IMPROVEMENT AND CHARACTERIZATION OF SOMATIC EMBRYOGENESIS IN DATE PALM (*Phoenix dactylifera* L.)

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Somatic embryogenesis is the process by which the somatic cells or tissues develop into differentiated embryos, and each fully developed embryo is capable of developing into a plantlet (young or miniature plant). Embryos can be obtained directly from either cultured explants (the organized structures, for example, leaf, hypocotyl, stem, anthers and pollen) or indirectly from callus, (an unorganized mass of parachymatous tissue derived from explant culture as a result of wound response) and isolated single cells in culture. The process of embryogenesis involves various stages of differentiation and development such as proembryo, globular, heart-shape and torpedo embryo (Kumar, 1999).

In fact, somatic embryogenesis is essentially similar in structure, function and biochemistry to that of zygotic embryogenesis (embryo derived from fertilized egg cell- the zygote), and somatic embryos share many characteristic features with zygote embryos; for instance, both are bipolar structures and originate from a single cell and do not show vascular differentiation. Nevertheless, somatic embryos are large in size and sometimes show more than two cotyledons (pluricotyledony) as compared to zygote embryos that are smaller and exhibit two cotyledons (in case of dicots). Significantly, a large number of embryos potential uses in rapid micropropagation of high value crops, including crops propagated by seeds.

Reducing auxins concentrations in the induction medium became a critical demand and necessary to reduce somaclonal variation probability and in the same time to produce direct somatic embryos Abo-El-Soaud *et al.*, (2002b). The purpose of this study that we are assess some parameters to determine the characteristics of embryo differentiation process where it's the neck of bottle to obtain the vital date palm plantlet.

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MATERIALS AND METHODS

This study was carried out at the period of 1995-1999 in the Laboratory of Plant Tissue Culture, Agriculture Development System project (ADS), Ministry of Agriculture. One segment shoot tip (quarter of shoot tip) of *Phoenix dactylifera* L., cv. Zaghloul was placed on the surface of MS (Murashige and Skoog, 1962) solidified nutrient medium contained 100 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D);3mg/l 2-isopentenyladenine (2ip) and 40 mg/l adenine sulfate.$2H_2O$ (Tisserat,1979),170 mg/l $NaH_2\ PO_4\ .2H_2\ O$;3g/l activated charcoal (AC). The initial explants and callus cultures were maintained at 27±2 °C in darkness through four subcultures (the first two subcultures employed for callus formation followed by two subculture for embryogenic callus formation, Abo-El-Soaud *et al.* (2002a).

Differentiation process was conducted by:

1. Transferring small masses of white friable embryogenic callus tissues (1-2 mm in diameter), from high auxin media to an MS-basal medium supplemented with various concentrations of NAA and 2iP, with activated charcoal (100 mg/l) and without activated charcoal.
2. MS-basal medium with or without activated charcoal containing 0.1 mg/l NAA and 0.0 mg/l 2iP was used as a control. Each treatment included 3 replicates in small Jars. Each Jar contained about 0.1 g embryogenic callus. All cultures were incubated in growth room, at 24 ± 1C° under 16 hour's daily exposure to low light intensity of 1000 lux illumination. The following experiment included 10 treatments using NAA at 0.1 mg/l and 2iP at 0.0, 0.1, 0.3, 0.5 and 1.0 mg/l, with or without activated charcoal (100 mg/l), which were added to MS- basal medium. The following data were recorded after 6 weeks from culture.
   a. Average number of embryos / culture.
   b. Average length of embryos / culture.
   c. Growth value $\frac{\text{Final fresh weight—Initial fresh weight}}{\text{Initial fresh weight}}$ by (Ziv, 1992).
   d. Type of embryos.

Picking up germinating embryos individually is important when they reached 5-10 mm in length. This process was continued until germination. This experiment aims to establish regenerative types developed within three months.

3. Types of somatic embryos were transferred to media of the same compositions (the same treatments) to proliferate the adventitious embryos (secondary embryos), or to germinate and develop shoots. All cultures were incubated in growth room at 27 ± 2C°, under 16 hour's daily exposure to moderate light intensity of 2000 lux. The same data, mentioned in the previous experiment were recorded after 6 weeks from culture.
4. The regenerative types of embryos were employed for multiplication stage and the individual plantlets for pre-rooting stage.

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 Steel and Torrie (1980).

RESULTS AND DISCUSSION

1. Effect of different concentrations of 2ip and activated charcoal on embryogenic callus differentiation, after 6 weeks in culture:

This experiment was carried out in order to evaluate the effect of different concentrations of cytokinin (2iP) + 0.1 auxin (NAA) with or without activated charcoal (AC) on the white embryogenic callus (Fig. 1), which produced from early stages of growth, when these components added to MS basal medium.

Data revealed that, a suitable treatment to differentiate the date palm embryogenic callus was 0.1 mg/l NAA + 0.1 mg/l 2iP with AC. Where, the number of embryos was 5.33 embryos/culture, as compared to control treatment, where it was 4.67 embryos/culture. Other treatments were less efficient than control treatment, where the number of embryos ranged from 1.33 to 2.00 embryos/culture (Table 1).

In respect to treatments without AC (Fig. 4), the best treatment was 0.1 mg/l NAA + 0.1 mg/l 2iP, where the number of embryos was 3.0 embryos/culture, as compared to control treatment (0.1 mg/l NAA + 0.0 mg/l 2iP without AC), where it was 1.0 embryos/culture. However, other treatments were more effective than control treatment, where the number of embryos ranged from 1.67 to 2.00 embryos/culture, with exception of 0.1 mg/l NAA + 1.0 mg/l 2iP treatment, where it was 0.67 embryos/culture.

On the other hand, a significant difference was observed between the differentiation media with AC and without AC (0.31), and then the average number of embryos for the treatments with AC was 3.07, while it was 1.67 for treatments without AC.

Data presented in Table (1) clearly reveal the effect of 0.1 mg/l NAA and different concentrations of 2iP (with or without AC) on growth value (G.V) of differentiated somatic embryos of date palm. The highest growth value was occurred in response to 0.1 mg/l NAA + 1.0 mg/l 2iP with AC (2.86), followed by 0.1 mg/l NAA + 0.1 mg/l 2iP with AC, where G.V was 2.84, compared to the control (2.06).

In regard to differentiation media without AC, the highest growth value was obtained on 0.1 mg/l NAA + 0.1 mg/l 2iP (3.95), compared to the control (2.05). Whereas, other treatments have not a significant differences among of them and were less effective than best treatment. No significant difference was observed in the differentiation media with and without AC.

In fact, several morphological forms of embryos were initiated and these have different potentials for regeneration of embryos. Along the way, abnormal structures would also be formed, on the strength of our observations; there are two different types of embryos: Repeated embryos, RE, (Fig. 2A) and Non-repeated embryos, NRE, (Fig. 2B).

Both of repeated (RE) and non-repeated embryos (NRE) percentages represented in Table (1). Data also show the effect of MS basal medium supplemented with 0.1 mg/l NAA + 0.0, 0.1, 0.3, 0.5 and 1.0 mg/l 2iP with or without 100 mg/l AC on white embryogenic callus to produce repeated embryos (the best type for multiplication stage) of date palm.

As respects differentiation media with AC, the better treatment to produce repeated embryos was 0.1 mg/l NAA + 1.0 mg/l 2iP compared to other treatment. However, it clearly observed that, a differentiation media with AC were superior in increasing repeated embryos percentage, when compared with differentiation media without AC, where it was 33.2% and 19.8%, respectively.

Generally, we can conclude that, a differentiation medium with AC was favorable for production of somatic embryos from white embryogenic callus of date palm cv. Zaghloul, compared to differentiation medium without AC. However, the average embryo length/culture depend on the presence of AC in nutrient medium and the percentage of NRE, where it was increased in presence of AC and increasing NRE percentage. These results are in line with those of Tisserat (1982) who mentioned that; embryogenic callus of date palm was planted on nutrient medium containing charcoal to produce somatic embryos. Also, Sharma *et al.*, (1984) found that somatic embryos were induced on modified MS medium containing activated charcoal at 0.3% (Dass *et al.*, 1989; Veramendi and Navarro, 1996). Al-Kharyi and Al-Maarri (1997) were used MS culture medium containing 1.5 g/l AC. Then better growth responses of the plant tissues are associated with the addition of AC to the medium (Fridborg *et al.*, Eriksson, 1975; Ebert and Taylor, 1990). On the other hand, these results are not in line with those of Zaid and Tisserat (1983) who reported asexual embryos produced on nutrient medium without charcoal. Therefore, using MS medium supplemented with 0.1 mg/l NAA + 0.1 mg/l 2iP with AC was superior for differentiation, where the average of number embryos was 5.33, average length of embryos was 8.33 mm and the growth value was 2.84. Under these conditions 50-60 mature embryos/g of embryogenic callus, reached 5-10 mm in length, after 6 weeks in culture. All of these embryos are capable to germinate and develop. Whereas, Veramendi and Navarro (1996) reported that under the optimal conditions 40-60 mature embryos/g of callus were obtained after four months in culture, using different genotypes and a germination rate of around 10% only.

In our experiment, at the high concentrations of 2iP (0.5 and 1.0 mg/l) especially in presence of AC, new colonies of nodular callus has appeared, where it increased by increasing 2iP concentration. Transfer of this nodular callus to fresh medium, containing 0.1 mg NAA only (control treatment) resulted in a large number of somatic embryos. Omission of NAA from the medium encouraged precocious elongation of the nodules before completion of their maturation. This resulted in the development of creamy colored weak plantlets.

Similar results were obtained by Mater (1986) who noted that, the best results and number of survived plantlets was obtained when free nodules were subcultures to medium containing 0.1 mg/l NAA. NAA at 0.1 mg/l enhanced maturation of the nodules and differentiation into small normal white plantlets (5-10 mm length). In the second subculture (experiments 2 and 3), individual embryos were transferred to fresh medium. Removal of germinating embryos at this stage stimulated further germination of other nodules in the culture vessels. This doesn't elicit nutritional competition among different developmental stages of embryos, then they are asynchronous cultures.

2. *Effect of different concentrations of 2ip and AC on proliferation of white repeated embryos of date palm, after 6 weeks in culture:*

This experiment was conducted to determine the effect of MS basal medium supplemented with 0.1 mg/l NAA and various concentrations of 2iP with or without AC (0.01%) on proliferation of white repeated embryos of date palm cv. Zaghloul, after 6 weeks in culture. Where embryo initials are successfully established, clusters of discreet globular and torpedo-shaped embryo structures developed and matured to fully formed embryos with a cotyledonary structure enclosing primary shoot and root meristems (Fig. 2A). Embryo development and germination form a continuous process (there is no dormant phase).

Thus, transferring embryogenic structures to optimal fresh medium continuously is very important (Fig. 5). If merely left, these primary embryo structures will germinate and develop shoots. However, if transferred to optimal fresh media and judiciously cut, proliferation of adventitious embryos occurs from the hypocotyl zone of the primary embryos. Adventitious embryos formation continues along embryo maturation and germination (Proliferation of secondary embryos) giving multiplying asynchronous cultures. Although, well-formed somatic embryos have a primary root meristem, not all germinated embryos develop a primary root.

Table (2) represents the effect of 0.1 mg/l NAA and different concentrations of 2iP with or without AC on the average number of embryos. It was clearly that, the average number of secondary embryos ranged from 1.33 to 3.33. Data also showed that, the best result was recorded at 0.1 mg/l NAA + 0.5 mg/l 2iP with AC, compared with control (0.1 mg/l NAA + 0.0 mg/L 2iP with AC), where it was 1.28. As well as, increasing 2iP concentration to 0.5 mg/l increased secondary embryos, which produced on the base of repeated embryos (RE), and then was decreased by increasing 2iP concentration.

Regarding the proliferation media without AC, the average number of secondary embryos ranged from 1.17 to 2.67, and the same trend was found for proliferating media with AC. There wasn't significant interaction.

Generally, presence of 2iP in proliferation media of embryogenic cultures without AC produce callus. Therefore, a lot of adventitious roots were observed. The results under discussion clearly show that, presence AC in the nutrient media was strongly stimulated the proliferation of secondary embryos. Also, reduce the callus, formation on the meristematic parts of repeated embryos. This may be due to adsorption of the excessive amounts of plant growth regulators. These results are in harmony with those of Tisserat (1979) and Zaid & Tisserat (1983) who mentioned that, the charcoal reduced the availability of hormonal substances. Also, Weatherhead *et al.* (1978) reported that the beneficial effects of AC are attributed to the removal of inhibitory substances from the media, produced either on autoclaving the media or by the tissue it self (Johansson and Eriksson, 1977).

The highest rate of proliferated cultures (secondary embryos and shoots), was occurred at 0.1 mg/l NAA + 0.5 mg/l 2iP with 100 mg/l AC, when added to MS basal medium. No callus or a little of callus on the meristematic parts of embryos, especially the coat of embryo was observed.

3. ***Effect of different concentrations of 2ip and AC on prolife.ation of white small embryos of date palm, after 6 weeks in culture:***

The individual embryos (NRE), which produced on differentiation media, were transferred to media of the same concentrations (in experiment 2) to complete their germination and produce whole plants (5-10 cm in length), where they could be transferred to pre-rooting media, (Fig. 3B). Occasionally, these embryos produce adventitious embryos or shoots on their base (PE), as shown in Fig. (3A).

No proliferated embryos were observed at the control treatment (0.1 mg/ NAA + 0.0 mg/l 2iP & AC) where, the average number of embryos was 0.0, as shown in Table 3. Data also showed that, other treatments produced adventitious embryos, as the number of embryos/ culture ranged from 1.33 to 7.67. However, there was a significant difference among different treatments (0.55). The best result was obtained at 0.1 mg/l NAA + 0.1 mg/l 2iP & AC, as the number of embryos/ culture was 7.67.

With regard to media without AC, a few embryos were able to proliferate adventitious embryos. Therefore, the number of embryos/ culture ranged from 1.33 to 2.33. The interaction between AC and treatment was significant (0.78). Generally, the proliferating medium with AC was more effective than proliferation medium without AC, where the average number of embryos was 3.13 and 1.07, respectively.

The average of embryo length ranged from 2.67 to 8.33 cm, on the medium containing AC, and from 3.0 to 6.0 on the media without AC. However, the presence of AC in nutrient media stimulated the embryos length, where the average of embryo length was 6.07 cm, whereas it was 4.13 for nutrient media without AC. There was a significant difference between AC and treatments (1.03).

Data in Table (3) shows the effect of different treatments on type percentage of cultured embryo [proliferated embryo (PE) or none proliferated embryo (NPE)]. The data indicated that, 0.1 mg/l NAA + 0.1 & 0.3 mg/l 2iP with AC treatments induced proliferation of adventive's embryos (shoots) at the base of cultured embryos, where the percentage of PE was 67% for both. However, the nutrient media with AC was better than nutrient media without AC, where the total percentage of PE was 40.0% and 19.8%, respectively.

The results under discussion revealed that, addition of AC in nutrient media, stimulated the adventitious embryos (shoots) proliferation at 0.1 mg/l NAA + 0.1 mg/l 2iP and shoot growth at control treatment (0.1 mg/l NAA + 0.0 mg/l 2iP). However, the presence of AC inhibited adventitious rooting at any concentration. Although, well formed somatic embryos have a primary root. The inverse was observed in nutrient media without AC, where the formations of adventitious roots in this stage are precocious. These observations are in line with Tisserat (1982) and Mater (1986).

The developmental stages of individual embryos (NPE and adventive's embryos) closely paralleled with the developmental stages of adventitious embryos were obtained by Tisserat (1979), who mentioned that, the developmental stages of adventitious embryos similar to the growth of excised zygotic embryos *in vitro*. Then, the apex of the cotyledon or suctorial haustorium's, degenerated in both the excised zygotic and adventitious embryos. Followed by, the cotyledonary sheath elongated several centimeters and produced the characteristic slit-shaped opening, which the plumule precedes. Following several weeks in culture, the plumule and the radical broke through the cotyledonary sheath.

On the other hand, the individual embryos which grown on nutrient medium without AC resulted in malformed shoots and the white callus was produced on cotyledonary sheath, as well as these germinating embryos produced a large number of adventitious roots at any concentration, as obvious in Table 1. Thus, the mean value of G.V for the treatments without AC was 9.25, compared to 4.68 for the treatments with AC.

Eventually, it can be concluded that the micropropagation method which employed in this stage could need two subcultures. The first, to differentiate of free nodules (which produced on media containing high levels of auxins with AC) and produce repeated embryos (RE) and non-repeated embryos (NRE) [small germinating embryos, with 5-10 mm in length] during the first 6 weeks. In the second subculture individual embryos (NRE) and multiplying types could be transferred to fresh medium of the same treatments. This process was schematic illustrated in Fig. 6. Removal of germinating embryos at the first subculture stimulated further germination of other nodules in the culture vessels.

The second subculture allowed fast growth of individual embryos and their development into whole plantlets (5-10 cm in length) after 6 weeks in culture, where transferred directly to pre-rooting stage (Abo-El-Soaud *et al.*, 1999).

Transferring of date palm plants derived from laboratory to the free-living conditions (*ex vitro*) is considered as a problem that hampers the success of such technique, but the fully developed embryos (miniature plants) were transplanted successfully in the greenhouse (Fig. 7).

SUMMARY

Nutrient medium composition and somatic embryos characterization are important factors affect the differentiation process of *Phoenix dactylifera* L. *in vitro*. During the differentiation process of date palm many somatic embryo shapes appeared. Determination of these embryos may be used to improve the date palm production *in vitro* and may reduce somaclonal variations. Two shapes of somatic embryos differentiated from the embryogenic callus (pro-embryos) were detected when cultured onto MS basal nutrient medium supplemented with 0.1 mg/l NAA, 0.1 mg/l 2ip and 100.0 mg/l activated charcoal, one of them called repeated embryo (RE) and the other called non-repeated embryo (NRE). The repeated embryo have more than one embryo simultaneously and have a self capability for proliferation but the non-repeated embryo directly differentiated to a single embryo which continued to grow into a distinct plantlet or form secondary embryos on the base, this depending on the culture medium composition. Many of shapes were collected and determined in a schematic illustration.

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Table (1): Effect of different concentrations of 2ip + 0.1 mg/l NAA with and without activated charcoal (AC) on embryogenic callus differentiation of date palm cv. Zaghloul, after 6 weeks in culture.

(A) Activated charcoal	(B) (mg/l)		Avg. number of embryos/ culture	Avg. length of embryos/ culture (mm)	*GV	Type of embryos (%)	
	NAA	2ip				RE	NRE
100 mg/l	0.1	0.0	4.67	3.67	2.06	33.0	67.0
	0.1	0.1	5.33	8.33	2.84	33.0	67.0
	0.1	0.3	2.00	4.33	1.34	00.0	100
	0.1	0.5	1.33	5.00	2.19	33.0	67.0
	0.1	1.0	2.00	2.00	2.86	67.0	33.0
Mean			3.07	4.67	2.26	33.2	66.8
0.0 mg/l	0.1	0.0	1.00	2.33	2.05	00.0	100
	0.1	0.1	3.00	4.33	3.95	33.0	67.0
	0.1	0.3	1.67	1.67	2.57	33.0	67.0
	0.1	0.5	2.00	4.00	2.40	33.0	67.0
	0.1	1.0	0.67	1.33	2.57	00.0	100
Mean			1.67	2.73	2.71	19.8	80.2
L.S.D at 0.05	A		0.31	0.56	N.S		
	B		0.50	0.89	0.78		
	AB		0.70	1.26	N.S		

*GV: growth value.

Table (2): Effect of different concentrations of 2ip + 0.1 mg/l NAA with and without activated charcoal (AC) on proliferation of white repeated embryos of date palm cv. Zaghloul, after 6 weeks in culture.

(A) Activated charcoal	(B) (mg/l)		Avg. number of embryos/ culture	Avg. length of embryos/ culture (cm)	*GV
	NAA	2ip			
100 mg/l	0.1	0.0	1.28	5.33	5.30
	0.1	0.1	1.33	5.00	5.46
	0.1	0.3	2.83	3.00	20.21
	0.1	0.5	3.33	2.33	4.96
	0.1	1.0	1.75	2.00	3.11
Mean			2.10	3.53	7.81
0.0 mg/l	0.1	0.0	1.00	3.67	6.50
	0.1	0.1	1.17	3.00	7.34
	0.1	0.3	2.00	2.67	10.77
	0.1	0.5	2.67	2.00	17.39
	0.1	1.0	1.33	2.00	6.55
Mean			1.63	2.67	9.71
L.S.D at 0.05	A		0.39	0.48	1.00
	B		0.63	0.76	1.59
	AB		N.S	1.08	2.24

*GV: growth value.

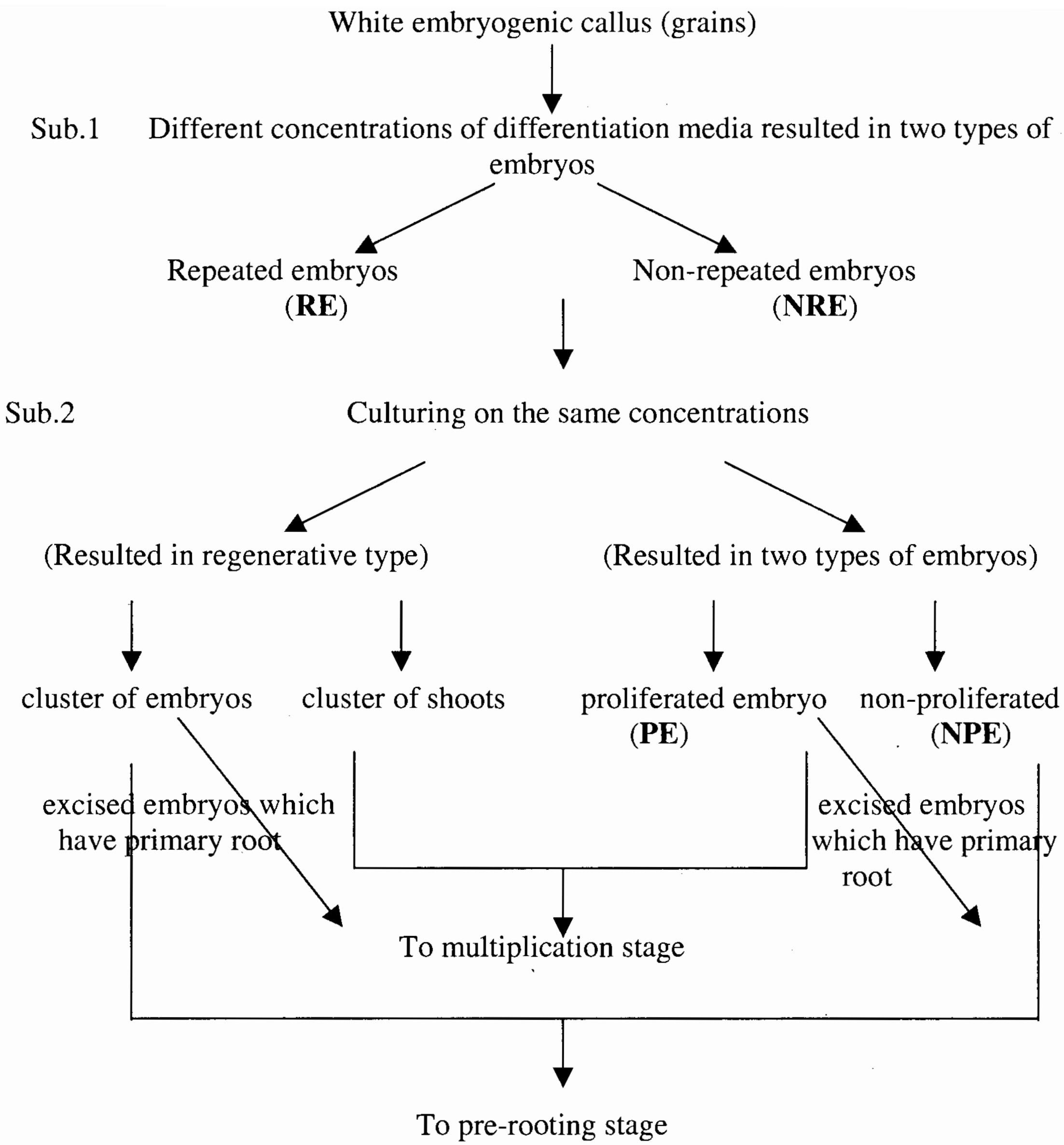

Fig. (6): Schematic illustration of embryogenic callus differentiation
Sub.1,2: Subcultures 1,2.