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REVIEW

Endosperm culture: a novel method for triploid plant production

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Abstract Triploid nature of endosperm is the characteristic feature of angiosperms and is formed as a result of triple fusion. Present review discusses the morphogenic response and production of triploid plantlets by endosperm culture. Both mature and immature endosperm used for culture initiation responded differently in cultures. A key factor for the induction of cell divisions in mature endosperm cultures is the initial association of embryo but immature endosperms proliferate independent of embryo. In almost all the parasitic angiosperms, endosperm shows a tendency of direct differentiation of organs without prior callusing, whereas in autotrophic taxa the endosperm usually forms callus tissue followed by differentiation of shoot buds, roots or embryos. The endosperm tissue often shows a high degree of chromosomal variations and polyploidy. Mitotic irregularities, chromosome bridges and laggards are the other important characteristics of endosperm tissues. Triploids are usually seed sterile and is undesirable for plants where seeds are commercially useful. However, in cases where seedlessness is employed to improve the quality of fruits as in banana, apple, citrus, grapes, papaya etc. the induction of triploid plants would be of immense use. Triploid plants have more vigorous vegetative growth than their diploid counterparts. Hence, in plants where the vegetative parts are economically useful, triploids are of good use. This review focuses on the progress achieved so far in endosperm culture to obtain triploid plants.

Keywords Endosperm culture · Triploids · Embryos · Differentiation · Embryogenesis

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Abbreviations

BA	N6-Benzyladenine
CH	Casein hydrolysate
CM	Coconut milk
CE	Corn extract
CWM	Cow's milk
DAP	Days after pollination
2, 4-D	2, 4-Dichlorophenoxyacetic acid
GA_3	Gibberellic acid
GJ	Grape juice
IBA	Indole-3-butyric acid

IAA	Indole-3-acetic acid
Kn	6-furfurylamino purine (kinetin)
MS	Murashige and Skoog medium
MT	Murashige and Tucker medium
NAA	α-Naphthalene acetic acid
PE	Potato extract
TDZ	Thidiazuron
TJ	Tomato juice
YE	Yeast extract
WM	White's medium

Introduction

Endosperm is a distinctive tissue in its origin, development and ploidy. It is formed as a result of fusion of three haploid nuclei—one from male gametophyte and the other two from female gametophyte. This phenomenon is called triple fusion and is very common in angiosperms. More than 80% of the flowering plants are having endosperms in their developing seeds, which provide nutrition to the growing embryo (Williams and De Latour 1980). Any abnormality in the development of endosperm may cause abortion of embryo resulting in sterile seeds (Johnston et al. 1980). Endosperm may be totally consumed by developing embryo leading to the formation of exalbuminous (non-endospermous) seeds or when it persists, the seed is called albuminous (endospermous). In albuminous seeds the persisting endosperm is used as a food source, which may contain proteins, starch or fats and the embryo can utilize this food during seed germination.

Triploid nature of endosperm is the characteristic feature of angiosperms. However, various ploidy levels are also observed in plants like *Butomopsis* sp. (diploid), *Fritillaria* sp. (pentaploid), *Acalypha indica* and *Peperomia* sp. (polyploid). In apomictic species of *Taraxacum* and *Erigeron* the endosperm develop autonomously, without fertilization of the secondary nucleus (Battaglia 1963). In another apomictic species *Brachiaria setigera*, the endosperm gave rise to triploid embryos in vivo and seedlings where six out of 675 aposporous embryo sacs of post-fertilization ovules contained endosperm-embryos (Muniyamma 1977).

Triploid plants are usually seed sterile and is undesirable where seeds are of commercial value. But in cases where the seedlessness is employed to improve the quality of fruits as in banana, apple, citrus, grapes, papaya etc. induction of triploid plants would be of immense use. Triploids have more vigorous vegetative growth than their diploid counterparts. Hence, in plants where the vegetative parts are economically useful triploids are of good use. For example, triploid *Populus tremuloides* contain superior pulp quality and is, therefore, preferred over its diploid counterparts (Bhojwani and Razdan 1996). Triploids of mulberry (*Morus* sp.), which are under cultivation in the Northern part of Japan, are known for their superior quality of leaves and disease resistance (Hamada 1963). The triploid plants of tomato produce larger and tastier fruits than natural diploids (Kagan-Zur et al. 1990). The triploid plants of rice (*Oryza sativa*) produced from endosperm showed broader leaves, a faster growth rate, and more of tillering than the normal diploid plants (Bajaj et al. 1980).

Traditionally, triploids are produced by hybridization between induced superior tetraploids and diploids. The first step in this process is to produce tetraploids by colchicine treatment of germinating seeds, seedlings or vegetative buds (Das et al. 1970; Dwivedi et al. 1986; Verma et al. 1986; Sikdar and Jolly 1994; Chakraborty et al. 1998). In most of these cases the rate of induction of tetraploids had been low (7–22%). Moreover, the treatment is lengthy and laborious. Once tetraploids have been produced, their cross with the diploid parent may not be successful in majority of the cases. In successful crosses the seed-set, seed germination and survival rate of the seedlings is low (Sikdar and Jolly 1995). Further, all sexually produced triploids do not behave uniformly, which may be due to segregation both at tetraploid level and subsequent population of crosses with putative diploid (Dandin 1990).

In contrast, in vitro regeneration of plants from endosperm offers a one step approach to triploid production. The sexually sterile triploids can also be bulked up in vitro by micropropagation. Attempts to grow endosperm tissue in cultures began in early 1930s and since then immature and mature endosperms of various angiosperm taxa (autotrophic as well as parasitic) have been successfully cultured. Lampe and Mills (1933, cited in La Rue 1936) first attempted to grow immature endosperm of *Zea mays* on a medium containing potato extract or young corn extract and observed minor proliferation of cells in

the vicinity of the embryo. Later La Rue and colleagues at University of Michigan, Ann Arbor, USA did extensive research in this field. In 1949 La Rue for the first time reported the possibility of obtaining a continuously growing tissues from the cultured immature maize endosperm (La Rue 1947, 1949). Following this report, tissue cultures were established from immature endosperm of *Asimina triloba* (Lampton 1952), *Lolium perenne* (Norstog 1956), *Cucumis sativus* (Nakajima 1962), *Triticum aestivum* (Sehgal 1974), *Malus pumila* (Mu et al. 1977), Citrus (Wang and Chang 1978), *Oryza sativa* (Nakano et al. 1975; Bajaj et al. 1980), *Hordium vulgare* (Sun and Chu 1981), *Asparagus officinalis* (Liu et al. 1987), *Acacia nilotica* (Garg et al. 1996), *Morus alba* (Thomas et al. 2000) and *Azadirachta indica* (Chaturvedi et al. 2003).

Induction of cell division and proliferation of mature endosperm was initially considered as a difficult task. The first report on proliferation of mature endosperm was reported by Rangaswamy and Rao (1963) in *Santalum album* followed by Mohan Ram and Satsangi (1963) in *Ricinus communis*. The mature endosperm culture was reported in a number of systems like *Croton bonplandianum* (Bhojwani and Johri 1971), *Jatropha panduraefolia*, *Ricinus communis* (Srivastava 1971a, b; Johri and Srivastava 1973) and *Actinidia deliciosa* (Machno and Przywara 1997; Goralski et al. 2005).

Factors controlling callus proliferation and plant regeneration

Endosperm at culture

Usually culture of endosperm needs the selection of seeds at proper stage of development. This is usually calculated as days after pollination (DAP) and it varies from plant to plant as 9–10 DAP in *Lolium perenne* (Norstog 1956), 8–11 DAP in *Zea mays* (Straus and La Rue 1954; Tamaoki and Ullstrup 1958), 8 DAP in *Triticum aestivum* and *Hordeum vulgare* (Sehgal 1974), 4–7 DAP in *Oryza sativa* (Nakano et al. 1975) and 17–20 DAP in *Morus alba* (Thomas et al. 2000). Free nuclear endosperm did not produce any callus and the intensity of response depends on the level of organization of endosperm cells.

Plant growth regulators and other supplements

Selection of a suitable basal medium, addition of proper growth regulators and other adjuvants are the decisive factors that determine the success of triploid plant development. The culture of immature endosperm requires yeast extract (YE), casein hydrolysate (CH), coconut milk (CM), corn extract (CE), potato extract (PE), grape juice (GJ), cow's milk (CWM) or tomato juice (TJ) despite a suitable medium and growth regulators. Murashige and Skoog (1962) basal medium was mostly used to initiate and improve the response in in vitro endosperm cultures. White (1963) basal medium (WM) was also employed in some cases. La Rue (1949) used various organic supplements like CE, PE, TJ, GJ, YE or CWM to raise endosperm callus cultures. Of these, TJ was found to be superior over other additives due to cytokinin-like activities (Sternheimer 1954). In some cases the callus growth was erratic and unpredictable in TJ supplemented medium as cytokinin-like activity of TJ decreases with the age of the tomato fruit (La Rue 1944; Bottomley et al. 1963).

Later it was found that the TJ could be replaced by YE (La Rue 1944; Tamaoki and Ullstrup 1958). YE induced callus proliferation was reported in *Zea mays* (Sehgal 1969; Zhu et al. 1988), *Croton* (Bhojwani and Johri 1971), *Jatropha panduraefolia* (Srivastava 1971a), *Lolium* (Smith and Stone 1973; Norstog 1956), *Ricinus communis* (Johri and Srivastava 1973), *Oryza sativa* (Nakano et al. 1975; Bajaj et al. 1980), *Coffea arabica* (Monoco et al. 1977) and *Juglans regia* (Tulecke et al. 1988).

Other additives like CH in *Exocarpus cupressiformis* (Johri and Bhojwani 1965), *Dendrophthoe falcata* (Nag and Johri 1971), *Nuytsia floribunda* (Nag and Johri 1971), *Putrangiva roxburghii* (Srivastava 1973), *Hordeum vulgare* (Sehgal 1974), *Achras sapota* (Bapat and Narayana swamy 1977), *Citrus grandis* (Wang and Chang 1978), *Prunus persica* (Liu and Liu 1980), *Actinidia chinensis* (Gui et al. 1982), *Actinidia urguta* × *A. deliciosa* (Kin et al. 1990) and CM in *Acacia nilotica* (Garg et al. 1996), and *Codiaeum variegatum* (Gayatri 1978) were also employed by different workers. Most of the immature endosperm culture needs the presence of one or more growth regulators for plant regeneration except *Petroselinum*, where MS basal medium is sufficient for endosperm embryogenesis (Masuda

et al. 1977). In majority of reports an auxin preferably 2, 4-D is necessary for callus induction.

In case of mature endosperm of parasitic plants the optimum callus growth was observed either on a cytokinin or a cytokinin in combination with an auxin and for autotrophic taxa, cytokinin, auxin, CH or YE is necessary. In most of the cases the time required to initiate proliferation varies from 10 to 20 days. But a presoaking of endosperm with GA_3 (5.78 μM) could reduce the time period from 10 to 7 days in *Ricinus* (Srivastava 1971b; Johri and Srivastava 1973).

Physical factors

This includes effect of temperature, light and pH on endosperm proliferation. But the fact is that very few workers concentrated on this aspect of endosperm culture and that also limited mainly to maize and caster bean. Straus and La Rue (1954) observed that corn endosperm develop better in dark than light condition. But in *Ricinus* reverse is the case where a continuous light period was found optimum for endosperm proliferation (Srivastava 1971b). In some cases, the endosperms were cultured along with the embryo and kept in the diffuse light with 16 h photoperiod. Light conditions facilitate the early germination of embryo, which can be removed easily due to their characteristic green colour (Thomas et al. 2000). In coffee, the endosperm callus grows better under 12 h light/dark conditions (Keller et al. 1972). In *Lolium* the light doesn't have any significant role on endosperm proliferation (Norstog 1956).

Not much research has been carried out till date with regard to the effect of temperature and pH on endosperm proliferation. In available literature the optimum temperature for endosperm growth was reported to be 25°C (Johri and Srivastava 1973). The pH varies from 4.0 for *Asimina* (Lampton 1952) to 5.0 for *Ricinus* (Johri and Srivastava 1973), 5.6 for *Jatropa* and *Putranjiva* (Srivastava 1971a, b, 1973) and 6.1 for *Zea mays* (Straus and LaRue 1954). In general, pH 5.5–5.8 seem to support the best growth of endosperm tissues in cultures.

The embryo factor

There is an absolute necessity of the so called "embryo factor" for the proliferation of endosperm (Bhojwani 1968; Srivastava 1971a, b). According to Brown et al. (1970) and Kagan-Zur et al. (1990) some factors contributed by the germinating embryo is required for the stimulation of mature and dried endosperm of caster bean and tomato, respectively. In general, it has been found that mature endosperm requires the initial association of embryo to form callus but immature endosperm proliferates independent of the embryo. However, in neem the association of the embryo proved essential to induce callusing of immature endosperm; the best explant was immature seeds (Chaturvedi et al. 2003; Figs. 1 and 2). Similar observation for mulberry was reported by Thomas et al. (2000; Fig. 3). However, the embryo factor can be overcome by the use of GA_3 as was observed in *Croton bonplandianum* (Bhojwani 1968) and *Putranjiva roxburghii* (Srivastava 1973). It is reported that during germination the embryo releases certain gibberellin like substances, which may promote the endosperm proliferation (Ogawa 1964; Ingale and Hageman 1965). However, the mature endosperms of *Achras sapota* (Bapat and Narayanaswamy 1977), *Santalum album* (Lakshmi Sita et al. 1980), *Emblica officinalis* (Sehgal and Khurana 1985) and *Juglans regia* (Cheema and Mehra 1982; Tulecke et al. 1988) could proliferate without the association of embryo or pre-soaking of them in GA_3.

Shoot regeneration

Organogenesis from endosperm tissue was first reported in *Exocarpus cupressiformis* (a member of the family Santalaceae) by Johri and Bhojwani (1965). They obtained shoot buds all over the endosperm explant on WM supplemented with IAA, Kn and CH. However, the omission of CH from the medium increased the number of shoot buds from 13 to 26.

The pathway of plant regeneration includes shoot-bud differentiation or embryogenesis directly from the explants or indirectly from proliferating callus (Table 1). In almost all the parasitic angiosperms, the endosperm shows a tendency of direct differentiation of organs without prior callusing, whereas in autotrophic taxa the endosperm usually forms callus tissue followed by differentiation of shoot buds, roots or embryos. Direct shoot regeneration from the cultured endosperm was observed in a number of semiparasitic angiosperms including *Exocarpus*, *Taxillus*, *Leptomeria*, *Scurrula* and *Dendrophthoe*.

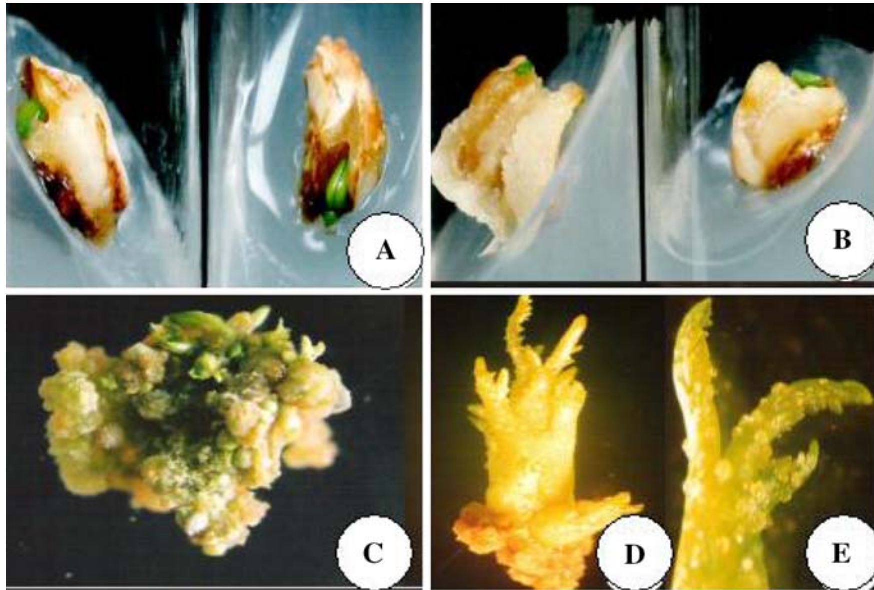
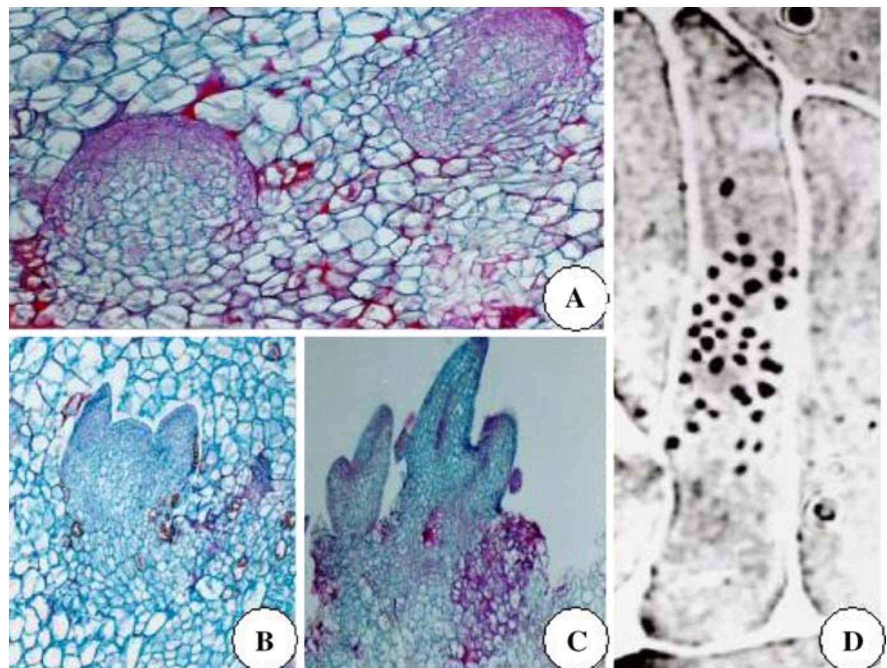


Fig. 1 (**a**) Immature seeds of *Azadirachta indica* on MS + NAA (5 μM) + BA (2 μM) + CH (500 mg/l). After 2 weeks seeds have split open and releasing the green embryos and callused endosperms. (**b**) White fluffy endosperm calli can be seen from the fully opened seeds after 3 weeks. Green embryos are lying at one end of the explants. (**c**) 6-week-old subculture of endosperm callus on MS + BA (5 μM), showing the differentiation of distinct shoots and nodules as well. (**d**) 3-week-old culture on MS + BA (0.5 μM), showing a healthy elongated shoot. (**e**) Same as **d**, enlarged view of shoot tip region showing numerous glands on the surface of leaves

Fig. 2 (**a**) Section of a 2-week-old regenerating endosperm derived callus of *A. indica* from MS + BA (5 μM), showing endogenous meristematic pockets. (**b**) Same as **a**, after 3 weeks, showing young endogenous shoot bud. (**c**) Section of a 4-week-old regenerating callus from MS + BA (5 μM), showing distinct shoot buds differentiated from peripheral vascularized nodules. One of the buds showing glands. (**d**) A cell from the root-tip of endosperm origin, showing triploid number of chromosomes ($2n = 3x = 36$)



Direct shoot regeneration occurred in *Taxillus* sp. on WM fortified with KN (46.5 μM) (Nag and Johri 1971). In *Exocarpus* an auxin (IAA, 5.71 μM) along with cytokinin (Kn, 4.65 μM) was required for direct shoot regeneration (Johri and Bhojwani 1965). Addition of zeatin in WM gave rise to green shoots

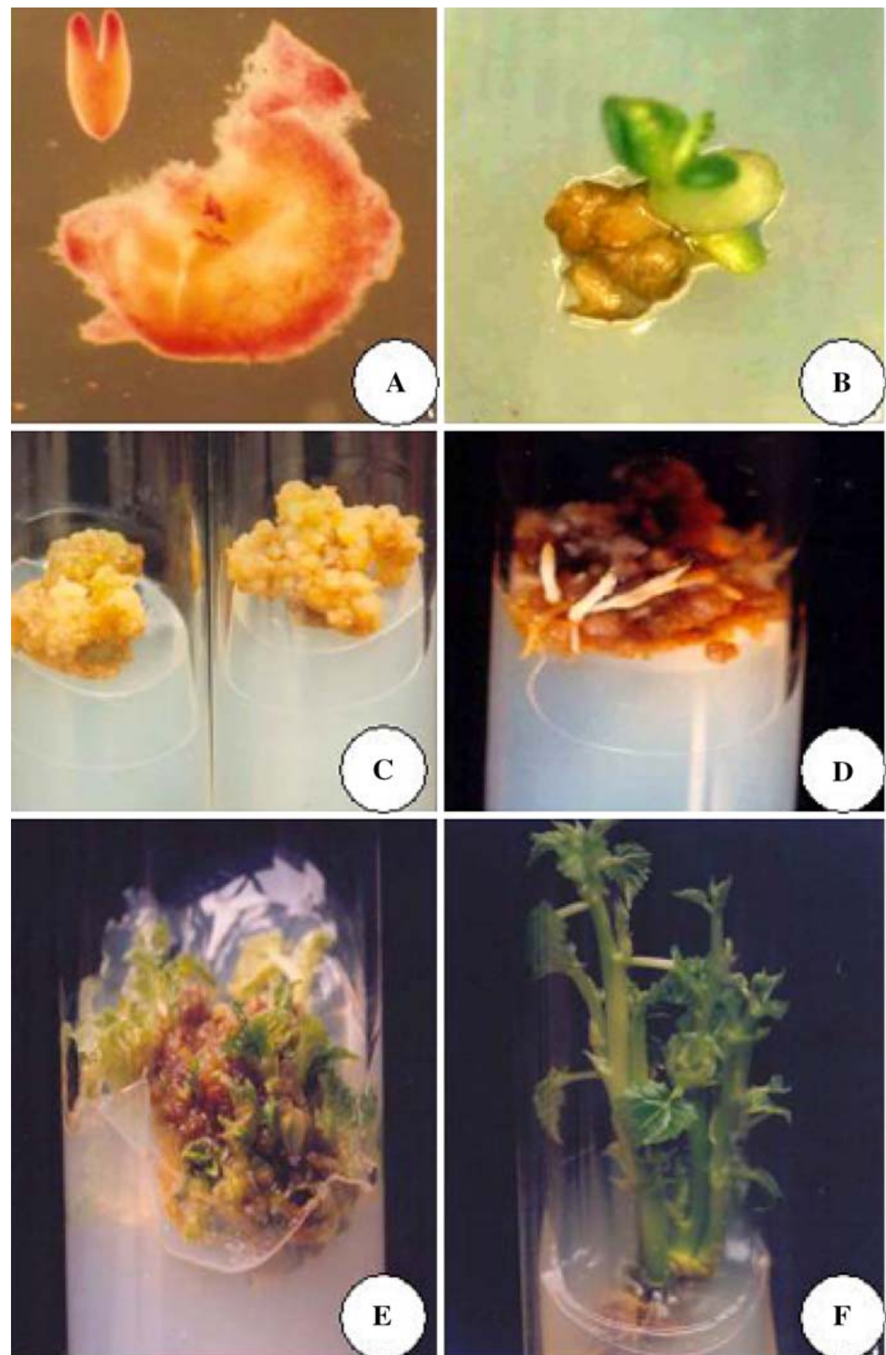


Fig. 3 (**a**) Endosperm and embryo of *Morus alba* 17–20 days after pollination. The seeds were dissected and stained with safranin. (**b**) Endosperm cultured along with embryo 7 days after culture on MS medium supplemented with BA (5 μM) and NAA (1 μM). The embryo had grown and cotyledon turned green. (**c**) Endosperm derived calli on MS medium supplemented with 2 μM 2, 4-D. (**d**) Root regeneration from endosperm calli on MS + IBA (1 μM). (**e**) Regeneration of shoots from endosperm derived calli on MS medium fortified with BA (10 μM) 30 days after culture. (**f**) Multiplication of endosperm derived shoots on MS medium supplemented with 7 μM BA

from the intact seed (i.e. endosperm with embryo) culture of *Scurrula pulverulenta* which on subculture produced characteristic haustoria (Bhojwani and Johri 1970). In *Taxillus vestitus*, shoot bud formation occurred on WM supplemented with IAA, Kn and CH after 7 weeks. Replacement of IAA with IBA could induce shoot regeneration in 55% cultures and haustoria in 60% cultures (Johri and Nag 1970). Here, the embryo had an adverse effect on bud differentiation from endosperm. Injury to the endosperm was found to be beneficial for shoot induction. Shoot buds first develop along the injured region. The position of the explant on medium plays a significant role in regeneration of shoot in *Taxillus* sp. When half split

Table 1 Morphogenic response of endosperm in culture

Sl. No.	Taxa	Family	Endosperm stage	Response	References
1.	*Zea mays*	Graminae	I	P	Straus (1954, 1960), Straus and La Rue (1954)
2.	*Zea mays*	Graminae	I	P	Tamaoki and Ullstrup (1958)
3.	*Cucumis sativus*	Cucurbitaceae	I	P	Nakajima (1962)
4.	*Santalum album*	Santalaceae	M	P	Rangaswamy and Rao (1963)
5.	*Exocarpus cupressiformis*	Santalaceae	M	D-s	Johri and Bhojwani (1965)
6.	*Ricinus communis*	Euphorbiaceae	M	P	Mohan Ram and Satsangi (1963)
7.	*Lolium perenne*	Poaceae	I	P	Norstog (1956), Norstog et al. (1969)
8.	*Zea mays*	Graminae	I	P	Sehgal (1969)
9.	*Scurrula pulverulenta*	Loranthaceae	M	D-s	Bhojwani and Johri (1970)
10.	*Ricinus communis*	Euphorbiaceae	M & I	P	Brown et al. (1970)
11.	*Taxillus vestitus*	Loranthaceae	M	D-s	Johri and Nag (1970)
12.	Nuytsia floribunda	Loranthaceae	M	P	Nag and Johri (1971)
13.	*Leptomeria acida*	Santalaceae	M	C-s	Nag and Johri (1971)
14.	*Taxillus vestitus*	Loranthaceae	M	D-s	Nag and Johri (1971)
15.	*Taxillus cuneatus*	Loranthaceae	M	D-sh	Nag and Johri (1971)
16.	*Dendrophthoe falcata*	Loranthaceae	M	C-sh	Nag and Johri (1971)
17.	*Croton bonplandianum*	Euphorbiaceae	M	Pr	Bhojwani and Johri (1971)
18.	*Jatropha panduraefolia*	Euphorbiaceae	M	D-sr	Srivastava (1971a)
19.	*Ricinus communis*	Euphorbiaceae	M	P	Johri and Srivastava (1972)
20.	*Lolium multiflorum*	Poaceae	I	Ps	Smith and Stone (1973)
21.	*Putranjiva roxburghii*	Euphorbiaceae	M	C-s	Srivastava (1973)
22.	*Codiaeum variegatum*	Euphorbiaceae	M	C-sr	Chikkannaiah and Gayatri (1974)
23.	*Triticum aestivum*	Graminae	I	P	Sehgal (1974)
24.	*Hordeum vulgare*	Graminae	I	P	Sehgal (1974)
25.	*Oryza sativa*	Graminae	I	D-sr	Nakano et al. (1975)
26.	*Nigella damascena*	Ranunculaceae	M	E	Sethi and Rangaswamy (1976)
27.	*Achras sapota*	Sapotaceae	M	P	Bapat and Narayanaswamy (1977)
28.	*Coffea arabica*	Rubiaceae	M	P	Monoco et al. (1977)
29.	*Petroselinum hortense*	Umbelliferae	M	D-sre	Masuda et al. (1977)
30.	*Pyrus malus*	Rosaceae	I	C-sr	Shihkin and Shuchiung (1977)
31.	*Codiaeum variegatum*	Euphorbiaceae	M	C-s	Gayatri (1978)
32.	*Malus pumila*	Rosaceae	I	C-s	Mu and Liu (1978)
33.	*Citrus grandis*	Rutaceae	I	E	Wang and Chang (1978)
34.	*Oryza sativa*	Graminae	M & I	D-sr	Bajaj et al. (1980)
35.	*Prunus persica*	Rosaceae	I	E	Liu and Liu (1980)
36.	*Santalum album*	Santalaceae	M	E	Lakshmi Sita et al. (1980)
37.	*Euphorbia geniculata*	Euphorbiaceae	I	C-r	Sehgal et al. (1981)
38.	*Hordeum vulgare*	Graminae	M	C-s	Sun and Chu (1981)
39.	*Actinidia chinensis*	Actinidiaceae	M	C-s	Gui et al. (1982)
40.	*Carya illinoensis*	Juglandaceae	M	C-r	Cheema and Mehra (1982)
41.	*Lycium barbarum*	Solanaceae	M	C-s	Gu et al. (1985)
42.	*Lycium chinensis*	Solanaceae	M	C-s	Gu et al. (1985)
43.	*Cocos nucifera*	Palmae	I	P	Kumar et al. (1985)

Table 1 continued

Sl. No.	Taxa	Family	Endosperm stage	Response	References
44.	*Emblica officinale*	Euphorbiaceae	M	C-s	Sehgal and Khurana (1985)
45.	*Annona squamosa*	Annonaceae	M	D-sr	Nair et al. (1986)
46.	*Asparagus officinalis*	Liliaceae	M	C-s	Liu et al. (1987)
47.	*Juglans regia*	Juglandaceae	I	C-sr	Tulecke et al. (1988)
48.	*Pyrus communis*	Rosaceae	I	C-s	Zhao (1988)
49.	*Zea mays*	Graminae	I	C-s	Zhu et al. (1988)
50.	*Zea mays*	Graminae	I	P	Felker and Goodwin (1988)
51.	*Coffea* sp.	Rubiaceae	I	E	Raghuramalu (1989)
52.	*Actinidia chinensis* × *A. melandra*	Actinidiaceae	I	C-s	Kin et al. (1990)
53.	*Actinidia urguta* × *A. deliciosa*	Actinidiaceae	M & I	C-s	Kin et al. (1990)
53.	*Lycopersicon esculentum*	Solanaceae	M	P	Zur et al. (1990)
55.	*Citrus* spp.	Rutaceae	M	E	Gmitter et al. (1990)
56.	*Zea mays*	Graminae	I	P	Manzocchi (1991)
57.	*Cocos nucifera*	Palmae	I	P	Ceniza et al. (1992)
58.	*Zea mays*	Graminae	I	P	Faranda et al. (1994)
59.	*Mallotus philippensis*	Euphorbiaceae	M	C-s	Sehgal and Abbas (1996)
60.	*Acacia nilotica*	Mimosaceae	I	E	Garg et al. (1996)
61.	*Actinidia* spp.	Actinidiaceae	M	C-sr	Machno and Przywara (1997)
62.	*Morus alba*	Moraceae	I	C-s	Thomas et al. (2000)
63.	*Azadirachta indica*	Meliaceae	I	C-s	Chaturvedi et al. (2003)
64.	*Actinidia deliciosa*	Actinidiaceae	M	C-s	Goralski et al. (2005)

C-sh—shoot and haustoria from endosperm callus

C-s—shoot from endosperm callus

C-sr—shoot and root from endosperm callus

C-r—root differentiation from endosperm callus

D-s—direct shoot differentiation

D-sr—direct shoot and root differentiation

D-sre—differentiation of shoot, root and embryos

D-sh—differentiation of shoot and haustoria

E—embryogenesis from endosperm callus

I—immature

M—mature

P—endosperm proliferation

Pr—endosperm proliferation and rooting

Ps—endosperm proliferation in suspension culture

T. vestitus endosperm without embryo was placed on medium with its cut surface in contact with the medium containing Kn (23.25 and 46.5 μM), 100% cultures produced shoots.

In *Dendrophthoe falcata* callusing occurred on WM fortified with IAA (14.5 μM) + KN (23.25 μM) after 6 weeks. The callus on subculture produced shoots, differentiation of which was considerably influenced by the addition of CH (2000 mg/l) (Nag and Johri 1971). In *Leptomeria acida* IBA proved more efficient than IAA in terms of rapid callus proliferation. However, on IAA supplemented medium the callus gave rise to shoots in 100% cultures (Nag and Johri 1971).

Callus proliferation from endosperm and subsequent shoot organogenesis was also reported in *Jatropa panduraefolia* (Srivastava 1971a), *Putranjiva roxburghii* (Srivastava 1973), *Codiaeum variegatum* (Chikkannaiah and Gayatri 1974), *Malus pumila* (Mu and Liu 1978; Shihkin and Shuchiung 1977), *Oryza sativa* (Bajaj et al. 1980), *Annona squamosa* (Nair et al. 1986), *Actinidia chinensis* (Kin et al. 1990), *Mallotus philippensis* (Sehgal and Abbas 1996), *Actinidia deliciosa* (Machno and Przywara 1997), *Morus alba* (Thomas et al. 2000), *Azadirachta indica* (Chaturvedi et al. 2003) and *Actinidia deliciosa* (Goralski et al. 2005).

In *Actinidia* species callus initiation occurred on MS medium supplemented with 2, 4-D (5.78 μM) and kinetin (Kn, 23.25 μM). Transfer of these calli to MS medium containing IAA (1.7 μM) and 2iP (24.60 μM) resulted in shoot and root organogenesis (Machno and Przywara 1997). In *Annona squamosa* the callusing of endosperm occurred on WM supplemented with two cytokinins (Kn and BA), an auxin (α-napthaleneacetic acid; NAA) and gibberellic acid (GA_3). But organogenesis of the callus occurred on Nitsch's medium (Nitsch 1969) supplemented with 8.88 μM BA and 2.7 μM NAA (Nair et al. 1986).

In *Mallotus philippensis* a continuously growing callus was obtained on MS medium supplemented with 2, 4-D (5.78 μM) + Kn (2.5 μM). These calli when subcultured on MS + BA (13.3 μM) + CH (1000 mg/l) gave rise to four types of morphologically distinct cell lines. Among these four lines, only the green compact cell line was responsive for organogenic differentiation. Shoot regeneration occurred in this callus when subcultured on MS medium fortified with BA (13.3 μM) + NAA (1.1 μM) (Syed Abbas 1993).

In rice, there was a striking difference in the growth response of immature and mature endosperm. Immature endosperm underwent two modes of differentiation i.e. direct regeneration from the explant or indirectly via intervening callus phase. In mature endosperm, shoot organogenesis was always preceded by callusing. Callus from mature endosperm was initiated on MS + 2, 4-D (9 μM) and, further, maintained on MS + 2, 4-D (4.5 μM). Shoot differentiation from callus occurred on MS + IAA (22.8 μM) + Kn (9.3 μM). The proliferation of immature endosperm and occasional shoot formation occurred on YE supplemented medium. Addition of IAA and Kn improved the response further (Bajaj et al. 1980).

Emergence of embryos from endosperm calli were observed in *Nigella damascena* (Sethi and Rangaswamy 1976), *Petroselinum hortense* (Masuda et al. 1977), *Citrus grandis* (Wang and Chang 1978), *Prunus persica* (Liu and Liu 1980), *Santalum album* (Lakshmi Sita et al. 1980), *Juglans regia* (Tulecke et al. 1988), *Coffea* sp. (Raghuramalu 1989), *Citrus sinensis* (Gmitter et al. 1990) and *Acacia nilotica* (Garg et al. 1996).

Sethi and Rangaswamy (1976) reported "embryoids" from endosperm cultures of *Nigella damascena* on MS medium supplemented with 2, 4-D. In *Citrus grandis* and *C. sinensis* callus initiation from endosperm occurred on MT medium (Murashige and Tucker 1969) and WM. But the frequency of callusing of endosperm was more in MT medium supplemented with 2, 4-D (9.06 μM), BA (22.20 μM) and CH (1000 mg/l). Embryoids were emerged from 2MT medium (a medium containing double MT mineral) containing GA_3 (5.78 μM) (Wang and Chang 1978). However, the optimum medium for embryogenesis was 2MT containing 5.78 μM GA_3, 1.1 μM BA, 2 mg/l adenine and 500 mg/l CH (Gmitter et al. 1990).

In parsley (*Petroselinum hortense*), the embryogenic callus and embryos were obtained by culturing endosperm tissues from germinating seeds on MS basal medium. The embryogenic potential of callus was observed for more than half a year through several passages (Masuda et al. 1977). Endosperm embryogenesis from the open pollinated seeds of *Juglans regia* cv. Manregian was reported by Tulecke et al. (1988). Embryos were formed on a modified medium (Tulecke et al. 1988) containing BA (4.44 μM), Kn (9.30 μM) and IBA (0.049 μM).

In *Acacia nilotica* immature endosperm produced nodular callus on MS medium supplemented with 2, 4-D (2.5 μM), BA (5 μM) and CH (500 mg/l). The third callus subculture on the same medium gave rise to several somatic embryos when the cultures were incubated in dark (Garg et al. 1996). Embryogenesis was asynchronous and embryos occurred in large clusters by interconnecting the adjacent embryos. Further germination of the embryos occurred on modified MS medium containing B5 major salts (Gamborg et al. 1968), glutamine, CH and CW.

Immature endosperms of neem (*Azadirachta indica*) showed good percentage (45%) of callus

proliferation on MS + 2, 4-D (5 μM), but best callusing (53%) occurred on MS + NAA (5 μM) + BA (2 μM) + CH (500 mgl^{-1}). When the callus was transferred to a medium containing BA or Kn, shoot buds differentiated from all over the callus. Maximum regeneration in terms of number of cultures showing shoot buds and number of buds per callus occurred in the presence of 5 μM BA (Chaturvedi et al. 2003). Thomas et al. (2000) observed callusing of mulberry endosperm on MS + BA + NAA + CH or YE. Shoot buds were emerged when the callus was transferred on a medium containing a cytokinin or a cytokinin and an auxin (NAA). The percent response was highest on BA and NAA containing medium. However, the number of shoots per explant was maximum when TDZ alone was used. In *Actinidia deliciosa*, MS medium supplemented with 9.06 μM 2, 4-D and 23.3 μM Kn developed callus with 80% efficiency. However, the regeneration of shoots observed only on MS medium supplemented with 2.27 μM TDZ (Goralski et al. 2005).

Histology

Histological studies of the proliferating endosperm of *Jatropa*, *Putranjiva* and *Ricinus* revealed that the embryo too enlarged and proliferated along with the endosperm but soon showed the sign of degeneration. In such cases the endosperm calli were transferred to a fresh medium to avoid any contamination from degenerated embryonal cells. The 4-week-old callus derived from endosperm cultures, proliferated into parenchymatous cells and 6-week-old callus showed tracheidal cells (Srivastava 1971a, b, 1973; Johri and Srivastava 1973). In *Santalum*, endosperm proliferation started after the formation of several meristematic layers below the epidermal region (Rangaswamy and Rao 1963). By carefully applying plant growth regulators the nodular outgrowths can be induced on the surface of the cultured endosperm as in case of *Osyris wightiana* (Johri and Bhojwani 1965) and *Putranjiva roxburghii* (Srivastava 1973).

Importance of tracheidal differentiation in the callus of endosperm cultures is that it facilitates organogenic differentiation. In families like euphorbiaceae, loranthaceae and santalaceae the endosperm tissues readily form tracheidal elements in cultures (Johri and Srivastava 1973; Johri and Bhojwani 1971). In *Emblica officinale*, tracheidal cells and cambium like cells organized into vascular strands or nodules in the differentiation medium while in callusing medium tracheidal cells remained scattered. Differentiation of vascular strand in the callus accompanied shoot bud formation (Sehgal and Khurana 1985).

In *Aleuritus fordii*, callus proliferated from endosperm explant consisted of large, compact and vacuolated cells. Tiny group of cells became distinct from adjoining large and vacuolated cells and became meristematic. These cells remained thin walled with dense cytoplasm and a large clear nucleus. Later the meristemoids developed in to dome shaped shoot apex, which produces leaf primordia (Syed Abbas 1993). In *Mallotus philippensis*, only the compact green callus under went differentiation. Such callus showed vasculature, developed protuberances and eventually gave rise to shoot buds. Small group of cells with deep-seated distinct meristematic loci were also observed in these calli, which later gave rise to dome-shaped shoot primordia endogenously (Sehgal and Abbas 1996).

In mulberry (*Morus alba*), histological analysis revealed that the older region of the callus comprised of highly vacuolated cells. Shoot buds differentiated from peripheral nodular structures, which comprised of compactly arranged highly cytoplasmic cells. Often a few layers of degenerating vacuolated cells were seen outside the shoot primordia. It is possible that the shoots originated inside the nodules and emerged after rupturing the surrounding tissues. The regenerating shoots showed vascular supply continuous with the vasculature of the callus (Thomas 2000).

Both exogenous and endogenous differentiation of shoots was observed in *Azadirachta indica*. The serial section of 2-week-old regenerating callus showed that many meristematic pockets developed from inside the callus, which developed into shoot buds after 3 weeks (Fig. 2). Histological analysis also revealed that the shoot buds emerged from peripheral tissues of the callus as well (Chaturvedi et al. 2003). In *Actinidia deliciosa* histological analysis of the freshly isolated endosperm revealed small intercellular spaces and cells were filled with storage materials. However, the calli derived from the endosperm were larger, vacuolated and lacked storage materials. In

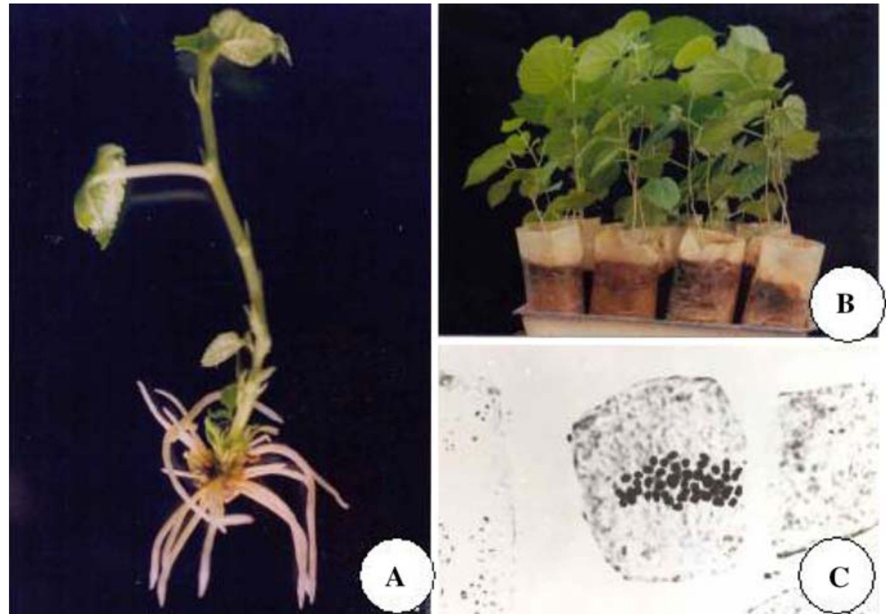


Fig. 4 (**a**) Rooting of endosperm derived shoots of *M. alba* on 1/2 MS medium with 7 μM IBA. Several roots have emerged from the basal cut end of the shoot. (**b**) The endosperm derived hardened plants after transferred to polythene bags containing garden soil. (**c**) A root tip cell of endosperm derived plant showing triploid number of chromosome ($2n = 3x = 42$)

older callus, daughter nuclei attached to newly formed cell walls were often observed, suggesting disturbances of cell division. The cells differed in size and shape and contained nuclei with variable numbers of nucleoli (Goralski et al. 2005).

Cytology

The endosperm tissue often shows a high degree of chromosomal variation and polyploidy. Mitotic irregularities, chromosome bridges and laggards are other important characteristic features of endosperm tissue. Some reports suggest that the cells of endosperm cultures showed ploidy higher than 3n as in the case of *Croton* (Bhojwani and Johri 1971), *Jatropha* (Srivastava 1971a) and *Lolium* (Norstog et al. 1969). Cytological observations of the endosperm callus derived from *Dendrophthoe falcata*, *Taxillus cuneatus* and *Taxillux vestitus*, showed diploid ($2n = 18$) and triploid ($3n = 27$) chromosomes (Johri and Nag 1974).

In addition to cytological observations of endosperm callus, chromosomal analysis of the regenerated plantlets were also studied in a number of systems. In *Juglans regia* two plants of endosperm origin were analysed for ploidy determination and both the plants showed triploid ($3n = 3x = 48$) number of chromosomes (Tulecke et al. 1988). In *Citrus*, stability at the ploidy level and chromosome number were observed all through the regeneration process and triploid ($2n = 3x = 27$) plantlets were recovered (Gmitter et al. 1990). In *Mallotus philippensis* the squash preparation of root tip cells of regenerated plants invariably showed triploid chromosome number ($3n = 3x = 33$) (Sehgal and Abbas 1996). The triploid nature of the endosperm-derived plants was determined by Feulgen cytophotometry in *Acacia nilotica* (Garg et al. 1996).

In mulberry (*Morus alba*), 7-month-old plants of endosperm origin were utilized for ploidy determination. All the ten plants analysed cytologically showed triploid number of chromosome ($2n = 3x = 42$) (Thomas et al. 2000; Fig. 4). The ploidy determination of 20 plants of *Azadirachta indica*, regenerated from endosperm, showed that 66% of the plants had triploid number of chromosome ($2n = 3x = 36$) and the rest 34% were diploids ($2n = 2x = 24$) (Chaturvedi et al. 2003). In *Actinidia deliciosa* three different ploidy levels viz. 3C, 6C and 9C were observed in cells of endosperm derived callus analyzed by flow cytometry. Analysis of the leaves of endosperm derived plants showed 45.7% fluorescence intensity peaks corresponding exactly to 3C whereas 42.2% exhibited peaks of fluorescence intensity representing C-values between 2C and 4C. Only 8.4% of the samples indicated 2C DNA content, and one sample showed 6C DNA content (Goralski et al. 2005).

Concluding remarks

Even though callus proliferation from endosperm was possible in several systems, the regeneration of shoots and complete plantlets was possible only to limited number of plants belonging to certain families. Like all other plant parts, endosperm can also respond the same way under in vitro conditions, irrespective of their genetic constitutions. Hence, it helped in changing the misconception that endosperm being a "dead tissue" has now been contradicted by several reports suggesting the full plant regeneration from endosperm. Despite the success of plant regeneration from endosperm cultures in a number of systems, this protocol for production of triploid plants remained unutilised mostly. It may be due to the difficulty in obtaining organogenic callus from mature or immature endosperms. The ploidy variation exhibited by endosperm derived plantlets is another difficulty which limits this technique. From the available reports it is clear that nature of endosperm, media, plant growth regulators, and other additives play very crucial role in endosperm proliferation as well as regeneration. More efforts should be focussed on endosperm regeneration from plants where seedlessness is employed to improve the quality of fruits and plants that has economically useful vegetative parts. Continued research in this field will definitely produce improved varieties of plants.

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