher rate (85%) than those derived from shoots cultured on liquid medium (40%). The results of this first experiment showed that despite that liquid medium resulted in longer plantlets; those developed on semi-solid medium were physiologically superior and survived well under glasshouse conditions.

Effects of PGRs on shoot elongation, rooting and plantlet acclimatization

Generally speaking, PGRs induced longer plantlets with more roots compared to PGR-free medium (CM1). The highest shoot length (19.2 cm) was observed on CM6 medium, which was containing NAA and kinetin. The average number of roots ranged from 3.9 (CM12 medium) to 5.9 (CM7 medium) while root length ranged from 5.20 cm (CM12 medium) to 6.20 cm (CM9 and CM10 media). There was no significant difference in shoot rooting among all tested media (Table 3). The ranges of physiological characteristics were: chlorophyll content, 11.7-14.4 CCI; chlorophyll fluorescence, 0.685-0.704; foliar surface, 9.01-10.19 cm²; and stomatal conductance, 10.3-15.6 mmol m⁻² s⁻¹. The survival rates ranged from 50 to 70%. Compared to the first experiment, the shoots obtained from CM1 medium exhibited a higher survival rate than those cultured on media containing PGRs. Consequently, these findings suggest using a PGR-free medium for shoot elongation and rooting since it permits cost saving as well as high survival after acclimatization to *ex vitro* conditions.

Table 2. Effect of medium texture on shoot elongation, rooting, physiological characteristics and plantlet acclimatization

Culture medium	Shoot length (cm)	Number of roots per shoot	Root length (cm)	Chlorophyll content (CCI)	Chlorophyll fluorescence	Foliar surface (cm ²)	Stomatal conductance (mmol m ⁻² s ⁻¹)	Plantlet acclimatization
CM1	13.15 \pm 1.36 a	3.3 \pm 0.82 a	3.15 \pm 1.23 a	31.6 \pm 3.00 a	0.726 \pm 0.034 a	13.03 \pm 0.69 a	34.6 \pm 7.05 a	85 \pm 36.6 a
CM2	16.55 \pm 2.30 b	3.7 \pm 1.49 a	3.45 \pm 1.04 a	12.3 \pm 0.97 b	0.633 \pm 0.053 b	7.35 \pm 1.26 b	16.4 \pm 7.48 b	40 \pm 50.3 b

Data are means \pm standard deviations. Means with different letters are significantly different at 0.05 level of probability.

Table 3. Effect of plant growth regulators on shoot elongation, rooting, physiological characteristics and plantlet acclimatization

Culture medium	Shoot length (cm)	Number of roots per shoot	Root length (cm)	Chlorophyll content (CCI)	Chlorophyll fluorescence	Foliar surface (cm ²)	Stomatal conductance (mmol m ⁻² s ⁻¹)	Plantlet acclimatization
CM3	15.60 \pm 2.57 a	4.4 \pm 1.07 a	5.30 \pm 1.18 a	11.7 \pm 0.68 a	0.694 \pm 0.043 a	9.93 \pm 1.16 a	10.3 \pm 1.62 a	65 \pm 48.9 a
CM4	15.10 \pm 2.47 a	4.5 \pm 1.35 a	5.70 \pm 1.16 a	13.1 \pm 1.38 ab	0.688 \pm 0.033 a	9.54 \pm 1.59 a	12.5 \pm 1.15 ab	60 \pm 50.3 a
CM5	15.30 \pm 1.70 a	5.5 \pm 1.51 a	5.60 \pm 0.84 a	12.7 \pm 1.46 ab	0.701 \pm 0.032 a	9.01 \pm 2.36 a	14.5 \pm 2.36 b	50 \pm 51.3 a
CM6	19.20 \pm 2.72 b	5.6 \pm 1.51 a	5.45 \pm 1.09 a	12.0 \pm 1.02 a	0.704 \pm 0.029 a	9.75 \pm 2.14 a	12.9 \pm 1.82 ab	60 \pm 50.3 a
CM7	15.90 \pm 2.22 a	5.9 \pm 2.23 a	5.80 \pm 1.58 a	13.1 \pm 0.95 ab	0.697 \pm 0.013 a	9.83 \pm 1.84 a	15.4 \pm 3.10 b	65 \pm 48.9 a
CM8	15.70 \pm 1.77 a	5.7 \pm 0.95 a	5.90 \pm 1.37 a	12.5 \pm 0.76 ab	0.699 \pm 0.015 a	9.45 \pm 2.47 a	13.7 \pm 1.89 b	70 \pm 47.0 a
CM9	17.20 \pm 1.93 ab	4.4 \pm 1.51 a	6.20 \pm 1.27 a	12.2 \pm 0.96 a	0.700 \pm 0.017 a	9.79 \pm 2.79 a	15.5 \pm 1.73 b	55 \pm 51.0 a
CM10	16.70 \pm 2.57 ab	5.7 \pm 1.49 a	6.20 \pm 1.21 a	13.8 \pm 0.96 bc	0.697 \pm 0.037 a	9.51 \pm 1.68 a	13.3 \pm 1.84 ab	60 \pm 50.3 a
CM11	17.20 \pm 2.66 ab	5.5 \pm 1.96 a	6.00 \pm 1.27 a	14.4 \pm 1.38 c	0.697 \pm 0.023 a	9.57 \pm 1.92 a	13.5 \pm 1.70 ab	60 \pm 50.3 a
CM12	15.70 \pm 2.26 a	3.9 \pm 0.99 a	5.20 \pm 1.11 a	12.1 \pm 0.83 a	0.685 \pm 0.068 a	10.19 \pm 2.28 a	15.6 \pm 5.62 b	70 \pm 47.0 a

Data are means \pm standard deviations. Means with different letters are significantly different at 0.05 level of probability.

Effects of PVP, L-glutamine, myo-inositol and adenine on shoot elongation, rooting and plantlet acclimatization

After 3 months of culture on media containing PVP, L-glutamine, myo-inositol and/or adenine, shoot length, root number and length ranged from 12.05 to 14.98 cm, 3.2 to 4.1 and 3.05 to 4.30 cm, respectively (Table 4). As regards to the physiological characteristics, the chlorophyll content ranged from 15.2 to 31.8 CCI, the chlorophyll fluorescence ranged from 0.740 to 0.795, the foliar surface ranged from 8.99 to 11.78 cm² and the stomatal conductance ranged from 10.5 to 33.5 mmol m⁻² s⁻¹. The observed survival rates were between 60% and 85% and therefore did not exceed that obtained on the control medium (85%). This result indicates that the addition of PVP, L-glutamine, myo-inositol and adenine, alone or in combination to the elongation-rooting medium does not improve the survival rate during acclimatization as compared to CM1 medium. This shows that there is no need to use PVP, L-glutamine, myo-inositol and adenine at 1 g L⁻¹ during shoot elongation and rooting.

Effects of carbon source on shoot elongation, rooting and plantlet acclimatization

In this experiment, there was no significant difference in terms of shoot length, root number and length when using different carbon sources. Shoot length ranged from 12.60 to 13.60 cm, root number ranged from 3.1 to 3.6 and root

length ranged from 2.95 to 3.85 cm. Expectedly, it was found that sucrose affects the physiological characteristics of young plantlets and increases their survival rate during acclimatization. In fact, the shoots cultured on CM28 medium (i.e. containing sucrose) had thicker and larger leaves (Fig. 1) than those cultured on the other media. In addition, chlorophyll content foliar surface and stomatal conductance were significantly higher in shoots cultured on CM28 medium. This was reflected in a higher survival rate (90%) during acclimatization (Fig. 2; Table 5).

The results of all these experiments suggest that CM28 medium, which was containing 6 g L⁻¹ agar and 30 g L⁻¹ sucrose, is the most effective for shoot elongation, rooting and plantlet acclimatization.

Correlation between physiological characteristics and plantlet acclimatization

As a final step of this study, the correlations between the measured physiological parameters and plantlet acclimatization using Pearson's correlation coefficients were assessed. Significant positive correlations were detected between chlorophyll content, chlorophyll fluorescence, foliar surface, stomatal conductance and plantlet survival during acclimatization (Table 6). This suggests that the physiological characteristics of young plantlets cultured *in vitro* affect their acclimatization to *ex vitro* conditions.

Table 4. Effect of PVP, adenine, L-glutamine and myo-inositol on shoot elongation, rooting, physiological characteristics and plantlet acclimatization

Culture medium	Shoot length (cm)	Number of roots per shoot	Root length (cm)	Chlorophyll content (CCI)	Chlorophyll fluorescence	Foliar surface (cm ²)	Stomatal conductance (mmol m ⁻² s ⁻¹)	Plantlet acclimatization
CM13	14.07 ± 3.07 a	3.9 ± 1.79 a	3.40 ± 1.24 a	22.0 ± 10.38 ab	0.788 ± 0.011 fg	9.63 ± 3.26 a	29.6 ± 15.08 cd	60 ± 50.3 a
CM14	14.24 ± 5.26 a	3.3 ± 1.34 a	3.60 ± 1.90 a	17.4 ± 9.22 ab	0.773 ± 0.033 cdef	10.15 ± 4.09 a	19.4 ± 7.00 abc	60 ± 50.3 a
CM15	13.89 ± 4.58 a	3.9 ± 1.66 a	3.25 ± 1.21 a	16.6 ± 7.70 a	0.785 ± 0.011 efg	11.1 ± 4.06 a	22.4 ± 12.43 bcd	65 ± 48.9 a
CM16	14.87 ± 3.01 a	3.8 ± 1.40 a	3.15 ± 0.88 a	15.2 ± 4.41 a	0.767 ± 0.021 cde	10.76 ± 3.94 a	15.0 ± 5.52 ab	60 ± 50.3 a
CM17	13.93 ± 4.19 a	3.5 ± 0.97 a	3.30 ± 1.09 a	24.6 ± 7.71 ab	0.789 ± 0.007 fg	11.78 ± 2.14 a	23.6 ± 6.10 bcd	80 ± 41.0 a
CM18	12.60 ± 3.10 a	3.8 ± 1.55 a	3.05 ± 1.64 a	17.5 ± 0.97 ab	0.774 ± 0.006 cdef	9.83 ± 2.76 a	26.1 ± 6.29 bcd	75 ± 44.4 a
CM19	14.35 ± 3.25 a	3.5 ± 1.35 a	3.85 ± 1.67 a	19.3 ± 12.70 ab	0.749 ± 0.016 ab	9.90 ± 2.02 a	22.1 ± 5.82 bcd	75 ± 44.4 a
CM20	12.05 ± 3.31 a	3.3 ± 1.06 a	3.95 ± 1.88 a	16.7 ± 1.07 a	0.759 ± 0.007 bc	9.28 ± 1.62 a	10.5 ± 0.71 a	80 ± 41.0 a
CM21	14.98 ± 3.81 a	3.7 ± 2.00 a	3.60 ± 1.29 a	24.8 ± 13.53 ab	0.779 ± 0.007 defg	9.77 ± 4.13 a	33.5 ± 5.88 d	80 ± 41.0 a
CM22	13.52 ± 2.12 a	3.2 ± 1.03 a	3.80 ± 1.65 a	18.9 ± 14.52 ab	0.795 ± 0.020 g	11.77 ± 1.35 a	32.6 ± 18.57 cd	75 ± 44.4 a
CM23	14.35 ± 2.50 a	3.8 ± 1.32 a	3.95 ± 1.85 a	16.4 ± 1.30 a	0.760 ± 0.006 bcd	10.27 ± 4.06 a	29.8 ± 11.07 cd	75 ± 44.4 a
CM24	14.92 ± 4.44 a	4.1 ± 2.23 a	3.30 ± 1.09 a	20.0 ± 10.70 ab	0.768 ± 0.016 cde	8.99 ± 2.13 a	30.1 ± 7.30 cd	70 ± 47.0 a
CM25	13.94 ± 2.82 a	3.6 ± 1.58 a	4.30 ± 2.00 a	28.3 ± 4.72 ab	0.779 ± 0.008 defg	10.31 ± 3.43 a	31.9 ± 9.86 cd	85 ± 36.6 a
CM26	14.33 ± 3.77 a	3.8 ± 2.04 a	3.80 ± 1.84 a	31.8 ± 16.86 b	0.780 ± 0.011 efg	11.51 ± 1.81 a	28.6 ± 3.83 cd	80 ± 41.0 a
CM27	14.76 ± 3.77 a	3.8 ± 2.44 a	4.10 ± 1.52 a	31.7 ± 11.47 b	0.740 ± 0.008 a	9.14 ± 1.53 a	29.4 ± 1.76 cd	75 ± 44.4 a

Data are means ± standard deviations. Means with different letters are significantly different at 0.05 level of probability.

Table 5. Effect of carbon source on shoot elongation, rooting, physiological characteristics and plantlet acclimatization

Culture medium	Shoot length (cm)	Number of roots per shoot	Root length (cm)	Chlorophyll content (CCI)	Chlorophyll fluorescence	Foliar surface (cm ²)	Stomatal conductance (mmol m ⁻² s ⁻¹)	Plantlet acclimatization
CM28	13.60 ± 2.01 a	3.6 ± 0.84 a	3.85 ± 0.85 a	30.0 ± 1.89 a	0.784 ± 0.015 a	13.29 ± 1.71 a	36.0 ± 2.35 a	90 ± 30.8 a
CM29	12.80 ± 1.62 a	3.2 ± 0.79 a	2.95 ± 1.28 a	28.0 ± 1.18 b	0.774 ± 0.026 a	11.77 ± 0.03 b	33.3 ± 3.86 a	70 ± 47.0 a
CM30	12.60 ± 1.51 a	3.1 ± 1.20 a	3.00 ± 1.15 a	27.2 ± 0.57 b	0.770 ± 0.022 a	11.29 ± 0.01 b	28.9 ± 5.21 b	75 ± 44.4 a

Data are means ± standard deviations. Means with different letters are significantly different at 0.05 level of probability.

Table 6. Pearson's correlation coefficients between physiological characteristics and plantlet acclimatization

Variables	Chlorophyll content	Chlorophyll fluorescence	Foliar surface	Stomatal conductance	Plantlet acclimatization
Chlorophyll content	1				
Chlorophyll fluorescence	,627**	1			
Foliar surface	,598**	,568**	1		
Stomatal conductance	,837**	,696**	,564**	1	
Plantlet acclimatization	,716**	,637**	,652**	,640**	1

** Correlation is significant at the 0.01 level (2-tailed).



Fig. 1. Shoot elongation and rooting after 3 months of culture on : **A** MS/2 medium supplemented with 1 mg L⁻¹ IBA, 1 mg L⁻¹ NAA, 1 mg L⁻¹ BAP, 1 mg L⁻¹ kinetin, 30 g L⁻¹ commercial granulated sugar and 6 g L⁻¹ agar (CM11); **B** PGR-free liquid MS/2 medium supplemented with 30 g L⁻¹ commercial granulated sugar (CM2); and **C** PGR-free MS/2 medium supplemented with 30 g L⁻¹ sucrose and 6 g L⁻¹ agar (CM28).



Fig. 2. Plantlet acclimatization: These plantlets were cultured on PGR-free MS/2 medium supplemented with 30 g L⁻¹ sucrose and 6 g L⁻¹ agar (CM28) during the elongation-rooting phase.

Discussion

This study investigated the effects of several factors on shoot elongation and rooting and plantlet acclimatization of date palm cv. 'Mejhoul'. In many plant species, including date palm, it has been reported that liquid medium promotes shoot elongation in comparison with semi-solid medium (Skidmore *et al.*, 1988; Suthar *et al.*, 2011; Mazri, 2012). This is in good agreement with the results of the present study. Rezali *et al.* (2017) reported that liquid media facilitate nutrient uptake which stimulates plant growth. This might be the reason behind the longer shoots obtained in liquid medium. However, it appears that the shoots cultured on liquid medium are fragile and most of them fail to survive under the glasshouse conditions. In fact, the findings of the present study indicated that semi-solid medium resulted in higher plantlet quality (in terms of leaf thickness, width and greening) and a significantly higher acclimatization rate as compared to the liquid one. This supports previous findings on date palm cv. 'Boufeggous' (Mazri, 2015). In date palm and banana micropropagation, it has been suggested to transfer shoots from liquid to a semi-solid medium for few weeks before being transferred to the glasshouse in order to increase their survival rate (Bhagyalakshmi and Singh, 1995; Mazri, 2012).

The results of the present study support the general statement that plant growth regulators promote *in vitro* shoot elongation and rooting. Auxins and cytokinins are the most important classes of PGRs (Machakova *et al.*, 2008). Accordingly, in many plant species, combinations of auxins and cytokinins were successfully used to stimulate shoot elongation and root induction. Auxins are known to control cell division and cell elongation and to promote adventitious root formation (Machakova *et al.*, 2008). Cytokinins are essential for plant cell division and are very effective in promoting shoot bud formation and proliferation (Van Staden *et al.*, 2008). GA₃ is a PGR that plays various roles. It may promote or inhibit shoot and root formation depending on the species, and has been successfully used for shoot elongation (Moshkov *et al.*, 2008). Our results indicated that GA₃ increases shoot and root lengths in comparison with PGR-free semi-solid medium (CM1). This is in good agreement with the findings of Khierallah and Bader (2007) who demonstrated that increasing GA₃ concentration increased shoot length in date palm cv. 'Maktoom'.

In the present investigation, PGRs resulted in longer shoots with higher number of roots compared to PGR-free semi-solid medium. However, the shoots cultured on PGR-free semi-solid medium showed greener, thicker and more vigorous leaves. This is in good agreement with previous findings on date palm cv. 'Najda' (Mazri and Meziani, 2013). Interestingly, it has been found that shoots that were cultured on CM1 medium exhibit higher survival rate during acclimatization than those cultured on PGR-containing media. Accordingly, the use of PGR-free medium for shoot elongation and rooting of date palm cv. 'Mejhoul' is recommended.

L-Glutamine, myo-inositol, adenine and PVP are widely used in plant tissue culture. L-Glutamine is an amino acid that promotes and maintains cell function (Newsholme *et al.*, 2003). In date palm micropropagation, L-glutamine was used to promote adventitious bud and somatic embryo proliferation (Zouine *et al.*, 2005; Zouine and El Hadrami, 2007; Mazri *et al.*, 2016). Myo-inositol is a vitamin that promotes cell division (Kiviharju *et al.*, 2005). Adenine, also known as vitamin B₄, has been used in plant tissue culture for its effects similar to those of cytokinins (Thorpe *et al.*, 2008; Van Staden *et al.*, 2008), while PVP is a widely used compound in date palm organogenesis to control tissue browning. In previous studies on cv. 'Mejhoul' organogenesis, L-glutamine and myo-inositol showed their beneficial effect during shoot bud multiplication (Mazri *et al.*, 2016) whereas PVP has been successfully used as an anti-browning agent during adventitious bud initiation and shoot bud multiplication (Meziani *et al.*, 2016). Herein, the effects of these compounds as well as adenine were evaluated on shoot elongation, rooting and plantlet acclimatization. However, none of them has improved the survival rate after transfer to *ex vitro* conditions. Accordingly, the use of a medium without these compounds is suggested. This will contribute to cost saving during the organogenesis process.

Carbon sources play both nutritional and osmotic roles in plant micropropagation, and supply the necessary energy for plant cell and tissue growth and morphogenesis (Neto and Otoni, 2003). Sucrose is by far the most used carbon source in plant micropropagation (Yaseen *et al.*, 2013). The data presented here show that shoot quality and plantlet survival after *ex vitro* acclimatization were improved by the addition of sucrose to elongation and rooting medium. This is in good agreement with previous results on date palm cv. '16-bis' (Mazri, 2014). Sucrose has been used for the micropropagation of many date palm cultivars through either somatic embryogenesis or organogenesis (e.g. Fki *et al.*, 2011; Mazri *et al.*, 2016; Mazri *et al.*, 2017). This may be due to its beneficial effect on adventitious bud and somatic embryo induction and proliferation.

Chlorophyll content and fluorescence, foliar surface and stomatal conductance are physiological characteristics that determine plant quality. To the best of our knowledge, this is the first study that measures these physiological parameters in date palm cv. 'Mejhoul' plantlets obtained *in vitro*. Our results indicated that the composition of the culture medium affects the physiological characteristics of date palm plantlets. This is in good agreement with results from the literature. In fact, in many other plant species, it has been found that medium composition and culture conditions affect the physiological characteristics of plants (e.g. Lu, 2005; Mohamed and Alsadon, 2010). In the present investigation, chlorophyll content and fluorescence, foliar surface and stomatal conductance showed a positive correlation with plantlet survival during acclimatization. This suggests that high values of these physiological parameters are reflected in high plantlet quality and high survival rate under *ex vitro* conditions. Accordingly, they can be considered as good indicators for successful transplantation.

Conclusions

Date palm organogenesis has gained recently much interest because of its high multiplication potential and production of true to type plantlets. This research demonstrated how culture conditions during the elongation-rooting phase affect shoot growth and their physiological characteristics as well as plantlet acclimatization. It has been found that some medium additives such as L-glutamine, myo-inositol, adenine, PVP and PGRs are not needed to achieve high levels of acclimatization. This is very important in developing a cost-effective regeneration system for date palm. Regarding carbon sources, sucrose has improved plantlet quality and increased the survival rate during acclimatization. However, it is important to carry out an economic analysis to determine whether its beneficial effect justifies its high cost.

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