



IBA and TDZ Induced Plant Regeneration of Date Palm through Immature Female Inflorescence Culture

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ABSTRACT

The present work discussed the effectiveness of Indole butyric acid (IBA) and Thidiazuron (TDZ) on establishment of plant regeneration of immature inflorescence of female date palm cv. Sewi. MS medium supplemented with IBA at 1, 2 and 4 mg l⁻¹ in combination with TDZ at 0.5, 1 and 2 mg l⁻¹ was tested to investigate their effects on direct and indirect regeneration for 8 months (two month interval). An additional objective of this study was to investigate the effect of culture media components on growth and development of the different resulted aggregates from the previous experiments separately as follows: effect of cytokinin combination treatments on shoot multiplication of direct shoot buds, effect of culture media components on multiplication and germination of direct somatic embryos and finally effect of Paclobutrazol on growth and development of callus cultures. The results showed three responses depend on TDZ and IBA concentrations and combinations. The highest percentage of direct shoot buds and direct embryos formation on MS supplemented with 2 mg l⁻¹ IBA combined with 1 mg l⁻¹ TDZ while 4 mg l⁻¹ IBA combined with 0.5 or 1 mg l⁻¹ TDZ gave highest significant callus formation percentages. The best shoot buds multiplication achieved at 1 mg l⁻¹ BA and 1 mg l⁻¹ kinetin. Direct somatic embryos produced a significant large number of secondary somatic embryos on 1/2 MS medium containing 0.5 mg l⁻¹ kinetin and 0.25 mg l⁻¹ 2iP, the best germination was found on 1/2 MS medium supplemented with 0.1mg l⁻¹



NAA and 1g l^{-1} activated charcoal. pbz at 0.2 mg l^{-1} to callus culture medium enhanced significantly secondary somatic embryo formation, 0.3 mg l^{-1} pbz gave the highest callus fresh weight. Direct and indirect healthy shoots rooted in $1/2\text{ MS}$ supplemented with 1 mg l^{-1} IBA.

Key words: Date palm, Immature inflorescence and Plant regeneration.

Abbreviations: MS: Murashige and Skoog (1962) basal medium; PGRs: plant growth regulators; TDZ: Thidiazuron; IBA: Indole butyric acid; NAA: α -naphthalene acetic acid; BA: 6-benzylaminopurine; 2-iP: N6-2-Isopentenyladenine; Kin.: Kinetin; Pbz: Paclobutrazol; PVP: polyvinylpyrrolidone; AC: activated charcoal; ABA: Abscisic acid.

INTRODUCTION

Tissue culture is the most technology method to provide large-scale propagation of healthy true-to-type date palm (*Phoenix dactylifera* L.) plants. Micropropagation of date palm can be achieved through either somatic embryogenesis or direct organogenesis. Somatic embryogenesis is characterized by the development of a somatic cell into an embryo with a bipolar structure, leading to shoot and root formation. On the other hand, organogenesis is characterized by the direct formation of adventitious buds on the explant which has lower multiplication efficiency than somatic embryogenesis but it permits the preservation of true-to-type of multiplied plants (Mazri and Meziani, 2015). The organogenesis technique comprises four steps: initiation of vegetative buds, bud multiplication, shoot elongation, and rooting. The first step plays the important role in the process of propagation (Abahmane, 2011). Several explant sources have been used in tissue culture of date palm including shoot tip (Abd-El Kareim *et al.*, 2013), roots (Madboly, 2007), and inflorescences (Stino *et al.*, 2015). Immature Inflorescence explants represent a quick and safe method for micropropagation of date palms and were used as an alternative source of explants (Gadalla *et al.*, 2015). During starting stage of date palm micropropagation using inflorescence explants, different types of responses were noticed, (a) direct green shoots (Abul-Soad *et al.*, 2011) (b) direct embryogenic cells which will differentiate after maturation into direct somatic embryos or direct shoots (Ibrahim and Hassan, 2006; Kriaa *et al.*, 2007) and (c) unfriable callus (Feki and Drira, 2007). El



Hadrami *et al.*, (1995) recorded that *in vitro* plant regeneration of date palm occurs through organogenesis depending on genotypes and hormonal manipulation. Composition and relative concentration of PGRs determine both the ability of the explant to respond as well as the mode of the morphogenic reaction. Depending on PGRs composition of a medium, somatic embryogenesis, organogenesis or axillary buds development was observed in cultures of zygotic embryos of *Juglans regia* (**Fernandez *et al.*, 2000**). The aim of the current study was to determine the role of TDZ and IBA on induction of direct organs, direct somatic embryos and callus formation. An additional objective of this study was to investigate the effect of culture media components on growth and development of the of the previous three aggregates, separately.

MATERIAL AND METHODS

This study was conducted at the Central Laboratory for Date Palm Researches and Development, Agricultural Research Center, Giza, Egypt.

Plant preparation and sterilization

Spaths of female date palm tree cv. Sewi were removed from mother trees grown in the orchard of the Central Laboratory of Date Palm Researches and development in the period of late February by using tapestry knife, and transferred directly to laboratory. Spaths were rinsed under running tap water and liquid soap for half an hour. Surface sterilization in laminar air flow started by using 50% Clorox (sodium hypochlorite NaOCl at 5.25%) containing 2 drops of tween-20 for 10 minutes. After that the sterilized female spaths were opened and the spiklets were isolated and sterilized by immersion in 0.1% mercuric chloride (HgCl₂) solution for 5 minutes. Sterilized spiklets were then rinsed with sterilized distilled water three times.

Experiment 1: Effect of IBA and TDZ on percentage of direct shoot buds, direct somatic embryos and callus formation of Date Palm cv. Sewi

Sterilized spiklets divided into small pieces each piece containing 4-5 florets (Plate 1-a) and cultured on **Murashige & Skoog medium, 1962 (MS)** containing 40 g l⁻¹ sucrose, 100 mg l⁻¹ glutamine, 5 mg l⁻¹ thiamine HCl, 1 mg l⁻¹ biotin, 2 g l⁻¹ polyvinylpyrrolidone (PVP), solidified with gelrite at 2 g l⁻¹ and supplemented with indole butyric acid (IBA) at (1, 2



and 4 mg l^{-1}) in combination with thidiazuron (1-Phenyl-3-(1,2,3-thiadiazol-5-yl)-urea) (TDZ) at ($0.5, 1$ and 2 mg l^{-1}). The pH of culture medium was adjusted to 5.7 and dispensed on small jars 200 ml at rate of 30 ml per jar and autoclaved on 121°C at 15 lbs/in^2 for 20 minutes. Explants from each treatment were re-cultured on the same media composition every two month for four times. All cultures were maintained at $27\pm 2^{\circ}\text{C}$ under dark condition. Percentages of shoot buds formation, direct somatic embryos and direct callus formation were recorded after this period.

Experiment 2: Effect of cytokinin combination treatments on direct shoot bud multiplication

Direct shoot buds formed in different starting media were transferred to control medium (free plant growth regulators) for 1 month for obvious shoot appearance (Plate 1-c) then clusters containing 2-3 shoots were cultured on 3/4MS supplemented with the following cytokinin combinations:

1. 1 mg l^{-1} Benzyl adenine (BA)+ 1 mg l^{-1} kinetin
2. 1 mg l^{-1} BA+ 1 mg l^{-1} isopentenyl adenine (2ip)
3. 1 mg l^{-1} 2ip+ 1 mg l^{-1} kinetin

After 3 months (1 month interval) shoot number/ shoot, shoot length (cm) and vitrified shoot/explant were recorded

Experiment 3: Effect of culture media components on direct somatic embryos multiplication and germination

Clusters of direct embryos (3-4 embryos) (Plate 1-e) were transferred to control medium after starting stage for 1 month and after that cultured on 1/2 strength MS medium containing 0.25 mg/l ABA and the following supplements:

- E1- 0.1 mg l^{-1} NAA+ 1 g l^{-1} activated charcoal
- E2- 0.1 mg l^{-1} NAA + 0.05 mg l^{-1} BA + 1 g l^{-1} activated charcoal
- E3- 0.5 mg l^{-1} kinetin + 0.25 mg l^{-1} 2iP

Clusters of somatic embryos were continued in previous media for nine weeks with regular transfer to fresh medium of the same supplements every three weeks. Number of secondary embryos, germinated embryos/embryo and embryo length (cm) were recorded.



Experiment 4: Effect of Paclobutrazol on growth and development of callus cultures

Paclobutrazol (2S,3S)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)pentan-3-ol is a heterocyclic nitrogen-containing compound and a plant growth retardant and triazole fungicide.

The obtained embryogenic callus (Plate 1-g) weighed (0.5g) cultivated on 1/2 MS medium containing 0.1 mg l⁻¹ NAA, 0.05 mg l⁻¹ BA, 40 g l⁻¹ sucrose, 1g l⁻¹ activated charcoal (AC) and solidified with agar at 6.0 g l⁻¹. Paclobutrazol (Pbz) was employed at 0.1, 0.2, 0.3, 0.4 or 0.5 mg l⁻¹, to obtain more development and embryo formation. Embryogenic callus on the medium without Pbz was used as a control. Cultures were maintained at 27±2°C, under total darkness for 12 weeks (6 weeks interval). Callus fresh weight and number of normal and vitrified embryos were calculated after 12 weeks.

Root formation of direct and indirect shoots

According to Hassan *et al.*, (2013), healthy formed shoots (5-7 cm) were cultured on 1/2 MS with 1.0 mg l⁻¹ indole butyric acid (IBA), 40 g l⁻¹ sucrose, 2mg l⁻¹ glycine, 5 mg l⁻¹ thiamine HCl, 1 mg l⁻¹ biotin and 1g l⁻¹ AC for 6 weeks and incubated under light intensity 54 μmol m⁻² s⁻¹ for 16 hr. light / day to form primary roots which shorten to 1-1.5 cm in length and sub-cultured on half MS liquid medium containing 0.2 mg l⁻¹ NAA+ 0.1 mg l⁻¹ paclobutrazol dispensed into test tubes (2.5 × 25 cm) at rate of 20 ml and incubated under 81 μmol m⁻² s⁻¹ light intensity for 16 hr. light / day for six weeks to form adventitious roots before transplanting to greenhouse

Statistical analysis:

Each treatment of different experiments contained three replicates and each replicate contain five jars. Data obtained were subjected to the analysis of variances of randomized complete design as recommended by Snedecor and Cochran, (1980).

RESULTS AND DISCUSSION

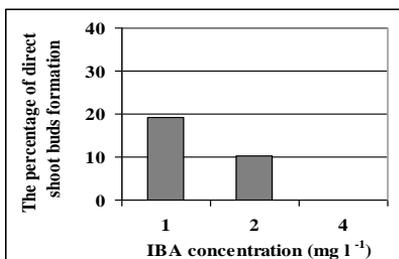
Experiment 1: Effect of IBA and TDZ on percentage of direct shoot buds, direct somatic embryos and callus formation of Date Palm cv. Sewi



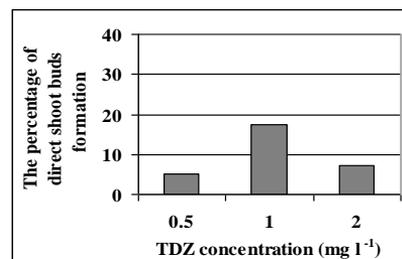
1- Effect of IBA and TDZ on percentage of direct shoot buds formation

a- Effect of IBA

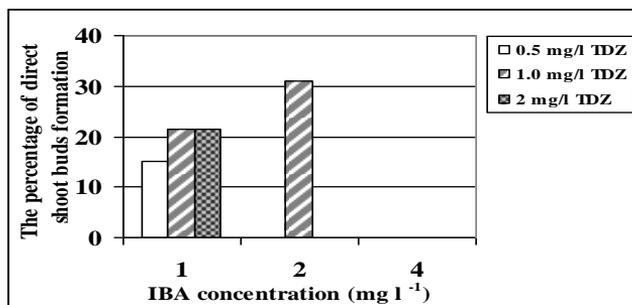
Data presented in **Fig. (1)** indicated that explants cultured on medium containing 1mg l^{-1} IBA statistically produced the highest percentage of direct shoot buds formation (19.27%), while higher concentration of IBA suppress completely shoot formation from immature inflorescence. According to **Al-Khateeb (2006)**, low PGRs concentrations promote the formation of buds while high concentrations induce abnormal growth without bud formation.



a- Effect of IBA



b- Effect of TDZ



c- Effect of interaction between IBA & TDZ

Fig. (1): Effect of IBA and TDZ on percentage of direct shoot buds formation

b- Effect of TDZ

Concerning TDZ, it is clear that 1mg l^{-1} statistically more responsible than 2 and 0.5mg l^{-1} as the percentages were (17.5, 7.13 and 5%, respectively).



c- Effect of interaction between IBA & TDZ

Interaction revealed that 2mg l⁻¹ IBA combined with 1mg l⁻¹ TDZ produced the highest significant percentage of direct shoot buds formation (Plate 1-b) compared with other combinations. In addition data clearly reflected that IBA at 4mg l⁻¹ was not suitable to form direct shoot in all tested concentration of TDZ. **Basalma *et al.*, (2008)** stated that frequency of shoot organogenesis may be increased with combinations of TDZ and IBA. Combinations of TDZ and IBA in the media revealed an efficient pathway for shoot proliferation in cotyledonary leaves of safflower. The use of IBA with TDZ might be best treatment to eliminate the secretion of phenolic substances and this effect might be also due to the oxidation of phenols by auxin oxidase. **Çöçü *et al.*, (2004)** stated that combination of TDZ and IBA induced shoot regeneration from cotyledonary nodes in *Calendula*.

2- Effect of IBA and TDZ on percentage of direct embryos formation

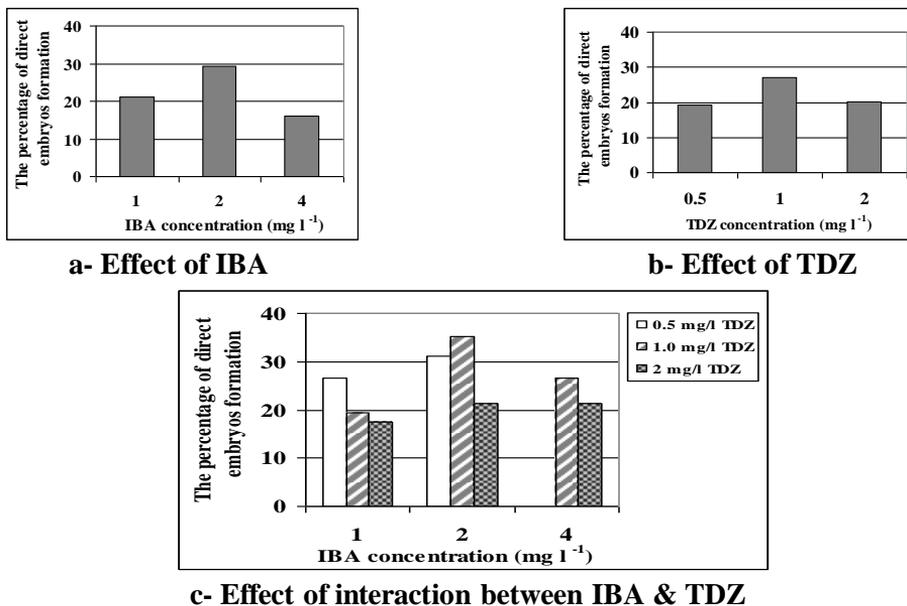


Fig. (2): Effect of IBA and TDZ on percentage of direct embryos formation of Date Palm cv. Sewi



a- Effect of IBA

Data in **Fig. (2)** revealed that the highest significant average percentage of explants producing direct embryos resulted from 2mg Γ^{-1} IBA (29.23%) followed significantly by 1mg Γ^{-1} was (21.17%), while the lowest significant percentages of explants producing direct embryos (16%) was noticed in 4mg Γ^{-1} IBA.

b- Effect of TDZ

With respect to TDZ factor, data showed that the addition of 1mg Γ^{-1} TDZ to induction medium gave the highest significant average percentage of direct embryo (27.10%) than other concentrations. These results are in accordance with observations of **Kahia *et al.*, (2016)** who reported that, cytokinins strongly enhanced induction and regeneration of somatic embryos from leaves derived from *in vitro* germinated seedlings of a *Coffea arabica* especially TDZ.

c- Effect of interaction between IBA & TDZ

With respect to the interaction, data show that the highest percentage of direct embryos (Plate 1-e) appeared with 2mg Γ^{-1} IBA combined with 1 mg Γ^{-1} TDZ (35.2 %) followed significantly by medium supplemented with 2mg Γ^{-1} IBA combined with 0.5 mg Γ^{-1} TDZ (31.1). On the other hand culture medium containing 4mg Γ^{-1} IBA with 0.5 mg Γ^{-1} TDZ failed to form any direct embryos. These results are in agree with those obtained by **Ghaniti *et al.*, (2010)** who mentioned that the optimization of *in vitro* somatic embryogenesis development depends on the right balance or the ratio of plant growth regulators.

3- Effect of IBA and TDZ on percentage of callus formation

Data in **Fig. (3)** revealed the effect of IBA and TDZ on callus formation percentage from sewi immature inflorescence.

a- Effect of IBA:

Respecting to IBA, it is clearly that the addition of 4mg Γ^{-1} IBA in respective order gave the highest significant callus formation percentage followed by 2 and 1mg Γ^{-1} with significant differences (33, 27 and 22.5%).

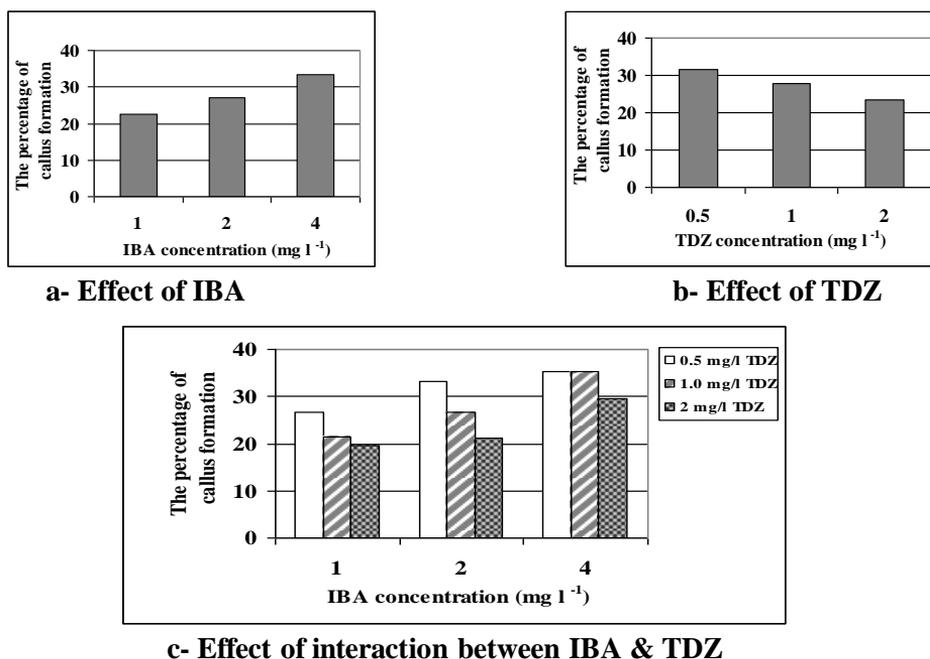


Fig. (3): Effect of IBA and TDZ on percentage of callus formation of Date Palm cv. Sewi

b- Effect of TDZ:

Referring to TDZ, data showed that, the highest significant percentage was observed at 0.5 mg l⁻¹ (31.7%) reduced gradually by increasing concentration to 1 and 2 mg l⁻¹ (27.73 and 23.37%, respectively).

c- Effect of interaction between IBA & TDZ

Culture media containing 4mg l⁻¹ IBA combined with 0.5 or 1mg l⁻¹ TDZ were most effective media to produce the highest significant percentages of callus formation (35.2%) (Plate 1-g). While the lowest significant percentage (19.5%) was noticed with culture medium containing 1mg l⁻¹ from IBA with 2mg l⁻¹ from TDZ.

Experiment 2: Effect of cytokinin combination treatments on direct shoot buds multiplication

Data in Table (1) and Plate (1-d) showed that the best shoot numbers was achieved on culture medium supplemented with 1 mg l⁻¹ BA



and 1mg l^{-1} kinetin (28.8 shoots) followed significantly by medium containing 1mg l^{-1} BAP and 1mg l^{-1} 2iP which yielded an average of 21 shoot numbers per explant after 2 months, while clear reduction in shoot numbers was observed when culture medium containing 1mg l^{-1} kinetin and 1mg l^{-1} 2iP which resulted the highest number of vitrified shoots 4.9 and the tallest shoots 2.6 cm, least 1.6cm shoot elongation and vitrified shoot 2.9 were found on medium containing 1mg l^{-1} BA and 1mg l^{-1} Kinetin. Our results are in accordance with observations of a number of researchers who mentioned that *in vitro* shoot buds multiplication is mainly phytohormone dependent. Cytokinins are generally known to reduce the apical meristem dominance

Table (1): Effect of cytokinin combination treatments on shoot number, shoot length and vitrified shoot numbers

cytokinin combination treatments				Shoot number	Shoot length (cm)	Vitrified shoot
BA	Kinetin	2ip	mg l^{-1}			
1	1	0		28.8	1.6	2.9
1	0	1		21.0	1.8	3.2
0	1	1		16.7	2.6	4.9
LSD at 0.05				2.2	0.3	0.5

and induce both axillary and adventitious shoots formation from meristematic explants (Madhulatha *et al.*, 2004). Khan and Bi, (2012) BA and Kinetin resulted in maximum shoot bud proliferation of date palm (cv. Dhakki) and significant number of direct shoot regeneration. cytokinins enhanced shoot proliferation due to their possible role in cell division and inhibition of apical dominance. Kathal *et al.*, (1988) reported more effective shoot proliferation in the presence of more than one type of cytokinin.

Experiment 3: Effect of culture media components on direct somatic embryos multiplication and germination

Data in Table (2) showed the effect of different culture media (E1, E2 and E3) on secondary embryo numbers (Plate 1-f), germinated embryo numbers and germinated embryo length after three re-cultures. It is clearly



that E3 proved to be highly effective for producing a significant large number of somatic embryos compared to the other media. Respecting to germinated embryo numbers, data reflected that somatic embryo germination and plantlet conversion usually take place in culture medium free cytokinin (E1) which was found to be more active as compared to E2 and E3 in SEs germination.

Table (2): Effect of culture media on multiplication and germination of direct and indirect embryos

Culture medium	Secondary embryo number	Germinated embryo number	Germinated embryo length (cm)
E1	37	27	4.12
E2	52	17	4.38
E3	67	12	2.50
LSD at 0.05	2.8	2.1	0.25

The highest significant value of germinated embryo length Table (2) was noticed with E2 (4.38cm) meanwhile reduced significantly to (4.12 cm) with E1, while E3 produced the lowest significant one (2.5 cm). The optimization of *in vitro* somatic embryogenesis development depends on the right balance or the ratio of plant growth regulators (**Ghaniti *et al.*, 2010**)

Zouine and Hadrami, (2007) found that, 0.05 mg l⁻¹ BAP on embryogenic suspension culture of date palm could be useful in terms of germination percentage of somatic embryos. **Hassan *et al.*, (2013)** reported that shoot formation of date palm different cultivars was best in culture medium containing 0.1 mg l⁻¹ NAA + 0.05 mg l⁻¹ BA+ 1.0 g l⁻¹ AC.



Experiment 4: Effect of Paclobutrazol on growth and development of callus cultures

Table (3): Effect of Paclobutrazol on callus development

Pbz conc. mg l ⁻¹	Callus fresh weight (g)	Somatic embryo number	Vitrified embryos
0.0	1.71	9	13
0.1	2.31	17	8
0.2	2.92	27	6
0.3	4.8	21	4
0.4	4.4	16	3
0.5	3.53	11	3
LSD at 0.05	0.16	2	2

Table (3) and (Plate 1-h) cleared that 0.3 mg l⁻¹ Pbz gave the highest callus fresh weight (4.8 g). Higher concentrations of Pbz reduced fresh weight but still be better than control treatment. Meanwhile the addition of Pbz at 0.2 mg l⁻¹ enhanced significantly somatic embryo number (Plate 1-i) in relation to the other concentration. Culture medium with Pbz at 0.5, 0.4 or 0.3mg l⁻¹ minimized significantly vitrified embryo. While using control treatment and 0.1 mg l⁻¹ pbz enhanced significantly vitrified embryo numbers. **Chen *et al.*, (2005)** reported that application of Pbz enhanced the formation and proliferation of meristematic clusters of Daylily (*Hemerocallis* spp.). Pbz at 0.33, 1.0 or 3.0 mg l⁻¹ increased somatic embryo growth of Loblolly pine (*Pinus tadea* L.) genotypes except one genotypes at 0.33 mg l⁻¹ Pbz that was similar to the control (**Pullman *et al.*, (2005)**). **Ibrahim *et al.*, (2011)** found that ABA and Pbz at 0.25 or 0.5 mg l⁻¹ stimulated germination of date palm somatic embryos.



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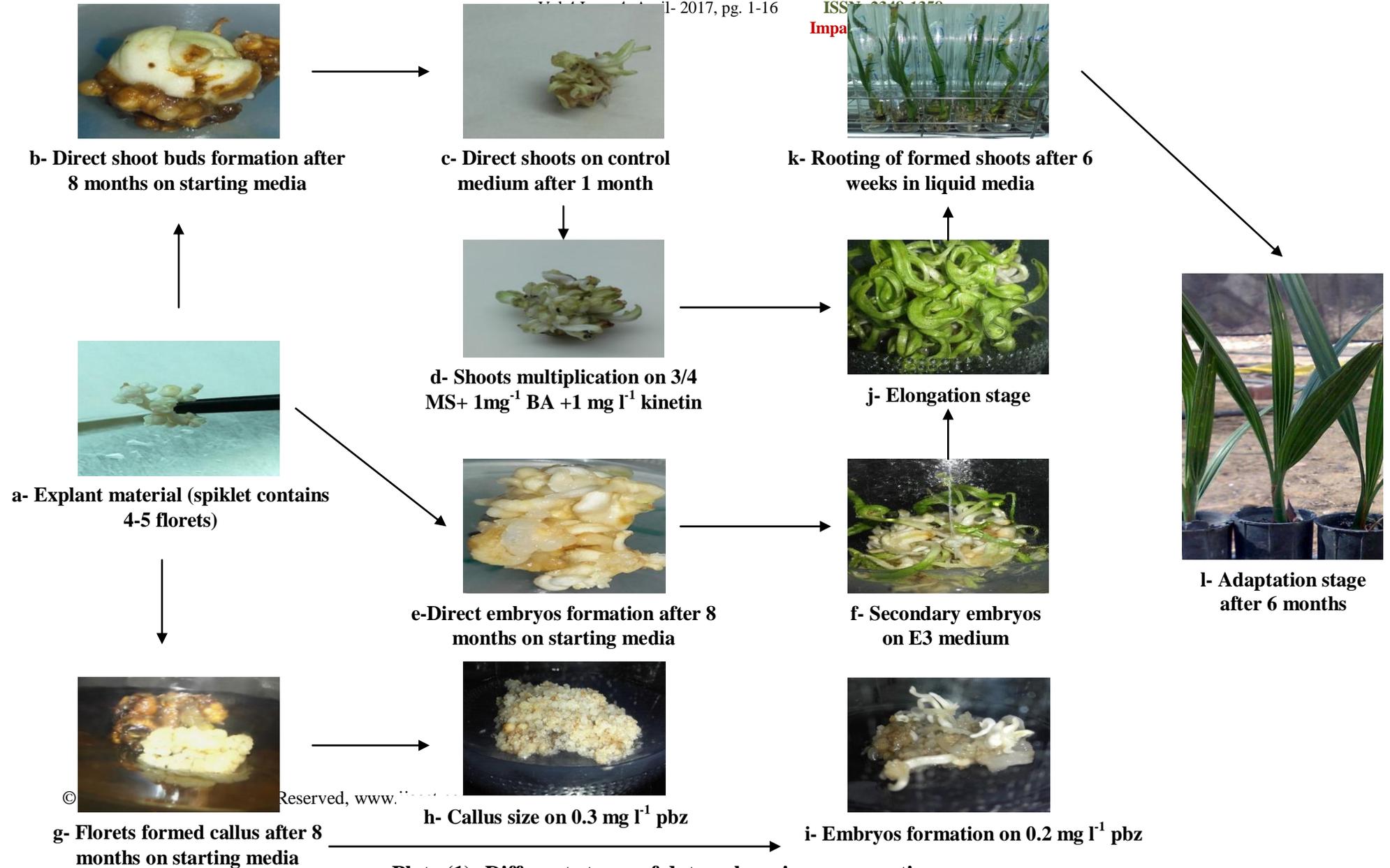


Plate (1): Different stages of date palm micropropagation



Conclusion

The present work represented an efficient protocol for *in vitro* regeneration of date palm cultivar Sewi immature female inflorescence using TDZ and IBA.

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