Effect of different growth regulators on production of somatic embryos from immature inflorescence of *Phoenix dactylifera*

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ABSTRACT

Immature inflorescences of date palm (Phoenix dactylifer) cv. Sewi were used as explants for in vitro culture to investigate the effect of plant growth regulators on inflorescence proliferation. The inflorescences on initiation stage cultured on solidified Murashige and Skoog (MS) basal medium supplemented with Picloram 1, 5, 10 and 15 mg l-1 for two re-cultures. Then, transferred into medium supplemented with different concentration of TDZ combined with NAA on proliferate stage. The optimal concentration for successful inflorescence growth was 5 or 10 mg l-1 Picloram and through studying the residuals effect of Picloram on inflorescences proliferation in the presence of three concentration of TDZ, it found that, 0.5 mg l-1 TDZ combined with 0.1 mg l-1 NAA was more effective to induce direct somatic embryos and gave the highest inflorescence proliferation percentage, while the high level of Picloram induced callus. Vegetative shoots formed into media containing 0.1 NAA mg l-1, 0.2 mg l-1 BA and 0.2 mg l-1 kin. All somatic embryos were converted successfully to healthy normal plantlets which could be transferred to greenhouse.

Keywords: *Phoenix dactylifera*, *in vitro* inflorescences, direct somatic embryos, Picloram, TDZ

Abbreviation: Picloram *(4-amino-3,5,6-trichloropicolinic acid)*,Thidiazuron (*N*-phenyl-*N*'-1,2,3-thiadiazol-5-ylurea),NAA(naphthaleneacetic acid), BA (6-Benzylaminopurine), kin (kinetin).

INTRODUCTION

Date palm, *Phoenix dactylifera* L., is one of the oldest fruit trees in the world and is mentioned in the Holy Qur'an and Bible. Date palm is one of the most important fruit trees in the Middle East and in the Saharan and Sub-Saharan regions of Africa. In some areas, this is the only tree which provides food, shelter and fuel to the communities. Dates are not only a staple food but are also an important export cash crop) Zaid and Hegarty 2006). Conventional propagation is by offshoots making it slow to establish new date palm plantations. Moreover, several genotypes do not produce offshoots while others are difficult to root. In addition seed-propagated palms do not bear true to type due to heterozygosis and require up to 7 years reaching fruiting stage. The need for rapid and efficient vegetative propagation systems for elite genotypes has therefore become urgent. Since 1970 intensive efforts have been undertaken into large-scale micropropagation of date palm using techniques such as somatic embryogenesis and organogenesis (Drira 1983; Drira and Benbadis 1985; Tisserat 1979; ElHadrami *et al*. 1998).

Exogenously supplied plant growth regulators are essential for somatic embryogenesis (Ammirato 1983). Most tissue culture studies of palms have focused on the effects of different auxin types and concentrations on various explants cultures as investigated in date palm (Othmani et al. 2009, Eke et al. 2005), macaw palm (Moura et al. 2009),

and coconut (Verdeil *et al.,* 1989and Verdeil *et al.,*1994). Picloram has recently been reported to be successful in Arecanut palm tissue culture in terms of callus and somatic embryo production. The continuous production of embryonic calli from the initial explants indicates the potential of the protocol for multiplication of palms (Karun et al. 2004).

Thidiazuron has been used successfully *in vitro* to induce adventitious shoot formation and to promote axillary shoot proliferation (Chin-Yi Lu, 1993).The ability of TDZ to stimulate cell division has been demonstrated in soybean callus.Apart from stimulating cell division, TDZ had also been shown to induce adventitious shoot formation from tobacco leaf discs and to stimulate radish cotyledon expansion (Thomas and Katterman, 1986).

The objective of this study was to investigate inflorescence proliferation of *Phoenix dactylifera* cv. sewi maintained in the immature phase and induced somatic embryos directly. We also systematically examined the residual effects of picloram and the change composition of media to another type of growth regulators like thidiazuron (TDZ) and naphthalene acetic acid (NAA) on proliferation percentage.

MATERIALS AND METHODS

Plant material

Two distinct steps were followed:

The first step had been done outside the laminar flow hood and before cutting the spathes, they were sprayed with 70% ethanol and burned for a few seconds to burn the external hairs. The second step took place under aseptic condition into laminar air flow hood. The spathes were gently opened with sterilized scalpel then the spikelets were soaked in mercuric chloride (Hg Cl2) at 0.1% for 15 min. The explants of inflorescences were rinsed three times with sterilized distilled water.

Spikelet of length (7-10 cm) were cut into 1-3 cm long pieces which each piece carries many florets (Fig.1a,b) Sidky and Eldawyati, 2012)

Inflorescence Initiation (first stage)

To establish direct somatic of *Phoenix dactylifera* cv. Sewi, we first cultured of Spikelets explants of length (7-10 cm) on half macro and full micro elements of Murashig and Skoog (1962) combined with 40 mg-1 adenine–Sulphate, 5mg-1 thiamin- HCl, 100 mg-l myoinositol, 200 mg-1 glutamine, 0.5 g-l activated charcoal, 50g-1 sucrose and 5.0 g-1 agar with different concentration of Picloram 1,5,10, and 15 mg l-1 . Effects of treatments were evaluated after two re-cultures (12 weeks). Data collection and re-culturing were performed at 6 weeks intervals. pH of each medium was adjusted to 5.7 ± 0.1 prior to addition of agar, the medium were distributed into culture small jars (150 ml), the jars were autoclaved at 121°C and 1.2 kg/cm² for 20 min. Culture of all treatments was incubated under complete darkness at 27± $2C^0$. Data were taken on swelling and browning rate.

Inflorescence proliferation (second stage)

In order to examine the effects of plant growth regulators on inflorescence proliferation, we added TDZ at 0.1, 0.5 and 1.0 mg l-1 combined with 0.1mg l-1 NAA to half MS basal medium the explants re-cultured every 6 weeks for twice. As soon as somatic embryos were germinated, it was transferred into MS solid medium containing 0.1 NAA mg/l + 0.2 mg/l BA+0.2 mg/l kin, 200 mg l-1 KH2PO4, 40 g l-1 sucrose and .0.3g l-1 AC (Sidky and Gadalla 2013). Plantlets were transferred to rooting media. Data were calculated after each culture as follows:

1. Number of direct somatic embryos.
2. Percentage of direct somatic embryos.
3. Callus initiation degree/explant.

(This data Scored visually according to Pottino (1981) as follow:

- Negative results (-) 1
- Average results (+) 2
- Good results (+++) 3

Rooting stage

Plantlets were cultured on 1/2 MS liquid medium supplemented with 1.0 NAA mg l-1, 200 mg l-1 mg/l KH2PO4, 40 g l-1 sucrose, 100 mg l-1 myoinsitol and 1g l-1 AC, and incubated under 6000 lux light(Fig.1g), then rooting and eventually successfully transplanted in the greenhouse.

Statistical analysis

The randomized factorial design was used and data were subjected to analysis of variance. Separation of means among treatments was determined using L.S.D test at 5% according to Snedecor and Cochran (1980).

RESULT AND DISCUSSIONS

Effects of picloram on Inflorescence Initiation

The immature flower buds were swelling after 6 weeks, some culture turned brown, but several of them enlarged and gave different response (Table 1 and Fig 1c). This culture had to be re-cultured after another 6 weeks to promote further growth. The highest swelling rate (73.33%) was obtained at 5 mg l-1 picloram concentrations. However; this is not statistically significant at 15 mg l-1 picloram (33.33%). Zimmerman, (1993) reported that the pro embryogenic callus were containing auxins to synthesize all the necessary genes to complete the globular stage. However, the auxins

were removed from the culture to make inactive genes or synthesize new gene products for the completion of embryo development. Kawahara and Komamine (1995) reported that, the exogenous auxins were involved in gene expression of early stages of somatic embryogenesis.

Similarly, browning rate differed according to picloram concentrations and ranged from 20.00% to 46.66%, the lowest browning rate was obtained at 5 mg.l-1 picloram concentration. While, 46.66% of these culture turned brown at1mg l-1picloram. 2, 4-D at levels higher than 30 x 10M inhibited callusing and enhanced browning of coconut embryos (Karunaratne and Periyapperuma 1989*)*.

Effects of TDZ on inflorescence proliferation.

Three concentrations of TDZ were tested combined with 0.1 mg l-1 NAA for their effect on date palm inflorescence proliferation (Table 2 and Fig.1e, f).

Twelve weeks after culture initiation on media containing auxin (Fig. 1d), inflorescence buds transferring into media containing cytokinin, Zimmerman, (1993) reported that the pro embryogenic callus were containing auxins to synthesize all the necessary genes to complete the globular stage. After six weeks we observed growth of structures like globular, the structures proliferation to direct somatic embryos after another six weeks (Fig. 1e,f) . Residual effect of picloram observed in (Table 2). 5mg l-1 or 10 mg l-1 picloram produced the highest direct embryos (4.44, 4.33 embryos / culture). The addition of a cytokinin resulted essential to promote growth of somatic embryos from immature flowers, direct somatic embryos occurred at all TDZ concentrations tested, the highest direct of somatic embryos was obtained after floral buds were cultured on medium containing 0.5 mg l-1 TDZ +0.1 mgl-1 NAA (4.41 embryos /culture).

There was also a significant interaction between TDZ concentrations and picloram concentrations. The interaction between 5mg l-1 picloram and 0.5mg l-1 TDZ+0.1 mgl-1 NAA gave the significant result of direct embryos (7 embryos /culture).These results suggested that changing the medium composition could significantly change the number of direct somatic embryos .This type of response shows similarities with the flower bud-like structures observed by Verron *et a*l. (1995) in the monocot lily of the valley, also in bamboo and ginseng explants, which have been shown to flower and thereof proliferate inflorescences *in vitro* in medium containing TDZ (Lin *et al*. 2003; 2004).

The effects of residual concentrations of picloram on the percentage of proliferation embryos are investigated in (Table 3, Fig.1f). Medium containing 10 mg l-1 picloram gave the maximum proliferation embryos response (84.44%). On the other hand, the highest proliferation embryos were obtained after floral buds were cultured on medium containing 0.5 mg l-1 TDZ (85.83%).

We also showed that the effect of picloram concentrations interaction with TDZ concentrations on proliferation embryos percentage. The highest percentage of proliferation embryos were occurred on medium ranged from 1 mg l-1 to 15 mg l-1 picloram concentrations with medium containing 0.5 mg l-1 TDZ.

The result of the study showed that proliferation embryos percentage, from the immature inflorescence was significantly affected by the changing the medium composition. Picloram concentration residues significantly with another type of growth regulator enhanced embryos proliferation. Lin et al.2006,, conclude that TDZ is essential for inflorescence proliferation. Somatic embryo like structures have been observed on walnut immature cotyledons cultured on woody plant medium containing TDZ (0.11 or 1.1 mg/liter) and 2,4-dichlorophenoxyacetic acid (2,4-D) Neuman *et al*.1988. More recently, somatic embryogenesis was reported in watermelon (Compton and Gray, 1992) and muskmelon (Gray *et al*, 1992), again on medium with TDZ and 2,4-D.

The results showed that effects of picloram concentrations on the callus induction from explants were significantly different in Table 4. We observed some flower buds produced callus, the highest callus induction obtained in the high level of picloram at 15 mg l-1 (1.88 degree/jar). On the other hand, transferring the initial explants to medium containing TDZ at 1.0 mg l-1 often increasing callus (1.66 degree/jar). We also showed that the effect of picloram concentrations interaction with TDZ concentrations on callus degree, the highest callus degree were occurred on medium containing 10 mg l-1 with medium containing 1.0 mg l-1 TDZ(2.00 degree/ jar). Ahmed *et al*.2011 reported that, the callus initiation didn't occur without the growth regulators. Induction of somatic embryos on medium containing picloram has also been reported in many species (Castillo *et al*. 1998; Mendoza and Kaeppler, 2002; Preeti and Kothari, 2004).

Acknowledgements

I am grateful to Professor Ezz Gadalla for his help of Statistical analysis and his collaboration.

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Tables

Table (1): Effect of different Picloram concentrations on the Swelling and Browning rate (%) on the first stage.

Picloram (mg l^{-1})	Swelling%	Browning%
1	53.33	46.66
5	73.33	20.00
10	46.66	40.00
15	33.33	26.66
Mean	51.66	33.33
L.S.D. at 0.05	23.07	17.62

Table (2): Effect of auxin residues on number of direct somatic embryos after transferring to cytokinin concentration.

TDZconcentration+	Previous Treatment Picloram (B)				
0.1 mg l^{-1} NAA (A)	1 mg l^{-1}	5 mg l^{-1}	10 mg l^{-1}	15 mg l^{-1}	Mean
0.1 mg l^{-1}	1.00	1.00	3.66	3.00	2.16
0.5 mg l^{-1}	2.00	7.00	5.33	3.33	4.41
1.0 mg l^{-1}	1.00	5.33	4.00	3.00	3.33
Mean	1.33	4.44	4.33	3.11	
L.S.D. at 0.05	A = 0.68	B = 0.79		AB = 1.38	

Table (3): Effect of auxin residues on proliferation embryos percentage after transferring to cytokinin concentration.

TDZconcentration+	Previous Treatment Picloram (B)				
0.1 mg l^{-1} NAA (A)	1 mg l^{-1}	5 mg l^{-1}	10 mg l^{-1}	15 mg l^{-1}	Mean
0.1 mg l^{-1}	30.00	56.67	76.67	63.33	56.66
0.5 mg l^{-1}	73.33	93.33	93.33	83.33	85.83
1.0 mg l^{-1}	40.00	83.33	83.33	53.33	64.99
Mean	47.77	77.77	84.44	66.66	
L.S.D. at 0.05	(A) = 6.63	(B) = 7.65		AB = 13.26	

Table (4): Effect of auxin residues on callus initiation after transferring to cytokinin treatments

TDZconcentration+	Previous Treatment Picloram (B)				
0.1 mg l^{-1} NAA (A)	1 mg l^{-1}	5 mg l^{-1}	10 mg l^{-1}	15 mg l^{-1}	Mean
0.1 mg l^{-1}	1.00	2.00	1.00	2.33	1.58
0.5 mg l^{-1}	1.33	1.33	1.66	1.66	1.49
1.0 mg l^{-1}	1.66	1.33	2.00	1.66	1.66
Mean	1.33	1.55	1.55	1.88	
L.S.D. at 0.05	(A) = 0.58	(B) = 0.68		AB = 1.17	

Figures

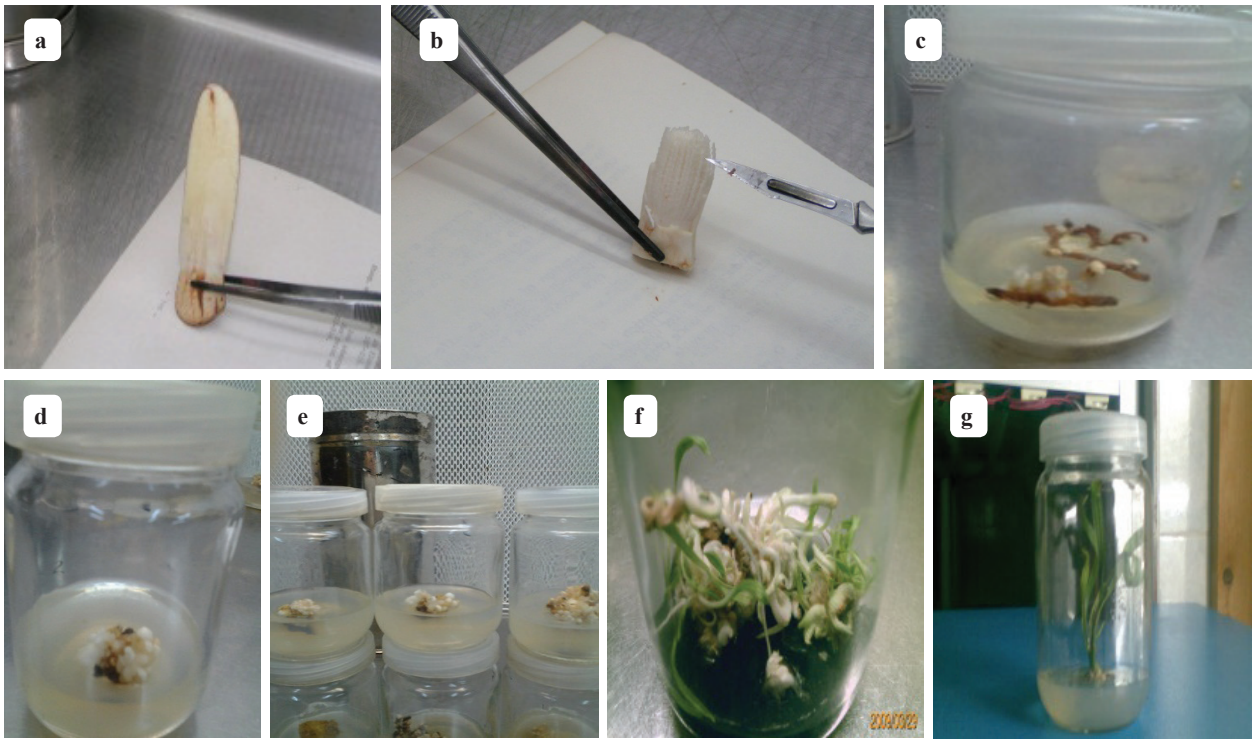

Fig. 1 a) The spathe from 7-10cm, b) Explant after remove the outer protective sheath, c) Swilling of explant, d) Initiation of Explant, e) Flower bud-like structures, f) Explant proliferation to embryos, g) Healthy plantlets