Introduction to
Plant Biotechnology
Second edition
H.S. Chawla
Part I

Plant Tissue Culture
Plants are the key to life on earth as they directly supply 90% of human calorie intake, and 80% of the protein intake, the remainder being derived from animal products, although these animals have also derived their nutrition from plants. Of the three thousand plant species which have been used as food by man, the world now depends mainly on around twenty crop species for the majority of its calories, with 50% being contributed by eight species of cereals. Minerals and vitamins are supplied by a further thirty species of fruits and vegetables. Most important of the staple foods are the cereals, particularly wheat and rice, with more than one-third of all cultivated land used to produce these two crops.

As the population continues to expand, concern has been expressed over the finite number of people world agriculture can support. An estimate of how many people could be supported by world food production can be obtained from consideration of the average daily calorie requirements and the net amount of grain yield from well-managed areas. It has been calculated that the earth can support about 15 billion people on a strictly vegetarian diet, or five billion on a mixed diet. The population is expected to be eight billion by 2001 and double by 2025.

The farming practices and crops cultivated today have developed over a relatively short span of time. Crop plants of today have changed in a number of ways so that they now bear very little resemblance to their wild type ancestors. These changes have come about through selection, either conscious or unconscious, for traits which are advantageous to the people growing the crops. Thus modern wheats do not disperse their seeds or legumes do not have pods which burst open. Today, varieties are the result of generation of plants cultivated under ideal conditions from the man's point of view.

From the beginning of crop cultivation to the late nineteenth century, all improvements in the species used were brought about by those who were directly involved, i.e. farmers themselves. In the following 100 years, the laws of genetic inheritance and rules governing species variation were laid down by Mendel, Darwin, and others, which redefined the breeding techniques by making them predictable and therefore quicker, more precise and more productive. Furthermore, despite the implementation of breeding techniques, the time taken to produce and test varieties is an important limiting consideration.

**NEW TECHNOLOGIES**

Over the past few years a number of methodologies have come that would seem to have much more to offer in terms of advancing current research in the plant sciences, and exploiting the knowledge gained to develop new crops. The first
of these areas is concerned with manipulation and subsequent growth of cells, tissues, organs and naked plant cells (protoplasts) in tissue culture. The second field is genetic engineering or recombinant DNA (rDNA) technology which has grown out very fast from the work initially carried out on microorganisms. With genetic engineering, scientists have more exact methods for breeding better livestock and crop varieties. This technology allows for the detailed manipulation of genes. These two areas of research have in recent years become associated with the general field of biotechnology and are potentially applicable to a wide variety of plant species, as well as offer a precision in manipulating genetic material. Recombinant DNA techniques in particular have already contributed much to the elucidation of basic mechanisms in plants at the molecular level.

Our knowledge of the structure and expression of the plant genome has come largely through the use of recombinant DNA or gene cloning techniques. This technology allows the isolation and characterization of specific pieces of DNA and by cloning the DNA sequences into bacterial cells, they can be replicated to yield large quantities for analysis. In addition to supplying much basic information concerning gene structure and expression, recombinant-DNA technology provides the opportunity for specifically manipulating genetic material, and moving such material among different organisms.

**ORIGIN OF BIOTECHNOLOGY**

The term Biotechnology was coined by Karl Ereky, a Hungarian engineer, in 1919. The origin of biotechnology can be traced back to prehistoric times when microorganisms were already used for processes like fermentation, formation of yoghurt and cheese from milk, vinegar from molasses, production of butanol and acetone from starch by *Clostridium acetobutylicum* or the production of antibiotics like penicillin from *Penicillium notatum*. However, biotechnology got a boost in the 1970's with the discovery of restriction enzymes which led to the development of a variety of gene technologies and is thus considered to be the greatest scientific revolution of this century. Biotechnology thus consists of a variety of techniques, designed to genetically improve and/or exploit living systems or their components for the benefit of man. In fact, biotechnology is the product of interaction between sciences of biology and technology. It is the technological exploitation and control of biological systems. Attempts have been made to define biotechnology and it has been interpreted in different ways by different groups of workers. However, the following definitions seems to be most appropriate.

i. The application of science and engineering in the direct or indirect use of living organisms, or parts or products of living organisms, in their natural or modified form.

ii. Biotechnology means any technological application that uses biological systems, living organism or derivatives thereof to make or modify products or processes for specific use (Convention on Biodiversity).

iii. The controlled use of biological agents such as microorganisms or cellular components for beneficial use (US National Science Foundation).

The term genetic engineering refers to a number of new techniques involving the transfer of specific genetic information from one organism to another. These techniques do not rely on sexual methods, but instead involve genetic manipulation at the cellular and molecular levels. These are the nonsexual methods for gene transfer.

An important aspect of all plant biotechnology processes is the culture of either the microorganisms or plant cells or tissues and organs in artificial media. One of the problems in conventional plant breeding is that the range of organisms among which genes can be transferred is severely limited by species barriers. The new technologies provide a better approach for defining and manipulation of targets,
and species-specific barriers are broken. They do not replace plant breeding but provide methods capable of achieving objectives not possible by other means.

**HISTORY**

The last three decades have seen a very rapid rise in the number of plant scientists using the techniques of organ, tissue and cell cultures for plant improvement. The term "plant tissue culture" broadly refers to the *in vitro* cultivation of plants, seeds, plant parts (tissues, organs, embryos, single cells, protoplasts, etc.) on nutrient media under aseptic conditions.

During the 1800s, the cell theory, which states that the cell is the basic structural unit of all living creatures, was very quick to gain acceptance. However, the second portion of the cell theory states that these structural units are distinct and potentially totipotent physiological and developmental units, failed to gain universal acceptance. The skepticism associated with the latter part was because of the inability of scientists such as Schleiden and Schwann to demonstrate totipotency in their laboratories. It was in 1902 that the well-known German plant physiologist, Gottlieb Haberlandt (1854–1945), attempted to cultivate plant tissue culture cells *in vitro*. He is regarded as the father of plant tissue culture. He clearly stated the desirability of cultivating the isolated vegetative cells of higher plants. He stated: "To 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 insights into the properties and potentialities which the cell as an elementary organism possesses. Moreover, it would provide information about the interrelationships and complementary influences to which cells within the multicellular whole organism are exposed." Haberlandt started his experiments in 1898 using single cells isolated from the palisade tissue of leaves, pith parenchyma, epidermis and epidermal hair of various plants. He grew them on Knop's salt solution with sucrose and observed obvious growth in the palisade cells. However, unfortunately none of the cells divided but this did not deter others to perform experiments. In the early 1920s workers again attempted to grow plant tissues and organs *in vitro*. Molliard in 1921 demonstrated limited success with the cultivation of plant embryos and subsequently Kotte (1922a, b), a student of Haberlandt in Germany and, independently Robbins (1922a, b) were successful in the establishment of excised plant root tips *in vitro*. However, in 1934, the pioneering work of growing excised roots of tomato *in vitro* for periods of time without theoretical limits was demonstrated by White (1934). Initially, White used a medium containing inorganic salts, yeast extract and sucrose, but later yeast extract was replaced by three B-vitamins viz. pyridoxine, thiamine and nicotinic acid. In the same year, Gautheret was engaged in experimentation with excised root tips and the cultivation of cambial tissue removed under aseptic conditions from *Salix caprea*, *Populus nigra* and other trees on Knop's solution containing glucose and cysteine hydrochloride and recorded that they proliferated for a few months. These studies continued, and in 1939, Gautheret, Nobecourt and White, published independently studies on the successful cultivation for prolonged periods of cambial tissues of carrot root (Gautheret, 1939), tobacco (White, 1939) and carrot (Nobecourt, 1939). These were the first true plant tissue cultures in the strict sense of prolonged cultures of unorganized materials. The methods and media now used are in principle modifications of those established by these three pioneers. All the three workers used meristematic cells to generate continuously growing cultures. Philip R. White (1943, 1954, 1963) and Roger J. Gautheret (1959) can be credited with providing a significant impetus to the field with the publication of their authoritative handbooks.

From 1939 to 1950, experimental work with root cultures drew attention to the role of vitamins
in plant growth and advanced the knowledge of the shoot-root relationship. During early 1950s, a number of lines of enquiry were initiated. The work of Miller and Skoog (1953) on bud formation from cultured pith explants of tobacco led to the discovery of kinetin. In 1952, Steward initiated work on cultured carrot explants and used coconut milk as a nutrient (Steward et al., 1952) that ultimately led to the discovery of embryogenesis (Steward et al., 1958). In 1953, Muir reported that if fragments of callus of Tagetes erecta and Nicotiana tabacum are transferred to liquid culture medium and the medium is agitated on a reciprocal shaker, then the callus fragments break up to give a suspension of single cells and cell aggregates (Muir, 1953). He further developed a nurse culture technique. An important technique of cloning large number of single cells of higher plants was developed by Bergmann (1960).

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 somatic embryos. Differentiation of plants from callus cultures has often been suggested as a potential method for rapid propagation. Ball in 1946 successfully raised transplantable whole plants of Lupinus and Tropaeolum by culturing their shoot tips with a couple of leaf primordia. The practical usefulness of this technique is credited to Morel and Martin (1952), who for the first time recovered virus-free Dahlia plants. Morel (1960) also realized the potential of this method for rapid propagation of virus-free orchids.

The release of protoplasts from root tip cells using a fungal cellulase in 0.6M sucrose was reported by Cocking in 1960. Protoplasts released by cell wall degrading enzymes have now been prepared from many plant tissues. The most universally used high-salt medium was developed by Skoog and his students (Murashige and Skoog, 1962). In addition to mineral salts, media contain an energy source, vitamins and growth regulators.

In 1970s, restriction enzymes were discovered, and the technique was developed by Lobban and Kaiser for joining two restriction fragments by ligase. Paul Berg, working at Stanford University, was the first to make a recombinant DNA molecule combining DNA from SV40 virus with that of lambda virus in 1972. However, realizing the dangers of his experiments, he terminated it immediately. He proposed, in what is known as "Berg Letter," a one-year moratorium on such research in order that safety concerns be worked out. In 1973 Stanley Cohen of Stanford University and Herbert Boyer of University of California produced world's first recombinant DNA organism. This set the foundation of modern biotechnology and genetic engineering. Genetic engineering became a reality when a man-made insulin gene was used to manufacture a human protein in bacteria. The 1980s saw the development of various genetic transformation techniques which revolutionized the rDNA technology and led to the development of transgenic plants for various crops.

It was in mid-1970s that techniques to sequence DNA were developed. Sanger et al. (1977) and Maxam and Gilbert (1977) were the first to report gene sequencing techniques. Sanger et al. (1977) sequenced virus φx 174 comprising 5375 nucleotides that code for 10 proteins. This was followed by report on complete DNA sequence of simian virus 40 (SV40). In recent years, entire genomes of a number of prokaryotes and eukaryotes have been sequenced. In 1990, the Human Genome Project (HGP) was launched to sequence the genome and identify all the genes. This laid the foundation for genomics.

Molecular breeding work started in 1980s with the development of the restriction fragment length polymorphism technique. The technique of DNA fingerprinting, based on the sequence of bases in segments of DNA, was introduced in 1986 by Jeffreys to identify individuals and it was first used as an evidence in the court room in USA in 1987. A large number of molecular
markers have been reported that led to the development of methodologies for mapping of genes on the chromosomes and in molecular-marker-assisted selection. Some of the contributions are mentioned below.

1902 First attempt of plant tissue culture (Haberlandt).
1904 Embryo culture of selected crucifers attempted (Hannig).
1922 Asymiotic germination of orchid seeds in vitro (Knudson).
1922 In vitro culture of root tips (Robbins).
1925 Use of embryo culture technique in interspecific crosses of Linum (Laibach).
1934 In vitro culture of cambial tissue of a few trees and shrubs, although failed to sustain cell division (Gautheret).
1934 Successful culture of tomato roots (White).
1939 Successful establishment of continuously growing callus cultures (Gautheret, Nobecourt and White).
1940 In vitro culture of cambial tissues of Ulmus to study adventitious shoot formation (Gautheret).
1941 Use of coconut milk containing a cell division factor for the first time in Datura (van Overbeek).
1941 In vitro culture of crown gall tissues (Braun).
1944 In vitro adventitious shoot formation in tobacco (Skoog).
1946 Raising of whole plants of Lupinus and Tropaeolum by shoot tip culture (Ball).
1950 Regeneration of organs from callus tissue of Sequoia sempervirens (Ball).
1952 Use of meristem culture to obtain virus-free dahlias (Morel and Martin).
1952 First application of micrografting (Morel and Martin).
1953 Production of haploid callus of the gynospertm Ginkgo biloba from pollen (Tulecke).
1954 First plant from a single cell (Muir et al.).
1955 Discovery of kinetin a cell division hormone (Miller et al.).
1957 Discovery of the regulation of organ formation by changing the ratio of auxin: cytokinin (Skoog and Miller).
1958 Regeneration of somatic embryos in vitro from the nucellus of Citrus ovules (Maheshwari and Rangaswamy).
1959 Regeneration of embryos from callus clumps and cell suspensions of Daucus carota (Reinert, Steward).
1959 Publication of first handbook on plant tissue culture (Gautheret).
1960 First successful test tube fertilization in Papaver rhoeas (Kanta).
1960 Use of the microculture method for growing single cells in hanging drops in a conditioned medium (Jones et al.).
1960 Enzymatic degradation of cell walls to obtain large number of protoplasts (Cocking).
1960 Filtration of cell suspensions and isolation of single cells by plating (Bergmann).
1962 Development of Murashige and Skoog nutrition medium (Murashige and Skoog).
1964 Production of first haploid plants from pollen grains of Datura (Guha and Maheshwari).
1968 Restriction endonuclease term coined to a class of enzymes involved in cleaving DNA (Meselson and Yuan).
1970 Selection of biochemical mutants in vitro by the use of tissue culture derived variation (Carlson).
1970 First achievement of protoplast fusion (Power et al.).
1970 Discovery of first restriction endonuclease from Haemophilus influenzae Rd. It was later purified and named HindIII (Smith).
1971 Preparation of first restriction map using HindIII enzyme to cut circular DNA of SV40 into 11 specific fragments was prepared (Nathans).
1971 Regeneration of first plants from protoplasts (Takebe et al.).
1972 First report of interspecific hybridization through protoplast fusion in two species of Nicotiana (Carlson et al.).
1972 First recombinant DNA molecule produced
using restriction enzymes (Berg et al.).

1972 Joining of two restriction fragments regardless of their origin produced by the same restriction enzyme by the action of DNA ligase (Mertz and Davis).

1972 Development of a procedure in which the appropriate enzyme can be added to fill in any single-stranded gap and the use of DNA ligase to join the two fragments thus giving rise to recombinant DNA (Lobban and Kaiser).

1972 Discovery of reverse transcriptase: In certain cancer-causing animal virus, genetic information flows in the reverse form (Temini).


1974 Cytokinins found capable of breaking dormancy in excised capitulum explants of Gerbera (Pierik et al.).

1974 Regeneration of haploid Petunia hybrida plants from protoplasts (Binding).

1974 Biotransformation in plant tissue cultures (Reinhard).

1974 Discovery that the Ti plasmid is the tumor inducing principle of Agrobacterium (Zaenen et al.; Larebeke et al.).

1975 Positive in vitro selection of maize callus cultures resistant to T toxin of Helminthosporium maydis (Gengenbach and Green).

1975 Development of the high resolution two-dimensional gel electrophoresis procedure, which led to the development of proteomics (O’Farrel).

1976 Shoot initiation from cryopreserved shoot apices of carnation (Seibert).

1976 Octopine and nopaline synthesis and breakdown found to be genetically controlled by the Ti plasmid of A. tumefaciens (Bomhoff et al.).

1977 Successful integration of the Ti plasmid DNA from A. tumefaciens in plants (Chilton et al.).


1977 Discovery of split genes (Sharp and Roberts).

1978 Somatic hybridization of tomato and potato resulting in tomato (Melchers et al.).

1979 Cocultivation procedure developed for transformation of plant protoplasts with Agrobacterium (Marton et al.).

1980 Use of immobilized whole cells for biotransformation of digitoxin into digoxin (Altermann et al.).

1980 Commercial production of human insulin through genetic engineering in bacterial cells (Eli Lilly and Co.).

1980 Restriction fragment length polymorphism (RFLP) technique developed.

1980 Studies on the structure of T-DNA by cloning the complete EcoRI digest of T37 tobacco crown gall DNA into a phage vector, thus allowing the isolation and detailed study of T-DNA border sequences (Zambryski et al.).

1981 Introduction of the term somaclonal variation (Larkin and Scowcroft).

1982 Incorporation of naked DNA by protoplasts resulting in the transformation with isolated DNA (Krens et al.).

1983 Polymerase chain reaction (PCR), a chemical DNA amplification process idea conceived (Kary Mullis).

1984 Transformation of tobacco with Agrobacterium; transgenic plants developed (De Block et al.; Horsch et al.).

1984 Development of the genetic fingerprinting technique for identifying individuals by analyzing polymorphism at DNA sequence level (Jeffreys).

1986 TMV virus-resistant tobacco and tomato transgenic plants developed using cDNA of coat protein gene of TMV (Powell-Abel et al.).
1987 Development of biolistic gene transfer method for plant transformation (Sanford et al.; Klein et al.).

1987 Isolation of Bt gene from bacterium (Bacillus thuringiensis) (Barton et al.).

1990 Formal launch of the Human Genome Project.

1990 Development of the random amplified polymorphic DNA (RAPD) technique (Williams et al.; Welsh and McClelland).

1991 Development of DNA microarray system using light directed chemical synthesis system (Fodor).

1995 Reporting by the Institute for Genomic Research of the complete DNA sequence of Haemophilus influenzae (Fleischmann et al.).

1995 Development of DNA fingerprinting by amplified fragment length polymorphism (AFLP) technique (Vos et al.).

1997 Sequencing of E. coli genome (Blattner et al.).

1998 Sequencing of the genome of a multicellular organism (Caenorhabditis elegans) (C. elegans sequencing consortium).

2001 Sequencing of human genome successfully completed (Human Genome Project Consortium and Venter et al.).