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In vitro germination of somatic embryos in date palm: Effect of auxin concentration and strength of MS salts

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To characterize the germination behaviour of somatic embryos in date palm (*Phoenix dactylifera* L.), embryos derived from callus cultured on hormone-free medium were inoculated on 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} naphthaleneacetic acid (NAA) or indole-3-butyric acid (IBA). The embryos either developed complete plantlets, or only shoots or roots. The results indicate a significant interaction between the experimental factors and response. Addition of IBA to the culture medium generally induced higher percentages of complete plantlets 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% and 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 MS medium. Regardless of the germination treatment, 80% of plantlets (192 plants) survived in soil. This study demonstrates the possibility of reducing the length of tissue culture protocols for date palm by merging the germination (shoot formation) and rooting to a one-step procedure.

DATE palm (*Phoenix dactylifera* L.), an economically important commodity, is a monocotyledonous tree widely cultivated in arid regions of the Middle East and North Africa. *In vitro* micropropagation is increasingly becoming an attractive alternative for large-scale propagation of date palm. *In vitro* plant regeneration of date palm occurs through organogenesis and somatic embryogenesis depending on genotype and hormonal manipulations. Explants, including zygotic embryos, shoot tips and lateral buds appear to be most responsive for *in vitro* culture of date palm[1]. Somatic embryogenesis from shoot tip-derived callus has been viewed as the most appealing process for date palm regeneration[2–7]. This method has proved feasibility and agronomic acceptability justifying scale-up of micropropagation for commercial purposes[8]. 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 be three months. The total incubation translates to periods reaching up to a year or more in some cultivars, to obtain complete plantlets. In addition, another six to 12 months are required in greenhouse nursery before transplanting to the field. Therefore, it is of paramount importance to evaluate the potential of reducing this period so as to enhance the feasibility of commercial micropropagation. This study therefore was conducted to examine the potential of germinating somatic embryos directly on rooting medium. This would allow merging the lateral two stages of culture system, shoot development and rooting.

Previous studies have shown that maturation of somatic embryo, 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[9–13]. Limited studies, however, addressed factors affecting the formation and germination of somatic embryos in date palm such as temporary sucrose starvation[5] and augmenting the culture medium with silver nitrate[14] or biotin[7]. The current study characterized the germination behaviour of date palm somatic embryos and plant establishment in response to salt strength of the medium and various concentrations of indole-3-butyric acid (IBA) and naphthaleneacetic acid (NAA).

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Shoot-tip explant preparation, callus initiation and somatic embryogenesis were conducted according to previously described procedures[7,14]. Callus maintained on a medium containing 10 mg l^{-1} NAA (53.7 μM) and 1.5 mg l^{-1} 2iP (7.4 μM) was induced to form somatic embryos by culturing on a hormone-free medium. The medium consisted of Murashige and Skoog (MS)[15] salts supplemented with (in mg l^{-1}) 170 NaH_2PO_4, 125 *myo*-inositol, 200 glutamine, 100 ascorbic acid, 100 citric acid, 1 thiamine-HCl, 1 nicotinic acid, 1 pyridoxine-HCl, 2 glycine, 1 calcium pentothenate, 1 biotin, and 30 g l^{-1} sucrose. The medium was solidified with 30 g l^{-1} agar (purified agar-agar/gum agar; Sigma), adjusted to pH 5.7 with 1 N KOH, dispensed in 125 ml flasks (25 ml per flask), capped with aluminum foil and autoclaved for 15 min at 121°C and 1×10^5 Pa (1.1 kg cm^{-2}). The cultures were incubated at 24 ± 3°C in 16-h photoperiods (50 μmol m^{-2} s^{-1}) and transferred to a fresh medium every four weeks for 12 weeks.

The resultant embryos, 1 to 2 cm long, were collected from regeneration cultures and inoculated vertically on the germination treatments. 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 IBA (0, 0.98, 1.97, 2.96, 3. 94, 4.93 μM). Other additives added to the germination medium were identical to those described above, but solidified with 2 g l^{-1} phytagel (Sigma).

The experiment was set up as a $2 \times 2 \times 6$ factorial design with salt concentration, auxin type and auxin concentration as main factors. Each germination treatment consisted of 50 embryos (10 embryos per flask). The response was assessed 12 weeks later, including number of embryos that formed complete plantlets, those that formed only shoots and those with only roots. Data were subjected to analysis of variance (ANOVA) and the means were separated, where appropriate, using the least significant difference (LSD) at 5% significance. Transformation of the proportion data was not necessary. Observations were confirmed by repeating the experiment twice.

Whole plantlets were transferred to soil to detect any correlation between *in vitro* germination treatments and survivability *ex vitro*. 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 five days, the covering was removed and after another three days, 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 weekly with 100 mg l^{-1} N–P–K fertilizer (20–20–20) and kept in the culture room for eight weeks after which they were transferred to a greenhouse. The number of plants survived in relation to the germination treatments was noted.

Somatic embryogenesis and germination leading to plant establishment in monocotyledonous species is usually accomplished on hormone-free medium, where both shoots and roots develop simultaneously[16]. 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[3,17,18]. In the current study, embryos cultured on germination media responded by forming either complete plantlets, shoots only or roots only. Germination commenced within four weeks, but the cultures were maintained for additional eight 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 germination, since full- and half-strength produced 48 and 43% plantlets respectively. The addition of auxins modified this effect causing a significant interaction between MS salts, auxin type and auxin concentration (Table 1). In general, data have shown that IBA resulted in higher percentages of complete plantlets compared to NAA, and that half-strength MS salt was superior to full strength

Table 1. Analysis of variance for the effect of MS medium strength, auxin type (IBA and NAA) and auxin concentration on germination response of date palm somatic embryos

		Embryos that formed plantlets		Embryos that formed shoots only		Embryos that formed roots only	
Factor	*df*	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 × auxin type	1	5200.83	0.0001	440.83	0.0001	2613.33	0.0001
MS strength × auxin concentration	5	150.83	0.0017	71.50	0.0131	271.33	0.0001
Auxin type × auxin concentration	5	2037.50	0.0001	488.83	0.0001	1323.33	0.0001
MS × auxin type × auxin concentration	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.

salt (Figure 1 *a*). When full-strength MS medium was used, the percentage of complete plantlets decreased as the concentration of NAA increased. At low NAA concentration, 0.2 mg l^{-1}, germination inhibition was not significant; however, as the concentration was increased to 0.4 mg l^{-1}, a significant reduction in the number of 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 additional decrease occurred and then levelled-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}. Germination inhibition associated with NAA was also observed with half-strength MS medium (Figure 1 *a*).

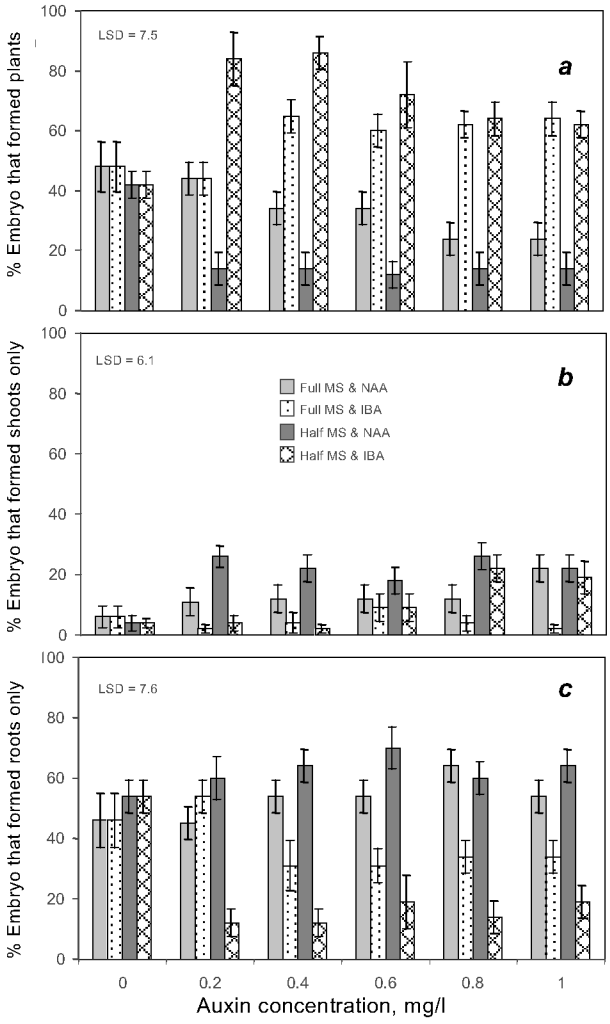

Figure 1. Effect of MS salt strength and auxin (NAA and IBA) concentration on germination of somatic embryos of date palm (*Phoenix dactylifera*). Percentage of embryos that formed complete plants (***a***), shoots only (***b***), and roots only (***c***).

In contrast, stimulation of embryos to produce complete plants was observed in response to increasing IBA concentration with full-strength MS salt (Figure 1 *a*). Adding 1 mg l^{-1} IBA caused no significant change in the percentage of complete plant formation. The best results were obtained when half-strength MS medium was augmented with 0.2 to 0.4 mg l^{-1} IBA (Figure 1 *a*). Compared to the hormone-free control, addition of 0.2 mg l^{-1} IBA resulted in a significant increase in the formation of whole plants. Further, increase in the concentration of IBA to 0.4 slightly increased the percentage of complete plant formation reaching a maximum of 86%. However, beyond this IBA concentration a reduction in the percentage of complete plant formation was observed. Data on the germination percentage of date-palm somatic embryos are unavailable in most previous literature. Nevertheless, Tisserat[6] obtained germination rate of 5 to 15%, and Veramendi and Navarro[5] reported 10%. 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 ANOVA, these two responses were significantly influenced by an interaction between MS salt strength, auxin type and auxin concentration (Table 1). Somatic embryos that formed only shoots without root systems ranged from 2 to 26% of the total embryos cultured (Figure 1 *b*). These shoots can be routinely rooted by subsequent transfer to a medium supplemented with 0.2 mg l^{-1} NAA[7,17]. The majority of the embryos forming shoots were associated more with NAA-containing treatments compared to IBA-containing treatments. In comparison to the NAA-free control, NAA at 1 mg l^{-1} caused a significant increase in shoot formation when full-strength MS was used. With half-strength MS, NAA at 0.2 mg l^{-1} significantly increased the percentage of embryos that formed shoots. Results have shown that at any given NAA concentration, except 1 mg l^{-1}, half-strength MS stimulated more embryos to develop shoots compared to the full-strength medium. 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 (Figure 1 *b*).

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 (Figure 1 *c*). In contrast, IBA reduced the percentage of embryos that formed roots, particularly on half-strength medium. Since no shoot growth developed from these embryos, they were considered unviable.

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 80% survivable rate (192 plantlets), irrespective of the germination treatment. The plants grew normally in soil and appeared to exhibit normal phenotype.

In conclusion, this study has 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 consists 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.

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ISSR and SSR markers based on AG and GA repeats delineate geographically diverse *Oryza nivara* accessions and reveal rare alleles

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***Oryza nivara* is one of the closest wild progenitors of *O. sativa*. Genetic diversity among 24 accessions of *O. nivara* from 11 states of India and four *O. sativa* varieties one each from Glaszmann's isozyme groups I, II, V and VI were analysed using ISSR- and SSR-PCR. The primers based on AG and GA repeats were informative; their resolving power ranged from 4.2 to 10.8 and polymorphism information content from 0.64 to 0.89. They could together enable grouping accessions on a geographical basis. Ten alleles out of 40 amplified at 6 loci were unique to an accession. Two accessions each from UP and Bihar and one from Madhya Pradesh were distinct from other accessions. *O. nivara* alleles in common with Jaya, Dular, Basmati 370 and Taipei 309 were identified. The use of such informative primers helps reduce time, cost and labour involved in studies on phylogeny and germplasm management.**

ORYZA nivara Sharma *et* Shastry (syn *O. rufipogon* annual form, AA, $2n = 24$) is the closest wild relative and progenitor of *O. sativa*. It grows around rice fields and in seasonal ponds in South and Southeast Asia[1]. In India, it grows abundantly in the Central Gangetic Plain and the Deccan plateau, sparsely in southern states and is rare in the northeastern states[2]. *O. nivara* has contributed substantially to rice improvement programmes. It provided a rare major dominant gene for resistance to grassy stunt virus[3] and is a new source of cytoplasmic male sterility[4]. The genetic diversity of Indian *O. nivara* is estimated to be high based on morphological characters[5]. Based on crosses and F_1 pollen sterility among different populations of Indian *O. nivara* and aus, aman, *japonica* and *javanica* ecotypes of *O. sativa*, it was proposed that aus and *japonica* types originated directly from two different populations of *O. nivara*, and introgression of *rufipogon* characters into aus might have given rise to aman ecotype[6]. There is no molecular evidence to support this hypothesis. On one hand, the diversity in *O. nivara* continues to be enriched by intercrossing with cultivars and other hybrid swarms (spontanea), all of which frequently grow around rice fields[1]. On the other hand, there is increasing concern about their extinction from native

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