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Impact of Osmotica and Plant Growth Regulators on Somatic Embryogenesis of Date Palm

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Abstract

An efficient somatic embryogenesis system is reported for date palm cv. Al-Fayda, a genotype resistant to the bayoud disease. Callus induction was achieved from adventitious bud explants cultured for 6 months on semi-solid Murashige and Skoog (MS) medium containing 4.5 µM 6-(dimethylallylamino) purine (2iP) and various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) or picloram. The highest somatic embryogenesis frequency (89%) was obtained on MS medium supplemented with 225 µM 2,4-D. Subsequently, embryogenic cultures were transferred to agitated liquid MS medium (maturation medium) containing various concentrations of mannitol, polyethylene glycol (PEG) or sorbitol. The highest rate of somatic embryo maturation (71.4 mature embryos per 100 mg callus) was achieved on the medium supplemented with 40 g l^{-1} PEG. Mature somatic embryos were then transferred to MS medium supplemented with gibberellic acid (GA_3) or 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) at various concentrations. The highest frequency of germination and conversion (26%) was obtained on the medium containing 5 µM NAA and 5 µM BAP. The developed plants were then transferred to *ex vitro* conditions, where a survival rate of 77.02% was observed. The regeneration protocol established in the present investigation will be used for mass propagation of date palm cv. Al-Fayda.

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Introduction

Date palm (*Phoenix dactylifera* L.) is a highly valuable crop species in the Middle East and North Africa (MENA) region.[1,2] This species is mainly cultivated for its highly nutritious fruits and for creating favorable conditions for agriculture in arid region.[2,3] Besides, date palm plays important economic roles by generating employment and significantly contributing to the income of local populations.[4] Unfortunately, the Moroccan palm groves are threatened by the wilt disease known as bayoud. Bayoud, which is caused by *Fusarium oxysporum* f. sp. *albedinis*, killed millions of date palm plants during the last century, causing a significant reduction in the populations of the best date palm varieties and the disappearance of many others.[5] To date, there is no effective chemical treatment to control bayoud.[1] The only way to fight this disease and to rehabilitate Moroccan palm groves is through selection and large-scale propagation of bayoud-resistant cultivars.

Since 1970s, researchers from the National Institute of Agronomic Research of Morocco (INRA) have carried out a series of prospection in the Moroccan oases in order to select date palm genotypes characterized by high fruit quality and resistance to bayoud. As a result, some interesting genotypes were selected, including Al-Fayda (INRA-1447). It was reported that cv. Al-Fayda produces fruits of excellent quality that are similar to those produced by cv. Jihel.[5]

Large-scale and mass multiplication of date palm can be achieved by employing *in vitro* techniques such as organogenesis or somatic embryogenesis.[6] In somatic embryogenesis, the somatic cells are developed and form complete embryos that are similar to the zygotic ones.[7] It involves the following steps: callus induction (in case of indirect somatic embryogenesis), embryogenesis expression, embryo maturation and germination, and then the formation of complete plants. The developed plants are transplanted *ex vitro*.[8,9] To the best of our knowledge, there is no published study describing this regeneration pathway in date palm cv. Al-Fayda.

Somatic embryogenesis was previously achieved from various date palm explants, but mostly from shoot tips and inflorescences.[10] Recently, explants derived from plant material maintained *in vitro*, such as adventitious buds, leaves, roots were also used, and a high embryogenic potential of adventitious bud explants was reported.[8,11] Such explants are pathogen-free, available independently of seasons and allow to avoid excessive use of offshoots and spathes.[8]

The main objective of the present work was to develop a regeneration system for date palm cv. Al-Fayda through somatic embryogenesis using adventitious buds as explants. Accordingly, the impacts of different osmotica and plant growth regulators (PGRs) on callus induction, somatic embryogenesis expression, embryo maturation and germination were investigated.

Materials and Methods

Plant Material

Adventitious buds of date palm cv. Al-Fayda induced and maintained *in vitro,*[12] and then they used for somatic embryogenesis. Before starting embryogenesis experiments, the adventitious buds were maintained during 3 months in PGR-free semi-solid and half-strength Murashige and Skoog medium (1/2MS)[13] in order to avoid the effect of previous PGRs on callus induction.

Callus Induction and Embryogenesis Expression

Adventitious buds of date palm cv. Al-Fayda were cut into small segments then cultured on semi-solid MS medium containing 4.5 µM 6-(dimethylallylamino) purine (2iP) and picloram or 2,4-dichlorophenoxyacetic acid (2,4-D) at three different concentrations: 45, 225 and 450 µM. For each treatment, 10 segments were used per jar containing 25 ml of induction medium. Each jar was counted as one, and 10 replications were made per treatment. After 6 months on the induction medium, the developed calli were transferred to PGR-free semi-solid MS medium for one month. All cultures were kept in dark conditions and were subcultured monthly.

Somatic Embryo Maturation

Embryogenic cultures (calli with globular embryos) disintegrated and cultured on liquid MS medium with 20, 30 or 40 g l^{-1} sorbitol, polyethylene glycol (PEG) or mannitol, respectively. In addition, a medium devoid of these osmotica was used as control. For

each treatment, 10 replications were used. The cultures were maintained under dark conditions for 12 weeks and were shaken at 60 rpm. The cultures were subcultured at intervals of three weeks.

Somatic Embryo Germination

Mature embryos were cultured on semi-solid MS medium supplemented with gibberellic acid (GA_3) or 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) at 2,5 or 5 µM. A PGR-free medium was used as control. In this experiment, 10 mature somatic embryos were cultured per jar (25 ml culture medium) and five replications were carried out. All cultures were kept under 16h photoperiod for 5 months with monthly subcultures.

Plant Acclimatization

The regenerated plants transferred in culture into the glasshouse.[14] Briefly, well rooted plants were taken out of culture media, then their roots were rinsed with tap water and dipped in a solution of Pelt 44 PM at the concentration of 1 g l^{-1} during 15 min. Thereafter, the plants were transplanted into bags containing a peat-gravel (1:1; w/w) mixture and covered with polyethylene for 15 days (27°C; 98% RH). The polyethylene was progressively removed to allow plant acclimatization.

Culture Conditions

The basal formulation of all culture media[13] was consisting of MS salts and vitamins. Besides, 30 g l^{-1} sucrose and 1 g l^{-1} activated charcoal were added to all culture media, while 6 g l^{-1} agar was added to the semi-solid ones. "The pH of all media was set to 5.7 then they were autoclaved (121°C, 25 min). The temperature of culture chambers was set to 25°C.".

Data Collection and Statistical Analysis

Callus formation rate was calculated after 6 months of induction. Somatic embryogenesis expression, which corresponds to the percentage of calli forming globular embryos (embryogenic cultures), was calculated after one month on PGR-free MS medium. Maturation of somatic embryos (mature embryos per 100 mg FW callus) and the percentage of somatic embryo germination were calculated after three months on maturation and germination media, respectively. Finally, plant acclimatization was calculated after 3 months in the glasshouse.

Data were subjected to analysis of variance using the software SPSS v. 21, and the means were separated by the Student-Newman-Keuls (SNK) test at 5% level of significance. Before analyzing, percentages were subjected to arcsine transformation.

Results

Effect of PGRs on Somatic Embryogenesis

Callus formation started during the first month of culture from the wounded areas before enveloping the entire explant. All calli were white and friable. After 6 months of culture, the callogenesis rate was 100% in all induction media. After transferring calli to expression medium (PGR-free and semi-solid MS medium), globular somatic embryos started to appear (Fig. 1a), with somatic embryogenesis frequencies ranging from 60 to 89%. The highest frequency was obtained in explants that were cultured on the medium supplemented with 225 µM 2,4-D. Increasing 2,4-D concentration to 450 µM significantly decreased the frequency of somatic embryogenesis (68%; Table 1). The explants that were cultured on media containing picloram exhibited somatic embryogenesis rates ranging from 60 to 83%. Based on these findings, 225 µM 2,4-D is recommended for somatic embryogenesis in date palm cv. Al-Fayda.

Table 1: Impact of plant growth regulators on somatic embryogenesis in date palm cv. Al-Fayda

Plant growth regulator (PGR) in induction medium	PGR concentration (µM)	Frequency of somatic embryogenesis (%)
2,4-D	45	71 ± 17.28 ab
2,4-D	225	89 ± 7.37 c
2,4-D	450	68 ± 13.98 ab
Picloram	45	74 ± 14.29 ab
Picloram	225	83 ± 11.59 bc
Picloram	450	60 ± 16.32 a

Somatic Embryo Maturation

Preliminary experiments showed that semi-solid culture media resulted in very low rates of somatic embryo maturation. Therefore, in this study, only agitated liquid media were used.

After 3 months of culture, the highest number of mature embryos (71.4; Fig. 1b) was obtained on the medium supplemented with 40 g l^{-1} PEG (Table 2). On the other culture media that were containing osmotica, the mean number of mature embryos ranged from 40.7 (20 g l^{-1} mannitol) to 67.6 (30 g l^{-1} PEG). Osmoticum-free medium exhibited a maturation rate of 27.7 mature somatic embryos per 100 mg callus. Accordingly, our results suggest the use of 40 g l^{-1} PEG in liquid MS medium for the maturation of somatic embryos of date palm cv. Al-Fayda.

Table 2: Impact of osmotica on the maturation of date palm cv. Al-Fayda somatic embryos

Osmoticum type and concentration in maturation medium (g l^{-1})			Mean number of mature embryos per 100 mg FW callus
Sorbitol	Mannitol	Polyethylene glycol	
0	0	0	27.7 ± 8.01 a
20	0	0	47.9 ± 7.95 c
30	0	0	56.2 ± 7.29 cde
40	0	0	62.3 ± 6.88 ef
0	20	0	40.7 ± 6.97 b
0	30	0	52.0 ± 8.47 cd
0	40	0	58.4 ± 11.14 de
0	0	20	52.2 ± 6.08 cd
0	0	30	67.6 ± 4.59 fg
0	0	40	71.4 ± 5.81 g

Table 3: Impact of different PGRs on somatic embryo germination

PGR type and concentration in germination medium (µM)			Germination frequency (%)
1-Naphthaleneacetic acid	6-Benzylaminopurine	Gibberellic acid	
0	0	0	2 ± 4.47 a
0	2.5	0	6 ± 5.47 ab
0	5	0	8 ± 10.95 ab
2.5	0	0	6 ± 5.47 ab
2.5	2.5	0	14 ± 5.47 bc
2.5	5	0	18 ± 4.47 bc
5	0	0	10 ± 10 abc
5	2.5	0	22 ± 8.36 bc
5	5	0	26 ± 8.94 c
0	0	2.5	16 ± 5.47 bc
0	0	5	20 ± 7.07 bc

Germination of Somatic Embryos and Plant Acclimatization

For somatic embryo germination (Fig. 1c), the effects of NAA, BAP and GA3 were evaluated. The highest germination frequency was 26% on MS medium supplemented with 5 µM NAA and 5 µM BAP (Table 3). On PGR-free medium, a very low germination frequency of 2% was observed. On the other media, the germination rates ranged from 6 to 22%. Besides, secondary embryogenesis was observed while

many embryos turned brown and die even though all germination media contained activated charcoal. Based on our results, the combination of 5 µM NAA and 5 µM BAP is recommended for the germination of somatic embryos of date palm cv. Al-Fayda. After 5 months on germination media, the developed plants were transplanted to *ex vitro* conditions, where the survival rate was 77.02% (Fig.1d).

Fig. 1: Somatic embryogenesis in date palm cv. Al-Fayda

a) Induction and expression of embryogenesis after 6 months on MS medium supplemented with 225 µM 2,4-D and 4.5 µM 2iP, and one month on PGR-free medium. b) Somatic embryo maturation after 3 months on liquid agitated MS medium supplemented with 40 g l-1 polyethylene glycol. c) Beginning of germination on MS medium containing 5 µM NAA and 5 µM BAP. d) Plant acclimatization

Discussion

Somatic embryogenesis is an interesting regeneration pathway for mass and rapid production of date palm plants characterized by resistance to bayoud. In fact, rapid and mass multiplication of resistant cultivars is the only practical way to rehabilitate Moroccan groves infested with this fungus.[15] In the present study, we reported for the first time ever a somatic embryogenesis process for cv. Al-Fayda. In date palm, somatic embryogenesis is generally induced from shoot tip explants and inflorescences.[16] In the present work, callus induction was achieved from adventitious bud-derived explants that were initiated and maintained *in vitro*. Such explants, which are pathogen-free and available throughout the year, were successfully used previously in other Moroccan date palm cultivars.[8,9,11] The findings of the present investigation indicated that adventitious bud explants have a high embryogenic potential, with a rate of up to 89%. This is in good agreement with our previous studies. In fact, the somatic embryogenesis rates from these same explants reached 86 and 78% in cvs. Najda and Mejhoul, respectively.[8,11]

It has been found that auxin type and concentration have a significant effect on somatic embryogenesis induction and expression in date palm cv. Al-Fayda. The highest somatic embryogenesis rate was observed in explants cultured on MS medium supplemented with 225 µM 2,4-D for 6 months, followed by one month on PGR-free medium. The auxin 2,4-D has been used in many date palm cultivars for callus induction. For example, in cv. Medjool (100 mg l^{-1}), cvs. Barhi and Khalas (10-100 mg l^{-1}), and cvs. Deglet Nur and Takerbucht (2-100 mg l^{-1}).[17,18,19] In the present study, 225 µM 2,4-D (49.73 mg l^{-1}) gave the highest somatic embryogenesis frequency. Increasing 2,4-D concentration to 450 µM decreased somatic embryogenesis while picloram showed lower frequencies than that obtained with 225 µM 2,4-D. Very few studies reported the use of the auxin picloram in date palm, and when used, it showed different results depending on the genotype. For example, picloram induced somatic embryogenesis in cvs. Bream, Najda and Mejhoul,[8,11, 20] whereas it failed to induce it in cv. Boufeggous.[21] Based on

our findings, 225 µM 2,4-D is recommended for somatic embryogenesis induction from adventitious bud-derived explants of cv. Al-Fayda.

Somatic embryo maturation was performed on liquid media supplemented with various concentrations of mannitol, sorbitol or PEG. In previous works, liquid media were suggested to stimulate somatic embryo maturation. For example, in date palm cv. Najda, the average number of mature somatic embryo per 100 mg FW calli was 16.2 on semi-solid medium while it reached 106.4 on liquid medium.[9] Similar findings were obtained with cv. Deglet Nour in which 100 mg FW callus produced 10 mature embryos on solid medium, whereas on liquid medium, the same amount of callus generated up to 200 mature embryos.[22] These findings suggest that liquid medium is suitable for date palm embryo maturation. The stimulatory effect of liquid media on somatic embryo maturation might be due to the uptake of nutrients by explants, which was reported to be more effective in the liquid state of culture medium.[23] Regarding mannitol, sorbitol and PEG, their beneficial effects on somatic embryo maturation were previously reported in many plant species such as holm oak, mung bean and papaya.[24, 25, 26] Our findings indicated that incorporating PEG in culture medium at the concentration of 40 g l^{-1} gave the highest number of mature embryos. This is in good agreement with previous results on the Moroccan date palm cultivars Mejhoul and Najda, in which PEG improved somatic embryo maturation.[9,11] The non-permeating osmoticum PEG was added to culture medium to improve the maturation of somatic embryos in numerous crop species and was reported to increase storage proteins and lipids in mature somatic embryos.[27] This may explain its beneficial effect on somatic embryo maturation. Based on our results, liquid MS medium containing 40 g l^{-1} PEG is recommended for somatic embryo maturation in date palm cv. Al-Fayda.

The germination of somatic embryos and their development into complete plants is a fundamental step of the somatic embryogenesis process. In the present study, NAA, BAP and GA_3 were used to stimulate mature embryo germination. The auxin NAA was successfully used for somatic embryo germination in many plant species such as *Mondia whitei, Bambusa arundinacea* and *Cordyline australis.*[28, 29, 30] In date palm, NAA was used alone,[21] in combination with BAP,[11] or in combination with other PGRs[18] to induce somatic embryo germination. The findings of the present study showed that combining 5 µM NAA and 5 µM BAP results in the highest somatic embryo germination frequency (26%). However, this germination rate is still lower than that observed in many other date palm cultivars. For example, in cv. Najda, the highest germination rate was 68%[9] while in cv. Boufeggous, it was 83%.[21] In cvs. Barhi and Khalas, germination rates of somatic embryos were 95.45%, and 94.68%, respectively.[18] These results highlight the effect of genotype on somatic embryo germination. Therefore, more experiments should be carried out to improve somatic embryo germination in date palm cv. Al-Fayda. Regarding plant acclimatization, a survival rate of 77.02% was observed. This is in good agreement with results in literature since high survival frequencies have been reported in date palm plants derived from both somatic embryogenesis[9,11] and organogenesis.[31,32]

Conclusion

This is the first report describing regeneration through somatic embryogenesis in date palm cv. Al-Fayda, a genotype selected for its high fruit quality and resistance to bayoud. In sum, somatic embryogenesis was achieved from adventitious bud-derived explants after 6 months of culture on semi-solid MS medium containing 225 µM 2,4-D and 4.5 µM 2iP, followed by one month on PGR-free MS medium. Somatic embryo maturation was higher in liquid MS medium containing 40 g l^{-1} PEG, while embryo germination was achieved on semi-solid MS medium containing 5 µM NAA and 5 µM BAP. After transferring regenerants to *ex vitro* conditions, a survival rate of 77.02% was observed. The findings of the present work are valuable to rehabilitate palm groves destroyed by the bayoud disease.

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Conflict of Interest

The authors declare no conflict of interest.

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