# An optimized protocol for direct shoot regeneration from shoot tips cultures of date palm (*Phoenix dactylifera L.)* cv. Hayani

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**Abstract:** This study was an attempt to develop an in vitro protocol for propagation of date palm Hayani cv via direct shoot regeneration system without callus formation. Combinations between plant growth regulators were used in order to achieve organogenesis and multiplication of date palm Hayani cv. Shoot tips were cultured on MS media supplemented with cytokinins namely BA and 2iP at different concentrations either individually or in combinations in the presence of 1.0 mg/ L of both NAA and NOA auxins. Results revealed that MS medium supplemented with 2.0 mg/ L 2iP and 3.0 mg/ L BA was the best for bud formation from shoot tip after 8 weeks of culturing at initiation stage. Sub-culturing the formed buds on solid MS multiplication medium supplemented with 0.5 mg/ L 2iP and 0.5 mg/ L BA gave the maximum number of shoots with appropriate length at multiplication stage. MS medium supplemented with 1.0 mg/ L $GA_3$ and 1.0 mg/ L NAA caused maximum shoot length at elongation stage compared with control and other treatments. Moreover, maximum rooting percentage, root number and root length was obtained when MS medium was supplemented with1.o mg/L IBA and 0.5 mg/L NAA.
[Edriss, M.H; Elghayaty, S.H.; Abdrabboh, G.A.; Elsharabasy, S.F. and G. E. Abd-kareiam**. An optimized protocol for direct shoot regeneration from shoot tips cultures of date palm (*Phoenix dactylifera* L.) cv. Hayani.** *World Rural Observ* 2016;8(2):19-24]. ISSN: 1944-6543 (Print); ISSN: 1944-6551 (Online). http://www.sciencepub.net/rural. 4. doi:10.7537/marswro08021604.

**Keywords:** Micropropagation**,** Date palm, Growth regulators, Organogenesis**,** Hayani cv**.**

## 1. Introduction

Date palm (***Phoenix dactylifera*** L.) the dioecious, monocotyledon species belonging to the family Arecaceae is a multipurpose tree having food, medicinal and ornamental importance. Date palm readily grows from seeds but half of the seedlings may turn out to be males and high proportion of inferior quality segregates (**Al-Khalifa, 2000**). Furthermore, seedlings take 6 to 10 years to fruit so male and female trees are not identifiable until flowering (**Othmani *et al*., 2009**). The vegetative multiplication of date palm is traditionally achieved by offshoots. This kind of propagation has limitations such as slow propagation rate, transmission of disease-causing pathogens and insects and production of offshoots in a limited number for a certain period in the life time of a young palm tree (**Gueye *et al*., 2009**). Hence, *in vitro* propagation is the only available alternative tool to produce disease free, uniform and good quality planting material to establish large scale cultivation within a short period of time Date palm is mainly *in vitro* multiplied through somatic embryogenesis (**Rashid and Quraishi, 1994; Fki *et al*., 2003; Al-Khateeb, 2008a; Othmani *et al*., 2009**). Most of the protocols for somatic embryogenesis of date palm made the use of high concentrations of 2, 4-dichlorophenoxy acetic acid (2, 4-D) in media which is known to be associated with genetic instability in regenerated plants. Furthermore, callogenesis is prerequisite for somatic embryogenesis in date palm (**Gueye *et al*., 2009**) which enhances the possibility of producing off type plants (**Saker *et al*., 2006**). Conversely, micropropagation through direct organogenesis lacking callus phase, has the advantage of producing highly identical plants in their vegetative characteristics, with the mother plant. Although there are few reports on direct organogenesis of date palm **(Hussain *et al*., 1995; Bekheet and Saker; 1998; Al-Khateeb, 2008b**), however these are confined to specific genotypes. Because of plant genotype specificity even closely related cultivars of date palm observed with variable growth behavior to the same culture conditions (**Al-Khayri and Al-Bahrany, 2004**). This distinct influence of genotypic specificity puts emphasis on the optimization of direct shoot proliferation protocol. Accordingly, the main aim of this study was to determine the best combination of plant growth regulators and culture growth conditions for stimulating the initiation and multiplication of adventitious buds directly from shoot tips (without callus formation) and then to elongate these buds to shoots and roots.

## 2. Materials and Methods

**Plant material and its primary preparation;** females of date palm cv. Hayani offshoots (3-4 years old) were obtained from a private farm located at Giza, Egypt. The primary preparation of explants was

done outside the laboratory by removing the roots, brown fibrous leaf sheaths and outer green mature leaves from the offshoots reducing the longer to 30 cm. In the laboratory, remaining mature leaves were removed gradually from the bottom to the top, exposing the white young leaves. The gradual removal of white young leaves and surrounding white fibrous leaf sheath resulted in 8 cm long shoot tips.

**Surface sterilization and dissecation of explants;** Shoot tips of date palm were treated with 80% (v/v) commercial bleach solution (5%w/v sodium hypochlorite, containing 1 drop of Tween-20 per 100 ml (as a surfactant) and stirred gently for 20 minutes. These shoot tips were rinsed in sterile distilled water for three minutes and then disinfested with 0.2 % (w/v) mercuric chloride solution for 20 minutes and finally rinsed thoroughly with sterile distilled water three times. The upper and lower most regions of shoot tips exposed to disinfectants were removed. The shoot tips of 6 cm long were shortened by removing the leaf primordial surrounding the meristematic region acropetally. The apical meristems were then removed and divided longitudinally into four equal segments and then cultured on initiation media.

**2.1. Initiation Stage**

The medium used in the initiation stage was composed of MS

(**Murashige and Skoog, 1962**) plus the following :Thiamine – HCl, 1.0mg/L, pyridoxine – HCl, 1.0 mg/L; adenine sulfate $2H_2O$, 50 mg/L; myo-inositol, 100 mg/L; $NaH_2$ $PO_4$. 2 $H_2O$, 170 mg/L; glutamine 200 mg/L; benzyl adenine (BA), isopentyladenine (2iP),in the presence of 1.0 mg/ L of both naphthalene acetic acid (NAA) and naphthoxy acetic acid (NOA), sucrose 30000 mg/L, agar-agar 7g /L as shown in (**Table 1**). The activated charcoal was replaced by 2g/L of poly venylpyroledone (PVP). The pH of the medium was adjusted to 5.7 with 0.1 N NaOH or HCl, before the addition of agar. Media were dispensed into culture jars with 25 ml in each, then covered with polypropylene caps. All vials with media were autoclaved at 121°C and 1.04 kg/cm² for 20 minutes. Apical meristems were cultured into the jars aseptically in a laminar air flow cabinet and cultures were incubated in the dark for one month to reduce phenolic secretions from the explant then all cultures were incubated in a culture room under low light intensity of 1000 lux for 16 hours daily at 27 ± 1°C for four weeks. Cultures were moved to fresh media four times at four week intervals until the buds had initiated, at which time data were recorded. There were five replicates of each treatment.

**2.2. Multiplication Stage**

The formed shoots were transferred to MS medium supplemented with BA and 2ip both added at concentrations of 0.0, 0.5, 1.0, mg/L in presence 1.0 mg/L of both NAA and NOA in order to multiply them. There were five replicates for each treatment. Cultures were incubated under the same conditions mentioned in initiation stage. Re-culturing was carried out every four weeks. Shoots number and shoot length (cm) were determined as growth parameters.

**2.3. Elongation Stage**

In order to increase the shoot length, shoots were transferred to MS medium supplemented with $GA_3$ at 0.0, 1.0 and10.0 mg/ L to elongate the obtained shoots. Five replicates for each treatment were used and data were recorded after eight weeks.

**2.4. Rooting Stage**

Resultant shoots from elongation stage were transferred to test tubes (one shoot / test tube) containing half strength MS medium and the following (in mg/L): Thiamine HCl 0.4, myo –inositol 100, sucrose 60000 and agar 7000. The auxins NAA and IBA were added to MS media separately or in combinations at concentrations of 0.1, 0.5, 1.0, and 1. 5 mg/L. Five replicates for each treatment were uesd, and cultures were incubated at 27 ± 1 ° C and 1000 lux light intensity for 16 hours daily. Rooting percentage, roots number and root length were taken as root growth parameter in this stage after eight weeks.

Table 1. Adding the co-application of 2iP and BA to MS media in the presence of 1mg /L of both NAA and NOA to induce direct shoot regeneration (Initiation stage).

| 2iP + BA (mg/ L) | NAA (mg/ L) | NOA (mg/ L) |
|---|---|---|
| 0.0 + 0.0 | 1.0 | 1.0 |
| 1.0 + 1.0 | 1.0 | 1.0 |
| 2.0 + 1.0 | 1.0 | 1.0 |
| 3.0 + 1.0 | 1.0 | 1.0 |
| 1.0 + 2.0 | 1.0 | 1.0 |
| 2.0 + 2.0 | 1.0 | 1.0 |
| 3.0 + 2.0 | 1.0 | 1.0 |
| 1.0 + 3.0 | 1.0 | 1.0 |
| 2.0 + 3.0 | 1.0 | 1.0 |
| 3.0 + 3.0 | 1.0 | 1.0 |

**3. Results and Discussion**

**3.1. Initiation Stage**

It was clear that the type and concentration of cytokinin affected the response percentage as well as the formation of buds were no response was noticed among explants cultured on media free from cytokinin (Table 2). The medium containing 2 mg/L 2ip plus 3.0 mg/L BA gave the best results in terms of growth response percentage (80%) and bud formation (4.6 bud), (Table 2 and Fig. 4A). These results showed importance of the combination between those two cytokinins in the initiation and development of buds.

There are some reports which support our results such as **Khierallah and Bader (2007)** who stated that in vitro direct bud formation of date palm var. Maktoom was achieved by culturing shoot tips for 16 weeks on MS medium supplemented with 2 mg/l 2ip, 1 mg/l BA, 1 mg/l NAA and 1 mg/l NOA. **Hegazy and Aboshama (2010**) reported that date palm axillary bud proliferation of Medjool cultivar occurred under dark condition after three subcultures on MS medium supplemented with 2ip (1 mg/l), kin (1 mg/l), BA (1 mg/l) and NOA (0.5 mg/l). When these shoot buds were transferred under light condition onto the same medium containing putrescine (150 mg/l), about 55 % of them showed direct somatic embryo formation. Likewise, direct shoot regeneration for date palm cv. Dhakki was reported by (**Khan and Bibi, 2012)** as they cultured shoot tips on MS media supplemented with 1 mg/l NAA, 3 mg/l 2iP and 3 mg/l BA for initiation and developing buds at initiation stage.

Table 2. Effect of MS media supplemented with 2iP and BA on some morphogenetic characteristics of date palm cv. Hayany after eight weeks.

| 2iP+BA (mg/ L) | NAA (mg/ L) | NOA (mg/ L) | Number of buds | Response (%) |
|---|---|---|---|---|
| 0.0 + 0.0 | 1.0 | 1.0 | 0.0 | 0.0 |
| 1.0 +1.0 | 1.0 | 1.0 | 1.2 | 10 |
| 2.0+1.0 | 1.0 | 1.0 | 0.0 | 0.0 |
| 3.0+1.0 | 1.0 | 1.0 | 0.0 | 0.0 |
| 1.0 +2.0 | 1.0 | 1.0 | 2.3 | 20 |
| 2.0 +2.0 | 1.0 | 1.0 | 0.0 | 0.0 |
| 3.0 +2.0 | 1.0 | 1.0 | 2.5 | 40 |
| 1.0+3.0 | 1.0 | 1.0 | 3.2 | 60 |
| 2.0 +3.0 | 1.0 | 1.0 | 4.6 | 80 |
| 3.0 +3.0 | 1.0 | 1.0 | 3.1 | 30 |
| L.S.D at 0.05 | | | 1.23 | 27 |

**3.2. Multiplication Stage:**

After direct emergence of 2-3 shoots, cultures were transferred to MS shoot multiplication media supplemented BA and 2ip either individually or in combinations. It was clear from the results (Table 3 and Figs 1+ 4B) that medium supplemented with 0.5 mg/l of both BA and 2iP possessed the best results regarding both shoot number and shoot length compared with control and other treatments. This result is consistent with other results where 2ip and BA have been used for in vitro multiplication of date palm (**Al-Marri and Al Ghamdi, 1997; Al-Khateeb *et al*., 2002**). Likewise, **Taha *et al*. (2001)** achieved an effective rapid method in vitro multiplication of shoot buds of date palm cv. Zaghlool. They used medium contained high levels of 2ip. However, in a study of micropropagation of date palm cv. Sukry, **Al-Khateeb (2006**) reported that low hormone concentrations promoted formation of new buds while high concentrations resulted in abnormal growth without any sign of budding or shoot formation. In vitro shoot buds multiplication is mainly phytohormone dependent. Cytokinins are generally known to reduce the apical meristem dominance and induce both axillary and adventitious shoots formation from meristematic explants (**Madhulatha *et al.*, 2004).**

Table 3. Effect of MS media supplemented with combination between 2ip and BA on shoot multiplication and shoot length of Hayani date palm cv. after eight weeks.

| 2iP + BA (mg/ L) | NAA (mg/ L) | NOA (mg/ L) | Shoot number | Shoot length cm) |
|---|---|---|---|---|
| 0.0 + 0.0 | 1.0 | 1.0 | 1.0 | 1.5 |
| 0.5 + 0.5 | 1.0 | 1.0 | 4.1 | 2.5 |
| 1.0 + 0.5 | 1.0 | 1.0 | 3.3 | 2.3 |
| 0.5 +1.0 | 1.0 | 1.0 | 2.1 | 1.8 |
| 1.0 +1.0 | 1.0 | 1.0 | 1.0 | 1.5 |
| L.S.D at 0.05 | | | 1.36 | 1.18 |

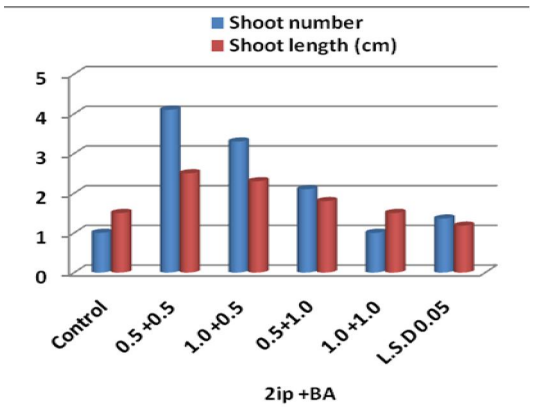


Fig 1. Effect of MS media supplemented with combination between 2ip and BA on shoot multiplication and shoot length of Hayani date palm cv. after eight weeks.

Table 4. Effect of various concentrations of $GA_3$ added to MS media on Hayani shoot elongation after eight weeks in presence 1 mg/L NAA

| $GA_3$ (mg/ L) | NAA (mg/ L) | Shoot length (cm) |
|---|---|---|
| 0.0 | 1.0 | 1.5 |
| 1.0 | 1.0 | 4.5 |
| 10.0 | 1.0 | 4.0 |
| L.S.D 0.05 | | 1.23 |

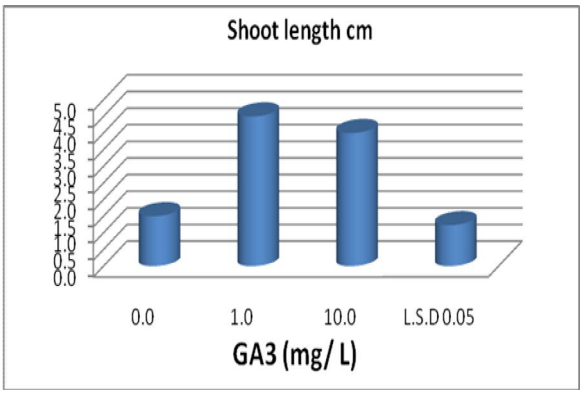


Fig 2. Effect of $GA_3$ added to MS media on Hayani shoot elongation after eight weeks in presence 1 mg/L NAA.

**3.3. Elongation Stage**.

$GA_3$ had a positive effect on the elongation of shoots produced in the multiplication stage (Table 4 and Figs 2 and 4 C). Shoot length increased with the increasing $GA_3$ concentration in the medium, but some malformations were noticed at 1.0 mg/L in spite of its superiority over other treatments. The average length of shoots cultured on this medium caused maximum values of shoot length (4.5 cm) compared with other treatments. This result directs our attention to the well know role of gibberellins in the elongation of the plant cells (**1PGSA, 1998**).

**3.4. Rooting Stage.**

Data presented in and Fig (3 and 4D) indicated that the addition of NAA led to an increase in rooting. Adding NAA at 0.5 mg/L resulted in the best rooting percentage (85%) and average root length (5.0 cm), which were significantly different from other treatments. No reduction in root length was observed with the increasing of auxin concentration except at high concentration (1.5 mg/l). It is known that auxins play an active role in root formation by the induction of root initials (**IPGSA, 1998**). Many researchers have mentioned the importance of NAA in the rooting of date palm shoots in vitro (Al-**Maari and Al-Gamdi, 1997**). Regarding IBA date presented in)Fig 3 (indicated that adding IBA at 1.0 mg/L resulted in the best rooting percentage (75%) and average root length (5. 0 cm) compared with other treatments. This result are in agreement with those reported by (**Aslam and Khan, 2009**). Cleared that root length (cm) of date palm was somewhat enhanced when the media were supplemented with IBA at 0.5 and 1.0 mg / L compared with medium free of auxin and other treatment. Our results plus indicated that the combination between IBA and NAA significantly enhanced rcoting characteristics, where both root formation number and root length (cm) recorded the highest values when shoots were cultured on MS media supplemented with 1.0 mg / L of both IBA and NAA, while the maximum root formation percentage (85%) and root length (5.0 cm) was obtained when shoots were cultured on MS media supplemented with 1.0 mg /L of IBA and at 0.5 mg /L of NAA com compared with other treatments. Also, **Bekheet (2013)** tested the effect of MS media supplemented with different auxins of IBA, NAA and IAA at 0.0 and 1.0 mg /L on rooting of some date palm cultivars and he found that the maximum root formation percentage and the highest root number obtained, when axillary shoots were cultured on MS media containing NAA at 1.0 mg / L in comparison to other auxins and control. This result indicated the superiority of adding combination between NAA + IBA over adding NAA or IBA individually to MS media.

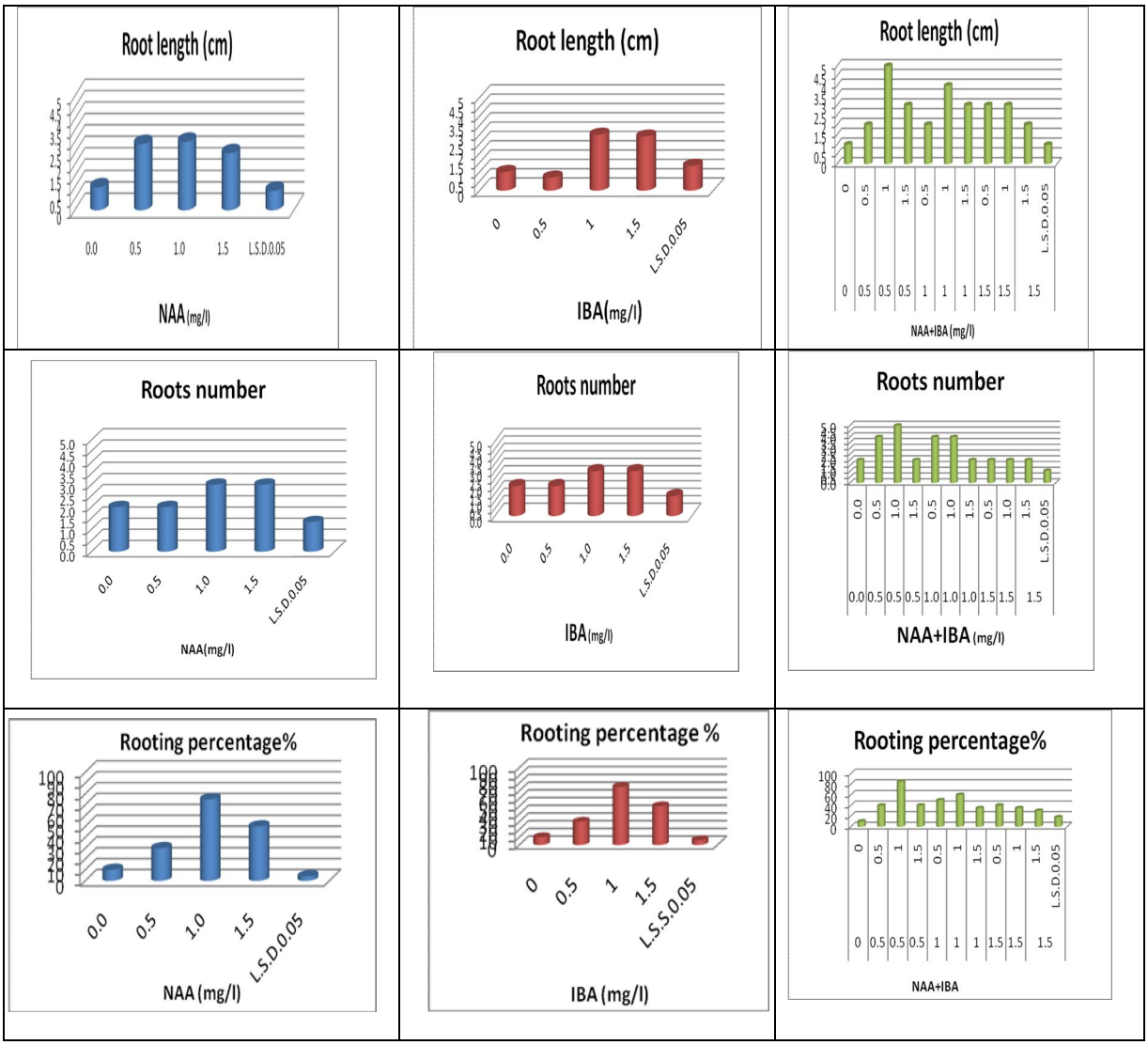


Fig 3. Effect of MS media supplemented with NAA and IBA either individually or in combinations on root characteristics of Hayani shoots after eight weeks.


Fig 4. *In vitro* propagation of date palm through direct shoot regeneration.
A) Direct shoot bud proliferation on MS medium + 2 mg/l 2iP and 3 mg/L BA.
B) Shoot multiplication on MS medium containing 0.5 mg/l 2iP + 0.5 mg/l BA.
C) Elongation of shoots on MS medium containing 1.0 mg/L GA3+ 1.0 mg/L NAA.
D) Root formation using MS medium supplemented with 1.0 mg/l IBA + and 0.5 mg/L NAA.

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5/25/2016