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REVIEW:

Isolation, Culture, and Fusion of Protoplasts: Problems and Prospects*

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Summary

The status of protoplast research in plants, including herbaceous and tree species, is reviewed. Methods for isolation, culture, and fusion of plant protoplasts are discussed. Protoplasts can be isolated and cultured from a wide range of higher plants, both herbaceous and tree species. However, sustained cell divisions leading to callus formation have been achieved only in a limited number of plant species. Plant regeneration from protoplasts is still a rare event and has been reported in some 12 genera, more than half of which belong to the family *Solanaceae*. Protoplast fusion leading to somatic hybrids in intraspecific interspecific, and intergeneric combinations have been reported in a limited number of plant species. Most somatic hybrids are genetically unstable and exhibit chromosomal and phenotypic variability. The availability of genetic variability in the somatic hybrids, and the fact that protoplasts are efficient experimental system for uptake of cell organelles, microorganisms, and macromolecules, offer far reaching possibilities for genetic modification and improvement of plant species.

Key words: Protoplast, isolation, culture, fusion, growth and differentiation, herbaceous and tree species, somatic hybrids, genetic variability, genetic modification, plant improvement.

Zusammenfassung

Es wird über den Stand der Protoplastenforschung bei Pflanzen, sowohl bei krautigen Arten als auch bei Baumarten berichtet. Methoden zur Isolierung, Kultur und Fusion von Protoplasten werden diskutiert. Bei einer Vielzahl von krautigen Arten und Baumarten ist die Isolierung und Kultur von Protoplasten möglich. Jedoch ist bei einer geringen Zahl von Arten die Bildung von Kallus, die nach anhaltender Zellteilung entstehen, erreicht worden. Die Regeneration von ganzen Pflanzen aus Protoplasten ist immer noch ein seltenes Ereignis und ist für Arten von 12 Gattungen berichtet worden, mehr als die Hälfte davon gehört zur Familie der *Solanaceae*. Von Protoplastenfusionen, die zu somatischen Hybriden innerhalb und zwischen Arten sowie zwischen Gattungen führen, sind nur in wenigen Arten berichtet worden. Die meisten somatischen Hybriden sind genetisch instabil und zeigen chromosomale und phänotypische Variabilität. Die Verfügbarkeit genetischer Variabilität in somatischen Hybriden und die Tatsache, daß Protoplasten als Versuchssysteme für die Aufnahme von Zellorganellen, Mikroorganismen und Makromolekülen geeignet sind, bieten viele Möglichkeiten für genetische Modifikationen und Verbesserungen von verschiedenen Pflanzenarten.

I. Introduction

Recent advances in organ, tissue, cell, and protoplast culture technology have opened up new avenues for con-

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ducting basic as well as applied genetic research in higher plants at the cellular level and have provided powerful new tools in the hands of biologists for generating, selecting, and propagating novel and economically important plant varieties. Although our understanding of basic cellular processes has been considerably enhanced through the in vitro culture of a variety of plant cells, progress in the application of tissue culture technology to remedy specific problems or in "genetic engineering" to produce economically important plant material has been relatively slow. This may be partly because cells or tissues from a large number of crop plants and forest tree species may be unresponsive to in vitro conditions as now provided, and partly because the tissue culture manipulations required to modify the genetic content of plant cells are still in early stages of development. Nevertheless, it is now possible to achieve rapid clonal propagation of plants, especially herbacious species and a few tree species through tissue culture technology. Attempts have been made to obtain new combination of genes by fusion of somatic cells of diverse species or by incorporation of foreign genetic information into cells of selected host plants. For the purposes of genetic modification of plant cells, protoplasts or naked wall-less plant cells, can serve as valuable experimental materials. Protoplasts are amenable to a variety of experimental manipulations that are generally difficult or not possible with intact plant cells, such as uptake of cell organelles, microorganisms, or foreign genetic material, or for the production of parasexual hybrids of widely divergent species that are sexually incompatible.

This review deals with the isolation, culture, and fusion of protoplasts in plants, including tree species, and critically examines the status of protoplast research in relation to genetic stability of somatic fusion products and its prospects in plant improvement.

II. Isolation of Protoplasts

A. *Source of Protoplasts*

Protoplasts can be isolated from a variety of plant tissues and organs, including leaves, shoot apices, roots, fruits, coleoptiles, aleurone layer of cereal grains, root nodules, microspore mother cells, microspore tetrads, and pollen tubes (see review Vasil and Vasil ,1980). Protoplasts have also been recently isolated from pollen grains of a gymnosperm (Duhoux, 1980), and an angiosperm (Radenbaugh *et al.*. 1980), although angiosperm pollen grains having a thick exine are not easily amenable to cell wall degregation procedures. In addition, protoplasts have also been isolated from plant callus and suspension cells. Isolating protoplasts from cultured cells has certain advantages as these cells are grown under aseptic and carefully controlled nutritional and physical conditions, and in many cases the requirements for their growth and morphogenesis are already known. Mesophyll tissues from leaves are, however, the most commonly used source of protoplasts. Leaves from young green-house grown plants or shoots grown *in vitro* provide a reasonably good protoplast-source material.

B. *Mechanical Methods*

Protoplasts have been isolated by mechanical methods for a long time. As early as 1892 Klercker attempted to isolate viable plant protoplasts by mechanical methods. The mechanical methods basically involve strip-cutting of plasmolysed plant tissues followed by induced osmotic swelling to release the protoplasts. The number of protoplasts obtained by mechanical methods is rather limited, and the procedure can be adapted for only a few types of tissues, which may not be necessarily suitable for continued growth in culture. The mechanical method is used only occasionally, but has a merit in that the unknown effects of macerating and cell wall degredation enzymes on protoplast and its contents are eliminated. A combination of mechanical and enzymatic procedures for isolation of protoplasts (Harada, 1973; Bui-Dang-Ha and Mackenzie, 1973) may retain the merit of both methods.

C. *Enzymatic Isolation*

One of the important early contributions to modern protoplast research was the development of enzymatic procedures for the isolation of protoplasts by Cocking (1960). Cocking used a relatively crude cellulase preparation from the fungus *Myrothecium verrucaria* to isolate protoplasts from tomato roots. Further modification of this procedure made it possible to obtain viable protoplasts in large numbers from a variety of plant tissues. A number of potent but crude enzyme preparations for maceration and cell wall digestion have become available in the last years. The most commonly used enzyme preparations include: cellulase from the fungus *Trichoderma viridae,* Macerozyme, from the fungus *Rhizopus* rich in pectinases and hemicellulases, and Driselase, from a basidiomycete, rich in cellulase and pectinase. In most instances the crude commercial enzyme preparations have been used without any further purification but some workers partially purify their enzymes by gel filtration. Very highly purified or crystalline enzyme preparations are less suitable for protoplast isolation, as these are unable to break down the chemically and structurally complex plant cell wall.

For the enzymatic isolation of protoplasts, two principal methods have been employed. In a sequential procedure, surface-sterilized young leaves are plasmolyzed and cut into small pieces following the removal of the lower epidermis. The plasmolyzed leaf pieces are next macerated with macerozyme, which releases the mesophyll cells. The mesophyll cells are then washed and incubated in cellulase to digest the cell wall and release the protoplasts (Takebe *et al.*, 1968). In the second, one-step method, which is most commonly used, a mixture of macerozyme (rich in pectinase) and cellulase is used for the maceration of cell as well as digestion of the cell walls to release the protoplasts (Power and Cocking, 1970). Further details of the protoplast isolation procedures can be found in "Plant Tissue culture Methods" edited by Gamborg and Wetter (1975), and in reviews by Bajaj (1977), Vasil and Vasil (1980), Gamborg et al. (1981).

Normally in plant cells, the protoplasts are protected from osmotic change by cell wall. Without the cell wall, the protoplasts become highly susceptible to changes in the osmotic environment. Therefore, the protoplasts must be protected against osmotic swelling and bursting during and after the removal of cell wall. Generally, this is easily accomplished by inclusion of osmotic stabilizers, as 0.4—0.8 M mannitol and/or sorbitol, glucose or sucrose in the enzyme solution, or with a combination of ionic as well as nonionic osmotica.

After the digestion of the cell walls, the protoplasts must be removed from enzyme solution as soon as possible. In most cases much the debris can be satisfactorily removed by filtration through stainless steel sieve or nylon filters or Mirocloth, followed by washing on Milipore filters or by short duration (1—5 min) low speed centrifugation (100 × g) to yield viable preparation of protoplasts. Viability of the protoplasts can be determined in various ways. Of course, the best test for viability is the regeneration of cell wall and the onset of cell divisions. The presence of active cytoplasmic streaming in freshly isolated protoplasts is a good indication of viability. Vital stains, such as Evans blue or flouorescein blue, have been employed to differentiate between viable and nonviable protoplasts (WIDHOLM, 1972; KANAI and EDWARDS, 1973; GLIMELIUS *et al.*, 1974; LARKIN, 1976).

III. Culture of Protoplasts

A. *Culture Media*

Following isolation, protoplasts can be suspended in suitable media and can be cultured in various ways. The nutrient requirements of isolated protoplasts are very similar to those of cultured cells and tissues. In the absence of cell wall, protoplasts tend to be very efficient in the uptake of nutrients from the medium. For this reason, the nutrient media used for culture of protoplasts are somewhat modified to contain reduced levels of inorganic substances. Various modifications of nutrient media developed by MURASHIGE and SKOOG (1962) and GAMBORG *et al.* (1968) have been successfully employed for the culture of protoplasts. KAO and MICHAYLUK (1975) and KAO (1977) used rather complex media for the culture of single protoplasts of *Vicia hajastana* and for fused protoplasts of tobacco and soybean respectively. Important ingredia in all the nutrient media are osmotic stabilizers and plant growth substances. Once cell wall regeneration and sustained cell activity have been initiated the cells can be transfered to media without osmotic stabilizers. For effective cell wall regeneration and cell division activity, several other factors must also be controlled. Important among these are the density of the protoplasts in the culture medium (1×10^3 to 1×10^5/ml), temperature (25°—30° C) and light, since some protoplast grow better in dark, while for others low light intensity appears to be essential. The inclusion of non-dividing but metabotically active, X-ray irradiated protoplasts (RAVEH and GALUN, 1975) or "nurse culture" (MENCZEL *et al.*, 1978) in the agar-medium apparently supports the growth of protoplasts.

B. *Methods of Culture*

Protoplasts are generally cultured in any of the following methods. Essentially these are modifications of the procedures established for the culture single cells or cell suspensions.

1. *Suspension or Drop culture:* Protoplasts are suspended in a medium at a density of about 1×10^5/ml and are cultured in a shallow layer (about 2 ml) in 25—50 ml flasks. The flasks may be kept stationary or shaken at low speed. KAO *et al.* (1971) developed an important modification of the suspension culture technique, called the liquid droplet method, which has been used successfully (GAMBORG and WETTER, 1975). Several 50 µl drops of protoplast suspension are placed in a petri dish, which is than sealed with parafilm and incubated.

2. *Plating:* Protoplasts suspended in a liquid medium are mixed gently but quickly with an equal amount of medium prepared in agar and kept at about 45° C in a water bath (NAGATA and TAKEBE, 1971). Small amounts of the medium are then poured into petri dishes, sealed and incubated. Several minor modifications of this method are also common.

3. *Microculture Chambers:* Microculture chambers are prepared by placing a droplet of 30 µl of nutrient medium containing one to several protoplasts on a slide and is enclosed by a cover glass resting on two other glasses placed on either side of the drop (VASIL and VASIL, 1973; DURAND *et al.*, 1973). The cultures are sealed with sterile parafin oil and incubated. Single protoplasts have also been cultured in Cuprak dishes (KAO, 1977; GLEBA, 1978). These procedures are especially useful for closely observing the growth of the protoplasts during culture.

IV. From Protoplasts to Plants

The first visible signs of growth in protoplasts involve the rearrangements of most cell organelles that become aggregated around the nucleus, and the formation of a new cell wall. The formation of cell wall starts minutes after isolation and culture of protoplasts (WILLISON and COCKING, 1975; WILLIAMSON *et al.*, 1977).

The first mitotic division in the reorganized cell takes place after 2—7 days of culture. Protoplasts isolated from nondividing cells, such as mesophyll, take longer to undergo first cell division in culture compared to those isolated from rapidly dividing cells from tissue culture (VASIL and VASIL, 1974). Multicellular clumps or cell colonies are generally formed within 1 to 3 weeks in culture, and these can be transfered to appropriate media either for callus formation or plantlet formation. The first plants regenerated from isolated protoplasts in culture were those from *Nicotiana tabacum* (TAKEBE *et al.*, 1971; NAGATA and TAKEBE, 1971). Subsequently a number of plant species have been regenerated from isolated protoplasts *(Table 1)*. An examination of *Table 1* reveals that, with a few exceptions, bulk of the regenerated species come from the family *Solanaceae*. Crop plants or economically important plant species are poorly represented and not a single tree species has so far been regenerated from isolated protoplasts. Nevertheless, these renerations studies listed in *Table 1* are enough to demonstrate the totipotency of cultured protoplasts. The number of species in which plants — or even sustained cell divisions — can be obtained is rather limited, and therefore major efforts are needed to expand this list to include a wider variety of plants, particularly important crop plant species belonging to the cereals, the legumes, and the tree species. At least some progress has been made in isolation and culture of protoplasts from some crop plants (see reviews by VASIL *et al.*, 1979; GAMBORG *et al.*, 1981).

V. Fusion of Protoplasts

There is widespread interest in plant protoplast research because it offers possibilities to carry out somatic cell fusions, where sexual crosses are incompatible, as well as for studies involving uptake of macromolecules, cell organelles, and microorganisms. These attributes make plant protoplast an ideal experimental system for studies on somatic cell genetics and for development of parasexual methods for the genetic modification of cell for plant improvement (VASIL *et al.*, 1979).

Table 1. — Plant regeneration from cultured protoplasts.

Species	References
Asparagus officinalis	Bui-Dang-Ha & Mackenzie, 1973
Atropa belladonna	Gosch et al. 1975
Brassica napus	Kartha et al. 1974
Brassica napus (haploid)	Thomas et al. 1976
Bromus inermis	Kao et al. 1973
Datura metel (haploid & diploid)	Schieder, 1977 a
Datura meteloides (haploid & diploid)	Schieder, 1977 a
Datura innoxia (haploid & diploid)	Schieder, 1975
Daucus carota	Grambow et al. 1972; Dudits et al. 1976 a
Hyoscyamus muticus	Lörz et al. 1979
Medicago sativa	Kao & Michayluk, 1980; Dos Santos et al. 1980
Nicotiana acuminata	Bourgin et al. 1979
Nicotiana alata (haploid)	Bourgin & Missonier, 1978
Nicotiana debneyi	Scowcroft & Larkin, 1980
Nicotiana glauca	Bourgin et al. 1979
Nicotiana langsdorffii	Bourgin et al. 1979
Nicotiana otophora	Banks & Evans, 1976; Bourgin et al. 1979
Nicotiana paniculata	Bourgin et al. 1979
Nicotiana plumbaginifolia	Bourgin et al. 1979
Nicotiana sauveolens	Bourgin et al. 1979
Nicotiana sylvestris	Bourgin et al. 1976; Nagy & Maliga, 1976; Banks & Evans, 1976
Nicotiana sylvestris x N. otophora-F_1	Banks & Evans, 1976
Nicotiana tabacum	Nagata & Takebe, 1971
Nicotiana tabacum (haploid)	Ohyama & Nitsch, 1972
Nicotiana tabacum x N. otophora-F_1	Banks & Evans, 1976
Pennisetum americanum	Vasil & Vasil, 1980
Petunia axillaris	Power et al. 1976 a
Petunia hybrida	Durand et al. 1973; Frearson et al. 1973; Vasil & Vasil, 1974
Petunia hybrida (haploid)	Binding, 1974
Petunia hybrida x P. parodii-F_1	Power et al. 1976 b
Petunia inflata	Power et al. 1976 a
Petunia parodii	Hayward & Power, 1975
Petunia parviflora	Sink & Power, 1977
Petunia violacea	Power et al. 1976 a
Ranunculus scleratus	Dorion et al. 1975
Solanum dulcamara	Binding & Nehls, 1977
Solanum melongena	Saxena et al. 1981
Solanum tuberosum	Shepard & Totten, 1977; Binding et al. 1978
Trifolium repens	Gresshoff, 1980

The fusion of protoplasts can occur spontaneously or it can be induced by mechanical, chemical and physical means.

A. *Spontaneous Fusion of Protoplasts*

Spontaneous protoplast fusions can occur in the mechanically or enzymatically isolated protoplasts. Spontaneous fusions in mechanically isolated protoplasts are known since 1909 (KÜSTER, 1909), but these fusions are uncontrolled, rare, and generally nonreproducible (MICHEL, 1937; HOFMEISTER, 1954). Spontaneous fusion of two or more adjoining protoplasts is common during enzymatic isolation of protoplasts owing to the expansion of their common plasodesmatal connections (POWER *et al.*, 1970; WITHERS and COCKING, 1972; SCHIEDER, 1976). Similar spontaneous fusion is also cery common during preparation of protoplasts from meiocytes (ITO, 1973; ITO and MAEDA, 1973).

B. *Induced Fusion of Protoplasts*

Isolated protoplasts can be induced to undergo fusions by several different procedures. Some of these are given below:

1. *Mechanically Induced Fusion:* This method involves bringing isolated protoplasts into intimate contact through micromanipulations and prefusion micropipette (MICHEL, 1937; SCHENK and HILDEBRANDT, 1971). The micropipette is partially blocked within one millimeter of the tip by a sealed glass rod, so that the protoplasts are retained and remain compressed by the flow of the liquid. By this method, occassional protoplast fusion products were observed in *Glycine max, Arachis hypogea,* and *Vinca rosea.*

2. *Chemically Induced Fusion:* At the turn of the century KÜSTER (1909) observed for the first time that mechanically isolated onion protoplasts plasmolysed with sodium salts underwent fusion on deplasmolysis. Employing the same principle, POWER *et al.* (1970) induced intra- and interspecific fusion of protoplasts following treatment with sodium nitrate. CARLSON *et al.* (1972) also employed sodium nitrate to fuse protoplasts of *Nicotiana glauca* with *Nicotiana langsdorffii* to produce parasexual hybrid plants for the first time. Later studies have shown that sodium nitrate-induced fusions are generally limited to protoplasts with near-identical osmotic characteristics, and that it has adverse effect on the viability of protoplasts and at best produces fusions in a very limited number of protoplasts (POTRYKUS, 1973; KELLER and MELCHERS, 1973; BURGESS and FLEMING, 1974). For these reasons it became necessary to develop procedures that would ensure high protoplast fusion frequencies. KELLER and MELCHERS (1973) obtained fusion frequencies of more than 25% by incubating isolated protoplasts in a nutrient media containing high concentrations of Ca^{2+} ions at a high temperature (37° C) and in a highly alkaline environment (pH 10.5). Another successful and now commonly used method for fusion of protoplasts was developed by KAO and his associates (KAO and MICHAYLUK, 1974; CONSTABEL and KAO, 1974) and WALLIN *et al.* (1974). This method is in part based on the use of Ca^{2+} ions, but with lower concentrations. A key step in the method involves agglutination of protoplasts with the aid of a high molecular weight polyethylene glycol (PEG). Protoplasts treated with PEG solution containing Ca^{2+} ions fuse during the elution and/or dilution of PEG with the protoplast culture medium. The fusion treatment must follow immediately after the removal of protoplasts from the enzyme solution for maximum agglutination and fusion, as cell wall regeneration occurs very rapidly. PEG-induced fusion is nonspecific and therefore useful for intra- and interspecific or intergeneric *(Table 2)* or interkingdom fusions between plant cells and animal cells *(Table 3).* A combination of the high Ca^{2+}, high temperature, and high pH method of KELLER and MELCHERS (1973) and the PEG procedure of KAO and MICHAYLUK (1974) may yet give the best results, as it not only ensures high fusion frequencies, but also seems to improve the survival of fusion products (BURGESS and FLEMING, 1974; KAO *et al.*, 1974; WALLIN *et al.*, 1974). With the aid of above procedure, it should now be possible to fuse protoplasts of any two higher plant species irrespective of their taxonomic relationship, or for fusion of plant protoplasts with animal cells.

Although the above methods may be highly effective in inducing high frequency of protoplast fusion, the effects of high concentration of Ca^{2+}, high temperature, high pH, and high molecular weight PEG on protoplast and the genetic apparatus remain largely unknown. For this reason, the mechanical method of protoplast fusion involving micromanipulation may have a merit because the unknown effects of PEG-related fusion procedures on protoplast are eliminated. If perfected, the micromanipulation method may, in principle, lessen our dependence upon the development of elaborate selection systems for the recovery of somatic fusion products.

In fusion experiments involving protoplasts from two different species, five different types of protoplasts are

Table 2. — Somatic hybridizations in plants.

Parents	References
A. Intraspecific somatic hybrids	
Nicotiana tabacum "v" + "s"	Melchers & Labib, 1974
Nicotiana tabacum "albino" + "susu"	Gleba et al. 1975
Nicotiana tabacum "albino" + "Ws1Ws2"	Kameya, 1975
Nicotiana tabacum "cms" + "fertile"	Belliard et al. 1977
Nicotiana tabacum "nia" + "cnx"	Glimelius et al. 1978
Nicotiana tabacum "albino" + "cms"	Gleba, 1979
Nicotiana tabacum "SR1" + "tumor"	Mullems et al. 1979
Datura innoxia "albino" + " albino"	Schieder, 1977 b
B. Interspecific somatic hybrids	
Nicotiana glauca + *Nicotiana langsdorffii*	Carlson et al. 1972; Smith et al. 1976; Chupeau et al. 1978
Petunia hybrida + *Petunia parodii*	Power et al. 1976 b
Nicotiana tabacum + *Nicotiana sylvestris*	Melchers, 1977
Daucus carota + *Daucus capillifolius*	Dudits et al. 1977
Nicotiana sylvestris + *Nicotiana knightiana*	Maliga et al. 1977
Nicotiana tabacum + *Nicotiana knightiana*	Maliga et al. 1978
Nicotiana tabacum + *Nicotiana rustica*	Nagao, 1978
Nicotiana tabacum "cms" + *Nicotiana sylvestris*	Zelcer et al. 1978
Petunia parodii + *Petunia inflata*	Cocking, 1978
Datura innoxia + *Datura stramonium*	Schieder, 1978
Datura innoxia + *Datura discolor*	Schieder, 1978
Nicotiana tabacum + *Nicotiana glauca*	Evans et al. 1980
Datura innoxia + *Datura sanguinea*	Schieder, 1980
Datura innoxia + *Datura candida*	Schieder, 1980
Petunia parodii + *Petunia parviflora*	Power et al. 1980
C. Intergeneric somatic hybrids	
Arabidopsis thaliana + *Brassica campestris*	Gleba & Hoffmann, 1978, 1980
Solanum tuberosum + *Lycopersicon esculentum*	Melchers et al. 1978
Aegopodium podagraria + *Daucus carota*	Dudits et al. 1979
Datura innoxia + *Atropa belladonna*	Krumbiegel & Schieder, 1979
Daucus carota + *Petroselinum hortense*	Dudits et al. 1980
D. Intergeneric somatic fusions (cell lines)	
Petunia hybrida + *parthenocissus tricuspidata*	Power et al. 1975
Petunia hybrida + *Atropa belladonna*	Gosch & Reinert, 1976, 1978
Nicotiana glauca + *Glycine max*	Kao, 1977
Glycine max + *Vicia hajastana*	Constabel et al. 1977
Petunia hybrida + *Vicia faba*	Binding & Nehls, 1978

found at the end of the fusion treatment *(Figure 1)*: unfused protoplasts of the two parental species, fusion products of genetically identical protoplasts, that is, intraspecific fusions (homokaryotic fusions), and fusion products of protoplasts of the two different species (heterokaryotic fusions). Nuclear fusion and formation of true hybrid cell in most experiments is at best an infrequent event and does not necessarily follow protoplast fusion. Unfortunately, no methods are known that will enhance nuclear fusion in the heterokaryon. However, nuclear fusion leading to true hybrid cell and hybrid plant formation following heterokaryotic protoplast fusion has been conclusively demonstrated in several plant species *(Table 2)*.

Table 3. — Somatic fusion experiments involving animal cells and plant protoplasts.

Cellular source	References
Chicken + Yeast	Ahkong et al. 1975
Human (HeLa) + Tobacco (GGLL)	Jones et al. 1976
Human (HeLa) + Carrot	Dudits et al. 1976 b
Human (HeLa) + Haplopappus	Lima-de-Faria et al. 1977
Chicken + Tobacco	Willis et al. 1977
Amphibia + Carrot	Davey et al. 1978
Human lymphocyte + Arabidopsis	Sidorov et al. 1978
Hamster + Tobacco (GGLL)	Mastrangelo & Mitra, 1981

HeLa- cell line derived from cervical cancer of a black female Henrietta Lack; Henrietta Lack died in fifties, but her (cancerous) cells have achieved immortality.
GGLL- tumor-forming amphidiploid *Nicotiana glauca-langsdorffii*.

VI. Selection of Somatic Fusion Products

As shown in *Figure 1*, a variety of different products, including homokaryotic and heterokaryotic fusion products, may be formed in fusion experiments involving protoplasts from two different species. Therefore, selection of true somatic hybrid products is a key problem in parasexual hybridization. The formation of true hybrid cells does not assure the recovery of hybrid tissues and plants, for it is generally the unfused protoplasts of both parental species, as well as homokaryotic products, that grow vigorously, and the few hybrid cells and their descendents are soon lost. Recovery of hybrid cells from cultures growing on normal nutrient media is highly unlikely. Selective nutrient media- which would promote a vigorous growth of the hybrid products or preferentially allow the growth of only the hybrid cells, would assure the recovery of somatic hybrids. Unfortunately, selective media for the recovery of hybrid cells are known only in a few instances, Therefore, it seemed necessary to develop genetic, biochemical and morphological markers, in addition to the *in vitro* growth requirements, for the recovery of somatic hybrid products. It is through the employment of these selection procedures that a number of perasexual hybrids in plants have been recovered *(Table 2)*.

A. *Vigorous Growth*

Selection system based on vigorous growth pattern of the somatic hybrid cells has the advantage in that this methods can be directly applied at an early stage of hybrid tissue development. Further, this selection system is independent of the complementation selection system requiring the presence of mutants, or the differential growth pattern of the parental cell lines. On a defined medium, the somatic hybrid fusion products either grow more vigorously compared to the cells from parental lines, or, only the somatic hybrid cells can grow well on a medium, whereas the parental cells are unable to grow at all on the same medium. This selection procedure, based on the growth pattern of the hybrid callus, has been employed for the recovery of somatic hybrids between *Nicotiana glauca* and *Nicotiana langsdorffii* (Carlson *et al.*, 1972; Smith *et al.*, 1976; Chupeau *et al.*, 1978) and certain somatic hybrids in *Datura* (Schieder, 1978, 1980).

B. *Complementation Selection*

This selection procedure requires the presence of non-allelic recessive mutants, affecting the same trait, in the parental cell lines. The fusion of protoplast from two non-allelic mutants, for example affecting chlorophyll synthesis, leads to somatic fusion products expressing the wildtype phenotype with respect to chlorophyll synthesis. Likewise the non-allelic auxotroph mutants can also be employed for isolation and recovery of wildtype autotroph somatic hybrids. Non-allelic chlorophyll mutants have been effectively utilized for the recovery of somaic hybrid plants in a number of the parasexual hybrids involving *Nicotiana, Petunia,* and *Datura (Table 2)*. A combination of the chlo-

rophyll-deficient mutant which is an autotroph, with wild-type green but auxotroph mutant has also been used for the recovery of somatic hybrids.

C. *Resistant Factors*

This selection method is based on the availability of dominant drug resistant factors in the cell lines involved in the somatic fusions. Each cell line is resistant to a specific drug and when protoplasts from these two lines are fused, somatic hybrids products are recovered based on double resistance to the two drugs in question. Such a selection system has been employed for the recovery of somatic hybrids between *Nicotiana sylvestris* + *Nicotiana knightiana* (Maliga *et al.*, 1977), and *Petunia parodii* + *Petunia hybrida* (Power *et al.*, 1976; *Table 2B*). A combination of drug resistance and auxotrophy of tumor cells was used as a basis of selection of somatic hybrids involving *Nicotiana tabacum* cell lines (Wullems *et al.*, 1979; *Table 2A*).

D. *Morphology*

Morphology of callus, plants and chromosomes as a basis of selection of somatic hybrid cell lines and plants has been successfully employed in some cases. For example intergeneric somatic hybrids plants between tomato *(Lycopersicon esculentum)* + potato *(solanum tuberosum)* were recovered on the basis of abnormal morphology of plants (Melchers *et al.*, 1978; *Table 2C*). Isolation of intergeneric somatic hybrid cell lines between *Vicia faba* + *Petunia hybrida* (Binding and Nehls, 1978) was based on the intermediate morphology of callus, and between *Petunia hybrida* + *Atropa belladonna* (Gosch and Reinert, 1976, 1978) was based on the differential chromosome morphology of the parental species *(Table 2D)*.

E. *Mechanical-Visual Method*

This method is based on the visual difference between the cell types involved in fusion and the recovery of their fusion products by mechanical means using micropipettes. Such a selection procedure was first introduced by Kao (1977) who fused green mesophyll protoplasts of *Nicotiana glauca* with callus-derived colorless protoplasts of *Glycine max*, and then isolated individual heterokaryons after 24—48 hours with micropipettes and cultivated them in Cuprak dishes containing many individual wells. In this way Kao isolated 20 hybrid cell lines. The same method was used for the isolation of somatic hybrids between *Arabidopsis thaliana* and *Brassica campestris* (Gleba and Hoffmann,

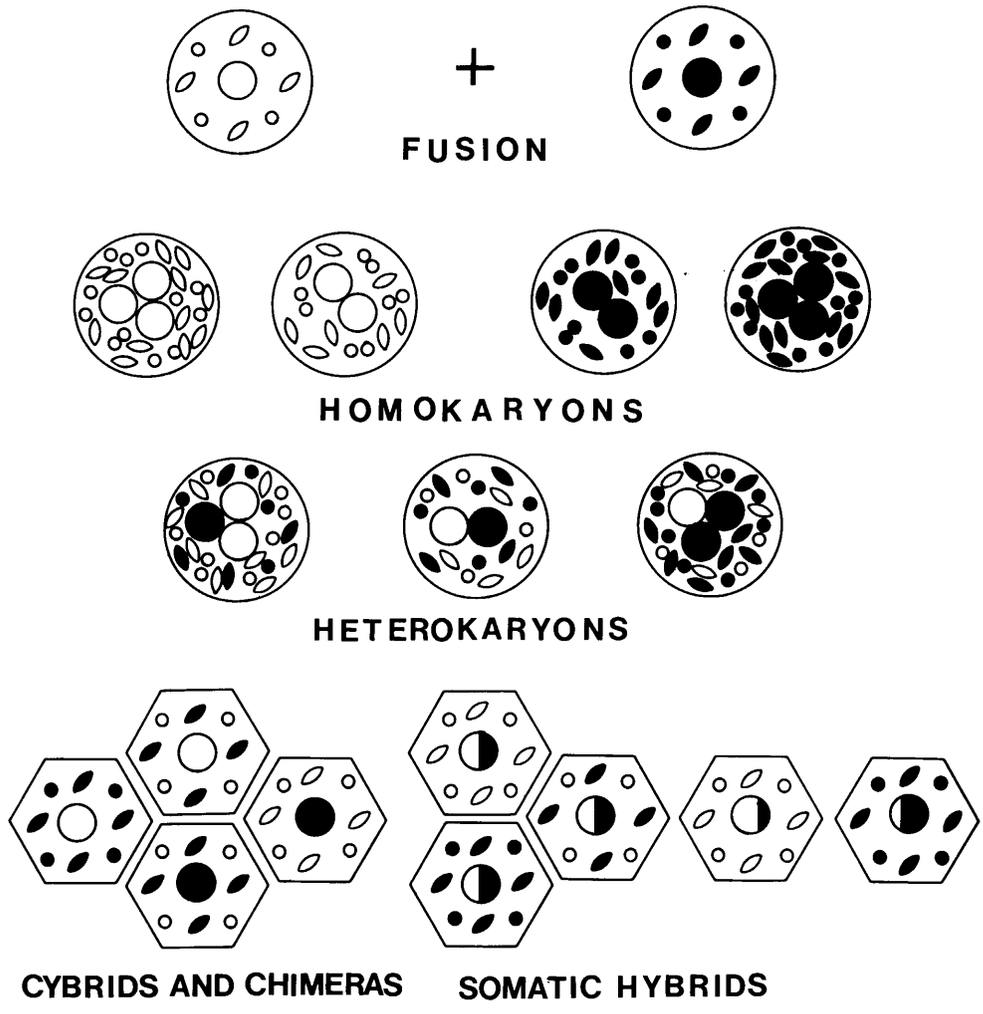

Figure 1. — Diagrams showing a range (but not all) of somatic fusion products possible following fusion of protoplasts of two different strains or species. Within the cells, nuclei are represented by large circles, mitochondria by small circles, and chloroplasts by elliptical figures. Nuclear fusions, segregation of nuclear and cytoplasmic components, and formation of somatic fusion products are illustrated. (Modified after Schieder and Vasil, 1980).

1978, 1980; *Table 2C)* to produce the so-called "Arabidobrassica".

The mechanical method, involving micropipettes for the recovery of somatic fusion products, has a distinct advantage over other selection procedures as it does not require the presence of mutants, which may not be readily available in all plant species. This is certainly true of a large number of economically important plants, including cereals, legumes, and tree species. Therefore, perfection of mechanical-micromanipulation methods, combined with fusion of visually different protoplasts, for example, obtained from leaf mesophyll and callus tissues, or from leaf mesophyll and other parts of the plant lacking chlorophyll, such as roots, or having another color pigment, would certainly offer opportunities for the recovery of somatic hybrid products in a variety of different plants, in which mutants for complementation selection are not readily available.

VII. Parasexual or Somatic Hybrids

After the discovery that protoplasts can be agglutinated and induced to fuse with the aid of polyethylene glycol, protoplasts have been fused between cell lines of the same species, or derived from different species or genera, or even plant protoplasts have been fused with animal cells to produce heterokaryons *(Table 3),* although plant + animal fusions products do not go beyond the stage of cell fusion. With the exception of one somatic plant hybrid involving fusion of haploid cell lines in *Nicotiana tabacum* (Melchers and Labib, 1974), all other parasexual hybrids are derived from fusion of somatic cells. Therefore, the initial fusion products (assuming fusion of two nuclei from two different species) should be essentially allotetraploids, and in theory should be genetically stable as is the case with the allotetraploids produced via the sexual channel. But in practice such is not the case. Most somatic hybrids show intermediate morphology of different traits examined, such as leaf shape, leaf area, petiole length, floral length, corolla morphology, root morphology tuber morphology, etc. (see Evans, 1981). No similar variability has been observed in comparable sexual hybrids, when available. The phenotypic and genetic variability in the somatic hybrids may result from a number of mechanisms. Some of these mechanisms are discussed below.

A. *Long Range Effect of Culture*

It is recognized that longterm culture of plant tissues may result in genetic instability. The causes for this variability are not entirely understood. But long range exposure of cells to very high levels of exogenously supplied phytohormones and other chemicals in the culture media (which are many-folds higher than the physiological endogenous concentration of these chemicals required for *in vivo* growth and differentiation) may be a major contributing factor to the genetic variability. The genetic variability of plants regenerated *in vitro* has been documented (Skirvin, 1978; Larkin and Scowcroft, 1981). This variability is particularly evident when individual protoplast-derived clones are screened (see Shepard *et al.* 1980).

B. *Chromosome Variability*

In great majority of somatic hybrids produced to date, genetic variability as monitored by chromosome instability has been observed. Although karyotype analysis has not been carried out in all the somatic hybrids produced, information on chromosome numbers are available on a number of cases. For example, in a parasexual intraspecific hybrid of *Nicotiana* chromosome number ranged from 47—100 (in a population of 108 parasexual hybrids), but the mode was 48 (the summation number) (Melchers and Sacristan, 1977). Carlson *et al.* (1972) counted a chromosome number of 42 (the summation number) in the three parasexual hybrids produced following protoplast fusion between *Nicotiana glauca* (2n = 24) and *Nicotiana langsdorffii* (2n = 18). On the other hand, Smith *et al.* (1976), examined chromosome of 23 somatic hybrids involving *Nicotiana glauca* + *Nicotiana langsdorffii* and reported a chromosome range of 56—64 (possibly resulting from triple fusions) in these plants; all recovered plants were aneuploid as no plants were recovered with the summation chromosome number af 42. The same hybrid combination was once again investigated by Chupeau *et al.* (1978). From the 48 colonies derived from protoplast fusion of *Nicotiana glauca* + *Nicotiana langsdorffii,* Chupeau *et al.* (1978) regenerated 6 plants, and 2 of 6 regenerated plants showed a chromosome number of 42 (the summation number) and were morphologically similar to the sexual amphidiploid *Nicotiana glauca* — *langsdorffii* (GGLL). This discrepancy in the chromosome numbers observed by the three sets of workers on the same somatic hybrid may have resulted partly from the effects of isolation — fusion — cultural environment (Vasil *et al.*, 1979) and partly from the selection procedures based on vigorous growth versus good growth of hybrid calli (Chupeau et al., 1978) on a complete media before transfering to media without hormones for a final selection of hybrid calli (GGLL develops „spontaneous" tumors, and its tissue explants are autonomous with respect to phytohormones *in vitro).* And finally, Evans *et al.* (1980) have reported that all the 25 clonal *Nicotiana glauca* (2n = 24) + *Nicotiana tabacum* (2n = 48) somatic hybrids carried a chromosome number of 72, expected in the amphidiploid.

Chromosome elimination and aneuploidy seems to be a common phenomenon in intergeneric protoplast fusion products (Gleba and Hoffmann, 1980). The chromosome elimination may be preferential or random. In the somatic hybrid cell lines of *Nicotiana glauca* + *Glycine max,* there is a preferential loss of *N. glauca* chromosomes (Kao, 1977). In another intergeneric somatic hybrid involving *Aegopodium podagraria* (2n = 42) *Daucus carota* (2n = 18) all the 42 chromosomes of *A. podagraria* were apparently lost and all plants regenerated from such fusion experiments showed a chromosome number of 18 (Dudits *et al.*, 1979). On the other hand, chromosme elimination appears to be random in *Lycopersicon esculentum* + *Solanum tuberosum* (Melchers *et al.*, 1978), *Vicia faba* + *Petunia hybrida* (Binding and Nehls, 1978), *Arabidopsis thaliana* + *Brasica campestris* (Gleba and Hoffmann, 1980); *Datura innoxia* + *Atropa belladonna* (Krumbiegel and Schieder, 1979), and *Petunia hybrida* + *Atropa belladonna* (Gosch and Reinert, 1978).

C. *Nuclear and Cytoplasmic Segregation*

One of the major causes of genetic instability of somatic hybrids may be in the segregation of nuclear and cytoplasmic organelles, such as plastids and mitochondria. Such segregations can produce populations of plants containing a range of nuclear cytoplasmic mixtures *(Fig. 1).* There is a basic difference between sexual and somatic hybridization. In the sexual hybridization, the male parent con-

tributes only (or almost only) nuclear genes, while the female parent contributes both nuclear genes and cytoplasm. However, no such unilateral exclusion of cytoplasm exists in the somatic hybridizations. All experiments to data suggest that somatic fusion products, with mixed cytoplasms initially, seggregate to one or the other parental cytoplasm type (at least with respect to plastids) either shortly after protoplast fusion or within 1—2 generations of self fertilization after plant regeneration (CHEN *et al.* 1977; IZHAR and TABIB, 1980).

The combination of cytoplasm from one parent and genome of the second parents, following nuclear segregation in the somatic fusion products, resulting in cybrid formation, has been used to transfer cytoplasmically controlled male sterility in somatic hybrids of *Nicotiana* (ZELCER *et al.*, 1978). Recent studies suggest that recombinant cytoplasms, particularly those for mitochondrial genomes (BELLIARD *et al.*, 1979), or possibly even plastid genomes, may be recovered following protoplast fusions. Consequently a wider range of nuclear - cytoplasmic hybrid combinations, resulting in enormous genetic variability, can be recovered following somatic hybridization compared to sexual hybridization.

VIII. Uptake Studies with Plant Protoplasts

Because of lack of cell wall, plant protoplasts seem to provide an efficient experimental system for uptake of foreign material, such as nuclei, cell organelles, microorganisms, and macromolecules *(Table 4)*. As far as these organelles, microorganisms, and DNA is concerned, little more than uptake has been reported so far. Convincing evidence for direct integration and expression of DNA or other organelles in the receptor protoplast has yet to be provided (VASIL *et al.*, 1979). However, transfer and expression of tumor inducing (Ti) DNA from bacterial plasmids into plant cells, via protoplast, has been demonstrated

Table 4. — Uptake studies with plant protoplasts.

Species	References
A. Uptake of organelles	
Uptake of isolated Petunia nuclei into Petunia protoplasts	Potrykus & Hoffmann, 1973
Uptake of isolated Hordeum nuclei into Zea protoplasts	Lörz & Potrykus, 1978
Uptake of isolated algal (Vaucheria) chloroplasts into Daucus protoplasts	Bonnet, 1976
Uptake of isolated Petunia chloroplasts into Parthenocissus protoplasts	Davey et al. 1976
Uptake of isolated Spinacea chloroplasts into Neurospora protoplasts	Vasil & Giles, 1975
B. Uptake of microorganisms	
Uptake of yeast and bacteria into Parthenocissus protoplasts	Davey & Power, 1975
Uptake of blue green algae into Nicotiana protoplasts	Burgoon & Bottino, 1976
Uptake of nitrogen-fixing bacteria (Azotobacter) into fungus (Rhizopogon) protoplasts	Giles & Whitehead, 1976
C. Uptake of DNA (host-vector system)	
Uptake of isolated Ti plasmid into Nicotiana protoplasts and transformation	Martin et al. 1979
Uptake of isolated Ti plasmid into Petunia protoplasts and transformation	Davey et al. 1980

Table 5. — Protoplast research in tree species.

Species	References
A. Isolation of protoplasts	
Acer pseudoplatanus	Rona & Grignon, 1972
Picea abies	Huhtinen & Winton, 1973; Chalupa, 1974
Pseudotsuga menziesii	Winton et al. 1975
Pinus taeda	Winton et al. 1975
Pinus echinata	Winton et al. 1975
Pinus contorta	Winton et al. 1975
Tsuga heterophylla	Winton et al. 1975
Populus x euramericana	Saito, 1976, 1980
Paulownia taiwaniana	Saito, 1976, 1980
Morus alba	Ohyama & Oka, 1975
Ulmus americana (haploid)	Redenbaugh et al. 1980
Cupressus arizona (haploid)	Duhoux, 1980
B. Isolation and culture of protoplasts	
Pseudotsuga menziesii	Kirby & Cheng, 1979
Pinus pinaster	David & David, 1979
Leucaena leucocephala	Venketeswaran & Gandhi, 1980
Sapium sebiferum	Venketeswaran & Gandhi, 1980
Copaifera multijuga	Venketeswaran & Gandhi, 1980
Biota orientalis	David et al. 1981
Picea excelsa	Strmen & Cierna, 1981
Populus tremula	Ahuja, unpub.
C. Fusion of protoplasts	
Paulownia taiwaniana + Populus x euramericana	Saito, 1980

(MARTIN *et al.*, 1979; DAVEY *et al.*, 1980). Only in one case have these studies progressed far enough to be of some practical significance. In a series of successful experiments GILES and WHITEHEAD (1975, 1976, 1977) have described the transfer of nitrogen-fixing bacteria *(Azotobacter vinelandii)*, with the aid of PEG, into isolated protoplasts of a fungus *Rhizopogon*. This fungus forms mycorhizal association with the roots of *Pinus radiata*. The important aspect of this work is that the modified strain of *Rhizopogon*, containing nitrogen-fixing bacteria *Azotobacter*, may provide some degree of nitrogen sufficiency, if this new strain can enter into symbiotic relationship with the *Pinus radiata* roots. Such an association has been claimed by GILES and WHITEHEAD.

IX. Protoplast Research in Tree Species

Although tree species of gymnosperms and angiosperms have been uniformely difficult to culture *in vitro*, recent reports on culture of somatic and haploid tissues, organs, *in vitro* differentiation of plantlets, as well as isolation and culture of protoplasts of several tree species are encouraging. Indeed, tissue culture research in the tree species lags far behind tissue culture research in the herbeceous plant species. This is partly due to the fact that tree species have been neglected for a long time, and partly because most tree species were unresponsive to *in vitro* conditions provided. However, this situation is changing rapidly and there is now sustained interest to recognize and promote tissue culture technology as an important tool for the microvegetative propagation and for attempting genomic modification of forest tree species, in addition to the time-honored tree improvement practices.

Protoplasts have been isolated from only a handful of tree species *(Table 5)*. Because of indequate knowledge of growth requirements of cells from tree species, it has not been possible to carry isolated protoplasts much further. Following isolation, only in a few cases the protoplasts have

been cultured to give rise to a small mass of cells. But efforts are being made to define the growth requirement of isolated protoplasts of tree species so that both growth and differentiation in the protoplast-derived callus can be obtained under controlled conditions.

X. Protoplast Research: Limitations and Prospects

In the early 1970's it became possible to isolate large amounts of viable plant protoplasts. During the last ten years much progress has been made in order to isolate, culture and produce somatic hybrids following fusion of protoplasts in a number of plant species, particularly those belonging to the family *Solanaceae*. The results from Solanaceaous plants model systems such as *Nicotiana, Petunia, Datura* and *Solanum*, have provided impetus to extend protoplast research to a number of different plant genera including legumes, cereals and tree species. Although it is now possible to isolate protoplasts from any plant species, sustained cell dividions leading to callus formation have been achieved only in a limited number of plant species. Plant regeneration from protoplasts is still a rare event, and has been reported in some 12 genera, more than half of which belong to the family *Solanaceae* represented by a number of species.

The most frequently quoted potential of the protoplast research is to produce somatic hybrids between plant species which are sexually incompatible or difficult to cross by sexual hybridization. First attempts to produce somatic hybrids were made on those plants in which sexual hybrids existed, so that parasexually produced plants could be compared with sexual hybrids. More recently parasexual hybridizations have been extended to intergeneric or interkingdom (between plant protoplasts and animal cells) to test the limits to the range of somatic fusion products. After the initial excitement, it became clear that although somatic hybridizations offer unlimited possibilities for combining sexually incompatible as well as widely divergent species, there will certainly be eventual limits to range in remoteness of types that can be combined parasexually and regenerated into *viable and stable hybrids*, just as there are limits to the range of combinations through sexual hybridizations. As it turns out, the somatic hybrids, investigated so far, show a wider range of phenotypic variability and genetic variability (as monitored by chromosomal instability) than has been observed in the comparable sexual hybrids. The intergeneric parasexual hybrids plants or cell lines are genetically even more unstable as chromosome elimination, random or preferential, seem to be a common phenomenon in the intergeneric fusion products. The preferential loss of chromosomes in certain intergeneric plant hybrids could be utilized for gene mapping, as been done in certain animal somatic hybrids. For example, in human and mouse somatic cell hybrids there is a preferential loss of human chromosomes, a phenomenon utilyzed for mapping genes on human chromosomes. The availability of genetic variability in the somatic plant hybrids also offers opportunities for isolation of novel nuclear or nuclear-cytoplasmic combinations for plant improvement as well as for understanding of problems of growth and differentiation.

The interkingdom somatic hybrids, involving fusions between plant protoplasts and animal cells, generated a lot of interest and curiosity. I do not believe that it was intended towards producing a first „Planimal" (a hybrid between a plant and an animal); it was merely an academic exercice to produce, at best, a heterokaryon for investigating the range and extent of genetic compatibility and relatedness. It should be mentioned that upto now only the fusion of cells has been observed in the interkingdom somatic hybrids, and fusion between plant and animal nuclei and the first division of the fused nuclei in the heterokaryon has not been observed (Mastrangelo and Mitra, 1981).

Table 6. — Protoplast research: Prospects.

1. Fusion of somatic cells of closely or distantly or unrelated species
2. Fusion of haploid cells of closely or distantly or unrelated species
3. Transfer of cytoplasmic genetic information
4. Uptake of cell organelles
5. Uptake of microorganisms
6. Uptake of genetic material (DNA or RNA)
7. Isolation of mutant cell lines
8. Understanding of growth and differentiation (through fusion experiments)
9. Harnessing of genetic variability from somatic hybrids

Protoplasts have also been used as experimental systems for uptake of cell organelles, microorganisms and DNA. Although viability and expression of foreign genetic material into host plant cells have not been convincingly demonstrated in all cases, these investigations on uptake of alien material offer opportunities for attempting "genetic engineering" in plants. In this respect, it is worthwhile to mention the transfer of nitrogen-fixing bacteria, *Azotobacter*, into a mycorrhizal fungus *Rhizopogon*, which enters into a symbiotic association with the roots of *Pinus radiata*. In addition, uptake of tumor-inducing (Ti) plasmid into plant protoplasts and expression of Ti genes, through transformation of the protoplast-derived cells, have also been demonstrated (Marton *et al.*, 1979; Davey *et al.*, 1980). Recent evidence suggests that at least part of the Ti plasmid DNA, relevant to cell transformation, seems to be integrated in the plant host cell genomes (Zambryski *et al.* 1980; Thomashow *et al.*, 1980).

Finally protoplast research seems to offer far reaching possibilities for conducting basic as well as applied research *(Table 6)*. A prerequisite for extending protoplast research to economically important plants, such as cereals, legumes and tree species, would be to carry out extensive organ, tissue, cell and protoplast culture research on these plants so as to define their *in vitro* growth and differentiation requirements. Only then shall we come closer to harvesting the fruits of protoplast technology.

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XII. References

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Variation in Mineral Nutrient content between young plants of Norway spruce provenances and clones*)

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Summary

A part of the results of a program to evaluate the possibilities of clonal identification in Norway spruce are presented. The material analysed are 3 years old clonal plants of 10 provenances with 50 clones each. The needle mineral nutrient content is discussed. There is a strict genetic control for the nutrient uptake, explaining roughly 80% of the total variation. About 75% of the genetic variation appear on clonal level and only about 25% on provenance level. There is no obvious correlation between height growth and anyone of the needle nutrient contents on clonal level. The great genetic variation within provenances can be explained as a reaction to a heterogeneous environment.

The necessity to maintain genetic variation in breeding material to reduce risks is stressed.

Key words: Norway spruce, genetic variation, mineral nutrient content, cutting propagation.

Zusammenfassung

Aus einem Programm, das zum Ziel hatte, die Möglichkeiten der Klonidentifikation bei Fichte *(Picea abies)* abzuklären, wird hier ein Teilmaterial, das den Nadelnährstoffgehalt behandelt, vorgestellt. Es wurden 3 Jahre alte Fichtenstecklinge von 10 Herkünften mit jeweils 50 Klonen analysiert. Von jedem Klon lagen 3 Wiederholungen vor.

Die Nährstoffaufnahme scheint starker genetischer Kontrolle zu unterliegen, die genetische Variation erklärt ungefähr 80% der insgesamt auftretenden Variation.

Von der genetischen Variation treten etwa 75% auf dem Klonniveau und 25% auf dem Herkunftsniveau auf. Es besteht keine Abhängigkeit zwischen Höhenwachstum und irgendeinem einzelnen Nadelnährstoffgehalt auf dem Klonniveau. Die erhebliche genetische Variation innerhalb von Herkünften kann als Reaktion der Population auf heterogene Umweltbedingungen erklärt werden. Hieraus wird in der Diskussion abgeleitet, daß es auch für forstliches Züchtungsmaterial notwendig ist, genetische Variation zu erhalten, um Risiken zu verringern.

1. Introduction

Within the frame of a research program which had the aim to test the possibilities for clonal identification in Norway spruce, we included mineral nutrient content of branches too.

In this program we included 10 provenances with 50 clones each and we measured morphological, physiological, chemical and biochemical traits. In this paper the results of the evaluation of the mineral needle nutrient content of the clones will be discussed.

2. Material and methods

All 1.500 plants were 3 years old nursery stock. Three plants of each of the 500 clones (10 provenances × 50 clones each) were analysed. The experimental material grew in a nursery in Escherode. Analyses were carried out at the

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