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Plant Regeneration from Mature Inflorescence of Date Palm by Using *Moringa* Extract

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Authors' contributions

This work was carried out in collaboration among all authors. Author A designed the study, performed in vitro work. Author B. wrote the study, statistical analysis, managed the literature searches. Author C preparation the inflorescence stock.

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ABSTRACT

Plant tissue culture is becoming an important technique for the massive propagation of date palm and the available source lead to the success of this process. The mature female inflorescence is a novel and available source for in vitro propagation of date palm, nowadays become needed at the large scale to usage of organic nature materials in agriculture practice which are rich in plant growth regulators as well as micro elements. This study describes in vitro plant regeneration from mature female inflorescence explants of evaluating date palm (*Phoenix dactylifera* L.) by using the methanol extract of *Moringa oleifera* leaves which has great potential to be used as a natural source of nutraceutical in micropropagation. Mature inflorescences explants were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of an extract of *Moringa* leaves with or without add TDZ at 0.2 and 0.6 BA mg/l. Indoles, phenols, proteins, amino acids, and carbohydrates were determined. The addition of different concentrations of an extract of *Moringa oleifera* leaves with TDZ at 0.2 and 0.6 BA mg/l gave the highest significant average percentage of callus formation, friable callus, germination of embryos and numbers of embryos comparing with other treatments. Also, given the highest significant values of free amino acids and carbohydrates were noted in callus and embryo germination stage.

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1. INTRODUCTION

Date palm (*Phoenix dactylifera* L.) the dioecious, monocotyledon species belonging to the family Arecaceae cultivated for its edible sweet fruit, the species is widely cultivated and is naturalized in many tropical and subtropical regions worldwide, only 50% of seedlings will be female and hence fruit bearing, and dates from seedling plants are often smaller and of poorer quality, otherwise many fruits seedlings trees cultivated in most areas that have good fruit characteristics, it may be concerning to the nearest cultivar find in this area [1], thus commercial plantations use cuttings (offshoots), plants grown from cuttings will fruit 2–3 years earlier than seedling plants, cuttings propagation has limitations such as slow propagation rate, limited number of cuttings for a certain period in the life time of a young palm tree [2,3,4], in addition propagation by offshoots led to lose the plant, Suckers or offshoots were produced during juvenile life cycle small numbers, to produce true-to-type progeny [5], for there the new method was found to get over problem, the inflorescences technique allow to undergo either in vitro somatic embryogenesis or organogenesis [6] and derived plants have shown normal growth and no abnormalities [7], moreover, Abul-Soad [8] proved that can be accomplished in a short time with minimal effort as compared to the traditional practice of using shoot-tip explants and excise the immature inflorescence without damage to the mother tree. In fact plant growth regulators have a particular factor on tissue culture success which involved in the regulation of cell division, tissue and organ differentiation [9], Auxin and cytokinin are crucial role to regulation of organ regeneration, and the concentration ratio between these hormones is critical for determine specific organogenesis processes (Su et al., 2011), naphthalene acetic acid (NAA), Picloram, Dicamba, 2,4,5-tricholorophenoxy acetic acid (2,4,5-T) and endogenous hormone metabolism play a key role in somatic embryogenesis in different plant species [10], Thidiazuron (N-phenyl-N-1,2,3-thiadiazol- 5- yl (urea) has successfully in vitro to induce adventitious shoot formation and axillary shoot proliferation [11], TDZ produces different regeneration responses depending on genotype and the starting explant [12], furthermore, TDZ involved in the synthesis of auxin by increasing the levels (IAA) [13], Zeatin is one of the most common forms which is naturally occurring cytokinin in plants which plays an important role in cell division and cell elongation that led to promote the growth of plants as well as has anti-aging potential and protective effects in plants [14], cytokinin promotes plant growth and has anti-aging potential also protective effects in plants [15]. Nowadays the workers research about natural product biochemical in plants found to be rich in nutrients and growth regulating hormones as extracts from these products, *Moringa oleifera* leaf extract (MLE) riched in amino acids, K, Ca, Fe, ascorbate and glutathione, and growth regulating hormones as zeatin which can be used as natural source of cytokinin that used as plant growth enhancer [16, 17,18] also *moringa* leaf extract rich in, carotenoids, phenols, potassium and calcium, so play as a plant growth promoting [19,20], Cytokinins can be enhancing food production and involved in cell growth and differentiation, zeatin is one form of the most common cytokinin forms naturally in plants, *Moringa* leaves were found to have high zeatin concentrations of between 5 µg and 200 µg/g of leaves [21], furthermore *moringa* leaf extract contain proteins, vitamins (such as A, B1, B2, B3, ascorbic acid and E), β carotene, amino acids phenolic compounds, sugars, and minerals (such as calcium, magnesium, sodium, iron, phosphorus and potassium) and several flavonoid pigments [22,23]. So the current study was conducted to test the effect of *moringa* leaf extract and different growth regulators to stimulate mature date palm inflorescence for produce valuable plants from valuable evaluated seedling.

2. MATERIALS AND METHODS

Mature inflorescences were collected at 1-15April 2017 from adult female mother palm from North Delta Egypt which previous evaluated [1] for used as explant *in vitro* culture in the Central Lab. For Research and Development of Date Palm at 2017-2018. Mature inflorescences were treated with different medium supplemented as *Moringa* extract and TDZ to enhancing for produced callus and embryos. The female inflorescences were removed with protective sheath (spathe) intact and refrigerated at 4C° until used.

2.1 Sterilization

Spathe of date palm were sterilized with washing tap water and soap careful, explants were surface-sterilized by using sodium hypochlorite

5.25% (Clorox) for 20 min., followed by three independent rinses each in sterile distilled water under aseptic conditions. Remove the outer sheath and base sliced the inflorescence horizontally into 2–3 cm segments, and each explant was cultured horizontally with a good contact with the surface of establishment media. *Moringa oleifera* leaf contents as Table 1.

2.2 Preparation of *Moringa oleifera* Extract

20 g of young *moringa* leaves was mixed with 675 ml of 80% ethanol as suggested by (Makker and Becker 1996), the suspension was stirred using a homogenizer to help maximize the amount of the extract. The solution was then filtered by wringing the solution using a mutton cloth. The solution was re-filtered using No. 2 Whatman filter paper. Using a method developed by Fuglie [30], the extract was diluted with distilled water at a 1:32 ratio (v/v), the extract was used within five hours from cutting and extracting (if not ready to be used, the extract or the solution prepared was stored at 0ºC and only taken out when needed for use).

2.3 Establishment Medium

The basal cultured medium consists of MS medium and vitamins [31] as establishment medium + 30 g/l sucrose + 200 mg/l glutamine + 170 mg/l KH2PO4. H2O + 100 mg/l myo-inositol + 40 mg/l adenine sulphate + 4 mg/l thiamine HCl + 5 mg/l biotin + 1 mg/l pyridoxine + 6 g/l gerlite. Adjust pH medium at 5.7 ± 0.1 by using KOH or HCl diluted solutions the medium was poured in jars at 40ml for each one and autoclaving at 121ºC for 20 minutes. the cultures were incubated in the darkness rooms at 27± 2Cºand transferred to fresh media every 8 weeks

Treatments

M0 - (Control treatment) as establishment medium
M1 - 10 ml/l *Moringa* leaf extract (MLE)
M2 - 15 ml/l *Moringa* leaf extract (MLE)
M3 - 20 ml/l *Moringa* leaf extract (MLE)
M4- 10 ml/l *Moringa* leaf extract (MLE)+ 0.2 mg/l TDZ + 0.6 mg/l BA (Zayed 2011)
M5 - 15 ml/l *Moringa* leaf extract (MLE) + 0.2 mg/l TDZ + 0.6 mg/l BA
M6 - 20ml/l *Moringa* leaf extract (MLE) + 0.2 mg/l TDZ + 0.6 mg/l BA

After this the forming somatic embryos were cultured on establishment medium supplemented with 0.1 mg/l NAA for shooting induction.

Following data were estimated at the end of experiment Browning degree was scored visually as [32] as follows

1-Negative results
2- below average
3- average
4- good

Callus formation percentage after 4-5 subculture

Friable callus percentage after 4-5 subculture

Numbers of embryo Fresh and dry weight of callus and embryos

2.4 Chemical Contents of Callus and Embryo

Explants were taken from callus and embryos to determination of Indoles as Larsen et al. [33] Phenols as A.O.A.C. [34] Proteins as Bradford [35]. Amino acids Moore and Stein [36]. Carbohydrates as Dubois et al. [37].

2.5 Experimental Design

Complete randomized block design with three replicates and three plantlets for each one, two growth seasons. Data were analyzed by analysis of variances (ANOVA) and the means were compared following t- test using L.s.d. values at 5% level [38].

3. RESULTS AND DISCUSSION

3.1 Morphology Attributes

The segments of mature date palm inflorescences were inoculated in a different culture medium supplemented with *moringa* leaf extract MLE to form callus showed apparent different degrees of browning, the establishment medium free hormone supplemented with 20 ml of MLE produced high degree of browning 3.0 followed by 15 and 10 ml of MLE (Table 2 and Fig 1) compared to control treatment, in spite of this results from medium free hormone, the browning degree was achieved from medium supplemented with MLE with 0.2 mg/l TDZ + 0.6 mg/l BA showed significant results 3.3 degree under 15 and 20 ml MLE without significant

differs in between, generally the establishment medium supplemented with 0.2 mg/l TDZ + 0.6 mg/l BA and different levels of *moringa* leaf extract MLE bringing highest browning 3.2 degree, in this concern, MS medium containing BAP and TDZ (Thidiazuron) caused browning of the Eucalyptus clones (Sappi and Mondi, South Africa) callus was observed in all cultures [39], *Phoenix dactylifera* L. callus induction was obtained with 5 mgl-1 BAP or with 5 mgl-1 TDZ and browning was recorded [40], browning was severely observed on the culture containing 10 µM and 15µM TDZ and 100% induction callus of Cynometra *cauliflora* after two weeks [41].

Formation percentage of callus from date palm inflorescence (Table 2) induced maximum percent under medium supplemented with MLE gradually from 10, 15 and 20 ml/l + 0.2 mg/l TDZ + 0.6 mg/l BA as follows 3.0,3.3 and 3.3 respectively, on the other hand medium with MLE and free hormone had attained lowest percentages of callus formation, Plant growth regulator with *moringa* leaf extract MLE induced significant high percent of callus formation upon medium free hormone, Abahmane (2011) and Roberto et al. [42] on strawberry and blueberry stated that 0.2 mg/l BA for callus formation from inflorescences date palm, 3 mg/l BAP, and 3 mg/l 2-iP caused initiation callus of date palm cv. Dhakki [43].

Attributed to medium free hormone supplemented with MLE on the average friable callus percentage that recorded significant results 50 and 49% respectively under control treatment and 10 ml/l MLE upon 15 and 20 ml/l which created 47.0% without significant differs in between, establishment medium containing 0.2 mg/l TDZ + 0.6 mg/l BA with 20 ml/l MLE bring out great average friable callus percent 70.7% comparing 50% for control treatment, Zayed [44] and Zayed and Ola [45] proved that callus of date palm inflorescences was induction produced by 1 mg/l TDZ.

Table 1. *Moringa oleifera* leaf contents

Total soluble protein (mg-1 g)	**1.40**	**Super oxide dismutase (SOD) EC number (1.15.1.1)**	**191.86 (IU min -1 mg -1 protein)**
Peroxidase (POD) EC number (1.11.1.7)	21.99 (IU min -1 mg-1 protein)	Catalase (CAT) EC number (1.11.1.6)	7.09 (IU min -1 mg -1 protein)
Total phenolic contents (mg g -1 GAE)	8.19	Ascorbic acid (m mole g -1)	0.36
Elements			
Nitrogen (%)	1.933	Phosphorus (%)	0.180
Potassium (%)	2.187	Calcium (%)	2.433
Magnesium (%)	0.012	Zinc (mg kg -1)	38.333
Copper (mg kg -1)	3.500	Iron (mg kg -1)	544.000
Manganese (mg kg -1)	49.667	Boron (mg kg -1)	21.333

Total soluble proteins (Bradford 1976); Enzymatic antioxidants catalase (CAT), peroxidase (POD) as [24]; Superoxide dismutase (SOD) [25]; Total phenolics [26]; Ascorbic acid [27]; Moringa oleifera leaf total nitrogen as [28]; Phosphorous, potassium, calcium, magnesium, copper, iron, manganese and zinc as (Ryan et al.*, 2001); Boron as [29]*

Table 2. Different medium affected morphology characteristics of date palm tissues *in vitro*

Treatments	**Browning degree**	**Callus formation %**	**Friable callus %**	**Numbers of embryos**	**Germination %**
M0	2.0	53.3	50.0	7.7	55.8
10 ml MLE (M1)	2.0	52.0	49.0	6.3	47.6
15 ml MLE (M2)	2.3	50.0	47.0	5.3	43.3
20 ml MLE (M3)	3.0	50.0	47.0	5.3	43.3
0.2 mg/l TDZ + 0.6 mg/l BA +10 ml MLE (M4)	3.0	67.3	69.0	16.3	65.6
0.2 mg/l TDZ + 0.6 mg/l BA +15 ml MLE (M5)	3.3	68.3	69.3	17.7	73.4
0.2 mg/l TDZ + 0.6 mg/l BA +20 ml MLE (M6)	3.3	69.3	70.7	18.3	76.5
L.s.d.	= 0.1	= 3.8	= 2.8	= 0.7	= 3.0

Can be notice from (Table 2) that the numbers of embryos under medium free hormones with different levels of MLE gave the fewer numbers of embryos in comparable with control treatment 7.7 embryos, the levels 15 and 20 ml/l MLE have left the significant little numbers 5.3 embryos without differs in between, furthermore the medium supplemented with different levels of MLE and hormones revealed the highest significant numbers of embryos as the treatment number (6) which gave significant numbers 18.3 embryos than treatment number (5) which produced 17.7 embryos, somatic embryo induced by short time treatment of TDZ [46], direct globular somatic embryo produced by thidiazuron (TDZ) alone or in combination with 2,4-D Sidky and Zaid [47], Somatic embryos of *Elaeis guineensis* (Jacq.) were found with BA supplemented medium Madhavan et al. [48], TDZ 0.1 mg/L produced somatic embryo of *Phoenix dactylifera* L. cv Hellawi., with auxin [49], direct embryogenesis induced by TDZ+BAP in culture of cotyledonary nodes of oil palm [50], highest percentage of embryogenic calli of Jojoba (*Simmondsia chinensis*) derived by using 1 or 4 mg L–1 TDZ containing medium Amal El Ashry et al. [51].

Percent of germination are provided in (Table 2) get higher under treatment 10 ml/l MLE (M1) 47.6% upon control treatment 55.8 % and the other levels of MLE 43.3 and 43.3 respectively without differs in between, medium supplemented with MLE and 0.2 mg/l TDZ + 0.6 mg/l BA take place the great percent of germination 65.6, 73.4 and 76.5 in the same order of M4, M5 and M6 compared of control treatment, low levels of TDZ induce the axillary shoot proliferation promote callus and somatic embryo formation [52,11], TDZ treatment produces different regeneration responses depending on genotype (Passey et al., 2003), 1 mg/l thidiazuron (TDZ); 0.5 mg/l 1, 6-benzoaminopurine (BAP); or 1 mg/l BAP, all plant growth regulator (PGR) occurred direct adventitious bud formation from the inflorescences of *Salix nigra* Marsh Satulyyra et al. [53], TDZ (10-10, 10-9 or 10-8 M) caused increasing somatic embryo germination of *Solanum tuberosum* [54], TDZ and BAP induced higher numbers of shoot per explant [55,56] on *Phalaenopsis aphrodite*), TDZ have the same effects of auxin and cytokinin that can be formation callus and shoot [57].

Based on the results of medium free hormones supplemented with MLE at different levels 10,15 and 20 ml/l on the fresh weight of date palm callus (Table 3).

which presented heavy callus under control treatment 0.55 g followed by different levels of MLE 0.33, 0.32 and 0.32 g respectively, moreover highest significant fresh weight recorded from medium supplemented by different levels of MLE 10,15 and 20 ml/l with 0.2 mg/l TDZ + 0.6 mg/l BA which produced 2.1,2.3 and 2.4 g respectively comparing control treatment, embryo fresh weight were decreased under all medium free hormones M1 ,M2 and M3 while fresh embryo weight was significantly highest under establishment medium + 0.2 mg/l TDZ + 0.6 mg/l BA + different levels 10,15 which haven't significant differences in between 3.8 g and 20 ml/l of MLE 3.9 g, same trend of embryo fresh weight was found with dry weights of embryo, results of callus fresh and dry weights explain the activity of callus to development as embryos and consumed the components for this goal.

Table 3. Fresh and dry weights of date palm callus and embryo *in vitro* as affected by different medium

Treatments	Callus fresh weight (g)	Callus dry weight (g)	Embryo fresh weight (g)	Embryo dry weight (g)
M0	0.55	0.24	0.31	0.14
10 ml MLE (M1)	0.33	0.14	0.29	0.12
15 ml MLE (M2)	0.32	0.14	0.28	0.11
20 ml MLE (M3)	0.32	0.12	0.28	0.10
0.2 mg/l TDZ + 0.6 mg/l BA +10 ml MLE (M4)	2.1	0.97	3.8	1.6
0.2 mg/l TDZ + 0.6 mg/l BA +15 ml MLE (M5)	2.3	0.99	3.8	1.7
0.2 mg/l TDZ + 0.6 mg/l BA +20 ml MLE (M6)	2.4	1.0	3.9	1.8
L.s.d.	= 0.5	= 0.2	= 0.9	= 0.3

From the previous results it can summarize the effects of medium supplemented by different levels of moronga leaf extract and 0.2 mg/l TDZ + 0.6 mg/l BA on the efficiency of in vitro tissue culture of mature date palm inflorescences, the analysis of variance revealed that all levels of *moringa* leaf extract with hormones were significantly increasing browning degree as well as enhancing the average callus formation percentage, average friable callus percentage, average numbers of embryos, average germination percent and fresh and dry weights of callus and embryo upon the medium supplemented *moringa* leaf extract free hormones also checked the chemical contents of callus and embryos (Table 4) that demonstrated the callus tissues found on the medium supplemented with different levels of *moringa* MLE contain the lowest quantity of indoles 0.15 mg/g f.w. related to 15 and 20 ml/l MLE without significant differences in between comparing 0.18 mg/g f.w. to control treatment, results on embryo indoles contents seems to have same tendency which contains little quantity than control treatment 0.23 mg/g f.w., medium supplemented with different levels of *moringa* leaf extract MLE + 0.2 mg/l TDZ + 0.6 mg/l BA displayed little significant contents of indoles in the callus tissues than control treatment, meanwhile highest significant indoles contents come out in the embryo tissues under medium supplemented with hormones, medium with 20 ml/l MLE with hormones contains 2.9 mg/g f.w. followed by 15 and 10 ml/l MLE 1.3 and 1.2 mg/g f.w. respectively,

Phenols contents (Table 4) of callus and embryo tissues on the medium free hormones with all *moringa* leaf extract MLE, increasing phenols contents on medium with 10 and 15 ml/l MLE haven't significant differs in between 0.8 mg/g f.w. increasing phenols contents found under 20 ml/l MLE for callus tissues while in case of embryo tissue decreased phenols under callus tissues, the treatment 15 and 20 ml/l MLE haven't variance 0.6 mg/g f.w. upon control treatment 0.4 and 0.5 mg/g f.w. respectively for callus and embryo, phenols callus contents under MLE levels and hormones have increasing phenols contents comparing to control treatment, whereas embryo tissue under 10 and 15 ml/l MLE with hormones take place insignificant differs in between of phenols 0.5 mg/g f.w., the level 20 ml/l MLE + 0.2 mg/l TDZ + 0.6 mg/l BA recorded 0.6 mg/l phenols comparing control treatment. Previous results indicated insignificant differences between medium free hormone or medium supplemented with hormones on the phenols contents of callus and embryo tissues.

Regarding to protein contents in the two tested tissues callus and embryo (Table 4) two tested medium MLE with or without hormones cleared decreasing effect of proteins contents for different tissues callus and embryo comparing to control treatment which have significant contents of proteins 2.5 mg/g f.w. for callus and 2.9 mg/g f.w. for embryo tissue, on the other hand the embryo tissues contain highest protein quantity 1.3,1.3 and 1.4 respectively for 10, 15 and 20 ml/l MLE + hormones upon the callus contents 0.78,0.82 and 0.95 mg/g f.w. in the same order of 10,15 and 20 ml/l MLE + hormones, additionally callus and embryo tissue contain lowest significant quantity of proteins than control treatment on the medium free hormones. Results on the proteins contents of callus tissue showed decreasing contents that's it may be consume for synthesis and structure of enzymes, hormones, plasma membranes and cell wall [58,59].

Results of amino acids callus and embryo tissues (Table 4) presented lowest contents of amino acids than control treatment 1.8 mg/g f.w. for callus and 2.4 mg/g f.w. for embryo tissue on the medium free hormones, the levels 10,15 and 20 ml/l MLE produced 1.6, 1.5 and 1.4 mg/g f.w. respectively for callus tissues and 2.2,2.3 and 2.1 mg/g f.w. respectively for embryo tissues, furthermore medium with different levels of MLE 10,15 and 20 ml/l + 0.2 mg/l TDZ + 0.6 mg/l BA produced highest contents of amino acids for two tested tissues, largest significant contents obtained by 20 ml/l MLE + hormones as 5.4 mg/g f.w. for callus and 8.2 mg/g f.w. for embryo tissue.

Different tested medium affected contents of carbohydrates of callus and embryo (Table 4) significant reduction was found under medium free hormones supplemented with *moringa* leaf extract MLE in comparable of control treatment which derived the highest carbohydrates contents of callus and embryo 2.7 and 2.9 mg/g d.w. in contrast with medium supplemented with levels of MLE + 0.2 mg/l TDZ + 0.6 mg/l BA, the level 20 ml/l MLE + hormones accord the great significant contents of carbohydrates 4.6 mg/g d.w. for callus and 3.5 mg/g d.w. for embryo above the control treatment 2.7 mg/g d.w for callus and 2.9 mg/g d.w. for embryo. Swamy et al. [60] stated that 20% banana extract 10% tomato extract, 10% carrot extract and 10% papaya extract increased total proteins and total

Table 4. Different medium affected chemical contents of date palm tissues *In vitro*

Treatments	Mg/g f.w.								Mg/g d.w	
	Indoles		Phenols		Proteins		Amino acids		Carbohydrates	
	callus	embryo	callus	embryo	callus	embryo	callus	embryo	callus	embryo
M0	0.18	0.23	0.4	0.5	2.5	2.9	1.8	2.4	2.7	2.9
10 ml MLE (M1)	0.16	0.21	0.8	0.5	0.81	1.1	1.6	2.2	2.5	2.3
15 ml MLE (M2)	0.15	0.21	0.8	0.6	0.81	1.1	1.5	2.3	2.4	2.3
20 ml MLE (M3)	0.15	0.19	0.9	0.6	0.80	1.0	1.4	2.1	2.4	2.5
0.2 mg/l TDZ + 0.6 mg/l BA +10 ml MLE (M4)	0.09	1.2	0.6	0.5	0.78	1.3	4.9	4.9	2.9	3.0
0.2 mg/l TDZ + 0.6 mg/l BA +15 ml MLE (M5)	0.1	1.3	0.8	0.5	0.82	1.3	2.4	4.9	3.1	3.1
0.2 mg/l TDZ + 0.6 mg/l BA +20 ml MLE (M6)	0.2	2.9	0.85	0.6	0.95	1.4	5.4	8.2	4.6	3.5
L.s.d.	= 0.1	= 0.3	= 0.2	= 0.1	= 0.6	= 0.5	= 0.9	= 0.8	= 0.5	= 0.4

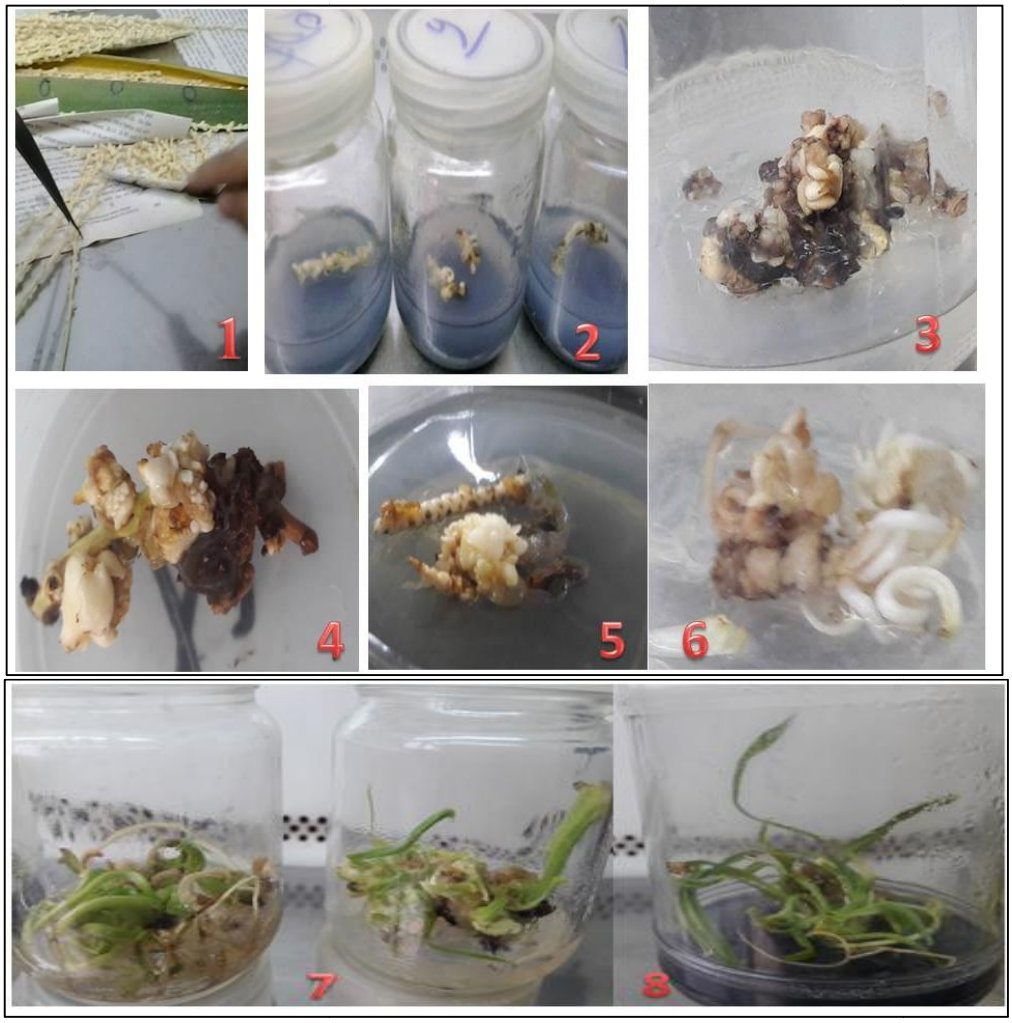

Fig. 1. Protocol of plant regeneration from mature female inflorescence of date palm
1= mature inflorescences explant 2 = Establishment stage 3&4&5 = Embryogenic stage
6= Emergence embryo 7 = starting of embryo elongation 8= shooting stage

carbohydrates of *Pogostemon cablin* Benth, Rasmia Darwesh et al. [61] found that 400 ppm date palm pollen grains extracts with hormones increased in vitro date palm embryos indole contents and proteins.

4. CONCLUSION

In overall the medium supplemented with different levels of *moringa* MLE and hormones can be significant rising indoles contents in the embryo tissues, proteins, amino acids and carbohydrates, while on the contrary phenols contents were found insignificant differs between medium free hormones and medium with hormones.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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