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Prem Kumar Dantu

Plant Tissue Culture: An Introductory Text

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Dedicated to the most Revered
Dr. M. B. Lal Sahab (1907–2002)
D.Sc. (Lucknow), D.Sc. (Edinburgh),
the visionary Founder Director of the
Dayalbagh Educational Institute, for
the inspiration and strength to
undertake and complete the task of
writing this book

Preface

Plant tissue culture (PTC) broadly refers to cultivation of plant cells, tissues, organs, and plantlets on artificial medium under aseptic and controlled environmental conditions. PTC is as much an art as a science. It is the art of growing experimental plants, selecting a suitable plant organ or tissue to initiate cultures, cleaning, sterilization and trimming it to a suitable size, and planting it on a culture medium in right orientation while maintaining complete asepsis. It also requires an experienced and vigilant eye to select healthy and normal tissues for subculture. PTC involves a scientific approach to systematically optimize physical (nature of the substrate, pH, light, temperature and humidity), chemical (composition of the culture medium, particularly nutrients and growth regulators), biological (source, physiological status and size of the explant), and environmental (gaseous environment inside the culture vial) parameters to achieve the desired growth rate, cellular metabolism, and differentiation.

The most important contribution made through PTC is the demonstration of the unique capacity of plant cells to regenerate full plants, via organogenesis or embryogenesis, irrespective of their source (root, leaf, stem, floral parts, pollen, endosperm) and ploidy level (haploid, diploid, triploid). PTC is also the best technique to exploit the cellular totipotency of plant cells for numerous practical applications, and offers technologies for crop improvement (haploid and triploid production, in vitro fertilization, hybrid embryo rescue, variant selection), clonal propagation (Micropropagation), virus elimination (shoot tip culture), germplasm conservation, production of industrial phytochemicals, and regeneration of plants from genetically manipulated cells by recombinant DNA technology (genetic engineering) or cell fusion (somatic hybridization). PTC has been extensively employed for basic studies related to plant physiology (photosynthesis, nutrition of plant cells, and embryos), biochemistry, cellular metabolism, morphogenesis (organogenesis, embryogenesis), phytopathology (plant microbe interaction), histology (cytodifferentiation), cytology (cell cycle), etc. Indeed the discovery of first cytokinin is based on PTC studies.

Thus, PTC is an exciting area of basic and applied sciences with considerable scope for further research. Considerable work is being done to understand the physiology and genetics of embryogenesis and

organogenesis using PTC systems, especially *Arabidopsis* and carrot, which are likely to enhance the efficiency of in vitro regeneration protocols. Therefore, PTC forms a part of most of the courses on plant sciences (Developmental Botany, Embryology, Physiology, Genetics, Plant Breeding, Horticulture, Sylviculture, Phytopathology, etc.) and is an essential component of Plant Biotechnology.

After the first book on "*Plant Tissue Culture*" by Prof. P. R. White in 1943, several volumes describing different aspects of PTC have been published. Most of these are compilations of invited articles by different experts or proceedings of conferences. More recently, a number of books describing the methods and protocols for one or more techniques of PTC have been published which should serve as useful laboratory manuals. The impetus for writing this book was to make available an up-to-date text covering all theoretical and practical aspects of PTC for the students and early career researchers of plant sciences and agricultural biotechnology. The book includes 19 chapters profusely illustrated with half-tone pictures and self-explanatory diagrams. Most of the chapters include relevant media compositions and protocols that should be helpful in conducting laboratory exercises. For those who are interested in further details, Suggested Further Reading are given at the end of each chapter. We hope that the readers will find it useful. Suggestions for further improvement of the book are most welcome.

During the past two decades or so research in the area of plant biotechnology has become a closed door activity because many renowned scientists have moved from public research laboratories in universities and institutions to the private industry. Consequently, detailed information on many recent developments is not readily available.

We would like to thank many scientists who provided illustrations from their works and those who have helped us in completing this mammoth task. The help of Mr. Jai Bhargava and Mr. Atul Haseja in preparing some of the illustrations is gratefully acknowledged.

October 2012

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About the Book

Plant tissue culture (PTC) is basic to all plant biotechnologies and is an exciting area of basic and applied sciences with considerable scope for further research. PTC is also the best approach to demonstrate the totipotency of plant cells, and to exploit it for numerous practical applications. It offers technologies for crop improvement (haploid and triploid production, in vitro fertilization, hybrid embryo rescue, variant selection), clonal propagation (micropropagation), virus elimination (shoot tip culture), germplasm conservation, production of industrial phytochemicals, and regeneration of plants from genetically manipulated cells by recombinant DNA technology (genetic engineering) or cell fusion (somatic hybridization and cybridization). Considerable work is being done to understand the physiology and genetics of in vitro embryogenesis and organogenesis using model systems, especially *Arabidopsis* and carrot, which is likely to enhance the efficiency of in vitro regeneration protocols. All these aspects are covered extensively in this book.

Historical Sketch

1

Gottlieb Haberlandt, a German botanist, made the first attempts to culture fully differentiated single cells isolated from the leaves of *Lamium purpureum*, petioles of *Eichhornia crassipes*, glandular hairs of *Pulmonaria mollissima*, and stamen hairs of *Tradescantia* in a simple nutrient solution of Knop. The purpose of this experiment was to achieve divisions in these cells and obtain complete plants from them to verify the concept of cellular totipotency inherent in the famous Cell Theory put forward by Schleiden (1838) and Schwann (1839). The cultured cells survived for up to 1 month and also increased in volume but did not divide. Although Haberlandt could not achieve his goals, his genius is apparent in his classic paper presented before the Vienna Academy of Science in Berlin in 1902 wherein he laid down, for the first time, several postulates and principles of plant tissue culture. He had proposed that cells in the plant body stop growing after acquiring the features required by the entire organism without losing their (cell's) inherent potentiality for further growth and are capable of resuming uninterrupted growth on getting suitable stimulus. He also put forward the view that it should be possible to obtain embryos from vegetative cells. With the passage of time, most of the postulates of Haberlandt have been confirmed experimentally, and therefore he is justifiably recognized as the father of plant tissue culture.

GOTTLIEB HABERLANDT
(1854-1945)

A new line of investigation was initiated by Hannig (1904) that later emerged as an important applied area of plant tissue culture. He excised nearly mature embryos of some crucifers and successfully cultured them to maturity on mineral salts and sugar solution. In 1925, Laibach made a very significant contribution when he demonstrated that in the cross *Linum perenne* x *L. austriacum* the hybrid embryos, which normally abort prematurely, could be rescued to obtain full hybrid plants by excising them from the immature seeds and culturing on nutrient medium. Embryo culture has since become a useful tool in the hands of plant breeders to obtain rare hybrids which otherwise fail due to post-zygotic sexual incompatibility (Chap. 11). Van Overbeek et al. (1940) demonstrated for the

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first time, the stimulatory effect of coconut milk on development of young embryos of *Datura*. It was possible only in 1993 that as small as 8-celled embryos of *Brassica juncea* could be cultured successfully using double-layer culture system and a complex nutrient medium (Liu et al. 1993). Almost the same time, Kranz and Lörz (1993) and Holm et al. (1994) succeeded in in vitro cultivation of excised in vitro and in vivo formed zygotes, respectively. However, this required the use of a nurse tissue.

In 1922, Kotte in Germany and Robbins in the USA suggested that the meristematic cells in shoot buds and root tips could possibly be used to initiate in vitro cultures. Their work on root culture, although not very successful, opened up a new approach to tissue culture studies. In 1932, White started his famous work on isolated root culture, and in 1934 he announced the establishment of continuously growing root cultures of tomato. Some of these root cultures were maintained, by periodic subcultures, until shortly before his death in 1968, in India. The medium initially used by White contained inorganic salts, yeast extract, and sugar. Yeast extract was later replaced with the three B vitamins, namely pyridoxine, thiamine, and nicotinic acid. This heralded the first synthetic medium, which was widely used as basal medium for a variety of cell and tissue cultures. During 1939–1950, Street and his students extensively worked on the root culture system to understand the importance of vitamins in plant growth and root-shoot relationship. The other postulate of Kotte and Robbins was realized when Loo (1945) established excellent cultures of *Asparagus* and *Cuscuta* shoot tips. Finally, Ball (1946) succeeded in raising whole plants from shoot tip (apical meristem plus a couple of leaf primordia) cultures of *Lupinus* and *Tropaeolum*.

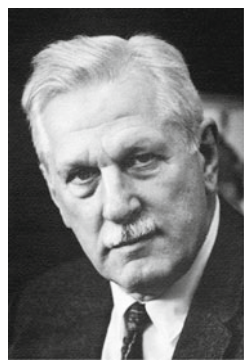
The discovery of auxin (Kogl et al. 1934) and recognition of the importance of B vitamins in plant growth (White 1937) gave the required impetus for further progress in the field of plant tissue culture. Using indoleacetic acid and B vitamins, Gautheret (1939) obtained continuously growing cultures from carrot root cambium. In the same year, White (1939) and Nobécourt (1939) reported the establishment of callus cultures from tumor tissue of the hybrid *Nicotiana glauca* × *Nicotiana langsdorffii* and carrot, respectively. These three scientists are credited for laying the foundation for further work in the field of plant tissue culture. The methods and media now used are, in principle, modifications of those established by these three pioneers in 1939. The first book on plant tissue culture, authored by White, was published in 1943.

PHILIP R. WHITE
(1901-1968)

ROGER J. GAUTHERET
(1910-1997)

PIERRE NOBÉCOURT
(1895-1961)

During 1950s Skoog and his co-workers, at the University of Wisconsin, USA made several major contributions toward the progress of plant tissue culture. Jablonski and Skoog (1954) tested several plant extracts to induce divisions in mature pith cells of tobacco and found yeast extract to be most suitable in this respect. Miller et al. (1955) isolated the first cell division factor from degraded sample of herring sperm and named it 6-furfurylamino purine, commonly called kinetin. Following this discovery, several natural and synthetic cytokinins were identified, of which benzylamino purine (BAP) is most widely used in plant tissue cultures. The availability of cytokinins made it possible to induce divisions in cells of highly mature and differentiated tissues, such as mesophyll and endosperm from dried seeds. With the discovery of auxins and cytokinins the stage was set for rapid developments in the field of plant tissue culture. The classic experiments of Skoog and Miller (1957) demonstrated chemical regulation of organogenesis in tobacco tissue cultures by manipulating auxin and kinetin ratio in the medium (Chap. 6). Relatively high concentration of auxin promoted rooting whereas higher levels of cytokinin favored shoot bud differentiation. In 1962, Murashige and Skoog formulated the now most extensively used plant tissue culture medium, popularly called MS medium. It contains 25 times higher salt concentration than the Knop's medium, particularly in NO_3^- and NH_4^+ ions (Thorpe 2007).



FOLKE SKOOG
(1908-2001)

TOSHIO MURASHIGE
(Born 1930)

The dream of Haberlandt of cultivating isolated single cells began to be realized with the work of Muir. In 1953, Muir demonstrated that by transferring callus tissues to liquid medium and agitating the cultures on a shaking machine, it was possible to break the tissues into small cell aggregates and single cells. Muir et al. (1954) succeeded in inducing the single cells to divide by placing them individually on separate filter papers, resting on the top of well-established callus cultures that acted as a nurse tissue, and supplied the necessary factors for cell division. Jones et al. (1960) designed a microchamber method for growing single cells in hanging drops of a conditioned medium (medium in which tissue has been grown for some time). This technique allowed continuous observation of the cultured cells. Using this technique, Vasil and Hildebrandt (1965) were able to raise complete plants starting from single cells of tobacco. An important biological technique of cloning large number of single cells was, however, developed in 1960 by Bergmann. It involved mixing single cell suspension with warm, molten agar medium, and plating the cells in a Petri dish where the medium solidified. This cell plating technique is now widely used for cloning cells (Chap. 4) and protoplast culture experiments (Chap. 14). The work of Kohlenbach (1966) came closest to the experiment of Haberlandt. He successfully cultured mature mesophyll cells of *Macleaya cordata* and obtained germinable somatic embryos (Lang and Kohlenbach 1975). Kohlenbach is also credited for providing convincing evidence that an isolated fully differentiated mesophyll cell of *Zinnia elegans* can directly differentiate(transdifferentiation) into a tracheary

element without cell division (Kohlenbach and Schmidt 1975). This provided a model system for detailed cytological, molecular, and genetic studies on the differentiation of tracheary elements by Komamine and his students (Chap. 5).

White (1934) during the course of his work with virus-infected roots observed that some of the subcultures were free of viruses. Limasset and Cornuet (1949) verified that lack of viruses in the meristematic cells is true not only for root tips but also for shoot tips. Taking a cue from this, Morel and Martin (1952) raised virus-free plants of *Dahlia* by meristem culture of infected plants. Shoot tip culture, alone or in combination with chemotherapy or/and thermotherapy, has since become the most popular technique to obtain virus-free plants from infected stocks (Chap. 16).

While applying the technique of shoot tip culture for raising virus-free individuals of an orchid, Morel (1960) realized the potential of this method for rapid clonal propagation. The technique allowed the production of almost 4 million genetically identical plants from a single bud in 1 year. This revolutionized the orchid industry, which was dependent on seeds for multiplication. This method of in vitro clonal propagation, popularly called micropropagation, was soon extended, with modifications, to other angiosperms. Toshio Murashige (USA) was instrumental in popularizing micropropagation for horticultural species. Micropropagation has now become an industrial technology, and several commercial companies round the world, including India, are using it for clonal propagation of horticultural and forest species (Chap. 17).

GEORGES MOREL
(1916-1973)

In 1958, Reinert (Germany) and Steward et al. (USA) demonstrated that plant regeneration in tissue cultures could also occur via embryogenesis. They observed differentiation of somatic embryos in the cultures of root tissue of carrot. These observations fascinated many scientists because in nature embryo formation is restricted to seeds. Backs-Hüsemann and Reinert (1970) achieved embryo formation from an isolated single cell of carrot. Somatic embryogenesis has been projected as the future method of rapid cloning of plants because: (a) the embryos are bipolar with root and shoot primordia, and (b) they can be converted into synthetic seeds by encapsulation in biodegradable substances for direct field planting (Chap. 7).

FREDERICK C. STEWARD
(1904-1993)

HERBERT E. STREET
(1913-1977)

ATSUSHI KOMAMINE
(1929-2011)

By the early 1960s, methods of in vitro culture were reasonably well developed, and the emphasis was shifting toward applied aspects of the technique. Cocking (1960) demonstrated that a large number of protoplasts could be isolated by enzymatic degradation of cell walls. He used culture filtrates of the fungus *Myrothecium verrucaria* to degrade cell walls. Takebe et al. (1968) were the first to use commercially available enzymes, cellulase, and macerozyme, to isolate protoplasts from tobacco mesophyll cells. In 1971, the totipotency of isolated plant protoplasts was demonstrated (Nagata and Takebe 1971; Takebe et al. 1970). At almost the same time, Cocking's group in the UK achieved fusion of isolated protoplasts using $NaNO_3$ (Power et al. 1970). Since then more efficient methods of protoplast fusion, using high pH-high Ca^{2+} (Keller and Melchers 1973), polyethylene glycol (Wallin et al. 1974; Kao et al. 1974), and electrofusion (Zimmermann and Vienka 1982) have been developed. These discoveries gave birth to a new field of somatic hybridization and cybridization (Chap. 14). Carlson et al. (1972) produced the first somatic hybrids between the sexually compatible parents *N. glauca* and *N. langsdorffii*. In 1978, Melchers and co-workers produced intergeneric somatic hybrids between sexually incompatible parents, potato and tomato, but the hybrids were sexually sterile. A unique application of protoplast fusion is in the production of cybrids, with novel nuclear-cytoplasmic combinations. This technique has already been used to transfer male sterility inter- and intraspecifically.

In India, tissue culture started in 1957 at the Department of Botany, University of Delhi under the dynamic leadership of P. Maheshwari. The emphasis was on in vitro culture of reproductive structures (ovary, ovule, nucellus, and embryo) of flowering plants. Some pioneering contributions were made at this school. Incidentally, one of the first International Conferences on plant tissue culture was held at the Department of Botany, University of Delhi in December 1961 (see Maheshwari and Rangaswamy 1963). Prompted by her success with intra-ovarian pollination (Kanta 1960), Kanta et al. (1962) developed the technique of test tube fertilization. It involved culturing excised ovules (attached to a piece of placental tissue) and pollen grains together on the same medium; the pollen germinated and fertilized the ovule. Using this approach, Zenkteler and co-workers (Poland) produced interspecific and intergeneric hybrids unknown in nature (see Bhojwani and Raste 1996; Zenkteler 1999). Kranz et al. (1990) reported a major breakthrough when they electrofused isolated male and female gametes of maize and 3 years later regenerated fertile plants from the in vitro formed zygotes (Kranz and Lörz 1993).

PANCHANAN MAHESHWARI
(1904-1966)

EDWARD C. COCKING
(Born 1931)

ERHARD KRANZ
(Born 1947)

In 1964, the Delhi school made another major discovery when Guha and Maheshwari demonstrated that in anther cultures of *Datura innoxia* the microspores (immature pollen) could be induced to form sporophytes (androgenesis). Bourgin and Nitsch (1967) confirmed the totipotency of pollen grains, and Nitsch and Norreel (1973) succeeded in raising haploid plants from isolated microspore cultures of *Datura innoxia.* Production of androgenic haploids by anther or microspore culture, now reported in several crop plants, has become an important adjunct to plant breeding tools and is being widely used by plant breeders (Chap. 8). Androgenesis also provides a unique opportunity to screen gametophytic variation at the sporophytic level. For some plants, where androgenesis is difficult or not possible, haploids can be obtained by culturing unfertilized ovules or ovaries (Chap. 9). San Noeum (1976) published the first report of gynogenic haploid formation in unfertilized ovary cultures of barley.

SIPRA GUHA-MUKHERJEE
(1938-2007)

SATISH C. MAHESHWARI
(Born 1933)

In 1965, Johri and Bhojwani reported for the first time differentiation of triploid shoots from the cultured mature endosperm of *Exocarpus cupressiformis*. It provides a direct, single step approach to produce triploid plants.

Regeneration of plants from carrot cells frozen at the temperature of liquid nitrogen (−196 °C) was first reported by Nag and Street in 1973. Seibert (1976) demonstrated that even shoot tips of carnation survived exposure to the super-low temperature of liquid nitrogen. This and subsequent successes with freeze preservation of cells, shoot tips and embryos gave birth to a new applied area of plant tissue culture, called in vitro conservation of germplasm. Cultured shoots could also be stored at 4 °C for 1–3 years. These methods are being used at several laboratories to establish in vitro repository of valuable germplasm.

The Pfizer Company made the first attempt for in vitro production of secondary metabolites on industrial scale during 1950–1960 for which Routin and Nickell (1956) obtained the first patent. Tulecke and Nickell (1956) first reported large-scale culture of plant cells in a 134 L bioreactor. Shikonin from cell cultures of *Lithospermum erythrorhizon* was the first in vitro produced phytochemical to be commercialized in 1983 by Mitsui Petrochemical Co., Japan (Curtin 1983). The other industrial compounds under commercial production through tissue culture are taxol and ginseng.

For long the variations observed in ploidy, morphology, pigmentation, and growth rates of cultured cells were ignored as mere abnormalities. Heinz and Mee (1971) published the first report of morphological variation in sugarcane hybrids regenerated from cell cultures. The agronomic importance of such variability was immediately recognized and the regenerants were screened for useful variations. During the next few years, *Saccharum* clones with resistance to various fungal and viral diseases as well as variation in yield, growth habit and sugar content were isolated (Krishnamurthi and Tlaskal 1974; Heinz et al. 1977). Larkin and Scowcroft (1981) reviewed the literature on

spontaneous in vitro occurring variation suitable for crop improvement, and termed the variation in the regenerants from somatic tissue cultures as somaclonal variation. Evans et al. (1984) introduced the term gametoclones for the plants regenerated from gametic cells. Several somaclones (Chap. 12) and gametoclones (Chap. 8) have already been released as new improved cultivars.

Based on his extensive studies on crown gall tissue culture, Braun (1947) suggested that probably during infection the bacterium introduces a tumor-inducing principle into the plant genome. Subsequently, Chilton et al. (1977) demonstrated that the crown galls were actually produced as a result of transfer and integration of genes from the bacteria *Agrobacterium tumefaciens* into the plant genome, which led to the use of this bacterium as a gene transfer system in plants.

ARMIN C. BRAUN
(1911-1986)

MARY-DELL CHILTON
(Born 1939)

The first transgenic tobacco plants expressing engineered foreign genes were produced by Horsch et al. (1984) with the aid of *A. tumefaciens*. Since 1988, biolistic gun, also called particle gun, has become a popular means to deliver purified genes into plant cells (see McCabe and Christou 1993). In 1986, Abel et al. produced the first genetically engineered plants for a useful agronomic trait. The list of genetically engineered varieties with useful traits has considerably enlarged, and since 1993 several transgenic varieties of crop plants, such as canola, cotton, maize, rice, tomato, and soybean, have been released. In 1996, nearly 5 million acres of biotech crops were sown, mainly in the USA and by 2007 these figures rose to 282 million acres in 23 countries (Vasil 2008). Efforts are now being made to genetically modify plants in such a way so as to utilize them as factories for producing desired biomolecules in large quantities (Chap. 15).

These, in brief, are some of the milestones in the history of plant tissue culture. Like any other area of science, plant tissue culture started as an academic exercise to answer some basic questions related to plant growth and development. However, over the years it has emerged as a tool of immense practical value. Plant tissue culture is being extensively used for clonal plant propagation, germplasm storage, production, and maintenance of disease-free plants and as a valuable adjunct to the conventional methods of plant improvement. Plant tissue culture techniques are also being extensively used in basic studies related to plant growth and development, cytodifferentiation, physiology, biochemistry, genetics, and pathology.

Plant tissue culture in India was started way back in 1957 at the Department of Botany, University of Delhi, India. Soon active centers of plant tissue culture were established at the Bose Institute, Kolkata, M.S. University, Vadodra, National Botanical Research Institute, Lucknow, and National Chemical Laboratory, Pune. The creation of the Department of Biotechnology (DBT) by the Government of India in 1986 gave a substantial boost to plant tissue culture research in this country. Many new tissue culture laboratories appeared in several traditional and agricultural universities and institutes across the country. DBT supported the establishment of plant tissue culture pilot plants at

Tata Energy Research Institute, New Delhi and National Chemical Laboratory, Pune in 1989, National Research Centre for Plant Biotechnology at IARI, New Delhi, in 1985, National Facility for Plant Tissue Culture Repository at National Bureau of plant Genetic Resources (NBPGR), New Delhi in 1986 and National Gene Banks of Medicinal and Aromatic Plants at NBPGR, New Delhi, Central Institute of Medicinal and Aromatic Plants, Lucknow, Tropical Botanic Garden and Research Institute, Thiruvananthapuram, and Regional Research Laboratories, Jammu in 1993.

In 1970, International Association of Plant Tissue Culture (IAPTC) was established to promote research and development in this area, and in 1971 it started publishing "IAPTC Newsletter" with one or two feature articles on a current topic, forthcoming events related to PTC, list of recent publications and highlights of major developments in the area. The association organizes international conferences once in 4 years in different parts of the globe. The association was renamed in 1998 as "International Association of Plant Tissue Culture and Biotechnology" and again in 2006 as "International Association of Plant Biotechnology". Similarly, the Newsletter of IAPTC was renamed in 1995 as "Journal of Plant Tissue Culture & Biotechnology". Now it is published as a part of the journal "In Vitro Cellular and Developmental Biology – Plant". For more detailed history of plant tissue culture see White (1943), Krikorian and Berquam (1969), Gautheret (1985), Bhojwani and Razdan (1996), Thorpe (2007) and Vasil (2008).

1.1 Landmarks/Milestones

1. 1902—Haberlandt presented the classic paper describing his pioneering attempt to culture isolated plant cells in a simple nutrient solution at a meeting of the Vienna Academy of Sciences in Germany.
2. 1904—Hannig initiated the work on excised embryo culture of several Crucifers.
3. 1922—Knudson demonstrated asymbiotic in vitro germination of orchid seeds.
4. 1925, 1929—Laibach demonstrated the practical application of embryo culture to produce interspecific hybrids between sexually incompatible parents (*Linum perenne* x *L. austriacum*).
5. 1934—White established continuously growing cultures of tomato root tips.
6. 1937—White formulated the first synthetic plant tissue culture medium (WM).
7. 1939—Gautheret, Nobécourt and White, independently, established continuously growing tissue cultures.
8. 1941—Van Overbeek introduced coconut water as a medium constituent by demonstrating its beneficial effect on in vitro development of immature embryos and callus formation in *Datura*.
9. 1946—Ball succeeded in raising whole plants from excised shoot tips of *Lupinus* and *Tropaeolum.*
10. 1947—Braun proposed the concept of tumor inducing principal (TiP) of *Agrobacterium tumefaciens* responsible for autonomous growth of crown gall tissue.
11. 1950—Braun demonstrated that Ti principal in *Agrobacterium tumefaciens* is transferred to plant genome naturally.
12. 1952—Morel & Martin developed the technique of meristem culture of *Dahlia* to raise virus-free plants from infected individuals.
13. 1954—Muir et al. succeeded in inducing divisions in mechanically isolated single cells cultured in the presence of a nurse tissue.
14. 1955—Miller et al. discovered the first cytokinin (kinetin) from autoclaved herring sperm DNA.
15. 1957—Skoog and Miller put forth the concept of chemical control of organogenesis (root and shoot differentiation) by manipulating the relative concentrations of auxin and kinetin.
16. 1958—Steward (USA) and Reinert (Germany), independently, reported the formation of embryos by the somatic cells of carrot (somatic embryogenesis).

17. 1960—Jones et al. successfully cultured isolated single cells using conditioned medium in microchamber.
18. 1960—Bergmann developed the cell plating technique for the culture of isolated single cells.
19. 1960—Morel described a method for rapid in vitro clonal propagation of orchids (micropropagation).
20. 1960—Cocking isolated plant protoplasts enzymatically.
21. 1962—Kanta et al. developed the technique of in vitro pollination; viable seed formation by in vitro pollination of naked ovules.
22. 1962—Murashige & Skoog formulated the most widely used plant tissue culture medium (MS).
23. 1964—Guha and Maheshwari produced the first androgenic haploid plants of *Datura* by anther culture.
24. 1965—Johri and Bhojwani demonstrated the totipotency of triploid endosperm cells.
25. 1965—Vasil and Hildebrand achieved regeneration of full plants starting from isolated single cells of tobacco.
26. 1966—Kohlenbach succeeded in inducing divisions in isolated mature mesophyll cells of *Macleaya cordata* which later differentiated somatic embryos.
27. 1970—Power et al. published the first report of chemical fusion of plant protoplast.
28. 1970—Establishment of International Association of Plant Tissue Culture (IAPTC).
29. 1971—Heinz and Mee reported somaclonal variation in the regenerants from callus cultures of sugarcane.
30. 1971—Takebe et al. achieved plant regeneration from isolated protoplasts of tobacco.
31. 1971—Newsletter of IAPTC launched.
32. 1972—Carlson et al. produced the first somatic hybrids by the fusion of isolated protoplasts of *Nicotiana glauca* and *N. langsdorffii*.
33. 1973—Nitsch and Norreel succeeded in producing haploid plants from isolated microspore cultures of tobacco.
34. 1973—Nag and Street succeeded in regeneration of plants from carrot cells frozen in liquid nitrogen (−196 °C).
35. 1974—Zaenen et al. identified *Ti* plasmid as the causative factor of *Agrobacterium tumefaciens* for crown gall formation.
36. 1974—Kao et al. and Walin et al. introduced PEG as a versatile chemical for the fusion of plant protoplasts.
37. 1974—Reinhard reported biotransformation by plant tissue cultures.
38. 1976—Seibert reported regeneration of shoots from cryopreserved shoot.
39. 1976—San Noeum reported the development of gynogenic haploids from the cultured unfertilized ovaries of barley.
40. 1977—Chilton et al. demonstrated that only a part of the *Ti* plasmid of *A. tumefaciens* is responsible for crown gall formation.
41. 1984—Horsch et al. produced the first transgenic plants of tobacco by co-culture of leaf discs with *Agrobacterium tumefaciens.*
42. 1986—Abel et al. produced the first transgenic plants with useful agronomic traits.
43. 1987—Sanford et al. invented the biolistic method of direct gene transfer into plant cells.
44. 1987—Fujita and Tabata developed commercial process for the production of shikonin by cell cultures of *Lithospermum erythrorhizon*.
45. 1993—Kranz et al. reported regeneration of full plants from in vitro fertilized eggs of maize (In Vitro Fertilization).
46. 1994—Holm et al. succeeded in raising full plants from excised in situ fertilized eggs (zygotes) of barley.
47. 1995-To date; the existing in vitro techniques were refined to enhance their efficiency and were applied to increasing number of plant species with different objectives.
48. 1995—IAPTC Newsletter developed into Journal of Plant Tissue Culture and Biotechnology.
49. 1998—IAPTC renamed as International Association of Plant Tissue Culture and Biotechnology (IAPTC & B).

50. 2006—IAPTC & B renamed as International Association of Plant Biotechnology (IAPB).

Suggested Further Reading

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General Requirements and Techniques

2

2.1 Introduction

A plant tissue culture laboratory, whether for research or for commercial purpose, should provide certain basic facilities, such as (i) washing and storage of glassware, plasticware and other labwares, (ii) preparation, sterilization and storage of nutrient media, (iii) aseptic manipulation of plant material, (iv) maintenance of cultures under controlled conditions of temperature, light and humidity, (v) observation of cultures and (vi) hardening of in vitro developed plants. The extent of sophistication in terms of equipment and facilities depends on the need and the funds available. Therefore, establishment of a new tissue culture facility requiring ingenuity and careful planning.

2.2 Requirements

2.2.1 Structure and Utilities

The construction of a laboratory from scratch is a costly affair but there is considerable scope for maneuverability with the design at the conceptional stage and in the selection of construction material. To begin with, a commercial laboratory is best housed in a pre-existing building with suitable modifications. After carefully examining the economic feasibility of the venture an independent facility may be erected. More often than not, for research work the tissue culture laboratory is carved out of the existing infrastructure, and several facilities/equipments are shared with other laboratories. A research facility should have at least four rooms: (i) *Washing Room,* for glassware washing, storage and autoclaving (ii) *Media Room*, for media preparation (iii) *Sterile Area*, for aseptic manipulation and (iv) G*rowth/Culture Room*, to maintain cultures under suitable environmental conditions. The culture room should also have a working table, a stereoscopic microscope and a good light source, preferably cool light (fiber optics), for observing cultures. The sterile transfer cabinets could be placed in the culture room or in a specially designed transfer room. In many research laboratories it is kept in an undisturbed area of a general lab.

In case the facility needs to be constructed, especially for a commercial setup, it would be desirable to locate the unit away from the city to avoid heavy pollution and vehicular vibrations. However, this may require transportation of the personnel. The location of the laboratory should not be near fields to avoid spurts of infection by the combines and threshers during the harvest seasons. The facility needs to be adequately protected from rains and winds as these carry spores, mites and thrips. Thermal insulation of the facility to conserve energy is another aspect requiring proper thought. One way is to have the transfer area and the growth rooms below ground level. In that case care must be taken to protect the lab from seepage and provide adequate ventilation. Alternately, these two rooms could have a double wall or built of hollow

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bricks with air trapped in between, which could be vented during summers.

A tissue culture facility requires large quantities of good quality water. At the designing stage itself adequate attention should be paid to the source of water and waste-water disposal, especially where sewer facilities are not available, keeping in view the local municipal rules for health and environment.

A tissue culture unit must have power backup to save cultures from getting contaminated in the event of power failure or load-shedding from the mains during aseptic manipulations. Valuable cultures may be lost because of temperature shocks in the growth room during electricity breakdowns/shutdown. The generator may be fitted with a self-starting switch.

It is of paramount importance that a tissue culture laboratory is clean and movement of materials from one area to another occurs with minimal backtracking. These aspects should be the guiding principle while designing the layout plan of various rooms, pass-through windows, doors and hallways. It is necessary and desirable to isolate the 'clean area', comprising of transfer room and growth room from rest of the 'unclean area' and it should be treated as 'restricted area', out of bounds for visitors and outsiders. In the passage between these two areas, especially in a commercial set-up, one is required to wash hands and feet and wear sterilized overcoats and headgear before entering the 'restricted area'. Generally, high standards of sanitation need to be maintained and these have to be more stringent where dust, pollen and small insects abound in the environment. It is a good idea to have paved pathways and shrubs around. High levels of cleanliness and freedom from extraneous materials could be achieved by having positive air pressure, at least in the 'clean area'. Depending on the necessity, a Class 1,000 or Class 10,000 standard should be maintained for the clean room. For the movement of material in (sterilized medium, instruments, water, etc.) and out of (glassware, old and infected cultures, tissue culture produced plants for hardening, etc.) the 'clean area' a window with double door hatch should be provided to maintain high asepsis in the 'clean area'.

As far as possible indigenously available construction material, equipment, apparatus, and instruments should be used for cost effectiveness and ease of maintenance. Innovativeness and indigenous fabrication will go a long way in reducing the costs.

2.2.2 Washing Room

Depending on the availability of funds and space the washing and sterilization areas may be in separate rooms or in a common room. In either case, the washing area should have adequate supply of good quality hot and cold running water and an acid and alkali resistant big sink. Adequate steel or plastic buckets and tubs are required for soaking culture vials and other labwares used in medium preparation. Brushes of various sizes and shapes are essential for cleaning glassware, while it is optional to have a dish washing machine. For a commercial set-up an industrial dish washer is desirable. The media room should have a hot air cabinet to dry the washed labware, an oven for dry sterilization, and a dust proof cupboard for storage of plastic and glass-ware. When washing is done in media room a temporary partition can be erected between the two areas to prevent splashing of soap solution and any other interference in the two activities. Alternately, timings of the two activities could be staggered. Where the autoclave is to be housed within the media room an isolated area with provision for ventilation through an exhaust should be chosen.

Even if good quality water is available it cannot be used for final washing of labwares or for medium preparation as it contains impurities such as inorganic and organic compounds, dissolved gases, particulate debris and microorganisms. Water could be purified through distillation, deionization or reverse osmosis. Sometimes a combination of two or more is required. Water purity is measured in terms of resistivity (ohms cm^{-1}) or its reciprocal, i.e.,

conductivity (mhos cm^{-1}). Water for tissue culture should ideally have a conductivity of 5.0 μmhos cm^{-1} although a conductivity level up to 15 μmhos cm^{-1} is acceptable. Deionized water may be used for teaching laboratories or for rinsing labware but for research and commercial purposes, water distillation apparatus, a reverse osmosis unit or a Mili-Q purification system needs to be installed. The choice between the three is one of quality of final water, speed of production and cost. For a research laboratory a glass distillation unit with a handling capacity of 1.5 to 2 L h^{-1} of water should be sufficient. For a commercial set-up or where high purity water is required a Mili-Q purification system that can provide 90 L h^{-1} may be used. Proper storage tanks should be provided for the purified water.

2.2.3 Media Room

The media room is the kitchen of the tissue culture facility. The media room is provided with a working table in the centre and benches along the wall, the tops of which are either covered with granite or laminated board (Fig. 2.1). The tables and benches should be at a height suitable for working while standing and the space below them could be fitted with drawers and cupboards for storage purposes. The benches are required for keeping balances, pH meter, magnetic stirrers, hot plates etc. A top loading electronic balance with tare for weighing large quantities and an analytical balance for small quantities of chemicals must be provided. The balances should be isolated in a small chamber if the media room also houses the autoclave. In a large commercial laboratory it will be of help to have an automatic media dispenser.

For short term storage of certain chemicals, plant materials, and stock solutions a refrigerator and a deep freeze are required. These could be kept in the corridor if sufficient space in the room is not available. A single electrode pH meter that can read conductivity also should be provided. For filter sterilization of medium or solutions of thermolabile compounds an aspirator or vacuum pump may be required. For steam sterilization an autoclave or a domestic pressure cooker, depending on the quantity to be sterilized, is needed.

For emergencies a fire extinguisher and a first aid kit should be kept in this room.

Fig. 2.1 Media preparation

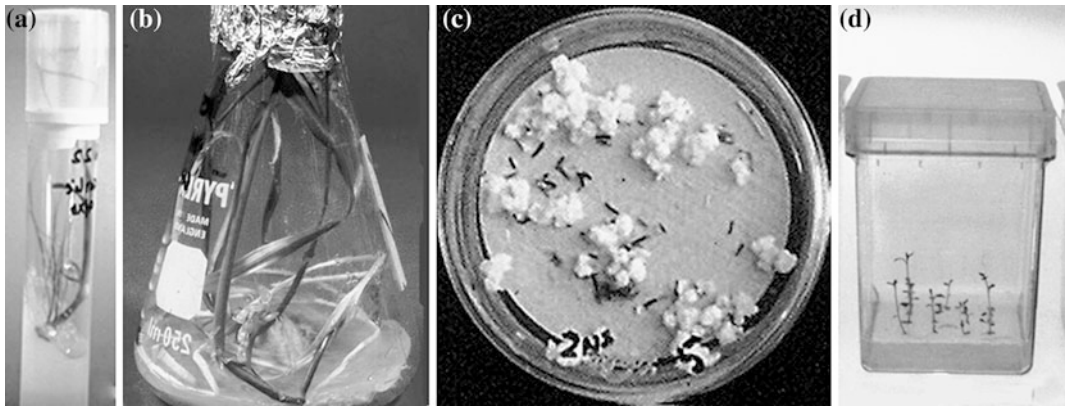

Fig. 2.2 Culture vials. **a** Culture tube with polypropylene cap. **b** Flask. **c** Petri plate. **d** Plastic jar (*Magenta box*)

2.2.4 Glassware/Plasticware

In a tissue culture laboratory culture vessels (Fig. 2.2) are required in bulk. Depending on the type of work, adequate supplies of these should be maintained. For standard tissue culture work rimless test tubes (25 × 150 mm) are widely used (Fig. 2.2a). The culture tubes are important for culture initiation and establishment even in a commercial set-up. For further mass multiplication larger containers such as jam bottles or other wide mouthed bottles are required. Erlenmeyer flasks have also been used as culture vessels (Fig. 2.2b). Only borosilicate or Pyrex glassware should be used.

Plastic culture vials, autoclavable and presterilized, have replaced glass culture vials to a large extent. A wide range of presterilized, disposable culture vials made of clear plastic, especially designed for protoplast, cell, tissue and organ culture, are available in the market under different brand names. The presterilized plastic Petri dishes (Fig. 2.2c), jars (Fig. 2.2d), screw cap bottles, and various cell culture plates come with their closures. For culture tubes and flasks, traditionally, non-absorbent cotton plugs wrapped in a single layer of cheesecloth have been used as a closure. Autoclavable, transparent polypropylene caps with a membrane built into the top are also available (KimKaps, Kimble, Division of Owens, IL). Cotton plugs provide excellent aeration but the medium dehydrates very fast. On the other hand, polypropylene caps reduce the rate of medium desiccation but increase moisture and gaseous accumulation within the container. However, it is important to ensure that the closure allows proper aseptic aeration and does not inhibit the growth of culture materials. In this regard it may be mentioned that Parafilm/Nescofilm, commonly used to seal Petri dishes, releases butylated hydroxytoluene, which is toxic to the cultured plant material (Selby et al. 1996). Alternately, one can use cling film for sealing, as 2-ethyl-1-hexanol released by it is not inhibitory to culture material.

Now it is possible to buy culture vessels made of different synthetic materials. Culture vessels made of polypropylene transmit 65 % light and those made of polycarbonate transmit almost 100 % light. Gas permeable fluorocarbonate vessels are available for use with plant materials that are sensitive to gas build up within the culture vials (Kozai 1991).

Besides culture vials, various other glass-/plasticware such as beakers, measuring cylinders, pipettes, etc. of various sizes are required for media preparation.

2.2.5 Transfer Room

In research laboratories the transfer hoods are placed in the growth/culture room or even in a quite corner of a general laboratory. However, in a commercial facility it is necessary to have separate transfer and growth room(s). There are