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(RESEARCH ARTICLE)



Optimization of auxin-cytokinin combination for rapid proliferation of adventitious buds of date palm cv. Aziza Bouzid

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Abstract

We evaluated the effects of various plant growth regulator (PGR) combinations on adventitious shoot bud multiplication of date palm cv. Aziza Bouzid. In the first experiment, we assessed the effect of four PGR combinations that have already shown good results in other date palm cultivars: half-strength Murashige and Skoog medium (MS/2) supplemented with 0.5 mg/L 2-naphthoxyacetic acid (NOA) and 0.5 mg/L kinetin (KIN); MS/2 supplemented with 0.2 mg/L NOA, 0.2 mg/L indole-3-acetic acid (IAA), 0.4 mg/L 6-(dimethylallylamino)purine (2iP) and 0.4 mg/L KIN; MS/2 supplemented with 0.5 mg/L 1-naphthaleneacetic acid (NAA) and 0.5 mg/L KIN and finally MS/2 supplemented with 0.5 mg/L indole-3-butyric acid (IBA) and 0.6 mg/L 6-benzylaminopurine (BAP). Our results showed no significant difference among the four culture media in terms of adventitious shoot bud multiplication (26.7-28.4 adventitious buds per explant). However, the medium containing 0.5 mg/L NOA and 0.5 mg/L KIN showed significantly lower rate of precocious rooting and thus this auxin-cytokinin combination was chosen for the second experiment. In the second experiment, we evaluated the effects of various NOA and KIN concentrations on adventitious shoot bud multiplication: 0, 0.25, 0.5 and 1 mg/L of each PGR, either alone or in combination. It was found that the combination of 0.5 mg/L NOA and 0.5 mg/L KIN is still the most appropriate for adventitious shoot bud multiplication. Shoot elongation and rooting were achieved on PGR-free MS/2 medium and a high acclimatization rate of 70% was observed in the greenhouse.

Keywords: Auxin; Adventitious buds; Cytokinin; Date palm; Organogenesis.

1. Introduction

Date palm (*Phoenix dactylifera* L.) is a plant species that has been cultivated for centuries in the Middle East and North Africa region [1]. In the recent years, and due to the high socio-economic, agronomic and ecological importance of date palm, this species is now cultivated in many other parts of the world such as Mexico, USA and Spain [2]. In fact, date palm contributes to the revenue of the population of arid and semi-arid zones since it provides livelihoods and creates employment. Besides, date palm is essential for food security, biodiversity preservation and desertification prevention in arid and semi-arid regions since it creates appropriate microclimatic conditions for agriculture.

Date palm is mainly cultivated for fruit production. Indeed, this plant species produces a very delicious and highly nutritious fruit. According to FAOSTAT [3], the annual worldwide production of date fruits exceeds eight million tons.

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Date palm is threatened by the bayoud disease, a very dangerous wilt disease caused by the fungus *Fusarium oxysporum* f. sp. *albedinis*. Bayoud has killed millions of plants of the best date palm cultivars and is still threatening many genotypes that produce fruits of high quality [4, 5]. To date, there is no efficient chemical compound to fight the bayoud disease. Thus, rapid and large-scale multiplication of date palm genotypes seems to be the best strategy to rehabilitate the groves destroyed by the bayoud disease, and to preserve the best cultivars that are sensitive to this fungus.

Date palm could be propagated by seeds, by offshoots or through *in vitro* culture techniques [6]. *In vitro* culture techniques, namely somatic embryogenesis and organogenesis, have been widely used in the last years by many countries since they have many advantages when compared to the conventional techniques [6]. Organogenesis is an *in vitro* propagation technique based on the formation of adventitious buds and their subsequent development into complete plants. In the last few years, this technique has gained much attention in date palm due to its high multiplication potential and the production of true-to-type plants [7]. Accordingly, successful regeneration through organogenesis was reported in some genotypes of high commercial values [8, 9, 10, 11]. However, it is well known that, in date palm, the development of a successful regeneration system through organogenesis depends strongly on genotype [12]. Regarding cv. Aziza Bouzid, a date palm genotype highly appreciated by the consumers, there is no published regeneration system through organogenesis.

In addition to plant genotype, the development of a successful regeneration system through organogenesis depends on many other factors, the most important of them is the plant growth regulators (PGRs) used in culture medium. Auxin and cytokinin are the most frequently used families of PGRs, and are generally added in combination. These exogenous hormones modify the concentrations of the endogenous ones, which affects cell division, growth and differentiation, and thus inducing morphogenesis [13]. Optimizing the auxin-cytokinin combination is a very important step for the successful development of any *in vitro* regeneration system, regardless of the plant species or genotype used.

In the present study, we aimed to evaluate the effects of various PGRs on adventitious shoot bud multiplication, as well as on the incidence of some undesirable physiological phenomena, namely hyperhydricity, tissue browning and precocious rooting. Accordingly, the impact of 19 different PGR combinations was assessed on organogenesis of date palm cv. Aziza Bouzid.

2. Materials and methods

2.1. Plant material

Organogenic cultures of date palm cv. Aziza Bouzid were obtained according to the induction protocol described by Beauchesne et al. [14]. Afterwards, the organogenic cultures were maintained in PGR-free half-strength Murashige and Skoog medium (MS/2) [15] for 3 months to avoid the effect of previous PGRs on our experiments. In the present study, each organogenic culture comprised 3 to 4 adventitious buds.

2.2. Culture medium

The basal medium used in all experiments was that of Murashige and Skoog [15] at half strength (MS/2): MS/2 macro-elements, MS microelements and MS vitamins. This basal medium was supplemented with 30 g/L commercial granulated sugar, 1 g/L polyvinyl-pyrrolidone (PVP) and solidified with 8 g/L of agar.

2.3. Experiment 1: Effect of various PGR combinations on adventitious shoot bud multiplication

In order to evaluate the effect of PGRs on adventitious bud multiplication of date palm cv. Aziza Bouzid, organogenic cultures were cultured on media containing four PGR combinations that have already shown interesting results on other date palm cultivars. These PGR combinations were as follows:

- Medium A: MS/2 supplemented with 0.5 mg/L 2-naphthoxyacetic acid (NOA) and 0.5 mg/L kinetin (KIN) [9].
- Medium B: MS/2 supplemented with 0.2 mg/L NOA, 0.2 mg/L indole-3-acetic acid (IAA), 0.4 mg/L 6-(dimethylallylamino)purine (2iP) and 0.4 mg/L KIN [11].
- Medium C: MS/2 supplemented with 0.5 mg/L 1-naphthaleneacetic acid (NAA) and 0.5 mg/L KIN (not published).
- Medium D: MS/2 supplemented with 0.5 mg/L indole-3-butyric acid (IBA) and 0.6 mg/L 6-benzylaminopurine (BAP) [16].

2.4. Experiment 2: Effect of different NOA and KIN concentrations on adventitious shoot bud multiplication

Based on the results of the first experiment, we evaluated the effects of various NOA and KIN concentrations on adventitious shoot bud multiplication. Accordingly, the concentrations of 0, 0.25, 0.5 and 1 mg/L of each PGR, either alone or in combination, were evaluated.

2.5. Shoot elongation, rooting and plantlet acclimatization

Shoot elongation and rooting were performed by culturing shoots on PGR-free MS/2 medium for 3 months as already suggested for many date palm cultivars [8, 9, 17]. Plantlets of 3 to 4 leaves and a good root system were acclimatized according to the protocol described by Mazri et al. [17, 18].

2.6. Culture conditions

The pH of culture media was adjusted to 5.7 before autoclaving at 121 ° C for 25 minutes. For each medium, we used two organogenic cultures per jar. Each jar was containing 40 ml of culture medium, and was considered as one repetition. In all experiments, we used 10 repetitions per treatment. The organogenic cultures were transferred monthly to a fresh medium.

The cultures were placed in a culture chamber under 16h photoperiod and 25 ± 1 ° C temperature.

2.7. Date collection and Statistical analysis

At the end of our experiments, we calculated the number of adventitious buds per explant, the percentages and intensity of hyperhydricity and tissue browning, the percentage of precocious rooting and the mean number of roots per organogenic culture.

For analysis, we adopted ANOVA and a completely randomized design. The analysis was carried out on the observed values except for percentage data, which were arcsine transformed before analysis.

Statistical analysis was performed by the statistical software SPSS for Windows (Version 26) and the means were separated using the Student-Newman-Keuls (SNK) test at the significance level of 5%.

3. Results and discussion

The findings of our study showed that the four PGR combinations evaluated in the first experiment did not show any significant difference regarding adventitious shoot bud multiplication. These PGR combinations, which have been recommended for other date palm genotypes (i.e. Najda, 16-bis and Mejhoul), have all shown very high multiplication rates, with averages ranging from 26.7 to 28.4 adventitious shoot buds per organogenic culture (Table 1).

Table 1 Effects of different PGR combinations on adventitious shoot bud multiplication, hyperhydricity, tissue browning and precocious rooting of date palm cv. Aziza Bouzid

Culture medium	Mean number of adventitious buds per explant	Hyperhydricity (%)	Intensity of hyperhydricity	Intensity of Tissue browning (%)	Intensity of tissue browning	Precocious rooting (%)	Mean number of roots per explant
A	26.9 ± 8.4 a	60 ± 50.2 a	+	70 ± 47.0 a	+	30 ± 47.0 a	0.4 ± 0.8 a
B	26.7 ± 8.2 a	60 ± 50.2 a	+	65 ± 48.9 a	+	55 ± 51.0 b	1.0 ± 1.1 a
C	28.4 ± 9.6 a	70 ± 47.0 a	++	75 ± 44.4 a	+	50 ± 51.2 b	0.9 ± 1.0 a
D	27.8 ± 5.1 a	75 ± 44.4 a	++	65 ± 48.9 a	+	60 ± 50.2 b	0.8 ± 0.8 a

Data are means ± standard deviation. Data in the same column followed by the same letter are not significantly different at 5% level (SNK). Hyperhydricity and tissue browning intensities: +, low intensity; ++, moderate intensity; +++, high intensity. PGRs in culture media: A, 0.5 mg/L NOA + 0.5 mg/L KIN. B, 0.2 mg/L NOA + 0.2 mg/L IAA + 0.4 mg/L 2iP + 0.4 mg/L KIN. C, 0.5 mg/L NAA + 0.5 mg/L KIN. D, 0.5 mg/L IBA + 0.6 mg/L BAP.

Several authors have indicated the need to add an auxin-cytokinin combination to culture medium for a rapid multiplication of adventitious shoot buds of date palm. In fact, exogenous hormones modify the concentrations of the endogenous ones [13]. This interaction of exogenous and endogenous hormones affects cell division, growth and

differentiation, and may thus promote morphogenesis as well as the multiplication and development of adventitious buds [19, 20]. In the present work, the choice of the auxin-cytokinin combinations was based on the results of previous researches on other date palm genotypes [9, 11, 16]. The combination of 0.5 mg/L NOA and 0.5 mg/L KIN showed an average of 23.5 shoot buds per explant in cv. Najda [9]. In cv. 16-bis, combining 0.5 mg/L IBA and 0.6 mg/L BAP gave 22.3 shoot buds per organogenic culture while in cv. Mejhoul, the combination of 0.2 mg/L NOA, 0.2 mg/L IAA, 0.4 mg/L 2iP and 0.4 mg/L KIN resulted in an average of 12.8 shoot buds per organogenic culture [11, 16]. By comparing these findings with those of the present work, we can notice that cv. Aziza Bouzid have a greater multiplication capacity than all the above-mentioned date palm cultivars (26.7-28.4 shoot buds per organogenic culture). This is due to the genotype effect. In fact, it is well known that, in many plant species, the response of plant cells and tissues cultured *in vitro* depends strongly on the genotype since the requirements in PGRs and other nutrients for *in vitro* morphogenesis vary among genotypes [21, 22].

Precocious rooting is an undesirable phenomenon observed during the multiplication of date palm adventitious buds. In the first experiment of the present study, the percentage of precociously formed roots ranged from 30 to 60%, depending on PGRs. Medium A (supplemented with NOA and KIN) showed the lowest rate of precocious rooting, with a significant difference to the other media (50-60%). This difference among the four PGR combinations evaluated is due to the type of auxins used. Indeed, it is well known that certain auxins are more potent than others regarding their effects on *in vitro* morphogenesis in general and rhizogenesis in particular. The stimulating effects of NAA and IBA on rooting have been previously reported on other date palm cultivars. For example, Khalas and Medjool [23, 24]. On the other hand, Fki et al. [25] reported the presence of a positive correlation between IBA concentrations and the average number of roots in date palm cv. Barhee. It is important to note that, during this multiplication phase of adventitious shoot buds, precocious rooting is undesirable. Accordingly, we generally try to use a culture medium that does not promote precocious rooting. In fact, precocious rooting is known to have a negative impact on adventitious shoot bud multiplication since the nutrients in the culture medium will be used for root formation instead of shoot bud multiplication [26]. Therefore, we strongly suggest the use of the combination NOA-KIN for the multiplication of adventitious shoot buds of date palm cv. Aziza Bouzid. Regarding the average number of roots per organogenic culture, it varied from 0.4 to 1, with no significant difference among the four culture media.

In the second experiment, we evaluated the effects of different concentrations of NOA and KIN on adventitious shoot bud multiplication. Indeed, we attempted to find the most appropriate concentrations of these two PGRs for cv. Aziza Bouzid. Our results showed that the average number of shoot buds per organogenic culture ranged from 10.7 in the PGR-free medium to 26.9 when 0.5 mg/L of each PGR was used (Table 2). Low concentrations of NOA and KIN showed lower number of shoot buds per organogenic cultures while increasing the concentrations of these two PGRs did not increase the average number of shoot buds. However, it significantly increased the percentage of precocious rooting. Thus, the combination of 0.5 mg/L NOA and 0.5 mg/L KIN seems to be the most appropriate for the multiplication of adventitious shoot buds of date palm cv. Aziza Bouzid (Fig. 1 A).

Regarding hyperhydricity and tissue browning, these are two phenomena widely encountered during the *in vitro* culture of date palm and which may cause significant losses [6, 8]. Hyperhydricity is mainly caused by high concentrations of ammonium ions and the use of liquid media [7, 26], while tissue browning is due to the high concentrations of caffeoylshikimic acids in date palm explants [27]. In our case, we noticed very high rates of hyperhydricity and tissue browning, even though their intensities were low to moderate. The hyperhydricity percentages ranged from 40 to 80% with low to moderate intensity, while the browning rates varied from 50 to 75%, with low intensity (Tables 1 and 2). The analysis of variance showed the absence of a significant impact of the different media on tissue browning. However, increasing the concentrations of NOA and KIN to 1 mg/L each significantly increased the percentage of hyperhydricity (Table 2). The effect of PGRs on hyperhydricity was already observed in other date palm cultivars [8, 9].

Shoot elongation and rooting (Fig. 2B) were carried out on PGR-free MS/2 medium as already suggested for many other date palm cultivars [8, 9, 17], and acclimatization was successfully achieved with a 70% survival rate after transferring the plantlets to the greenhouse (Fig. 2C).

Table 2 Effects of different NOA-KIN concentrations on adventitious shoot bud multiplication, hyperhydricity, tissue browning and precocious rooting of date palm cv. Aziza Bouzid

PGRs in culture medium	Mean number of adventitious buds per explant	Hyperhydricity (%)	Intensity of hyperhydricity	Tissue browning (%)	Intensity of tissue browning	Precocious rooting (%)	Mean number of roots per explant
PGR-free medium	10.7 ± 3.5 a	40 ± 50.2 a	+	50 ± 51.2 a	+	5 ± 22.3 a	0.05 ± 0.2 a
0.25 mg/L NOA	14.7 ± 4.9 b	50 ± 51.2 ab	+	60 ± 50.2 a	+	15 ± 36.6 ab	0.2 ± 0.5 abc
0.5 mg/L NOA	17.8 ± 4.9 c	70 ± 47.0 ab	++	60 ± 50.2 a	+	30 ± 47.0 ab	0.5 ± 0.8 abc
1 mg/L NOA	18.3 ± 6.2 cd	60 ± 50.2 ab	+	55 ± 51.0 a	+	50 ± 51.2 b	0.8 ± 0.8 bc
0.25 mg/L KIN	19.1 ± 4.5 cd	45 ± 51.0 ab	+	60 ± 50.2 a	+	10 ± 30.7 ab	0.1 ± 0.3 a
0.25 mg/L NOA + 0.25 mg/L KIN	23.1 ± 4.1 efg	45 ± 51.0 ab	+	65 ± 48.9 a	+	25 ± 44.4 ab	0.3 ± 0.5 abc
0.5 mg/L NOA + 0.25 mg/L KIN	23.4 ± 4.4 efg	55 ± 51.0 ab	+	60 ± 50.2 a	+	35 ± 48.9 ab	0.4 ± 0.5 abc
1 mg/L NOA + 0.25 mg/L KIN	23.9 ± 3.3 efg	65 ± 48.9 ab	+	55 ± 51.0 a	+	50 ± 51.2 b	0.7 ± 0.8 abc
0.5 mg/L KIN	20.3 ± 4.1 cde	55 ± 51.0 ab	+	60 ± 50.2 a	+	15 ± 36.6 ab	0.15 ± 0.3 ab
0.25 mg/L NOA + 0.5 mg/L KIN	22.0 ± 2.9 def	60 ± 50.2 ab	+	65 ± 48.9 a	+	25 ± 44.4 ab	0.25 ± 0.4 abc
0.5 mg/L NOA + 0.5 mg/L KIN	26.9 ± 8.4 g	60 ± 50.2 ab	+	70 ± 47.0 a	+	30 ± 47.0 ab	0.4 ± 0.8 abc
1 mg/L NOA + 0.5 mg/L KIN	25.0 ± 3.6 fg	60 ± 50.2 ab	+	70 ± 47.0 a	+	50 ± 51.2 b	0.85 ± 0.9 c
1 mg/L KIN	20.3 ± 4.1 cde	60 ± 50.2 ab	+	65 ± 48.9 a	+	25 ± 44.4 ab	0.25 ± 0.4 abc
0.25 mg/L NOA + 1 mg/L KIN	24.2 ± 3.2 efg	60 ± 50.2 ab	+	70 ± 47.0 a	+	35 ± 48.9 ab	0.35 ± 0.4 abc
0.5 mg/L NOA + 1 mg/L KIN	25.1 ± 2.2 fg	70 ± 47.0 ab	+	65 ± 48.9 a	+	45 ± 51.0 ab	0.55 ± 0.6 abc
1 mg/L NOA + 1 mg/L KIN	23.7 ± 3.5 efg	80 ± 41.0 b	++	75 ± 44.4 a	+	50 ± 51.2 b	0.8 ± 0.8 bc

Data are means ± standard deviation. Data in the same column followed by the same letter are not significantly different at 5% level (SNK). Hyperhydricity and tissue browning intensities: +, low intensity; ++, moderate intensity; +++, high intensity



Figure 1 Effects of PGRs on adventitious shoot bud multiplication and subsequent development in date palm cv. Aziza Bouzid. A: Adventitious shoot bud multiplication on MS/2 medium supplemented with 0.5 mg/L NOA and 0.5 mg/L KIN. B: Shoot elongation and rooting on PGR-free MS/2 medium. C: Plantlet acclimatization.

4. Conclusion

Our findings showed that the combination of 0.5 mg/L NOA and 0.5 mg/L KIN is the most appropriate for the multiplication of adventitious shoot buds of date palm cv. Aziza Bouzid. However, we have noticed high hyperhydricity and tissue browning percentages. Accordingly, it is important to evaluate the effect of other factors in order to reduce the incidence of these two phenomena, for example the use of a different basal formulation of culture medium and the evaluation of other antioxidants at different concentrations.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of interest.

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