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Abstract

Variability in callus growth and somatic embryogenesis in response to silver nitrate ($AgNO_3$) among date palm (*Phoenix dactylifera* L.) genotypes was investigated. Callus was cultured on MS medium containing 53.7 μM NAA and 7.4 μM 2iP and supplemented with $AgNO_3$ at 0, 12.5, 25, 37.5, 50, 62.5, 75, 87.5, or 100 μM. Subsequently, somatic embryogenesis was induced by transferring callus to hormone-free MS medium containing corresponding concentrations of $AgNO_3$. Callus growth of cv. Barhee, Naboot Saif, Ruzaiz, and Hillali was significantly promoted in response to 37.5 μM $AgNO_3$ but optimum growth was obtained at 50 μM except for cv. Hillali the optimum was 62.5 μM. In contrast, cv. Khusab produced significant callus weight increase at 12.5 μM but maximum growth was obtained at 62.5 μM. Similarly, callus proliferation preceding somatic embryo formation during the regeneration stage as well as the frequency somatic embryogenesis and the number of resultant embryos varied significantly among cultivars and depended upon $AgNO_3$ concentration. Regeneration percentage was significantly enhanced in all genotypes except cv. Hillali was unaffected. Optimum $AgNO_3$ concentrations were 62.5, 50, 37.5 μM for cv. Barhee, Hillali, and Ruzaiz, whereas 12.5 μM was optimum for cv. Naboot Saif and Khusab. Significant increase in the number of resultant somatic embryos was observed in cv. Barhee, Naboot Saif, and Ruzaiz in response to 75, 12.5, and 37.5 μM but the optimum concentrations were 75, 87.5, and 75 μM $AgNO_3$, respectively. Embryo number in cv. Hillali and Khusab was unchanged or decreased.

The resultant embryos were tested for germination efficiency to form complete plantlets. To test the behavior of somatic embryos in date palm, embryos were cultured on hormone-free medium consisting of full- or half-strength Murashige and Skoog (MS) medium supplemented with 0, 0.2, 0.4, 0.6, 0.8, and 1 mg l^{-1} of naphthaleneacetic acid (NAA) or indole-3-butyric acid (IBA). Embryo germination responses were development of either complete plantlets, shoots only, or roots only. Results indicated a significant interaction between the experimental factors in relation to these responses. Addition of IBA to the culture medium generally induced higher percentages of complete plantlets as compared to NAA at any given concentration. The optimum treatment that maximized the percentage of complete plant formation, 86%, consisted of half-strength MS medium containing 0.2 to 0.4 mg l^{-1} IBA. Somatic embryos that formed only shoots ranged from 2% to 26%; the majority of which were associated with NAA-containing treatments. Generally, NAA enhanced the percentage of embryos that formed only roots, irrespective of medium strength; whereas, IBA was inhibitory particularly on half-strength medium. Regardless of the germination treatment, 80% of the plantlets survived in soil.

This study identified the optimum silver nitrate levels for in vitro culture of five important commercial date palm cultivars and demonstrated the possibility of reducing the length of tissue culture protocol by merging the germination (shoot formation) and rooting stages instead of the customary two-step procedures.

1. Introduction

Date palm (*Phoenix dactylifera* L.), 2n = 36, is a member of the monocot family Arecacea. It is a dioecious species bearing male and female flowers on separate plants. All parts of date palm tree can be utilized either directly as food or indirectly through industrial processing. The date fruit is a nutritious source of sugars, inorganic salts, and vitamins. Sugars comprise more than 70% of the fruit making it an excellent source of energy. The dates are also a good source of iron, potassium, calcium, magnesium, sulfur, copper, and phosphorous. Vitamins present in the date fruit include thiamine, riboflavin, biotin, niacin, folic acid, and ascorbic acid. In addition to its nutritional value, over 800 uses have been recorded including the manufacturing of compact wood, paper, and glue. The ripe dates are extensively used for extraction of date syrup, production of jams, ice cream, baby food, and soft drinks (Al-Bakr, 1972).

Cultivation of date palm is concentrated between latitudes 10° and 30° north of the Equator. It is widely cultivated in the arid regions of the Middle East and North Africa with the greatest production in Iraq, Saudi Arabia, Iran, Pakistan, and Egypt. The total number of date palms in the world is 88.5 million with production of 2.6 million metric tones. Saudi Arabia contains 8% of the world date palms with 15% of the total world production.

The date palm can be propagated sexually by seeds and asexually by offshoots. Propagation by seeds is used basically for breeding. Seed-derived female palms usually produce dates of inferior and heterogeneous quality, unsuitable for commercial marketing. These plants are usually characterized by a long juvenile phase as compared

to those derived form offshoots. Seed propagation cannot be used to clone desired cultivars, since cross-pollination in the date palm always results in new varieties of unknown characteristics (Omar et al., 1992).

Propagation by offshoots, axillary buds that grow from the trunk of the tree, is more widely used since they produce true-to-type trees. Offshoots are produced only in the early life of the date palm, the first 10 to 15 years, and the offshoots are produced at a very limited number although this number varies among cultivars. The offshoots must remain attached to the parent tree for 2 to 3 years until an adequate root system develops. The process of offshoot separation from the parent is often difficult, expensive, and a large number of the offshoots my not survive (Al-Bakr, 1972; Omar et al., 1992).

It is evident that a rapid clonal propagation protocols is necessary for date palm commercial expansion since asexual propagation by offshoot is a slow method. Tissue culture offers a promising means of propagation for date palms. In addition to its suitability for propagation, in vitro culture techniques offer means for the genetic improvement of date palms. These techniques include selection of somaclonal variants, protoplast fusion, and genetic transformation, the applicability of which rests upon the capability of plant regeneration from in vitro cultures.

Tissue culture is growing plant tissues in vitro on a defined culture medium under aseptic conditions and controlled environment. Plant tissues such as seed, leaf, bud, root, or flower parts can be used to initiate the cultures and are referred to as explant. The tissues are surface sterilized using ethanol and sodium hypochlorite and then cultured on a sterile culture medium dispensed in glass containers hence in vitro. The medium consists of macro- and microelements in addition to sucrose as a carbon source, vitamins,

plant growth regulators, and a solidifying agent such as agar. The appropriate type and concentration of growth regulators promote a specific response such as shoot multiplication, callus proliferation, or rooting. Tow pathways for plant regeneration are possible, somatic embryogenesis and organogenesis depending on the growth regulators and genotype. Both modes of regeneration were observed in various explants of date palms (Abo El-Nil et al., 1989; Al-Ghamdi, 1993; Ammar and Benbadis, 1982; Khan et al., 1982; Tisserat, 1991, 1982a; Omar et al. (1992); Zaid, 1989). Somatic embryogenesis has proved feasibility and agronomic acceptability justifying scale-up of micropropagation for commercial purpose (Smith and Aynsley, 1995). Somatic embryogenesis from shoot tip-derived callus viewed as the most appealing process for date palm regeneration (Sharma et al., 1984; Das et al., 1989; El-Hadrami and Baziz, 1995; Veramendi and Navarro, 1996, Tisserat, 1982; Al-Khayri, 2001).

Plant regeneration from date palm explants was first attained by Ammar and Benbadis (1977, 1982). The phenomenon of somatic embryogenesis in callus cultures of date palm was later reported by Reynolds and Murashige (1979) and by Tisserat (1979). Different type of explants have been used to achieve clonal propagation of date palms (Khan et al., 1982). Immature zygotic embryos (Reynolds and Murashige, 1979; Mater, 1983) and mature zygotic embryos (Reuveni, 1979; Zaid and Tisserat, 1984) have been used as a source of callus cultures. Shoot tip and lateral buds have been frequently used to initiate callus and subsequent asexual embryogenesis (Tisserat, 1982a,b; Sharma et al., 1986; Omar, 1988; Omar and Novak, 1990). Shoot tips and lateral buds have been also used for shoot bud proliferation via organogenesis (El-Hennawy and Wally, 1987; Tisserat, 1984). Omar (1988) induced callus from shoot tips and lateral buds cultured on

a medium containing 3 mg/L 2-iP, 100 mg/L 2,4-D and 3 g/L charcoal. Subsequent transfer of the callus to a hormone-free medium triggered somatic embryogenesis and the formation of complete plants. The development of adventitious buds directly and subsequent plant formation from cultured lateral buds and from shoot tips was observed (Tisserat, 1982a,b, 1984). Callus cultures was successfully initiated from highly differentiated tissues such as leaf segments excised from young seedlings and young offshoots (Eeuwens, 1978; Tisserat, 1979; Zaid, 1984). Only limited success with inflorescence explant has been obtained (Omar et al., 1992).

Despite various efforts, available systems are inefficient for certain cultivars and totally ineffective for others. Moreover, studies aimed at examining the effect of various tissue culture factors that may improve regeneration are relatively limited (Tisserat, 1982; Veramendi and Navarro, 1996). This, perhaps, is a consequence of the low efficiency and extremely lengthy period, up to a year, associated with date palm regeneration. Furthermore, the systems are not applicable to all cultivars, rather they are genotype specific and only applicable to certain genotypes. The recalcitrant nature of date palm to in vitro manipulation coupled by its long tissue culture procedures that may reach a full year, it is imperative to investigate tissue culture factors to enhance the regeneration frequency. Efficient regeneration system is required not only for mass propagation but also to facilitate genetic manipulation of date palm using biotechnological approaches, such as genetic engineering and selection of useful somaclonal variants. An important factor that have not been investigated in date palm is the role of ethylene inhibitors in the culture medium on callus proliferation and subsequent plant regeneration, which is the topic of the current proposal.

Considerable evidence have suggested that ethylene, a gaseous phytohormone accumulating in culture vessels, exerts influence on growth and differentiation of plant in vitro cultures depending upon genotype and culture stage (Biddington, 1992; Dimasi-Theriou et al., 1993; Kumar et al., 1998; Nissen 1994). This important in-vitro factor is unexplored in date palm. Ethylene is known to inhibit and sometimes stimulate in vitro growth and morphogenesis depending upon the species and culture stage (Kumar et al., 1998). Consequently, inhibitors of ethylene action, such as silver nitrate ($AgNO_3$), also induce variable in vitro response. Variability of responses to silver nitrate can even occur between genotypes within the same species. Diverse in vitro influence was reported for the ethylene antagonists silver nitrate ($AgNO_3$), a potent ethylene-action inhibitor. In some cases silver nitrate was shown to have adverse effect on shoot regeneration in *Saccharum* spp. hybrids (Taylor et al., 1994), callus production in *Rubus* spp. hybrids (Tsao and Reed, 2002), and somatic embryogenesis in *Coffea canephora* (Hatanaka et al., 1995). In other instances, however, silver nitrate was shown to exert a stimulatory effect on callus proliferation in *Oryza sativa* (Adkins et al., 1993), *Zea mays* (Songstad et al., 1991), and *Buchloe dactyloides* (Fei et al., 2000). Additionally, silver nitrate promoted shoot regeneration in *Vigna unguiculata* (Brar et al., 1999), *Capsicum annuum* (Hyde and Phillips, 1996), *Arachis hypogaea* (Pestana et al., 1999), *Raphanus sativus* (Pua et al., 1996), and *Manihot esculenta* (Zhang et al., 2001). Enhanced somatic embryogenesis was also observed in *Picea glauca* (Kong and Yeung, 1995) and *Daucus carota* (Roustan et al., 1990) in response to silver nitrate.

The current investigation was conducted to test the effect of adding silver nitrate at various concentrations starting with the callus stage and continuing throughout the

regeneration stage. Moreover, this study involved evaluating the genotypic response of five commercially important date palm cultivars since variability in response to silver nitrate can occur not only between species but also among genotypes within the same species (Songstad et al., 1991; Tsaso and Reed, 2002). The outcome of this study may help overcome the recalcitrant nature of date palm, a persistent limitation in date palm commercial propagation despite previous efforts aimed at improving its regeneration (Al-Khayri, 2001; Tisserat, 1982; Veramendi and Navarro, 1996).

A typical somatic embryogenesis protocol for date palm involves a series of consecutive stages beginning with callus induction, embryogenic callus multiplication, somatic embryo formation, somatic embryo germination (shoot formation from embryos), and finally rooting. The complexity of the system is magnified by the requirement for different hormonal compositions and lengthy incubation periods associated with each stage, which can reach 3 months. This lengthy incubation characteristic translates to periods reaching up to a year, or more in some cultivars, to obtain complete plantlets; in addition, another 6 to 12 months greenhouse nursery before transplanting to the field. Therefore, it is of paramount importance to evaluate the potential of reducing this period, especially to enhance the feasibility of commercial micropropagation of date palm since the cost of operational resources is directly related to the time consumed before plantlets are ready for consignment. In an effort to reduce the length of culturing and thus expediting the recovery of plantlets, this study was conducted to examine the potential of germinating somatic embryos directly on rooting medium. This would allow merging the lateral two stages of the culture system, shoot development and rooting.

Previous studies have shown that somatic embryo maturation, germination, in vitro rooting, and plant establishment can be influenced by various in vitro factors including solidifying agent, auxin concentration, and medium strength in tissue culture systems of numerous plant species (Huang et al., 1992; Klimaszewska and Smith, 1997; Kooi et al., 1999; Sha et al., 1999; Pretto and Santarem, 2000). Limited studies were encountered addressing factors affecting formation and germination of somatic embryos in relation to date palm. In this respect, studies have shown that somatic embryogenesis of date palm was stimulated by temporary sucrose starvation (Veramendi and Navarro, 1996), and by the augmenting the culture medium with silver nitrate (Al-Khayri and Al-Bahrany, 2001) and biotin (Al-Khayri, 2001).

The current study also involved characterization of the germination behavior of the resultant somatic embryos and plant establishment in date palm in response to medium salt strength and various concentrations of indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA).

2. Materials and Methods

2.1. Culture initiation and callus induction

Explant isolation and culture procedures were according to procedures previously described earlier (Al-Khayri, 2001; Al-Khayri and Al-Bahrany, 2001). Three-year-old offshoots of five commercially important date palm (*Phoenix dactylifera* L.) cultivars including Barhee, Hillali, Naboot Saif, Ruzaiz, and Khusab were separated from mother

trees and the outer leaves were removed to expose shoot tip regions. Excised tips were immediately placed in a chilled antioxidant solution consisting of ascorbic acid and citric acid, 150 mg/L each, to prevent browning. Shoot tips were surface sterilized in 70% ethanol for 1 min, followed by 15 min in 1.6% w/v sodium hypochlorite (30% v/v Clorox, commercial bleach) containing 3 drops of Tween 20 (Sigma Chem Co, St. Louis, MO) per 100 ml Clorox solution, and then rinsed with sterile distilled water four times. The explant tissue was replaced in a sterile chilled antioxidant solution during explant excision. The tissue surrounding the shoot tips was removed until the leaf primordia were exposed and detached at the base. Shoot tip terminal, about 1cm long, was sectioned longitudinally into four sections. Terminal tip sections and whole leaf primordia were individually cultured on initiation medium.

The basal medium consisted of MS salts (Murashige and Skoog, 1962) supplemented with (per L) 170 mg NaH_2PO_4, 125 mg *myo*-inositol, 200 mg glutamine, 2 mg glycine, 1 mg biotin, 1 mg thiamine-HCl, 1 mg nicotinic acid, 1 mg pyridoxine-HCl, 1 mg calcium pantothenate, 30 g sucrose, and 7 g agar (purified Agar-agar/Gum agar) (Sigma). Growth regulators and activated charcoal were added according to the culture phase Culture initiation medium contained 100 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) (452.5 μM), and mg L^{-1} 2-isopentenyladenine (2iP) (14.7 μM), and 1.5 g L^{-1} activated charcoal (acid-washed, neutralized) (Sigma). These cultures were maintained in darkness at 24 ± 3ºC for 12 wk during which they were transferred at a 3-wk interval. The entire expanding explants with resultant callus were transferred to callus proliferation medium that contained 10 mg L^{-1} naphthaleneacetic acid (NAA) (53.7 μM), 30 mg L^{-1} 2iP (147 μM), and 1.5 g L^{-1} activated charcoal. These cultures were

maintained for 3 wk at 24 ± 3ºC and a 16-h photoperiod (50 μmol·m^{-2}·s^{-1}) provided from cool-white fluorescent lamps. Subsequent culture stages were exposed to the same temperature and light regime. To proliferate embryogenic callus, the explants were transferred to a medium containing 10 mg L^{-1} NAA (53.7 μM), 6 mg L^{-1} 2iP (29.6 μM), and 1.5 g L^{-1} activated charcoal. These cultures were maintained for 9 wk during which they were transferred at a 3-wk interval. Callus was separated from original explants and multiplied on a medium containing 10 mg L^{-1} NAA (53.7 μM) and 1.5 mg L^{-1} 2iP (7.4 μM) for 16 wk to obtain sufficient callus proliferation to study the effect of $AgNO_3$.

All media were adjusted to pH 5.7 with 1 *N* KOH and dispensed in 25 x 150-mm tubes (125 ml per tube) capped with plastic closures for the first two passages of the initiation stage, and in 125-ml flasks (25 ml per flask) capped with aluminum foil for subsequent stages. The medium was autoclaved for 15 min at 121°C and $1x10^5$ Pa (1.1 kg cm^{-2}).

2.2. Influence of silver nitrate on callus growth

To examine the genotypic response of callus growth to $AgNO_3$, callus from multiplication cultures was transferred to a fresh callus multiplication medium augmented with $AgNO_3$ at 0, 2, 4, 6, 9, 11, 13, 15, and 17 mg L^{-1} (0, 12.5, 25, 37.5, 50, 62.5, 75, 87.5, and 100 μM). Each culture flask was inoculated with 0.25 g callus and incubated at the dark for 6 wk during which the cultures were transferred once 3 wk after inoculation. Growth determination in response to $AgNO_3$ was based on callus fresh weight obtained at the end of this incubation period.

2.3. Influence of silver nitrate on somatic embryogenesis

To evaluate the regeneration capacity of the callus, resultant callus growth was transferred to a hormone-free medium to encourage embryo development. Regeneration medium was augmented with the corresponding concentration of $AgNO_3$ identical to that added to the callus medium. The cultures were maintained at 24 ºC ± 3 ºC and a 16-h photoperiod of cool-white fluorescent light (50 μmol m^{-2} s^{-1}). After 6 wk of culturing and prior to embryo formation, the calli were weighed to determine the effect of $AgNO_3$ treatments on callus proliferation on the hormone-free regeneration medium. After additional 14 wks, the resultant embryos were counted.

2.4. Embryo germination and plant development

Embryos, 1 to 2 cm long, were collected from the hormone-free regeneration 3-month-old cultures and inoculated vertically on the germination treatments. To test the effect of inorganic salts and auxins concentration the germination media consisted of either full- or half-strength MS salts supplemented with 0, 0.2, 0.4, 0.6, 0.8, and 1 mg l^{-1} of either NAA (0, 1.07, 2.15, 3.22, 4.28, 5.37 μM) or indole-3-butyric acid (IBA) (0, 0.98, 1.97, 2.96, 3. 94, 4.93 μM). Other additives added to the germination medium were identical to those used through out but solidified with 2 g l^{-1} phytagel (Sigma) and contained no activated charcoal.

2.5. Acclimatization and plant establishment

For acclimatization, the plantlets were removed from the tubes, the agar was carefully washed from the roots, placed in a beaker containing enough water to keep the roots submerged, and covered with plastic bags to maintain humidity. After 5 d, the covering was removed and the plantlets were kept in water for another 3 d, after which they were treated with 500 mg l^{-1} Benlate fungicide and planted in 5-cm plastic pots containing potting mix (1 soil: 1 peat moss: 1 vermiculite). The plantlets were watered with 100 mg l^{-1} N-P-K fertilizer (20-20-20) weekly and kept in the culture room for 8 weeks after which they were transferred to a greenhouse for further growth.

2.6. Experimental design

The influence of silver nitrate was evaluated in an experiment that was setup as a 5 x 9 factorial design with cultivar and $AgNO_3$ concentration as the main factors. Each $AgNO_3$ treatment consisted of 10 culture flasks per cultivar. Data obtained included callus weight during callus growth phase, callus weight during regeneration phase, percentage of regeneration, and embryo number. Data were subjected to analysis of variance (ANOVA) and the means were separated, where appropriate, using the least significant difference (LSD) at 5% significance. Observations were confirmed by repeating the experiment twice.

Germination of somatic embryos were tested in an experiment that was setup as a 2 x 2 x 6 factorial design with salt concentration, auxin type, and auxin concentrations

are the main factors. Each germination treatment consisted of 50 embryos cultured at 10 embryos per flask. The responses assessed 12 weeks later included number of embryos that formed complete plantlets, number of embryos that formed shoots only, and number of embryos that formed roots only. Data were subjected to analysis of variance (ANOVA) and the means were separated, where appropriate, using the least significant difference (LSD) at 5% significance.

3. Results and Discussion

3.1. Effect of silver nitrate on callus growth during callus stage

Variable influence of $AgNO_3$ on callus proliferation has been demonstrated in a number of species (Adkins et al., 1993; Fei et al., 2000; Songstad et al., 1991; Tsao and Reed, 2002). Such information in relation to date palm was reveled in the current study that has shown a stimulatory effect of $AgNO_3$ on callus growth. The degree of stimulation differed among genotypes and was dependent upon the concentration of $AgNO_3$ as revealed by a two-way interaction in the analysis of variance (Table 1). Callus growth , expressed in fresh weight, of cv. Barhee, Naboot Saif, and Ruzaiz was significantly promoted in response to adding as low as 37.5 μM $AgNO_3$ to the callus medium (Fig. 1 A, C, D). Further increase of $AgNO_3$ stimulated gradual increase in callus growth, reaching maxima at 50 μM. At higher $AgNO_3$ concentrations, however, these cultivars behaved differently. Callus of cv. Barhee showed significant reduction in growth in response to 62.5 μM, with no significant affect at higher concentrations. In comparison,

Table 2. Analysis of variance for callus weight during callus phase, callus weight during regeneration phase, and embryo number in response to the addition of silver nitrate to callus phase, regeneration phase, neither phase, or both phases.

	Callus weight during callus phase			Callus weight during regeneration phase			Regeneration %			Embryo numb	
Source	df	MS	*p*-value	df	MS	*p*-value	df	MS	*p*-value	df	MS
Cultivar (CV)	1	0.084	0.0011	1	10.789	0.0001	1	2756.250	0.0005	1	15.763
$AgNO_3$ in callus stage (Ag callus)	1	0.052	0.0096	1	1.197	0.0001	1	2256.250	0.0010	1	897.117
CV x Ag callus	1	0.001	0.8043	1	0.040	0.1351	1	56.250	0.4458	1	3.359
$AgNO_3$ in regeneration stage (Ag regeneration)	NA	NA	NA	1	0.267	0.0001	1	6.250	0.7960	1	2.433
CV x Ag regeneration	NA	NA	NA	1	0.239	0.0001	1	56.250	0.4458	1	0.067
Ag callus x Ag regeneration	NA	NA	NA	1	0.030	0.1915	1	156.250	0.2182	1	1581.473
CV x Ag callus x Ag regeneration	NA	NA	NA	1	0.079	0.0353	1	56.250	0.4458	1	150.320
Error	76	0.007		152	0.018		8	87.500		112	54.899

P-values less than 0.05 are significant; NA indicates non-applicable.

Fig. 1. Effect of silver nitrate concentrations on callus weight during callus growth stage in date palm genotypes.

cv. Naboot Saif showed no further change in response to higher $AgNO_3$ concentration. Whereas, cv. Ruzaiz showed significant reduction at 75 µM, with no significant affect on callus growth beyond this level. Similarly, the minimum $AgNO_3$ concentration that stimulated callus growth in cv. Hillali was 37.5 µM and the optimum was 62.5 µM but not significantly different from 50 µM (Fig. 1 B). Further increase in $AgNO_3$ concentration resulted in reduction of callus weight but the difference was non-significant. In contrast to the other genotypes tested, cv. Khusab showed significant callus growth increase at lower $AgNO_3$ concentration, 12.5 µM (Fig. 1 E). Further increase of $AgNO_3$ caused no significant change in callus weight of cv. Khusab up to 62.5 µM, the level at which maximum callus growth was obtained. At higher $AgNO_3$ concentrations, significant reduction in callus weight was observed.

Overall, date palm callus growth gradually increased as the $AgNO_3$ concentration increased, however, the optimum concentration depended upon the cultivar and ranged from 50 to 62.5 µM. Similarly, Adkins et al. (1993) found that increasing $AgNO_3$ concentration caused a gradual increase in callus growth of *Oryza sativa* reaching a maximum at 50 µM beyond which reduction in callus growth when callus was grown in the light. However, when incubated in the dark a gradual reduction occurred in callus growth in response to increasing $AgNO_3$ concentrations from 10 to 100 µM. Conversely, silver nitrate significantly reduced callus growth in *Rubus* spp. at 59 µM (Tsoa and Reed, 2002) and in *Saccharum* spp. at 29 to 118 µM (Taylor et al., 1994).

3.2. Effect of silver nitrate on callus growth during regeneration stage

Transferring embryogenic callus to a hormone-free regeneration medium is a customary procedure used to encourage somatic embryogenesis in date palm. This step is usually associated with initial proliferation of callus followed by redifferentiation into somatic embryos, which occurs 12 to 16 wk after callus transfer to regeneration medium. Similar to callus growth during the callus stage, callus proliferation during regeneration stage was also influenced by the concentration of $AgNO_3$. This finding was previously observed in date palm cv. Barhee (Al-Khayri and Al-Bahrany, 2001). The current study, refined the optimum concentration by increasing $AgNO_3$ at smaller increments than previously tested (0, 25, 50, 75, or 100 μM $AgNO_3$). Perhaps more importantly, several date palm cultivars were involved in the present investigation. Similar to the growth response during callus stage, data revealed that $AgNO_3$ influence on callus growth during regeneration stage varied significantly among cultivars and depended upon the concentration as indicated by a two-way interaction in the analysis of variance (Table 1).

The minimum concentration of $AgNO_3$ that induced significant callus weight increase in cv. Barhee was 37.5 μM (Fig. 2, A). Further increase of $AgNO_3$ concentrations to 50 and 62.5 μM caused gradual increase in callus weight reaching maximum callus growth at 75 μM beyond which a significant reduction was observed. In comparison, cv. Hillali and Ruzaiz required a minimum of 50 μM $AgNO_3$ to stimulate significant increase in callus weight (Fig. 2 B, D). This $AgNO_3$ concentration coincided with the maximum callus growth in cv. Hillali; whereas, in cv. Ruzaiz the optimum concentration was 62.5 μM. In both cultivars, higher $AgNO_3$ concentrations caused gradual reductions in callus growth with a significant reduction at 75 to 100 μM.

Fig. 2. Effect of silver nitrate concentrations on callus weight during regeneration stage in date palm genotypes.

The lowest concentration of $AgNO_3$ tested, 12.5 μM, was sufficient to induce a significant increase in callus growth in cv. Naboot Saif and Khusab in comparison to their respective controls (Fig. 2 C, E). This concentration coincided with the maximum callus growth in cv. Khusab; whereas, in cv. Naboot Saif the optimum concentration was 50 μM. In both cultivars, higher $AgNO_3$ concentrations up to 87.5 μM caused no further change in callus growth; however, at 100 μM callus growth significantly diminished.

3.3. Effect of silver nitrate on percentage of somatic embryogenesis

Although considerable information on the effect of $AgNO_3$ on organogenesis of various species has been encountered, comparatively limited studies involved somatic embryogenesis (Al-Khayri and Al-Bahrany, 2001; Hatanaka et al., 1995; Kong and Yeung, 1995; Roustan et al., 1990; Fuentes et al., 2000; Dias and Martins, 1999; Biddington et al., 1988). These studies indicated that the action of silver nitrate on somatic embryogenesis was species dependent but they often lacked data on the effect of $AgNO_3$ on the percentage of regeneration. The current study has shed light on the effect on the percentage of regeneration and demonstrated that the response variability is not only species specific but also occurs among various genotypes within a species. Furthermore, this study has shown that the addition of $AgNO_3$ at a certain concentration modified regenerability of various date palm genotypes differently as indicated by a significant two-way interaction revealed by analysis of variance (Table 1). The optimum $AgNO_3$ concentration for regeneration enhancement in cv. Barhee was 62.5 μM, resulting

Fig. 3. Effect of silver nitrate concentrations on percentage of somatic embryogenesis in date palm genotypes.

in 2.4 folds as compared to the control (Fig. 3 A). Somatic embryogenesis percentage in cv. Hillali and Ruzaiz (Fig. 3 B, D) was least modified by the addition of silver nitrate. Although, 50 µM $AgNO_3$ slightly increased the regeneration percentage in cv. Hillali, the affect was insignificant. In cv. Ruzaiz, the only concentration that elicited response was 37.5 µM giving 3 times the regeneration percentage as non-treated callus. In cv. Naboot Saif and Khusab, as low as 12.5 µM $AgNO_3$ stimulated significant improvement in the percentage of somatic embryogenesis giving 1.3 folds as compared to the control (Fig. 3 C, E). Increasing $AgNO_3$ concentration to 37.5 µM in cv. Naboot Saif, and up to 75 µM in cv. Khusab, unmodified the regeneration response; however, at higher concentrations, a significant decline in the regeneration percentage was observed.

3.4. Effect of silver nitrate on number of somatic embryos

In order of embryo profusion of the non-treated controls cv. Naboot Saif (33 embryos) ranked first, followed by Khusab (20 embryos), cv. Barhee (12 embryos), cv. Hillali (7 embryos), and Ruzaiz (5 embryos); this sequence parallels that associated with the regeneration percentages. These numbers were modified by the addition of silver nitrate as has been previously shown with cv. Barhee, where silver nitrate was added to the regeneration medium only (Al-Khayri and Al-Bahrany, 2001). The highest number of embryos obtained in that study was 6 embryos per 0.25 g callus inoculum on regeneration medium containing 75 µM silver nitrate. Similarly, the current study has shown that 75 µM $AgNO_3$ was optimum, but the resultant embryo number increased to

25 embryos per the same amount of initial callus inoculum. This increase may be attributed to the presence of silver nitrate throughout the culture where previously silver nitrate addition was confined to the regeneration medium. Silver nitrate at 75 μM appeared to be the optimum for somatic embryogenesis in cv. Barhee, since changing silver nitrate concentration at smaller-increments than previously tested again gave optimum results at this level.

The current study has revealed that the optimum concentration varies according to genotype as indicated by a significant two-way interaction between genotype and $AgNO_3$ concentration in the analysis of variance (Table 1). In response to silver nitrate, modifications involved either inhibition, no affect, or stimulation of embryo number (Fig. 4 A-E). In cv. Hillali, embryo number was inversely related to the concentration of sliver nitrate; however, this inhibition was insignificant as compared to the control (Fig. 4 B). Embryo number of cv. Khusab was unaffected, as compared to the control, by the addition of $AgNO_3$ up to 50 μM but at higher concentrations, significant reductions were observed (Fig. 4 E). The other genotypes tested exhibited increased embryo number in response to silver nitrate. The minimum silver nitrate concentrations that induced significant improvement in embryo number of cv. Barhee, Naboot Saif, and Ruzaiz were 75, 12.5, 37.5 μM, respectively while the optimum concentrations were 75, 87.5, and 75 μM, respectively (Fig. 4 A, C, D). At concentrations higher than the optima, significant reduction in embryo number was observed in both cv. Naboot Saif and Ruzaiz, whereas cv. Barhee was unchanged.

Although, several studies have shown that silver nitrate influence may vary among species, the current study has demonstrated differences in response and optimum

concentrations among genotypes within the same species. In *Z. mays*, Songstad et al. (1988) observed a 12-fold increase in plant regeneration from callus treated with 100 μM silver nitrate. In *D. carota*, Roustan et al. (1990) have shown that silver nitrate induced doubling of number of somatic embryos in response to 10 μM. On the contrary, working with *D. carota* Nissen (1994) found that 10 μM $AgNO_3$ completely inhibited somatic embryogenesis with no indication of stimulation within the range tested (0 to 20 uM). Somatic embryogenesis in *C. canephora* has been shown to be completely inhibited at concentrations as low as 5 μM silver nitrate (Hatanaka et al., 1995).

To summarize, genotypic-dependent variability in date palm to the prescribed in vitro protocol was demonstrated. Variations were observed in callus proliferation and regeneration efficiency. This variation was also expressed in the presence of silver nitrate, which either stimulated or inhibited growth and morphogenesis depending upon the concentration and genotype. It was evident that a concentration optimal for callus growth may not coincide with that best for somatic embryogenesis. The information obtained in this study provides an insight for the improvement of date palm micropropagation. Exploration of the effect of other ethylene antagonists may be worthy of future investigation.

3.5. In vitro germination of somatic embryos

The final tissue culture developmental stage leading to the formation of complete plantlets involves rooting of in-vitro-derived shoots in case of organogenesis, or germination of embryos in somatic embryogenesis pathway. Commonly, embryogenesis

Fig. 4. Effect of silver nitrate concentrations on number of somatic embryos in date palm genotypes.

and germination leading to plant establishment in monocotyledonous species is accomplished on hormone-free medium, where both shoots and roots develop simultaneously (Swati et al., 2001). In date palm, however, embryos cultured on a hormone-free medium often produce shoots only and require another step for rooting and shoot elongation, usually on a medium enriched with NAA (Das et al., 1999; Tisserat, 1994; Omar and Novak, 1990). In the current study, embryos cultured on germination media responded by forming either complete plantlets, shoots only, or roots only. Germination commenced within 4 weeks but the cultures were maintained for additional 8 weeks to maximize the number of responding embryos.

The percentage of embryos that formed complete plantlets ranged from 12% to 86% depending upon the treatment. When the medium was devoid of growth regulators, the strength of MS salt had no significant effect on the germination of date palm somatic embryos into complete plants, since full strength and half strength produced 48% and 43%, respectively. The addition of auxins modified this effect causing a significant interaction between MS salt strength, auxin type, and auxin concentration in relation to the development of complete plants (Table 2). The interaction between the experimental factors was expressed in the different response pattern exhibited by the embryos in relation to changing auxin concentration that was dependent upon the MS salt strength and auxin type. In general, the addition of IBA to the culture medium resulted in higher percentages of complete plantlets as compared to NAA; whereas, half-strength MS salt was superior to full-strength medium (Fig. 5). When full-strength MS medium was used, the percentage of embryos that formed complete plantlets decreased as the concentration of NAA increased (Fig. 5 A). At low NAA concentrations, 0.2 mg l^{-1}, germination

Table 2. Analysis of variance for the effect of MS medium strength, auxin type (IBA and NAA), and auxin concentrations on the germination responses of date palm somatic embryos.

Factor	df	Embryos formed plantlets		Embryos formed shoots only		Embryos formed roots only	
		MS	P-value	MS	P-value	MS	P-value
MS medium strength	1	300.83	0.0047	187.50	0.0056	13.33	0.5479
Auxin type	1	37807.50	0.0001	4440.83	0.0001	16333.33	0.0001
Auxin concentration	5	461.50	0.0001	388.83	0.0001	495.33	0.0001
MS strength x Auxin type	1	5200.83	0.0001	440.83	0.0001	2613.33	0.0001
MS strength x Auxin conc	5	150.83	0.0017	71.50	0.0131	271.33	0.0001
Auxin type x Auxin conc	5	2037.50	0.0001	488.83	0.0001	1323.33	0.0001
MS x Auxin type x Auxin conc	5	666.83	0.0001	120.83	0.0003	883.33	0.0001
Error	96	35.83	-	23.33	-	36.67	-

P-Values less than 0.05 are significant.

inhibition was not significant; however, as the concentration was increased to 0.4 mg l^{-1}, a significant reduction in the number of embryos that formed complete plants was observed. Further increase of NAA to 0.6 mg l^{-1} caused no further reduction, but at 0.8 mg l^{-1} NAA an additional significant decrease occurred then leveled off at 1 mg l^{-1}. These observations suggest that NAA was inhibitory to the germination processes of date palm somatic embryos at the levels tested. It may be necessary to test NAA concentrations lower than 0.2 mg l^{-1} to reveal the influence of low levels of NAA on somatic embryo germination. Germination inhibition associated with NAA was also observed with half-strength MS medium was used (Fig. 5 B), where the percentages of somatic embryos that formed complete plants on all NAA-containing treatments were significantly reduced as compared to the hormone-free control. In fact, these treatments resulted in the lowest percentage of complete plant formation, 14%. When half-strength MS was used, increasing NAA concentration caused no further reduction on the percentage of plant formation.

In contrast, stimulation of embryos to produced complete plants was observed in response to increasing IBA concentration when full-strength MS salt was used (Fig. 5 C). This increase, however, showed significant differences in comparison to the control, only when the concentration of IBA reached 0.8 mg l^{-1}. Further increase of IBA to 1 mg l^{-1} caused no significant change in the percentage of complete plant formation. The best results were obtained when half-strength MS medium was augmented with IBA (Fig. 5 D). As compared to the hormone-free control, the addition of 0.2 mg l^{-1} IBA resulted in a significant increase in the formation of whole plants. Further increases in the concentration of IBA to 0.4 slightly increased the percentage of complete plant formation

Fig. 5. Effect of MS salt, NAA, and IBA concentrations on the percentage of date palm embryos that formed complete plants. ***A*** Full strength MS plus NAA; ***B*** Half strength MS plus NAA; ***C*** Full strength MS plus IBA; ***D*** Half strength MS plus IBA.

reaching a maximum of 86%. Although, beyond this IBA concentration, a reduction in the percentage of complete plant formation was observed; nevertheless, the percentages of embryos that formed complete plants remained higher than the hormone-free control. Data on the germination percentage of date palm somatic embryos are unavailable in most previous literature. Nevertheless, Tisserat (1982) obtained germination rate of 5 to 15%, and Veramendi and Navarro (1996) reported germination rate of 10%. As compared to previous rates, the current study clearly demonstrated a significant improvement in the germination of date palm somatic embryos.

A proportion of somatic embryos failed to germinate into complete plantlets. Instead, they formed either shoots or roots. According to the analysis of variance, these two responses were significantly influenced by an interaction between MS salt strength, auxin type, and auxin concentration (Table 2). Somatic embryos that formed only shoots without root systems ranged from 2% to 26% of the total embryos cultured (Fig. 6). These shoots can be routinely rooted by subsequent transfer to a medium supplemented with 0.2 mg l^{-1} NAA (AL-Khayri 2001; Tisserat, 1984). The majority of the embryos forming shoots were more associated with NAA-containing treatments (Fig. 6 A, B) as compared to IBA-containing treatments (Fig. 6 C, D). In comparison to the NAA-free control, including 0.2 to 0.8 mg l^{-1} NAA in the medium slightly stimulated the embryos to form shoots but at 1 mg l^{-1} a significant increase in shoot formation was observed when full-strength MS was used (Fig. 6 A). Using half-strength MS, NAA significantly increased the percentage of embryos that formed shoots in response to 0.2 mg l^{-1}, with no further increases in response to higher NAA concentrations (Fig. 6 B). Results have shown that at any given NAA concentration, except 1 mg l^{-1}, half-strength MS (Fig. 6 B)

Fig. 6. Effect of MS salt, NAA, and IBA concentrations on the percentage of date palm embryos that formed shoots only. ***A*** Full strength MS plus NAA; ***B*** Half

strength MS plus NAA; ***C*** Full strength MS plus IBA; ***D*** Half strength MS plus IBA.

stimulated more embryos to develop shoots as compared to full-strength medium (Fig. 6 A). In contrast, IBA had significant effect on shoot formation only at 0.8 and 1 mg l^{-1} when added to half-strength MS medium (Fig. 6 C, D).

The remaining somatic embryos that failed to form complete plantlets or shoots developed roots only. These ranged from 12 to 70 %, depending upon the hormonal supplement and MS salt strength. Generally, NAA enhanced root formation irrespective of medium strength (Fig. 7 A, B). In contrast, IBA reduced the percentage of embryos that formed roots particularly on half-strength medium (Fig. 7 C, D). Since no shoot growth developed from these embryos, they were considered of no use.

Embryos that germinated into complete plants exhibited well-developed shoot and root systems. They required 2 to 3 months to reach 8 to 10 cm, a suitable size for transfer to soil. Acclimatization conditions yielded nearly 80% survivable rate irrespective of the germination treatment. The plants grew normally in soil and appeared to exhibit normal phenotype.

To summarize, this experiment demonstrated the possibility of reducing the length of the date palm tissue culture protocols by merging the germination and rooting stages. This eliminates the need to transfer shoots obtained from somatic embryos to rooting medium. The optimum treatment suitable to produce maximum number of complete plants consisted of half-strength MS salt supplemented with 0.2 to 0.4 mg l^{-1} IBA. Examining the effectiveness of this procedure in other date palm genotypes is worthy of future research. Moreover, although this study involved two auxins, the effect

of other auxins such as IAA on the germination of date palm somatic embryos may provide additional pertinent information to enhance date palm micropropagation.

Fig. 7. Effect of MS salt, NAA, and IBA concentrations on the percentage of date palm

embryos that formed roots only. ***A*** Full strength MS plus NAA; ***B*** Half strength MS plus NAA; ***C*** Full strength MS plus IBA; ***D*** Half strength MS plus IBA.

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5. Finance Summary

The project required the purchase of certain supplies as outlined in the proposal. The following table lists the materials purchased, the amount proposed and the amount actually spent. These materials have been purchased during the first phase of the project as dated. Partial salary for research assistance (12 months) was provided. The remaining amount is needed for research assistance and researchers rewards.

Description	**Reference**	**Date**	**Proposed Amount, SR**	**Actual Amount Provided, SR**
Equipments	265/13/A	7/3/1422	20,000	19,800
Materials	342/13/A	5/4/1422	18,000	17,989
Computer	228/13/A	25/2/1422	7,000	7,000
Miscellaneous	278/13/A	13/3/1422	11,000	10,000
Research assistants salary		25/9/2001 25/3/2002	6000 7200 10800	6000 7200 0
Researcher rewards			52800	0
Publications			3000	0
Total			**135,800**	**67,989**