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Full Length Research Paper

Date palm (*Phoenix dactylifera* L.) *in vitro* morphogenesis in response to growth regulators, sucrose and nitrogen

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Studies were conducted to test the effect of different growth regulators, sucrose and nitrogen on *Phoenix dactylifera* L. explants cultured on Eeuwen's basal medium. Naphthalene acetic acid (NAA) was very effective for callus induction. Addition of cytokinins (BAP and Kinetin) to NAA containing media did not enhance actual callus growth. Sucrose influenced callus production. Depending on the auxin concentration of media, callus production could be supported by sucrose within the range 15 - 105 g/l but the optimum sucrose concentration in the medium in all cases, as determined by size of callus was 30 g/l. NAA and sucrose tended to interact at relatively high levels of sucrose (45 – 90 g/l) to produce roots in culture. KNO_3 was essential as a source of nitrogen for callogenesis and optimum callus formation was observed at 50 mM (combined nitrogen).

Key words: *Phoenix dactylifera*, *in vitro* culture, phytohormone, sucrose.

INTRODUCTION

The date palm fruit is an important commodity of world trade with the leading producer countries being from North Africa, the Middle East and Asia. The single largest producer is Iran (18%), the largest exporter is United Arab Emirates (37%), while the largest importer is India (38%) (Botes and Zaid, 2002).

Date palm is an out-crossed, perennial monocotyledon which is very heterozygous. In addition, date palm is dioecious. Consequently, date palm is not usually propagated by seed for commercial planting but by offshoots. The limitations of that method however, are that the average sucker production per palm per lifetime is low, restricted mainly to the juvenile years and the suckers are difficult to root. Some genotypes do not produce suckers at all. For date palm production therefore, *in vitro* multiplication is very useful because it provides a means of overcoming difficulties of producing large numbers of relatively homogenous female date palm seedlings.

There have been previous reports of date palm micropropagation through somatic embryogenesis (Tisserat, 1979; Sharma et al., 1984; Daquin and Letouze, 1988; Letouze et al., 2000) as well as by organogenesis (Rhiss et al., 1979; Beauchesne, 1982). However, refinements to the protocols have continued to be made.

In vitro callus induction, growth and differentiation are controlled by the type and concentration of plant growth regulators added to the basal medium as well as the interaction between auxins and cytokinins in culture media, (Rao et al., 1973; George and Sherrington, 1984). Other workers have observed however, that some morphogenetic responses were not influenced by growth regulators only, but that growth regulators could interact with other media components such as sucrose and nitrogen (Welander, 1976; Wetherall and Dougall, 1976; Jeannin et al., 1995; Ahn et al., 1996). The mechanism of auxin and cytokinin mediated morphogenesis has been the subject of extensive studies using different tissues and organs of various plant species (Murashige, 1974; Paranjothy, 1984; Lioseau et al., 1995).

While some of these reports contain information on *in vitro* morphogenesis in date palm, there is only limited information targeted at determining the effects of auxins

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; **2-ip**, 6.γ.γ.dimethylallylamino purine; **NAA**, Naphthalene acetic acid.

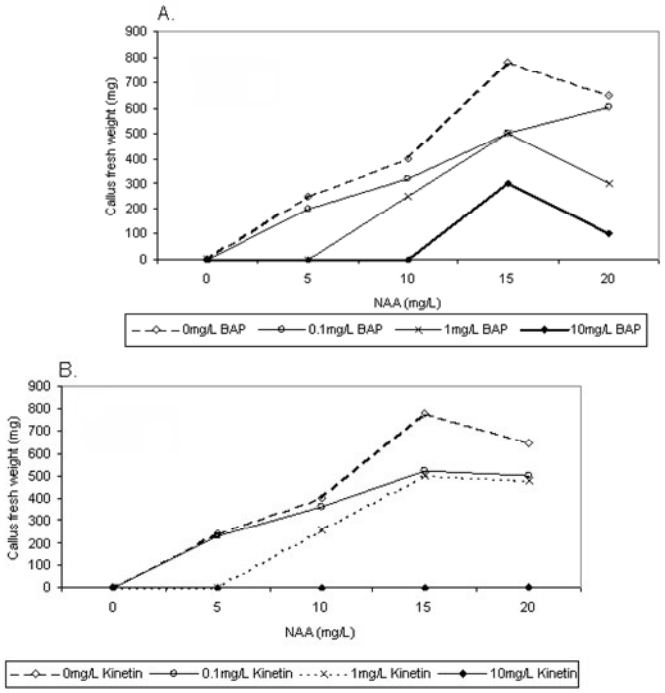

Figure 1. Effect of combinations of cytokinin (A) BAP and (B) kinetin) with auxin (NAA) on date palm leaf explant morphogenesis measured as mean fresh weight of callus produced.

and cytokinins with other media components on *P. dactylifera* in culture. This work was therefore aimed at investigating the effects of supplementing the basal medium with different growth regulators and testing different levels of other media components such as sucrose and nitrogen on *Phoenix dactylifera* leaf explants cultured *in vitro*.

MATERIALS AND METHODS

Culture conditions

The medium used for this study was that developed by Eeuwen (1976) while the explants used were young unopened leaves taken from the apical growing region. Sterilization was according to standard procedures involving commercial sodium hypochlorite solution. The pH of the media was adjusted to 5.7 and 0.8% agar was added to each and melted. The different media were dispensed into culture tubes, autoclaved and allowed to cool to ambient temperature. Sterilized date palm leaf explants were inoculated on the media and incubated in the dark at 27°C.

Phytohormone treatments

The basal medium was supplemented with hormones. Three auxins, NAA (naphthalene acetic acid), IAA (indole acetic acid), IBA (indole butyric acid) and two cytokinins BAP (benzyl-amino purine) and kinetin were used. The auxin concentrations were as follows: 0, 5, 10, 15 and 20 mg/L while the cytokinins were at 0, 0.1, 1.0 and 10 mg/L. For each auxin-cytokinin combination, all the possible combinations of the various concentrations of these two plant growth regulators, 20 treatments in all, were used.

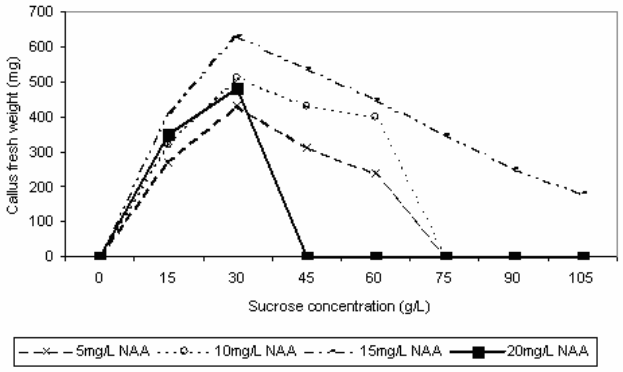

Figure 2. Effect of sucrose and NAA concentration on callus induction measured as mean weight (mg) of callus obtained per treatment.

Sucrose concentrations

Growth medium was supplemented with various concentrations of sucrose as energy source. The sucrose concentrations ranged from 0 to 100 mg/L. In a related experiment, these concentrations were also used in combination with NAA (5, 10, 15 and 20 mg/L). All combinations of the various concentrations of NAA and sucrose resulted in 32 treatments.

Combinations of different nitrogen sources

The Eeuwen's medium used was supplemented with two different sources of nitrogen: inorganic nitrogen as potassium nitrate (NO_3^-) and organic nitrogen as ammonium chloride (NH_4^+). The concentrations used varied between 0 and 100 mM.

RESULTS

Callus was initiated mainly in media with NAA alone or in combination with BAP or Kinetin. Callus initiation from *P. dactylifera* leaves was observed at various concentrations of NAA within the range 5 - 20 mg/L. Optimum concentrations of NAA for callus initiation was however observed to be 15 mg/L (Figure 1). The addition of cytokinin, BAP or Kinetin 0.1 - 10 mg/L to the culture containing different NAA levels did not stimulate further callus proliferation. The use of the hormones IAA and IBA, either alone or in combination with BAP or Kinetin was not effective in initiating callus on *P. dactylifera* leaf explants as no callus formation was observed.

Leaf explants grown on Eeuwen's medium at 27°C with different NAA concentrations (5 - 20 mg/L) and sucrose levels (15 - 105 g/L) initiated callus at all the NAA concentrations. At 5 mg/L NAA, callus was initiated on the leaf explant at sucrose concentrations of 15 - 60 g/L (Figure 2). Callus weight increased with increased levels of sucrose up to 30 g/L and decreased thereafter. No callus was observed at sucrose concentrations of between 75 and 105 g/L. The same pattern of callus initiation and growth was observed at 10 mg/L NAA at 27°C. At 15

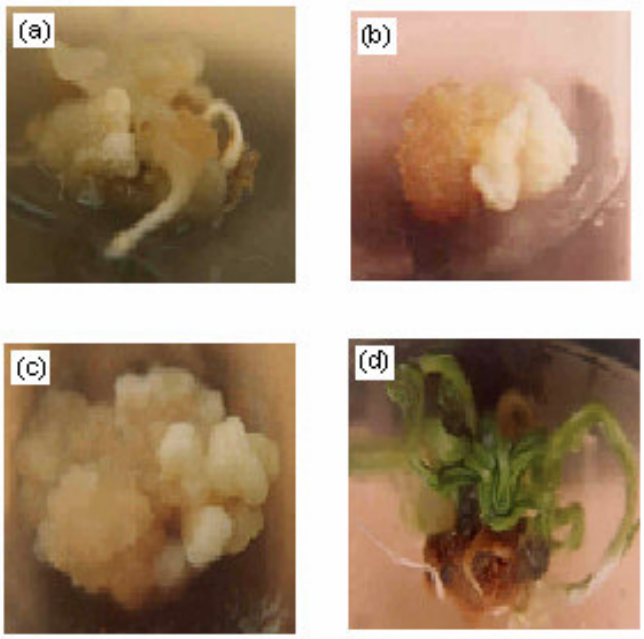

Figure 3. (a) Callus induced from date palm leaf explant, cultured on Eeuwen's medium containing 30 g/L sucrose and (b, c) initiation of somatic embryos and (d) mass of plantlets. Profuse rooting induced by increasing levels of NAA-sucrose combina-tions in culture medium.

mg/L NAA however, where optimal callus initiation and growth was at 30 g/L sucrose, there was callus at the other sucrose levels, 45 -105 g/L. In contrast, at 20 mg/L NAA, no callus was observed between 45 g/L and 105 g/L sucrose although there was callus initiation at 30 g/L sucrose.

Roots developed from leaf explants cultured at 27°C on Eeuwens' medium supplemented with NAA (5 - 15 mg/L) and sucrose (45 - 90 g/L). While a single or few roots were produced at low NAA concentrations (5 mg/L) and 90 g/L sucrose, profuse root development, (Figure 3), occurred at higher NAA combined with lower sucrose concentrations.

Leaf explants formed pneumathode-like structures at 5 - 20 mg/L NAA at different sucrose concentrations. At 5 mg/L NAA, these pseudo shoots were formed on the leaf explants at sucrose concentrations of between 60 and 90 g/L, while at 20 mg/L NAA, they were observed at 75 - 90 g/L sucrose. However, the highest number of these structures was observed at 10 - 15 mg/L NAA and 45 g/L sucrose.

In the experiment with different sources of nitrogen, callus was produced in media supplemented with potassium nitrate alone or in combination with ammonium chloride. Callus initiation and growth on media containing potassium nitrate was enhanced by the addition of ammonium chloride. Optimum callus growth was observed at 50 mM (combined) nitrogen (Table 1). While pneumathode-like structures were formed on media supplemented with NO_3^- in combination with NH_4^+, they were absent in media containing NH_4^+ alone as nitrogen source. In slightly prolonged culture, somatic embryo development was observed from leaf explants in media supplemented with NO_3^- and NH_4^+.

Table 1. Effect of varying concentrations of organic and inorganic nitrogen on callus initiation and growth measured as callus mean weight (mg).

KNO_3 (mM)	NH_4 (mM)			
	0	10	20	40
0	0	0	0	0
10	0	220±5.3	180±2.6	0
20	316±4.5	570±6.1	145±2.8	50±0.3
40	250±3.6	920±10.3	90±1.1	20±0
60	200±3.6	720±9.1	60±1.0	20±0
80	170±2.6	200±3.1	0	10±0

DISCUSSION

This study was aimed at testing the effect of supplementing basal Eeuwen's medium with growth regulators, sucrose and different nitrogen sources on the morphogenesis of *Phoenix dactylifera* leaf explants cultured *in vitro*. Callus initiation and growth were markedly stimulated by a relatively high auxin concentration, 15 mg/L NAA either alone or in combination with cytokinin (BAP or Kinetin). This relatively high NAA concentration has however proved equally successful for leaf explants of *Elaeis guineensis* (Odewale et al., 1996). The addition of cytokinin to the culture medium containing near optimal levels of NAA did not promote further growth. However, when compared on equimolar basis, BAP, in combination with NAA was less effective than kinetin with NAA. This agrees with the findings of Smith and Thomas (1973), Reuveni and Liciem-Kipnis (1974), who reported kinetin to be more effective than either BAP or zeatin in stimulating explant and callus growth of *Phoenix* or *Elaeis*.

P. dactylifera callus could be induced at the different sucrose concentrations used. Callus fresh weight increased with sucrose concentration up to 0.1 M and then declined. Eeuwen (1976) reported that fresh weight of explants from coconut and date palms cultured *in vitro* increased with sucrose concentration up to 0.2 M and then declined whereas dry weight continued to increase up to 0.4 M. However, the observed optimal sucrose concentration of 30 g/L was the same suggested by Tisserat (1982).

From the results, the requirement of both a high nitrate (NO_3^-) and a reduced form of nitrogen (NH_4^+) is demonstrated. It appears that NH_4^+ alone cannot serve as the sole nitrogen source for callogenesis for date palm leaf culture while the contrary is true for NO_3^- alone. These

differences could be attributed to an observation that NH_4^+ nitrogen can only serve as the sole source of nitrogen in a medium at a pH close to neutrality (Sheat et al., 1959). The lack of callus initiation and growth of *P. dactylifera* leaf tissue supplied with only (NH_4^+) nitrogen may thus have been as a result of the initial low pH of the medium and the possible drift during culture, towards even more acidic conditions. Street (1966) suggested that at low pH, (NH_4^+) nitrogen either interferes with the uptake of essential inorganic elements or alternatively, enhances the leakages of essential nitrogenous metabolites. Stimulation of callus initiation by the addition of NO_3^- to NH_4^+ containing media suggests that this addition either reduced the pH drift or checked the unfavorable effects of (NH_4^+) nitrogen under acidic conditions. It could also be that the presence of the two nitrogen sources in culture is essential for monocotyledonous species (Kaul and Sabharval, 1972), including the date palm (Reuveni and Liciem-Lipnis, 1974).

Optimal callus growth, which was observed at 50 mM nitrogen (40 mM NO_3^- and 10 mM NH_4^+ at 27°C contrasts with the nitrogen concentration of 30 mM (20 mM NO_3^- and 10 mM NH_4^+) or 60 mM (40 mM NO_3^- and 20 mM NH_4^+) proposed by Eeuwens (1976) and Murashige and Skoog (1962) media, respectively. Consequently, a modified medium can be proposed to include this 50 mM nitrogen, which will support callus initiation and growth of leaf explants of *P. dactylifera* very satisfactorily.

The role of NAA as an effective auxin in root formation has been established in many plants such as *Asparagus* (Harada, 1973), *Petunia* (Rao et al., 1973) and *Torenia* (Kamanda and Harada, 1979). Sangwan and Harada (1975) reported that NAA was essential in promoting profuse root formation from *in vitro* cultured explants of *Artirrhinum majus* L. In this study however, root formation was promoted by NAA-sucrose interactions. The fact that sucrose could be causative of root initiation has also been reported for other plants. Van Telgen et al., (1992) and Welander (1976) also reported that root formation was stimulated in sugar beet hypocotyls by increased sucrose when IAA was present in high concentrations (10 mg/L). This is consistent with results of the present work showing profuse root formation at sucrose: NAA ratios of 6:1 and 4:1. From the results of this study a medium can be proposed to incorporate high sucrose and moderate levels of NAA that will satisfactorily support the growth of roots in date palm plantlets cultured *in vitro*. The establishment of an effective root system from shoots grown *in vitro* is essential for subsequent success during acclimatization of plantlets to autotrophic conditions.

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