Micro propagation of Date Palm (*Phoenix dactylifera* L.) var. Zaghlol via Direct Organogenesis

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ABSTRACT

This study was an attempt to develop an *in vitro* protocol for propagation date palm *(Phoenix dactylifera* L.) Zaghlol cv via direct shoot regeneration system. Combinations between plant growth regulators were used in order to achieve this goal. Shoot tips were cultured on MS medium supplemented with cytokinins namely BA or KIN at different concentrations either individually or in combinations with NAA at 0.0, 1.0, 2.0 and 4.0 mg /L in the presence of 1.0 mg/ L NOA. Results revealed that MS medium supplemented with BA or KIN at 1.0 mg /Lin combination with NAA at 1.0 mg/ L 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 medium supplemented with 1.0 mg/ L of BA and KIN gave the maximum number of shoots with appropriate length at multiplication stage. MS medium supplemented with 10 mg/ L of GA_3 caused the maximum shoot length at elongation stage compared with control and other treatments. Moreover, maximum rooting percentage, root number and root length were obtained as MS medium supplemented with 0.5 mg/L IBA and 1.0 mg/L NAA was used.

Keywords: In vitro**,** shoot tips, growth regulators, direct pathway**.**

Introduction

Date palm (***Phoenix dactylifera*** L.) tree is one of the important fruit crops cultivated in arid and semi-arid regions. It has tremendous advantages resilience, require a limited inputs, long-term productivity and multiple purposes attributes (**Bircher, 1990**). Due to the high degree of genetic heterozygosity and dioecious nature of date palm, sexual propagation method cannot be used for propagation of true-to-type cultivars. It propagated vegetatively by the offshoots which arise from the base of mother plants. This method of propagation is limited since mother plant produces 15 to 20 offshoots during palm's life, depending on the cultivar and this reduces considerably the expansion of the existing palm (**Zaid, and de Wet, 1999**). The vegetative multiplication of date palm is traditionally achieved by offshoots. This kind of offshoots propagation has limitations such as slow propagation rate, transmission of disease-causing pathogens & insects and production a limited number of offshoots for a certain period in the life time of a young palm tree (**Gueye *et al*., 2009**). Conversely, the most advantage of micropropagation via organogenesis is getting in vitro plantlets highly identical in their genetic and vegetative characteristics with the mother plant (**Tisserat, 1984; Aaouine 2000)**. The organogenesis technique consists of three stages: initiation of vegetative buds; bud multiplication; and rooting. The success of this technique is highly dependent on the success of the first stage (**Abahmane, 2011**). In vitro propagation of date palm from several genotypes through organogenesis has been studied using various meristematic explants including shoot tips and lateral buds (**Al Khateeb, 2006**).. The growth regulators incorporated in the media are used at the lowest possible concentration (**Zaid and de Wet 1999**). Furthermore, since shoots are directly initiated from mother tissue without passing through a callus phase, the plantlets produced are supposed to be true-to-type (**Kunert *et al.*, 2003**). The best time period for starting in vitro culture from offshoots is between the end of date fruit harvest and the start of the next flowering stage (**Beauchesne *et al.*, 1986**). 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 (**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 **(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 aim of this study was to determine the best combination of plant growth regulators and culture growth conditions to stimulate the initiation and multiplication of adventitious buds directly from shoot tips and then to proliferate shoots and roots.

Materials and Methods

Plant material and its primary preparation; females date palm cv. Zghalol offshoots (3-4 years old) were obtained from a private farm located at Elsharkia , 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 size 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 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 primordia surrounding the meristematic region acropetally. The apical meristems were then removed and divided longitudinally into four equal segments and then cultured on initiation media.

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 PO4. 2 H_2O, 170 mg/L; glutamine 200 mg/L, MS media was also supplemented with BA(Benzyl adenine) , KIN(Kinetin) and NAA (Naphthalene acetic acid) either individually or in combination at different concentrations in the presence of 1.0 mg /L of NOA as shown in Table 1, sucrose 30 g/L and agar-agar 7 g /L. The activated charcoal was replaced by 2 g/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. Medium was dispensed into culture jars with 25 ml in each, then covered with polypropylene caps. All vials with medium were autoclaved at 121°C and 1.04 kg/cm² for20 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 was recorded. The measurements include shoot number and shoot fresh weight (g) and collected from five replicates of each treatment.

Table 1. Supplementations of KIN, BA and NAA either individually or in combinations to MS media in the presence of 1.0 mg /L of NOA during initiation stage.

Treatment number	BA + NAA (mg/ L)	Treatment number	KIN + NAA (mg/ L)
1	0.0 + 0.0		0.0 + 0.0
2	0.0 + 1.0	19	0.0 + 1.0
3	0.0 + 2.0	20	0.0 + 2.0
4	0.0 + 4.0	21	0.0 + 4.0
5	1.0 + 0.0	22	1.0 + 0.0
6	1.0 + 1.0	23	1.0 + 1.0
7	2.0 + 1.0	24	2.0 + 1.0
8	4.0 + 1.0	25	4.0 + 1.0
9	2.0 + 0.0	26	2.0 + 0.0
10	1.0 + 2.0	27	1.0 + 2.0
11	2.0 + 1.0	28	2.0 + 1.0
12	1.0 +2.0	29	1.0 +2.0
13	2.0 + 2.0	30	2.0 + 2.0
14	4.0 + 2.0	31	4.0 + 2.0
15	4.0+2.0	32	4.0+2.0
16	1.0 +4.0	33	1.0 +4.0
17	2.0 + 4.0	34	2.0 + 4.0
18	4.0 + 4.0	35	4.0 + 4.0

2. Multiplication Stage

The obtained shoots will be transferred to MS medium supplemented with BA and KIN both added at concentrations of 0.0, 1.0, 2.0 and 4.0 mg/L. There were five replicates for each treatment. Cultures were incubated in a culture room under similar condition of initiation stage Re-culturing was carried out every four

weeks. Shoots number and shoot fresh weight (g) were determined as growth parameters.

3. Rooting stage

3.1. Elongation phase

Shoots will be transferred to MS medium supplemented with GA_3 at 0.0, 1.0, 5.0 and 10.0 mg / L and NAA at 0.0 0.1, 0.5 and 1.0 mg / L either individually or in combinations to elongate the obtained shoots. Five replicates for each treatment were used and data were recorded after eight weeks.

3.2 Formation phase

Resultant shoots from the previous phase will be 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 60 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 2.0 mg /L. There were five replicates for each treatment, and cultures were incubated in a culture room at 27 ± 1 ° C and 1000 lux light intensity for 16 hours daily for four weeks. Rooting percentage, roots number and root length (cm) were taken as root growth parameters in this stage after eight weeks.

A complete randomized block design was followed and analysis of variance (ANOVA) was performed using one way ANOVA Co-stat software according to **Stern (1991).**

Results and Discussion

1. Initiation Stage.

It was clear that the type and concentration of cytokinin affected the response percentage as well as the formation of buds (Figs from 1 to 8). No significant differences between all concentrations of BA and NAA were noticed on shoot number of date palm zaghlol cv, while MS media supplemented with 1.0 mg /L of both BA and NAA possessed the highest values of all parameters such as shoot number and shoot fresh weight (g). Adding 1.0 mg /L of BA, KIN and NAA possessed highest than of control of shoot fresh weight (g) in comparison with other treatments. (Figs. 1, 2, 3 and 4). These results indicated the superiority of BA over cytokinin (KIN) and auxin (NAA) for the initiation and development of buds. There are some reports which support our results such as **Khan and Bibi, (2012)** who reported that the medium supplemented with 0.5 mg / L of BAP, 0.5 mg/l Kinetin and 1 mg/l NAA proved to be effective for achieving optimum number of shoots with appropriate length of Dhakki date palm cv compared with control and other treatments. **Hegazy and Aboshama (2010**) reported that axillary bud proliferation of Medjool date palm cultivar occurred under dark condition after three subcultures on MS medium supplemented with 2ip (1.0 mg/ L), kin (1.0 mg/ L), BA (1.0 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. **Khierallah and Bader (2007)** 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. Likewise, direct shoot regeneration for date palm cv. Dhakki was reported by (**Khan and Bibi, 2012)** as they cultured shoot tips onMS media supplemented with 1 mg/l NAA, 3 mg/l 2iP and 3 mg/l BA for initiation stage. Moreover, the growth regulators added to medium played an important role in the growth and differentiation of explants (**Tisserat, 1984**). Shoot proliferation also depends upon the balance of cytokinins and auxins. The increase in shoot proliferation might be due to the physiological role of BA which is considered to be the most widely used cytokinin in the micropropagation industry due to its effectiveness and affordability **(Bairu *et al.*, 2007)**. As cytokinins are breaking apical dominance agents, so they influence the success of multiplication in vitro. **(Silva *et al.*, 2003)**.

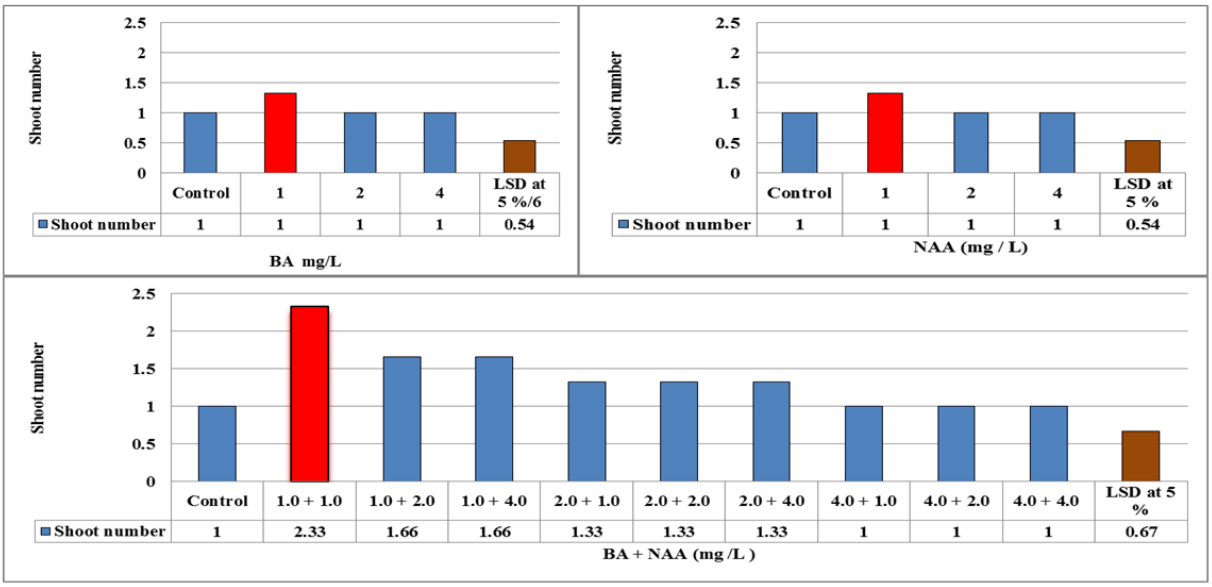

Fig. 1. Effect of supplementation MS medium with BA and NAA either individually or in combination on shoot number of date palm zghalol cultivar at initiation stage.

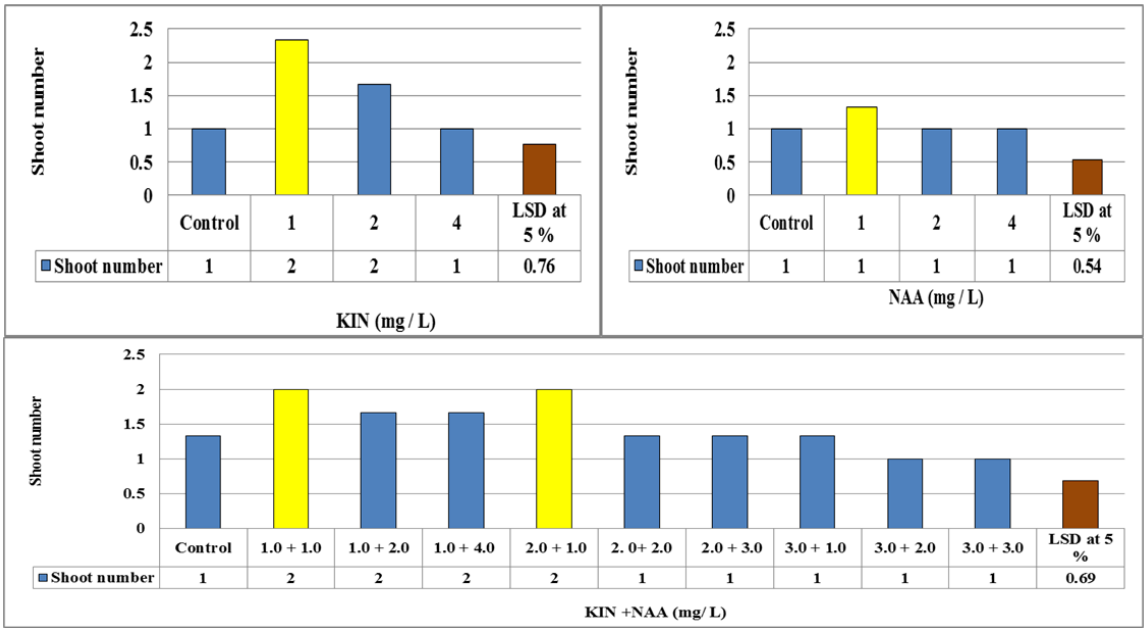

Fig. 2. Effect of supplementation MS medium with KIN and NAA either individually or in combination on shoot number of date palm zghalol cultivar at initiation stage.

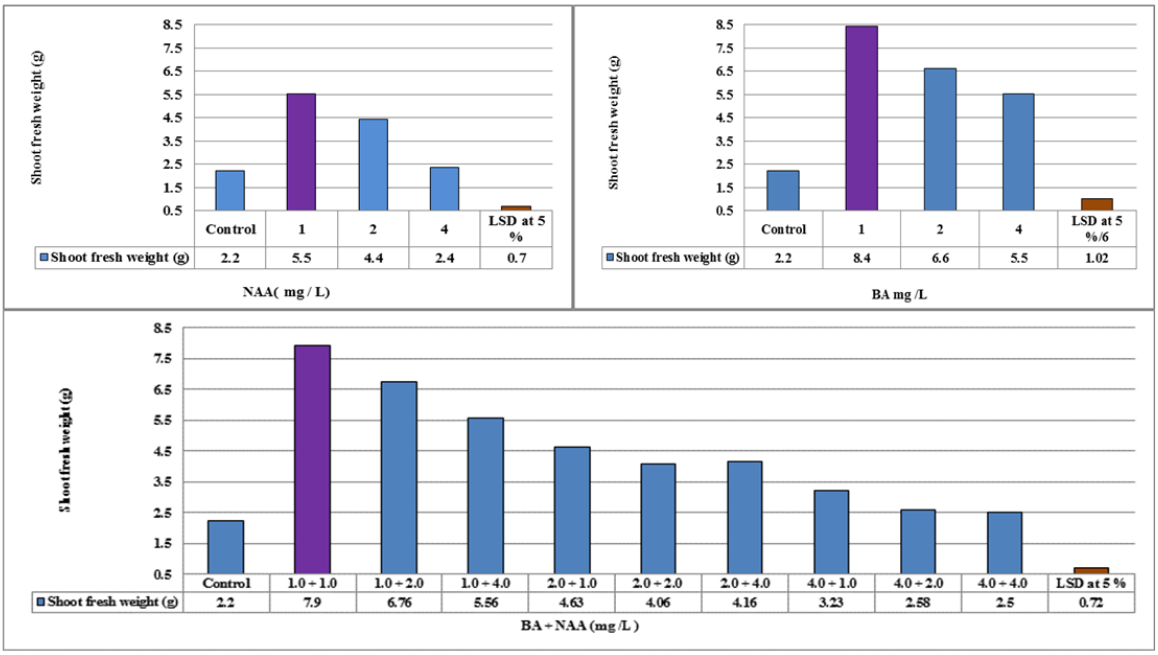

Fig. 3. Effect of supplementation MS medium with BA and NAA either individually or in combination on shoot fresh weight (g) of date palm zghalol cultivar at initiation stage.

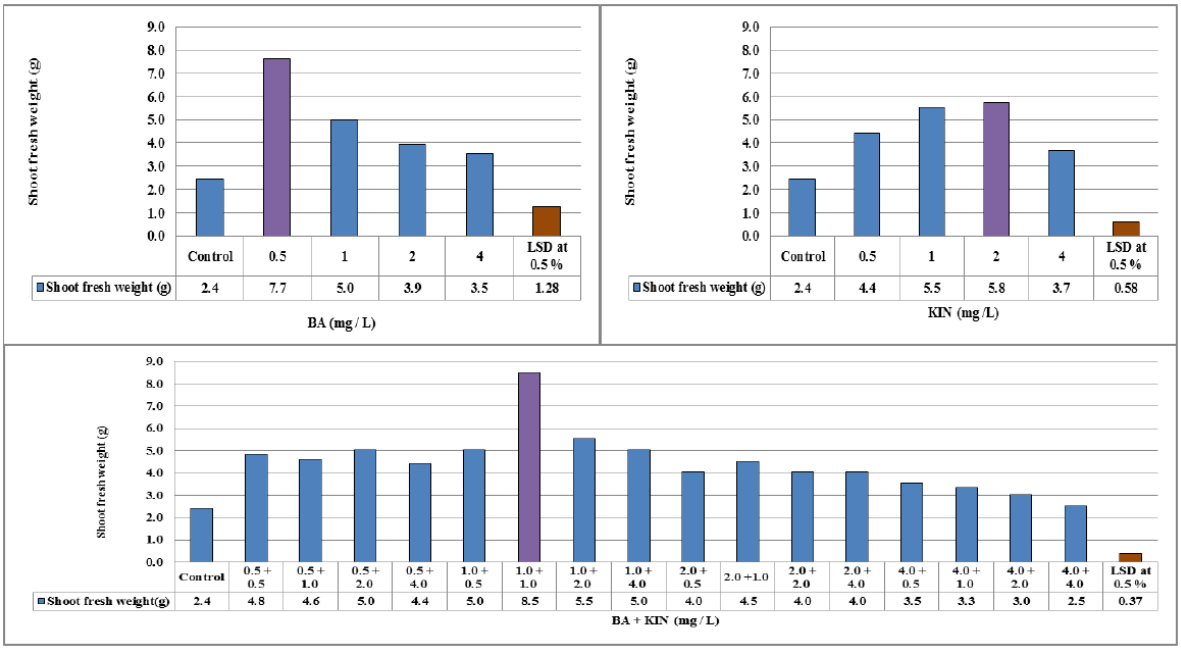

Fig. 4. Effect of supplementation MS medium with KIN and NAA either individually or in combination on shoot fresh weight (g) of date palm zghalol cultivar at initiation stage.

3.2. Multiplication Stag:

After direct emergence of 2-3 shoots, cultures were transferred to MS shoot multiplication media supplemented BAP and KIN either individually or in combinations. It was clear from the results (Figs 5 and 6). MS media supplemented with BA at any concentration was superior to control treatment. In this regard, adding BA at 1.0 mg /L to MS media showed the highest values of shoot number and shoot fresh weight (g). Similar results were obtained regarding addition of KIN to MS media where KIN at any concentration caused values highest than those of control. on the other hand, addition of co-application of BA and KIN both 1.0 mg /L caused the maximum values of shoot number and shoot fresh weight (g) in comparison with control and all other treatments. This result is consistent with other results where KIN and BA have been used for in vitro multiplication of date palm (**Khan and Bibi, 2012; Bekheet 2013**). In addition, **Bekheet, (2013**) who reported that adding

three types of cytokinins to the growth media such as BA , KIN and 2iP at different concentrations from 0.5 to 5.0 mg /l enhanced shoot bud formation of date palm cv. Zaghlool compared with control. The reported that maximum shoot bud formation achieved when micro- shoots of date palm cv. Zaghlool were cultured on MS media complemented with 2iP at 5.0 mg /L and KIN at 2.0 mg /L compared with control and other treatments. Likewise, **Taha *et al.*, (2001)** achieved an effective rapid method of 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. Sukkary, **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).** Some authors have reported that shoot multiplication occurs on media characterized by auxin/ cytokinin ratios >1 **(Loutfi and El Hadrami 2005).** The increase in shoot proliferation may be due to kind and concentration of cytokinin. A range of cytokinins (Kinetin, Zeatin, 2iP and BAP) have been used for the purpose of micropropagation **(Bhojwani and Razdan 1992).** Currently, BAP is the most widely used cytokinin in the micropropagation industry due to its effectiveness and affordability **(Bairu *et al.*, 2007)**. The influence of cytokinins on tissue or organ cultures can be differed based on the kind of culture, the variety of plant and the age of explant. It also reported that BA is required at low concentrations ranging from 0.5 to 2.5 mg / L **(Thorpe *et al.*, 2008)**. Cytokinins 2iP, Kin and BA are rarely used in shoot proliferation of fruit varieties **(Hsia and Korban, 1997; Jaakola *et al.*, 2001 and Góralski *et al.*, 2005).**

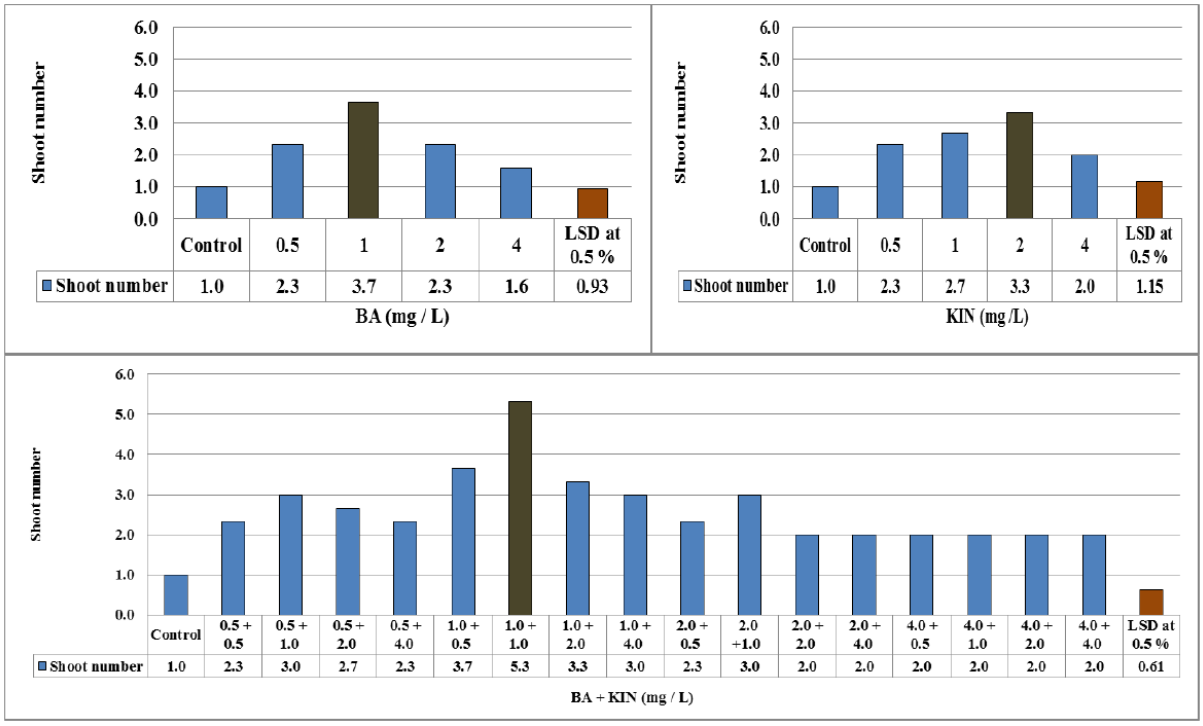

Fig. 5. Effect of supplementation MS medium with BA and KIN either individually or in combination on shoot number of date palm zghalol cultivar at multiplication stage.

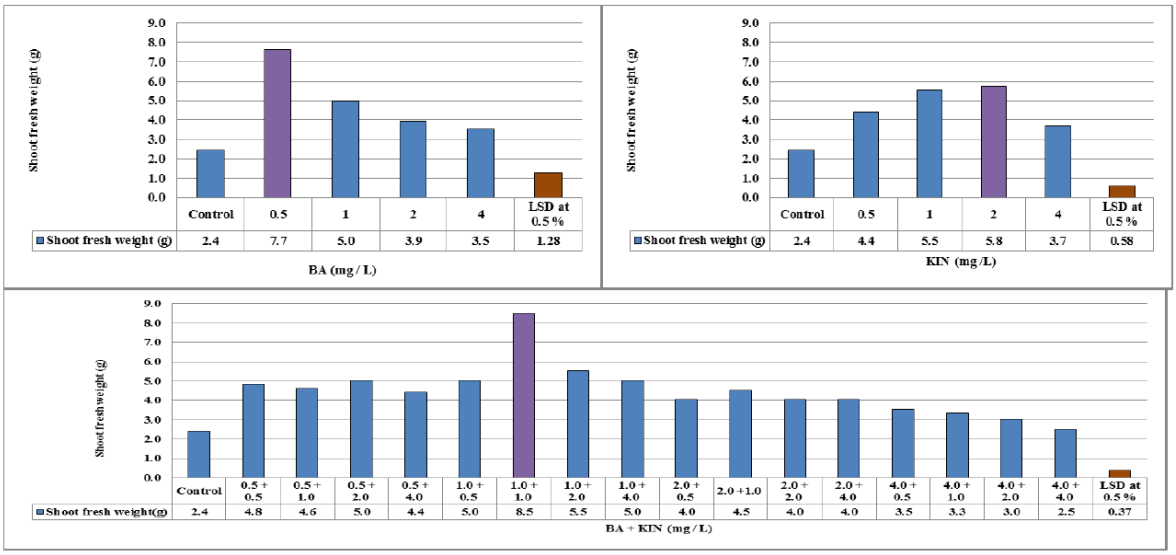

Fig. 6. Effect of supplementation MS medium with BA and KIN either individually or in combination on shoot fresh weight (g) of date palm zghalol cultivar at multiplication stage.

3.3. Rooting stage

3.3.1. Elongation phase.

GA_3 had a positive effect on the elongation of shoots previously produced in the multiplication stage (Fig.7). Data indicated that the highest shoot length (cm) were obtained when micro-cultures of date palm zghalol cv were cultured on MS media supplemented with GA_3 at 10 mg /L followed in descending order by those of 1.0 mg /L GA3 + 0.5 mg /L of NAA compared with control and other treatments. This result directs our attention to the well know role of gibberellins in the elongation of the plant cells (**1PGSA, 1998**). Gibberellins at 2.0 mg /L can also be incorporated into the culture medium in this stage but for no more than 15 days (**Beauchesne *et al.*, 1986**). A study conducted on Zaghloul and Sewi cvs. showed that NAA at 0.1 mg/l has a pronounced effect on shoot length (**El Sharabasy *et al.*, 2001**). Generally, shoot elongation requires transfer of shoots from the multiplication medium to another medium with a high auxin/cytokinin ratio (**Loutfi and Chlyah, 1998**).

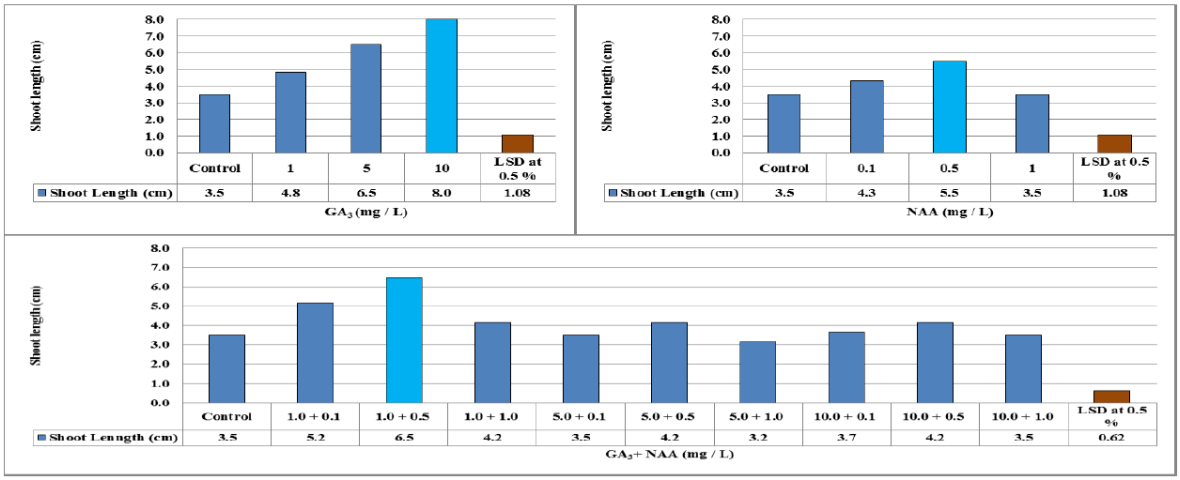

Fig. 7. Effect of supplementation MS medium with GA_3 and NAA either individually or in combination on shoot length (cm) of date palm zghalol cultivar at elongation phase.

3.3.2. Formation phase.

Data presented in Fig 8 indicated that the addition of NAA and IBA either individually or in combinations to MS media led to an increase percentage in rooting in comparison with control. Adding IBA at 2.0 mg/L to the growth media possessed the highest rooting percentage of date palm zghalol cv in comparison with control and other concentrations. Results also showed that MS media supplemented with NAA at 1.0 mg /L gained the highest root formation percentage compared with control and other treatments. Data also showed that supplementing MS media with combination between IBA at 0.5 and NAA at 1.0 mg /L led to an increase

root formation percentage in comparison with control and other treatments. It is known that auxin plays 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**). They reported that adding IBA at 1.0 mg/L resulted in the best rooting percentage (75%) and average root length (5. 0 cm). They also, reported that the highest root formation and the best root length of date palm cultivars obtained when shoots were cultured on MS media supplemented with IBA at 1.0 mg / L. However, **Aslam and Khan (2009)** found that a rooting percentage of 87.34% was obtained on solid MS medium supplemented with 24.6 mM of IBA. They also reported that root length was higher when MS liquid medium was used. They also reported that the combination between IBA and NAA enhanced morphogenetic 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. Auxins are involved in the many woody plants; IBA is commonly used to promote root initiation **(Torrey, 1976).** Auxin induced number of responses which involved cell division, cell enlargement, protein and nucleic acids synthesis which are concomitants of auxin-induced growth and changes in wall plasticity of plant cell and increase the apical dominance as there are essential and rapid processes involved in growth and elongation **(Wilkins, 1989).**

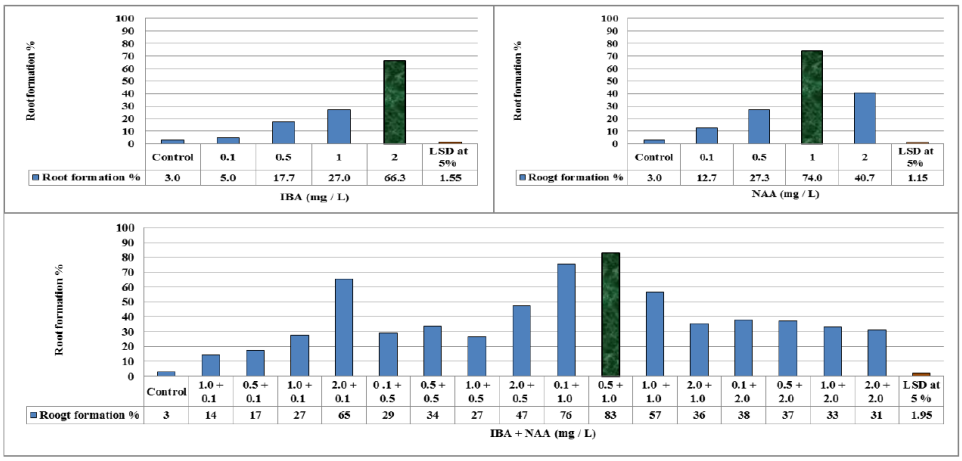

Fig. 8. Effect of supplementation MS medium with IBA and NAA either individually or in combination on root formation percentage of date palm zghalol cultivar at root formation phase.

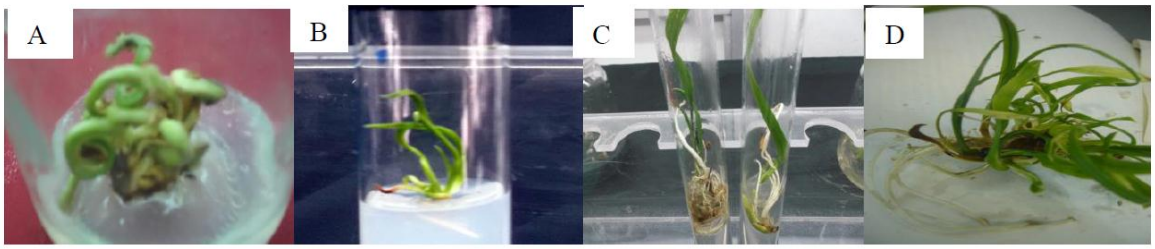

Fig 9. In vitro propagation of date palm through direct regeneration
A) Direct shoot bud proliferation on MS medium with 1.0 mg /L of BA and KIN.
B) Shoot multiplication on MS medium containing 1.0 BA and 2.0 mg /L KIN.
C) Shoot Elongation on MS medium containing 1.0 mg/L of GA3 and NAA.
D) Root formation using MS medium supplemented with 0.5 mg /L IBA and 2.0 mg /L NAA.

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