Table 6. — Ranks of survival, height and flowering of grafts in relation to age classes/provenances and 11 selected rootstocks out of 17 rootstocks used as mentioned in *Table 1a*. A high survival percent, a low height and a high number of flowering ramets correspond to a low rank and the desired property of a rootstock.

Rootstocks		Survival 1982						Height 1979						Flowering 1982					
Type	No	8	20	36	40	71	96	8	20	36	40	71	96	8	20	36	40	71	96
Dwarf	1	1	3	2	2	12.5	3	5	16	15	9	15	15	5	9	1	9	3	2
	2	2	12,5	17	17	16.5	15	8	14	17	17	9	14	5	9	10	9	4	10.5
	3	6	15	13	13	16.5	11.5	7	7	9	15	17	11	5	9	10	9	12	10.5
	5	4	1	4.5	4	5	7	4	15	13	16	13	12	5	9	10	9	2	3
	8	–	17	9	13	7	10	–	9.5	3	1	7	8	–	9	10	9	12	10.5
	19	–	12.5	3	9	6	13	–	2	6	2	4	3	–	9	10	9	12	10.5
Normal	10	7	4	8	6.5	10.5	2	2	6	14	12	5	9	5	9	10	9	12	1
	12	–	7	10.5	5	2	6	–	8	5	10.5	10	17	–	9	10	9	12	10.5
	13	–	5.5	1	1	1	1	–	13	4	13.5	12	4	–	9	2	9	12	10.5
	15	–	10.5	7	8	3	9	–	1	2	5	1	2	–	9	10	9	1	10.5
	16	–	2	10.5	3	4	4	–	3	1	6	2	7	–	9	10	9	12	10.5

ramets. March till mid-May treatments and nursery control did not flower. So, at the same time we could find an approximation for the time of flower initiation which took place in 1981 between the 15 of May and the 15 of June *(Table 5)*.

4. Conclusions

For the seed orchard practice we need a rootstock which increases survival, reduces the height growth and stimulates flowering and cone production of a graft (see Heybroek *et al.* 1976) at a time when late frosts can no longer damage the flowering strobili. Unfortunately this latter intention to influence the flushing behaviour could not so far be observed on the tested clonal material. Although the ortets of the clonal rootstocks used vary considerably concerning this character, the inherited provenance and/or tree effects could not be influenced by one rootstock from the tested 17. We have to consider this problem as open. But if we rank the results of survival 1982, length of scion 1979 and flowering 1982 in this way that low values correspond to a high survival percent, short scions and a high number of flowering grafts *(Table 6)* some preliminary indications on the possible suitable clonal rootstocks can be obtained, which underline our results (Krusche *et al.* 1976, 1977). The high survival of grafts on dwarf rootstocks of *Picea glauca* 'Conica' and *Picea abies* 'Clanbrasiliana' an early start of flowering and a relatively high number of flowering ramets on these rootstocks indicate that both should be especially observed. The influence on flowering, fruit setting and compatibility will be the most important criteria for recommending the first standard Norway spruce rootstocks in the future. Other criteria of less weight are shoot growth and flushing.

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Protoplast Research in Woody Plants*)

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Abstract

The status of woody plant protoplast research is reviewed. Protoplasts have been isolated and cultured from several woody plant genera of angiosperms and gymnosperms. Sustained cell divisions, colony/callus formation have been observed in the protoplast cultures of a few woody species. With the exception of *Citrus* species, plant regeneration from the protoplast cultures of other woody plants has not been achieved so far. Protoplast fusions have been attempted in a few woody genera. Somatic hybridization techniques seemingly offer new options for combining genotypes, isolation of novel and new genotypes, understanding of growth and differentiation, and genetic improvement of woody plants.

*) Based on a seminar given at The International Conifer Tissue Culture Workgroup, Second Meeting, Tacoma, Washington, U.S.A., September 1983.

Key words: Protoplasts, isolation and culture, fusion, woody plants.

Zusammenfassung

Es wird über den Stand der Protoplastenforschung bei holzigen Pflanzen berichtet. Bei einigen Gattungen der Angiospermen und Gymnospermen sind Protoplasten isoliert und kultiviert worden. Anhaltende Zellteilungen, und Kolonie-/Kallusbildung wurden in den Protoplastenkulturen bei einigen Baumarten beobachtet. Außer bei *Citrus* Arten, sind Pflanzenregeneration von Protoplastenkulturen anderer Baumarten bisher nicht erreicht worden. Fusionen von Protoplasten sind in einigen Holzpflanzen-Gattungen versucht worden. Die Technik der somatischen Hybridisation bietet scheinbar neue Möglichkeiten für die Kombination von Genotypen, die Isolierung von neuartigen und neuen Genotypen, das Verstehen des Wachstums sowie der Differenzierung und für die genetische Verbesserung der Holzpflanzen.

Introduction

In recent years protoplasts have been routinely isolated from a number of plant species. Under appropriate conditions, protoplast cultures have led to cell wall formation, cell divisions, colony/callus formation, and finally plantlet regeneration (Vasil and Vasil 1980; Ahuja 1982; Davey 1983). In some 61 species plants have been regenerated from protoplasts (Binding *et al.* 1982), and this list continues to expand. However, cereal crops and woody species are among the recalcitrant plant species which are difficult to regenerate from the protoplast cultures.

Isolated protoplasts from several herbaceous plant species, particularly those from the family *Solanaceae,* have been fused to yield intraspecific, interspecific, and intergeneric somatic hybrids (Vasil, Ahuja, and Vasil 1979; Schieder and Vasil 1980; Gamborg *et al.* 1981; Ahuja 1982; Harms 1983). However, recovery of genetically stable somatic hybrids, having the summation chromosome number of the parental species involved, still remains an unresolved problem. Genetic and phenotypic variability seems to be a common feature of most somatic hybrids investigated so far. The presence of genetic variability, on the other hand, offers opportunities for selection of hitherto new and novel 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 or generally not possible with plant cells having a cellulose wall, such as uptake of nuclei, organelles, microorganisms, chromosomes or fragments of chromosomes carrying specific genes, or macromolecules as DNA or RNA. Therefore, for the purposes of genetic modifications of plant cells, protoplasts can serve as valuable experimental materials (Cocking *et al.* 1981; Ahuja 1982).

Protoplasts have been isolated from several woody plant species of gymnosperms and angiosperms, but cultured in a few (see Ahuja 1981, 1982). Sustained cell divisions have not been commonly observed in the protoplasts cultures of woody plants. This may be partly due to lack of informations on the parameters affecting growth and differentiation of woody protoplasts/cells, and partly due to the fact that cells of long-lived woody plants may be relatively less labile and consequently less responsive, as compared to cells from short-lived herbaceous plant species, under *in vitro* conditions. With the exception of *Citrus* species (Vardi *et al.* 1982), it has not been possible so far to regenerate whole plants from protoplast cultures of other tree species. The present paper examines the status of protoplast research in woody plants, in particular the regenerative potential and prospects of genetic modification in tree species.

Isolation of protoplasts

Protoplasts have been isolated from leaves, cotyledonary tissues, callus cultures and cell suspension cultures of several woody plant species *(Table 1A).* The protoplast yields may be variable depending upon the sterilization proce-

Table 1. — Protoplast research in woody plants.

Species	Reference
A. Isolation of protoplasts (preparatory studies)	
Acer pseudoplatanus	Rona & Grignon, 1972
Picea abies	Huhtinen & Winton, 1973
Pseudotsuga menziesii	Winton et al. 1975
Pinus taeda	Winton et al. 1975
Pinus echinata	Winton et al. 1975
Tsuga heterophylla	Winton et al. 1975
Morus alba	Ohyama & Oka, 1975
Populus x euramericana	Saito, 1980a
Paulownia taiwaniana	Saito, 1980a
Ulmus americana (haploid)	Radenbaugh et al. 1980
Cupressus arizona (haploid)	Duhoux, 1980
Leucana leucocephala	Venketeswaran & Gandhi, 1980
Sapium sebifera	Venketeswaran & Gandhi, 1980
Copaifera multijuga	Venketeswaran & Gandhi, 1980
Betula pendula	Steinhauer et al. 1980
Santalum album	Lakshmi Sita & Shobha Rani, 1983
Populus tremuloides	Verma & Wann, 1983
Betula platyphylla	Smith & McCown, 1983
Rhododendron sp.	Smith & McCown, 1983
Ulmus sp	Dorion et al. 1983
Larix decidua	Ahuja, this study
Quercus petraea	Ahuja, this study
B. Culture of protoplasts	
Pseudotsuga menziesii	Kirby & Cheng, 1979; Kirby, 1982
Pinus pinaster	David & David, 1979; David et al. 1982
Biota orientalis	David et al. 1981
Picea excelsa	Strmen & Cierna, 1981
Citrus sp	Vardi et al. 1982
Alnus glutinosa	Huhtinen et al. 1982
Alnus indica	Huhtinen et al. 1982
Pinus contorta	Hakman & von Arnold, 1983
Populus tremula	Ahuja, 1983a, b
Populus tremuloides	Ahuja, 1983b
Fagus sylvatica	Ahuja, 1983c
C. Fusion of protoplasts	
Paulownia taiwaniana + *Populus x euramericana*	Saito, 1980b
Citrus sp + *Citrus* sp	Vardi et al. 1983
Populus tremula + *Populus tremuloides*	Ahuja, this study
Populus tremula + *Fagus sylvatica*	Ahuja, unpub.

dures, composition of the cell wall degredation enzyme solutions, the age and the physiological state of the source material (for example, leaves). Different osmotic stabilizers (0.4 to 0.7 M mannitol, sorbitol, glucose, or sucrose) have been included in the enzyme solutions for the isolation procedures. Following incubation in the enzyme solutions (which may vary from 4 to 22 hours) in dark at controlled temperatures, the sieved protoplasts have been centrifuged at various speeds (50 to 350 g) for pelleting or floating them in the cell/protoplast wash (CPW) solutions.

In most of the studies with protoplast isolation in woody plants, leaves from young plants or juvenile/regenerative tissues/cells have been employed. Since we are interested in the area of mature micropropagation, we have developed a relatively simple system for the isolation of viable protoplasts from young leaves of mature trees, ranging in age from 15 to 70 years (AHUJA 1983 b, c). We developed this method for isolating aspen and beech protoplasts, and are extending it to other tree species. Essentially this method consists of cutting 40 to 50 cm long branches with dormant buds in winter-early spring from mature trees and store them in a cold room maintained at 4° C. Such branches can be stored for a period of several months without appreciable loss of bud viability. Before protoplast isolation, the branches with dormant buds are placed in controlled chambers (temperature 22—24° C; light 3400—4000 lux; humidity 60—70%) with their cut ends dipped in tap water. In aspen, the bud break occurs within a week, and after 8—10 days the first leaves can be harvested for protoplast isolation. In this way a constant supply of young leaves can be obtained for protoplast work. These leaves do not have a thick cuticle, and slight scraping of the lower surface is adequate for the release of protoplasts in low levels of cell wall maceration/degredation enzyme solutions (AHUJA 1983 a, b). By employing this method, we have been able to procure a yield of 10^6 protoplasts per gram of leaf tissue in aspen. At least/this method offers options for isolating protoplasts that may be relatively more genetically stable as compared to those derived from tissue/callus/cell suspension cultures in which somaclonal variation may be present.

In addition to isolation of mesophyll protoplasts by our method in European aspen *(Populus tremula)*, quaking aspen *(Populus tremuloides, Fig. 1A)*, and beech *(Fagus sylvatica, Fig. 1B)*, our preliminary studies with other forest tree species, such as oak *(Quercus petraea, Fig. 1C)*, birch *(Betula pendula)*, larch *(Larix decidua, Fig. 1D)*, and Norway spruce *(Picea abies)* have indicated that this method has potential, but optimal conditions for protoplast isolation must be worked out.

Viability of the protoplasts can be determined in various ways. The presence of active cytoplasmic streaming in freshly isolated protoplasts is a good indication of viability. Vital stains, such as Evans blue or fluorescein blue, have been employed to differentiate between viable and non-viable protoplasts (WIDHOLM 1972; LARKIN 1976). Of course, the best test for the viability is the regeneration of cell wall and the onset of cell divisions.

Culture of protoplasts

Following isolation, protoplasts have been cultured in several woody plant genera *(Table 1B)*. Since tree species are generally cross pollinated, they are highly heterozygous in nature. This also reflects in their *in vitro* growth and differentiation requirements (AHUJA 1983 d), and therefore makes the task of developing a common woody plant medium extremely difficult. A broad spectrum medium for the *in vitro* growth and differentiation of tissues, cells or protoplasts from woody plants hovers in the minds of many of us, but it only remains an illusion at the present time. Because of genetic differentiation between the woody species, the growth and differentiation requirements are quite different between the genera, species, subspecies, or clones of a single species. Therefore, variously modified media have been employed for the culture of woody tree protoplasts (see references in *Table 1B)*. Following culture, cell divisions have been observed in the protoplast cultures of *Picea excelsa* (STRMEN and CIERNA 1981), *Pinus contorta* (HAKMAN and VON ARNOLD 1983), *Fagus sylvatica* (AHUJA 1983 c); cell colony/cluster formation in *Pinus pinaster* (DAVID and DAVID 1979; DAVID *et al.* 1982), *Biota orientalis* (DAVID *et al.* 1981), *Populus tremula* (AHUJA 1983 a, b), *Populus tremuloides* (AHUJA 1983 b); cell cluster/callus formation in *Pseudotsuga menziesii* (KIRBY and CHENG 1979 ; KIRBY 1982), and *Alnus glutinosa* and *Alnus indica* (HUHTINEN *et al.* 1982); and finally plant regeneration has been claimed in *Citrus* species (VARDI *et al.* 1982).

In all these studies, the protoplasts were obtained from juvenile plants or callus/cell suspension cultures. We have demonstrated that developmentally viable protoplasts can be isolated from young leaves of mature trees even when they are 70 or more years old (AHUJA 1983 c). It would appear that the important criteria for *in vitro* rejuvenation/regeneration seems to be the stage of development of the leaf or the cell type, rather than the age of the tree per se.

In addition to normal range protoplasts, we have also observed exceptionally large protoplasts, previously designated megaprotoplasts (AHUJA, 1983 a), in *Populus tremula (Fig. 1E)* and *Populus tremuloides* (AHUJA, 1983 a, b), *Fagus sylvatica* (AHUJA 1983 c), *Quercus petraea,* and *Larix decidua (Fig. 1F)*. The megaprotoplasts are usually densely packed with chloroplasts as compared to normal protoplasts. The megaprotoplasts are 2—5 times greater in diameter as compared to average protoplasts. In terms of total volume, the ratio between normal and megaprotoplasts would work out to be 1:8 to 1:125. The megaprotoplasts are developmentally viable and undergo budding/cell divisions just like normal protoplasts. The origin of megaprotoplasts is not known. We have considered the possibility that megaprotoplasts may be the artefacts of the incubation procedure, since they were observed in the enzyme solution before centrifugations, and thus could have arisen following fusion between protoplasts. However, it would require many fusions to yield protoplasts of such dimensions. Another intriguing fact about megaprotoplasts is that they are transient in their existence, since they could only be isolated from the young leaves. There were hardly any megaprotoplasts in the older leaves. It remains an illusive possibility that megaprotoplasts develop at a specific stage of leaf development in the decidous tree species. We plan to investigate the possible origin of megaprotoplasts by inclusion of EGTA that seems to inhibit the expansion of plasmodesmatal connections during incubation period to check on the fusion hypothesis. Leaf anatomy and chromosome constitution of megaprotoplasts may also shed some light on their origin.

Fusion of protoplasts

The fusion of protoplasts can occur spontaneously or it can be induced by mechanical or chemical means. The commonly used method for protoplasts fusion employs

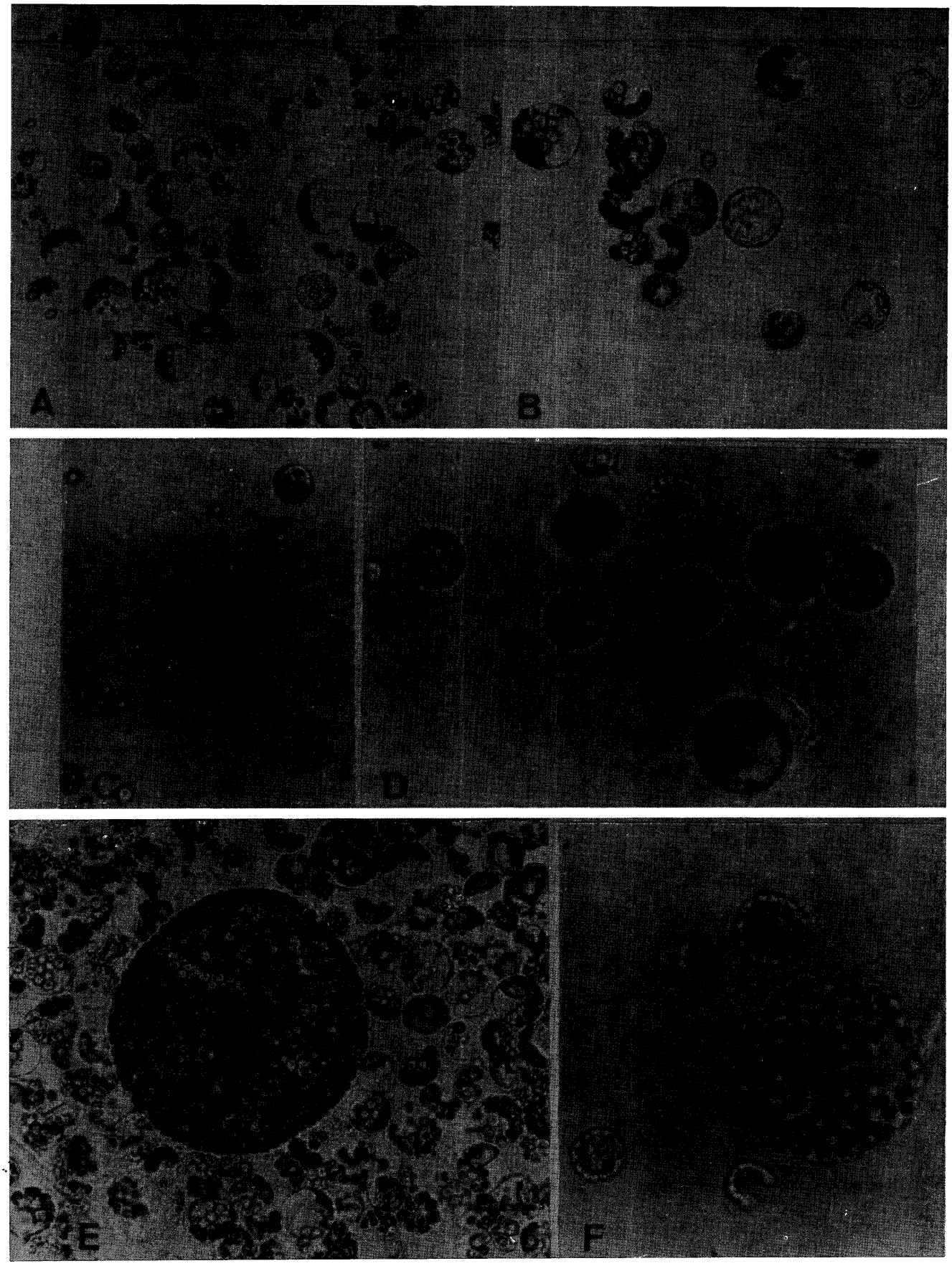

Figure 1. — Freshly isolated mesophyll protoplasts from forest tree species: *Populus tremuloides* (A), *Fagus sylvatica* (B), *Quercus petraea* (C), *Larix decidua* (D); normal and megaprotoplasts in *Populus tremula* (E), and in *Larix decidua* (F). x560

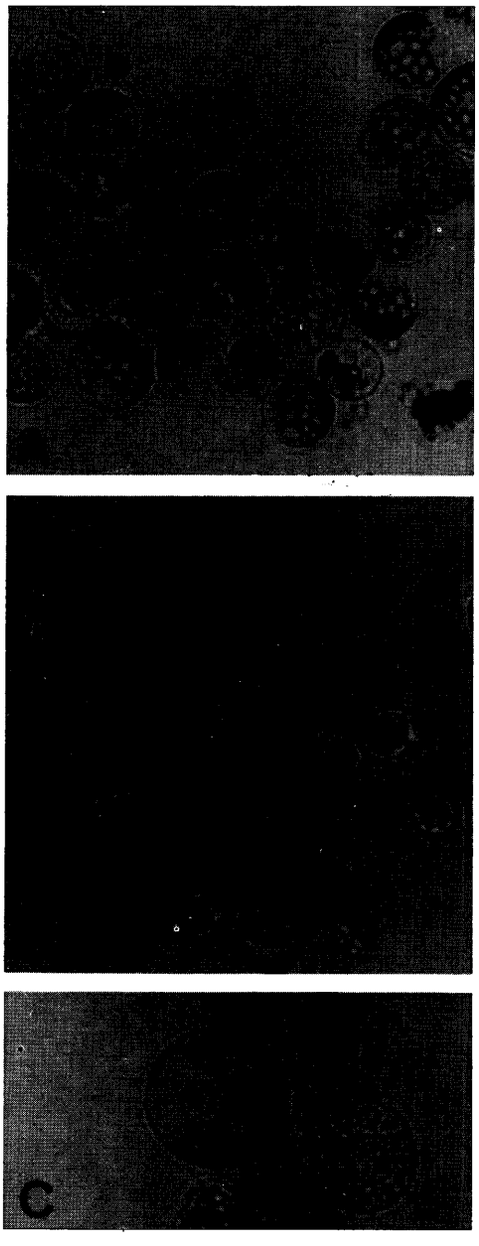

Figure 2. — Freshly isolated mesophyll protoplasts from *Populus tremula* (A), *Populus tremuloides* (B, from slightly younger leaves), and fusion products (C). A and B x560; C x1120.

polyethylene glycol (PEG), an agglutinating agent (Kao and Michaluk 1974). Protoplasts have been fused in widely divergent herbaceous plant genera, followed by plant regeneration in a number of them. By contrast, protoplast fusions have been attempted only in a couple of woody plant species. Saito (1980 b) fused protoplasts of *Paulownia taiwaniana* and *Populus* × *euramericana* and observed homokaryons and heterokaryons. Protoplast fusion experiments were recently outlined in *Citrus* species in order to produce somatic hybrids and cybrids (Vardi *et al.* 1983). We have attempted PEG induced fusions (Kao 1982) in *Populus tremula* and *Populus tremuloides (Fig. 2),* and between *Populus tremula* and *Fagus sylvatica,* in order to explore the fate of fusion products and to find out if somatic hybrids can be exploited for overcoming the barriers to *in vitro* regeneration in recalcitrant tree species.

Woody plant protoplasts: problems and prospects

Although tissues from woody species of angiosperms and gymnosperms are generally difficult to grow and differentiate *in vitro,* recent reports on the culture and regeneration studies from organs, tissues, cells and protoplasts are encouraging. Tissue culture research in the woody plant species has lagged behind that of herbaceous plant species in the past. However, this gap has been considerably reduced in the last few years, because of sustained active interest in the tissue culture technology of woody plants. There are still a number of problem areas in the physiology and morphogenesis of woody plant tissues. Regenerative problems may be due to physiological state of the tissue, age of the explant material source, the genotype, and the cultural conditions. In terms of regenerative potential, the protoplasts are somewhat more recalcitrant than the tissues or organs from the woody plants. Nevertheless, recent reports on successful culture of protoplasts from woody plant species are significant. But much basic research needs to be done in this area.

Protoplast research in woody plants seems to offer new options for genetic improvement programs. Woody plants typically flower after an extended vegetative phase and therefore, sexual hybridization and seed collection can only be made after that phase, which may last from several to many years. Parasexual hybridizations may allow exchange of genetic information between sexually immature elite trees, or possibly between sexually incompatible or sterile woody plants, or between woody and herbaceous plants. These studies may provide clues to *in vitro* growth and differentiation of woody plants for the purposes of micropropagation, and may also lead to the production of new and novel genotypes (from gene transfers or protoclonal variability) of improved quality.

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Short Note: Isolation and Culture of Mesophyll Protoplasts from Mature Beech Trees

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Abstract

Protoplasts were isolated from young spring leaves of mature (70 years old) beech *(Fagus sylvatica* '*Atropunicea*' and '*Zlatia*') trees. In addition to normal protoplasts, exceptionally large (Mega) protoplasts were also observed. The mega protoplasts were upto 64 times greater in volume compared to average normal protoplasts. Following culture, the beech protoplasts regenerated cell wall and exhibited budding and cell division. These studies have shown that mesophyll protoplasts from mature beech trees are developmentally viable, when they are isolated at an appropriate stage of leaf development.

Key words: Beech *(Fagus sylvatica)*, mature trees, mesophyll protoplasts, mega protoplasts, isolation, culture, cell division.

Zusammenfassung

Aus jungen, im Frühjahr ausgetriebenen Blättern von ungefähr 70jährigen Buchen *(Fagus sylvatica* '*Atropunicea*' und '*Zlatia*') wurden Protoplasten isoliert. Dabei sind außerordentlich große (Mega-) Protoplasten beobachtet worden. Die Megaprotoplasten hatten ein Volumen bis zu 64 mal größer als das der durchschnittlich großen normalen Protoplasten des Mesophylls. In der Kultur haben die Buchenprotoplasten Zellwände gebildet und Zellsprossungen und Zellteilungen sind erfolgt. Die Untersuchung zeigt, daß Protoplasten des Mesophylls alter Buchen in ihrer Entwicklung lebensfähig sind, wenn sie in einem bestimmten Entwicklungsstadium der Blätter isoliert werden.

Introduction

Protoplasts have been isolated from several forest tree species, but cultured in a few (see Ahuja, 1981, 1982). Cell wall regeneration and cell division have been observed in the protoplast cultures of *Pinus pinaster* and *Biota orientalis* (David *et al.* 1981), *Picea excelsa* (Strmen and Cierna, 1981), *Populus tremula* (Ahuja, 1983), and colony/callus