Chapter-6

Sapindus emarginatus

INTRODUCTION:

Present day problems related in the areas of agriculture and forestry are of great concern to mankind. Increase in population, deforestation, the continual loss of agricultural land to housing and industry and problems such as salinity put the mankind into a serious predicament. Today *in-vitro* culture methods have reached a level of sophistication that can solve many problems in the area of forestry and agriculture within a reasonable period. It provides means not only for the mass multiplication of the existing stocks but also for the production of elite plants which are resistant to pests, unfavourable conditions, disease, displaying increased photosynthetic efficiency etc.

The traditional but massive reforestation programs through conventional methods can no longer be a viable alternative because of the long juvenile phase and life span of many forest trees. The conventional methods have their limitation especially when large quantities of propagules are required. The micropropagation technique offers an alternative possibility and the technique has been successfully applied to many of the tree species. However, the micropropagation system must produce large numbers of uniform plants that are genotypically and phenotypically the same as the origin; plant from which they are produced (Evans 1990). The micropropagation of mature trees is important for reforestation programs since it allows multiplication of superior genotypes whose characteristics have been evaluated in the field. Breeding programs achieve genetic gains for desired traits due to additive genetic variation, but non-additive variation, which can exceed 50 % of the total genetic variability, can only be exploited through cloning of mature trees (Hasnain & Cheliak 1986). Further, cloning elite trees increase genetic gain per unit time (Cheliak & Rogers 1990).

Most of the natural propagation takes place through seeds but the progenies produced are highly heterogeneous and also there is no surety of the quality of propagules in advance. So to avoid these problems, vegetative propagation includes cuttings of vegetative parts, layering, grafting and budding. These methods have advantage over seed propagation like faster asexual multiplication and bypassing of undesirable long seed propagation. The most important technique, which is used to make large number of clonal plants, is micropropagation. The method of micropropagation basically

involves regeneration of shoots from meristematic cells or tissues (Ignacimuthu 1996). By definition, micropropagation refers to *in vitro* clonal propagation of plants. Through the use of various plant growth regulators the plants are made to proliferate through induced growth of apical and axillary bud, production of adventitious shoots or through the formation of somatic embryos. However, it sometimes refers specially to axillary bud proliferation.

The micropropagation of elite stock with the right combination of characters will help in genetically upgrading the plant population. This approach can fruitfully be employed for multiplication of plantation crops, timber trees, aromatic and medicinal plants etc. The problem associated with conventional seed and vegetative propagation methods can be mitigated to a large extent by using this tissue culture technology and propagules can be produced at a faster rate especially of an elite stock.

The widespread use of micropropagation has many advantages:

- Rapid large-scale generation of disease-free propagules from selected elite clone(s)
- When classical methods of *in vivo* vegetative propagation prove inadequate, *in vitro* cloning is an important tool in speeding up the propagation.
- Adult plant material, which often cannot be cloned *in vivo*, van, be rejuvenated *in vitro* and then propagated.
- Growth of *in vitro* propagated plants is often stronger than those cloned *in vivo* (mainly due to rejuvenation).
- By *in vitro* propagation, expensive and laborious methods such as grafting and budding on a rootstock can be proved absolute.
- It also enables us to isolate and clone the spontaneous and induced mutants.
- It is immune to variable environmental conditions, therefore round the year production is feasible.
- *In vitro* storage and propagation facilitates the creation of gene-bank (preservation of valuable plant material) and storage under pathogen free condition in a relatively small area. By use of low temperature storage and freezing, the time spent on cloning as well as space required can be drastically decreased.
- This technique allows us to do genetic manipulations, which would be impossible when no methods for regenerating protoplast, cells and tissue exist.

The micropropagation system involves several methods that have been developed to propagate plants in vitro (Razdan 1993). However, following methods are used for micropropagation:

1. Forced shoot proliferation from axillary or apical buds
2. Production of adventitious buds and
3. Somatic embryogenesis

In the later two methods, organized structure arises either directly on the explants or indirectly from callus. Though multiplication through axillary bud produces the least number of propagules it remains the most widely used method in commercial micropropagation. This technique produces true-to-type plantlets as the explants used involve organized meristem. The adventitious bud formation has a greater potential for producing plantlets, as bud primordia may be formed on any part of the inoculated explant. Unfortunately, somatic embryogenesis, which has the potential of producing the largest number of plantlets, presently can only be induced in relatively few species. Somatic embryogenesis either direct or indirect is very useful in rapid tree multiplication and in tree crop improvement. And this system has many advantages over the other ones mentioned above. Millions of embryos can be obtained from a few grams of tissue. Since the somatic embryos are bipolar structures, the plants regenerated from them have well developed tap root system (superior to adventitious root as in other cases), and rooting phase can be obviated as the embryos directly germinate into plants. These embryoids can be encapsulated and treated like seeds, which can be, stored (Redenbaugh *et al* 1988). This technique has not been used for practical purposes because of low frequency of germination. If these two barriers are overcome than it can be exploited commercially. Synchronization and mass production of somatic embryos is now possible by physical and chemical treatments, which includes sieving the suspension through different grades of sieves and exposure to ABA. By manipulating the media factors and by incorporating increased osmoticum it is possible to prevent abnormalities and precocious germination and thus their germination rate can also be enhanced.

Table-6.1: Reports on somatic embryogenesis and encapsulation in tree species:

Species	Explants	References
Acacia coa A. Gray	Hypocotyl	Skolmen 1986
Aesculus hippocastamum	Flower bud	Capuana & Debergh,1997
Abizia amara (Roxb.) Boivin	Hypocotyl	Tomar & Gupta 1986
Albizia richardiana	Hypocotyl	Tomar & Gupta 1986
Azadirachta hippocastamum	Leaves	Su *et al* 1997
Cralaeggus oxyacantha	Apical and subapical meristems	Piccioni & Standardi 1995
Fraxinus americana	Leaflets	Bates *et al* 1992
Ginkgo biloba	Zygotic embryos	Laurain *et al* 1996
Hardwickia binata	Green pods	Das *et al* 1995
Malus alba	Apical,axillary meristems	Rout & Das 1993
Malus pumila	Micropropagated buds Encapsulated	Piccioni 1997
Morus indica	Micropropogated buds	Bapat & Rao 1990,1993
Picea glauca	Leaf,stem(Callus, embryogenesis, synseeds)	Attree *et al* 1994
Populus deltoides	Nodal explant	Adholeya & Cheema 1990
Populus sps.	Leaf tissue	Micher & Bower 1991
Santalum album	Leaves (somatic embryos) Axillary bud encapsulation	Bapat *et al* 1990 Bapat 1993
Sapindus trifoliatus	Leaves	Unnikrishnan *et al* 1990

***Sapindus* (soapnut):** *Sapindus emarginatus* Vahl commonly known as soapnut, belonging to Fam- Sapindaceae is an economically important tree of tropical region. In India it is a native of South India, common on the coast and in the open forests at low elevation. It is a good avenue tree and is occasionally cultivated around villages in West Bengal, Bihar, Madhya Pradesh, Uttar Pradesh. In Gujarat, the fruits of these trees are gathered from Dang forests as minor forest produce. This tree serves as a major source of saponin obtained from the pericarp of its fruits and is used in the manufacture of soaps and detergents.

Medicinal uses of the fruits are:

1. The pulp of the fruit is sweetish and afterwards very bitter. Thus it is hot dry, tonic and alexipharmic.
2. Externally it may be applied by plastering with vinegar, on the bites of reptiles, and to scrotulous swellings.
3. The root is useful as an expectorant.
4. It is also useful in diarrhea and cholera.
5. Pessaries made of kernel of the seed are used to stimulate the uterus during childbirth and amenorrhoea.
6. The pericarp of the pulp and kernel of the fruit is emetic nauseant and expectorant when taken orally.
7. The kernel oil besides this is used as ***hard oil*** in soap making.
8. The black shell of the seed yields a fair amount of dye, suitable for dyeing light shades on merchanted cotton, wood and silk, for dyeing leather, in coloring shoe creams and polishes for staining wood (Anonymous 1952).

MATERIALS AND METHODS:

Plant material:

The tree: *Sapindus emarginatus* (soapnut) located in the Botanical garden of the M. S. University of Baroda served as a primary source (Fig-13). The tender leaves procured from this tree were used as an explant to initiate callus. The leaves were collected and were washed throughly under running tap water (30 min) with few drops of teepol (5 min), followed by 4 – 5 rinses of distilled water. The leaves, under sterile conditions were treated with 0.1 % (w/v) $HgCl_2$ and thereafter washed with several rinses of sterile distilled water. The leaves earlier pre-treated with Bavistin-dimethyl carbendazim (1 %) a fungicide and an antibiotic Rifampcin (0.1 %).

Callus induction:

The leaves were cut along with the mid-rib into approximately 1 x 1 cm^2 pieces (Fig-14 a) and inoculated on semi-solid MS solid medium supplemented with 2 % sucrose, 2 uM 2-4,D and 0.5 uM KIN. The initiated callus was subcultured at 3 – 4 week intervals.

Fig-13 *Sapindus emarginatus* tree (≈ 60 year old) growing in the MSU Botanical garden

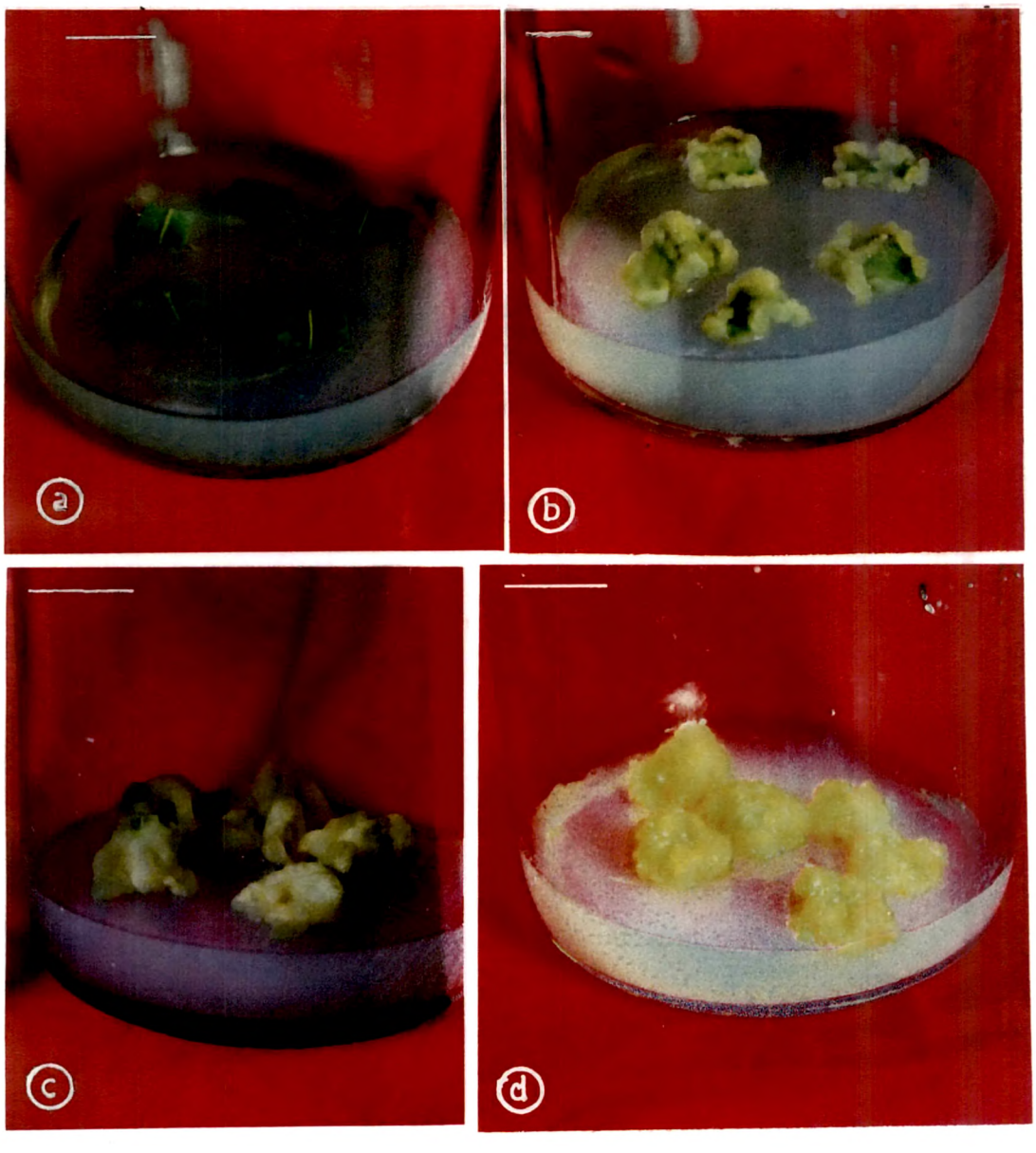
Fig-14 Induction of callus in *Sapindus*

a) leaf explants innoculated on MS medium containing BAP and KIN and

b) Note callus initiation from cut surfaces and vein region

c) further proliferation of callus

d) callus growing after 3 weeks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Somatic embryogenesis:

Desai et al 1986 and Unnikrishnan et al 1990 have already reported somatic embryogenesis in *Sapindus trifoliatus*. The induction of somatic embryogenesis was achieved with gradual decrease in 2,4-D levels and keeping the level of KIN constant. Here three different combinations of growth regulators were tried using BAP and KIN (Fig-15).

Table-6.2: Different combinations tried for somatic embryogenesis

	Concentration (uM)		Response
	BAP	KIN	
A	0.0002	0.001	Creamish coloured callus, showed less number of embryos
B	0.0004	0.001	Callus turned profusely green giving rise to large number of embryos
C	0.0005	0.001	Comparatively less development of somatic embryos.

Hence, in the present studies for embryogenesis, medium supplemented with 0.0004 uM BAP and 0.001 uM KIN and the same was used even for liquid cultures (Fig-16 a & b).

Synchronization:

For synchronization of somatic embryos, the suspension was sieved physically through a passage of sieves having varied mesh size (4.8×10^5 um^2, $1.8 \times 10^5 um^2$ and 1.1×10^5 um^2). And for further maturation and desiccation the somatic embryos were subjected to various concentrations of sucrose and ABA (0.5,1 & 10 uM).

Maturation:

And for further maturation and desiccation the somatic embryos were subjected to various concentrations of sucrose (2-8 %).

Encapsulation:

The mature embryos were encapsulated in 4 % Sodium alginate solution in MS basal

Fig-15 Effect of different concentrations of BAP & KIN tried on the nature of callus and formation of somatic embryos

a) BAP 0.0002 uM & KIN 0.001 uM

b) BAP 0.004 uM & KIN 0.001 uM

c) BAP 0.0005 uM & KIN 0.001 uM

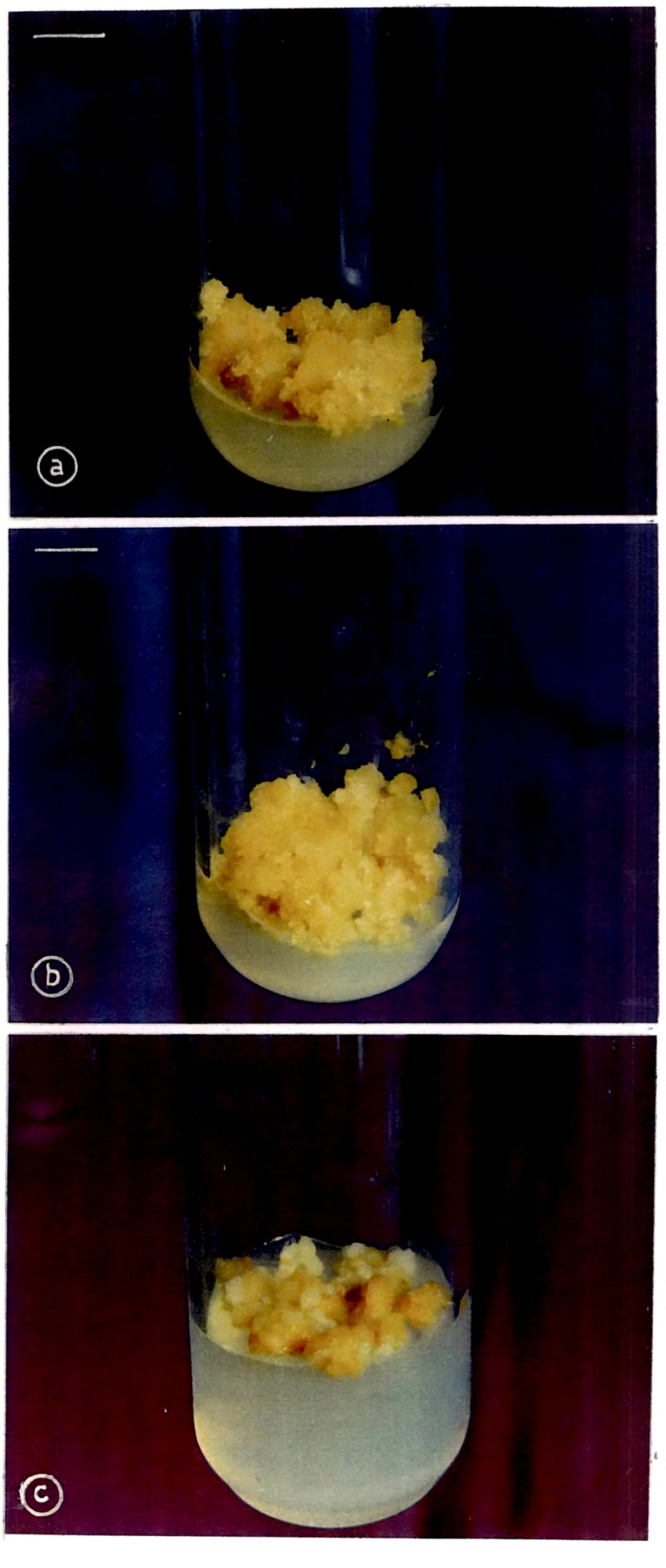
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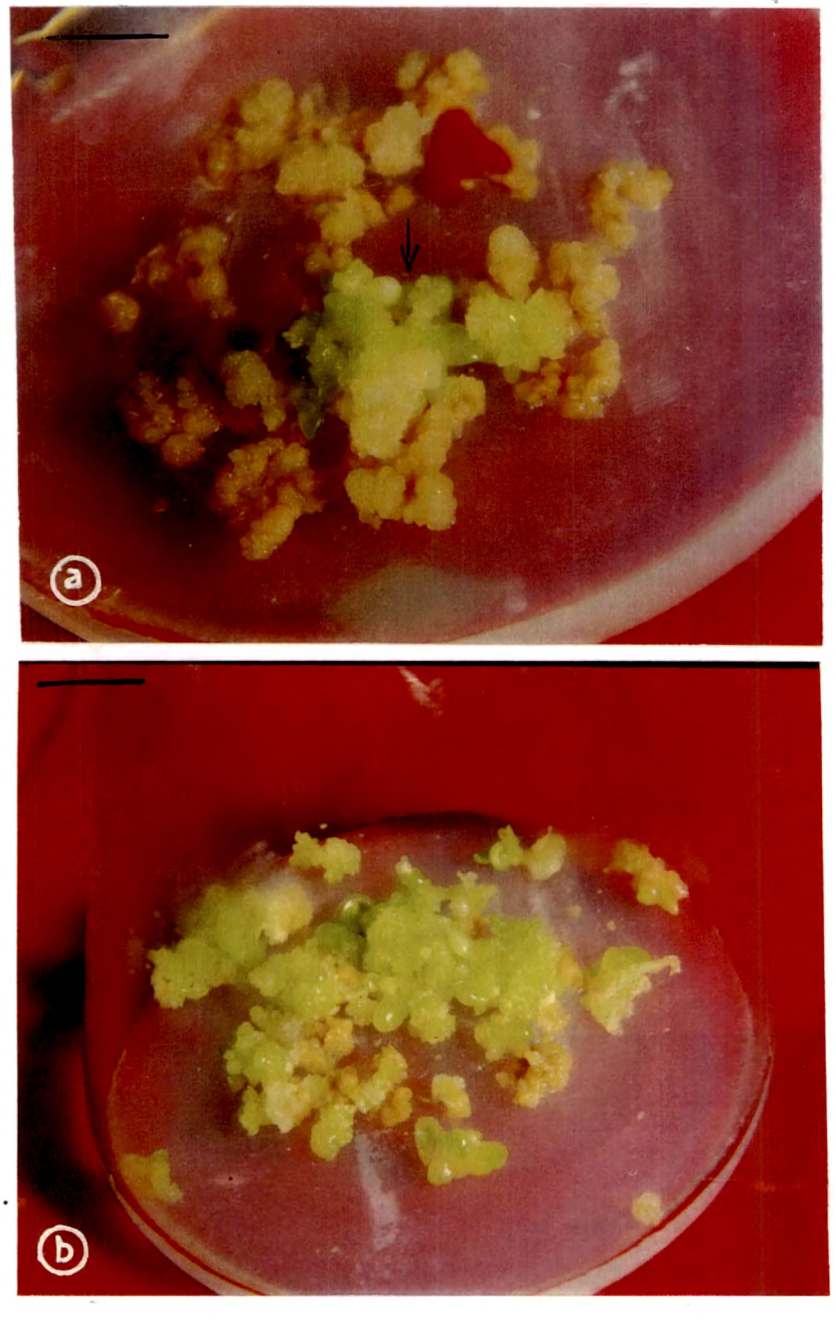
Fig-16 Formation of embryos from embryogenic callus

a) Note the greening of embryos

b) Further proliferation of embryos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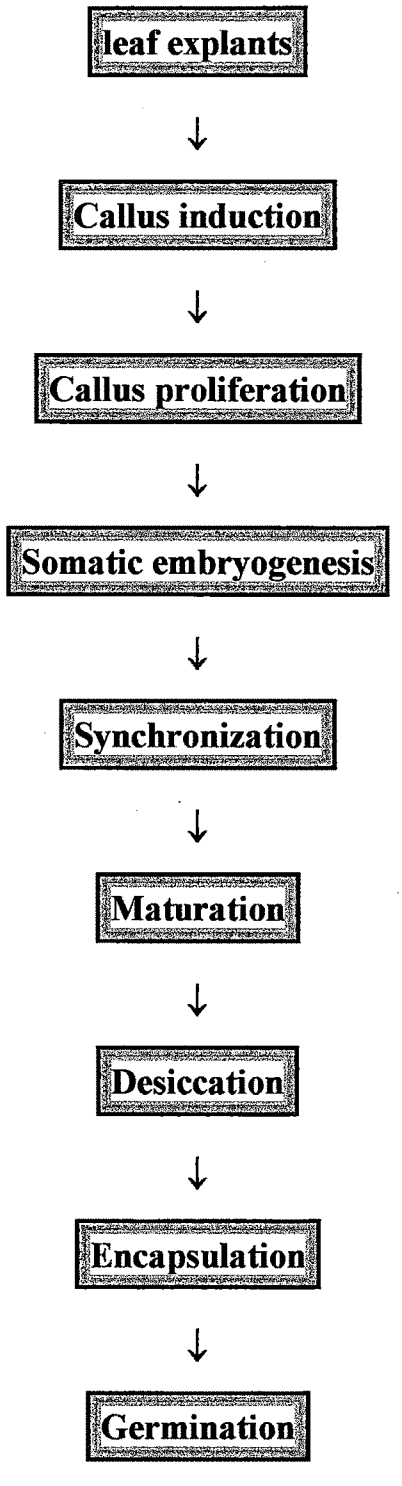
liquid medium and artificial or synthetic seeds, in form of beads, were prepared by dropping individual embryos in $CaCl_2$ solution (0.6 uM) using wide-mouth pipette. After 15 – 20 min incubation the hardened beads were washed with sterile distilled water and were kept on sterile filter paper, dried properly, stored (at low temperature) and were inoculated on MS basal solid medium.

Germination:

The synseeds so formed were germinated on MS basal semisolid medium having 3 % sucrose. The percentage germination of synseeds was noted after 7,14 & 21 days respectively

Fig-6: Basic protocol followed for the production of artificial seeds of *Sapindus emarginatus*



RESULTS AND DISCUSSION:

The friable yellowish callus obtained on MS medium supplemented with 2 uM 2,4-D and 0.5 uM KIN was allowed to proliferate (3-4 weeks). After one or two subculture it was transferred to MS basal liquid medium supplemented with 0.0004 uM BAP and 0.001 uM KIN for the induction of somatic embryogenesis. For synchronization, the cell suspension was passed through a series of stainless steel sieve of different mesh size (4.8×10^5 um^2; 1.8×10^5 um^2 and 1.1×10^5 um^2).

There are two possible causes of dormancy *in vitro*:

1) Dormancy that is typical for zygotic embryos may also be expressed by somatic embryos of the same species *in vitro*.
2) Dormancy may be artificially induced by aspects of the culture environment such as exogenously applied growth regulators (Gray & Purohit 1991).

ABA has been used to physiologically synchronize embryogenesis. ABA might interfere with the functions of endogenous growth regulators, causing a shift to conditions that favour embryogenesis. Also ABA has been documented to function as an osmoregulator by causing cell water content to decrease, leading to more normal development (Gray & Purohit 1991). ABA was incorporated into the medium in concentration range of 0.5, 1 and 10 uM after 30 days of incubation in the embryogenic medium having the presence of heart shaped embryos (Fig-17).

The collected form of suspension after 30 days of incubation in the embryogenic medium was passed through different grades (4.8×10^5 um^2 –Grd-1,1.8×10^5 um^2 – Grd-2 and 1.1×10^5 um^2 Grd-3) of stainless sieve mesh and further transferred to MS basal liquid medium containing different concentrations of ABA. The percentage synchronization was noted having the presence of mature cotyledonary embryos subjected to different concentration of ABA after 7,14 and 21 days of culture. ABA concentration of 10 uM was most effective showing 38.6 % of mature somatic embryos after 21 days of incubation whereas 10 uM ABA proved to be inhibitory in terms of conversion of embryoids after 21 days of culture (Table-6.3).

Fig-17 Different stages of embryoids in *Sapindus*

a) globular

b) intermediate stage Note arrow for the beginning of a notch

c) heart stage

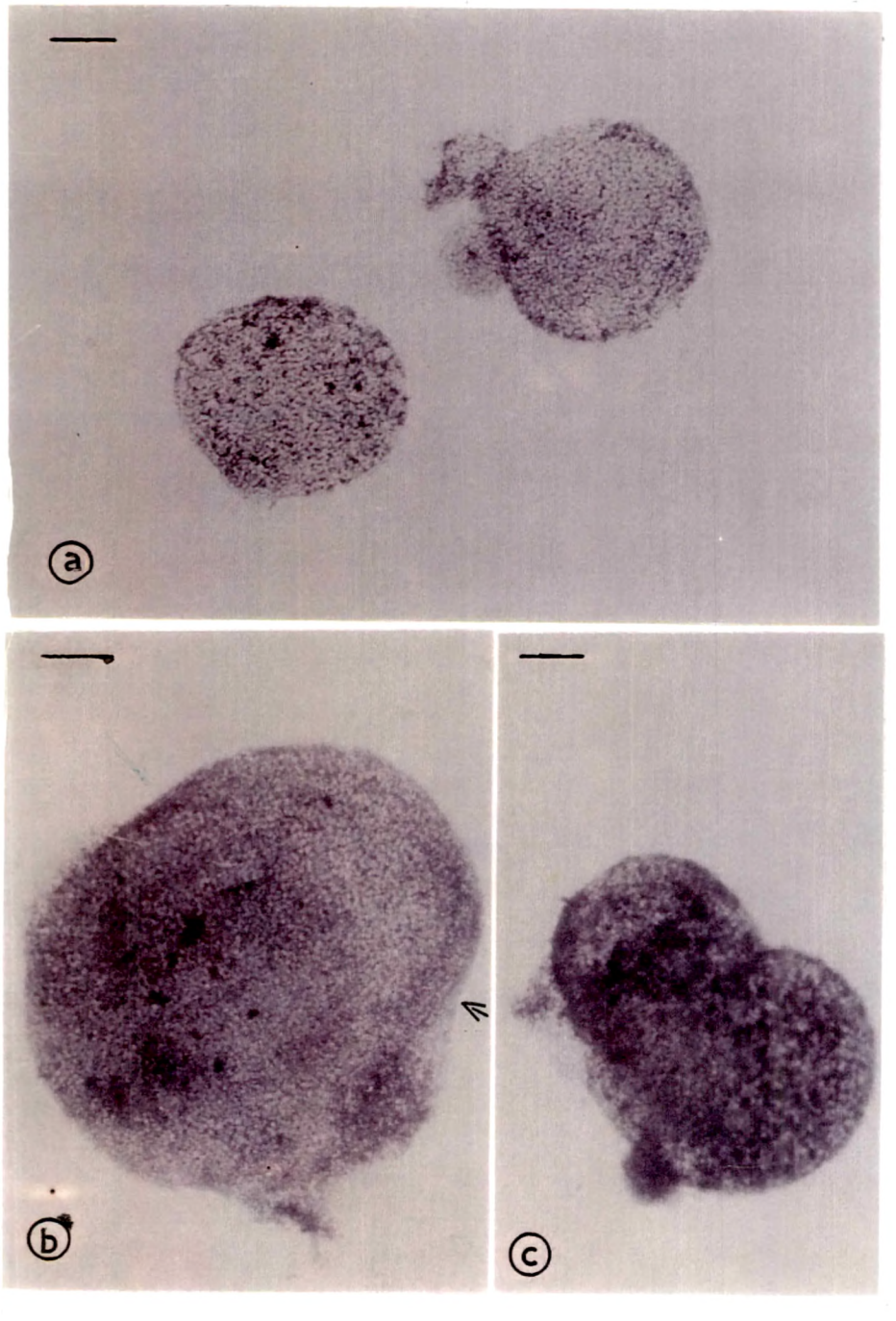

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Table-6.3: Effect of ABA on Synchronization (number of embryoids) for formation of embryoids in *Sapindus emarginatus*

Concentration of ABA (uM)	Days	Synchronization (%)		
		Grd.1	Grd.2	Grd.3
Control		0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
0.5	7 days	11.6 ±1.6 [b]	21.6 ±2.8 [bc]	13.3 ±3.3 [c]
	14 days	13.3 ±2.8 [b]	25.0 ±1.6 [bc]	14.8 ±2.8 [c]
	21 days	15.0 ±1.6 [b]	31.6 ±1.6 [ab]	15.0 ±2.8 [c]
1.0	7 days	11.6 ±2.8 [b]	31.6 ±2.8 [ab]	21.6 ±1.6 [b]
	14 days	15.0 ±5.2 [b]	50.0 ±0.6 [ab]	28.3 ±4.4 [a]
	21 days	29.6 ±0.8 [a]	38.6 ±0.8 [ab]	32.6 ±1.4 [a]
10	7 days	12.6 ±0.6 [b]	21.3 ±0.5 [bc]	11.0 ±0.5 [c]
	14 days	11.3 ±0.8 [b]	16.0 ±0.5 [c]	13.0 ±0.5 [c]
	21 days	14.4 ±1.2 [b]	12.0 ±3.0 [c]	14.2 ±0.5 [c]

- means within the column followed by the same superscript letter are not significantly different ($P \leq 0.05$, Duncan's multiple range test)
- values are the mean percentage of three independent experiments
- Grd.1-4.8 x 10^5 um^2,Grd.2-1.8 x 10^5 um^2 and Grd.3-1.1 x 10^5 um^2

Somatic embryos of alfalfa after dehydration and treatment with ABA showed 90 – 100 % conversion. After 1 year of dried storage without humidity control, 60 % of the embryos germinated into seedlings (Senaratna *et al* 1990). Ability of the alfalfa embryos to withstand dehydration was due to ABA pretreatment which induced a state of desiccation tolerance. ABA applied at 10 uM to 14-day old embryos was most effective. Specific pulse-timed applications of ABA in conjunction with controlled drying approximated aspects of the developing seed environment. Provision of desiccation tolerance to somatic embryos clearly demonstrates that they are capable of entering complex developmental pathways normally associated with those of seed embryos when proper environmental conditions are provided.

The ability of ABA to promote the accumulation of embryo specific proteins in zygotic embryos is well established. Crouch and Sussex (1981) demonstrated that ABA promoted embryo growth and protein accumulation in *in vitro* cultures rapeseed embryos. Later Ackerson (1984) provided the evidence that ABA is indeed necessary for normal embryogenesis and ABA selectively inhibited the synthesis and translation of certain m-RNAs only. It is conceivable therefore that ABA does not restrict storage protein synthesis (Ackerson1984), but suppresses at the same time the synthesis of germination enzymes (Ihle & Dure 1972) thereby inhibiting precocious germination. The enhancement in the frequency of germination of ABA treated embryoids can be attributed to the increased protein content compared to the control embryoids, because it is already shown that ABA can influence the synthesis of embryo specific m-RNAs (Dure 1985; Galan *et al* 1986 & Quatrano 1986). Also ABA not only promoted the accumulation of storage proteins but also inhibited precocious germination of somatic embryos in interior spruce (Roberts et al 1990) and microspore derived embryos of *Brassica napus* (Holbrook et al 1990).

Table-6.4: Effect of sucrose on maturation of somatic embryos

Sucrose (%)	Germination (%)
Control	
2	0.0 ±0.0
3	10.6 ±0.6 [c]
4	32.4 ±1.7 [a]
5	29.3 ±0.6 [a]
6	23.0 ±2.5 [ab]
7	12 6 ±1.4 [c]
8	11.0 ±0.5 [c]

- means within the column followed by the same superscript letter are not significantly different ($P < 0.05$, Duncan's multiple range test)
- values are the mean percentage of three independent experiments

After the exposure of embryoids to the different concentrations of sucrose in the medium for germination 4 % sucrose favoured embryo maturation and almost 33 % germination was noted compared to control after a period of 10 days. However the

exposure of embryoids in higher concentration of sucrose proved to be inhibitory and the rate of embryo maturation in reduced to almost 11 % (Table-6.4).

The physiological processes involved in seed development mean that at maturity the zygotic embryos are bipolarized, extremely rich in starch and protein reserves and also fully plasmolysed. Somatic embryos display none of these characteristics at the end of their development, accounting for their unsuitability for germination and subsequent plant conversion (Etienne *et al* 1993). Effect of sucrose concentration is both nutritive and osmotic and the increased level of sucrose concentration seems to stimulate endogenous levels of ABA synthesis (McKersie et al 1990).

To document the maturation of embryoids

1) Morphological: normal somatic embryoids should possess well-defined axis with two cotyledons and
2) physiological: normal somatic embryos germinate into seedling (Fig-18 a-c) .

The promotory effect of sucrose on the maturation of somatic embryos of soapnut might be due to the involvement of ABA in response to the osmotic stress. Eventhough the effect of sucrose was less pronounced as only 33 % of somatic embryos underwent germination the frequency of germination was high compared to the control. Under osmotic stress it was reported that the induction of ABA synthesis is promoted in spinach (Creelman & Zeewart 1985) and in suspension cultures of grape pericarp (Loveys *et al* 1975). Thus the effect of increased concentration of sucrose in the medium and embryo maturation may be the result of ABA synthesis under osmotic stress and this in turn favoured the synthesis of embryo specific proteins mediated by ABA, followed by the germination of the embryoids.

Crouch (1982) observed that accumulation of a 12 S storage protein in hypocotyl and pollen-derived somatic embryos of Brassica was necessary for obtaining efficient plantlet conversion. Likewise, the matured somatic and zygotic embryos of alfalfa were found to be rich in a 11S storage protein (Staurt et al 1985; Staurt & Redenbaugh 1987). However, direct addition of ABA in to the media is much more effective in in regulating embryo maturation. Ammirato (1986) echoed the same view in the case of carrot.

Fig-18 Germination of embryo in *Sapindus*

a) Induction of first leaf

b) growth of first leaf

c) further growth Note (arrow) the cotyledonary leaves

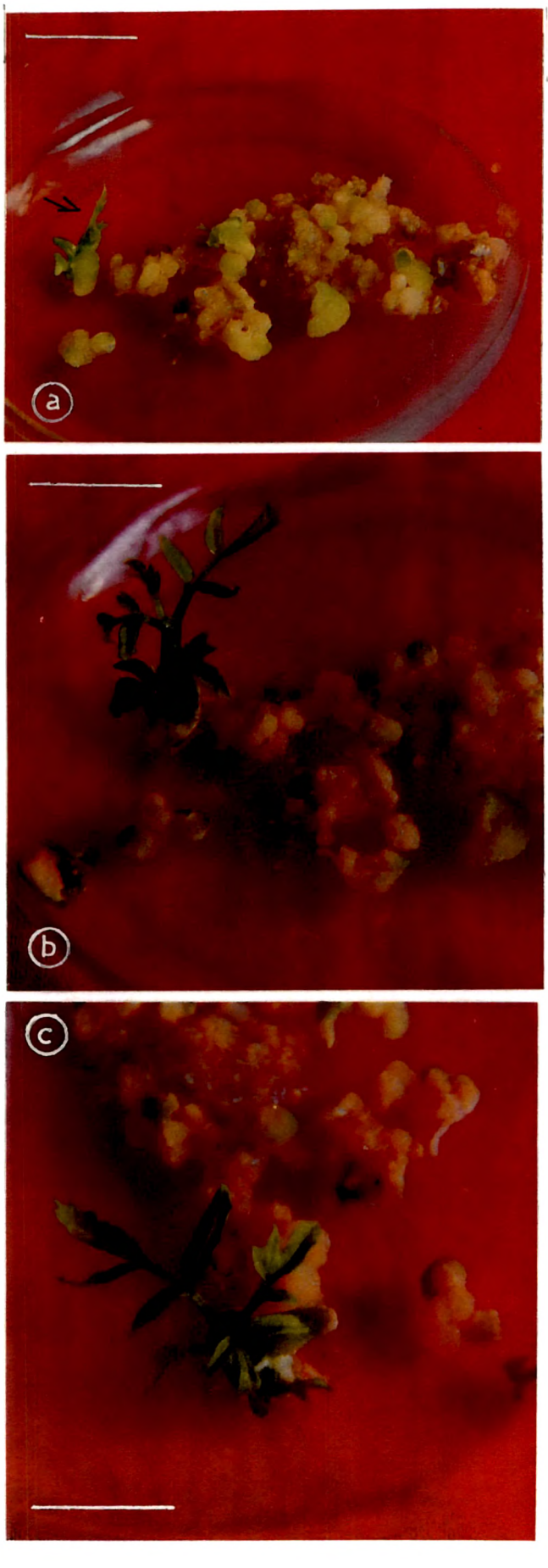
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Table-6.5: Germination of synseeds of *Sapindus emarginatus*

No. of Days	Germination (%)
Control	100.00 ± 0.0
7	77.66 ± 1.4
14	65.00 ± 1.1
21	40.00 ± 1.1
28	19.00 ± 0.5
35	05.40 ± 0.8

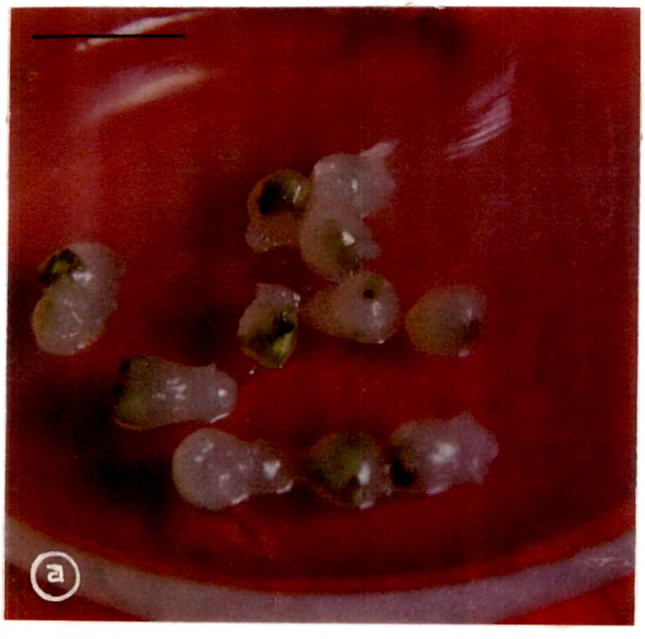
- values are the mean percentage of three different experiments

In the present system the somatic embryos undergo a slow desiccation under the effect of ABA and maturation process when subsequently exposed to sucrose. These somatic embryos were encapsulated as discussed above, and they showed about 40 % germination after 21 days of incubation on MS basal (solid) medium (Table-6.5) (Fig-19 a & b). The additon of 1 mmol m^{-3} ABA to the medium containing 341 mol m^{-3} sucrose increased the germination rate and improved conversion into plants. The use of such an ABA treatment followed by desiccation has been found to be effective in improving the germinability of somatic embryos of *Picea glauca-engelmanii, Picea sitchensis* (Roberts *et al* 1991) and oilseed rape (Senartna *et al* 1991).

Fig-19 Synseeds in *Sapindus*

a) well-formed synthetic seeds placed for germination

b) & c) various stages of germination synthetic seeds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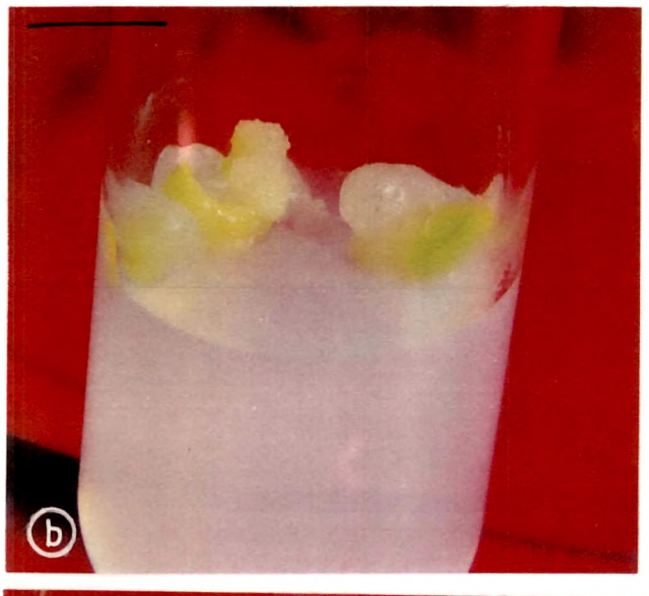
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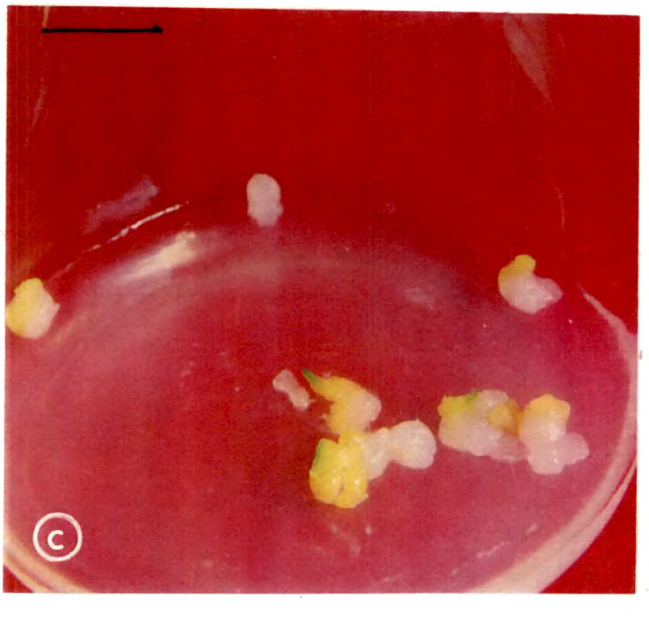
c