Influence of cytokinins and medium texture on organogenesis and plantlet regeneration in date palm (*Phoenix dactylifera* L.) cv. Najda

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Abstract

Najda cv. is an important date palm cultivar due to its high fruit quality and resistance to bayoud, a disease that killed million plants of date palm. The in vitro propagation of cv. Najda through organogenesis is hampered by some physiological disorders, namely hyperhydricity, tissue browning and precocious rooting. In order to achieve efficient and large-scale propagation of true-to-type plantlets of cv. Najda, we evaluated the effect of different cytokinin types and concentrations (6-benzylaminopurine (BAP), 6-(dimethylallylamino) purine (2iP) and thidiazuron (TDZ), at 0.1, 0.2, 0.3, 0.4 or 0.5 mg/L), as well as that of medium texture (semi-solid and liquid) on adventitious shoot bud multiplication, hyperhydricity, tissue browning and precocious rooting. The optimal culture conditions for shoot bud proliferation and subsequent development were observed on semi-solid half-strength Murashige and Skoog (MS/2) medium supplemented with 0.5 mg/L 1-naphthaleneacetic acid (NAA) and 0.4 mg/L BAP, where an average of 23.8 shoot buds per explant, low rate of hyperhydricity (22.5%), moderate tissue browning (55.0%) and low percentage of precocious rooting (20.0%) were observed. Shoot elongation and rooting were performed on semi-solid and liquid plant growth regulator (PGR)-free MS/2 medium. Shoots were then transferred to the glasshouse, where the survival rate reached 95 %. The findings of the present study will be highly beneficial to rehabilitate the Moroccan palm groves infested by bayoud.

Keywords: cytokinin, date palm, hyperhydricity, organogenesis, precocious rooting, tissue browning.

Effets des cytokinines et de la texture du milieu de culture sur l'organogenèse et la régénération des plantules du palmier dattier (*Phoenix dactylifera* L.) cv. Najda

Résumé

Najda est une variété marocaine du palmier dattier de grande importance en raison de la qualité de ses fruits et sa résistance au bayoud, une maladie causée par le champignon *Fusarium oxysporum* f. sp. *albedinis* et qui constitue une menace sérieuse pour la phoeniciculture nationale. La micropropgation de cette variété par organogenèse est entravée par certains problèmes d'ordre physiologiques notamment la vitrification, le brunissement des tissus et l'enracinement précoce. Afin d'assurer une multiplication rapide et massive des plants de cette variété, nous avons évalué l'effet de différents types et concentrations de cytokinine (6-benzylaminopurine (BAP), 6-(diméthylallylamino) purine (2iP) et thidiazuron (TDZ), à 0,1, 0,2, 0,3, 0,4 ou 0,5 mg/L), ainsi que celui de la texture du milieu de culture (semi-solide et liquide) sur la multiplication des bourgeons adventifs, la vitrification, le brunissement des tissus et l'enracinement précoce. Les conditions de culture optimales pour la multiplication des bourgeons et leur développement ont été observées sur le milieu semi-solide de Murashige et Skoog dilué de moitié (MS/2) additionné de 0,5 mg/L d'acide 1-naphtalénacétique (ANA) et de 0,4 mg/L de BAP, où une moyenne de 23,8 bourgeons par explant, un faible taux de vitrification (22,5%), un taux de brunissement modéré (55,0%) et un faible pourcentage d'enracinement précoce (20,0%) ont été observés. L'élongation et l'enracinement des pousses ont été réalisés sur le milieu MS/2 dépourvu de substances de croissance, à l'état semi-solide et liquide. Les plantules obtenues ont été ensuite transférées sous serre, où un taux de survie élevé de 95% a été observé. Les résultats de la présente étude seront très bénéfiques pour la réhabilitation des palmeraies marocaines infestées par le bayoud.

Mots-clés : Brunissement des tissus, cytokinine, enracinement précoce, organogenèse, palmier dattier, vitrification.

تأثير أنواع الستوكينين وبنية وسط النمو على التكاثر البرعمي ونمو نباتات نخيل الثمر (*Phoenix dactylifera* L.) صنف النجدة

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ملخص

يعتبر صنف النجدة أحد أهم أصناف نخيل الثمر بسبب جودة فواكهه العالية ومقاومته لمرض البيوض، وهو المرض الذي تسبب بقتل الملايين من أشجار نخيل الثمر في المغرب. تعتبر تقنية التكاثر البرعمي داخل الأنابيب تقنية فعالة للإنتاج السريع لنباتات صنف النجدة .لكن مع الأسف فإن هذه التقنية تعرف عدة مشاكل فسيولوجية كالتزجيج، اسمرار الأنسجة والتجذير المبكر. من أجل تحقيق تكاثر سريع لشتلات صنف النجدة، قمنا في هذا البحث بدراسة تأثير عدة أنواع من هرمونات نمو النباتات المنتمية لصنف الستوكينين بتراكيز مختلفة على النحو التالي: 6-benzylaminopurine (BAP)،-6 (dimethylallylamino) purine(2iP) وthidiazuron (TDZ)، بتركيز 0.1، 0.2، 0.3، 0.4 أو 0.5 ملغ/لتر، كما قمنا كذلك بدراسة تأثير بنية وسط النمو (شبه صلبة وسائلة) على التكاثر البرعمي، التزجيج، اسمرار الأنسجة والتجذير المبكر. أظهرت نتائج هذا البحث أن الظروف المثلى لتكاثر ونمو البراعم تتجلى في وسط النمو موراشيغ وسكوج شبه الصلب، المخفف التركيز إلى النصف (MS/2) والمحتوي على 0.5 ملغ/لتر NAA و0.4 ملغ/لترBAP، والذي أنتج معدل 23.8 برعم، إضافة إلى معدل تزجيج ضعيف (22.5٪)، معدل اسمرار متوسط (55.0٪) وتجذير مبكر ضعيف (20.0٪). بعد ذلك، تم إجراء استطالة البراعم على وسط نمو MS/2 شبه صلب وسائل، بدون هرمونات النمو، ثم تم نقل البراعم إلى البيت الزجاجي، حيث 95٪ من النباتات أكملت نموها بشكل طبيعي. نتائج هذه الدراسة ستكون مفيدة للغاية في إعادة تأهيل حقول النخيل المغربية آلتي ينتشر فيها مرض البيوض.

الكلمات المفتاحية: *الستوكينين، نخيل الثمر، التزجيج، التكاثر البرعمي، اسمرار الأنسجة، التجذير المبكر.*

Introduction

Date palm (*Phoenix dactylifera* L.) is a plant species with high socioeconomic and agronomic interest because of its high productivity and its adaptation to the conditions of arid and semi-arid regions (Sedra, 2011). In Morocco, date palm is the most economically important plant in oasis areas, thus contributing to preserving a fragile arid ecosystem threatened by desertification (Sedra and Lazrek, 2011). Besides, date palm significantly contributes to the income of the oasis populations, preserves biodiversity and creates favorable conditions for agriculture (Sedra, 2015; Al-Khayri et al., 2018).

The bayoud disease, caused by the fungus *Fusarium oxysporum* f. sp. *albedinis*, is by far the most serious disease affecting date palm cultivation in North Africa (Sedra and Lazrek, 2011). Indeed, bayoud has decimated more than 12 million date palm plants in Morocco and Algeria during the last century (Saker, 2011). Selecting and planting date palm genotypes resistant to bayoud is, up to date, the most efficient strategy to control bayoud and to rehabilitate Moroccan date palm groves (Ferry, 2011).

In order to rehabilitate Moroccan groves ravaged by bayoud, new resistant cultivars were selected by the researchers of the National Institute of Agronomic Research of Morocco (INRA). Among the selected cultivars, cv. Najda (INRA-3014) was well accepted by farmers and consumers (Sedra, 2011). In vitro propagation of this cultivar through organogenesis is able to provide a large number of plants and to preserve the genetic conformity of regenerants (Kunert et al., 2003; Al Khateeb, 2006; Jain, 2012; Mazri et al., 2019). In fact, organogenesis has been used in the recent years for large-scale propagation of true-to-type plantlets of the best date palm cultivars such as Boufeggous, Mejhoul and Al-Fayda (Mazri, 2015; Meziani et al., 2015, 2016; Mazri et al., 2016, 2019). As a result, the best date palm genotypes that are sensitive to bayoud are planted in bayoud-free areas in order to preserve them, while those resistant to bayoud are used to rehabilitate the groves already infested by *Fusarium oxysporum* f. sp. *albedinis*.

Regarding cv. Najda, a regeneration protocol via organogenesis was published (Mazri and Meziani, 2013). However, some physiological disorders that decrease the proliferation capacity of adventitious shoot buds, namely hyperhydricity, tissue browning and the precocious rooting were noticed. These same problems were already reported in many other date palm cultivars (McCubbin and Zaid, 2007; Al Khateeb, 2008; Mazri, 2015; Meziani et al., 2016; Mazri et al., 2019). Nevertheless, studies to reduce their incidence are scarce. Besides, the occurrence of these phenomena is genotype-dependent. Thus, more investigations should be carried out to optimize the culture medium depending on the genotype used.

Cytokinins are a family of phytohormones widely used in plant cell and tissue culture to promote cell division, differentiation and growth (Van Staden et al., 2008). They are highly effective in stimulating adventitious shoot bud induction and multiplication (Van Staden et al., 2008). Accordingly, they have been used in the organogenesis process of many plant species, including date palm (Mazri, 2015; Meziani et al., 2015; Mazri et al., 2019). There are two structurally different groups of cytokinins that are generally used for plant micropropagation: (i) adenine derivatives, among which 6-benzylaminopurine (BAP) is the most frequently used due to its high effectiveness and

low cost; and (ii) phenylurea derivatives, among which thidiazuron (TDZ) is the most frequently used since it has a potent caulogenesis effect (Van Staden et al., 2008).

The purpose of the present investigation was to improve the multiplication and regeneration process of date palm cv. Najda through organogenesis. Accordingly, we evaluated the effects of different cytokinin types and concentrations (BAP, 6-(dimethylallylamino) purine (2iP) and TDZ at 0.1, 0.2, 0.3, 0.4 or 0.5 mg/L) as well as culture medium texture (semi-solid or liquid) in order to determine the best conditions that are able to reduce the above-mentioned physiological disorders while ensuring high proliferation of adventitious shoot buds.

Materials and methods

Culture medium and conditions

The basal culture medium used in all experiments was that of Murashige and Skoog (Murashige and Skoog, 1962) at half-strength (MS/2), consisting of MS/2 macro-elements, MS/2 microelements and MS/2 vitamins. All culture media were supplemented with 30 g/L sucrose and 1 g/L polyvinylpyrrolidone (PVP). The semi-solid culture media were solidified with 8 g/L agar. The pH was adjusted to 5.7 then culture media were autoclaved for 25 min at 121°C. The cultures were kept at 25 $\pm$ 1 °C under a 16 h photoperiod and were transferred to fresh medium at one-month intervals.

Plant material and experiments

Experiments were conducted in 2017 at the Regional Center of Agronomic Research of Marrakech (CRRA-Marrakech).

Offshoots of date palm cv. Najda (3-year-old) were collected from open-field-grown plants located in the experimental station Zagora of the National Institute of Agronomic Research (INRA, CRRA-Errachidia). Shoot tips were extracted and disinfected according to Meziani et al. (2016), and the shoot tip explants were cultured following the protocol of Beauchesne et al. (1986).

Organogenic cultures derived from shoot tip explants were used in our experiments. Each organogenic culture contained five adventitious buds. The explants were cultured for 3 months (The multiplication phase) on either semi-solid or liquid MS/2 medium. Media were supplemented with 0.5 mg/L 1-naphthaleneacetic acid (NAA) and various concentrations (0.1, 0.2, 0.3, 0.4 or 0.5 mg/L) of BAP, 2iP and TDZ (Table 1). Elongation and rooting were performed by isolating and culturing shoots of 5 cm in length on plant growth regulator (PGR)-free semi-solid or liquid MS/2 media for 3 months.

Well-developed plantlets were taken from the culture medium and acclimatized according to the protocol described by Mazri et al. (2018a).

Table 1: Culture media used for shoot bud proliferation

Medium	Semi-solid MS/2					Liquid MS/2				
Cytokinin (mg/L)	0.1	0.2	0.3	0.4	0.5	0.1	0.2	0.3	0.4	0.5
BAP	BS1	BS2	BS3	BS4	BS5	BL1	BL2	BL3	BL4	BL5
2iP	IS1	IS2	IS3	IS4	IS5	IL1	IL2	IL3	IL4	IL5
TDZ	TS1	TS2	TS3	TS4	TS5	TL1	TL2	TL3	TL4	TL5

Data collection and statistical analysis

During the multiplication phase, we calculated the average number of shoot buds per explant, the percentage of hyperhydricity, the percentage of tissue browning, the percentage of precocious rooting and the average number of precociously formed roots. Besides, we visually estimated shoot bud morphology, as well as the intensity of hyperhydricity and tissue browning as low (+), moderate (++), high (+++) and very high (++++).

During the elongation-rooting phase, the collected data were the average shoot length, shoot greening, and the average number and length of roots. The plantlets were then acclimatized to ex vitro conditions, and the survival rate was calculated after three months in the glasshouse.

In all experiments, we placed two organogenic cultures/shoots per jar, which was considered as one replicate, and each treatment was replicated ten times. The experiments were carried out using a completely randomized design. Data were analyzed using multivariate analysis of variance (ANOVA) at the 5% significance level. The software used was SPSS version 16.0, and mean data were compared using the Student-Newman-Keuls (SNK) test. All percentage data were arcsine transformed before analysis.

Results and discussion

Effect of cytokinins and medium texture on shoot bud multiplication

After 3 months of culture, BS4 and BS5 media, which were solidified and supplemented with 0.4 and 0.5 mg/L BAP, respectively, showed the best effects during the multiplication phase in terms of adventitious shoot bud number and morphology. The average number of shoot buds per explant in these media was 23.8 and 24.1, respectively, with significant difference with all the other culture media (Tables 2, 3 and 4). Moreover, the explants cultured on BS4 and BS5 media showed greener, thicker and more vigorous shoot buds (Fig. 1). The use of other cytokinins resulted in a lower shoot bud multiplication rate. For example, when 2iP was added to semi-solid MS/2 medium, the highest mean number of shoot buds per explant was 19.0 at the concentration of 0.5 mg/L (Table 3). On the other hand, TDZ was not appropriate for shoot bud multiplication of cv. Najda. Indeed, the highest number of shoot buds per explant was 12.2 in TS5 medium (Table 4). According to many authors (Gaspar et al., 1996; Feher et al., 2003; Gaj, 2004), the exogenous application of cytokinins promotes the production of endogenous cytokinins, and thus increases their concentrations in

the cultured explants. This results in cell division, growth and morphogenesis. However, this effect varies according to the exogenous cytokinin type and concentration (Guo et al., 2011). Based on our findings, the combination of 0.5 mg/L NAA and 0.4-0.5 mg/L BAP resulted in higher shoot bud proliferation (23.8-24.1 shoot buds per explant) than 0.5 mg/L 2-naphthoxyacetic acid (NOA) and 0.5 mg/L kinetin (23.5 shoot buds per explant), which was previously reported in date palm cv. Najda (Mazri and Meziani 2013).

Figure 1: Shoot bud multiplication on MS/2 medium supplemented with 0.5 mg/L NAA and 0.4 mg/L BAP

Our results showed that, increasing the cytokinin concentration in culture medium generally increased the number of shoot buds per explant (Tables 2, 3 and 4). However, it is well known that high cytokinin concentrations may result in undesirable physiological disorders such as hyperhydricity and tissue browning, which affect the multiplication and regeneration potential of adventitious shoot buds (Hussain et al., 2001; Khierallah and Bader, 2007; Aslam and Khan, 2009). Hence, it is important to well optimize the cytokinin concentration of the culture medium. Accordingly, in the present study, the highest cytokinin concentration used was 0.5 mg/L.

Combining auxins and cytokinins was suggested for the multiplication of adventitious shoot buds of many date palm cultivars (Mazri and Meziani, 2013; Mazri, 2015; Meziani et al., 2015; Mazri et al., 2019). Our findings showed that optimizing the cytokinin type and concentration is crucial for efficient shoot bud multiplication of cv. Najda. The effect of cytokinin on in vitro shoot bud proliferation of date palm has been demonstrated in other cultivars and different cytokinins were suggested, mainly 2iP (Al Khateeb, 2006; Khierallah and Bader, 2007) and kinetin (Al Kaabi et al., 2001; Mazri and Meziani, 2013). The present study showed that BAP at 0.4 and 0.5 mg/L can also be used since

it allows high proliferation with morphologically superior shoot buds. 2iP showed intermediate results while TDZ appeared not appropriate for cv. Najda. This is consistent with the results reported in cv. Boufeggous (Mazri, 2015), but is not consistent with the findings of Hussain et al. (2001) who recommended TDZ for in vitro shoot proliferation of date palm. These conflicting results highlight the different requirements of different date palm cultivars in terms of cytokinins.

Regarding medium texture, our findings showed that the use of liquid media resulted in the formation of fragile shoot buds with low greening intensity. Furthermore, the number of regenerated shoot buds was generally lower than that observed in semi-solid media, when the same cytokinin type and concentration were used. For example, in BS5 medium the average number of shoot buds per explant was 24.1, while it was significantly lower (16.2) in BL5 medium (Tables 2, 3 and 4). These findings are consistent with those of Khierallah and Bader (2007) who evaluated the effects of different medium textures (semi-solid and stationary liquid) on in vitro shoot bud proliferation of cv. Maktoom.

Table 2. Effect of BAP on shoot bud proliferation, hyperhydricity, tissue browning and precocious rooting

Culture medium	Semi-solid MS/2					Liquid MS/2				
BAP (mg/L)	0.1	0.2	0.3	0.4	0.5	0.1	0.2	0.3	0.4	0.5
Shoot bud number	13.7 ± 3.4 def	14.2 ± 3.1 def	16.6 ± 3.2 f	23.8 ± 3.1 h	24.1 ± 2.3 h	11.3 ± 4.3 bcd	12.5 ± 2.7 bcd	13.6 ± 2.9 cdef	14.5 ± 4.4 def	16.2 ± 4.2 ef
Shoot greening	+++	+++	+++	++++	++++	+	+	+	+	+
Hyperhydricity (%)	20.0 ± 34.0 abc	25.0 ± 38.0 abcd	22.5 ± 34.3 abcd	22.5 ± 25.5 abcd	25.0 ± 41.3 abcd	30.0 ± 41.0 abcde	32.5 ± 43.7 abcdef	32.5 ± 24.4 abcdef	35.0 ± 43.2 abcdef	35.0 ± 46.1 abcdef
Intensity of hyperhydricity	+	+	+	+	+	+	+	++	++	++
Browning (%)	55.0 ± 51.0 abcdefg	57.5 ± 37.2 abcdefgh	52.5 ± 37.9 abcdefg	55.0 ± 39.4 abcdefg	60.0 ± 50.2 bcdefgh	20.0 ± 34.0 a	30.0 ± 44.1 ab	32.5 ± 46.6 abc	35.0 ± 46.1 abc	40.0 ± 50.2 abcd
Intensity of browning	+	+	+	+	+	+	+	+	+	+
Precocious rooting (%)	10.0 ± 30.7 a	20.0 ± 41.0 ab	22.5 ± 41.2 ab	20.0 ± 41.0 ab	20.0 ± 41.0 ab	0.0 ± 0.0 a	0.0 ± 0.0 a	10.0 ± 30.7 a	12.5 ± 31.9 a	20.0 ± 41.0 ab
Root number	0.1 ± 0.3 a	0.2 ± 0.4 a	0.3 ± 0.6 a	0.5 ± 1.0 a	0.5 ± 1.3 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.2 ± 0.6 a	0.2 ± 0.6 a	0.4 ± 1.3 a

Data are means ± standard deviation. Data in the same line followed by the same letter are not significantly different at the 5% significance level. Intensity of shoot greening, hyperhydricity and tissue browning were visually estimated as: + low, ++ moderate, +++ high, ++++ very high

Table 3. Effect of 2iP on shoot bud proliferation, hyperhydricity, tissue browning and precocious rooting

Culture medium	Semi-solid MS/2					Liquid MS/2				
2iP (mg/L)	0.1	0.2	0.3	0.4	0.5	0.1	0.2	0.3	0.4	0.5
Shoot bud number	13.6 ± 3.4 cdef	14.5 ± 3.0 def	14.7 ± 3.2 def	14.7 ± 1.9 def	19.0 ± 4.1 g	10.0 ± 4.0 b	11.5 ± 4.6 bcd	12.5 ± 3.0 bcd	13.3 ± 3.3 cde	14.1 ± 4.9 def
Shoot greening	++	++	++	++	+++	+	+	+	+	+
Hyperhydricity (%)	50.0 ± 51.2 bcdef	60.0 ± 50.2 cdefg	65.0 ± 48.9 defg	67.5 ± 46.6 efg	65.0 ± 48.9 defg	65.0 ± 48.9 defg	65.0 ± 36.6 defg	75.0 ± 25.6 g	72.5 ± 25.5 fg	75.0 ± 41.3 g
Intensity of hyperhydricity	++	+++	+++	++++	++++	+	++	++	+++	++++
Browning (%)	70.0 ± 41.0 cdefghi	75.0 ± 25.6 defghi	80.0 ± 34.0 efghi	90.0 ± 30.7 ghi	90.0 ± 30.7 ghi	42.5 ± 46.6 abcde	45.0 ± 48.3 abcde	47.5 ± 37.9 abcde	45.0 ± 45.5 abcde	50.0 ± 51.2 abcdef
Intensity of browning	++	++	++	++	++	+	+	+	+	+
Precocious rooting (%)	20.0 ± 41.0 ab	25.0 ± 38.0 ab	30.0 ± 47.0 ab	32.5 ± 43.7 ab	50.0 ± 51.2 b	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	7.5 ± 18.3 a	10.0 ± 30.7 a
Root number	0.3 ± 0.8 a	0.3 ± 0.4 a	0.3 ± 0.5 a	0.4 ± 0.5 a	1.2 ± 1.5 b	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.1 ± 0.3 a	0.1 ± 0.4 a

Data are means ± standard deviation. Data in the same line followed by the same letter are not significantly different at the 5% significance level. Intensity of shoot greening, hyperhydricity and tissue browning were visually estimated as: + low, ++ moderate, +++ high, ++++ very high

Statistical analysis showed significant effects of cytokinins and medium textures on shoot bud proliferation. However, BS4 and BS5 media showed no significant difference (P>0.05) in term of the number of shoot buds per explant. Accordingly, the concentrations of 0.4 and 0.5 mg/L of BAP in semi-solid MS/2 medium seemed to be the most appropriate for cv. Najda (Tables 2).

Effect of cytokinins and medium texture on hyperhydricity

Hyperhydricity is a physiological disorder observed in many plant species (Barakat and El-Sammak, 2011; Badr-Elden et al., 2012). In date palm, hyperhydricity was observed in both somatic embryogenesis (McCubbin and Zaid, 2007) and organogenesis (Mazri et al., 2019). Liquid media, PGRs (mainly cytokinins) and mineral salts (especially ammonium ions) were all reported to increase hyperhydricity in date palm cultures, which can lead to irreversible loss of the regenerative capacity of explants (Sreedhar et al., 2009; Mazri, 2015; Mazri et al., 2019). Our findings showed that the occurrence of hyperhydricity depends on both cytokinin and medium texture. Generally, the use of 2iP resulted in higher hyperhydricity percentages than BAP and TDZ (Tables 2, 3 and 4; Fig. 2 A). This is in good agreement with the findings of Mazri (2015). Regarding medium texture, the effect on hyperhydricity varied depending on the cytokinin added to culture medium. When BAP and 2iP were used, liquid media showed higher hyperhydricity percentages than the semi-solid ones, which confirms the results reported by Al Khateeb (2006), Al Khateeb (2008), Fki et al. (2011) and Mazri et al. (2019). However, when TDZ was used, the liquid state showed lower hyperhydricity percentages than the semi-solid one (Table 4). These results highlight the interaction effect of cytokinin type, concentration and medium texture on explant hyperhydricity. Thus, cytokinin and medium texture play a significant role on date palm hyperhydricity and should be well optimized to reduce the occurrence of this phenomenon.

Effect of cytokinins and medium texture on tissue browning

Tissue browning is another physiological disorder encountered during date palm organogenesis. According to Loutfi and El Hadrami (2005), tissue browning is caused by the high content of caffeoylshikimic acids in date palm explants.

Generally speaking, tissue browning was observed in all culture media (20-100%). The difference was observed in its percentage and intensity that were significantly higher when TDZ was used (Table 4 and Fig. 2 B). This phenomenon could be the cause of low shoot bud proliferation in media containing TDZ, since Zaid (1987), El Hadrami et al. (1995), Al Khayri (2005) and Mazri (2015) reported that tissue browning decreases the potential of date palm multiplication in vitro. Thus, we advise against the use of TDZ in cv. Najda organogenesis, despite that this cytokinin was suggested for the micropropagation of many plant species (Mazri et al., 2013; Mazri et al., 2018b).

Regarding medium texture, the liquid state showed lower browning percentages than the semi-solid one, except for TDZ, in which high levels of browning were observed in both medium textures (85-100%; Table 4). This confirms that TDZ is a causal factor of tissue browning, regardless of the other medium components and culture conditions. According to Abohatem et al. (2011), there is a lack of information on the relationship between the cytokinin concentration, tissue growth, phenol content, peroxidase activity and the oxidative browning process of date palm tissues. According to Meziani et al. (2016), tissue browning is positively correlated with the peroxidase activity of date palm explants.

Table 4. Effect of TDZ on shoot bud proliferation, hyperhydricity, tissue browning and precocious rooting

Culture medium	Semi-solid MS/2					Liquid MS/2				
TDZ (mg/L)	0.1	0.2	0.3	0.4	0.5	0.1	0.2	0.3	0.4	0.5
Shoot bud number	5.5 ± 1.5 a	5.8 ± 1.8 a	6.0 ± 2.0 a	7.0 ± 2.5 a	12.2 ± 4.1 bcd	5.0 ± 0.0 a	5.5 ± 0.6 a	5.9 ± 1.4 a	6.5 ± 1.7 a	10.5 ± 2.7 bc
Shoot greening	+++	+++	+++	+++	++++	+	+	+	+	+
Hyperhydricity (%)	30.0 ± 47.0 abcde	45.4 ± 48.3 bcdef	65.0 ± 48.9 defg	70.0 ± 47.0 fg	65.0 ± 48.9 defg	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	10.0 ± 20.5 ab	20.0 ± 37.6 abc
Intensity of hyperhydricity	+	+	+	+	+	+	+	+	+	+
Browning (%)	85.0 ± 28.5 fghi	90.0 ± 26.1 ghi	90.0 ± 30.7 ghi	95.0 ± 15.3 hi	100.0 ± 0.0 i	95.0 ± 15.3 hi	95.0 ± 15.3 hi	95.0 ± 15.3 hi	100.0 ± 0.0 i	100.0 ± 0.0 i
Intensity of browning	++	++	+++	++++	++++	++	++	+++	++++	++++
Precocious rooting (%)	10.0 ± 30.7 a	10.0 ± 30.7 a	12.5 ± 31.9 a	15.0 ± 36.6 a	20.0 ± 37.6 ab	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
Root number	0.1 ± 0.3 a	0.1 ± 0.3 a	0.1 ± 0.3 a	0.1 ± 0.3 a	0.2 ± 0.4 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a

Data are means ± standard deviation. Data in the same line followed by the same letter are not significantly different at the 5% significance level. Intensity of shoot greening, hyperhydricity and tissue browning were visually estimated as: + low, ++ moderate, +++ high, ++++ very high

Our findings showed that the use of BAP in liquid medium resulted in lower tissue browning percentages than semi-solid medium. However, the semi-solid media containing 0.4 and 0.5 mg/L BAP showed significantly higher number of morphologically superior shoot buds than liquid media. Accordingly, semi-solid MS/2 medium supplemented with BAP at 0.4 or 0.5 mg/L is recommended for the multiplication of shoot buds of cv. Najda.

Effect of cytokinins and medium texture on precocious rooting

In date palm organogenesis, root formation is undesirable during the proliferation of adventitious shoot buds. Indeed, root induction inhibits shoot bud multiplication as the components of culture medium are used for root formation. Generally, root formation is due to high levels of auxins. The findings of the present study indicated that cytokinins and medium texture may also affect precocious rooting. In fact, percentage of rooted organogenic cultures and the average number of roots were higher in the culture media supplemented with 2iP (Tables 2, 3, 4 and Fig. 2 C). On the other hand, precocious rooting was generally lower in liquid media than in the semi-solid ones. Furthermore, the use of TDZ decreased root formation. Based on our results, auxins are not the only factor behind precocious rooting during date palm organogenesis. This is in good agreement with the results reported in date palm cv. Boufeggous (Mazri, 2015). Regarding BAP, the use of this cytokinin in semi-solid MS/2 medium showed low percentages of precocious rooting (<22.5%) with a low average number of roots per organogenic culture (<0.5). Based on all these findings, MS/2 medium supplemented with BAP at 0.4-0.5 mg/L is the best treatment for the multiplication of adventitious shoot buds of date palm cv. Najda.

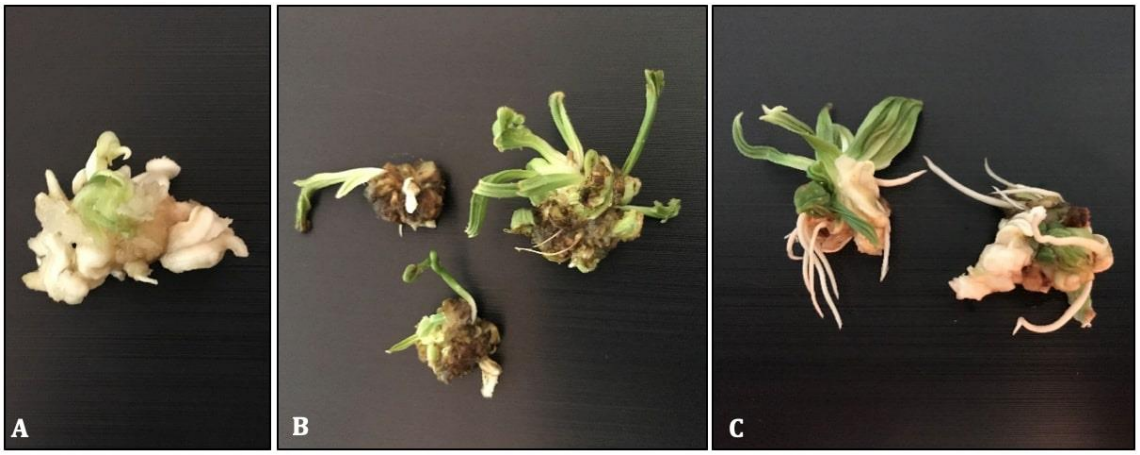

Figure 2: Different physiological disorders observed during shoot bud multiplication. **A.** Hyperhydricity of organogenic culture. **B.** Tissue browning. **C.** Precocious rooting.

Shoot elongation, rooting and plant acclimatization

Shoot elongation and rooting were carried out on PGR-free medium as suggested by Mazri and Meziani (2013). In fact, in date palm cv. Najda, the use of PGRs during shoot elongation and rooting has a negative impact on subsequent plantlet acclimatization (i.e. low survival rate) (Mazri and Meziani, 2013). Thus, shoots (≥5 cm long) were separated individually and transferred to PGR-free MS/2 medium.

The findings of the present study showed that not all proliferation media gave shoots larger than 5 cm. Accordingly, only those cultured on BS3, BS4, BS5, IS5, BL3, BL4, BL5 and IL5 media were used for elongation and rooting. During this phase, we observed that shoot elongation as well as root formation and length depended on the proliferation medium (Table 5). The shoots previously cultured on BS4 and BS5 media showed the highest values of almost all the parameters studied, except for root number which was higher in IS5 medium. Shoots reached their maximum length when they were transferred from BS5 medium (12.7 cm). The highest average root length (3.3 cm) was also observed in shoots previously cultured on BS5 medium, while the highest average number of roots per shoot (4.6) was observed in shoots previously cultured on IS5 medium. On the other hand, the shoots previously cultured on semi-solid media exhibited greener leaves than those cultured on the liquid ones (Fig. 3 A). This may reflect a higher chlorophyll content (Mazri et al., 2019; Meziani et al., 2019). Based on our results, we recommend the use of semi-solid media for date palm cv. Najda organogenesis.

Figure 3: Shoots developed on media supplemented with 0.4 mg/L BAP. **A1.** shoot development on semi-solid MS/2 medium. **A2.** shoot development on liquid MS/2 medium. **B.** Plantlet acclimatization.

Regarding acclimatization, a high survival rate of healthy and vigorous plants (Fig. 3 B), reaching 95%, was observed in the shoots previously cultured on semi-solid media. Shoots cultured in liquid media were fragile and showed a low survival rate (<35%) (Table 5). This is in good agreement with the results obtained on cvs. Boufeggous and Mejhoul by Mazri (2015) and Meziani et al. (2019), respectively.

Table 5: Effect of multiplication and elongation-rooting media on shoot development and subsequent acclimatization

Proliferation medium	BS3	BS4	BS5	IS5	BL3	BL4	BL5	IL5
Elongation medium	Semi-solid MS/2				Liquid MS/2			
Shoot length (cm)	11.5 ± 3.8 ab	12.6 ± 2.2 b	12.7 ± 3.2 b	10.0 ± 3.3 a	11.1 ± 2.6 ab	12.3 ± 0.8 b	12.4 ± 2.4 b	9.1 ± 3.7 a
Shoot greening	++++	++++	++++	+++	++	++	++	++
Root number	3.4 ± 2.2 ab	3.9 ± 1.1 b	4.2 ± 2.5 b	4.6 ± 1.6 b	2.5 ± 1.1 a	2.8 ± 0.7 a	2.9 ± 2.0 a	3.2 ± 1.5 ab
Root length (cm)	2.9 ± 1.2 a	3.2 ± 1.5 a	3.3 ± 1.4 a	3.0 ± 1.7 a	2.5 ± 2.0 a	2.7 ± 1.1 a	2.8 ± 1.5 a	2.6 ± 1.3 a
Survival rate during acclimatization (%)	85.0 ± 24.1 c	95.0 ± 15.8 c	90.0 ± 31.6 c	75.0 ± 35.3 b	30.0 ± 25.8 a	35.0 ± 24.1 a	30.0 ± 25.8 a	25.0 ± 26.3 a

Data are means ± standard deviation. Data in the same line followed by the same letter are not significantly different at the 5% significance level. Greening intensity was visually estimated as: + low, ++ moderate, +++ high, ++++ very high

Conclusions

In the present study, the combination of 0.5 mg/L NAA and 0.4 mg/L BAP in semi-solid MS/2 medium improved shoot bud multiplication of date palm cv. Najda (23.8 shoot buds per explant) and reduced the occurrence of undesirable physiological disorders, i.e. hyperhydricity (22.5%), tissue browning (55%) and precocious rooting (20%). Shoot elongation and rooting were achieved on semi-solid MS/2 medium and the plantlets were successfully acclimatized to the glasshouse conditions (95% survival rate). Our findings highlighted the impacts of different cytokinins and medium textures on cv. Najda organogenesis and recommended the use of agar-solidified medium containing BAP at 0.4 mg/L. The results of the present study will be beneficial for rapid and large-scale propagation of healthy and vigorous plantlets of Najda, a date palm cultivar resistant to the bayoud disease.

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