Chapter 1

Introductory History

One of the most important biological events in the life cycle of an organism is fertilization, which involves the fusion of two gametes of opposite sex or strain resulting in the formation of a zygote. From this single-celled zygote originates the entire multicellular and multiorganed body of a higher organism; may it be a flowering plant or a human body. In a flowering plant, for example, structures as morphologically and functionally diverse as underground roots, green photosynthesizing leaves, and beautiful flowers all arise from the single-celled zygote through millions of mitoses. The latter process is a type of cell division characterized by identical products. Theoretically, therefore, all the cells in a plant body, whether residing in the flowers, conducting tissues or root tips, should have received the same genetic material as originally present in the zygote. All this would then suggest that there must be some other factor(s) superimposed on the genetic characteristics of cells which bring about this vast variation expressed by the genetically identical cells. The process involved in the manifestation of these variations is called differentiation. The morphological differentiation is actually preceded by certain cellular and subcellular changes. A pertinent question that arises at this stage is: whether the cellular changes underlying differentiation of various types of cells are permanent and, consequently, irreversible, or whether it is merely a social feature in which a cell undergoes an adaptive change to suit the functional need of the organism in general and the organ in particular. The fact that during the normal life cycle of a plant a cell which has differentiated into a palisade cell dies as a palisade cell and an epidermal cell does not revert to meristemic state may suggest that the events leading to differentiation are of a permanent nature. However, the classic experiments of Vochting on polarity in cuttings, carried out in 1878, suggest otherwise. He observed that all cells along the stem length are capable of forming roots as well as shoots, but their destiny is decided by their relative position in the cutting. The best way to answer this question and understand more about the inter-relationship between different cells of an organ and different organs of an organism would, however, be to remove them from the influence of their neighbouring cells and tissues and grow them in isolation on nutrient media. To put it in the words of the great German botanist Gottlieb Haberlandt (1854–1945), now aptly regarded as the father of plant tissue culture, 'To

GOTTLIEB HABERLANDT
(1854 - 1945)

PHILIP R. WHITE
(1901 - 1968)

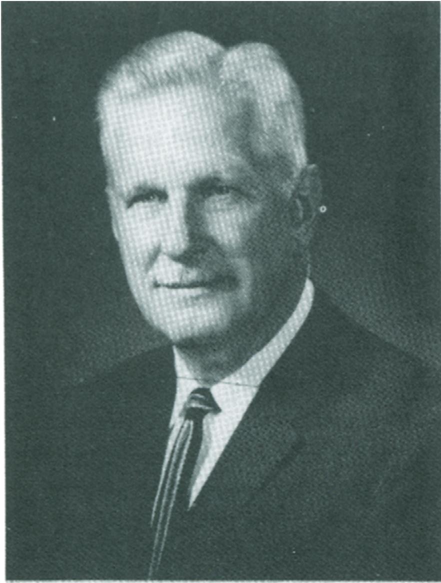
PANCHANAN MAHESHWARI
(1904 - 1966)

ROGER J. GAUTHERET
(Born 1910)

my knowledge, no systematically organized attempts to culture isolated vegetative cells from higher plants in simple nutrient solutions have been made. Yet the results of such culture experiments should give some interesting insight to the properties and potentialities which the cell as an elementary organism possesses. Moreover, it would provide information about the inter-relationships and complementary influences to which cells within a multicellular whole organism are exposed'. Haberlandt was the first person to culture isolated, fully differentiated cells as early as 1898 and the above lines are cited from the English translation of his classic paper presented in 1902 in which he described the results of his pioneering experiments (Krikorian and Berquam, 1969).

For his experiments Haberlandt (1902) chose single isolated cells from leaves. He used tissue of *Lamium purpureum* and *Eichhornia crassipes*, the epidermis of *Ornithogalum* and epidermal hairs of *Pulmonaria mollissima*. He grew them on Knop's (1865) salt solution with sucrose, and observed obvious growth in the palisade cells. In the first place they remained alive for up to 1 month. They grew in size from an initial length/width of 50 μm/27 μm to up to 180 μm/62 μm, changed shape, thickening of cell walls occurred, and starch appeared in the chloroplasts which initially lacked it. However, none of the cells divided. Some of the reasons for this failure were that he was handling highly differentiated cells and the present-day growth hormones, necessary for inducing division in mature cells, were not available to him. Charles Darwin once remarked 'I am a firm believer that without speculation there is no good or original research'. Despite the failure to achieve his goal, Haberlandt made several predictions in his paper of 1902. With the passage of time most of these ideas were confirmed experimentally, proving Haberlandt's broad vision and foresight. It was unfortunate that Haberlandt did not test his postulates experimentally or else several discoveries could have been made much earlier. Instead, he devoted his time to 'sensory physiological investigations'.

It would be worthwhile mentioning here some of the postulates of Haberlandt (1902). Despite the fact that he could not demonstrate the ability of mature cells to divide, he was clear in his mind that in the intact plant body the growth of a cell simply stops after acquiring the features required to meet the need of the whole organism. To this effect he states: 'This happens not because the cells lose their potential capacity for further growth, but because a stimulus is released from the whole organism or from particular parts of it'. 'The isolated cell is capable then of resuming uninterrupted growth'. Haberlandt had also perceived the concept of growth hormones, which he called 'growth enzymes', and felt these are released from one type of cells and stimulate growth and devel-



FOLKE SKOOG
(Born 1908)

GEORGES MOREL
(1916 - 1973)

HERBERT E. STREET
(1913 - 1977)

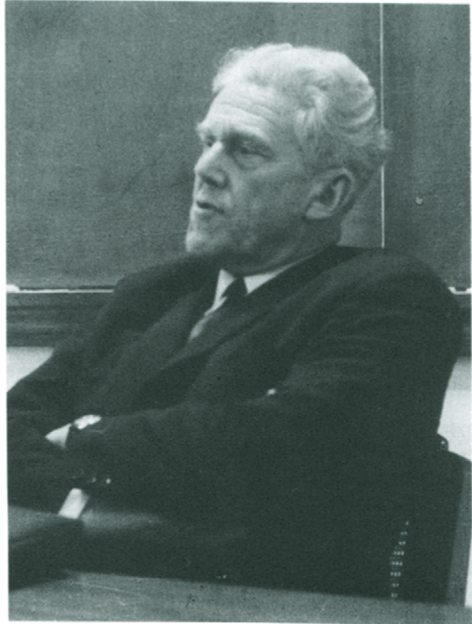
JEAN P. NITSCH
(1921 - 1971)

opment in other cells. Based on the observations of Winkler (1901) that pollen tubes stimulate growth in ovules and ovary, Haberlandt suggested '... it would be worthwhile to culture together in hanging drops vegetative cells and pollen tubes; perhaps the latter would induce the former to divide'. He continues, 'One could also add to the nutrient solutions used an extract from vegetative apices or else culture the cells from vegetative apices. One might also consider utilization of embryo sac fluids'. Haberlandt finally states 'Without permitting myself to pose further questions, I believe, in conclusion, that I am not making too bold a prediction if I point to the possibility that, in this way, one should successfully cultivate artificial embryos from vegetative cells. In any case, the technique of cultivating isolated plant cells in nutrient solutions permits the investigation of important problems from a new experimental approach.'

From the time Haberlandt presented his paper in 1902 until about 1934 hardly any progress was made in the field of plant tissue culture as conceived by Haberlandt. In 1904, however, Hannig had initiated a new line of investigation which later developed into an important applied area of in vitro techniques. Hannig excised nearly mature embryos of some crucifers (*Raphanus sativus*, *R. landra*, *R. candatus*, *Cochlearia danica*) and successfully grew them to maturity on mineral salts and sugar solution. He also tested, although unsuccessfully, the embryo sac fluid to support the growth of excised embryos. Proving one of the predictions of Haberlandt true, in 1941 Van Overbeek and co-workers demonstrated for the first time the stimulatory effect of coconut milk (embryo sac fluid) on embryo development and callus formation in *Datura* (Van Overbeek et al., 1941). Actually, this work proved a turning point in the field of embryo culture, for it enabled the culture of young embryos which failed to grow on a mixture of mineral salts, vitamins, amino acids and sugar. Subsequent detailed work by Raghavan and Torrey (1963), Norstog (1965) and others led to the development of synthetic media for the culture of younger embryos (see Raghavan, 1976a). However, until recently only post-globular embryos could be cultured ex-ovulo. Younger embryos either did not survive or exhibited callusing. Recently, Liu et al. (1993a) described a double layer culture system and a complex nutrient medium which supported embryogenic development of excised early globular (35 μm) embryos of *Brassica juncea*. Even more spectacular is the development of germinable embryos from naked 'zygote' formed by in vitro fusion of male and female gametes (Kranz and Lorz, 1993).

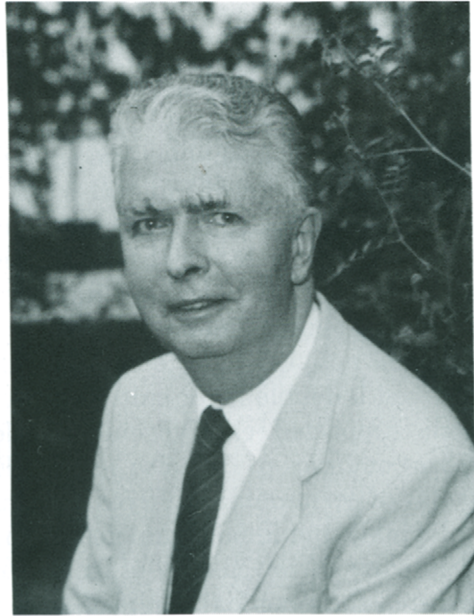
Fairly early in the history of embryo culture, Laibach (1925, 1929) demonstrated the practical application of embryo culture in the field of plant breeding. He isolated embryos from non-viable seeds of the cross *Linum perenne* × *L. austriacum* and reared them to maturity on a nutri-

FREDERICK C. STEWARD
(1904-1993)



GEORGE MELCHERS
(BORN 1906)

ARMIN C. BRAUN
(BORN 1911)



EDWARD C. COCKING
(BORN 1931)

ent medium. He also provided special impetus for further work in the area by stating, 'In any case, I deem it advisable to be cautious in declaring combination between higher plants to be inviable after fertilization has taken place and after they have begun to develop. Experiments to bring the aborted seed to development should always be undertaken if it is desirable for theoretical or practical reasons. The experiments will not always be successful, but many a result might be obtained by studying the conditions of ripeness of the embryo and by finding out the right time for the preparing out of the seed.' It should be mentioned here that to date several hybrids have been reared through embryo culture which would otherwise have failed due to embryo abortion (see Raghavan, 1976a).

As mentioned earlier, for a considerable time after Haberlandt's classic paper, work continued on organized structures. Pioneering work on root culture appeared during this period. In 1922, working independently, Robbins (USA) and Kotte (a student of Haberlandt in Germany) reported some success with growing isolated root tips. Further work by Robbins and Maneval (1924) enabled them to improve root growth, but the first successful report of continuously growing cultures of tomato root tips was made by White in 1934. Initially White used a medium containing inorganic salts, yeast extract and sucrose, but later yeast extract was replaced by three B-vitamins, namely pyridoxine, thiamine and nicotinic acid (White, 1937). On this synthetic medium, which has proved to be one of the basic media for a variety of cell and tissue cultures, White maintained some of the root cultures initiated in 1934 until shortly before his death in 1968. During 1939–1950 extensive work on root culture was undertaken by Street and his students to understand the role of vitamins in plant growth and shoot–root relationship.

The two important discoveries made in the mid-1930s which gave a big push to the development of plant tissue culture technique were: (a) identification of auxin as a natural growth regulator, and (b) recognition of the importance of B-vitamins in plant growth. In 1934, Gautheret had cultured cambium cells of some tree species (*Salix capraea*, *Populus nigra*) on Knop's solution containing glucose and cysteine hydrochloride and recorded that they proliferated for a few months. The addition of B-vitamins and IAA considerably enhanced the growth of *Salix* cambium. However, the first continuously growing tissue cultures from carrot root cambium were established by Gautheret in 1939. In the same year White (1939a) reported the establishment of similar cultures from tumour tissue of the hybrid *Nicotiana glauca* × *N. langsdorffii*. Gautheret and White, together with Nobecourt, who had independently reported the establishment of continuously growing cultures of carrot in the

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(BORN 1930)

INDRA K. VASIL
(BORN 1932)

same year, 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 the three pioneers in 1939. Although continuously growing cultures could be established in 1939, the tissues used by all the three workers included meristematic cells.

The induction of divisions in isolated mature and differentiated cells had to wait the discovery of another growth regulator. Skoog (1944) and Skoog and Tsui (1951) had demonstrated that in tobacco pith tissue cultures the addition of adenine and high levels of phosphate increased callus growth and bud formation even in the presence of IAA which otherwise acted as bud-inhibitor. However, the division of cells occurred only if vascular tissue was present; pith cells alone did not show any division (Jablonski and Skoog, 1954). Actually, the importance of the association of vascular tissue for inducing cell divisions in mature parenchyma cells of potato tuber was demonstrated by Haberlandt as early as 1913. In their search to replace the need for vascular tissue, Jablonski and Skoog tested several plant extracts by either adding them to the nutrient medium or injecting them into the tissue. One of the substances most effective in this respect was yeast extract (YE), which had enabled White (1934) to establish the first continuously growing root cultures. However, for cell division the active component of YE was not B-vitamins, but

something with properties common to purine. Based on this observation, when DNA was tested in place of YE it proved to be an enormously richer source of activity than any other substance tested before for cell division in pith tissue. Initially the activity was noticed in old samples of DNA, but it could also be produced by autoclaving weakly acid slurries of freshly isolated DNA (Miller et al., 1955b). Miller et al. (1955a) separated the first known cytokinin from the DNA of herring sperm and named it kinetin. At present, many synthetic as well as natural compounds with kinetin-like activity are known. The availability of these substances, collectively called cytokinins, has made it possible to induce divisions in cells of highly mature and differentiated tissue, such as mesophyll and endosperm from dried seeds.

At this stage, the dream of Haberlandt was realized only partially, for he foresaw the possibility of cultivating isolated single cells. Only small pieces of tissue could be grown in cultures. Further progress in this respect was made by Muir (1953). He demonstrated that by transferring callus tissues of *Tagetes erecta* and *Nicotiana tabacum* to liquid medium and agitating the cultures on a shaking machine it was possible to break the tissue into single cells and small cell aggregates. Muir et al. (1954) also succeeded in mechanically picking single cells from these shake cultures (suspension cultures) as well as soft callus tissues, and making them divide by placing them individually on separate filter papers resting on the top of a well-established callus culture. Apparently the callus tissue, which was separated from the cell only by thin filter paper, supplied the necessary factor(s) for cell division. This nurse culture method was very similar to the untested idea of Haberlandt wherein he suggested growing single cells along with pollen tubes so that the former may receive cell division stimulus from the latter. In 1960 Jones et al. designed a microculture method for growing single cells in hanging drops in a conditioned medium (medium in which tissue has been grown for some time). The advantage of this technique was that it allowed continuous observation of the cultured cells. Using this technique but replacing the conditioned medium by a fresh medium, enriched with coconut milk, Vasil and Hildebrandt (1965) raised whole plants starting from single cells of tobacco. An important biological technique of cloning large number of single cells of higher plants was, however, developed in 1960 by Bergmann. He filtered the suspension cultures of *Nicotiana tabacum* and *Phaseolus vulgaris* and obtained a population containing about 90% free cells. These were incorporated into a 1 mm layer of solidified medium containing 0.6% agar. In this experiment some of the single cells divided and formed visible colonies. This technique is now widely used for cloning cells, and in protoplast culture experiments.

The free cells thus far cultured successfully were derived from actively growing tissues in cultures. It was indeed the work of Kohlenbach in 1966 that came closest to Haberlandt's experimental material and objectives. Kohlenbach successfully cultured mature mesophyll cells from *Macleaya cordata*. The tissue obtained from these cells subsequently differentiated somatic embryos.

In 1957, Skoog and Miller put forth the concept of hormonal control of organ formation (Fig. 5.6). In this classic paper, they showed that the differentiation of roots and shoots in tobacco pith tissue cultures was a function of the auxin-cytokinin ratio, and that organ differentiation could be regulated by changing the relative concentrations of the two substances in the medium; high concentrations of auxin promoted rooting, whereas high levels of cytokinin supported shoot formation. At equal concentrations of auxin and cytokinin the tissue tended to grow in an unorganized fashion. This concept of hormonal regulation of organogenesis is now applicable to a large number of plant species. However, the exogenous requirement of growth regulators for a particular type of morphogenesis varies, depending on the endogenous levels of these substances in the tissue in question.

The differentiation of whole plants in tissue cultures may occur via shoot and root differentiation or, alternatively, the cells may undergo embryogenic development to give rise to bipolar embryos, referred to as 'somatic embryos' in this book to distinguish them from zygotic embryos. The first reports of somatic embryo formation from carrot tissue appeared in 1958–1959 by Reinert (Germany) and Steward (USA). To date, numerous plant species have been reported to form somatic embryos. In some plants, like carrot and buttercup, embryos can be obtained from virtually any part of the plant body.

Until the mid-1970s hormonal manipulation in the culture medium remained the main approach to achieve plant regeneration from cultured cells and it proved very successful with many species. However, some very important crop plants, such as cereals and legumes, did not respond favourably to this strategy and were, therefore, declared recalcitrant (Bhojwani et al., 1977a). In 1972, Saunders and Bingham reported that different cultivars of alfalfa varied considerably in their regeneration potential under a culture regime. More detailed studies by Bingham and his associates (Bingham et al., 1975; Reisch and Bingham, 1980) demonstrated that regeneration in tissue cultures is a genetically controlled phenomenon. Genotypic variation has been since observed in several plant species; it occurs between varieties and, in outbreeding crops, within varieties. The success in obtaining regeneration in tissue cultures of forage legumes has been mainly due to a shift in the emphasis from

medium selection to genotype selection. Similar success with cereals became possible only after the physiological state of the explant was recognized as another important factor affecting regeneration. In this group of plants the regeneration potential is largely restricted to immature embryos (Green and Phillips, 1975; Vasil and Vasil, 1980). Vasil and his associates, at the University of Florida, demonstrated that embryogenic cultures of most cereals can be established using immature embryos as the explant, and such cultures are suitable for protoplast isolation and culture as well as genetic manipulation of these plants (Vasil and Vasil, 1986; Vasil, 1988; Vasil et al., 1992). Immature embryos have also proved to be an ideal explant to raise embryogenic cultures of numerous other herbaceous and woody species, including Gymnosperms.

Establishment of suspension cultures of plant cells in liquid medium, similar to microbes, in the mid-1950s prompted scientists to apply this system for the production of natural plant products as an alternative to whole plant. The first attempt for the industrial production of secondary metabolites in vitro was made during 1950–1960 by the Pfizer Company (see Gautheret, 1985) and the first patent was obtained in 1956 by Routien and Nickell. However, not much progress in this area was made for many years. Apparently, the industrial production of secondary metabolites required large scale culture of cells. In 1959, Tulecke and Nickell published the first report of plant cell culture in a 134 l reactor. Noguchi et al. (1977) used 20 000 l reactor for the culture of tobacco cells. Since plant cells are different from microbes in many respects the reactors traditionally used in microbiology had to be modified to suit plant cell culture. Several different kinds of bioreactors have been designed for large scale cultivation of plant cells (see Chapters 4 and 17). The technology for mass culture of plant cells is now available but slow growth of plant cells, genetic instability of cultured cells, intracellular accumulation of secondary products and organ-specific synthesis of secondary products are some of the problems making tissue culture production of industrial compounds uneconomical. Despite these problems in several cases cell cultures have been shown to produce certain metabolites in quantities equal to (first reported by Kaul and Staba, 1967) or many fold greater than (first reported by Zenk, 1978) the parent plant. In 1979, Brodelius et al. developed the technique of immobilization of plant cells so that the biomass could be utilized for longer periods, besides its other advantages. Culture of 'hairy roots', produced by transformation with *Agrobacterium rhizogenes*, has been shown to be a more efficient system than cell cultures for the production of compounds which are normally synthesized in roots of intact plants. The first tissue culture product to be commercialized, by Mitsui Petrochemical Co. of Japan, is Shikonin from

cell cultures of *Lithospermum erythrorhizon* (Curtin, 1983). In 1988, another Japanese company (Nitto Denko) started marketing ginseng cell mass produced in culture (Misawa, 1994).

Differentiation of plants from callus cultures has been suggested as a potential method for rapid propagation of selected plant species because hundreds and thousands of plants can be raised from a small amount of tissue and in a continuous process. But this method suffers from one serious drawback that cells in long-term cultures are genetically unstable. A more important technique, which was later to become a viable horticultural practice, was developed by Ball in 1946. He successfully raised transplantable whole plants of *Lupinus* and *Tropaeolum* by culturing their shoot tips with a couple of leaf primordia. However, the demonstration of the practical usefulness of this important technique must be credited to Morel who, with Martin (Morel and Martin, 1952), for the first time recovered virus-free *Dahlia* plants from infected individuals by excising and culturing their shoot tips in vitro. The basis of this approach is that even in a virus-infected plant the cells of the shoot tip are either free of virus or carry a negligible amount of the pathogen. This technique of shoot tip culture, alone or in combination with chemotherapy or thermotherapy, has since then been widely used with a variety of plant species of horticultural and agronomic importance and has become a standard practice to raise virus-free plants from infected stocks (see Chapter 15).

While applying the technique of shoot-tip culture for raising virus-free individuals of an orchid, Morel (1960) also realized the potential of this method for the rapid propagation of these plants. The technique allowed the production of an estimated 4 million genetically identical plants from a single bud in a period of 1 year. Until this time orchid propagation was done by seeds. A serious problem inherent in this method is the appearance of a great variation in the progeny. Seeing a tremendous advantage in the technique, the commercial orchidologists soon adopted this novel technique as a standard method for propagation. This contribution of Morel not only revolutionized the orchid industry, but also gave impetus to the utilization of shoot-bud culture for rapid cloning of other plant species.

Murashige was instrumental in giving the techniques of in vitro culture a status of a viable practical approach to propagation of horticultural species. He worked extensively for the popularization of the technique by developing standard methods for in vitro propagation of several species ranging from ferns, to foliage, flower and fruit plants. Indeed, Murashige's name became intimately associated with the technique. Incidentally, the principle of the technique being used for in vitro propagation of most flowering plants is very different from that used for orchids.

It is based on another important finding made in 1958 by Wickson and Thimann. They showed that the growth of axillary buds, which remain dormant in the presence of terminal buds, can be initiated by the exogenous application of cytokinins. The implication of this is that one could induce the release of lateral buds on a growing shoot with an intact terminal bud by growing the shoot in a medium containing cytokinin. This would release buds from apical dominance not only on the initial stem segment, but also those on the lateral branches developed from it in cultures, giving rise to a bushy witch's broom-like structure with numerous shoots. Individual branches from this cluster can be made to repeat the process of shoot multiplication to build up innumerable shoots in a rather short period. Routinely, a portion of the total shoots may be rooted in another medium to get full plantlets ready for transfer to soil through careful handling.

Axillary bud proliferation is widely practised for in vitro propagation of plants because it ensures maximum genetic uniformity of the resulting plants but from economic considerations this method is not very attractive as it is slow and labour intensive. Therefore, attention is being given to developing somatic embryogenic systems for mass propagation of plants as it offers the possibility of rapid multiplication in automated bioreactors, with low inputs. Since the first attempt of Backs-Husemann and Reinert (1970) to scale-up somatic embryogenesis in carrot using a 20 l carboy, different types of bioreactors have been tested (see Chapter 6). For poinsettia embryo production, Preil (1991) used a round bottom 2 l bioreactor in which stirring was achieved by vibrating plates and bubble-free O_2 was supplied through a silicon tubing which was inserted as a spiral of 140 cm total tube length. For mechanical planting of somatic embryos in the field the concept of synthetic seeds has been proposed. Currently, two types of synthetic seeds, viz. desiccated and hydrated, are being developed in which somatic embryos are individually encapsulated in suitable compounds (see Chapter 6).

Regeneration of plants from carrot cells frozen at the temperature (–196°C) of liquid nitrogen 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 success with freeze preservation of cells, shoot tips and embryos gave birth to a new applied area of tissue culture, called germplasm storage (Chapter 18). Cultured shoots/plantlets can also be stored at 4°C for 1–3 years. These methods are being applied at several laboratories to establish in vitro repository of valuable germplasm.

The spontaneous occurrence of variation in tissue cultures with regard to the ploidy, morphology, pigmentation and growth rates had been ob-

served for quite some time. Changes to auxin habituation was reported by Gautheret (1955). However, for long these variations were ignored as mere abnormalities. The first formal report of morphological variation induced in tissue cultures was published from the Hawaiin Sugar Planter's Association Experimental Station. Heinz and Mee (1971) reported variation in sugarcane hybrids regenerated from cell cultures. The agronomic importance of such variability was immediately recognized and the regenerants were screened for useful variation. 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). In the following 5–6 years useful variants of crops, such as geranium (Skirvin and Janick, 1976a,b) and potato (Shepard et al., 1980), were obtained from tissue culture derived plants. However, it was the article by Larkin and Scowcroft (1981) which drew the attention of tissue culturists and plant breeders to tissue culture as a novel source of useful genetic variation. They proposed the term 'somaclonal variation' for the variation detected in plants regenerated from any form of culture and termed the regenerated plants as somaclones. Evans et al. (1984a) introduced the term 'gametoclones' for the plants regenerated from gametic cells. During the past decade scientists have examined their tissue cultures and the plants regenerated from them more critically and confirmed that tissue culture can serve as a novel source of variation suitable for crop improvement. Several somaclones and gametoclones have already been released as new improved cultivars (see Chapter 9).

By the early 1960s, methods of in vitro culture were reasonably well developed and the emphasis was shifting towards applied aspects of the technique. Around this time the Botany School at the University of Delhi, led by P. Maheshwari, became actively engaged with in vitro culture of reproductive organs of flowering plants (see Maheshwari and Rangaswamy, 1963). Prompted by her success with 'intra-ovarian pollination' (Kanta, 1960), Kanta developed the technique of 'test-tube fertilization' (Kanta et al., 1962). In essence, it involves culturing excised ovules and pollen grains together on the same medium; the pollen germinates and fertilizes the ovules. In theory, this technique could be applied to overcome any sexual incompatibility for which reaction occurs in the stigma and/or style. Using this approach, Zenkteler and co-workers (Zenkteler, 1967, 1970; Zenkteler et al., 1975) developed interspecific (*Melandrium album* × *M. rubrum*) and intergeneric (*M. album* × *Silene schafta*) hybrids unknown in nature. Similarly, self-incompatibility in *Petunia axillaris* could be overcome following this method. Therefore, for almost a decade this simple technique to overcome sexual incompatibility barriers

remained overshadowed by more sophisticated techniques of somatic hybridization and genetic engineering which were gaining popularity with the scientists during this period. A renewed interest in the technique of in vitro pollination occurred in the mid-1980s, when a number of laboratories used this technique to produced some rare hybrids (see Chapter 10). A major breakthrough in this area was made at the beginning of 1990s when Kranz et al. (1990) reported electrofusion of isolated male and female gametes of maize and, 3 years later (Kranz and Lorz, 1993), plant regeneration from the fusion product. The naked 'zygote' formed embryo and eventually fertile plants (see Chapter 10). This is the first and so far the only demonstration of in vitro fertilization in higher plants.

The role of haploids in breeding and genetics of higher plants had been emphasized for a considerable time but the restricted availability of such individuals, with the gametic number of chromosomes (half of that present in body cells), did not allow their full exploitation. In 1966, Guha and Maheshwari demonstrated the possibility of raising large numbers of androgenic haploid plantlets from pollen grains of *Datura innoxia* by culturing immature anthers. Later work by Bourgin and Nitsch (1967) confirmed the totipotency of pollen grains. They raised full haploid plants of tobacco. By the use of this technique, several promising new varieties of tobacco, rice and wheat have been introduced.

In 1970, Kameya and Hinata reported callus formation in isolated pollen cultures of *Brassica* sp. A couple of years later C. Nitsch and her associates, at the CNRS, France, succeeded in raising haploid plants from isolated microspore cultures of *Nicotiana* and *Datura* (Nitsch and Norreel, 1973; Nitsch, 1974). Initially, a nurse tissue was used to culture isolated microspores (Pelletier and Durran, 1972; Sharp et al., 1972) but soon it was possible to culture them on synthetic media. With the refinement of culture techniques and media it has become possible to raise androgenic plants by isolated microspore culture on synthetic media for a large number of species. So far pollen plants have been obtained by anther/pollen culture for over 134 species and the techniques are being used in plant breeding programmes (see Chapter 7). Isolated microspore culture of *B. napus* has emerged as a model system to study cellular basis of androgenesis (see Chapter 7).

Although the number of haploid cells in an ovule are very limited, it is possible to produce parthenogenetic or apogamous haploids by unfertilized ovary/ovule culture. It was first reported in barley by San Noeum (1976). To date gynogenetic haploids have been reported for about 19 species (see Chapter 7).

Although the isolation of protoplasts (Klercker, 1892) and their fusion (Kuster, 1909) were reported almost 100 years ago it was to the credit of

Cocking (1960) whose work introduced the concept of enzymatic isolation of plant protoplasts. He had used culture filtrate of the fungus *Myrothecium verrucaria*, but in 1968 cellulase and macerozyme became commercially available and isolation of large quantities of viable protoplasts by enzymatic degradation of cell wall soon became a routine technique (see Chapter 12). By 1970 it was demonstrated that isolated protoplasts are capable of regenerating a new wall (Pojnar et al., 1967) and the reconstituted cell is capable of sustained divisions (Kao et al., 1970a; Nagata and Takebe, 1970). In 1971 the totipotency of isolated protoplasts was demonstrated (Nagata and Takebe, 1971; Takebe et al., 1971). At almost the same time, Cocking's group at the University of Nottingham achieved fusion of isolated protoplasts using $NaNO_3$ (Power et al., 1970). These two observations, totipotency of protoplasts and induced fusion of protoplasts, gave birth to a new field of plant tissue culture, viz. somatic hybridization. This was one of the most active areas of research from 1970 to the mid-1980s because of its potential application in crop improvement by genetic manipulation of somatic cells. During this period, more efficient methods of protoplast fusion, using as 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), and improved culture methods and media were developed. Also, regeneration of plants from protoplasts of a large number of species was achieved.

The first somatic hybrids between *Nicotiana glauca* and *N. langsdorffii* was produced in 1972 by Carlson and his co-workers. However, these two species could be crossed sexually. In 1978, Melchers et al. produced an intergeneric hybrid between sexually incompatible parents, potato and tomato, but the somatic hybrid was sexually sterile. It was soon realized that although somatic hybrids could be produced between highly unrelated parents but such wide hybrids would not be agronomically useful. The technique of protoplast fusion is now being used to produce asymmetric hybrids, wherein only a part of the nuclear genome of the donor parent is transferred to the recipient parent. A novel application of protoplast fusion is in the production of cybrids with novel nuclear-cytoplasmic combinations. This technique has already been utilized to transfer male sterility inter- and intra-specifically (see Chapter 13).

The property of isolated protoplasts to take-up organelles and macromolecules prompted several scientists to employ this system for genetic transformation of plants by feeding them with purified DNA but it did not meet with much success (Bhojwani and Razdan, 1983). The field of genetic engineering, which refers to insertion of selected gene(s) for genetic modification of plants, became reality with the development of *Agrobacterium tumefaciens* based vectors. Smith and Townsend (1907)

had shown that this gram negative soil bacteria causes crown gall disease in some plants. Based on his observation that crown gall tissue displayed the tumorigenic character for autonomous growth on salt-sugar medium, even in the absence of the bacterium, Braun (1947) suggested that probably during infection the bacterium introduces a tumour-inducing principle in the plant genome. Transfer of bacterial genetic material into the crown gall cells was also proposed by Morel (1971) based on his observation that the crown gall cells acquired the new trait for the synthesis of opines, some novel amino acids. The elusive DNA was identified as a large plasmid (Ti-plasmid) found only in a virulent strain of the *A. tumefaciens* (Zaenen et al., 1974). The utility of the bacteria as a gene transfer system in plants was first recognized when Chilton et al. (1977) demonstrated that the crown galls were actually produced as a result of the transfer and integration of genes from the bacteria into the genome of plants. Barton et al. (1983) demonstrated that heterologous DNA inserted into the T-DNA of Ti-plasmid could be transferred to plants along with the existing T-DNA genes. With refinement of the *A. tumefaciens* system in the early 1980s, research to produce genetically engineered plant varieties blossomed. Efficient plant transformation vectors were constructed by removing the phytohormone biosynthesis genes from the T-DNA region and thereby eliminating the ability of the bacteria to induce aberrant cell proliferation (Fraley et al., 1985). The first transgenic tobacco plants expressing engineered foreign genes were produced with the aid of *A. tumefaciens* (Horsch et al., 1984). Since then derivatives of this bacteria have proved to be an efficient and highly versatile vehicle for the introduction of genes into plants and plant cells. Most of the transgenic plants produced to date were created through the use of this system. However, this transformation system is species-specific; it does not work with most monocotyledons which include the major cereals. Therefore, during the last decade the arsenal of the transformation system has been expanded to include free DNA delivery techniques, such as electroporation, particle gun and microinjection, which are not species limited and can be used with cells, tissues and organized structures. Of these, particle gun, also called microprojectile bombardment or biolistic, is the most promising DNA delivery system for plants. In 1986, the first plants were genetically engineered for a useful agronomic trait (Abel et al., 1986). During the last decade, the list of genetically improved varieties produced by this molecular breeding method has considerably enlarged (see Chapter 14).

These, in brief, are some of the milestones in the development of the techniques of plant tissue culture. Like any other area of science, it started as an academic exercise to answer some questions related to

plant growth and development, but proved to be of immense practical value, as an aid to plant propagation, raising and maintenance of high health-status plants, germplasm storage, and a valuable adjunct to the conventional methods of plant improvement.