Quest Journals
Journal of Research in Agriculture and Animal Science
Volume 9 ~ Issue 2 (2022) pp: 07-14
ISSN(Online) : 2321-9459
www.questjournals.org

Research Paper

Impact of cysteine and glutamine on callus growth and somatic embryo formation of dry date palm (*Phoenix dactylefira*) cv. Ghorm Ghazal

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ABSTRACT
Date palm is of the most important agricultural activities in Egypt, spessially the oases, the eastern desert and Aswan. The date palm is considered as the important crop in Egypt with a perspective to grow and development. Although, there are an excellent and high-quality varieties, the expansion of palm production suffers from many obstacles, such as the limited traditional propagation, in addition to the limited offshoots production and the rare of distinguished varieties. The present investigation is the first work on in vitro propagation of an elite date palm (Phoenix dactylifera L.) cv. Ghorm Ghazal grown at Siwa oasis through somatic embryogenesis. This study was conducted to examine different concentrations of cysteine and glutamine on callus initiation, embryogenic callus formation and embryos number of date palm cv. Ghorm Ghazal. Shoot tip explants were cultured on modified Murashige and Skoog (MS) medium supplemented with 100 mg/l of 2,4-dichlorophenoxy acetic acid (2,4-D), 3 mg/l isopentenyl adenine (2iP) ,folic acid at 0.1mg/l, 0.5mg/l biotin and different concentrations of cysteine and glutamine at 0 ,50,100,150, 200 and 250 mg/l for each . The best medium for callus initiation and formation was modified MS medium supplemented with 200 mg/l glutamine. The highest percentage of embryogenic callus formation was obtained on modified MS medium, supplemented with 10 mg/l 2, 4-D, 3 mg/l 2iP, 0.5 mg/l folic acid ,1.0 mg/l biotin and glutamine at 200 mg/l. This treatment also recorded increase in the embryogenic callus fresh weight. Moreover, the maximum number of embryos was (embryos /jar) on the modified MS medium supplemented with 0.1 mg/l NAA, 0.5 mg/l folic acid ,1.0 mg/l biotin and 200 mg/l glutamine, compared control treatment. Organic substances seem to be a limiting factor in in vitro date palm culture and their addition as amino acids (cysteine and glutamine) have a beneficial effect. The results showed that glutamine stimulated growth of callus and formation of somatic embryos better than cysteine, while cysteine inhibited the browning better than glutamine.

Keywords: *Date palm, Siwa oasis, Somatic embryogenesis, Embryogenic callus, Cysteine, Glutamine.*

I. INTRODUCTION

The date palm (*Phoenix dactylifera* L.) is one of an ecologically, economically and socio-economically important fruit species grown in arid regions of the West Asia ,Middle East and North Africa and some dispersed areas of Europe and America (Al-Khalifaha and Shanavaskhan, 2012) and (Haider et al., 2013).It is tolerant to adverse environmental conditions (Johnson,2011). In addition, date palm trees distributed at Nile Valley, Oases and desert districts in Egypt. Siwa Oasis is located in the Libyan desert 300 km south of the Mediterranean coast and the nearest city is Marsa Matrouh, about 70 km east of the current Libyan border (Batsti et al., 2018) and its one of the important areas in Egypt for producing excellent cultivars of date palm. These cultivars are well adapted to the local environmental conditions. Despite the prime importance of date palms, which constitute the main cash crop, Ghorm Ghazal cultivar has a great economic importance as a distinguished dry variety in Siwa oasis, Egypt.The traditional method of date palm propagation is by offshoots (Al-Khalifah and Shanavaskhan, 2012). However, the offshoots number produced from the mother tree that are used for vegetative propagation is limited, which hinders the expansion of the cultivated area for date palms. Therefore, in vitro propagation is a quick alternative method for obtaining large numbers of superior date palm cultivars.Several attempts have been made for the in vitro propagation of date palm cultivars based on somatic embryogenesis or organogenesis. Somatic embryogenesis has been achieved by Othmani et al.(2009), Al- Khayri (2011), Ageel and Elmeer (2011), Al-Khateeb and Ghazzawy (2015) ,Baharan (2015) , Diab (2015) , Ibrahim et al., (2017) recently, Kumar et al., (2020)

and Elsayh (2022). Embryogenic callus induction in date palm is influenced by different parameters such as genotype, explant type, induction period and plant growth regulators (PGRs).Several studies have shown effects of date palm genotype and cultivar for *in vitro* propagation (Al- Khayri and Al- Bahrany, 2004) ,the improved induction of date palm somatic embryos by abscisic acid (Hassan, 2007), effect of basal salt media formulations on callus growth and somatic embryogenesis (Al- Khayri ,2011), also, Influence of polyethylene glycol and gibberellin on somatic embryogenesis (Al-Khateeb and Ghazzawy ,2015) and callus formation and differentiation by folic acid and biotin (Diab,2015). For media composition, amino acids are important supplement and widely used in tissue culture systems (Benson,2000 and El-Sharabasy et al.,2012). Amino acids prove an organic form of nitrogen (reduced state), which are readily metabolized by plant cells, stimulating faster cell growth and development (Bader and Khierallah,2009). Therefore, the additional amino acids appear to have the potential to enhance to some extent the roles of suitable nitrogen source. Exogenously added amino acids play an important role in plant tissue culture but culture media of existing regeneration protocols are scarcely supplemented with amino acids. Effect of various amino acids on shoot regeneration of sugarcane *Sacchrum officinarum* L.(Asad et al., 2009). Amino acids have been used as organic nitrogen source in *in vitro* cultures of several species as date palm ,pineapple and rice to enhance somatic embryogenesis and regeneration(El-Shiaty et al.,2004 ;Hamasaki et al., 2005, Grewal et al., 2006 and Mariani et al.,2014). It has been suggested that positive effect of organic nitrogen, in comparison to that of inorganic sources is associated to enhanced mobility of the former at a lower energy cost than the later (Kim and Moon, 2007). Glutamine was reported to stimulate growth and somatic embryos formation in date palm (Jasim and Saad ,2001). Therefore, L-cysteine and/or its oxidation product, L-cysteine, are essential amino acids in cell culture. Therefore, the objective of the present study is to investigate the impact of cysteine and glutamine on callus initiation and embryogenic callus formation of date palm cv. Ghorm Ghazal. To our knowledge, this is the first report on the *in vitro* propagation of date palm Ghorm Ghazal cultivar through somatic embryos, in which two amino acids were used and their effect on the callus growth and development were studied.

II. MATERIALS AND METHODS

This study was carried out in the plant Tissue Culture weeks. Laboratory, Genetic Resources Department, Desert Research Center, Cairo, Egypt.

Plant Material: Date palm (*Phoenix dactylifera* L.) cv. Ghorm Ghazal offshoots, a well-known superior cultivar grown at Siwa Oasis region (Fig.1A); collected from healthy, disease-free mother plants, 3-5 years old, ranging in weight from 5-7 kg and about 50-70 cm in length were used as source of explant materials (Fig.1B).

Explants Preparation and Sterilization: The selected off shoots were cleaned and the outer large leaves and fibers were carefully and gradually removed until the appearance of the shoot tip zone. The apical shoot with a few of primordial leaves was used as explant material. Explants were washed by running tap water for one hour and then immediately placed in a chilled antioxidant solution consisting of 150 mg/l ascorbic acid and 1500 mg/l citric acid for 30 minutes to prevent explants browning. Explants were surface sterilized in 70% ethanol percentages for one minute followed by immersion in 0.5 g/l mercuric chloride (HgCl) for 5 minutes and thoroughly washed 2 with sterilized distilled water. After that, the sheathing leaf base was removed and the explants were re-sterilized by commercial Clorox (5.25% sodium hypochlorite, NaOCl) with two drops of tween-20 per 100 ml solution, firstly by40% Clorox for 15 minutes and thoroughly washed with sterilized distilled water for two times and secondly by 50% Clorox for 20 minutes. Then, they were rinsed with sterilized water for four times. Outer soft leaves were carefully removed to obtain shoot tip composed of apical meristem and 4-6 primordial leaves (Fig.1C). The shoot tips (about5-7 mm in length) were sectioned longitudinally into four to eight sections and cultured individually on a culture medium.

The Basic Nutrient Medium: The basic salts and vitamins of MS medium (Murashige and Skoog ,1962) were used and modified by the addition of adenine sulfate 40 mg/l, sodium dihydrogen orthophosphate ($NaH_2 PO_4$)170 mg/l, thiamine-HCL 0.5 mg/l, nicotinic acid 1.0 mg/l, sucrose 30 g/l and gelrite 2 g/l. The medium was adjusted to pH 5.7 ± 0.1 with 1N HCl or KOH, dispensed into small jars (200 ml) filled with 35 ml per jar and autoclaved under 1.05 kg/cm pressure at 121°C for 20 min.

Culture Conditions: All cultures were incubated at constant temperature 27±2°C under complete darkness and recultured onto the same fresh medium every six weeks.

Callus Initiation: To study the effect of cysteine and glutamine on the callus initiation. Sterilized shoot tip explants were (Fig.1D); cultured on modified MS medium supplemented with 100 mg/l 2,4- dichlorophenoxy acetic acid (2,4-D), 3 mg/l 2- isopentenyl adenine (2iP), 3 g/l activated charcoal (AC) ,0.1 mg/l folic acid, 0.5 mg/l biotin(Diab,2015)and different concentrations of cysteine and glutamine 0,50,100,150, 200 and 250mg/l for each. Each treatment consisted of twelve replicates; each replicate was represented by one explant. After six weeks, survival, swelling and callus initiation percentages were recorded

Callus Formation: Friable callus were divided into pieces approximately 0.5 - 1cm. The pieces were cultured individually in small jars (200 ml) containing 35 ml of the same media used, in the previous experiment under the same conditions for six weeks. Each treatment contains three replicates and each one contained four jars. Callus formation percentage were recorded.

Embryogenic Callus Formation: Calli produced from the previous step were transferred to the modified MS medium supplemented with 10 mg/l 2, 4-D, 3 mg/l 2iP, 2g/l(AC), 0.5 mg/l folic acid, 1.0 mg/l biotin(Diab,2015) and different concentrations of cysteine and glutamine at 0 ,50,100,150,200 and 250 mg/l for each. Each treatment consisted of twelve jars. Cultures were transferred to fresh medium every six weeks. The percentage of embryogenic callus formation was recorded after three months.

Embryogenic Callus Growth Rate: To evaluate callus growth rate in response to different concentrations of cysteine and glutamine at 0,50,100,150,200 and 250 mg/l for each were tested. In all treatments, 0.3 g of white friable callus was cultured in each small jar. Each treatment contains three replicates and each one contained four jars. To determine callus growth rate, the calli were weighted after eight weeks.

Somatic Embryogenesis Growth: Embryogenic callus and embryogenesis in response to. cystiene and glutamine. To test the effect of amino acids on embryogenic callus proliferation and embryos number, callus from the maintenance cultures were transferred to modified MS medium hormone-free supplemented with different combination of cystiene and glutamine at 0,50,100,150,200 and 250mg/l for each. Each treatment contained three jars. Experimental Design and Statistical Analysis: A complete randomized design was employed in all of the experiments. Variance analysis of data was carried out using ANOVA program for statistical analysis according to Snedecor and Cochran (1980). Duncan's multiple range test (Duncan,1955) was employed for means comparisons.

III. RESULTS

Callus Initiation

Effect of cysteine and glutamine on Callus Initiation:

The effect of different concentrations of the selected amino acids on survival, swelling and callus initiation of date palm (Phoenix dactylifera L.) cv. Ghorm Ghazal with resulted from cultured explants for 6 weeks were presented in Table 1. It was found that, swelling percentage was responded differently to the used cysteine and glutamine treatments. In this concern, swelling percentage ranged from66.7 to 100%. The highest significant swelling percentage 100% was recorded by using cysteine at the concentration of 150 mg/l and glutamine at 200 mg/l (Fig. 1E). While, the lowest significant swelling percentage 66.7% was recorded for free medium (control). cysteine at50 mg/l have.

Table 1: Effect of different concentrations of cysteine and glutamine on swelling and callus initiation percentage of date palm (*Phoenix dactylifera* L.) Ghorm Ghazal cultivar

Treatments concentration (mg/l)		Swelling	Callus initiation
Cysteine	Glutamine	**(%)**	**(%)**
0.0	**0.0**	66.7 e	50.0 h
50	**0.0**	75.0 d	55.6 f
100	**0.0**	91.7 b	63.6 d
150	**0.0**	100.0 a	83.3 b
200	**0.0**	91.7 b	72.7 c
250	**0.0**	83.3 c	55.6 f
0.0	**50**	83.3 c	50.0 h
0.0	**100**	91.7 b	54.5 g
0.0	**150**	91.7 b	72.7 c
0.0	**200**	100.0 a	90.9 a
0.0	**250**	83.3 c	60.0 e

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$

depressing effect on swelling percentage (75%). Other treatments had positive effect and good results on swelling percentage. Concerning of effect of cysteine and glutamine on callus initiation, data indicated that, callus initiation percentage ranged between 50 to 90.9%. It was found that the highest significant callus initiation (90.9%) was recorded by using MS medium supplemented with 200mg/l glutamine followed by (83.3%) for 150mg/l cysteine. While, the lowest significant callus initiation (50%) was recorded with control treatment and 50mg/l glutamine. From the obtained results, it can be concluded that modified MS medium containing 0.1 mg/l folic acid and 0.5 mg/l biotin and supplemented with200mg/l glutamine was the best medium for callus initiation (Fig.

1F). While, all cysteine concentrations gave less significant browning than all glutamine treatments and control treatment, they gave more browning (data note shown).

Callus Formation:

Effect of cysteine and glutamine on Callus Formation:

The results of the effect of different concentrations of cysteine and glutamine on callus formation percentage were presented in Table 2 and Fig.1G. Data clearly indicate that, all treatments used gave callus formation. The percentage of callus formation ranged between 33.3 and 88.9%. Using modified MS medium supplemented with 200mg/l glutamine gave the highest percentage of callus formation (88.9%). This value was significantly higher than any other value resulted from other treatments used.

Table2: Effect of different concentrations of cysteine and glutamine on callus formation percentage of date palm (*Phoenix dactylifera* L.) Ghorm Ghazal cultivar

Treatments concentration (mg/l)		**Callus formation (%)**
Cysteine	Glutamine	
0.0	**0.0**	33.3 f
50	**0.0**	44.4 e
100	**0.0**	55.6 d
150	**0.0**	77.8 b
200	**0.0**	55.6 d
250	**0.0**	55.6 d
0.0	**50**	44.4 e
0.0	**100**	55.6 d
0.0	**150**	77.8 b
0.0	**200**	88.9 a
0.0	**250**	66.7 c

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$

Embryogenic Callus Formation:

To test the effect of amino acids (cysteine and glutamine) on embryogenic callus formation, friable calli were transferred to modified MS basal nutrient medium supplemented with 10 mg/l 2, 4-D, 3 mg/l 2iP ,0.5 mg/l folic acid ,1.0 mg/l biotin and different concentrations of the two selected amino acids. Results present in Table 3 and Fig.1H showed that the highest significant percentage of embryogenic callus formation were obtained with modified MS medium containing 200mg/l glutamine, which recorded 87.5 %. This percentage was followed by 150 mg/l cysteine (83.3%), then cysteine at 200 mg/l and glutamine at 100 and 150 mg/l which recorded 75%. While, the lowest significant percentage of embryogenic callus was recorded with control treatment (41.7%). The percentage of embryogenic calli was increased gradually with an increase in cysteine and glutamine concentrations, while the concentration 200 and 250mg/l cysteine and glutamine at 250 mg/l caused a decrease in embryogenic callus. From the above mentioned results, it can be concluded that, modified MS medium supplemented with 200mg/l glutamine was the best medium for embryogenic callus formation.

Table3: Effect of different concentrations of cysteine and glutamine on the percentage of embryogenic callus of date palm (*Phoenix dactylifera* L.) Ghorm Ghazal cultivar

Treatments concentration (mg/l)		Embryogenic callus formation (%)
Cysteine	Glutamine	
0.0	**0.0**	41.7 g
50	**0.0**	50.0 f
100	**0.0**	58.3 e
150	**0.0**	83.3 b
200	**0.0**	75.0 c
250	**0.0**	50.0 f
0.0	**50**	58.3 e
0.0	**100**	75.0 c
0.0	**150**	75.0 c
0.0	**200**	87.5 a
0.0	**250**	66.7 d

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$

Embryogenic Callus Growth Rate:

Studies on the effect of amino acids on callus growth rate of date palm are rare. Data presented in Table 4 showed that the effect of cysteine and glutamine at different concentrations on embryogenic calli fresh weights. Highly effective in stimulating high embryogenic callus production was 200mg/l glutamine which recorded the maximum value 3.819 g/jar (Fig.1I) compared with 250 mg/l glutamine (1.743g/jar) and cysteine at 50 and 250mg/l (1.533 and 1.492 g/jar),respectively without significant difference in between. While, the lowest significant value of embryogenic callus fresh weight was recorded on control treatment (0.839g/jar).After 8 weeks culture duration cv.Ghorm Ghazal callus grew better on modified MS medium containing 200mg/l glutamine as compared to the other tested treatments. These responses were more accurately depict the effect of glutamine concentration.

Table 4: Effect of different concentrations of cysteine and glutamine on the embryogenic callus growth of date palm (*Phoenix dactylifera* L.) Ghorm Ghazal cultivar after eight weeks

Treatments concentration (mg/l)		Embryogenic callus fresh weight (g/jar)
Cysteine	Glutamine	
0.0	**0.0**	0.839 i
50	**0.0**	1.533 h
100	**0.0**	2.633 e
150	**0.0**	2.941 c
200	**0.0**	2.072 f
250	**0.0**	1.492 h
0.0	**50**	2.093 f
0.0	**100**	2.761 d
0.0	**150**	3.322 b
0.0	**200**	3.819 a
0.0	**250**	1.743g

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$

Somatic Embryogenesis: The influence of amino acids on somatic embryos number from embryogenic callus was presented in Table 5 and Fig.1J showed that the highest number of embryos (7.83embryos/jar) was formed with 200mg/l glutamine. While, the high concentration for both amino acids, cysteine and glutamine at 250 mg/l decreased the number of embryos (3.17 and 3.67 embryos / jar), respectively. Data about the effect of both cysteine and glutamine on number of formed somatic embryos showed that all treatments improved number of embryos compared with control treatment which recorded 3.0embryos/jar. Therefore, glutamine was stimulated growth of callus and formation of somatic embryos better than cysteine, while cysteine inhibited the browning better than glutamine (data not shown).

Table 5: Effect of different concentrations of cysteine and glutamine on somatic embryos number of date palm (*Phoenix dactylifera* L.) Ghorm Ghazal cultivar after twelve weeks

Treatments concentration (mg/l)		**Mean number of embryos/culture**
Cysteine	Glutamine	
0.0	**0.0**	3.00 j
50	**0.0**	3.83 h
100	**0.0**	4.83 f
150	**0.0**	6.67 c
200	**0.0**	5.17 e
250	**0.0**	3.17 k
0.0	**50**	4.20 g
0.0	**100**	6.00 d
0.0	**150**	7.33 b
0.0	**200**	7.83 a
0.0	**250**	3.67 i

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$

IV. DISCUSSION

The current study has clear that modified MS medium containing 0.1mg/l folic acid ,0.5 mg/l biotin and supplemented with two amino acids, cysteine and glutamine at different Concentrations, to test their effect on callus initiation, formation and embryogenic callus formation Ghorm Ghazal cv. Concerning the effect of vitamins on callus and embryogenic callus formation Al-Khayri (2011) and Diab (2015) reported that the exogenous vitamins such as thiamine , folic acid and biotin are essential for both callus initiation and induction .Scott et al.(2000) also reported that foliates, a class of pterdine compounds are essential for normal growth and differentiation. Studies on vitamin and amino acid requirements of date palm in vitro cultures are ashived (El-Shiaty et al.,2004). Accordingly, the present study has shown that folic acid combined with biotin, cysteine and glutamine significantly affected somatic embryogenesis of date palm. Accordingly, the present study,the results revealed the effectiveness of glutamine to promote callus initiation ,embryogenic callus formation and significantly affected somatic embryo formation of date palm *in vitro* culture. This might be due to the rapid uptake of reduced nitrogen which provided by this amino acid (Al- Khayri and Al- Bahrany, 2001). Glutamine and glutamic acid are directly involved in the assimilation of NH4+. A direct supply of these amino acids should therefore enhance the utilization of both nitrate and ammonium nitrogen and its conversion into amino acids (Bader and Khierallah ,2009) .The addition of glutamine in date palm tissue culture media increased callus quality and somatic embryos formation (Jasim and Saad,2001). In another study, Salehi et al., (2017) tested the effects of some amino acids (glutamine, proline, alanine, phenylalanine, cysteine and methionine) at different concentrations (0, 50, 100, 150 and 200 mgl^{-1}) on the growth of hazel callus. Results indicated that using MS medium supplemented with any studied concentration of glutamine, alanine or methionine improved callus growth, but the maximum RGR and RFWG of callus were obtained by the use of 50 $mg\ l^{-1}$ of glutamine, alanine or methionine. While, MS medium supplemented with any concentration of proline, phenylalanine or cysteine

improved callus growth. Although, Increased rate of callus growth by amino acid supplements have been reported(El-Sharabasy et al.,2012).Amino acids are an accessible nitrogen source for plant cells and can be absorbed much more readily than inorganic in the same medium(Thom et al.,1981. According to the results of some studies, Armstrong and Green(1985) amino acids are not necessary ingredient for many cultural purposes but their addition to the medium can compensate for medium deficiency or provide an accessible source of nitrogen to cultured cells or tissues. With ammonium ion uptake, plant tissues use adenosine triphosphate (ATP) as an energy source to convert it into amino acids (Durzan ,1982). Therefore, the presence of suitable amino acids in the medium may save some ATPs.

In conclusion, in date palm tissue culture, adding amino acids to the culture medium is critical. In addition to measuring their demands, this research has highlighted the need for cysteine and glutamine as medium additives. The best callus initiation and development were achieved using a modified MS medium supplemented with 0.1 mg/l folic acid, 0.5 mg/l biotin, and 200 mg/l glutamine. The greatest results in embryogenic callus development, callus growth, and embryogenic callus formation were achieved with 0.5 mg/l folic acid, 1.0 mg/l biotin, and 200 mg/l glutamine. As a result, glutamine promoted callus development and somatic embryo generation more than cysteine, whereas cysteine prevented browning better than glutamine.

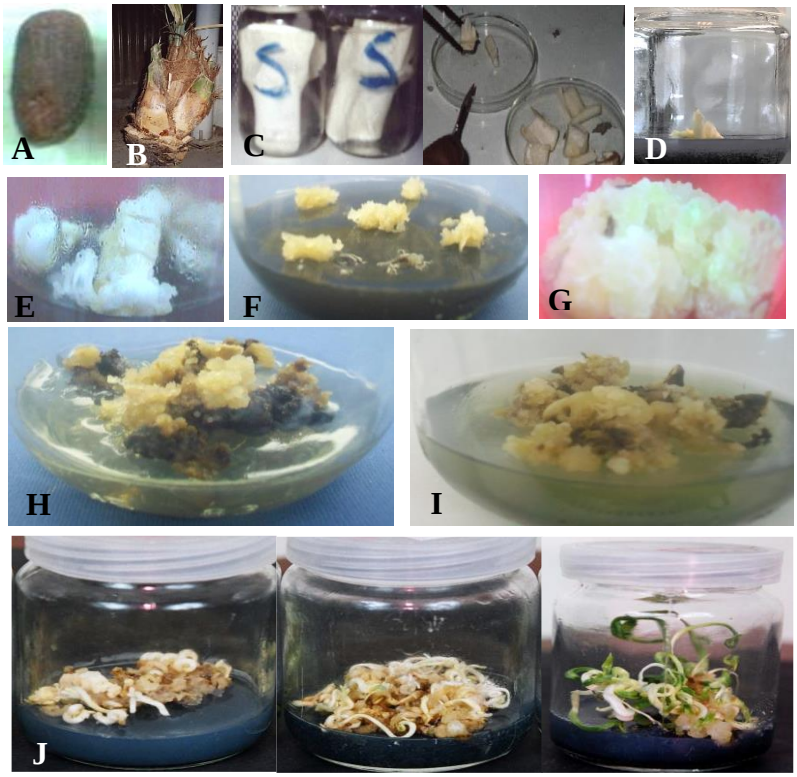

Fig.(1): Effect of modified MS medium supplemented with vitamin; folic acid, biotin and 200 mg/l glutamine on callus induction embryogenic callus development of date palm Ghorm Ghaza cultivar**: (A)** Fruit of superior cultivar; **B)** Offshoots before the excision of the apical part of the stem; **C)** Explant isolation from shoot tip; **D)** Explant establishment on culture modified medium ; **E)** Swelling of shoot tip explants; **F)** Callus initiation; **G)** Callus formation; **H)** Embryogenic callus formation; **I)** Embryogenic callus growth after 8 weeks**; J)** Development of somatic embryos

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