TWENTY FOURTH BIRBAL SAHNI MEMORIAL LECTURE

Plant tissue culture and biotechnology

B.M. Johri, P.S. Srivastava & Madhumati Purohit

B.M. Johri, Central Reference Library, University of Delhi, Delhi 110 007, India.

P.S. Srivastava & Madhumati Purohit, Department of Biotechnology, Hamdard University, Hamdard Nagar, New Delhi 110 062, India.

Johri BM, Srivastava PS & Purohit M 1997. Plant tissue culture and biotechnology. *Palaeobotanist* **46** (3): 134-159.

Plant tissue culture has progressed steadily ever since its inception in 1902. The initial experiments related to various tissues that could sustain prolonged in vitro conditions. The differential response of the cultured tissues under variable chemical milieu provided the necessary impetus to utilize the technique in a profitable manner. Over the years efficacy of the technique became apparent when noticeable in vitro morphogenic responses could be used to unravel the mysteries of growth and differentiation. Expectedly, therefore, any morphogenic event expressed in vitro could be correlated to the specific components of the nutritive medium.

By 1970s the applicability of the technique came to be realized with the possibility of exploring somatic hybridization, micropropagation of recalcitrant species, haploid and triploid plants, and finally genetic manipulations. Today, plant tissue culture has become an integral part of biotechnology and is being routinely employed for the improvement of crops and legumes—the back-bone of human nutrition that can also aid in the amelioration of malnutrition of millions of sufferers. The ultimate success with the transfer of 'nif'-gene to non-leguminous plants would help save millions of dollars in chemical fertilizers which can then be profitably used for the welfare of the human race.

Key-words—Tissue culture, Haploids, Triploids, Micropropagation, Fertilization Control, Biotechnology.

IN the following description the genesis of the technique as conceived by the German scientist Haberlandt (1902) and the present status of plant tissue culture and its role in biotechnology is elaborated. Plant tissue culture is a rapidly advancing area of research, and it is possible to control growth, development and differentiation of almost all parts (explants) of plants on suitable nutrient media (many exceptions). From the beginning of the 20th century, orchid propagation through culture of seeds and plant parts has been a thriving profession (see Prakash & Pierik, 1993).

HISTORICAL

In 1902, Haberlandt (Figure 1) suggested the concept of totipotency of plant cells. He himself did not succeed in culturing plant cells, but predicted that on appropriate nutrient media the cells will divide, grow and differentiate. His predictions have been amply justified and demonstrated (see Johri, 1971).

Discoveries made in the 1930s by P.R. White (Figure 2), R.J. Gautheret (Figure 3) and P. Nobécourt laid the foundation for further work in tissue culture. In 1934, Gautheret succeeded in culturing cambial

Figure 1 — G. Haberlandt (Germany) — Father of Plant Tissue Culture. For the first time he cultured isolated tissues and organs.

Delivered by B.M. Johri on November 14,1994, at the Birbal Sahni Institute of Palaeobotany, Lucknow. I (BMJ) feel honoured for the invitation to deliver the 24th Professor Birbal Sahni Memorial Lecture.

Figure 2—P.R. White (Maine, NY, USA). Initiated the technique of continuous culture of explants on a defined chemical medium that provided the necessary impetus for successful culture of several other explants. He also formulated the culture medium used by various investigators.

cells of tree species including *Salix capraea* and *Populus nigra*, and observed their proliferation for a few months, on Knop's solution containing glucose and casein hydrolysate (CH). He (Gautheret, 1939), for the first time, established continuously-growing tissue cultures from carrot and Jerusalem artichoke root cambium. At the same time, White (1939) also reported the establishment of such cultures from tumour tissue of the hybrid *Nicotiana glauca* x *N. langsdorfii*. Similar results were obtained independently by Nobécourt (1937). In sub-

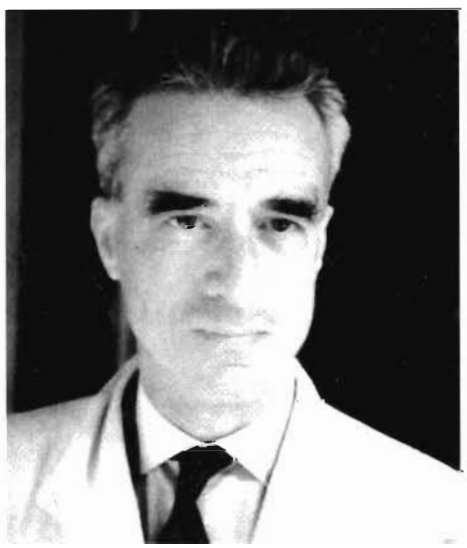


Figure 3—R.J. Gautheret (Sorbonne, Paris). Cultured lateral meristem and established the continuous culture procedure.

Figure 4—A.C. Hildebrandt (Wisconsin Univ., Madison, USA). Successfully demonstrated single cell suspension culture and its ultimate regeneration into plantlets.

sequent years further research carried out on other plants was also supportive of the concept of totipotency. Vimla and Hildebrandt (1965; Figure 4) demonstrated the totipotency of isolated single cells of tobacco that regenerated plantlets in cultures.

In 1975, Skoog (Figure 5) and Miller suggested that the differentiation of various organs is under the controlled mechanism of exogenously supplied hormones to the medium. Later, with Murashige (1962), the most widely used medium was formulated.

Nitsch (1951; Figure 6) also formulated the nutrient medium for in vitro culture of selected explants. He could achieve success in culturing isolated ovaries and anthers.

One of the most striking features of plant tissue culture has been the isolation and fusion of protoplasts (Cocking, 1972). The important practical application of protoplast culture/somatic hybridization was successfully applied to tobacco and other plants by Melchers (1977; Figure 7). He demonstrated the fusion of protoplasts of tomato and potato. The hybrid developed and produced flowers and fruits called 'pomato', and the suckers 'topato', the latter were thick and flat, full of starch grains (equivalent of potato).

A few biotechnological studies during yesteryears may be mentioned. Pasteur (1866; see Purohit 1994), the famous French Scientist and a great

Figure 5—F. Skoog (Wisconsin Univ., Madison, USA). Discovered Kinetin and (alongwith Murashige) formulated the most extensively used culture medium that has proved successful for the induction of morphogenic response in various explants. Skoog (alongwith Miller) also demonstrated the appropriate ratio of auxin and cytokinin for selective regeneration of shoots, roots or both.

saviour of human suffering, was approached by the French wine-makers seeking his advice to prevent the contamination of wine. Pasteur discovered that

Figure 6—J.P. Nitsch (Gif-Sur-Yvette, France). Besides the culture of ovaries, he also accomplished haploid regeneration through anther culture. The medium formulated by him is used in the in vitro culture of selected explants.

Figure 7— G. Melchers (Max Planck Institute, Tubingen, FRG). Besides several other achievements, he suggested the concept of somatic hybrids that could blend the economic features of two useful species; the concept of 'pomato' and 'topato' was proposed.

if the broth was maintained at a specific temperature, bacterial contamination did not occur. Thus, the wine industry was saved.

An English bacteriologist, Alexander Fleming (1929; see Irving & Herric, 1949) was working (in a London hospital) on *Staphylococcus* and *Streptococcus*. After a short holiday, when he returned to his laboratory, he noticed that in a particular petri dish the bacterial colonies looked unhealthy and were fringed by a somewhat semi-transparent area. From the contaminated colonies, Fleming was able to prepare an extract to contaminate fresh bacterial colonies. He reached the correct conclusion that the extract was antibacterial. The extract when administered to a female patient in the hospital arrested bacterial infection which caused bed-fever after child birth. It took almost four years when the organism, which restricted the growth of bacteria, was identified as *Penicillium;* its metabolic product as 'Penicillin'. After several years an organization was set up at Rome for industrial production of Penicillin; the collaborators were Florey and Chain. For this life-saving drug, Sir Alexander Fleming, Sir Howard W. Florey and Ernst B. Chain shared the Nobel Prize in 1945.

Another biotechnical episode relates to Tamiya (1959)—a Japanese physiologist, who set up a large-scale centre (near Tokyo) for the commercial culture

of *Chlorella*. *Chlorella,* in 1950s and 1960s, was considered to be an excellent source of protein for human needs. After harvest, the small-scale experimentally produced *Chlorella* was dehydrated, decolorized, deodorized and powdered. For a trial, the *Chlorella* powder was used as a filler in cookies for human consumption. The people who ate the cookies never felt any unusual taste or smell. Finally, Tamiya set up an industrial plant (with large, open tanks filled with liquid nutrient medium) that proved unsuccessful due to recurrent contamination.

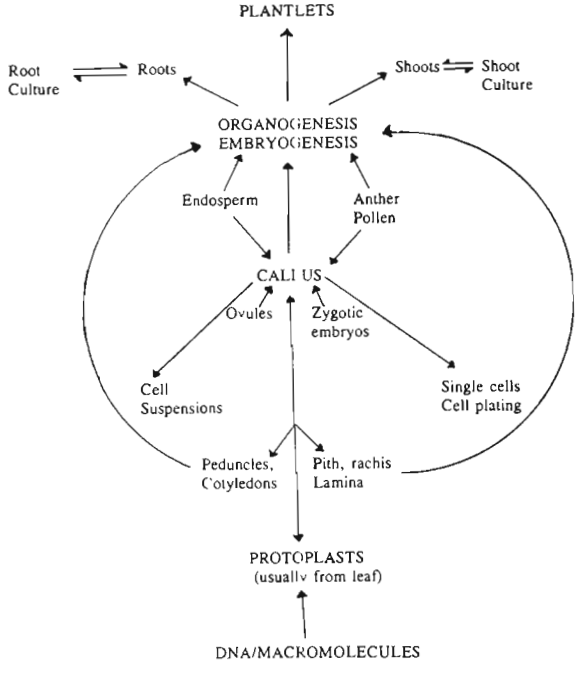
In 1950s and 1960s, Tulecke (1957; USA) succeeded in a mass suspension culture of pollen-derived cells of *Ginkgo*. The tube nucleus in the pollen grain divided and the daughter nuclei also divided ad infinitum. In spite of all efforts, there was no differentiation of tissues and organs.

Nitsch (France) also raised a mass culture of cells of the edible part of apple but the cells lost all the sugar they had at the beginning and no further sugar was synthesized in the cells (Pers. comm.). The usual storage product in cultured cells is starch; rarely oil.

There has been considerable work on the tissue and organ culture of bryophytes, pteridophytes (excellent material for experimental studies) and gymnosperms. A very spectacular example is the growth and differentiation of the excised young embryos of the fern, *Todea barbara*. This is the only fern known in which the egg and younger proembryos can be isolated from the venter of the archegonium and independently cultured. The growth and differentiation was comparable to in vivo growth and development (DeMaggio & Wetmore, 1961).

Maximal attention has been devoted to the growth, development and differentiation of organs, tissues, cells, and protoplasts of flowering plants (Angiosperms). These are cultured on suitable nutrient media — liquid media (stationary or agitated), and on semi-solid (gelled with agar) media. The glassware for culture, media, and explant (plant part for culture), must be fully aseptic. The cultures are grown under controlled temperature, light and humidity. The mass culture of cells and tissues is utilized to obtain chemicals (secondary metabolites; some used as medicines) and a large number of regenerants for large-scale propagation of plants (see Razdan, 1995).

When organs and tissues are cultured, the cells divide and often form a callus (mass of undifferentiated cells). The callus can be grown (by repeated sub-cultures) for as long as one desires. The callus differentiates and produces roots, shoots and plantlets (see Text-figure 1). The latter can be transferred to pots, and then to the field (see Srivastava & Steinhauer, 1981a, b). Using plant tissue culture technique, disease-free *Chrysanthemum, Dianthus, Solanum tuberosum* (see Quack, 1977), and disease resistant and transgenic (genetically engineered) tobacco (see Prakash & Pierik, 1993) plants have been developed. In cultures the explant may or may not produce callus, embryos, roots, shoots, and plantlets. The determining factor is the nutrient media on which the culture is raised. Through plant tissue culture, it has now become possible to obtain food additives such as carotenoids from *Daucus*, betamine from *Beta* and crocin from *Crocus;* metabolites of food, for example, capsicin from *Capsicum* and stevioside from *Stevia* (see Ravishankar &



Text-figure 1—In vitro propagation. Various manipulations through tissue culture technique (after Mantell *et al.*, 1985).

Venkataraman, 1993), products of medicinal value, and in addition, in the improvement in processing for enhancement of quality and quantity of food products, and bio-pesticides (see Berlyn, 1984; Beiderbeck & Knoop, 1988).

Presently, almost every University and many Research Institutes are engaged in plant tissue culture research. It is now possible to grow all types of young and mature cells, and tissues and organs of different plants on appropriate nutrient media. In some cases if the explants are not amenable to culture, it is usually due to deficiency in the medium and suitable stage of development and physiological status of the explant.

NUTRIENT MEDIA

The nutrient media contain a number of major and minor inorganic salts, organic compounds to provide nitrogen and carbon, growth promoters—auxins, cytokinins, gibberellins, and complex organic compounds like casein hydrolysate, yeast extract and coconut water (milk). If aseptic conditions are not maintained all through the period of growth, the cultures become infected with bacteria and fungi. Such cultures have to be promptly discarded.

Depending on the endogenous level of metabolites, some tissues grow on simple media containing only organic salts and a carbon source. But most tissues require vitamins, amino acids and growth substances. The commonly used media are those devised by White (1954), Murashige and Skoog (1962), Nitsch (1969), and others. For every tissue the most promotive medium has to be selected.

Quite often the investigators use undefined medium. As soon as the growth and differentiation has been attained by omitting one or more ingredients, a defined nutrient medium can be formulated so that any further omission of any ingredient will affect growth and differentiation. For the multiplication of agricultural and horticultural crops, forest trees, medicinal plants, and others, nutrient media have to be synthesized in relation to the age of explant, and maximal growth and differentiation in minimal time.

The multiplication of selected elite clones from the specific parent lines in *Eucalyptus* and *Tectona* (Gupta *et al.*, 1980,1981), or from the hybrids, is obtained through micropropagation or somatic embryogenesis. The production of disease-resistant and disease-free regenerants, improved varieties of plants and hybrids are all included in 'Biotechnology'.

The tissue culture procedures permit the study of physiology, genetics, molecular aspects, and effects of specific nutrients (individually and collectively). In some instances low temperature treatment of explants (before culture) results in much better growth and differentiation as in anther cultures of a number of species (see Srivastava & Johri, 1988).

With a short incubation period (after implanting the explant on nutrient media), the superficial cells—especially the injured cells at cut ends of the explant—divide and redivide forming a mass of cells (callus). In many cases the callus does not differentiate, but differentiation can be brought about by transferring the undifferentiated tissue to root and shoot-forming media, leading to differentiation of plantlets such as in *Artemisia, Delonix* and others (Gulati *et al.*, 1996; Gupta *et al.*, 1996).

OVARY CULTURE

To study the precise requirements for fruit development and fruit physiology, Laibach and Kribben (1949) devised a method by which young flowers or fruits of *Cucumis sativus* could be excised and grown under aseptic conditions on a nutrient medium.

Following the first attempt by LaRue (1942), considerable success has been achieved in culturing the pollinated ovaries (Figure 8) of several species (Sachar & Kanta, 1958; Johri & Sehgal, 1966; Guha & Johri, 1966). In culture the ovaries often fail to grow into full-sized fruits (in the restricted space of culture vial). Ito (1961, 1966) developed a partial sterile

Abbreviations used—Ad - Adenine; BAP6 - benzylamino purine; BM - Basal medium; CH - Casein hydrolysate; 2,4 - D - 2,4 - dichlorophenoxyacetic acid; GA - Gibberellin; IAA - Indole-3 - acetic acid, IBA - Indolebutyric acid; 2-ip-2 isopentenyl adenine; KN - Kinetin; M - Molar; MS - Murashigé and Skoog's medium; NAA - L naphthalene acetic acid; Wk/s - Week (s); WM - White's medium; YE - Yeast extract.

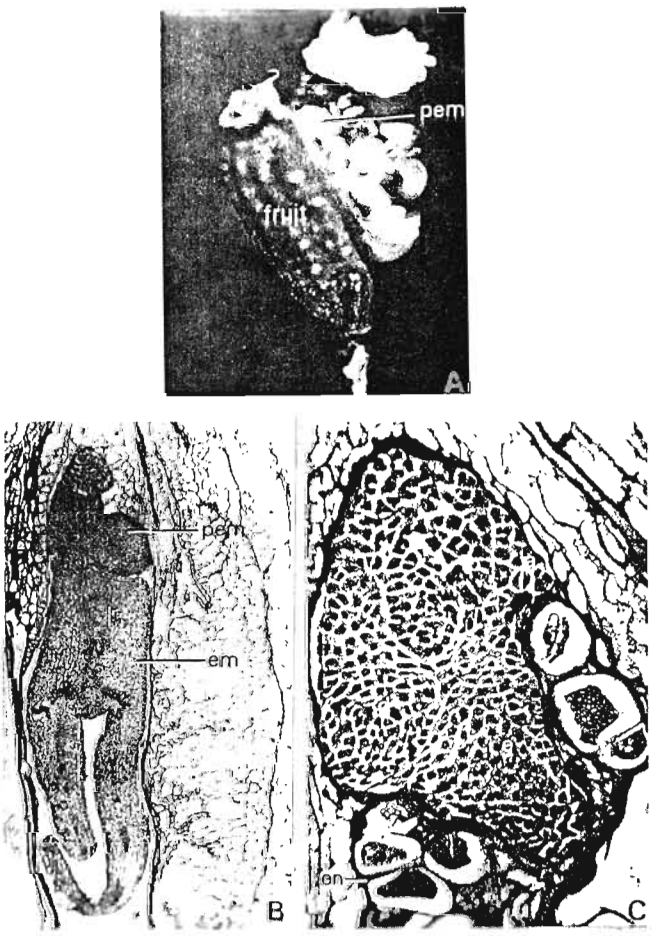


Figure 8— A-C *Anethum graveolens*, ovary culture. **A.** Ruptured fruit, 20 wks after inoculation and BM+YE (500 mg/1), shows a polyembryonal *(pem)* mass; **B.** 5-wk-old culture (longisection mericarp), grown on BM +CH (1000 mg/1.) shows mature embryo *(em)* and accessory proembryos (*pem*); **C,** longisection of upper part of mericarp, 4 wks after inoculation, cultured on BM+CH (1000 mg 1), shows five proembryonal masses, note endosperm *(en)* cells (after Johri & Sehgal, 1963.)

culture technique to overcome this problem. The ovary cultue has dual objectives; to obtain viable hybrids from normally unsuccessful crosses, and to overcome seed dormancy (Tukey, 1938). Subsequently, various growth factors which influence the in vitro culture of ovary have also been studied (Rau, 1956; Sachar & Kanta, 1958; Chopra, 1962). Significant role of floral organs in fruit development has been emphasized by several workers (P. Maheshwari & Lal, 1961; Chopra, 1958; Guha & Johri, 1966).

Ovary culture has been successfully employed to overcome various other impediments such as the failure of pollen germination on the stigma, or the slow and insufficient growth of pollen tube, as well as precocious abscission of flower (Rao, 1965; Shivanna, 1965). Through ovary culture technique, polyembryony could also be induced successfully in various species (Johri & Sehgal, 1966; Sehgal, 1972; Mitra & Chaturvedi, 1972). The culture of ovaries of apomicts has helped in understanding the nature of stimulus provided by pollination. Investigations on ovary culture revealed two important aspects: (a) detached ovaries are capable of autonomous growth and bear fertile seeds, and (b) growth pattern in vivo and in vitro is comparable (Rangan, 1982).

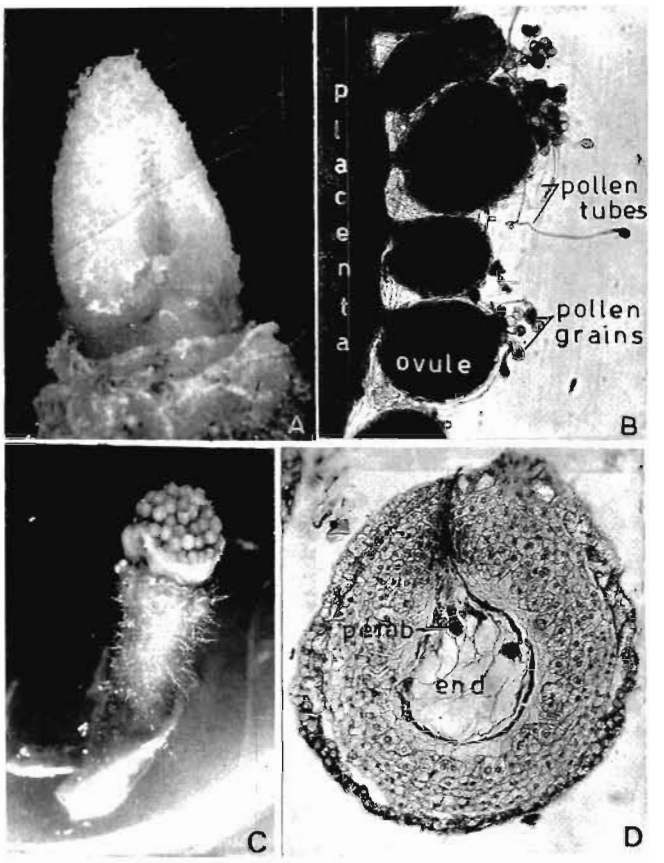


Figure 9—A-D. *Petunia axillaris*, placental pollination. **A.** Both placentae of ovary with its entire mass of ovules dusted with pollen; **B.** free-hand transection through self-pollinated placentae, 24 days after culture; note marginal portion of one placenta, four ovules, many pollen grains and pollen tubes; **C.** 24 days after placental, self-pollination; note mature seeds; **D.** longisection of young seed, 7 days after self-pollination; note the proembryo *(pemb)* and endosperm *(end)*. (after Rangaswamy & Shivanna, 1971).

OVULE CULTURE

In comparison to ovary culture, not much attention has been paid to ovule culture. The first report on ovule culture dates back to 1932 when White cultured the ovules of *Antirrhinum;* he obtained haploid callus. LaRue (1942) was unsuccessful in obtaining mature seeds through ovule culture of *Erythrontum*. The first successful report of ovule culture leading to fertile mature seeds is by Nirmala Maheshwari (1958) in *Papaversomniferum*. She cultured the fertilized ovules of *P. somniferum* on a nutrient medium containing kinetin and observed that kinetin triggered the growth and differentiation of embryo in ovule. However, this initial rate of growth was not maintained for long and the length of embryos in cultured ovule was less than that of embryos in nature. The additive role of placental tissue in the growth and maturation of seeds has been emphasized by Chopra and Sabharwal (1963) in pollinated ovlues of *Gynandropsis gynandra.*

The growth of embryo is related to the age of the ovule at culture (Siddiqui, 1964; Guha & Johri, 1966). Eid *et al.* (1973) concluded that the difference in the rate of growth is associated with the developmental stage of endosperm present in the ovule in culture. This has also been demonstrated that the osmotic concentration has a significant influence on the growth of excised embryos (Mauney, 1961; P. Maheshwari & Rangaswamy, 1965). In 1971, Uchimiya obtained haploid callus by culturing unfertilized ovules of *Solanun melongena.* This opens up new vistas in regeneration of haploid plants of maternal origin.

The major contribution on ovule has been test-tube pollination and fertilization (Kanta *et al.,* 1962; Kanta & P. Maheshwari, 1963; P. Maheshwari & Kanta, 1964). The work on culture of ovule and seeds of angiospermic parasites (Rangan & Rangaswamy, 1968) enables unravelling the intricacies of host-parasite relationship and development of embryos in seeds that are shed at immature stage. For plant breeders ovule culture can be an indispensible tool in obtaining seedlings from crosses which are normally unsuccessful because of abortive embryos. Similarly, success with test-tube fertilization opens up new avenues in hybridization programmes, specially in overcoming incompatibility barriers.

NUCELLUS CULTURE

Besides zygotic embryos, the adventitious embryos are also of much value since they are genetically uniform and reproduce the characters of maternal plant/parent without inheriting the variations brought about by gametic fusion. In addition, embryos originating from the nucellar tissue in *Citrus* are not only of help in obtaining virus-free plants but also of great advantage in citri-culture for propagating desirable varieties.

Through tissue culture technique, factors affecting the formation of adventitious embryos have been studied. Nucellar embryony has been reported from a natural polyembryonic species of citrus *(C. monocarpa)* by Rangaswamy (1958). He concluded that 'freed from the restraining influence of the integuments and grown on a suitable medium the nucellar tissue of *Citrus monocarpa* becomes activated to unlimited growth, and yields a continuous supply of nucellar embryos". Bitters *et al* . (1972) extended these studies to several other mono- and polyembryonic, as well as seedless varieties of *Citrus.*

Through a series of experiments on nucellar embryony, it has been concluded that the lack of stimulus of pollination and /or fertilization can no longer be considered as a limiting factor for nucellar embryogenesis (Button & Bornman, 1971; Mitra & Chaturvedi, 1972).

Most of the studies focus on nucellar culture of both mono- as well as polyembryonic type of *Citrus* varieties. Attempts to induce nucellar embryony in *Luffa cylindrica* and *Trichosanthes anguina* (Rangaswamy & Shivanna, 1975) have been unsuccessful. Nucellar polyembryony has been induced in *Vitis vinifera* (Mullins & Srinivasan, 1976) and *Cynanchum vincetoxicum* (Haccius & Hausner, 1976). All these taxa do not show natural polyembryony.

Although several embryos and plantlets can be obtained from a single cell of nucellus, the percent-

age of success varies from species to species. To make the technique more viable and widely applicable, it is advisable first to study the physiological changes underlying embryogenesis. To induce adventive embryony in those plants in which it does not occur in nature, and its control in those plants in which it exists as a normal feature, offers a challenging problem.

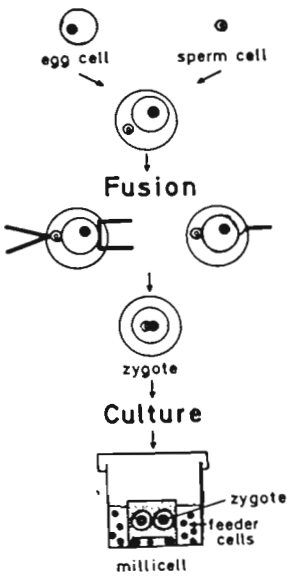
IN VITRO POLLINATION AND FERTILIZATION

The fusion of male and female gametes to form a zygote serves as a very valuable source of new combinations of genetic material. Through tissue culture technique it has become possible to overcome natural incompatibility, and to introduce new characteristics of genetic information from more distantly-related species, thus widening the gene pool (Kranz & Lörz, 1993).

For fertilization at the single cell level, the isolation of viable gametes is prerequisite. The gametes are genetically coded so that new genetic combinations occur in zygotes, and the embryos. These progenitor cells are therefore the most useful source for studies in embryogeny. Using floral explants, ovaries, ovules and mature pollen coupled with embryo rescue (Stewart, 1981), in vitro pollination (including placental pollination) and fertilization have been achieved leading to the production of new hybrids (Zenkteler, 1990). In vitro-formed zygotes have 'natural competence' for division (Figure 9) and can be used for understanding the cell cycle and differentiation.

The fertilization process at the cellular level relates to isolation of sperms and egg cells. This has been achieved by various workers in *Nicottana, Torenia, Zea* and *Plumbago.* Successful attempts have also been made for artificial fertilization in *Torenia* by injecting sperm cells directly into the embryo sac. Through this technique, effects of non-gametic cells on the development of artificially produced zygote can also be expressed effectively.

Kranz and Lörz (1994) have studied (Figures 10-11; Text-figure 2) in vitro fertilization through fusion of isolated protoplasts of single egg and sperm cell of maize in a mannitol solution (400-430 mosmol/kg H_20) containing 0.05 M $CaCl_2$ at pH 11.0 followed by



Text-figure 2—*Zea mays,* electrofusion-mediated in vitro fertilization. Isolated sperm and egg cells were transferred to fusion droplets, and pairs of gemetes were fused electrically after dielectrophoretical alignment on one of the electrodes. For culture, the fusion products were transferred individually to "Millicell" in sets surrounded by feeder cells (after Kranz *et al.*, 1991:courtesy Kranz).

cell division of the fusion product (the zygote). The electrofusion-mediated in vitro fertilization using isolated gametes is a logical extension of this work (Kranz & Lörz, 1990; Kranz & Dresselhas, 1996; Kranz *et al.*, 1991).

The detection of development of specific gene in only one or a few reproductive cells or zygote (via PCR-polymerase chain reaction), and also the transfer of foreign genes into gametes (via electroporation), as well as into zygotes (via microinjection), also

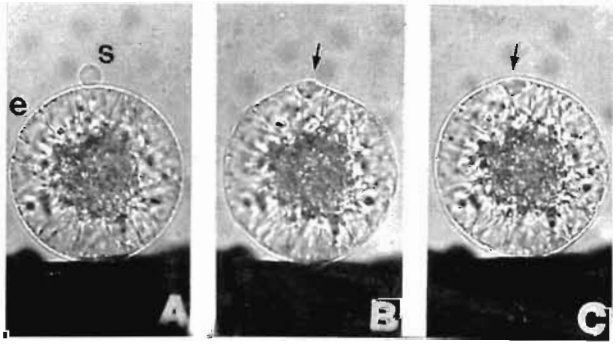


Figure 10—**A-C** *Zea mays,* fusion of sperm *(s)* cell protoplast and egg cell *(e)* protoplast in a 400 mosmol mannitol solution containing 0.05 M $CaCl_2$ at pH 11.0; bar=10 µm. **A.** Adhesion of the egg *(e)* and the sperm *(s)* cell protoplast; **B.** fused egg cell protoplast and sperm cell protoplast; **C.** microcallus (after 5 days of fusion) (after Kranz & Lörz, 1994; courtesy: Kranz).

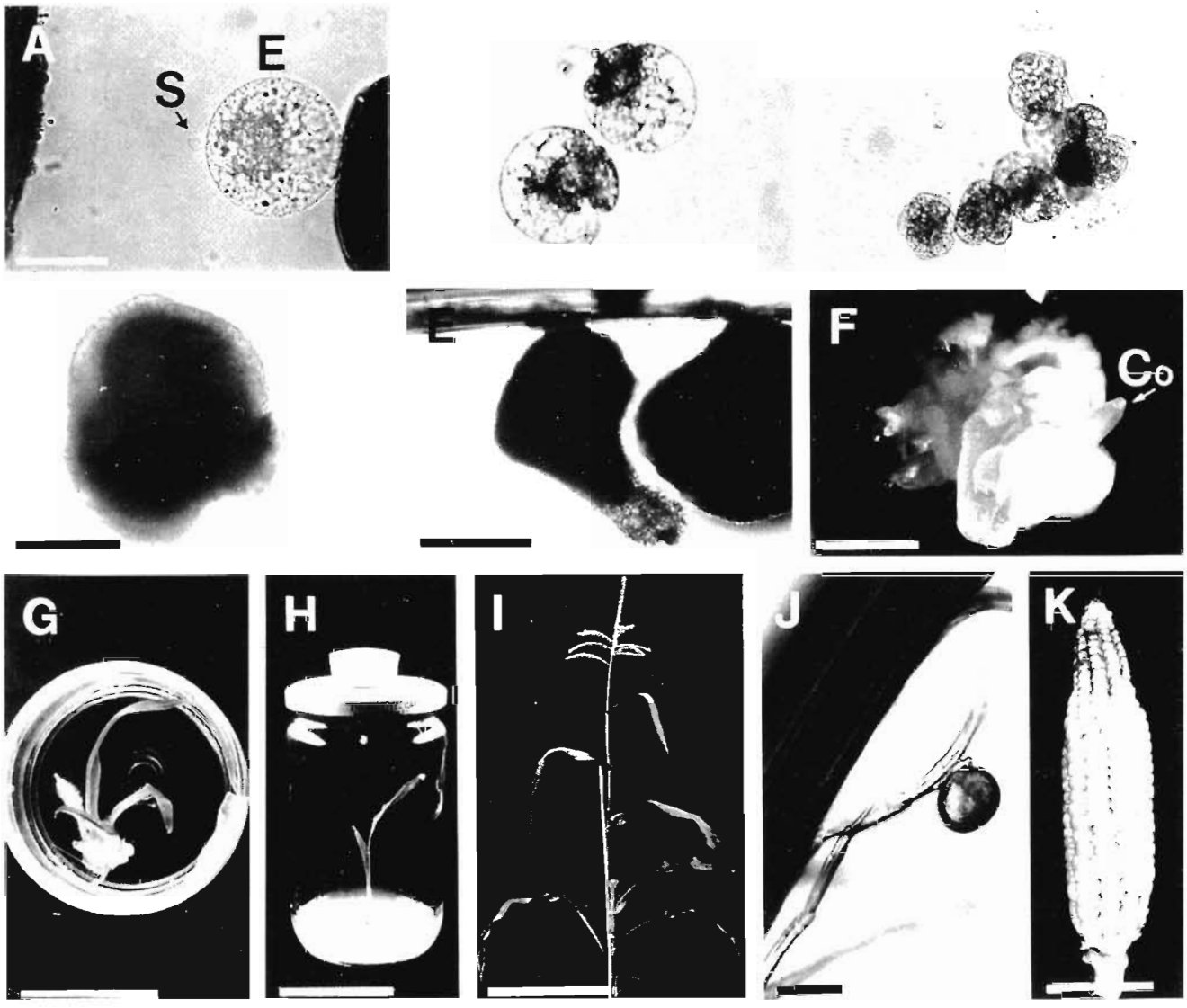


Figure 11—A-K. *Zea mays*, in vitro fertilization, through electrofusion. **A.** Alignment of egg cell protoplast (E) with a sperm cell protoplast (S); bar-50 cm; **B.** first cell division (after 42 hrs of fusion); **C.** multicelled structure (after 5 days of fusion); bar- 100 cm; **D.** polarized multicellular structure with an outer cell layer at one pole, and vacuolate cells at the other end (after 12 days of fusion); bar-200 μm; **E.** transition-phase embryo (after 14 days of fusion); bar-200 μm; **F.** compact white and green tissue, arrow indicates coleoptile *(Co)*(after 30 days of fusion); bar-4 mm, **G.** plantlets after 35 days of fusion; bar-2 cm; **H.** plantlet after 39 days of fusion; bar-6 cm; **I.** flowering plantlet after 39 days of fusion; bar-6 cm; **J.** self-pollination, one hour after pollen grain deposition, the pollen tube penetrated the red-colored trichome, the grain contents move into the style; bar-100 μm, **K.** cob after 148 days of fusion; bar-4 cm (after Kranz & Lörz, 1993; courtesy Kranz).

appears to be promising (Kranz *et al.*, 1991), as synchronized single cells can be used.

Besides, transmission of alien cytoplasm to study biparental inheritance of cytoplasm-determined characters, has also been achieved through the above method. However, in higher plants using non-electrical approaches are necessary to study cell-cell interaction: adhesion, fusion, and recognition (Russell, 1992; Chasan, 1992; 1993; Goodman, 1993; Faure *et al.*, 1993).

Regeneration as well as karyology of complete plantlet via zygotic embryogenesis of single maize zygote produced by in vitro fusion of isolated single gametes has already been reported by Kranz and Lörz (1993). With the cultivation of in vitro-formed zygote, transformation becomes the first priority.

Figure 12—*Quercus lebani*, embryo culture. Differentiation of embryoids from cultured middle segment of embryos on MS + IAA (2.0 ppm) + 2-ip (5.0 ppm) + adenine sulphate (30.0 ppm) + CH (1000 ppm), 8-wk-old (unpublished; courtesy Srivastava).

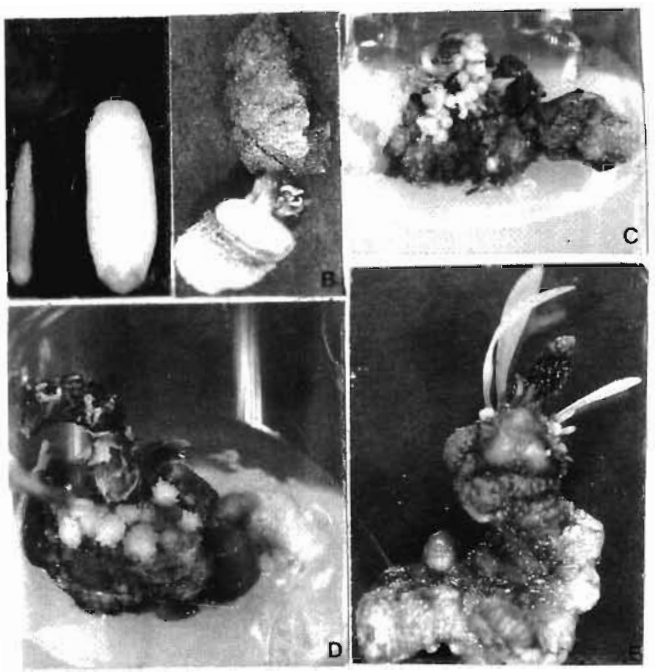


Figure 13—**A-E** *Dendrophthoe falcata*, embryo culture. **A.** Mature embryo without and with endosperm; **B.** 10-wk-old embryo grown on WM + IAA (1 ppm) +YE (500 ppm) shows callused cotyledons (upper region); also note the proliferated radicular end (basal region); **C.** 10 (right side) and 20-wk-old (left side) callus on WM + IAA (0.5 ppm) + CH (500 ppm) shows a large number of papillate structures; **D.** 15-wk-old proliferated seedling with buds from embryo cultured with the endosperm; **E.**20-wk-old seedling shows a large number of accessory leaves (after Johri & Bajaj, 1963).

Subsequently, it would then be possible to manipulate gametes individually (prior to their fusion), thus opening up a new field of study in experimental embryology and biotechnology.

EMBRYO CULTURE

Interest in embryo culture dates back to the work of Hannig (1904). He cultured embryos (of different age) of *Raphanus* spp. and *Cochlearia danica* and obtained transplantable seedlings. Embryo culture provided information on the physiology of development and growth of the embryo.

A stimulus for further progress in this field was provided by Laibach (1925) who demonstrated the use of this technique in all those crosses where viable seeds are not formed. He suggested to excise the embryos and grow them in a nutrient medium.

The embryo culture technique has been utilized for raising hybrids of unsuccessful crosses. Hybridity of the surviving plantlets reared by embryo culture has been established through karyotype mapping, and isozyme profiles. The technique has also been utilized for the induction of polyembryony as in *Quercus* (Figure 12) and *Dendrophthoe* (Figure 13). The technique of embryo culture has also been successfully employed in raising plants from interspecific or intervarietal crosses as in *Lilium* (North, 1975), *Allium* (Doezel *et al.*, 1980), *Solanum* (Sharma

Figure 14—Synthetic seeds. Embryos of alfalfa encapsulated in alginate beads. Each capsule is 4-6 mm in diameter and contains one embryo (after Fuji *et al.*, 1987)

et al., 1980), and cherry (Ivanicka & Pretova, 1980), and intergeneric hybrids *Triticum* and *Aegilops* (Cheuca *et al.*, 1977), barley and rye (Pickering & Thomas, 1979), and wheat and rye (Taira & Larter, 1978).

Morphogenesis of cultured embryos is significantly influenced by the exogenous supply of growth regulators. Both indolebutyric acid (IBA) and α-naphthalene acetic acid (NAA) increase the number of roots in the embryo of *Iris* (Stoltz, 1977). This technique has proved useful in comparison of growth rate of embryos developing in vivo and in vitro, and physiological and biochemical changes in the developing seeds.

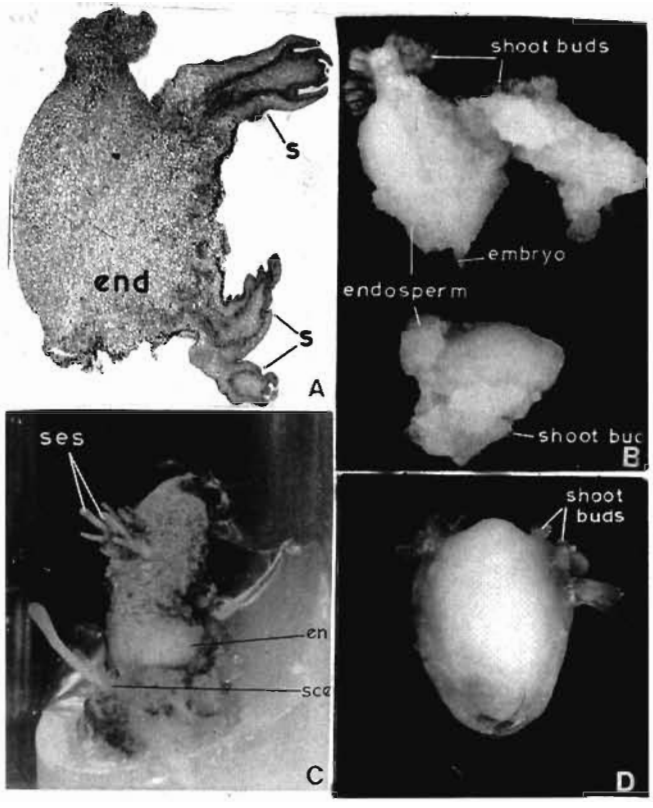


Figure 15—A-D. Endosperm culture. **A.** *Exocarpus cupressiformis*, section of endosperm (*end*) with differentiated shoot buds (*s*) on WM + IAA (2.0 ppm) + KN (5.0 ppm) + CH (500 ppm), 6-wk-old (after Johri & Bhojwani, 1965); **B.** *Leptomeria acida*, differentiation of shoot buds in 12-wk-old sub-cultured endosperm callus on WM + IAA (2.5 ppm) +KN (5.0 ppm) + CH (1000 ppm) (after Nag & Johri, 1971); **C.** *Dendrophthoe falcata*, 15-wk-old culture with shoot and many leaves, on WM + IAA (5.0 ppm) + KN (10.0 ppm) + CH (1000 ppm) (after Nag & Johri, 1971); **D.** *Taxillus vestitus*, 8-wk-old culture of endosperm-half on WM + BAP (2.0 ppm) (after Nag & Johri, 1971).

SYNTHETIC SEEDS

Synthetic or artificial seeds are encapsulated (originally only the somatic embryos produced in tissue cultures) in a synthetic polymer coating which functions as 'endosperm' and seed-coat (Figure 14). Besides the somatic embryos, now even the 'vegetative regenerants/propagules' capable of developing into a complete plantlet, shoot meristems, apical and axillary shoot buds of *Mentha arvensis* and *Morus indica* and callus-derived adventitious buds can also be encapsulated as in *Atropa belladonna, Dioscorea floribunda* and *Hyoscyamus muticus*. This is a new concept which is applicable even to crops which are not seed-sown (see Shekhawat *et al.*, 1995).

The encapsulating agents are agar, polyox, alginate, gelrite, carageenasa, polyacrylamide, nitrocellulose and ethylcellulose. Sodium alginate is commonly used due to its solubility at room temperature and formation of a complete permeable gel with calcium chloride (see Prakash & Pierik, 1993). The 'beads' (synthetic seeds) can be stored at 4°C in dark in sterile dry petri plates sealed with parafilm. For germination, the beads are rinsed with sterile water, and surface dried.

The production of synthetic seeds of somatic embryos enclosed in a protective covering has been proposed as a 'low-cost-high-volume' propagation system. The inherent benefits of this system are inbuilt in the production of a large number of somatic embryos, and the use of conventional seed-handling technique for embryo delivery (see Prakash & Pierik, 1993).

Figure 16—A-C. *Solanum* sp., interspecific hybrid. **A.** *S. tuberosum*, dihaploid parent (24 chromosomes); **B.** tetraploid somatic hybrid (48 chromosomes); **C.** *S. brevidens*, diploid parent (24 chromosomes). (after Jones, 1988).

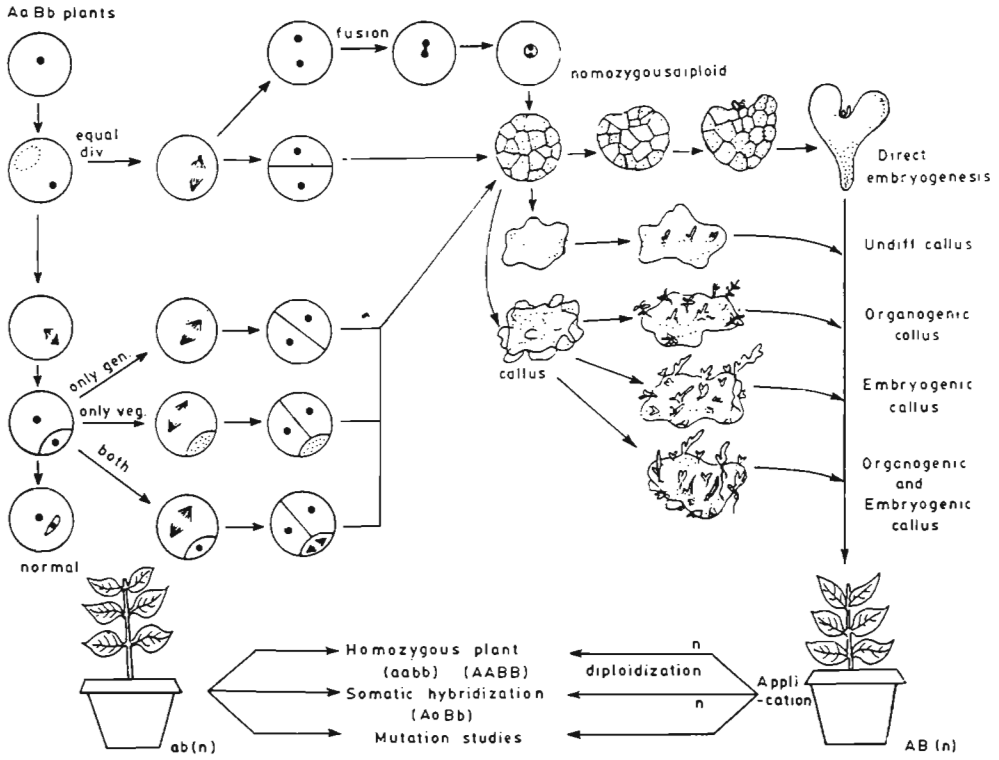
The synthetic seeds have a distinct advantage over micropropagation, such as economy, space, culture medium, time and expenditure, allow easy transportation, transplantation, and storage, and conservation of propagules (see Shekhawat *et al.*, 1995).

HAPLOID PLANTS THROUGH ANTHER/ POLLEN CULTURE

The haploid plants are of great value for breeding and study of fundamental genetics of higher plants. In angiosperms haploid cells are present only in anthers and ovules. The anthers, carrier of progenitor of male gametes, are much easier to excise. Starting from a single cell and ending in a whole organism passing through a series of cell divisions and differentiation, the microspores have now been shown to be totipotent (Text-figure 3).

The culture of pollen grains of higher plants was first attempted by C.D. LaRue (1954). Since then the totipotent nature of pollen grains has been exploited with varying success. The first successful differentiation of haploid plants was reported by Sipra Guha and S.C. Maheshwari in 1964 in anther cultures of *Datura innoxia* and *D. stramonium*. Subsequent studies have aimed at optimizing the different physical and chemical factors for obtaining maximal number of plantlets from pollen (in the cultured anthers). Besides other factors (physical and chemical), prechilling treatment of the flower buds is essential for successful regeneration of plantlets from pollen. Generally, pollen cultured prior to or after the first mitosis, give a positive response by producing typical embryoids or callus masses. During embryogenic process, the pollen either divides into two unequal cells (vegetative and generative) and both or only one of them takes part in the development of embryoid/plantlet, or the pollen divides into two equal cells, and the derivatives of both cells contribute in plantlet formation (Text-figure 3; Srivastava & Johri, 1988).

Androgenesis occurs when a microspore or pollen grain is induced to shift from a gametophytic



Text-figure 3—Anther culture. Differentiation of embryoids from pollen grains, possible pathways in embryogenic pollen grains, and utilization of regenerated haploid plantlets (*DIV*—division, *GEN*—generative cell, *VEG*—vegetative cell, *UNDIFF*—undifferentiated) (after Srivastava & Johri, 1988).

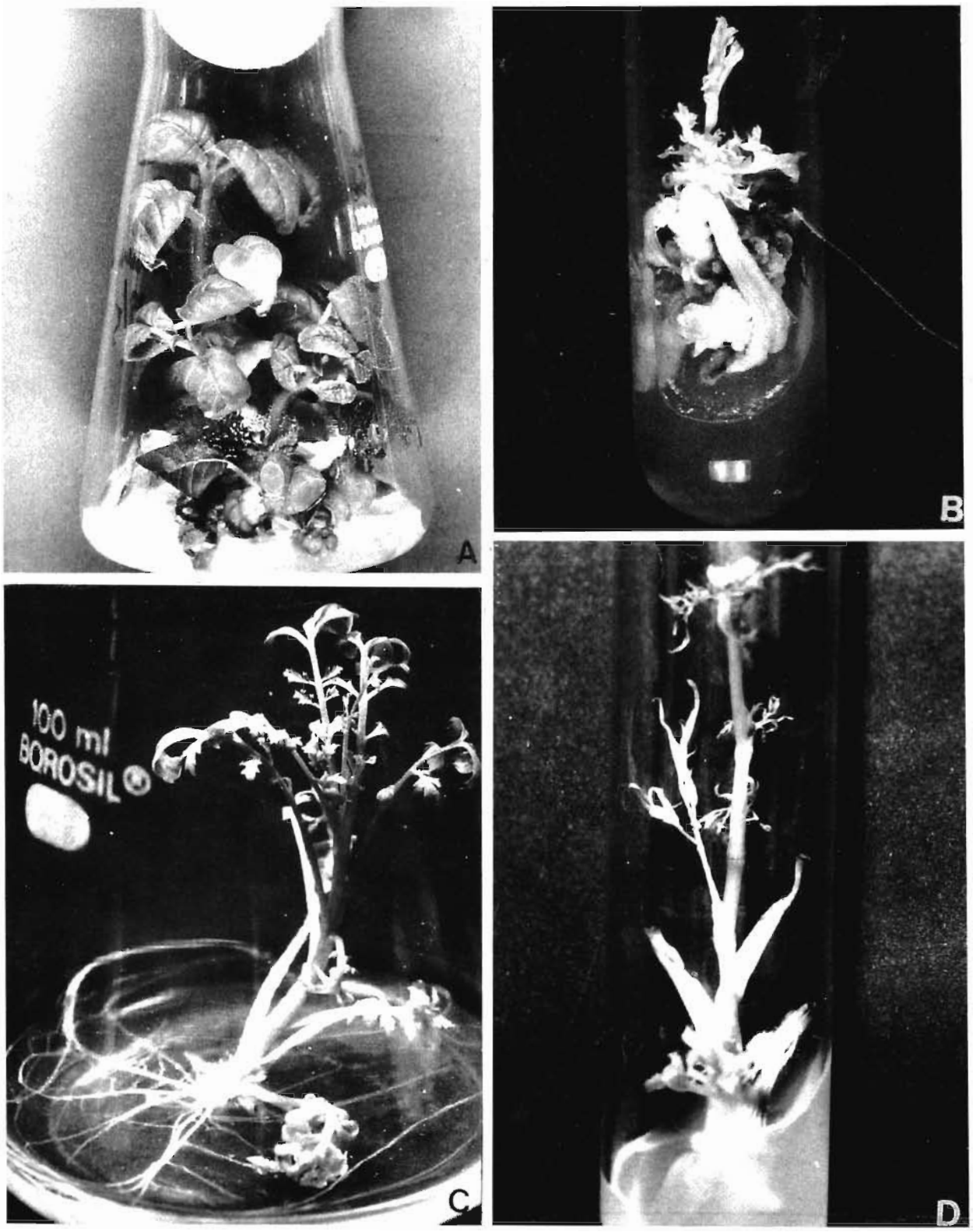


Figure 17—A-D. In vitro multiplication of medicinal plants. **A**. *Datura innoxia*, 12-wk-old androgenic cultures on MS + glutamine (100 ppm); **B**. *Ammi majus*, 6-wk-old regenerated callus on MS + IAA (0.5 ppm) + KN (2.0 ppm) + adenine (40 ppm) + CH (500 ppm); **C**. *Lepidium sativum*, 8-wk-old plantlet on MS + IBA (0.1 ppm); **D**. *Ammi majus*, 12-wk-old plantlet with flower buds, on MS + glutamine (100 ppm). (A, after Srivastava *et al.*, 1993; B,D, after Purohit *et al.*, 1995; C, after Pande *et al.* unpublished).

to sporophytic pathway. The single-celled pollen grains represent an ideal system to analyze the embryogenic process in angiosperms. Since the haploids contain only one set of chromosomes, the genes present, even the recessive ones, are expressed in phenotype. However, through diploidization of these haploids, homozygous and fertile *Nicotiana* are readily obtained, enabling the selection of desirable gene combinations. Employing this technique, four breeding lines of tobacco differing in alkaloid yield (Collins *et al.*, 1974), and homozygous recombinants of *Hyoscyamus niger* with enhanced alkaloid content, have been obtained (Corduan & Spix, 1978).

Androgenic haploids and the homozygous diploids are of much interest to plant breeders and geneticists. In the production of new mutant forms, haploids provide excellent material for experimentation as in *Nicotiana tobacum* (Nitsch, 1969) and *Brassica napus* (Hoffmann, 1978). Yet another application in one-step transfer of genotypes of inbred lines into cytoplasm that causes male sterility has been observed in haploids of cereals, e.g., maize (Goodsell, 1961; Kermicle, 1973).

Factors controlling pollen embryogenesis of higher plants (Vasil & Nitsch, 1975) can be studied by culturing the isolated microspore. The technique ensures the production of more isogenic progenies in *Nicotiana tabacum* than anther culture and, therefore, is more efficient in mutagenic studies (see Vasil, 1980).

After the discovery of pollen haploids, 35 years ago by Guha and SC Maheshwari and its great utility in breeding programmes, the advances in this area have been rather spectacular. The main obstacle, however, facing anther cultures is the lack of response from pollen grains of several crop plants and other species of economic importance. To overcome the problem, Wernicke *et al.* (1978) developed a technique for the isolation of potential embryogenic

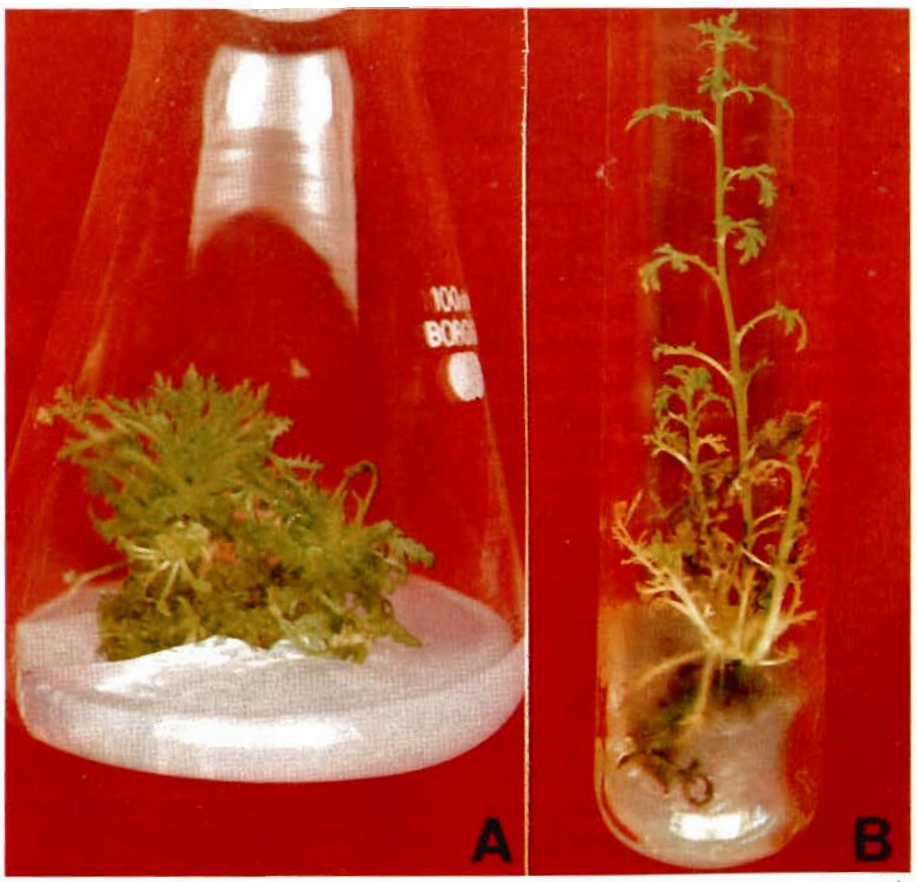


Figure 18—A-B. *Artemisia annua, micropropagation.* **A.** Multiple shoot differentiation on MS + BAP (3.0 ppm) + G^3A_3 (0.1. ppm) **B.** direct regeneration from flower buds on MS + NAA °0.1 ppm) + BAP (3.0 ppm) (after Gulati *et al.*, 1996).

pollen grains of *Nicotiana* from the microspore population which enhances the haploids in low-yielding species.

ENDOSPERM CULTURE

Unlike gymnosperms, the development of nutritive tissue (endosperm) in angiosperms is postponed until after fertilization (triple fusion) and is usually triploid. Improper development of endosperm may lead to the abortion of embryo as it has a direct and dynamic influence on the differentiation of embryos. The first report on the culture of endosperm of maize is by LaRue (1947); only callus developed but differentiation did not occur.

While investigating the factors affecting the growth of endosperm, the essentiality of nitrogen source other than minerals was also realized. It was confirmed that casein hydrolysate (CH) or yeast extract (YE) are essential supplements for the endosperm culture of *Cucumis* (Nakajima, 1962). The level of cell organization in the developing endosperm has indicated a regulatory role in callus formation.

The culture of mature endosperm is more responsive than the immature endosperm (see Figure 15). Most of the reports on endosperm culture are from semi-parasitic angiosperms. A few autotrophic taxa subsequently provided good results (Bhojwani, 1966; Masuda *et al.*, 1978). In cultures the differentiation of root, shoot, haustoria and plantlets establish the totipotency of the triploid endosperm tissue. The failure of mature endosperm to proliferate, if cultured without the embryo, emphasises the concept of 'embryo factor'. Srivastava (1971) has shown that the 'embryo factor' can be replaced by pretreatment of endosperm with GA_3. For example, in *Scurrula* and *Taxillus* shoots emerge directly from the endosperm explant (see Johri, 1971) whereas in *Jatropha* and *Putranjiva* regeneration is preceded by callusing (see Srivastava & Johri, 1978).

Organ differentiation in endosperm cultures has been demonstrated in parasitic (see Johri, 1971) as well as autotrophic species: *Croton* (Bhojwani, 1966), *Jatropha* (Srivastava, 1971), *Putranjiva* (Srivastava, 1973), *Oryza* (Nakano *et al.*, 1975), apple (Mu *et al.*, 1977) and other taxa.

Shoot buds differentiate directly without prior callusing in the cultured mature endosperm of semi-parasitic taxa as in *Scurrula* and *Taxillus*. In the autotrophic members, the endosperm usually forms a callus mass followed by the differentiation of shoot buds or roots, as in *Jatropha* and *Putranjiva* .

The only reports on the differentiation of roots and plantlets from the callus of the immature endosperm are in rice (Nakano *et al.*, 1975) and apple (Mu *et al.*, 1977).

PROTOPLAST CULTURE AND SOMATIC HYBRIDS

Since isolated protoplasts are generally free from one another, they constitute the nearest possible

Figure 19—A-B. *Betula pendula*. **A.** Shoot bud culture to show regeneration of numerous plantlets after 8 wks on MS + IAA (2.0 ppm) + BAP (5.0 ppm) + Ad (20.0 ppm); **B.** leaf culture to show differentiation of a number of leafy shoots, after 5 wks on MS + NAA (2.0 ppm) + Zeatin (2.0 ppm) + Ad (30.0 ppm) (after Srivastava & Steinhauer, 1981a).

approach to isolate cell suspension of higher plants and, as such, are increasingly used in several areas of plant cell biology. The most promising use of protoplast culture is the production of genetically-engineered plants involving the fusion of protoplasts from different genetic backgrounds, and regeneration into an intact hybrid plantlet exhibiting the characters of both parents (Figure 16). Somatic cell fusion or 'parasexual hybridization' is a novel approach to develop rare hybrids of sexually incompatible and sterile parents. Cocking and his group at Nottingham (England) demonstrated the use of $NaNO_3$ in protoplast fusion. The production of heterokaryon was first reported by Power *et al.* (1970), from maize. A critical factor in studies on induced-fusion between two unrelated protoplasts relates to the 'selective markers' to identify the heteroplastic fusion products.

The successful production of a mature interspecific hybrid plant *(Nicotiana glauca* X *N. langsdorfit)* by fusion of leaf protoplasts has been reported by Carlson *et al.* (1972). Melchers *et al.* (1978) could raise intergeneric hybrid, the so-called 'pomato' and 'topato', between potato (*Solanun tuberosum)* and tomato *(Lycopersicon esculentum)*. The hybrid flowered and fruited, and the fruits resembled tomato. Instead of the usual 'round' potato tubers, however, the underground 'suckers' were thick and elongated and stored abundant starch. In this case the analysis of ribulose triphosphase carboxylase proved to be a convenient marker to demonstrate the hybrid nature of the plant.

Quite recently, the scientists at the University at Solan (India) have produced a hybrid between tomato *(Lycopersicon esculentum)* and 'Shimla mircha' (*Capsicum frutescense)*. Rattan and Sharma–the two agricultural scientists, have produced this hybrid. The new hybrid is tolerant to early blight and brick-eye rot. These investigators claim that the hybrid could be grown in the plains and mid-hills. Rattan also points out that the 'hybrid' has a thick skin and stands better transportability.

L. Zenkteler (Poznan, Holland) using in vitro pollination and fertilization, obtained an intergeneric hybrid between *Melandrium* and *Nicotiana.* Further studies have not been attempted.

DISEASE-FREE PLANTS

Plants are prone to systemic infection with fungi, bacteria and viruses, and are also attacked by insects, pests and nematodes. A clean and healthy stock can be used for propagation through conventional means, but can be difficult and costly. Plant tissue culture eliminates these systemic diseases in *Begonia, Carnation, Asparagus* and *Prunus*, etc. (see Quak,1977; Prakash & Pierik, 1993) but do not guarantee freedom from diseases.

The first step in obtaining disease-free culture is to ensure that the mother plant (from which explants are taken) does not have any disease symptom.

Theoretically, the use of antibiotics seems feasible to overcome the contamination problems. But unfortunately, plant tissues are sensitive to antibiotics and show variable responses according to their genotype. The damage is mainly to the plastids or mitochondria within the plant cells (Zamski & Umiel, 1978), and prolonged exposure of cells or tissues to antibiotics can result in the development of resistance through mutation in cytoplasmic genes or cytoplasmic DNA (Maliga *et al.*, 1973;Umiel & Goldner, 1976). In other cases antibiotics increase the growth rate of cultured tissues, sometimes in a spectacular way. Such results have been reported with penicillin (see George & Sherrington, 1984), streptomycin and tetramycin (Nickell, 1952), and phosphomycin (Phillips *et al.*, 1981).

There is a variable concentration of virus in the cells of an intact plant with the assumption that apical shoot and root meristems may contain scanty or no virus at all, micropropagation is used as an effective means of removing virus infection. This can be achieved by culturing apical meristem or nucellus as in *Citrus* (see Prakash & Pierik, 1993).

GENETIC STABILITY

The physical make up and appearance of a plant (its phenotype) is governed by its genotype (genetic constitution) and the maintenance of genetic constitution of a particular clone is (so-called genetic stability) an important factor in tissue culture. Besides, occasionally originating from genetically vari-

able mother plant tissue, genetic variation may arise directly in culture. Endoreduplication is more prevalent in cultures that have been maintained for a long period.

Another reason is the failure of spindle formation during mitosis or the occurrence of abnormal multipolar instead of usual bipolar spindles. Synthetic plant growth regulators are implicated in

Figure 20—A-D. *Delonix regia*, regeneration from leaf callus. **A.** Compact green callus after 8 wks on MS + NAA (0.1 ppm) + BAP (3.0 ppm); **B.** shoot initiation after 10 wks on MS + NAA (0.1 ppm) + BAP (3.0 ppm); **C.** multiple shoot differentiation after 6 wks on MS + IAA (2.0 ppm) + KN (5.0 ppm); **D.** further growth of regenerants on MS + IBA (1.0 ppm) (after Gupta *et al.*, 1996).

Figure 21—P. Maheshwari (Univ. of Delhi). He initiated in vitro studies at the Department of Botany, University of Delhi, and helped many institutions to undertake such studies. He and his group, for the first time, suggested the technique of in vitro pollination and fertilization; and also guided work on in vitro culture of various reproductive organs.

inducing genetic changes in tissue cultures (see Thorpe, 1980).

Genetic variation, the somaclones have been induced by tissue culture. Also, new strains of plants from callus, 'calliclones', have been generated. A gradual increase in the proportion of cells with a typical chromosome complements is considered to be one of the main reasons why tissue cultures of many plant species lose regeneration ability with progressive age (see Bayliss, 1980).

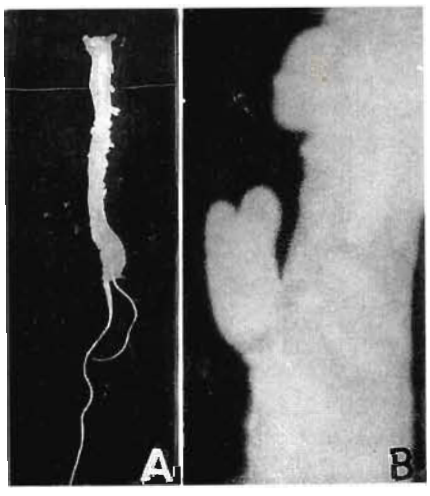


Figure 22—A-B. *Ranunculus sceleratus*, shoot bud culture. **A.** regenerated plantlet, stem studded with embryoids; **B.** part of stem with fully-differentiated dicotyledonous embryo (after Konar & Nataraja, 1969).

To overcome this problem one has to monitor the phenotypic, cytological and molecular level characteristics, and ascertain their correlation with the phenotypes at an early stage. Suspected variations at an early stage can be screened by various means, i.e., chromosome counts in mitotic cell, measurement of the DNA per cell, chloroplast counts in stomatal guard cells, measuring stomatal size (de Klerk, 1990), through isozyme and protein patterns (O'Connel *et al.*, 1986; Karp *et al.*, 1987; Kobayashi, 1987; Maheshwaran & Williams, 1987). Such tissues/plants become eliminated from the clone. Maintaining genetic stability is the quintessential factor in meeting the ever-rising demand of in vitro-raised plants of economic value.

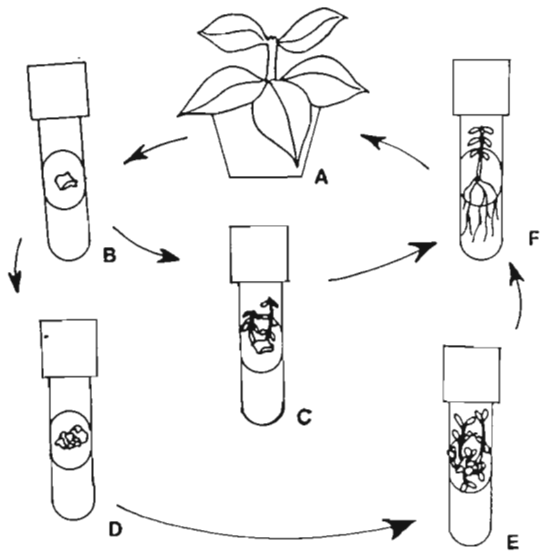
APPLICATIONS

The use of plant tissue culture for the production of industrial products has long been of much interest: drugs, breweries, milk products, flavours, fragrances, essential oils, pigments, sweetners and others. These chemicals are the secondary metabolites produced during culture. Plant cell cultures have advantages, as compared to intact plants (Figures 17, 18; Text-figure 4). The rate of cell growth in cultures and biosynthesis of chemicals is quite high in a short period. Suspension culture is a very effective mechanism of incorporating precursors into cells than in whole plants. Natural sources for the manufacture of these products are not enough to meet the ever-growing need of industrial production, although it has been possible to exploit the technique for large-scale production of selected plants (Figures 19, 20; Text-figure 5). Bioreactors are now being utilized to grow mass culture of cells.

The industrial potential of plant cell cultures is unlimited and will be exploited more and more as the years go by (see Prakash & Pierik, 1993; Razdan, 1995). A fairly large number of industrial concerns have been established in India and abroad, to exploit the in vitro culture methods for commercial benefits.

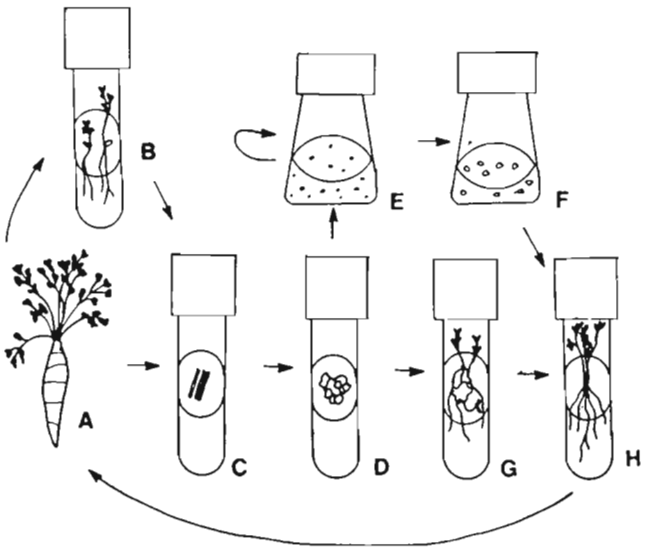
PLANT TISSUE CULTURE STUDIES AT THE UNIVERSITY OF DELHI

At the Department of Botany, University of Delhi, tissue culture researches were started in 1955-



Text-figure 4—A-F. *Nicotiana tobacum*. **A**. Plant grown in green-house; **B**. leaf section inoculated on nutrient medium; **C**. shoot differentiation directly from the epidermal cells explant on medium containing 1M BA (but no 2, 4-D); **D**. differentiation of callus from leaf section cultured on agarized nutrient medium containing 4.5 M 2, 4-D; **E**. shoot developed from the callus transferred to medium containing BA (but no 2, 4-D); **F**. fully differentiated plantlets on harmone-free medium (after Tisserat, 1985).

56, under the leadership of Professor P. Maheshwari, FRS (Figure 21). The investigations



Text-figure 5—A-H. *Daucus carota*, Somatic embryogenesis. **A**. Carrot plant; **B.** seedling; **C-D**. callus develops in 4 wks; **E-F**. suspension culture from callus; in liquid medium (without 2,4-D), somatic embryos differentiate; **G**. callus and plantlets on agarized medium, on transfer to medium without 2,4-D also produces somatic embryos and plantlets; **H**. rooted plantlets (after Tisserat, 1985).

mainly related to test the efficacy of the technique in morphogenic expression of various explants. In *Cuscuta* (Baldev, 1959) the vegetative shoot apex could be transformed into floral apex by controlling light/dark regime.

In vitro pollination and fertilization was demonstrated by P. Maheshwari and Kanta (1964) in *Eschscholzia* and *Argemone* spp., and Shivanna (1965) in *Petunia*. In due course normal seeds with endosperm and embryo are produced (see Shekhawat *et al.*, 1995). Several workers from the Botanical Institute, Prague (Czechoslavakia), and Zenkteler (Poznan, Poland) worked at the Delhi University to familiarise themselves with the in vitro pollination and fertilization techniques. Later at Poznan, Zenkteler (1970) successfully raised intergeneric hybrids between *Melandrium album* (Caryophyllaceae) and *Datura innoxia* (Solanaceae).

During 1956-1966, the pollinated ovaries of several plant species including *Aerva tomentosa, Anethum graveolens, Foeniculum vulgare, Hyoscyamus niger* and *Tropaeolum majus* were successfully cultured (Shekhawat *et al.*, 1995). Chopra (1962) reported development of parthenocarpic fruits, of the same size as obtained in vivo, from unpollinated ovaries of *Althea rosea* cultured on modified Nitsch's nutrient medium (NM) with IBA (20mg/1) or kinetin (KN) (0.5mg/1). With the success in ovary culture it became possible to culture ovules at even earlier stages. Nirmala Maheshwari (1958) obtained mature seeds of *Papaver somniferum* by culturing excised ovules containing zygote or two-celled pro-embryo. Sachar and Kapoor (1959) cultured pollinated and unpollinated ovules of *Zephyranthes*. The stimulatory role of placental tissue in the growth of ovules of *Gynandropsis gynandra* was demonstrated by Chopra and Sabharwal (1963).

A significant discovery is the development of haploids from pollen grains (through anther culture) in *Datura innoxia* (Guha & S.C. Maheshwari, 1964). This technique has been widely used to raise haploids (especially in rice in China) for genetical studies.

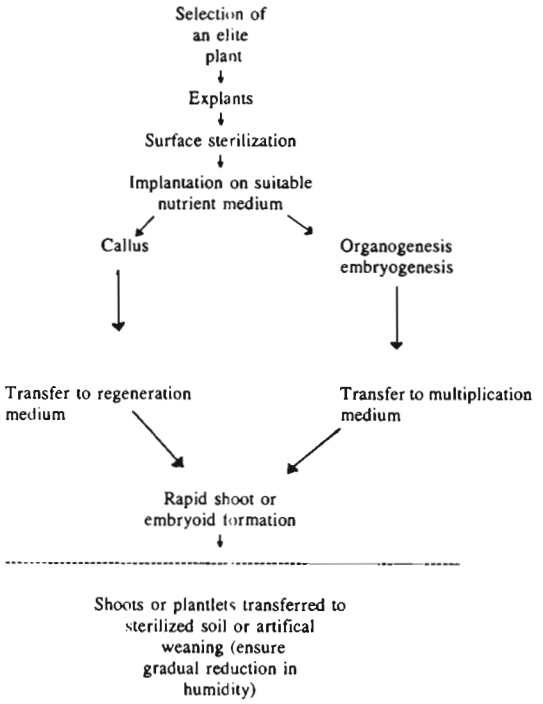
Another important achievement of the Delhi School is the demonstration of the triploid shoots

differentiated in endosperm cultures of *Exocarpus cupressiformis* (Santalaceae) by Bhojwani (Johri & Bhojwani, 1965; see Shekhawat *et al.*, 1995). Srivastava succeeded in raising triploid plantlets in mature endosperm cultures of *Jatropha panduraefolia* and *Putranjiva* (Euphorbiaceae) (Johri & Srivastava, 1973; Shekhawat *et al.*, 1995).

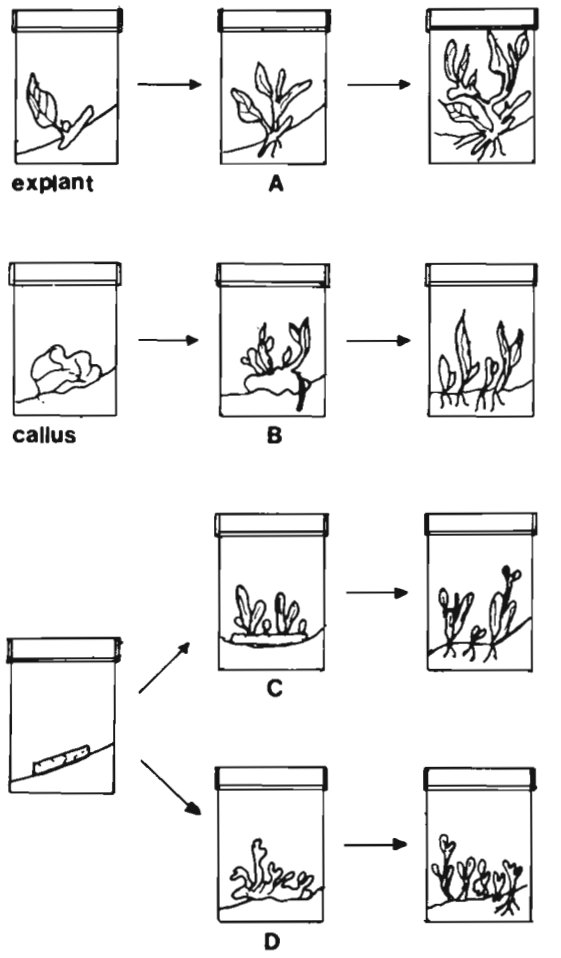
Many other plant parts have also been cultured. Of these, the embryo culture of Loranthaceae has yielded much new information. Differentiation of epidermal embryoids (Figure 22) on the stem of in vitro-raised plantlets, in floral bud cultures of *Ranunculus* was reported by Konar and Nataraja (1965).

CONCLUSIONS

Cellular totipotency—a porperty endowed only to plant cells, is the basis of micropropagation. The discovery and subsequent use of plant hormones—auxins, cytokinins and gibberellins, further strengthened the concept of organogenic differentiation. The widespread use of tissue culture technique has many advantages (Text-figures 6, 7) like in vitro cloning (Table 1) which is an important tool in speeding up propagation, and the production of disease-free plants, hybrid plants, haploids through anther and ovule culture, and also triploids through endosperm culture. The compatibility barriers can also be overcome through in vitro fertilization and embryo rescue.



Text-figure 6—Schematic representation to show in vitro procedures for raising shoot and plantlets.



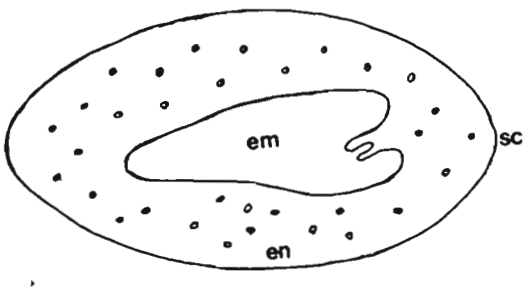
Text-figure 7—A-D. Micropropagation through culture of axillary buds. **A.** growth of axillary bud; **B.** differentiation of adventitious buds on callus; **C.** direct differentiation of buds on explant; **D.** induction of somatic embryogenesis.

Table 1—Clonal Propagation Systems

Greenhouse cuttings	a) Maintains genetic uniformity; b) Rooting of plantlets generally required prior to plantation; c) High cost per plantlet; d) Size of mother plant determines multiplication rate.
Micropropagation	a) Ensures genetic uniformity; b) Acclimatization of plantlets essential prior to transplantation; c) Cost per plantlet high; d) Multiplication rate relatively low.
Artificial seeds	a) Maintains genetic uniformity; b) Direct delivery of propagules to the field; c) Cost per plantlet low; d) Rapid multiplication.

To cope with the ever-increasing human population, the need of the hour is to increase the food production which must receive the highest priority. Plant tissue culture is a powerful and potential tool which would help in replacing the entire population of low-yielding and diseased plants. With proven positive economic impact, raising in vitro plants offers immense scope for further exploitation of the technique. The production of secondary metabolites of medicinal interest is now a favourite area of research. Even after several decades of research in this exciting area, the problem of low productivity and instability of plant cells in cultures continue to be the main bottleneck. Rapid industrialization and urbanization have imbalanced our ecosystem. In order to save our rich heritage and genetic diversity of economically valued plants, there is an urgent need to either alter the environment that is incomprehensible or raise such plants so as to suit the polluted environment. Raising stress-tolerant plants through in vitro technique is a boon to this problem.

The potentiality of synthetic seeds (Text-figure 8) lies in their vital role as rapid, inexpensive and universal clonal delivery system to propagate plants (Table 2). However, the problems encountered in their production and application need further research.



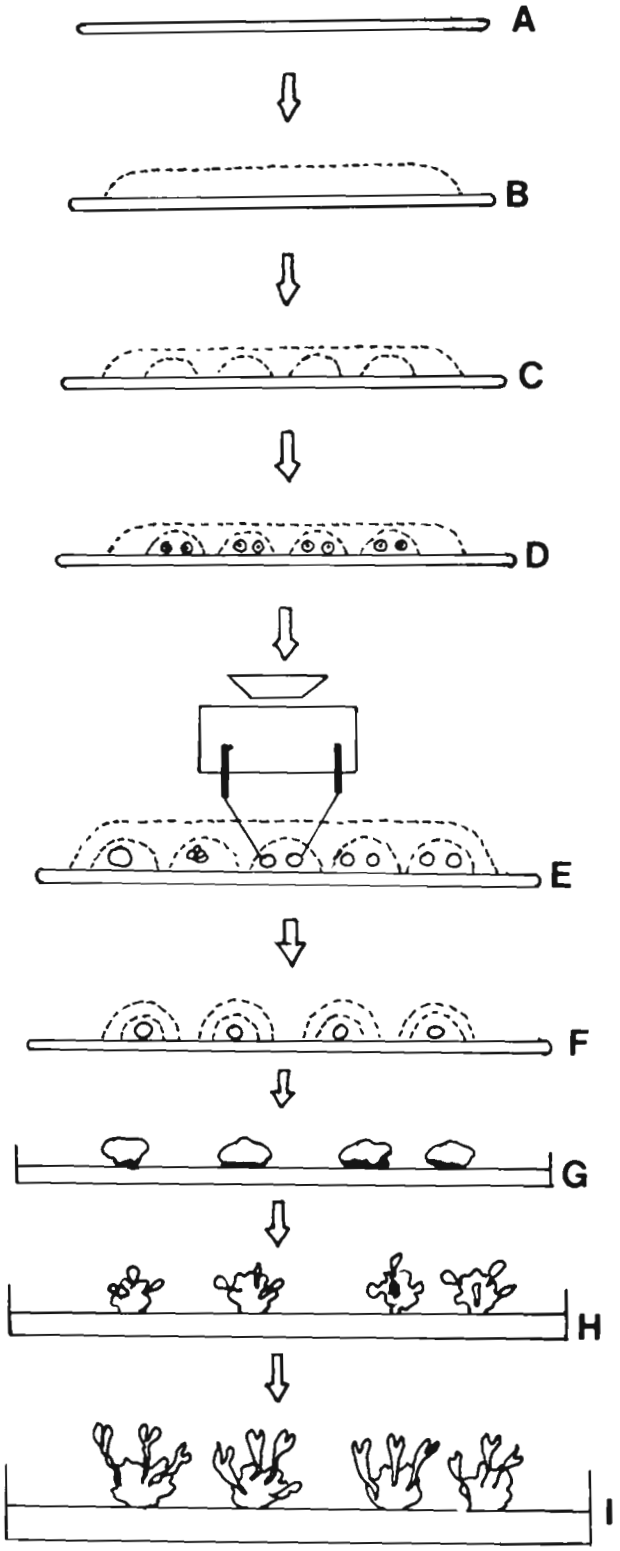
Text-figure 8—Synthetic seed. Somatic embryo (em) is encapsulated, in 'artificial endosperm' (en) covered with 'artificial seed-coat' (sc). In vitro differentiated shoot buds can also be encapsulated (after Fujii *et al.*, 1987).

Table 2—Crops that may benefit from artificial seeds

Hybrid Rice	Rapid propagation of F, hybrids.
Potato	a) Due to genetic instability, true seed can not be used; b) Propagation by cut tuber pieces are prone to diseases; c) Low storability of tuber pieces is a major problem in the tropics.
Geraniums	a) True seed costs are high; b) Vegetatively propagated.
European seedless Cucumber	a) True seed is expensive.
Garlic	a) Vegetatively propagated cloves have high carryover of virus and nematodes.
Gerbera/Daisy	a) Cost high.

Besides, one of the most striking developments witnessed in the field of tissue culture is the isolation, culture and fusion of protoplasts. A more recent approach is the genetic manipulation and regeneration of these cultured or fused protoplasts (Text-figure 9). Progress in recombinant DNA technology for the production of transgenic plants has been so spectacular that by the turn of the century we may hope our dream coming true for growing crops which have been manipulated to market specifications by adding, subtracting or modifying the threads of life (DNA).

Thus, during the last 50 years or so, following plant tissue culture procedures, significant advances have been made in understanding the growth, development and differentiation of plant parts. In future much attention will have to be paid to the isolation of male and female gametes of flowering plants and their use as tools of biotechnology, regeneration of isolated cells and protoplasts following genetic transformation, molecular biology and biochemical mechanisms of embryogenesis, production of artificial seeds, in vitro preservation of germplasm, and application of plant cell and tissue culture for understanding the basic features of differentiation. Tissue culture techniques will be used for screening and inducing disease susceptibility and resistance, and in breeding salt- and metal-resistant crops.



Text-figure 9—A-I. Procedure for electrofusion of protoplasts. **A.** Silicon coverslip; **B.** mineral oil; **C.** fusion microchamber, **D.** pairs of protoplast in the microchamber; **E.** Positioned micro-electrodes to induce fusion of the protoplasts; **F.** fused protoplasts in the microchamber; **G.** callus derived from the fusion product; **H.** differentiation/embryogenesis; **I.** further growth of shoots/plantlets (after Jones, 1988).

Attention will also have to be paid to produce triploid trees for rapid growth and biomass production, and increasing genetic base. Micropropagation of elite trees for tree improvement should also be given high priority. It is essential to device desired tissue culture techniques for individual crop plant species.

'Plant Tissue Culture' and 'Biotechnology' will be increasingly used in studies on Physiology, Biochemistry, Cell and Molecular Biology, Protoplasts and Somatic Hybrids, Micropropagation, Genetics and Plant Breeding, and Space Research.

ACKNOWLEDGEMENTS

We are grateful to Professor Hans Kranz (Hamburg University) for providing some illustrations and literature, Professor S.S. Bhojwani (Delhi University) and Professor M.M. Johri (Tata Institute of Fundamental Research, Bombay) for literature.

REFERENCES

Baldev B 1959. In vitro responses of growth and development in *Cuscuta reflexa* Roxb. *Phytomorphology* **9** : 316-319.

Bayliss MW 1980. Chromosomal variation in plant tissues in culture. *In*: Vasil IK (Editor)—*Int. Review of Cytology. Suppl IIA. Perspectives in plant cell and tissue culture*: 113-114. Academic Press, New York.

Berlyn J 1984. Plant cell cultures—A future source of natural products? *Endeavour* n.ser. **8**: 5-8.

Beiderbeck R & Knoop B 1988. Enhanced production of secondary substances : Addition of artificial accumulation sites of cultures. *In*: Bajaj YPS (Editor)—*Biotechnology in agriculture and forestry, medicinal and aromatic plants* I. **4**: 11-12. Springer-Verlag, Berlin.

Bhojwani SS 1966. Morphogenetic behaviour of mature endosperm of *Croton bonplandianum* in culture. *Phytomorphology* **16**: 349-353.

Bitters WP, Murashige T, Rangan TS & Nauer E 1972. Investigations on established virus-free *Citrus* plants through tissue culture. *In*: Price WC (Editor)—*Proc. 5th Int. Conf. Organ Citrus Virologists*: 267-271. Univ. of Florida, Gainesville, USA.

Button J & Bornman CH 1971. Development of nucellar plants from unpollinated and unfertilized ovules of Washington Navel orange in vitro *J. S. Afr. Bot.* **37**: 127-134.

Carlson S, Smith HH & Dearing RD 1972. Parasexual interspecific plant hybridization. *Proc. Natl. Acad. Sci. USA* **69** :2292-2294.

Chasan R 1992. Frontiers in fertilization. *Plant Cell* **4**: 369-372.

Chasan R 1993. Test-tube plants : The first of a new crop? *Plant Cell* **5**: 718-719.

Cheuca M-C, Cauderon Y & Tampe J 1977. Technique d'obtention d'hybrides Ble tendre x *Aegilops* par culture in vitro d'embryons immatures. *Ann. Amelior Plantes* **27:** 537-719.

Chopra RN 1958. In vitro culture of ovaries of *Althaea rosea* Cav. *In*: P. Maheshwari P (Editor)— *Modern developments in plant physiology*. 87-89. Univ. of Delhi.

Chopra RN 1962. Effect of some growth substances and calyx on fruit and seed development of *Althaea rosea* Cav. *In: Symp. Plant embryology*: 170-181. CSIR, New Delhi.

Chopra RN & Sabharwal PS 1963. In vitro culture of ovules of *Gynandropsis gynandra* (L.) Brig. and *Impatiens balsamia* L. *In*: Maheshwari P & Rangaswamy NS (Editors)—*Symp. Plant tissue and organ culture*. Int. Soc. Plant Morphologists, Delhi Univ.

Cocking EC 1972. Plant cell protoplasts—Isolation and development. *Rev. Plant Physiol.* **23:**29-50.

Collins GB, Legg PD & Kasperbater MJ 1974. Use of anther-derived haploids in *Nicotiana* : Isolation of breeding lines differing in total alkaloid content. *Crop Sci.* **14:** 77-80.

Corduan G & Spix C 1978. Anther culture of a hybrid of the genus *Hyoscyamus*: A rapid method to obtain homozygous recombinants. *In* : Alfermann AW & Reinhard E (Editors)—*Production of natural compounds by cell culture method.* : 295-302. Symp. Plant cell culture, Munich.

DeKlerk GJ 1990. How to measure somaclonal variations. *Acta bot. neerl.* **39:** 129-144.

DeMaggio AE & Wetmore RH 1961. Morphogenetic studies on the fern *Todea barbara* : Experimental embryology. *Am. J. Bot.* **48**:551-56.

Dolezel J, Novak FJ & Luzny J 1980. Embryo development and in vitro culture of *Allium cepa* and its interspecific hybrids. *Z. Pflanzenzüg* **85:** 177-184.

Eid AA, Delanghe E & Waterkyn L 1973. In vitro culture of fertilized cotton ovules: The growth of cotton embryo. *Cellule* **69:** 361-371.

Faure JE, Mogensen L, Dumas C, Lörz H & Kranz E 1993. Karyogamy after electrofusion of single egg and sperm cell protoplasts from maize; cytological evidence and time course. *Plant Cell* **5**: 747-755.

Gautheret RJ 1934. Culture du tissue cambial. *C.R. Acad. Sci. Paris* **198:** 2195-2196.

Gautheret RJ 1939. Sur la possibilite de realiser la culture indefinite des tissus de tubercules de carotte. *C.R. Acad. Sci. Paris* **208**:118-120.

George EF & Sherrington PD 1984. Plant propagation by tissue culture: Handbook and Directory of Commercial Laboratories: 88-118. England.

Goodman B 1993. A 'shotgun wedding' finally produces test-tube plants. *Science* **261**:430.

Goodsell SF 1961. Male sterility in corn by androgenesis. *Crop Sci.* **1**:227-228.

Guha Sipra & Johri BM 1966. In vitro development of ovary and ovule of *Allium cepa* L. *Phytomorphology* **16**:353-364.

Guha Sipra & Maheshwari SC 1964. In vitro production of embryos from anthers of *Datura*. *Nature* **204**:497.

Gulati A, Bharel S, Abidin MZ, Jain SJ & Srivastava PS 1996. In vitro micropropagation and flowering in *Artemisia annua*. *J. Plant Biochem. Biotechnol.* **5**:31-35.

Gupta N, Jain SK & Srivastava PS 1996. In vitro micropropagation of a multipurpose leguminous tree—*Delonix regia*. *Phytomorphology* **64**: 267-275.

Gupta PK, Nadgir AL, Mascarenhas AF & Jagannathan V 1980. Tissue culture of forest trees: Clonal multiplication of *Tectona grandis* L. (Teak) by tissue culture. *Plant Sci. Lett.* **17**:259-268.

Gupta PK, Mascarenhas AF & Jagannathan V 1981. Tissue culture of forest trees : Clonal propagation of mature trees of *Eucalyptus citriodora* Hook. by tissue culture. *Plant Sci. Lett.* **20**:195-201.

Haberlandt G 1902. Cultureversuche mit isolierten pflanzenzellen. *Sitzungsber, Math. Naturwiss Kl Kais Akad Wiss., Wien.* **111**: 69-92.

Haccicus B, Hausner G 1976. Ein transplanties-barer embryogener Callus aus Nucellusgenweb von *Cynanchum vincetoxicum* und die Rolle gloubularer Vorstadien in der Entwicklungsgeschichte nichtzygotischer Embryonen. *Protoplasma* **90**: 265-282.

Hannig E 1904. Zur Physiologic pflanzlicher Embryonen. I. Über die kultur von Crucifere-Embryonen ausserhalb des Embryosacks. *Bot. Ztg* **62:**45-80.

Hoffmann E 1978. Mutation and selection of haploid cell culture systems of rape and rye. *In*: Alfermann AW & Reinhard E (Editors)—*Production of natural compounds by cell culture methods*: 319-329. Int. Symp. Plant Cell Culture, Munich.

Iriving GW & Herrie HT 1949. *Antibiotics*. Chemical Publ. Co., Inc, Booklyn, USA.

Ito I 1961. In vitro culture of ovary and seed in orchids. *Ph.D. Thesis*. Kyoto Prefect Univ., Kyoto, Japan.

Ito I 1966. In vitro culture of orchids. (I) Effects of sugar, peptone, and coconut milk upon the growth of ovary of *Dendrobium mobile*. *Sci. Rep. Kyoto Prefect Univ. Agric.* **18**:38-50.

Ivanicka J & Pretova A 1980. Embryo culture and micropropagation of cherries in vitro. *Scient. Hortic.* **12**:77-82.

Johri BM 1971. Embryogenesis in tissue cultures. *In: Les Cultures de Tissue de Plantes* : 260-289. CNRS, Paris, No. 193.

Johri BM & Bajaj YPS 1963. In vitro response of the embryo of *Dendrophthoe falcata* (L.f.) Ettings. *In:* Maheshwari P& Rangaswamy NS (Editors)—*Symp plant tissue and organ culture*: 292-301. Int. Soc. Plant Morphologists, Univ. of Delhi.

Johri BM & Bhojwani SS 1965. Growth responses of mature endosperm in cultures. *Nature* **208:** 1345-1347.

Johri BM & Sehgal CB 1966. Chemical induction of polyembryony in *Anethum graveolens* L. *Naturwissenschaften* **50**:47-48.

Johri BM & Srivastava PS 1973. Morphogenesis in endosperm cultures. *Z. Pflanzenphysiol* **70:** 285-304.

Jones M 1988. Fusing plant protoplasts. *TIBTECH* **6:** 153-158.

Kanta K & Maheshwari P 1963. Intraovarian pollination in some Papaveraceae. *Phytomorphology* **13:** 230-237.

Kanta K, Rangaswamy NS & Maheshwari P 1962. Test-tube fertilization in a flowering plant. *Nature* **194:** 1214-1271.

Karp A, Wu QS, Steels SH & Jones MGK 1987. Chromosome variation in dividing protoplasts and cell suspensions of wheat. *Theor. Appl. Genet.* **74:** 140-146.

Kermicle JL 1973. Androgenesis and the indeterminate gametophyte mutation: Source of the cytoplasm. *Maize Genet. Cytol.* **17:** 655-666.

Kobayashi S 1987. Uniformity of plant regenerated from orange protoplasts. *Theor. Appl. Genet.* **74:** 10-14.

Konar RN & Nataraja K 1965. Experimental studies in *Ranunculus sceleratus* L. in vitro. *Acta bot. Neerl.* **18:** 680-699.

Konar RN & Nataraja K 1969. In vitro control of floral morphogenesis in *Ranunculus sceleratus* L. *Phytomorphology* **14:** 558-563.

Kranz E & Dresselhaus T 1996. In vitro fertilization with isolated higher plant gametes. *Trends Plant Sci. Rev.* **1**: 82-89.

Kranz E & Lörz H 1990. In vitro fertilization of single, isolated gametes, transmission of cytoplasmic organelles and cell reconstitution of maize (*Zea mays* L.) : *In:* Nijkamp HJJ, Van der Plas LHW & Van Aartrijk (Editors)—*Progress in plant cellular and molecular biology*. VII. 24-29. Kluwer Academic Publishers, Dordercht (Netherlands).

Kranz E & Lörz H 1993. In vitro fertilization with isolated, single gametes results in zygotic embryogenesis and fertile maize plants. *Plant Cell* **5:** 739-746.

Kranz E & Lörz H 1994. In vitro fertilization of maize by single egg and sperm cell protoplast fusion mediated by high calcium and high pH. *Zygote* **2**:125-128.

Kranz E, Bautor J & Lörz H 1991. Electrofusion-mediated transmission of cytoplasmic organelles through the in vitro fertilization process, fusion of sperm cells with synergids and central cells, and cell reconstitution in maize. *Sex Plant Reprod.* **4:** 17-21.

Laibach F 1925. Das Taubwerden von Bastardsamen und die kunstliche Aufzucht fruh absterbender bastardembryonen. *Z. Bot.* **17:** 417-459.

Laibach F & Kribben FJ 1949. Der Einfluss von Wuchsstoft auf die Bildung Mannlicher und Weiblicher Buten bei eier Monozischen Pflanze *(Cucumis sativus* L). *Ber. dtsch Bot. Ges.* **62:** 53-55.

LaRue CD 1942. The rooting of flowers in culture. *Bull. Torrey bot. Club* **69**: 332-341.

LaRue CD 1947. Growth and regeneration of the endosperm of maize in culture. *Am. J. Bot.* **34:** 585-568.

LaRue CD 1954. Studies on growth and regeneration in gametophytes and sporophytes of gymnosperms. *Brookhaven Symp. Biol.* **6:** 187-208.

Maheshwaran G & Williams EG 1987. Uniformity of plants regenerated by direct somatic embryogenesis from zygotic embryos of *Trifolium repens*. *Ann. Bot. (London)* **59**: 93-97.

Maheshwari Nirmala 1958. In vitro culture of excised ovules of *Papaver somniferum*. *Science* **127:** 342.

Maheshwari P & Kanta K 1964. Control of fertilization. *In:* Linskens HF (Editor)—*Pollen physiology and fertilization:* 187-194. North-Holland Publ., Amsterdam.

Maheshwari P & Lal M 1961. In vitro culture of excised ovules of *Papaver somniferum* L. *Phytomorphology* **11** : 307-314.

Maheshwari P & Rangaswamy NS 1965. Embryology in relation to physiology and genetics. *In*: Preston RD (Editor)—*Advances in Botanical Research* **2:** 219-321. Academic Press, London.

Maliga P, Sz-Breznovits A & Marton L 1973. Streptomycin-resistant plants from callus culture of haploid tobacco. *Nature (New Biol.)* **244**: 29-30.

Mantell SH, Matthews JA & Mckee RA 1985. *Principles of plant biotechnology-An introduction of genetic engineering in plants.* Blackwell Sci. Pub. Oxford, London, Edinburg.

Masuda K, Koda Y & Okazawa Y 1978. Callus formation and embryogenesis of endosperm tissue of parsley seed cultured on hormone-free medium. *Physiol. Plantarum* **41:** 135-138.

Mauney JR 1961. The culture in vitro of immature cotton embryos. *Bot. Gaz.* **122:** 205-209.

Melchers G 1977. Microbial techniques in somatic hybridization by fusion of protoplasts. *In:* Brinkley BR & Porter KR (Editors)—*Int. Cell Biol. 1976-1977*: 207-215. Rockfeller Univ. Press, Boston, Mass, USA.

Melchers G, Sacristan MD & Holder AA 1978. Somatic hybrid plants of potato and tomato regenerated from fused protoplasts. *Carlsberg Res. Commun.* **43**: 203-218.

Mitra GC & Chaturvedi HC 1972. Embryoids and complete plants from unpollinated ovaries and from ovules of in vivo-grown emasculated flower buds of *Citrus* spp. *Bull Torrey bot. Club* **99:** 184-189.

Mu S, Liu S, Zhou Y, Qian N, Zhang P, Xie H, Zhang F & Yan Z 1977. Induction of callus from apple endosperm and differentiation of the endosperm plantlet. *Sci. sin.* **20:**370-377.

Mullins MG & Srinivasan C 1976. Somatic embryos and plantlets from an ancient clone of the grapevine (cv. Cabernet-sauvignon) by apomixis in vitro. *J. Exp. Bot.* **27**: 1022-1030.

Murashige T & Skoog F 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plantarum* **15:** 473-497

Nag KK & Johri BM 1971 Morphogenic studies on endosperm of some parasitic angiosperms. *Phytomorphology* **21**: 202-208.

Nakajima T 1962. Physiological studies of seed development, especially embryonic growth and endosperm development. *Univ. Osaka Prefect* ser. B **13**: 13-48.

Nakano H, Tashiro T & Maeda E 1975. Plant differentiation in callus tissue induced from immature endosperm of *Oryza sativa*. *Z. Pflanzenphysiol.* **76**: 444-449.

Nickell LG 1952. Stimulation of plant growth by antibiotics. *Proc. Soc. Exp. Biol. Med.* **80**: 615.

Nitsch JP 1951. Growth and development in vitro of excised ovaries. *Am. J. Bot.* **38**: 556-576.

Nitsch JP 1969. Experimental androgenesis in *Nicotiana*. *Phytomorphology* **19:** 389-404.

Nobècourt P 1937. Cultures en serie de tissue Vegetaux sur milieu artificiel. *C.R. Seanc. Soc. Bid.* **205** : 521.523.

North C 1975. Embryo culture as an aid to breeding in *Lilium*. *Acta Hortic.* **47**: 187-191.

O'Connel MA, Hosticka LP & Hanson MR 1986. Examination of genome stability in cultured *Lycopersicon*. *Plant Cell Rep.* **5:** 276-279.

Phillips R, Arnott SM & Kaplan SE 1981. Antibiotics in plant tissue culture: Rifampicin activity controls bacterial contaminants without effecting the growth of short-term explant cultures of *Helianthus tuberosus*. *Plant Sci. Lett.* **21**: 235-240.

Pickering RA & Thomas HM 1979. Crosses between tetraploid barley and diploid rye. *Plant Sci. Lett.* **16:** 291-296.

Power JB, Cummins SE & Cocking EC 1970. Fusion of isolated plant protoplasts. *Nature* **225:** 1016-1018.

Prakash J & Pierik RLM (Editors) 1993. *Plant biotechnology: Commercial prospects and problems*: 1-289. Oxford & IBH Publ Co. Pvt Ltd., New Delhi.

Purohit M, Pande D, Datta A & Srivastava PS 1995. In vitro flowering and high xanthotoxin in *Ammi majus* L. *J. Plant Biochem. Biotechnol.* **4:** 73-76.

Purohit SS 1994. *Microbiology: Fundamentals and applications.* Agro Botanical Publ., Bikaner (India).

Quak F 1977. Meristem culture and virus-free plants. *In:* Reinert J & Bajaj YPS (Editors)—*Applied and fundamental aspects of plant cell, tissue and organ culture:* 598-600. Springer-Verlag, Berlin.

Rangan TS *1982.* Ovary, ovule and nucellus culture. *In:* Johri BM (Editor)—*Experimental embryology of vascular plants:* 105-130. Springer-Verlag, Berlin, Heidelberg, New York.

Rangan TS & Rangaswamy NS 1968. Morphogenic investigations on parasitic angiosperms. I. *Cistanche tubulosa* (Orobanchaceae). *Can. J. Bot.* **46:** 263-266.

Rangaswamy NS 1958. In vitro culture of nucellus and embryos of *Citrus. In:* Maheshwari P (Editor)—*Proc. Symp. Modern developments in plant physiology:* 104-106. Univ Delhi.

Rangaswamy NS & Shivanna KR 1971. Overcoming self-incompatibility in *Petunia axillaris* (Lam.) BSP.II. Placental pollination in vitro *J. Indian bot. Soc.* **50A:** 286-296.

Rangaswamy NS & Shivanna KR 1975. Nucellus culture of two cucurbits - *Luffa cylindrica* and *Trichosanthes anguina. Ann. Bot. (London)* **39**: 139-196.

Rao PS 1965. The in vitro fertilization and seed formation in *Nicotiana rustica* L. *Phyton (Hungary)* **2:** 183-196.

Rau MA 1956. Studies on the growth in vitro of excised ovaries- I, Influence of colchicine on the embryo and endosperm in *Phlox drummondi* Hock. *Phytomorphology* **6:** 90-96.

Ravishankar GA & Venkataraman LV 1993. Role of plant cell culture in food biotechnology: Current trends, limitations and future prospects. *In.* Prakash J & Pierik RLM (Editors)—*Plant biotechnology: Commercial prospects and problems:* 255-274. Oxford & IBH Publ Co. Pvt. Ltd, New Delhi.

Razdan MK 1995. *An introduction to plant tissue culture.* Oxford & IBH Publ Co. Pvt. Ltd., New Delhi.

Russell SD 1992. Double fertilization. *Int. Rev. Cytol.* **140:** 357-388.

Sachar RC & Kanta K1958. Influence of growth substances on artificially cultured ovaries of *Tropaeolum majus* L. *Phytomorphology* **8:** 202-218.

Sachar RC & Kapoor M 1959. In vitro culture of ovules of *Zephyranthes. Phytomorphology* **9:** 147-156.

Sehgal CB 1972. In vitro induction of polyembryony in *Ammi majus* L. *Curr. Sci.* **41:** 263-264.

Sharma'DR, Chowdhury JB, Ahuja V & Dankhar BS 1980. Interspecific hybridization in genus *Solanum*— A cross between *S. melongena* and *S. khasianum* through embryo culture. *Z. Pflanzenzütg* **985**: 248-253.

Shekhawat NS, Johri BM & Srivastava PS 1995. Morphogenesis and plant tissue culture. *In:* Johri BM (Editor)—*Botany in India: History and Progress:* 307-356. Oxford & IBH Publ. Co. Pvt Ltd, New Delhi.

Shivanna KR 1965. In vitro fertilization and seed formation in *Petunia violacea* Lindl. *Phytomorphology* **15:** 183-185.

Siddiqui SA 1964. In vitro culture of ovules of *Nicotiana tobacum* L. var. NP **31**. *Naturwissenschaften* **51:** 517.

Skoog F & Miller CO 1975. Chemical regulation of growth and organ formation in plant tissues cultured in vitro. *Symp. Soc. Exp. Biol.* **11:** 118-131.

Srivastava PS 1971. In vitro induction of triploid roots and shoots from mature endosperm of *Jatropha panduraefolia. Z.Pflanzenphysiol.* **66:** 93-96.

Srivastava PS 1973. Formation of triploid 'plantlets' in endosperm cultures of *Putranjiva roxburghii. Z. Pflanzenphysiol* **69:** 270-273.

Srivastava PS & Johri BM 1978. Pollen embryogenesis. *J. Palynol.* **23-24**: 83-99.

Srivastava PS & Steinhauer A 1981a. Isozymes in differentiating shoot bud cultures of *Betula pendula* Roth. *Z. Pflanzenphysiol.* **103**: 341-346.

Srivastava PS & Steinhauer A 1981b. Regeneration of birch plants from catkin tissue cultures. *Plant Sci. Lett.* **22:** 379-386.

Srivastava PS, Purohit M, Pande D & Datta A 1993. Phenotypic variation and alkaloid content in the androgenic plantlets of *Datura innoxia. Phytomorphology* **43:** 209-216.

Stewart JMD 1981. In vitro fertilization and embryo rescue. *Environ Exp. Bot.* **21**: 301-315.

Stoltz LP 1977. Growth regulator effects on growth and development of excised mature *Iris* embryo in vitro. *Hortic. Sci.* **12:** 495-496.

Taira T & Larter EN 1978. Factors influencing development of wheat-rye hybrid embryos in vitro. *Crop Sci.* **18:** 348-350.

Tamiya H 1959. Process of growth of *Chlorella* studied by the technique of synchronous culture. *In: Symp. Algology*: 1-10. New Delhi.

Thorpe TA 1980. Organogenesis in vitro: Structural, physiological and biochemical aspects. *In:* Vasil IK (Editor)—*Perspective in plant cell and tissue culture. Suppl II A:* 71-111. Academic Press, New York.

Tukey HB 1938. Growth patterns of plants developed from immature embryos in artificial culture. *Bot. Gaz.* **99**: 630-665.

Tulecke W 1957. The pollen of *Ginkgo biloba:* In vitro culture and tissue formation. *Am. J. Bot.* **44:** 602-608.

Uchimiya H, Kameya T & Takahashi N 1971. In vitro culture of unfertilized ovules in *Solanum melongena* and ovaries in *Zea mays. Japan J. Breed.* **21**: 247-250.

Umiel N & Goldner R 1976. Effects of streptomycin on diploid tobacco callus cultures and the isolation of resistant mutants. *Protoplasma* **89:** 83-89.

Vasil IK (Editor) 1980. International Review of Cytology. Suppl II A. *Perspectives in plant cell tissue culture.* Academic Press, New York.

Vasil IK & Nitsch C 1975. Experimental production of haploids and their uses. *Z. Pflanzenphysiol.* **76:** 191-212.

Vasil Vimla & Hildebrandt AC 1965. Differentiation of tobacco plants from single isolated cells in microculture. *Science* **150:** 189-192.

Wernicke W, Harms CT, Lörz H & Thomas E 1978. Selective enrichment of embryogenic microspore populations. *Naturwissenschaften* **65:** 540-541.

White PR 1932. Plant tissue culture: A preliminary report of results obtained in the culturing of certain plant meristems. *Arch. Exp. Zellforsch. besconders Gewebeziich* **12:** 602-620.

White PR 1939. Controlled differentiation in a plant tissue culture. *Bull. Torrey Bot. Club* **66:** 507-513.

White PR 1954. *The cultivation of animal and plant cells.* Ronald Press, New York.

Zamski E & Umiel N 1978. Streptomycin resistance in tobacco. II. Effects of the drug on the ultrastructure of plastids and mitochondria in callus cultures. *Z.Pflanzenphysiol.* **88:** 317-325.

Zenkteler M 1970. Test-tube fertilization of ovules in *Melandrium album* Mill with pollen grains of *Datura stramonium* L. *Experientia* **26:** 661-662.

Zenkteler M 1990 . In vitro fertilization and wide hybridization in higher plants. *Crit. Rev. Plant Sci.* **9**: 267-279.