1 Introductory History

Development of the science of tissue culture is historically linked to the discovery of the cell and subsequent propounding of the cell theory. More than 260 years ago, Henri-Louis Duhamel du Monccau's (1756) pioneering experiments on wound healing in plants demonstrated spontaneous' callus formation on the decorticated region of elm plants. His studies, according to noted biologist Gautheret (1985), could be considered a 'foreword' for the discovery of plant tissue culture. Further contributions to plant tissue culture could be attributed to the cell doctrine, which implicitly admitted that a cell is capable of autonomy and even demonstrated the potential for totipotency. The development of the multicellular or multiorganed body of a higher organism from a single-celled zygote supports the totipotent behaviour of a cell. Germination of spores into complete individuals is an obvious feature of cells in haplobiontic plants; similarly, epidermal cells of *Begonia* are transformed into new begonia plants. In the animal kingdom, a cell from any part of a *Hydra* may give rise to a new individual. Although these aspects of cellular behaviour point to the totipotent nature of some plant or animal cells, many somatic cells do not produce complete organisms since they form multicellular tissues or organs and are highly differentiated. They are mostly irreversible and lose the meristematic activity. One needs, therefore, to understand more about the interrelationships between the various cells of a tissue and the various organs of an organism.

There must be some factor(s) superimposed on theoretically genetically identical cells which brings about certain cellular or subcellular changes leading to morphological differentiation. Trécul (1853) observed callus formation in a number of decorticated trees. He published excellent pictures of callus sections and established that apart from the cambium, the medullary rays, phloem, and youngest xylem elements also contribute as raw materials in the development of tissue culture. An interesting observation made by Vöchting (1878) suggested 'polarity' as a characteristic feature guiding the development of plant fragments. In his classical experiments on stem cuttings, Vöchting observed that the upper portion of a piece of stem always produced buds and the basal region callus or roots. Further, the grafting experiments which he undertook among species of *Opuntia, Salix, Beta* and other trees, demonstrated that behaviour of a tissue is not altered by contact with other tissue because the dependence of morphogenetic capacity on hereditary internal factors is very strict. Wiesner (1884) proposed a general theory that suggested the existence of organ-forming substances distributed in a polar fashion. Minimal size of explants could be another factor

that determined the potential for differentiation. Small pieces of explants isolated from buds, roots and stems were placed by Rechinger (1893) on a surface of moistened sand. Pieces thicker than 20 mm were able to produce buds or even plants whereas smaller explants (with vascular elements) proliferated without organisation and those less than 1.5 mm (representing no more than 21 cell layers) failed to grow.

These experiments were really outlines that ultimately paved the way for studying the role of *in vitro* cultures for understanding the theoretical and practical aspects of plant tissue culture.

1.1 Concept of Cell Culture

German botanist Gottlieb Haberlandt (1902) developed the concept of *in vitro* cell culture. He was the first to culture isolated, fully differentiated cells in a nutrient medium containing glucose, peptone, and Knop's salt solution. Using palisade cells of *Lamium purpureum,* pith cells from petioles of *Eicchornia crassipes,* glandular hair of *Pulmonaria* and *Utrica,* stamen hair cells of *Tradescantia,* stomatal guard cells of *Ornithogalum,* and other plant materials, Haberlandt realised that asepsis was necessary when culture media are enriched with organic substances metabolised by micro-organisms. In his cultures, free from microcontamination, cells that were able to synthesise starch as well as increase in size survived for several weeks. However, Haberlandt failed in his goal to induce these cells to divide. Despite drawbacks, this plant physiologist made several predictions about the requirements for cell division under experimental conditions in 1902, which have been confirmed with the passage of time. Haberlandt is thus regarded as the father of tissue culture.

1.2 Development of Tissue Culture

Many plant physiologists have tried to obtain multiplication of isolated cells but failed. Hannig (1904) initiated a new line of investigation involving the culture of embryogenic tissue, which later became an important applied area of investigation, using *in vitro* techniques. He excised nearly mature embryos of some crucifers *(Raphanus sativus, R. landra, R. caudatus,* and *Cochlearia donica)* and successfully grew them to maturity on mineral salts and sugar solution. Winkler (1908) cultivated segments of string bean and observed some cell divisions but no proliferation. More promising results were obtained in the same year by Simon, who achieved success in the regeneration of a bulky callus, buds and roots from poplar stem segments. In effect, Simon may be credited with having established the basis for callus culture and to some extent even micropropagation.

1.2.1 Root-tip Culture

A new approach to tissue culture was conceived simultaneously in 1922 by Kotte (Germany) and Robbins (USA). They postulated that a true *in vitro* culture could be made easier by using meristematic cells, such as those that operate in the root tip or bud. Small excised root tips of pea and maize cultivated by Kotte in a variety of nutrients containing salts of Knop's solution, glucose and several nitrogen compounds (such as asparagine, alanine, and yeast extract) grew successfully for two weeks. Robbins, working independently, maintained his maize root tip cultures for a longer period by subculturing them, but the growth gradually diminished and the cultures were ultimately lost. An important breakthrough for continuously growing root tip cultures came from White (1934, 1937), who initially used yeast extract in a medium containing inorganic salts and sucrose but later replaced yeast extract by three B vitamins, namely, pyridoxine, thiamine and nicotinic acid. White's synthetic medium later proved to be one of the basic media for a variety of cell and tissue cultures.

The experiments on root cultures raised an interesting question on the chemical nature of yeast extract. Bonner (1937) demonstrated the importance of thiamine in yeast extract; eventually it was found that thiamine could be replaced by its two components, thiazole and

pyrimidine. Many workers continued efforts to develop a complete medium for ensuring prolonged growth of root cultures. In this regard, mention may be made of the contributions by Bürstrom (1953), Street and associates (1951, 1952, 1954: see Gautheret (1985), who demonstrated the importance of copper, manganese, iodine and chelating agents on root metabolism, and Sheat et al. (1959) who obtained information on the effect of NH_4^+ and amino acids.

Although the root culture technique helped in solving many morphological, physiological and pathological problems, it was soon realised that cultures should lead to organogenesis in order to fulfil Haberlandt's objectives.

1.2.2 Embryo Culture

Very early in the history of tissue culture, Laibach (1925, 1929) demonstrated the practical applications of zygotic embryo culture in the field of plant breeding, although Hannig was the first to initiate work along these lines. Laibach raised zygotic embryos isolated from non-viable seeds of *Linum perenne* × *L. austriacum* to maturity on a culture medium and obtained hybrids which in natural course died out due to embryo abortion. Later, Van Overbeek et al. (1941) used coconut milk (embryo sac fluid) for embryo development and callus formation in *Datura*, which proved a turning point in the field of embryo culture. These studies gave impetus to further work in the area and to date several hybrids have been raised through embryo culture which could not otherwise be obtained due to embryo abortion. Further embryo culture has significant application in plant biotechnology and clonal propagation. Feeder cell and "double medium" culture methods in particular improve the survival of zygotes and proembryos thus proving as invaluable tools in embryo rescue method (Haslam and Yeung 2011).

1.2.3 Stem-tip Culture

Like embryo culture, stem-tip cultures yielded success in producing whole plants. Loo (1945) obtained excellent cultures from stem tips of dodder and *Asparagus*, while the following year an ingenious method was devised by Ball (1946) to identify the exact part of the shoot meristem that gives rise to a whole plant. Thus shoot-tip culture is now used extensively in plant propagation industries and production of disease free plants throughout the world (*see* Chapter 15).

1.3 Role of Auxin

Successes achieved in tissue culture would be regarded as incomplete without an understanding of the role of auxins since earlier attempts involving the culture of isolated cells, root tips, or stem tips ended in the development of calluses. There remained two goals to fulfil the predictions of Haberlandt. First, the callus obtained from explants had to be perpetuated for unlimited periods and, second, the regenerated calluses had to undergo organogenesis to form whole plants. Gautheret (1934) cultured cambium cells of some tree species (*Acer pseudoplatanus, Ulmus campestre, Robinia pseudoacacia,* and *Salix caprea*) on the surface of a medium (Knop's solution containing glucose and cysteine hydrochloride) solidified with agar. After two months he observed the proliferation of callus from these cells which, however, ceased six months later due to distinct nutrient deficiency. Meanwhile, Snow (1935) demonstrated that indoleacetic acid (IAA—a growth substance discovered by Went in 1926) and occurring naturally in plant tissues stimulated cambial activity, and Gaurheret, following these observations, found that the addition of this auxin enhanced the proliferation of his cambial cultures, making it possible to prepare subcultures. White (1939) reported similar results in the cultures from tumour tissues of the hybrid *Nicotiana glauca* × *N. langsdorffii* and Nobécourt (1939) established continuously growing cultures of carrot slices. Finally, the possibility of cultivating plant tissues for an unlimited period, using media enriched with auxins, was announced independently by Gautheret, White, and Nobécourt in 1939. In

plant tissue culture medium presence of auxin is now must for growth and differentiation.

1.4 Discovery of Cytokinin

Caplin and Steward (1948) reported for carrot explants that coconut milk enhanced more proliferation of callus than did auxin. In a medium enriched with this natural extract, Morel (1950) obtained indefinite growth with respect to monocotyledonous tissues of *Amorphophallus rivieri, Sauromatum guttatum,* gladiolus, iris and lily. Even the cultures of royal fern tissue evinced a similar response with coconut milk. Van Overbeek et al. (1941) had suggested earlier that liquid endosperm such as coconut milk would be a good medium for embryo culture. The fact that in coconut milk the proliferation of a tumoural type of callus was more enhanced than in auxin indicated that the milk contained a stimulating substance that was not auxin. This prompted researchers to ascertain the chemical nature of this non-auxinic substance found in plant tissues. Skoog (1944), Skoog and Tsui (1951) demonstrated that adenine stimulates cell division and induces bud formation in tobacco tissue even in the presence of IAA (which normally acts as a bud inhibitor). This convinced Skoog and collaborators that nucleic acids which contain substances such as adenine influence tissue proliferation. With the collaboration of Miller and associates, he undertook experiments with nucleic acids extracted from herring sperm, calf thymus and yeast. In 1955, Skoog and collaborators (Miller et al. 1955) finally isolated from autoclaved yeast extract, a derivative of adenine (6-furyl arninopurine), named *kinetin.* A substance with kinetin-like properties was also detected in young maize endosperm (Miller 1961), which was isolated by Letham (1963) and named *zeatin.* It was also verified that a similar substance called *ribosylzeatin* occurred in coconut milk (Letham 1974). Now many synthetic as well as natural compounds with kinetin-like activity are known which show bud-promoting properties. These substances are collectively called *cytokinins* and are used to induce divisions in cells of highly mature and differentiated tissues (such as mesophyll or endosperm from dried seeds) to induce shoot differentiation even in the presence of auxin in cultures. Topolins are naturally occurring aromatic cytokinins and becoming popular in tissue culture studies because of positive effects of organogenic differentiation (Adeyami et al. 2012).

1.5 Hormonal Control of Organ Formation

Skoog and Miller (1957) proposed the concept of hormonal control of organ formation. Their classic experiments on tobacco pith cultures showed that root and bud initiation were conditioned by a balance between auxin and kinetin. High concentration of auxin promoted rooting whereas proportionately more kinetin initiated bud or shoot formation. Unequal proportion of auxin and cytokinin led to unorganised growth of the tissue. The discoveries of gibberellin and abscisic acid relatively altered this concept as a multiplicity of factors are responsible for control of organ formation. The determination of organogenesis also depends upon the source of plant tissue, environmental factors, composition of media, polarity, growth substances and other factors and, therefore, may not be restricted to hormonal balance only. These aspects are discussed in Chapters 5 and 6.

1.6 Improvement of Media

The advances made in tissue culture technology are directly related to the mineral content and overall composition of the culture media. Initially, tissue culturists used Knop's mineral solution (Gautheret 1942, Nobécourt 1937) and White's medium supplemented with various trace elements. Gradually, other workers tried to develop media differing essentially in mineral content. For example, Murashige and Skoog (1962) proposed a solution for tobacco tissues with a concentration of salts 25 times higher than in Knop's solution. This medium allowed five to seven times more active growth than other media. Following this conclusive improvement,

other researchers suggested new media for very special purposes. Increase in NO_3 content was proposed to facilitate flower initiation (Margara et al. 1967), whereas Zn and B were considered important in the formation of whole plants. The various media generally used in tissue culture of plants are described in Chapter 3 and in some other chapters, where considering their subject of topic media with special nutrient requirements have been mentioned.

For large-scale cultures and germplasm cryopreservation alternatives to gelling agent agar, mostly used in media preparation, have been suggested for reduction of culture media costs (Saad and Elshahed 2012, Lalitha et al. 2014)

1.7 Preparation and Cloning of Single Cell Cultures

During the period 1934-1954, the main focus remained on the growth and development of callus cultures from explants under *in vitro* conditions. However, the principles of tissue culture envisaged by Haberlandt on the basis of cell theory and the related concept of totipotency were sidetracked. Two aspects of these principles required experimental verification: first, the multiplication of a single cell *in vitro* and, second, the development of a whole plant from proliferated tissue of this cell.

Sanford et al. (1948) initiated studies on single cell cultures by demonstrating divisions in animal cells using conditioned media (media in which tissue has been growing for sometime). The isolated cell was placed in a very thin glass tube and the tube plunged into a medium containing numerous living cells. Some unknown substance excreted by the surrounding cells in the medium influenced the isolated cell in the tube to divide. Muir (1953) demonstrated that on transfer of callus tissues of *Tagetes erecta* and *Nicotiana tabacum* to a liquid medium and subsequent agitation in a shaking machine it was possible to break down the callus tissue into single cells and small cell aggregates. The following year Muir and associates (1954) applied the 'conditioning principle' to induce divisions in isolated single cells of *Tagetes* and tobacco growing in shake (suspension) cultures. A single isolated cell was placed on a filter paper positioned on top of a callus mass. About 8% of these isolated single cells on the filter paper multiplied and formed colonies (Fig. 1.1). Earlier, Steward et al. (1952) had designed a shaking apparatus which allowed dissociation of tissues to form cell suspension cultures. In these cultures crowds of isolated single cells or cell clumps were formed which could further multiply while remaining suspended in the medium under constant shaking. With improvement in culture vessel designs like bioreactors cells from suspension cultures are cultivated on mass scale which happens to be important basic requisite for industrial production of secondary metabolites (Chapter 17). Plant regeneration in species which earlier did not respond to tissue cultures has also been possible using suspension cultures. Additionally cell suspensions have increasing role in protoplast isolation, somatic hybridization /cybridization and genetic transformation.

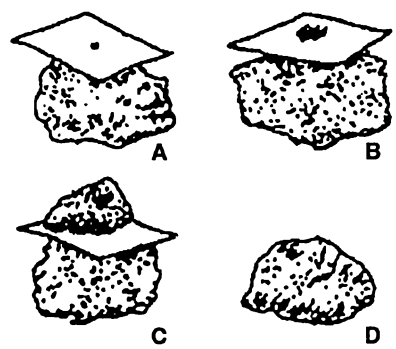

Fig. 1.1 First example of single-cell culture technique diagrammed from the work of Muir et at. (1954). A: Single cell placed on a small piece of filter paper positioned on top of a callus tissue; B: Isolated cell multiplies; C: Division of cells leads to the development of a callus tissue; D: Callus regenerated from single-cell culture.

The plating technique for cloning a large number of isolated single cells was developed by Bergmann in 1960. He filtered suspension

cultures of *Nicotiana tabacum* and *Phaseolus vulgaris* and obtained a population of nearly 90% free cells. This was followed by blending these free cells in a medium containing 0.6% agar, not completely cold, and finally plating in a petri dish where the medium solidified. In this experiment some of the single plated cells divided and formed visible colonies. This technique was later widely used, particularly for cloning isolated single protoplasts.

Jones et al. (1960) designed a microculture method using hanging drops of free cells in a conditioned medium. This facilitated continuous observation of cells growing in cultures. Using the hanging drop method, Vasil and Hildebrandt (1965) substituted fresh medium enriched with coconut milk and NAA (1-naphthaleneacetic acid) for 'conditioning' and observed divisions in isolated tobacco hybrid cells.

1.8 Regeneration of Single Cell to Whole Plant

The success achieved thus far in single cell cloning created great enthusiasm for research on the prospects of raising whole plants from a single cell. An important breakthrough was achieved in 1965 when Vasil and Hildebrandt observed that colonies arising from cloning of isolated cells of the hybrid *Nicotiana glutinosa* × *N. tabacum* regenerated plantlets (Fig. 1.2). Another aspect that helped in plant regeneration was the induction of somatic embryogenesis. Under suitable conditions tissues obtained from free cells in carrot suspension cultures (Steward et al. 1966) and isolated mesophyll cells of *Macleaya cordata* (Kohlenbach 1966) differentiated somatic embryos. These embryos on subsequent culture developed into normal plants. The phenomenon of somatic embryogenesis leading to plantlet formation in cultures was later reported in many species and its potential application in synseed technology presently is well recognized (Nower 2014, Siew et al. 2014, *see* also Chapter 7). All these discoveries contributed to the establishment of totipotency of somatic cells under experimental conditions, thereby accomplishing the goals set by Haberlandt.

1.9 Practical Applications and Recent Advances

Soon after working out the theoretical aspects of *in vitro* cultures, plant biologists made efforts to realise the practical applications of plant tissue culture technology. Success achieved has been spectacular since tissue culture techniques can now be gainfully employed to a range of plant groups, e.g., cereals and grasses, legumes, medicinals, vegetable crops, oil seeds, fruit crops, floriculture, clonal forestry, etc. (see Vasil and Thorpe 1994, Bhojwani and Razdan 1996, Smith 2000, 2013). From a survey of the literature, it is apparent that principal applications are based on advancements made in the areas of morphology, biochemistry, pathology and genetics and agricultural biotechnology.

1.9.1 Morphological Aspects

Tissue culture, to begin with, would be a good means for understanding the factors responsible for cell differentiation and organ formation. Based on histological observations of callus development, Trécul (1853) pointed out that establishment of a tissue culture involves the process of cell dedifferentiation. A differentiated cell is induced *in vitro* to divide, thereby providing a method for dedifferentiation. Buvat (1944, 1945) devised a scheme according to which dedifferentiation followed two steps: (1) regression to the cambial stage and (2) return to the cytological structure of primary meristematic cells. The first stage affects highly differentiated cells while the second is accompanied by the organisation of bud or root meristems.

Cell cultures provide valuable information on morphogenesis and plant development. Studies on molecular, physiological and biochemical aspects of cells in culture have contributed to an in-depth understanding of cytodifferentiation, organogenesis and somatic embryogenesis (Fukuda 1997, Thomson and Thorpe 1997, Zhou et al. 2000, Kondo et al. 2015).

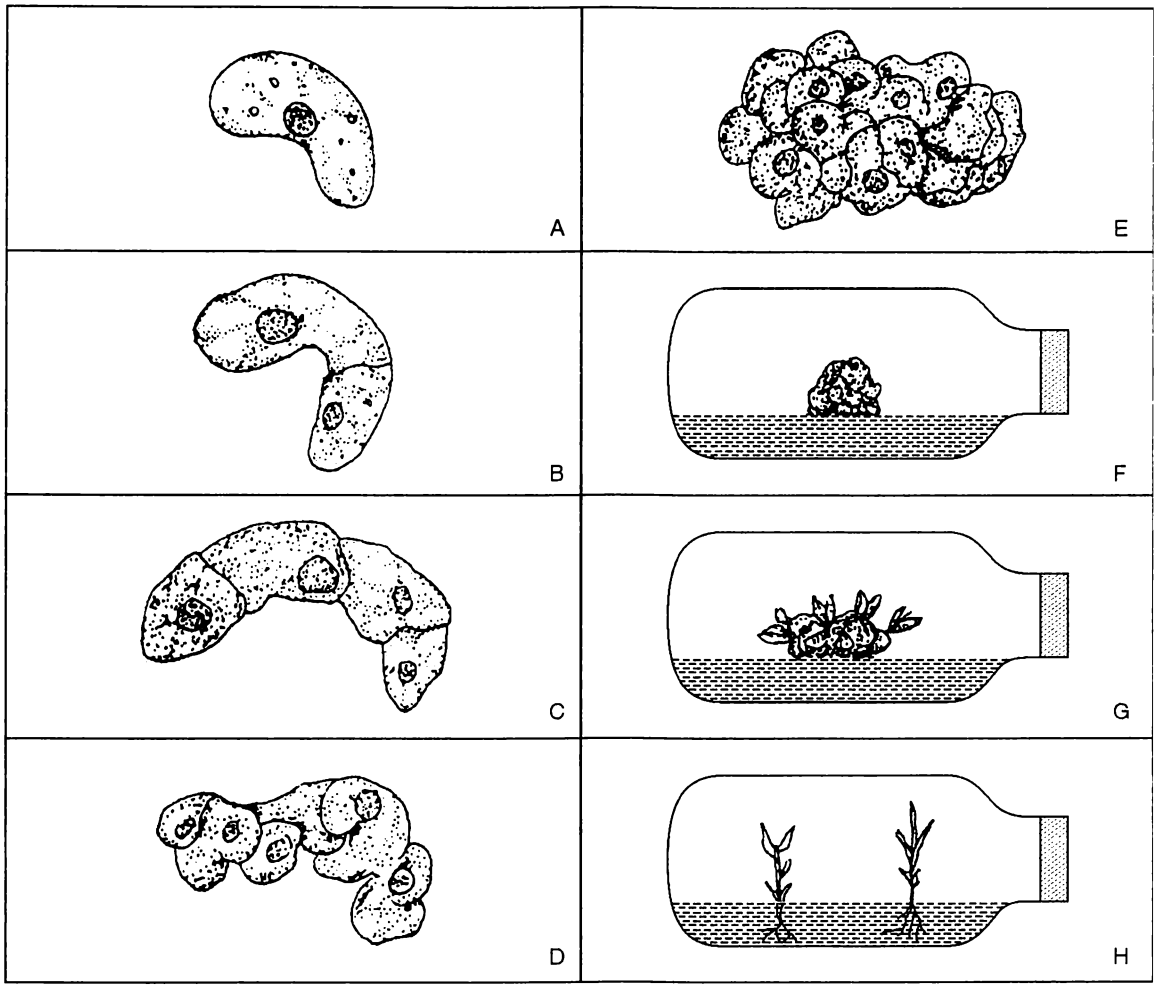

Fig 1.2 Regeneration of a single cell to whole plant. A: Isolated single cell cultured *in vitro;* B-E: Multiplication of this cell leading to the development of a callus; F: Subculture of the callus on a nutrient medium solidified with agar; G: Proliferation of shoots in the callus cultured on a medium containing a high amount of cytokinin; H: Shoots transferred onto a medium without growth substances to induce root formation; I: *In vitro* regenerated plant transplanted to soil inside a pot (diagrammed from the work of Vasil and Hildebrandt 1965).

Several theories have been proposed for the organ formation in tissue cultures. Of these, hormonal control of organogenesis, proposed by Skoog and Miller in 1957, is the most popular. This has already been described (Section 1.4). The regeneration of whole plants in tissue cultures may occur via shoot or root differentiation. Alternatively, the cells may undergo embryogenic development to give rise to bipolar embryos, also called *embryoids.*

Another important morphological application of plant tissue culture is micropropagation. Small amounts of tissue can be used to raise hundreds or thousands of plants in a continuous process. Ball (1946) successfully raised transplantable whole plants of *Lupinus* and *Tropaeolum* by culturing their shoot meristem (tip). The potential for this work was soon realised by Morel (1963) for rapid propagation of orchids *Cymbidium* and *Odontoglossum.* The advantage

of using this method was that about 4 million genetically identical plants could be obtained from a single bud. Paradoxically, orchid propagation of seeds showed greater variation in the progeny. Murashige (1978a,b) in USA developed standard methods of propagation *in vitro* of species ranging from ferns to foliage, flower and fruit plants, His name is intimately associated with this technique. The principle of the technique used for *in vitro* propagation is based on the proliferation and growth of numerous axillary buds which normally remain dormant in the presence of terminal bud due to apical dominance. Use of cytokinin exogenously in the culture medium releases buds from apical dominance both on initial shoot segment and subsequent lateral branches developed in shoot cultures. Thus a bushy-witch's broom like structure develops with numerous shoots which may be individually rooted to obtain plants. This new method of vegetative propagation is exploited intensively in horticulture and the nursery industry for rapid clonal propagation of many dicotyledons, monocotyledons and gymnosperms (*see* for details Chapter 16).

Attempts are also directed at development of somatic embryogenesis systems for mass propagation of plants. A rapid multiplication of somatic embryos is possible in automated bioreactors with low inputs. These embryos can be singly encapsulated in suitable chemical compound for use as 'synthetic seeds' (*see* Chapter 7). Different types of bioreactors have been tested and successfully used to scale-up embryogenesis, e.g., carrot, poinsettia (Redenbaugh 1993), or other plant cell and tissue cultures (Eibl and Eibl 2008).

1.9.2 Production of Secondary Metabolites

The industrial production of secondary metabolites using cell cultures was initiated during the period 1950-1960 by the Pfizer Company with the assistance of G.N. Nickell, a distinguished expert on plant tissue culture. These early attempts were disappointing and little progress was made on this aspect of applied plant tissue culture. From 1975, the fundamental aspects of the subject were explored carefully and intensively. Rapid progress in these investigations attracted many workers and important results were obtained with many species for engineering cells for secondary metabolite production (Verpoorte et al. 1998). The technology is now available to the industry; the commercial production of shikonin, ginseng saponins, berberidine, taxol, seiko saponins, and polysaccharides (from *Polyanthes tuberosus*) have been particularly encouraging (*see* Chapter 17).

1.9.3 Production of Pathogen-free Plants

Eradication of virus has been an outstanding contribution of tissue culture technology. The history of virus eradication began when White (1934) observed that virus-infected root subcultures frequently gave rise to cultures free from virus. In his opinion, the root meristem was deprived of virus and, therefore, it was possible to eliminate the pathogen in cultures provided the transferred part of the explant did not include a sufficient amount of the old tissue. Morel and Martin (1952, 1955) recovered virus-free *Dahlia* and potato plants from cultures obtained by cultivating the shoot meristem of infected plants. This showed that even in infected plants the cells of shoot tips are either free of virus or carry a negligible amount of the pathogen. This technique is economical and used very frequently. Meristem-tip culture is often coupled with thermotherapy or chemotherapy for virus eradication (*see* Chapter 15). Cryotherapy and electrotherapy are also emerging as potential techniques of virus elimination (Wang et al. 2009, Falah et al. 2009)

1.9.4 Germplasm Conservation

Plant germplasm is traditionally stored in the form of seeds, tubers, roots, bulbs, corms, rhizomes, buds, cuttings, etc. Field plots, nurseries and greenhouses are other sources of maintaining germplasm or gene banks. A number of difficulties are, however, encountered

while applying these modes of conservation. Important crop species produce recalcitrant seeds with early embryo degeneration (Reed and Chang 1997, Pritchard et al. 2000). Besides, the maintenance of field collections, nurseries or greenhouses are expensive with the further disadvantage of plants being vulnerable to insects, pathogens and various climatic hazards. About 2000-60,000 species of higher plants are believed endangered, rare and threatened with extinction (Bajaj 1995). According to FAO (2009) most of these are crop plants. Plant tissue culture is being developed as an effective means of germplasm conservation since a low maintenance *in vitro* germplasm storage collection provides a cost effective alternative to growing plants under field collections, nurseries, or greenhouses (Taylor 1997, Razdan and Cocking 1997, 2000; Engelmann 2012). Conservation of plant genetic resources *in vitro* is dealt comprehensively in Chapter 18.

1.9.5 Genetic Manipulations

The role of cell and tissue culture in plant genetic manipulations has been increasingly recognised. Heredity variations can be observed in cell colonies or plants regenerated *in vitro,* which may later express at the time of vegetative multiplication or sexual reproduction. The principal aspects of genetic manipulation are outlined below.

(I) Genetic Variability

Pioneering investigations (Gautheret 1955, Nobécourt 1955) on tissue cultures showed that cells in long-term cultures are genetically unstable. Many workers have confirmed that a tissue colony is a mosaic of different kinds of cells. Some of these, when cultivated separately, produce unorganised colonies while others regenerate plants frequently affected by morphological variations. Thus, tissue cultures are a direct source of genetic variability. Caryological investigations of cultured cells have revealed abnormal mitosis. The unstable chromosome numbers ultimately result in the variability of tissue cultures leading to somaclonal and gametoclonal variant selection (*see* Chapter 14) and commercial release of some crop varieties.

(II) *In Vitro* Pollination and Fertilisation

In the 1960s, the Botany School at the University of Delhi became actively engaged with the *in vitro* culture of reproductive organs of flowering plants. The initial success with intraovarian pollination led to the subsequent development of test-tube fertilization or *in vitro* Fertilization (IVF). This technique, developed by Kanta et al. (1962), involves culturing excised ovules and pollen grains in the same medium to make it easier for the germinating pollen to fertilise the ovule under *in vitro* conditions. Through the application of this technique the incompatibility barriers existing at the sexual level can be overcome, particularly those for which reaction occurs at the stigma or style. This method has been successfully employed to overcome self-incompatibility in *Petunia axillaris.* Similarly, interspecific *(Melandrium album* × M. *rubrum)* and intergeneric *(M. album* × *Silene schafta)* hybrids, hitherto unknown in nature, were obtained by Zenkteler and co-workers in 1975. Obtention of germinable embryos from 'naked zygote' developed *in vitro* by fusion of male and female gametes (Kranz and Lörz 1993) has been a landmark of this technique. IVF system for rice using electrofusion is also reported to be successful (Okamoto 2011).

(III) Induction of Haploidy

Another major contribution by the Botany School of Delhi University that attracted worldwide attention is the application of tissue culture to synthesise haploid plants. Normally, somatic cells of higher plants have a diploid chromosome number while reproductive cells (gametes) are haploid. In 1966, Guha and Maheswari cultured immature anthers of *Datura innoxia* (Solanaceae family) and were able to raise embryoids and

plantlets. These plants turned out to be haploid; apparently they had been released from microspores within the anthers. This opened the field of androgenesis. In the following year Bourgin and Nitsch (1967) confirmed the totipotency of pollen grains by raising full haploid plants of tobacco, rice and wheat. Haploids by anther culture are now reported to have been raised in 250 species belonging to 40 families (Mishra and Goswami 2014). Experimental evidence points out that the pollen populations are basically dimorphic and only 'smaller' pollen are capable of forming haploids. These pollen occur in low frequency and appear to be different from the majority destined to form gametes (Rashid 1983, Cho and Zapata 1990).

Induction of haploid plants from unpollinated ovaries and ovules (gynogenesis) is another subsequent advance in plant tissue culture and experimental embryology. San Noeum (1976) reported her first result on *in vitro* culture of ovary isolated from *Hordeum vulgare* and, thereafter, many other workers have obtained gynogenic haploid plants from cultured ovaries and ovules of tobacco, wheat, rice, lilium, maize, mulberry and other plants (*see* Chapter 8). This demonstrates that not only the microspore, but also the megaspore or female gametophyte of angiosperms can be triggered *in vitro* to sporophytic development, thus making way for an alternative approach to haploid plant breeding. It may be noted, however, that in some cases anther culture has given rise to diploid, polyploid and aneuploid plants. Due to these erratic and sporadic results, this technique cannot supplant the conventional methods of plant breeding.

(IV) Somatic Hybridisation

Isolation, regeneration and fusion of plant protoplasts *in vitro* is another area with potential for genetic manipulation. Somatic hybridisation, an asexual hybridisation procedure using isolated somatic protoplasts (wall-less cells), is now being developed as a new tool in plant breeding. Very early, Klercker (1892) isolated protoplasts and the first fusion was achieved in 1909 by Küster, a noted cytologist, who established that some salt solutions facilitated this process. This gave an indication that hybridisation by fusion of protoplasts could be a distinct possibility in future. Products of fusion of two protoplasts (heterokaryon) could be cultured to regenerate a somatic hybrid plant of desired genotype. New genetic variations might also be introduced as a consequence of cytoplasmic interactions during the process of fusion in protoplasts. The tempo of somatic hybridisation as a technique in higher plants accelerated after the pioneering work of Professor Edward C. Cocking (1960) in the UK, who demonstrated the feasibility of isolating a large number of protoplasts by incubating a small amount of tissue with the enzyme cellulase. Soon divisions were reported in isolated protoplasts leading to regeneration of plants (Nagata and Takebe 1971). Having established totipotency in plant protoplasts, the next endeavour was to fuse the regenerating protoplasts *in vitro* to produce hybrid plants. Carlson et al. (1972) obtained the first somatic hybrid by fusion of protoplasts isolated from *Nicotiana glauca* with *N. langsdorffii*. This attracted many people to this field of research and attempts were made to obtain somatic hybrid plants from species between which hybrids could not be produced by means of sexual crosses. Some success has been achieved in obtaining somatic hybrid plants between sexually compatible and incompatible species; the relative merits of this technique are discussed in Chapter 12. However, the role of cybridisation in bringing about subtle genetic variations by partial genome transfer in plants has been increasingly recognised.

(V) Genetic Transformation

The current importance of genetic transformation of cells by uptake of exogenous DNA has generated enormous interest in harnessing

the advantages offered by plant tissue and cell culture technology. This consists of four steps: insertion, integration, expression and replication of foreign DNA inside the host cell. The principle of transformation is based on the mechanism of infection by viruses, but cell function is so greatly disturbed by this procedure that the host dies subsequently. Since 1976, several researchers have reported that various eukaryotic genes introduced in bacteria are able to express their specific activity in the host and that it may be possible to obtain a similar transformation of eukaryotic cells. In nature, *Agrobacterium tumefaciens* can transfer genetic information to plant cells in the form of a plasmid that promotes tumoural formation (crown gall). Using recombinant DNA technology it was possible to introduce the gene for kanamycin resistance to the plasmid DNA of *A. tumefaciens* and the modified plasmid was later incorporated into tobacco protoplasts. Plants from the transformed tobacco protoplasts ultimately expressed for kanamycin resistance.

Attempts have now been made to genetically modify plants by introducing other useful genes for crop improvement, such as resistance to insects, weeds, and plant diseases, or in study of mechanism of gene action. Genetically modified (GM) plants are obtained by DNA transfer via vector-independent or vector-dependent means. Direct DNA transfer by electroporation, electrofusion, microinjection, microprojectile bombardment are vector independent and used for transformation of protoplasts and bacterial cells. Some of these methods are also used in callus cultures. In vector-mediated gene transfer, the genes of interest are inserted on plant expression binary vectors (Schardl et al. 1987) in 'sense' or 'antisense' orientation and these gene constructions mobilised into agrobacterium for transformation of cells, tissues, organ cultures or whole plant parts (floral dip: Clough and Bent 1998). Vectors for RNAi interference as well as the expression of polygenic traits have been constructed in genetic manipulation of crops aimed at their improvement (Stewart 2008).

The development of tissue culture techniques has thus a long and complicated history. Initial efforts concentrated on understanding the various aspects of plant growth and development. Success regarding the plant regeneration *in vitro* was achieved only during the first quarter of previous century when basic methods for plant tissue culture were established. Soon it was discovered that this new area of plant biology had practical value for commercial and agricultural plant propagators as well as in conservation of valuable germplasm (Razdan and Cocking 1997, 2000). Plant tissue culture is currently finding increased applications in study of molecular approaches followed in biotechnology. Success achieved in commercial plantings of GM crops (corn, soybean, cotton, canola and potato) and production additives (enzymes from genetically engineered microorganisms) for use in processed products like soft drinks, cakes, cheese, bread, meat, fish, etc. account for 90% common foodstuffs likely to contain GM components in USA (see Trigiano and Gray 2000, 2011). The production of GM crops has, however, been a matter of concern in certain parts of the world and issues involved are debatable. Yet plant tissue culture methods play a vital role in biotechnological improvement of plants particularly the agricultural crops. GM cotton (Bt cotton) introduced in India is estimated to result in increase of the yield by 50% (300kg/ha in 2002-2003 to 567kg/ha in 2007-2008), whereas in China GM crops with reduced (70%) application of pesticides have been produced (see Kumar and Loh 2012). These examples and other examples mentioned in Chapter 13 are claimed to have contributed significantly to socio-economic benefits for farmers in some countries.