Chapter-7

Emblica officinalis

INTRODUCTION:

One of the major limitations to the applications of somatic embryogenesis for propagation and genetic manipulation of hardwoods is a low multiplication rate ie the low number if field plantable clonal plantlets produced per embryogenic culture. Many reports of somatic embryogenesis in hardwood trees indicate that somatic embryos have produced, but the cultures fail to demonstrate continued embryo production. Generally, these reports of direct embryogenesis, where for example, individual somatic embryos arise from explanted zygotic embryo cotyledon tissues. Often only a single population of embryos are produced, some of which may mature and convert into plantlets. If this is the case, then the culture represents a dead end with regard to clonal propagation (Merkle 1995). In some cases, however, these primary somatic embryos fail to mature and instead give rise to successive cycles of embryo production. Usually the new embryos arise from epidermal or subepidermal cells of certain regions of the primary embryos. This cyclic production of new generations of somatic embryos is known as repetitive, recurrent or secondary embryogenesis (RSE). It is this phenomenon that gives somatic embryogenesis its great potential for mass propagation and gene transfer, since a single culture undergoing repetitive embryogenesis is theoretically capable of generating an unlimited number of somatic embryos (Merkle 1995, Raemakers *et al* 1995).

Somatic embryos have also shown to be an excellent source for secondary embryos. It is associated with the loss of integrated group control and initiate new somatic embryos (Williams & Maheswaran 1986). In most cases somatic embryos develop unto pre-embryonic masses (PEM's) or globular embryos without differentiation into organs.

When developing a regeneration system to be used in gene transformation, desirable characteristics include reliability and high frequency. A major advantage of developing a somatic embryogenic system is the potential for cultures to undergo secondary embryogenesis and long term regeneration of the transformed individuals. This ability can also be used in gene transformation and selection protocols, where repetitive embryogenesis can be used to eliminate transgenic chimerics (Mathews *et al* 1992, Mc Granahan *et* al 1990). Furthermore, RSE could be integrated into artificial seed production technology (Fujii *et al* 1989; Mc Kersie *et al* 1989; Slade *et al* 1989;

Redenbaugh & Walker 1990) as a system capable of providing an unlimited number of somatic embryos (Parrot & Bailey 1993).

Applications of Secondary somatic embryogenesis:

1. In crops with a long life cycle, as for example woody species, preservation of embryogenic lines can be a cost effective maintenance protocol until the lines have been tested in field conditions. Selected lines can then be multiplied in large quantities by secondary embryogenesis.
2. Immature embryos of interspecific plants from incompatible crosses (involving wild and cultivated plants to introgress resistance genes in economically important cultivars, in which post fertilization barriers may often prevent maturation of embryos) may be rescued by culturing them for secondary embryogenesis and thus the clonal multiplication of selected plant.
3. Secondary somatic embryogenesis can be used for the production of secondary metabolites from somatic embryos in species where the zygotic embryos contain commercially important metabolites.
4. An epidermal cell origin of somatic embryos is more suited to be used in conjunction with plant transformation than a mesophyll origin. It is evident that an individual cell origin is beneficial as compared to multiple origin. In such cases the transformed cells have the opportunity to act independently from neighboring cells thus generating completely transformed plants rather than chimeras.
5. Repetitive somatic embryogenic system if employed for genetic transformation with ***Agrobacterium*** or particle bombardment method, yields stable non-chimeric transgenenic plantlets. The transformed embryos can be, further, induced to form unlimited number of transformed somatic embryos through repetitive embryogenesis.
6. The partly transformed embryos can be made solid transformants after several cycles of RSE under selective conditions.
7. If primary somatic embryos have to be induced on tissues, like zygotic embryos, floral organs etc, to be used for transformation, the explants might not be available round the year. On the other hand secondary somatic embryos can be maintained round the year.

8. Efficiency of transformation is higher in RSE than primary embryos.
9. Due to the very high rate of embryo formation in RSE, it can be used for large-scale multiplication of plant material at rates higher than that by any other micropropagation system.
10. Doubling of chromosome number of haploid embryogenic lines and its multiplication is much easier with RSE.
11. The repetitive embryogenic system is of potential use in the synthesis of metabolites in bioreactors such as pharmaceuticals and oils, which are naturally synthesized only during organized zygotic embryogenesis or such differentiated tissues.

Table-7.1: Reports on secondary somatic embryogenesis

Plant	Explant	Reference
Apium graveolens	Leaf (I)	Nadel *et al* 1989,1990
Arachis hypogea	Zygotic embyro (I)	Baker & Wetzstein 1992
Brassica campestris	Zygotic embryo (D)	Maheswaran & Williams 1986
B. napus	Microspore,anthers (D)	Thomas & Wenzel 1975
Camellia japonica	Zygotic embryo (D)	Vietez & Barciela 1990
Carum carvi	Petiole (I)	Ammirato 1977
C.sinensis	Ovule (D)	Kochba & Spiegal-Roy 1972
Daucus carota	Zygotic embryo (B)	Smith & Krikorian 1989
Fagus sylatica	Zygotic embryo (I)	Vietez & Barciela 1982
Helianthus annuus	Zygotic embryo (D)	Finer 1987
Juglans major	Zygotic embryo(D)	Cornu 1988
Magnolia spp	Zygotic embryo (B)	Merkle & Wiecko 1990
Medicago sativa	Zygotic embryo (I)	Lupotto 1982
Prunus persica	Zygotic embryo (D)	Bhansali *et al* 1990
Rauvolfia vomitoria	Leaf (I)	Tremouillauz-Guiller & Chenieux 1991
Trifolium repens	Zygotic embryo (D)	Maheswaran & Williams 1986

* origin of emhryogenesis B both direct and indirect, D direct and I indirect

Emblica (Aonla): *Emblica officinalis* Gaertn. is commonly known as Aonla. Aonla is a member of Euphorbiaceae family. It is a small or medium sized deciduous tree with smooth, greenish grey, exfoliating bark. Leaves are feathery with small narrowly oblong pinnately arranged leaflets. Fruits is depressed globose, fleshy and obscurely 6 lobed. It is usually propagated by seeds; it may also be propagated vegetatively by budding, grafting and cutting. The plant is sensitive to frost and drought. The fruit is green when tender changing to light yellow or brick red colour when mature. It is sour and astringent and is occasionally eaten raw. Aonla fruit is probably the richest known natural source of vitamin-C tannin containing gallic acid, elagic acid and glucose in its molecule are naturally present in fruit. This prevents or retards the oxidation of the vitamin and renders the fruit a valuable property of antiascorbutic in the fresh as well as in the dry condition.

Medicinal values of Aonla-

1. Aonla fruit has been held in high esteem in indigenous medicine. It is acrid, cooling, refrigerant, diuretic and laxative.
2. It is the main ingredient of an Ayurvedic preparation called Chyawanprash, a health tonic which has been indicated as a rejuvenating preparation and recent studies have shown its action as an oxygen (free radicle) scavenger.
3. The raw fruit is eaten as aperient.
4. Dried fruit is useful in hemorrhage, diarrhea and dysentery. *Emblica* also is used in many compound preparations.
5. The exudation from incisions on the fruit is used as an external application for inflammation of the eye.
6. The flowers are cooling, refrigerant and aperient.
7. The root and bark are astringent.
8. The fruits are used in the preparation of writing inks and hair dyes.
9. The dried fruits are used in detergent and is used as shampoo for the hair.
10. The seeds are used in the treatment of asthma-bronchitis and biliousnes. They contain a fixed oil, phosphatides and a small quantity of essential oil with a characteristic odour.
11. The fruit barks and leaves are rich in tannin.
12. Immature fruit are used as fodder for cattle.

Fig-20 *Emblica officinalis* tree

a) Note the mature tree

b) Note the heavy bearing of fruits

a
b

13. The leaves contains brownish yellow colouring matter used in dyeing tusser and mulberry silk and wool as when it is used with iron mordant a black colour is produced.
14. The wood is red, hard and close-grained. It is used for agricultural implements, poles and furniture work. It is durable under water and is also used for fuel and for making charcoal .

Somatic embryogenic system in *Emblica officinalis* will have a lot of application for improvement and large-scale production of plants. The large-scale propagation of Aonla through somatic embryogenesis will have immense use in propagation of the plant as the conventional methods are not efficient. Also complete micropropagation protocols through axillary bud proliferation are yet to be standardized in this plant for commercial utilization. From experience it was found that the rooting of cutting as well as *in vitro* regenerated shoots was very difficult which led to the low survival rates of plantlets.

If somatic embryogenic system is developed in *Emblica officinalis*, the survival rate of the plantlets will be higher as both root and shoot poles are there in somatic embryos similar to that of a zygotic embryo. The somatic embryogenic system can also be used for production of synthetic seeds as propagules. The system will help in improvement of the varieties using protoplast isolation and fusion, selection against salt, drought, pest diseases and for genetic manipulation. The selection for quantitative traits like higher nutrient value, low fiber content, high antioxidant activity (and hence increased medicinal properties), higher production of female flowers in branchlets (leading to higher production and yield of fruits) etc will be easier with somatic embryogenesis. The somatic embryogenesis also has importance in basic studies in the plant. The zygotic embryos of the plant undergo a rest period before further development (Bajpai & shulka 1990). It will stay in this quiescent state for 4-6 months before the commencement of further development. Though some biochemical examination was carried out, the reason for this quiescence is not yet been unravelled. The somatic embryogenic system will definitely be useful for research in this line.

MATERIALS AND METHODS:

Somatic embryos were induced directly on cultured zygotic embryos of *Emblica officinalis* on MS medium supplemented with 0.4 uM NAA and 0.4 uM AS (Remakanthan, 2000). These primary somatic embryos were further multiplied on basal medium by secondary embryogenesis for several cycles. These secondary embryos undergoing recurrent secondary embryogenesis were used as the inoculum material for the present studies (Fig-21 a). For embryo proliferation the primary somatic embryos were transferred to basal MS medium. Embryos continue to germinate and grow further in the same medium (Fig-21 b-c)

With the aim of controlling the RSE and to enhance germination of embryos, some manipulation were carried out in media (concentration of media components/media addenda) and by plant growth regulators like ABA (0.05–0.2 uM) and 2,4-D (0.1–0.3 uM) and BAP (0.1, 0.2 and 0.3).

Encapsulation of the embryos was carried out by individually picking up the embryo dipped in the sodium alginate (4 %) matrix and dropping them one by one into $Cacl_2$ (0.6 mM) solution. The synseeds so formed were germinated when transferred toagar-based MS medium.

RESULTS AND DISCUSSION:

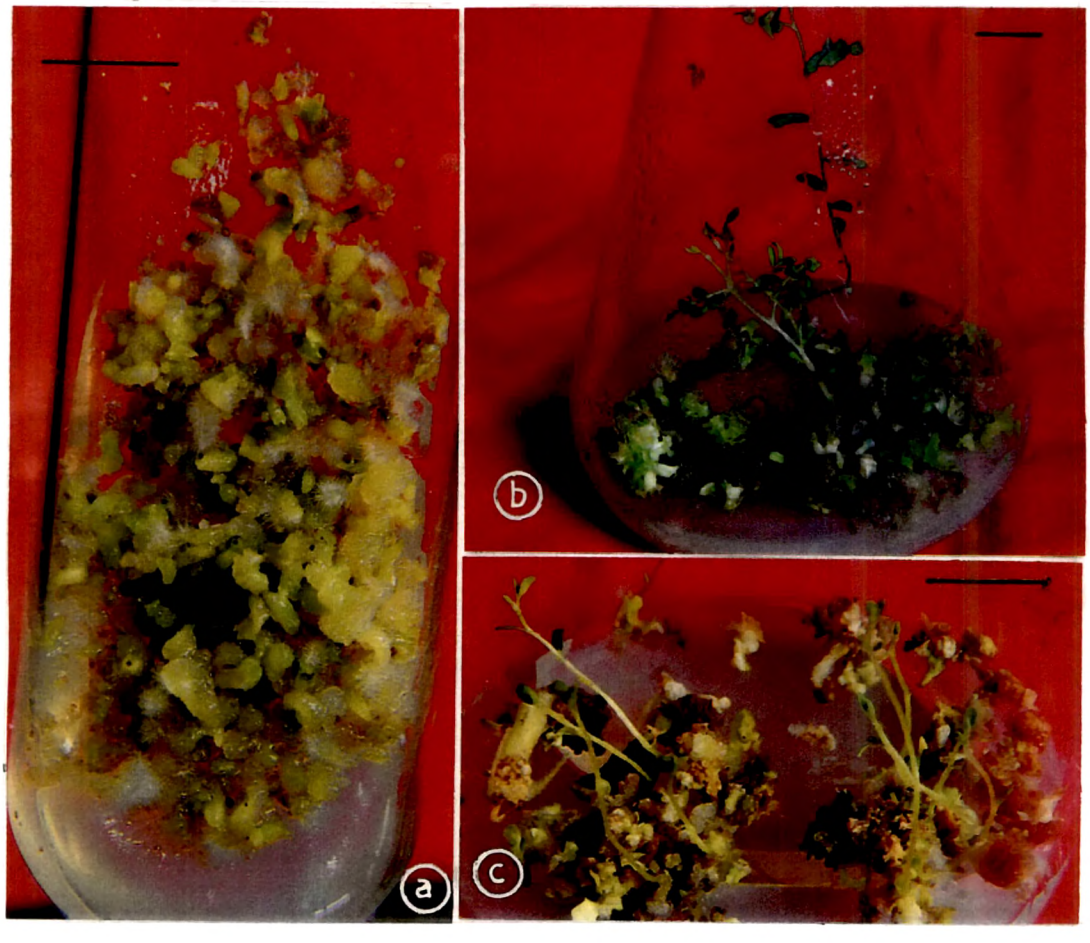
Secondary somatic embryogenesis offers the possibility of large-scale multiplication of plant material. The propagative potential depends on the duration of a cycle of secondary embryogenesis and on the number of embryos produced. Micropropagation by cuttings has a relatively low multiplication rate. With somatic embryogenesis, in principle, discrete propagules are produced which possess the developmental program to grow into a complete shoot without additional shooting or rooting steps, which are necessary in micropropagation, by cuttings. Redenbaugh *et al* (1986) have proposed to encapsulate somatic embryos in an artificial seed coat and use this as a synthetic seed for direct deliverance to the greenhouse of field.

Fig-21 Somatic embryogenesis in *Emblica*

a) Secondary somatic embryogenesis in Aonla

b) germination of embryoids

c) further growth of plantlets on germination


a
b
c

Effect of 2,4-D on secondary embryos of *Emblica officinalis*

According to Raemaker *et al* (1997) the auxins significantly reduced the number of somatic embryos produced. In *Cassava,* at a concentration of 4.5 uM 2,4-D very few secondary embryos were formed.

Table-7.2: Effect of 2,4-D on secondary embryos of *Emblica*

Conc of 2,4-D (uM)	No. of Developing embryos	
	After 2 weeks	After 5 weeks
Control	14.0 ± 0.36	30.0 ± 0.09
0.05	17.2 ± 0.37	36.4 ± 0.23
0.1	13.0 ± 0.35	26.6 ± 0.19
0.2	7.6 ± 0.53	34.3 ± 0.19

* values are the mean percentage of three independent experiments

In the present study with *Emblica officinalis* there was no significant difference in the number of secondary embryos produced after 2 weeks at 0.05 and 0.1 uM concentration of 2,4-D but a slight reduction in the number of embryos was seen at the higher concentration of 2,4-D (0.2 uM) (Table-7.2). However prolonged exposure to 2,4-D resulted in callusing.

For the development of somatic embryos into plants, first the process of embryo proliferation has to be stopped. With auxin induced embryogenesis this should be accomplished by omission of auxin or lowering the concentration. In some species it continued for one or two cycles of secondary embryogenesis (Vasil & Vasil 1981), probably because of the carry over effect of auxin.

Effect of ABA on secondary somatic embryos of *Emblica officinalis*

Maturation is a transitory, frequently indispensable stage between embryo development and embryo germination phases (Quatrano 1987). Stimulation of biochemical events of the *in ovulo* environment by the addition of osmotically active substances of abscisic acid (ABA) to the culture medium has been found to be

effective especially in wheat (Carman 1988) and *Picea glauca* (Attree *et al* 1991). Similarly ABA caused reduced neomorphism, prevented secondary somatic embryogenesis, decreased the conversion of embryos to plantlets *in vitro*, and increased the uniformitiy of resultant embryos (Ammirato1987).

ABA a general plant growth inhibitor was tried to control RSE for the potential use of secondary embryogenesis. The secondary embryos were subjected to different concentrations of ABA in liquid medium (supported by Whatmann filter paper No. 1) as stationary cultures.

Table-7.3: Effect of ABA of secondary embryos of *Emblica*

Conc of ABA (uM)	No. of Developing embryos	
	After 2 wks	After 5 wks
Control	15.00 ± 0.36	36.00 ± 0.22
0.1	13.83 ± 0.29	17.50 ± 0.16
0.2	07.4 ± 0.29	10.20 ± 0.25
0.3	8.80 ± 0.36	10.66 ± 0.04

* values are the mean percentage of three independent experiments

The effect of ABA on number of embryos within two weeks was not significantly different in comparision with control and 0.5 uM. There was some difference in the number of developing somatic embryos and after 5 weeks considerable reduction in the RSE was noticed compared to control. Incorporation of ABA at different concentration in the media slowed but did not induce any cotyledonary stage embryos. Embryos, which were produced, were too small and colourless. There is a possibility that ABA might have arrested the development of embryos at the globular stage itself, so that further development got hampered at the conentration of 0.2 and 0.3 uM. However, transfer of globular embryos to a medium with ABA improved maturation in Carum carvii (Ammirato 1997).

In *Carum carvi* (Ammirato, 1977) and *Medicago* spp (Parrott & Bailey 1993) ABA stopped proliferation. In *Camellia japonica* (Vieitez & Barciela 1990) the use of GA_3 stopped embryo proliferation. ABA has been found to induce more synchronized cell

cultures of Caraway (Ammirato 1977) and Celery (Nadel *et al* 1990) and also to reduce neomorphism, prevent secondary embryogenesis, decrease the conversion of embryos to plantlets in vitro by increasing the uniformity of resultant embryos.

Effect of BAP on secondary somatic embryos of *Emblica:*

There is a large variation of growth regulators used to induce somatic embryogenesis in dicot species. BA was used most frequently, followed by KIN, Zeatin and TDZ.

Table-6.4: Effect of BAP on secondary embryos of *Emblica*

Conc of BAP uM	No. of Developing embryos	
	After 2 wks	After 5 wks
Control	15.00 ± 0.36	20.00 ± 0.0
0.1	15.16 ± 0.35	19.33 ± 0.9
0.2	14.25 ± 0.34	16.00 ± 0.29
0.3	8.80 ± 0.18	16.00 ± 0.31

* values are the mean percentage of three independent experiments

Within 2 weeks no significant difference was noticed in control and 0.1, 0.2 uM of BAP. At 0.3 uM a little difference was noticed. After 5 weeks no significant difference in the number of embryo was noticed in control as well as in different BAP treatments. The treatment of secondary embryos of Aonla with growth regulator 2,4-D and ABA to control RSE did not show any significant effect although 0.3 uM of 2,4-D showed some effect initially but on continuos exposure to (5 weeks) there was no significant difference in the development of embryos.

Germination of synseeds of *Emblica* :

The mature secondary somatic embryos were encapsulated with 4% sodium alginate and $CaCl_2$ 0.6 uM as mentioned. The encapsulated synthetic seeds each containing a secondary embryo were germinated on different strength of basal medium (one-half, one-fourth and full strength).

Table-5.6: Germination of synseeds on MS basal medium

Basal medium (Strength)	No. of encapsulated embryos	No. of Germinating synseeds	
		After 12 days	After 30 days
Full	4	37.0 ± 0.054	48 ±0.052
1/2	4	30.0 ± 0.033	32 ±0.062
1/4	4	13.0 ± 0.11	18 ±0.138

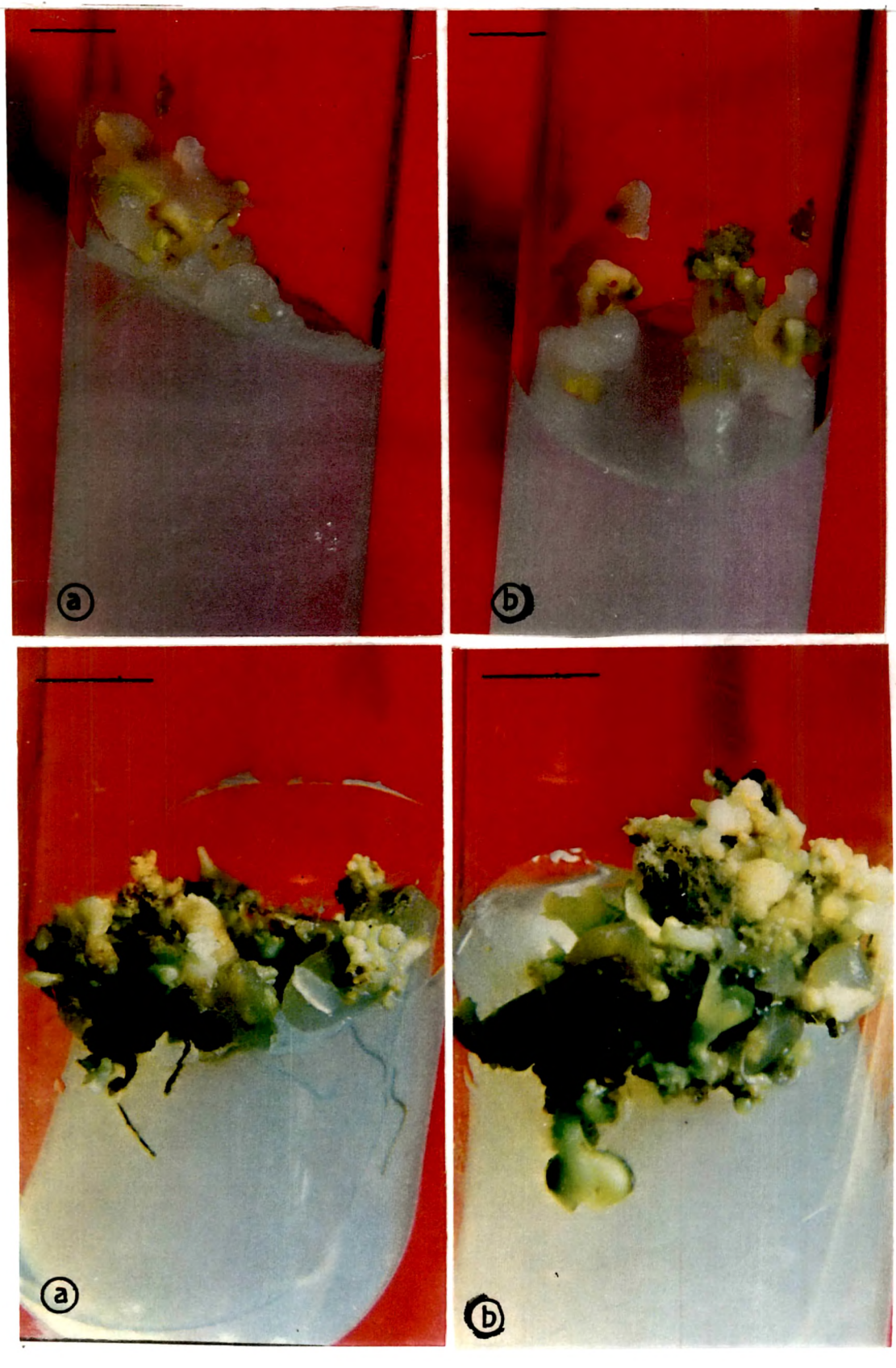
* values are the mean percentage of three independent experiments

In *citrus* often at levels less than full strength medium (lower salt concentration), the results suggest that different stages of embryogensis may require different types of basal salts (Merkel *et al* 1995). In the present study within 12 days there was a difference in the number of embryos in full-strength and half-strength MS media. But in one-fourth strength considerable decrease in number of embryos were seen. After 30 days some difference was noticed in full strength, half strength and one-fourth strength. One-fourth strength gave significantly less number of embryos even after 30 days as compared to full- strength (Fig-22 a & b).

Fig-22 Synthetic seeds of *Emblica*

a) germinating synseeds on one-fourth strength medium (after 2 weeks & 5 weeks)

b) germinating synseeds on full-strength medium (after 2 weeks & 5 weeks)


a
b
a
b