PERSPECTIVES IN PLANT BIOTECHNOLOGY

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ABSTRACT

The current status of biotechnology in plant improvement is reviewed. *In vitro* techniques have been successfully exploited for clonal propagation of a number of plant species, including forest trees. Meristem cultures in a number of crop plants have led to virus-free plants. Future application of biotechnology to plants include: isolation and maintenance of haploidy, early selection against biotic and abiotic factors, utilization of somaclonal variation, tissue gene banks, somatic hybridization, and genetic engineering.

PROGRESS in science depends on the advent of new technologies. Biotechnology, which has been referred to as a "revolution in applied biology", encompasses a broad spectrum of technologies for application to living organisms for the benefit of mankind. In the area of plant biotechnology, recent *in vitro* techniques have provided new methods for clonal propagation and for attempting somatic hybridization between species and genera that are difficult to cross by sexual means. Somatic hybridization, which involves fusions between non-gametic cells, and genetic engineering techniques have opened up possibilities for enlarging the genetic base and recovery of new combinations of genes. In addition, there are several other areas of biotechnology that hold potential for plant genetics, breeding and improvement. In this article the application of biotechnology to plants will be reviewed.

MICROPROPAGATION

Vegetative propagation has certain advantages over generative reproduction. It offers prospects not only for conservation of the genotype of the donor plant in its clones, but also for selection and maintenance of additive and nonadditive gene effects, thus increasing genetic gain. Through normal sexual reproduction only the additive gene effects can be utilized. On the other hand, the nonadditive gene effects arising from interaction of genes, are not normally transmitted through sexual reproduction. The nonadditive gene effects can give rise to exceptional individuals within superior families (in the outcrossed plants), and these can be captured, along with additive gene effects, by vegetative propagation or by crossing homozygous lines.

The conventional methods of vegetative propagation are by rooting of vegetative parts. Generally, stem cuttings, root suckers, needle fascicles, or meristematic leaves are used for propagation. However, the number of plants that can be propagated in a given season may be relatively small. The limiting factors for large scale vegetative propagation are often the inadequate supply of the improved genotype and the available space. Furthermore, in many tree species such as oak, beech, *Eucalyptus*, and most conifers, the woody cutting from the mature trees are generally difficult to root. In still others, such as aspens (*Populus tremula*, *P. tremuloides* and their hybrids) the root primordia may be lacking or difficult to induce in the woody cuttings. To overcome such problems, *in vitro* techniques offer prospects for large scale clonal propagation of plants. Micropropagation (vegetative propagation by *in vitro* techniques) has several advantages over conventional methods of vegetative propagation:

1. Higher multiplication rates.
2. Lower requirements for space.

3. Propagation throughout the year.
4. Greater degree of control over chemical and physical environmental factors.
5. Possibilities of rejuvenation from mature tissues.

Although initially callus cultures were employed for plant regeneration, now mostly organ cultures (shoot apex, bud meristem, embryo and cotyledon) are in vogue for rapid clonal propagation. A large number of plant species have been propagated by *in vitro* techniques[1,2]. However, these lists continue to expand. That is not to say that all plant species can be readily propagated by *in vitro* techniques. In fact, certain agricultural crops, such as legumes, and forest tree species are generally difficult to propagate by tissue culture. And tissue from mature broad-leaved tree species and conifers are still very difficult to grow and differentiate *in vitro*[3].

For large scale clonal propagation it is necessary to develop a micropropagation method(s) that is relatively simple, so that it can easily be adapted to nursery practices. It should give a high multiplication rate and be cost-effective. Most importantly it should ensure, to a large extent, the genetic stability of the propagules[4]. Although such an ideal micropropagation method may not be available for all plant species, but several aspects of this method have been worked out in a number of plant species, in particular in horticultural crops, and forest tree species[3].

Basically a micropropagation method employing bud meristem involves the following steps: 1) conditioning of the meristem, 2) growth and proliferation of microshoots, 3) rooting of microshoots and 4) transfer of plantlets into pots. This 4-step micropropagation method (figure 1), with minor modifications, has been employed for clonal propagation of a large number of plant species. This 4-step micropropagation method[5] has been variously simplified to reduce the number of steps in order to make the micropropagation method cost-effective (figure 1). From four, it has been possible to evolve an effective 2-step micropropagation method[6] involving meristem conditioning and proliferation of microshoots on one medium and rooting of microshoots directly in a soil-free potting mixture. By employing this 2-step micropropagation method, the cost can be reduced and also the exposure of tissues to unnatural *in vitro* conditions can be minimized. The 2-step micropropagation method has been successfully employed by us for rapid and large scale clonal propagation of aspens (*Populus tremula*, *P. tremuloides*, and their hybrids)[6,7]. The micropropagated plants are then hardened under controlled conditions, where relative humidity is gradually lowered, and later transplanted under field conditions[4–6]. The transition from test tube condition (almost 100 per cent relative humidity) to field conditions (approximately 50 per cent relative humidity or less) must be gradual, otherwise there would be a high mortality of plantlets.

SOMACLONAL VARIATION

For commercial micropropagation of plants it is essential that the propagules are "true-to-type". Although *in vitro* propagation techniques are expected to conserve the genotype of the donor plant in its clones, this is not always observed[8,9]. Variation which occurs in plants regenerated from cultured cells or tissues, termed somaclonal variation[10], has been observed for morphological, biochemical, and genetic traits. Somaclonal variation may either be transient or heritable (monogenic or polygenic). The value of somaclonal variation in crop plants has been adequately reviewed[10–12]. Here only a few examples will be mentioned. Useful variants have been detected in: 1) sugarcane[13,14] for high sucrose content and disease resistance, 2) potato[15,16] for growth habit, tuber colour, maturity date, tuber uniformity, and disease resistance, 3) rice[17,18] for tiller number, seed protein, and yield, 4) wheat[19,20] for grain colour, height, tiller number, and yield and 5) tomato[11] for fruit colour, fruit pedicel, and disease resistance.

PRODUCTION OF VIRUS-FREE PLANTS

Meristem cultures have not only been extensively employed for clonal propagation of plants,

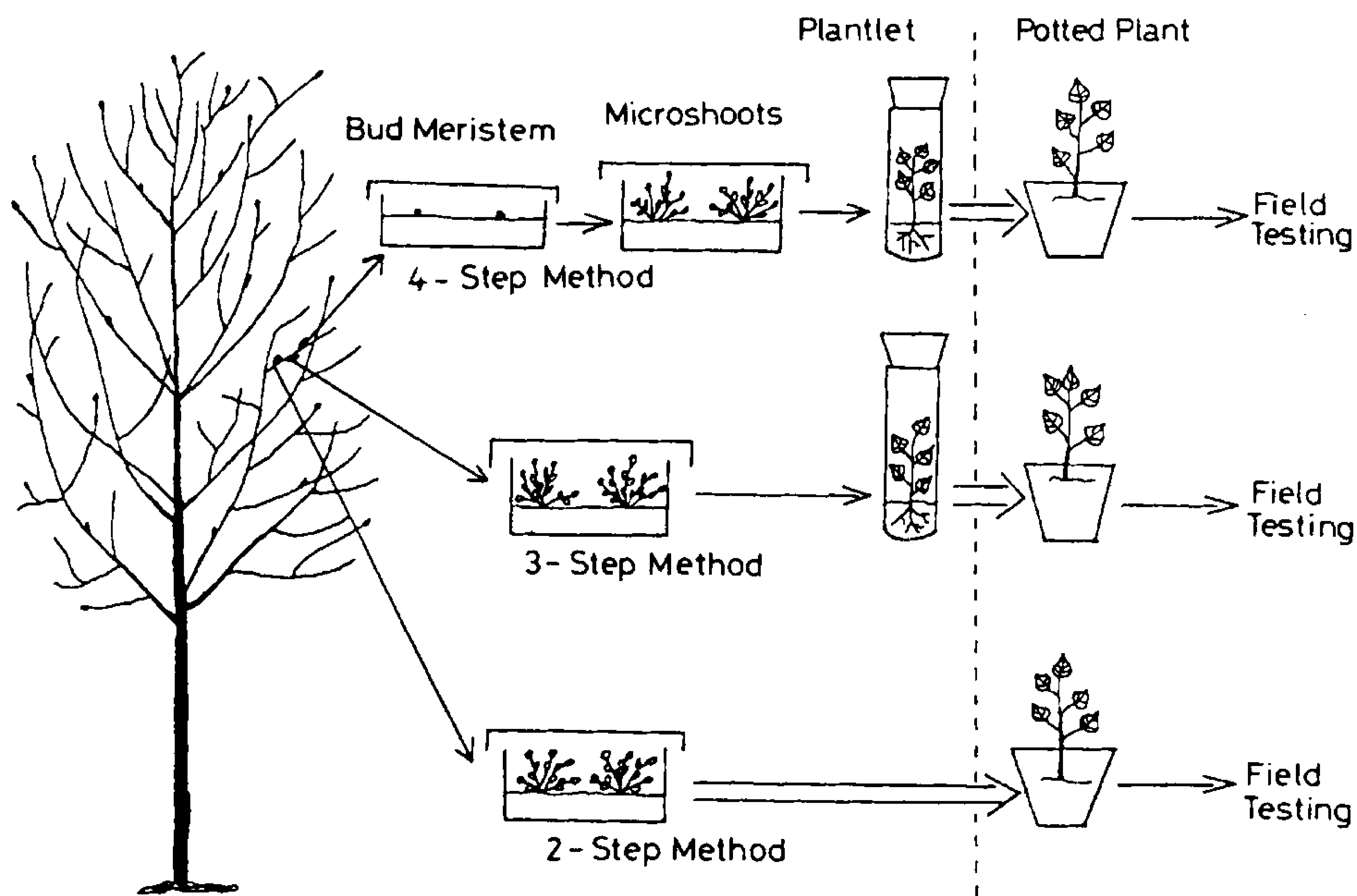

Figure 1. Diagram showing 4-step, 3-step, and 2-step micropropagation methods. By simplification of micropropagation procedure from 4-step to 2-step, the costs may be reduced and efficiency of clonal propagation improved.

but also for production of virus-free plants. In most of the systemic viral diseases, the meristem contains the least quantity of virus particles. In many plant species, virus-free plants have been produced by meristem culture alone or in combination with thermotherapy (heat treatment). Growing of virus infected plant at relatively high temperature (35 to 40°C) for 3 to 4 weeks generally restricts virus multiplication and movement, thus facilitating a greater number of cells in the meristem to be virus-free. This heat treatment is then followed by meristem culture for production of virus-free plants. A number of agricultural crops have been freed of virus pathogens by *in vitro* techniques, including potato, cassava, strawberry, sugarcane, pea, cauliflower, and banana[21].

Virus-free plants produced by *in vitro* techniques are only virus-free but not virus-resistant. Therefore, it is necessary to grow virus-free plants on locations where the virus in question is not prevalent. It should be pointed out that a number of viruses are difficult to eradicate by the existing thermotherapy-meristem culture techniques.

HAPLOIDY

Haploid tissue cultures have been established by culture of anther in many plant species, or by culture of female gametophyte (haploid endosperm) in conifers. Haploid cell lines are valuable for the production of diploid homozygous lines, and for the utility of these lines for the production of specific genotypes following hybridization. The application of anther culture for development of new varieties has been demonstrated in wheat and rice[22] and tobacco[23].

Haploid cultures from *Brassica napus* have been used for selection of disease resistant variants[24]. The development of techniques for individual pollen grains should facilitate mutant selection at a cellular level.

Haploid tissues are prone to undergo changes in the ploidy levels during cell proliferation *in vitro*. In most instances, diploids, or tetraploids and mixaploid cells may be present in the long term haploid cultures.

PRESERVATION OF GERMPLASM

Plant germplasm resources are threatened by man-made changes in the environment. These include destruction of habitats, indiscriminate felling of trees, and pollution of the environment. In particular, forest tree species are endangered because of long-range effects of unfavorable environmental conditions resulting from acidic depositions, unfavorable weather, as well as diseases and pests. In the circumstances, it is important to preserve the vast pool of genetic variability for later exploitation in the future plant breeding and improvement programmes. In recognition of this need, active programmes are under way for conservation of seed, pollen, and vegetative parts – in clonal orchards and tissue banks.

Germplasm can be stored *in vitro* by one of the two approaches: slow growth or cryopreservation. Plant tissue cultures are generally grown at temperatures ranging from 24 to 28°C, and require transfer to fresh media at least once a month. By lowering the temperature to 10°C or even upto 20°C, a significant reduction in the growth can be achieved. Meristem cultures have been stored at lower temperatures for upto one year or longer without transfer in potato[25], strawberry[26], grape[27], *Chrysanthemum*[28], and aspen[29]. Slow growth may also be achieved by addition of mannitol at concentrations around 0.2 M, as in potato[30]. However, slow growth approach seems to be suitable only for shoot tip cultures/plantlets, and that too for only a few years.

Cryopreservation involves storage of tissues at very low temperature, generally at −196°C (liquid nitrogen). At this temperature tissues can be stored for indefinite periods. The aim of storage at very low temperature is to halt metabolic processes, so that the material remains genetically stable for a long time. Cryopreservation could also bring considerable saving in the cost for equipment and personnel, and at the same time reduce the risk of loss of material due to contamination or failure of equipment. Freezing may be slow or rapid. In the slow freezing process, the cells are first cooled at a controlled rate to −30 to −40°C, and then stored in liquid nitrogen. On the other hand, rapid freezing is carried out by directly immersing the cells in liquid nitrogen. Dimethyl suloxide (DMSO) or glycerol have been used most frequently as cryoprotectants, that is, chemicals which protect the cells from freezing and thawing injury.

Meristems and cells from a number of plant species have been successfully cryopreserved[31,32]. Survival (effective growth) of cryopreserved cells lie around 50 per cent, and that of meristems (growth and regeneration) ranges from 0 to 100 per cent[32]. This might suggest that reliable techniques have to be developed for cryopreservation of different plant species/genotypes.

EARLY SELECTION TESTS

In vitro techniques can be employed for selection of genotypes resistance to bacterial or fungal parasites, toxins from parasites, herbicides, insecticides and chemical and gaseous pollutants. The selection tests may be carried out with callus or organ cultures for isolation of tissues resistant or tolerant against a given biotic or abiotic factor. The selection procedure normally takes months or years under greenhouse or field conditions. Under *in vitro* conditions, on the other hand, such selections can be carried out in a matter of weeks or at the most a few months. In a recent study, resistance against blister rust was investigated, in a relatively short time, in the callus cultures of white pine[33].

Following selection of resistant genotypes *in vitro*, they could be micropropagated and later tested under greenhouse and field conditions in

the presence of the harmful agent in question. It is not entirely inconceivable, that in certain cases, the observed resistance against a harmful factor in the test tube, may not necessarily hold under the natural *in situ* conditions.

SECONDARY PRODUCTS

Plant cells are small factories for the production of valuable chemicals, such as alkaloids, steroids, and terpenoids. A number of these compounds have medicinal properties. High yielding cell lines have been obtained by screening cultures in a large number of plant species[34]. However, large scale profitable production of secondary products has not been accomplished. Therefore, research has to be focused on refining cultural conditions, selection and stabilization of high yielding lines, and automation of the production procedures.

SOMATIC HYBRIDIZATION

In recent years protoplasts have been routinely isolated and cultured in a number of plant species[35,36]. In more than 60 species, plants have been regenerated from protoplasts[37], and this list is continuely expanding. However, cereal crops and woody plants are among the recalcitrant plant species that are still difficult to regenerate from the protoplast cultures.

Protoplasts from several herbaceous plant species have been fused to yield intraspecific, interspecific and intergeneric somatic hybrids[35,38,39]. Genetic and phenotypic variability seems to be a common feature of most protoplast regenerants and somatic hybrids, thus generating enormous amount of somaclonal variation. In the somatic hybrids, genetic variation appears to arise from changes/assortments in the nuclear genome as well as in the cytoplasmic orgenelles (mitochondria and chloroplasts). Thus somatic hybridization has potential for: a) production of fertile amphidiploids between sexually incompatible species, b) production of heterozygous lines within a species which is normally vegetatively propagated, and c) transfer of limited parts of the genomic elements, particularly cytoplasmic organelles.

Since protoplasts from most cereal crops and forest tree species are still difficult to grow and differentiate *in vitro*, it has not been possible to effectively utilize somatic hybridization techniques for production of new genotypes.

In addition to their potential for production of somatic hybrids between widely divergent or sexually incompatible plant species, protoplasts are also amenable to a variety of experimental manipulations, that are difficult with plant cells (having cell walls). These include uptake of nuclei, organelles, microorganisms, chromosomes or fragments of chromosomes, or macromolecules as DNA and RNA.

GENETIC ENGINEERING

Recent molecular techniques collectively known as "genetic engineering" have been employed for incorporation of foreign genetic material, DNA, into animal and plant cells. The gene transfers are carried out with the help of a plasmid-vector system or by direct microinjection of DNA into cells. Before attempting gene transfers, specific genes need to be characterized, sequenced, isolated, and cloned. A number of genes have been cloned in crop plants[40]. These include amylase gene from barley, seed storage protein genes and leghaemoglobin gene from soybean, and Thaumatin gene from Thaumatococcus. However, very little information is available regarding the structure and function of commercially important genes in the agricultural crops and forest tree species. As it turns out, most of the commercially important traits are governed by polygenes. The polygenes are highly complex and poorly understood. This might present certain problems, since recombinant DNA technology may work best for traits that are controlled by single genes or a block of closely linked genes. Nevertheless, there are several single gene controlled traits under study for genetic engineering.

There are at least four basic approaches to genetic manipulations by cloned genes: a) introduction of desirable alien genes from micro-

organisms into plant cells, b) removal, modification, and return of the modified gene into the same or different plant species, c) transfer of plant genes into bacteria for production of plant products on a commercial scale, and 4) transfer of genes from one microorganism to another that has a symbiotic association with a crop species.

Herbicide resistance is one area where attempts are being made to transfer resistance to crop plants through recombinant DNA technology. In this regard, it should be mentioned that major crops are already tolerant to certain herbicides. For example, corn is tolerant to Atrazine, and wheat and barley are tolerant to Glean. But these herbicides would kill just about anything else that is broad-leaved, including soyabean or other legumes, tobacco, or oil seed crops. Atrazine tolerance gene is located in the chloroplast DNA, and attempts to transfer this gene have not been entirely successful, since there is no way to genetically engineer chloroplast at the present time.

Herbicide resistance genes have also been identified in bacteria. Scientists at Calgene located in Davis, California have isolated mutant gene from Salmonella and *Escherichia coli* that confer glyphosate (Roundup) resistance, and Roy Chaleff and his associates at Du Pont, Wilmington, Delaware, have isolated a Glean tolerant gene from Salmonella. Both these research organizations have transferred these herbicide resistance genes to tobacco, by recombinant DNA technology, and are testing tobacco plants for resistance to Glean or Roundup under field conditions[41].

In the meanwhile researchers at Monsanto Agricultural Product Company, St. Louis, Missouri have inserted endotoxin gene from *Bacillus thurigenesis* in *Pseudomonas fluorescens*, which is a corn root colonizer. Endotoxin is a potent insecticide for lepidoteral pests. The recombinant bacterium may be freeze-dried and coated directly onto corn seeds before planting. Although recombinant strain of bacterium is a "prototype product" from Monsanto[42], it would be interesting to find out its performance under field conditions.

Thaumatin, a sweet-tasting plant protein, is considerably sweeter than sucrose on a weight-for-weight basis. Thaumatin gene has been cloned and expressed in *E. coli*[43]. Production of Thaumatin in fermenters on a commercial scale would certainly be a potential application of genetic engineering.

PERSPECTIVES

The immediate application of biotechnology to plants is in rapid and reliable clonal propagation of selected genotypes. Micropropagated plants from a number of plant species, including forest tree species, have been brought out of the laboratories into fields for commercial exploitation. Although organ cultures, in particular meristem cultures, have yielded propagules that exhibit relative genetic and morphogenetic stability, other types of cultures, for example callus cultures, may not always produce "true-to-type" plants. Somaclonal variation has been detected in a large number of plant species, and offers a new source of genetic variation for crop improvement. Tissue culture techniques may be utilized for early selection of variant regenerants that are resistant against biotic and abiotic factors, and for production of valuable medicinal compounds.

Meristem cultures have also been employed for production of virus-free plants, and for tissue gene banks. These tissue banks would be valuable for storage of endangered plant germplasm, in particular forest tree species. Man-made chemical pollutants are exerting strong selection pressure on the plant populations. Long-lived forest tree species are under cumulative stress of a variety of different abiotic and biotic factors, and therefore are showing the damage in the form of "Waldsterben" (dying forests) in Europe and North America. For endangered species two approaches could be explored: long-term tissue storage at $-196°C$, and induction of novel genetic variation to enrich the gene pool.

Somatic hybridization, which generated a lot of interest and enthusiasm for production of genetically stable somatic hybrids that cannot be produced by sexual hybridization, has not been

successfully exploited in the agricultural crops. This may be due to the fact that it has been difficult to regenerate plants from the protoplast cultures of these recalcitrant plant species. Production of *genetically stable* somatic hybrids may be yet another technological problem that needs to be resolved. Therefore, reliable and reproducible protocols for protoplast regeneration have to be developed in the crop plants and forest tree species.

Genetic engineering offers prospects for genetic modification of agricultural crops as well as the microorganisms associated with them. Application of recombinant DNA technology for plant improvement[44] will depend on the following:

a. Identification of the gene (DNA sequence) to be transferred to a commercial plant species.
b. Isolation and cloning of the gene of interest.
c. Transfer of gene via a vector system, or by direct microinjection of DNA into protoplast or cell.
d. Integration, transcription, and translation of the transferred gene in the recipient cell.
e. Multiplication (by sexual or asexual means) of the genetically engineered plant.

Results from the Biotechnology Firms in the United States of America indicate that it is possible to transfer specific genes from bacteria into plant cells. Whether such genes are integrated and function as integral genes of the host plant remains to be elucidated. Nevertheless, the economic and environmental benefits expected from the use of recombinant organisms in agricultural crops are potentially enormous. By employing recombinant DNA technology it might be possible to create novel genotypes that: a) are tolerant/resistant to diseases, pests, frost, salinity, herbicides, and pesticides, b) utilize fertilizer more efficiently so that there is little or no run off in the soil, c) exhibit increased photosynthetic efficiency, d) can fix atmospheric nitrogen, even if they are non-leguminous plants, and e) exhibit improved nutritional quality of seed storage proteins. At the same time, potential risks of genetic engineering in production of entirely new genotypes (for example, tuberless potato, or fruitless tomato) and microorganisms (that may become harmful) should be fully assessed.

In the final analysis, commercial application of biotechnology to agricultural crops and forest tree species will depend on the reliability and reproducibility of a technique, and the possibility of partial or complete automation, and not to forget the cost factors.

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