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REVIEW

Regeneration from protoplasts—a supplementary literature review

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INTRODUCTION

Protoplast technology is important in, on the one hand, the application of various cellular methods in plant breeding (i.e. somatic hybridization, cybridization, direct DNA transfer via polyethylene glycol (PEG), electroporation, micro-injection), and various fundamental studies (e.g. on membrane transport, cell compartmentation, the cytoskeleton in relation to the cell cycle and cell division) on the other hand. For practical applications, plant regeneration from protoplasts is a prerequisite.

In 1989, regeneration from protoplasts was listed (Roest & Gilissen 1989) for 214 higher plant species (Spermatophyta), representing 97 genera and 31 families. Since then, regeneration procedures for more than 100 higher plant species have been reported. These include many economically important agricultural and horticultural crops, as well as

woody plant species. In this paper these new species are listed, supplemented with specific information on the donor tissue used, the culture technique applied, and the type of development of the regenerants. In addition, recent achievements in fundamental aspects of protoplast research, which gradually provide further insight into the genetical, physiological and ultrastructural background of the phenomenon of totipotency of plant cells, will be briefly reviewed.

PLANT SPECIES

During the last 3 years, procedures have been published for regeneration from protoplasts of 106 higher plant species, belonging to 49 genera and 18 plant families, via somatic embryogenesis, i.e. the direct or indirect (through callus) regeneration of somatic embryos, and via organogenesis, i.e. the indirect regeneration of shoots, bulblets and plantlets (Table 1). This brings the total number of plant species for which regeneration has been achieved to 320, representing 146 genera and 49 plant families. Table 1 lists the first publication in which the regeneration procedure for a given plant species was described. The Solanaceae had the highest number of responding plant species, which increased from 67 to 76, representing 13 genera. Regeneration was reported in 29, 23, 7, and 6 species belonging to the genera *Solanum*, *Nicotiana*, *Petunia* and *Lycopersicon* respectively.

Ornamental plant species which can now be regenerated from protoplasts include: saffron (*Crocus sativus*, Iridaceae), lily (*Lilium formolongi*, Liliaceae), honeysuckle (*Lonicera nitida*, Caprifoliaceae), carnation (*Dianthus caryophyllus*, Caryophyllaceae), *Oxalis glaucifolia* (Oxalidaceae), lupin (*Lupinus mutabilis* × *hartwegii*, Papilionaceae), statice (*Limonium perezii*, Plumbaginaceae), cyclamen (*Cyclamen persicum*, Primulaceae), primrose (*Primula malacoides*, Primulaceae), and roses (*Rosa persica* × *xanthina* and *R. rugosa*, Rosaceae).

Woody plant species displayed a substantial increase in the number of regenerable species during the last 3 years. In the Gymnospermae, the number of regenerable species increased from two to nine, including those belonging to the genera *Abies*, *Larix* and *Pseudotsuga*. In the Angiospermae, various tree species were found to be regenerable from protoplasts: *Rauvolfia vomitoria* (Apocynaceae), eucalypt (*Eucalyptus* sp., Myrtaceae), oriental planetree (*Platanus orientalis*, Platanaceae), poplars (*Populus* sp., Salicaceae), tree of heaven (*Ailanthus altissima*, Simarubaceae), and elm (*Ulmus campestris*, Ulmaceae).

Further, many agricultural and horticultural crops can also be regenerated from protoplasts: Italian millet (*Setaria italica*) and great millet (*Sorghum vulgare*) (Gramineae), onion (*Allium cepa*, Liliaceae), kiwi (*Actinidia deliciosa*, Actinidiaceae), Japanese persimmon (*Diospyros kaki*, Ebenaceae), cotton (*Gossypium hirsutum*, Malvaceae), adsuki bean (*Phaseolus angularis*) and French vetch (*Vicia narbonensis*) (Papilionaceae), passion fruit (*Passiflora edulis*, Passifloraceae), buckwheat (*Fagopyrum esculentum*, Polygonaceae), plums and cherries (*Prunus* sp., Rosaceae), coffee (*Coffea* sp., Rubiaceae), calamondin and satsuma (*Citrus* sp., Rutaceae), cocoa (*Theobroma cacao*, Sterculiaceae), and celery (*Apium graveolens*, Umbelliferae).

However, several important plant species, such as banana (*Musa* sp., Musaceae), coconut (*Cocos nucifera*), date (*Phoenix dactylifera*) and oil palm (*Elaeis guineensis*) (Palmae), grape (*Vitis vinifera*, Ampelidaceae), groundnut (*Arachis hypogaea*, Papilionaceae), mango (*Mangifera indica*, Anacardiaceae), oat (*Avena savita*), and rye (*Secale cereale*) (Gramineae), papaya (*Carica papaya*, Caricaceae), pineapple (*Ananas comosus*, Bromeliaceae), rubber (*Hevea brasiliensis*, Euphorbiaceae), tea (*Camellia*

japonica, Theaceae), and yam (*Dioscorea sativa*, Dioscoreaceae), still remain recalcitrant or have not yet been investigated for their ability to regenerate from protoplasts.

PROTOPLAST REGENERATION

Several factors at the level of explants and cells, as well as external and morphogenetic factors, play a decisive role during isolation, culture and regeneration of protoplasts. Some recent data on these factors and their involvement in the regeneration process are discussed.

Plant factors

Genotype. In many plant species protoplast regeneration appears to be strongly dependent on the genotype. Izhar & Power (1977) concluded that different genes control the different developmental stages of leaf protoplasts of *Petunia*. In tomato (*Lycopersicon esculentum*), after crossing with *L. peruvianum* and segregation analysis, it was shown that the regeneration capacity from established callus cultures is controlled by two dominant genes (Koornneef *et al.* 1987). In *Solanum phureja*, Cheng & Veilleux (1991) proposed that the genetic basis for callus development from protoplasts is controlled by two independent dominant loci. In alfalfa (*Medicago sativa*), it was possible to achieve somatic embryo formation in a non-responsive genotype by introduction and expression of genes active in hormonal regulation, i.e. *rol* B and *rol* C genes of *Agrobacterium rhizogenes* (Dudits *et al.* 1991).

Donor tissue. The low reproducibility of protocols for protoplast isolation, culture and regeneration in many plant species may be due to the great physiological variation of donor plants, especially if grown under greenhouse conditions. By pretreatment of donor plants under controlled conditions in a growth chamber, cell division and subsequent regeneration capacity of cultured tobacco and soybean protoplasts were considerably improved (Negaard & Hoffmann 1989). Also in *Lycopersicon* species, growth conditions of source plants influenced protoplast division (Tabaeizadeh *et al.* 1984): both a reduction of the daylength from 16 to 9 h and a cold-treatment at 4°C of the donor plants, significantly increased the plating efficiency. It was suggested that this cold-treatment provoked mitotic activity of the protoplasts.

To overcome the problem of low reproducibility, protoplasts are usually isolated from plant material grown *in vitro* under controlled conditions. In many plant species belonging to the Gymnospermae and Gramineae, the use of embryogenic cell suspensions and callus cultures as protoplast donor tissues resulted in plant regeneration. In some cases, specific donor tissues, i.e. shoot tips (derived from *in-vitro* cultured shoots), embryos and anthers or pollen, have been successfully used for regeneration from protoplasts (Table 1).

A comparison of different plant donor tissues with respect to the early differentiation of somatic embryos from protoplasts of *Helianthus annuus* has been made by Dupuis *et al.* (1990). Only hypocotyl-derived protoplasts divided and gave rise simultaneously to microcalli and somatic embryos, whereas no cell divisions were observed in cotyledon- or leaflet-derived protoplasts. The number of protoplasts that could be isolated increased from the base to the top of the hypocotyl. Although the plating efficiency was similar for protoplasts isolated from various parts of the hypocotyl, differentiation into somatic embryos was enhanced only in protoplasts derived from the basal parts.

Table 1. Higher plant species (Spermatophyta) that gave development of various types of regenerants from protoplasts of different donor tissues after using several culture techniques

Taxon	Donor tissue*	Culture technique†	Regenerant development‡	Reference
Gymnospermae				
Pinaceae				
Abies alba	eC	D	C→E	Lang & Kohlenbach (1989)
Larix decidua	eC	S	C→E	Von Aderkas (1992)
× *eurolepis*	eC;eSc	L;S	C→E→P	Klimaszewska (1989)
Picea abies	eSc	N;S	C→E→P	Gupta *et al.* (1990)
mariana	eSc	L;S	E	Tautorus *et al.* (1990)
Pinus caribaea	eSc	L	C→E	Laine & David (1990)
kesiya	eC	?	C→E	Kumar & Tandon (1991)
Pseudotsuga menziesii	eSc	S	C→E	Gupta *et al.* (1988)
Angiospermae				
MONOCOTYLEDONAE				
Amaryllidaceae				
Hemerocallis fulva	P	?	C→E	Zhou (1989)
Araceae				
Caladium bicolor	L	L;S	C→E;S→P	Jing & Wang (1991)
Gramineae				
Agrostis alba	eSc	S	C→E→P	Asano & Sugiura (1990)
Festuca rubra	eSc	N;S	C→E→P	Zaghmout & Torello (1990)
Oryza rufipogon	eSc	S	C→E→P	Baset *et al.* (1991)
Paspalum dilatatum	eSc	S	C→E→P	Akashi & Adachi (1992)
scrobiculatum	eSc	L;sS	C→E→P	Nayak & Sen (1991)
Setaria italica	eC	L	C→E→P	Dong & Xia (1989)
Sorghum vulgare	eSc	L	C→S→P	Wei & Xu (1990)
Iridaceae				
Crocus sativus	Sc	B;N	C→S→P	Isa *et al.* (1990)
Liliaceae				
Allium cepa	L	L	C→B;S→P	Wang *et al.* (1986)
Lilium formolongi	eC	B	C→E→P	Mii *et al.* (1991a)
DICOTYLEDONAE				
Actinidiaceae				
Actinidia deliciosa	L;St	?	P	Cai *et al.* (1991)
	C;L	LoS	C→E;S→P	Oliviera & Pais (1991,1992)
Apocynaceae				
Rauvolfia vomitoria	L	L	C→E;S→P	Trémouillaux-Guiller & Chénieux (1991)

Araliaceae				
Panax ginseng	E	B;L;S	C→E→P	Arya *et al.* (1991)
Caprifoliaceae				
Lonicera nitida	L	L	C→S→P	Ochatt (1991a)
Caryophyllaceae				
Dianthus barbatus	L	B	C→S→P	Nakano & Mii (1992)
caryophyllus	L	B	C→S→P	Nakano & Mii (1992)
chinensis	L	B	C→S→P	Nakano & Mii (1992)
plumarius	L	B	C→S→P	Nakano & Mii (1992)
Compositae				
Brachycome iberidifolia	L	B;L	C→S→P	Malaure *et al.* (1990)
Callistephus chinensis	L	B;L	C→S→P	Pillai *et al.* (1990)
Centaurea cyanus	L	B	C→S→P	Pillai *et al.* (1990)
Chrysanthemum coronarium	?	?	P	Xia (1990)
× *hortorum*	L	L;S	C→S→P	Sauvadet *et al.* (1990)
Cichorium intybus × *endivia*	L	L	C→E→P	Sidikou-Seyni *et al.* (1992)
Felicia bergeriana	C;L	L;S	C→S→P	Malaure *et al.* (1990)
Helianthus petiolaris	Co	B	C→S→P	Chanabe *et al.* (1991)
giganteus	L	B;L	C→E→P	Krasnyanski *et al.* (1992)
Senecio × *hybridus*	L	D	C→S→P	Pillai *et al.* (1990)
Convolvulaceae				
Ipomoea trifida	L	?	P	Suga *et al.* (1990)
triloba	L;St	L	C→S→P	Liu *et al.* (1991)
Cruciferae				
Brassica chinensis	?	?	P	Li (1991)
Capsella bursa-pastoris	Sc	?	C→E→P	Bonfils *et al.* (1991)
Diplotaxis muralis	L	L	C→E;S→P	Sikdar *et al.* (1990)
Ebenaceae				
Diospyros kaki	L	S	C→S→P	Tao *et al.* (1991)
Gentianaceae				
Eustoma grandiflorum	L	B	C→S→P	Kunitake *et al.* (1990)
Geraniaceae				
Pelargonium × *domesticum*	L	LoS	C→S→P	Dunbar & Stephens (1991)
Labiatae				
Pogostemon cablin	Sc	LoS	C→S→P	Sakurai & Kawachi (1990)
Linaceae				
Linum catharticum	?	?	C→S→P	Ling & Binding (1992)
marginale	Sh	LoS	C→S→P	Zhan *et al.* (1989)
Malvaceae				
Gossypium barbadense	Co;R	L	C→E;S→P	Elshihy & Evans (1986)
hirsutum	eSc	B?	C→E→P	Chen *et al.* (1989), She *et al.* (1989)

Table 1. (*Cont'd*)

Taxon	Donor tissue*	Culture technique†	Regenerant development‡	Reference
Myrtaceae				
Eucalyptus sp.	Sh	N	C→S→P	Ito *et al.* (1990)
Oxalidaceae				
Oxalis glaucifolia	C	L;S	C→S→P	Ochatt *et al.* (1989)
Papilionaceae				
Coronilla varia	Co	L	C→E;S	Lu *et al.* (1986)
Glycine argyrea	Co;L	D	C→S→P	Hammatt *et al.* (1989)
Lotus pedunculatus	Co	L	C→S→P	Pupilli *et al.* (1990)
Lupinus mutabilis × *hartwegii*	L	L	C→S	Schäfer-Menuhr (1989)
Phaseolus angularis	L	L	C→E;S→P	Ge *et al.* (1989)
Stylosanthes macrocephala	Co	L	C→S→P	Vieira *et al.* (1990)
scabra	Co	L	C→S→P	Vieira *et al.* (1990)
Trifolium lupinaster	Sc	L	C→S→P	Zhao *et al.* (1991)
Vicia narbonensis	Sh	S	C→E→P	Tegeder *et al.* (1991)
Passifloraceae				
Passiflora edulis	L	?	C→S→P	Manders *et al.* (1991)
Platanaceae				
Platanus orientalis	L	L	C→S→P	Wei *et al.* (1991)
Plumbaginaceae				
Limonium perezii	Sc	S	C→S→P	Kunitake & Mii (1990a)
Polygonaceae				
Fagopyrum esculentum	Co	L	C→E;S→P	Adachi *et al.* (1989)
Primulaceae				
Cyclamen persicum	eC	B	C→E→P	Otani *et al.* (1989)
Primula malacoides	Sc	S	C→S→P	Mii *et al.* (1990)
Rosaceae				
Prunus avium	L	D;L;sS	C→S→P	Ochatt (1991b)
cerasifera	L	D;L;sS	C→S→P	Ochatt (1992)
spinosa	L	D;L;sS	C→S→P	Ochatt (1992)
Rosa persica × *xanthina*	eSc	S	C→E→P	Matthews *et al.* (1991)
rugosa	eC	B	C→E	Kunitake & Mii (1990b)
Rubiaceae				
Coffea arabica	eC	S	C→E→P	Yasuda *et al.* (1986)
	eSc	L	C→E→P	Acuna & de Pena (1991)
canephora	E	L	C→E→P	Schöpke *et al.* (1987,1988)
Rutaceae				
Citrus jambhiri	?	?	P	Li (1991)
madurensis	eC	LoS;S	C→E→P	Ling *et al.* (1989)
unshiu	eC	L	C→E→P	Ling *et al.* (1990), Kunitake *et al.* (1991a,b)

Salicaceae				
Populus alba	L	L?	C→S→P	Sasamoto & Hosoi (1990)
alba × *glandulosa*	L	L	C→S→P	Park & Son (1988)
glandulosa	L	L	C→S→P	Park *et al.* (1990)
nigra	C	L	C→S→P	Lee *et al.* (1987)
nigra × *maximowiczii*	L	L	C→S→P	Park & Son (1989,1992)
sieboldii	L	L?	C→S→P	Sasamoto & Hosoi (1990)
tomentosa	?	?	P	Li (1991)
Simarubaceae				
Ailanthus altissima	C	D;L;S	C→S→P	Park & Lee (1990)
Solanaceae				
Duboisia myoporoides	Sc	L;N	C→S→P	Kitamura *et al.* (1989)
Lycium barbarum	C;L	L	C→S→P	Ratushnyak *et al.* (1989,1990)
Lycopersicon hirsutum	L	L	C→S→P	Montagno *et al.* (1991)
Nicotiana glutinosa	L	?	C→E→P	Liu & Xu (1988)
Solanum capsicibaccatum	L	L	C→S→P	Xu *et al.* (1991)
commersonii	L	L;sS	C→S→P	Cardi *et al.* (1990)
hjertingii	L	L	C→S→P	Xu *et al.* (1991)
integriforium	L	?	C→S→P	Asao *et al.* (1989)
sanitwongsei	L	?	C→S→P	Asao *et al.* (1989)
Sterculiaceae				
Theobroma cacao	Sc	L	C→E	Kanchanapoom & Kanchanapoom (1991)
Tiliaceae				
Corchorus capsularis	Co;L	L	C→E	Saha & Sen (1992)
Ulmaceae				
Ulmus campestris	L	?	P	Dorion *et al.* (1991)
Umbelliferae				
Angelica dahurica	?	?	P	Li (1991)
sinensis	?	?	P	Li (1991)
Apium graveolens	?	?	C→E→P	Wan *et al.* (1988)
Anthriscus sylvestris	?	?	P	Li (1991)
Bupleurum scorzonerifolium	eC	sS	E→P	Xia *et al.* (1992)
Heracleum moellerdorfii	?	?	P	Li (1991)
Peucedanum prearuptorum	?	?	P	Li (1991)
terebinthaceum	?	?	P	Li (1991)

*C, callus; Co, cotyledon, hypocotyl; E, embryo; L, leaf; P, anthers, pollen; R, root; Sc, suspension cells; Sh, shoot; St, stem; e, embryogenic; ?, unknown.
†B, bead type; D, droplet (20–200 µl); L, liquid medium; LoS, liquid over solid medium; N, nurse, feeder; S, solid medium; s, semi; ?, unknown.
‡C, callus; B, bulblet; E, somatic embryo; P, plantlet; S, shoot; ?, unknown.

The protoplast source can also influence the type of regeneration. Protoplasts isolated from seedling roots of *Medicago sativa* cv. Adriana showed plant regeneration via direct somatic embryogenesis, whereas protoplasts from leaves and hypocotyl-derived suspension cultures initially formed an intermediate callus on which somatic embryos could be induced (Pezzotti *et al.* 1984).

Density gradient centrifugation was used in rice for the separation of relatively uniform protoplasts from a heterogeneous population (Masuda *et al.* 1989). The fraction with the highest specific gravity contained many cytoplasm-rich protoplasts and showed the highest plating efficiency (up to 0·7%). The fraction with the lowest specific gravity, which mainly contained transparent protoplasts with large vacuoles, rarely underwent continued divisions. Similar results have been reported for *Citrus* (Tusa *et al.* 1990) and *Larix* × *eurolepis* (Klimaszewska 1989). Protoplasts can also be separated electrophoretically. A population of pea protoplasts separated in this manner, appeared to be viable and able to divide after subsequent culture (Koonen & Jacobsen 1991). In these examples, however, a stimulatory influence of the treatment itself cannot be excluded.

Protoplasts, even originating from the same organ, can show differences in competence to cell division and subsequent plant regeneration (cell heterogeneity). In this regard, the phenomenon of polysomaty is important. In the majority of the Angiospermae the differentiated tissues are polysomatic, i.e. they contain a mixture of cells with diploid and polyploid nuclei (D'Amato 1952). Polyploidy can interfere with the isolation, culture and plant regeneration from protoplasts (Uijtewaal 1987; Huang & Chen 1988).

The complexity of cellular competence to cell division and regeneration has been a major subject of discussion in various fundamental studies on protoplast regeneration (see abstracts of the VIIIth International Protoplast Symposium at Uppsala in 1991). These studies can provide further knowledge on the cellular characteristics and processes involved in the development of a complete plant from a single protoplast.

Cellular factors

In the process of protoplast regeneration, five categories of cellular phenomena can be distinguished: *stress response*, the self-defence mechanism of the plant cell; *repair mechanism*, repair of membrane damage and of membrane protein systems, formation of a new cell wall and restoration of the cytoskeleton; *dedifferentiation*, morphological and functional adaptations of organelles and cytoplasm; *cell division*, induction of the cell cycle, continuous cell divisions and callus formation; and *morphogenesis*, induction of organized cell growth and differentiation. The first four processes, discussed below, show a considerable degree of autonomy. However, the way in which these individual processes interact, determines the ultimate success of the entire regeneration process. The latter process will be described in the section 'Morphogenesis'.

Stress response. In recent years, stress response processes have received much attention in protoplast research (see poster abstracts in the proceedings of the 1991 Congress of the International Society for Plant Molecular Biology at Tucson and the VIIIth International Protoplast Symposium at Uppsala in 1991). Cells have the competence to react to external stimulation or elicitation (e.g. wounding, infection, heat and UV-light) by a very rapid induction of self-defence mechanisms. During the protoplast isolation procedure, cell-wall degrading enzymes appeared to be important stress-inducing agents as they can produce activated oxygen, which causes lipid peroxidation, resulting in decrease of the fluidity and concomitant leakage of the cytoplasmic membrane (Ishii 1988). This type of

membrane damage was negatively correlated with the regeneration ability of sunflower (*Helianthus annuus*) protoplasts (Biedinger & Schnabl 1991). Cells generally respond to these and other stresses with a rapid production of enzyme systems, involved in the initiation of various metabolic pathways, such as the phenyl-propanoid route, resulting in the formation of phyto-alexins and structural polymers like lignin, as demonstrated in alfalfa (Dixon *et al.* 1991). Also genes coding for ubiquitin proteins, extensin-like proteins, peroxidases and proteinase-inhibitors, became activated immediately after protoplast isolation (Criqui *et al.* 1991). In freshly isolated tobacco protoplasts, the production of two chitinases, two osmotin proteins and a glucanase was demonstrated (Meyer *et al.* 1991).

Some of the stress responses appear to be less favourable for isolated protoplasts. The occurrence of lipoxygenase activity, that produced lipid peroxides, caused oxidative damage to membranes in *Beta vulgaris* protoplasts (Krens *et al.* 1990). Another detrimental stress response reaction on potato protoplast viability and development was the production of ethylene (Perl *et al.* 1988).

The nature of the stress response of the protoplasts to the isolation procedure and the applied culture conditions is an important factor which determines the further expression of their cellular totipotency. Therefore, more knowledge on these response processes will be essential for an understanding of the success or the failure of the following processes involved in plant regeneration from protoplasts.

Repair mechanism. Protoplast isolation starts with the removal of the cell wall by hydrolytic enzymes. During isolation, various cellular structures will be lost, like the cell wall, or disturbed, like the protein systems of the plasma membrane. Due to the absence of the cell wall, which is the modelling factor of the cell, changes occurred in the orientation and organization of the various elements of the cytoskeleton in the protoplasts. In addition, the protoplast isolation procedure generally results in the disturbance of cell polarity (Simmonds 1991).

The plasma membrane contains protein complexes, responsible for the transport of organic compounds and ions in and out the cell, and for the recognition of a variety of external signals. Since most cell wall degrading enzymes are more or less contaminated with proteolytic enzymes, these membrane protein complexes are disturbed partly or completely (Lin 1985; Morris 1985). Isolated protoplasts can only function after resynthesis of these protein complexes.

The ability of the individual protoplast to repair its cytoplasmic membrane and protein components in it, the cytoskeleton and the cell wall, greatly determines the success of its further development.

Dedifferentiation. Various dedifferentiation processes occur depending on the original cytology of the isolated protoplast. For example, protoplasts isolated from potato tuber tissue contain large amyloplasts. Cell division was observed only after metabolization of the starch, which was completed after approximately 7 days (Jones *et al.* 1989). The dedifferentiation process of chloroplasts in tobacco protoplasts could span over several cell cycles (Nagata & Yamaki 1973; Gigot *et al.* 1975), and was not necessarily linked to the initiation of the first cell divisions. Mesophyll protoplasts normally contain numerous chloroplasts. After isolation of tobacco protoplasts, the chloroplasts showed remarkable changes: their volume decreased and crystalloid inclusions and thylakoids disappeared. Also the cytoplasm changed in these protoplasts: the central vacuole disappeared, the

cellular volume increased considerably and the cell became rich in cytoplasm containing many ribosomes. In addition, the nucleus, mostly condensed before and during the isolation procedure, increased in volume and showed less condensed chromatin during the initial culture period (Bergounioux *et al.* 1986; 1988). The protoplast dedifferentiated into a meristematic-like cell.

Cell division. The cytoskeleton connects the various organelles and other cytoplasmic elements of the cell with each other and with the plasma membrane. The cytoskeleton and the cell wall are involved in cell division and cell differentiation (Derksen *et al.* 1990).

Microtubules play an important role in plant morphogenesis because they participate in regulating cell shape and determining the plane and site of cell division (Simmonds 1991). The correlation between microtubule organization and cell division has been demonstrated by Fowke & co-workers (1990) in embryogenic protoplast cultures of white spruce (*Picea glauca*) and by Dijak & Simmonds (1988) during direct somatic embryogenesis from mesophyll protoplasts of *Medicago sativa.*

The formation of a complete new cell wall generally takes 2 days. Protoplasts isolated from rapidly dividing suspension cells of *Vicia hajastana* divided within this period, which resulted in many severe abnormalities in the distribution of the genetic material over the daughter cells (Simmonds 1991). These results confirm the essential role of the cell wall in the cell division process. In addition, the presence of a (repaired) dense network of cortical microtubules appeared to be a prerequisite for the reorientation of the nucleus and the reoccurrence of cell division.

The competence of a protoplast to undergo cell division is also dependent on the cell cycle phase. In *Nicotiana plumbaginifolia* leaf tissue, mainly cells in the G2-phase showed high cell division activity after protoplast isolation (Magnien *et al.* 1982). Comparable results were obtained in cotton, where protoplasts isolated from cotyledons showed a strong positive correlation between G2-phase and the competence for cell wall formation and cell division; cells in G1-phase did not appear to be competent for these processes (Firoozabady 1986). In *Petunia* leaf protoplasts, synthesis of RNA was resumed 18 hours after isolation. In addition, an increase in the RNA concentration above a critical level was necessary to induce DNA replication and cell division (Bergounioux *et al.* 1988).

Due to cell heterogeneity, different types of calli developed from a single population of protoplasts. Compact, friable and intermediate types of calli were distinguished in leaf protoplast cultures of various monohaploid potato genotypes (Uijtewaal *et al.* 1987).

External factors

Culture medium. The basal culture medium and its supplements can play a decisive role in the regeneration of protoplasts (Roest & Gilissen 1989). The low viability of protoplasts of *Lycopersicon pennellii* could be correlated with high ethylene production and increased cell sap osmolality (Rethmeier *et al.* 1991). Moreover, the choice of cell wall degrading enzymes influenced the release of ethylene. Addition of the ethylene-inhibitor silver thiosulphate to the culture medium improved yield, viability and regeneration of protoplasts of *Lycopersicon pennellii* (Rethmeier *et al.* 1991) and potato (Perl *et al.* 1988; Möllers *et al.* 1992). Addition of *o*-acetyl-salicylic acid also repressed the formation of ethylene in isolated protoplasts. This repression resulted in an increased plating efficiency of protoplasts of potato (Perl *et al.* 1988), barley (Holme *et al.* 1991) and *Lolium* (Creemers-Molenaar *et al.* 1992). However, in the latter species, this increase was not related to a proportionally increased regeneration frequency.

The addition of the anti-oxidants polyvinylpirrolidone (pvp) and glycine to the enzyme mixture as well as to the culture medium resulted in a higher yield of viable mesophyll protoplasts in *Prunus avium* (Ochatt 1991b). A combination of the anti-oxidants glutathione, glutathione-peroxydase and phospholipase, also increased the plating efficiency and the growth of microcallus from protoplasts of *Lolium perenne* (Creemers-Molenaar & Van Oort 1990). In *Beta vulgaris*, the addition of the anti-oxidant *n*-propylgallate (*n*-PG) to the medium, which inhibits the activity of lipoxygenase, proved to be essential for successful protoplast culture and shoot regeneration (Krens *et al.* 1990).

Physical environment. The importance of the physical environment (e.g. plating density, pH, temperature and light) for culture and regeneration of protoplasts has been reviewed by Maheshwari *et al.* (1986). Electro-stimulation, i.e. the application of a low-voltage treatment, resulted in an enhanced division of protoplasts of *Medicago sativa* (Dijak & Simmonds 1988), *Trifolium subterraneum* (Li *et al.* 1990), *Solanum dulcamara* (Chand *et al.* 1988) and *Solanum viarum* (Chand 1991).

In barley (*Hordeum vulgare*) green plantlets have been regenerated from protoplasts derived from suspension cultures (Yan *et al.* 1990), and in rice (*Oryza sativa*) the plating efficiency has been increased from cell suspension-derived protoplasts (Lin *et al.* 1991), after heat shock treatment (45°C) for 5–8 min followed by a cold treatment in ice water (0°C) for 10 s.

Culture technique. The induction of cell division and regeneration of plated protoplasts is highly dependent on the culture system applied. Compared with plating in liquid medium, embedding of protoplasts in agarose beads and discs resulted in increased plating efficiencies. The application of agarose for protoplast cultures has been reviewed (Dons & Colijn-Hooymans 1989). Recently, embedding protoplasts in alginate (droplets or thin layers) or gellan gum (gelrite) also improved plating and regeneration efficiency in species like *Hordeum vulgare* (Eigel & Koop 1989; Yan *et al.* 1990), *Oryza sativa* (Datta *et al.* 1990a), *Vitis labruscana* and *Vitis thunbergii* (Mii *et al.* 1991b), *Brassica napus* and *Nicotiana tabacum* (Eigel & Koop 1989) and *Nicotiana plumbaginifolia* (Verhoeven *et al.* 1990). Embedding protoplasts in calcium alginate beads at room temperature increased the plating efficiency in *Medicago* and *Nicotiana* (Larkin *et al.* 1988). When compared with plating in agarose, embedding protoplasts in calcium alginate beads has two advantages: (i) the absence of an elevated temperature treatment in the latter procedure; and (ii) the possibility of using sodium citrate to dissolve the calcium alginate matrix. This enabled easy recovery of the entrapped cells or (micro)calli by a gentle dissolution of the gel layers and beads (Smidsród & Skjåk-Bræk 1990), which is much more complicated in agarose-solidified media.

The proportion of dividing protoplasts can also be improved by ultrafiltration of contaminant macromolecules (including glucose, minor sugars and sugar alcohols, etc.) from the culture medium, as demonstrated for *Medicago sativa* and *Nicotiana tabacum* (Davies *et al.* 1989).

The importance of the use of feeders or nurse culture techniques has been demonstrated in various crops. In oat (*Avena sativa*), feeders from graminaceous plants promoted protoplast proliferation, while feeders from dicotyledonous plant species suppressed protoplast division (Hahne *et al.* 1990). In *Brassica oleracea*, a simple versatile feeder layer system has been developed (Walters & Earle 1990). A nurse culture technique has been used for *Trifolium subterraneum* (Li *et al.* 1990) and a mixed nurse plating technique for

Festuca rubra (Zaghmout & Torello 1990). Regeneration in *Citrus* was achieved via co-culture with embryogenic cells (Tusa *et al.* 1990). In barley (*Hordeum vulgare*), tobacco (*Nicotiana tabacum*) and rape (*Brassica napus*), individual protoplasts were regenerated using feeder systems (Eigel & Koop 1989; Schäffler & Koop 1990). Conditioned medium can also be applied for an increase in the plating efficiency of cultured protoplasts, as demonstrated in perennial ryegrass (*Lolium perenne*) (Creemers-Molenaar *et al.* 1992).

Recently, a microscopic device connected to a cell finder system has been developed (Verhoeven *et al.* 1990). This system can be applied to determine the position of agarose-immobilized cells and protoplasts, and for various analyses, i.e. the development of individual protoplasts isolated from different sources, the effect of feeders and vital staining, the formation of micronuclei and fusion of microprotoplasts, the selection of fusion products, selection of cells or protoplasts with high production of secondary metabolites, micro-injection, fate of introduced organelles or chromosomes, and time-lapse analysis. In addition, the computerized hydraulic system of Eigel & Koop (1989), enables microscopic selection of single protoplasts from protoplast populations and subsequent regeneration studies.

Plant regeneration from individual protoplasts can be achieved via culture in microdroplets of medium or by the application of feeder systems. Using such culture systems, regeneration from individual protoplasts has been obtained for different *Solanum* species (Hunt & Helgeson 1989), *Hordeum vulgare, Nicotiana tabacum* and *Brassica napus* (Eigel & Koop 1989), as well as for fusion products of defined protoplast pairs in *Nicotiana* (Spangenberg *et al.* 1990) and products of microfusions between defined protoplast and cytoplast pairs in *Nicotiana tabacum* (Spangenberg *et al.* 1991).

In addition, regeneration of shoot buds and plants has been achieved for protoplasts isolated from cryopreserved cell lines of rice (*Oryza sativa*) (Meijer *et al.* 1991), and from cryopreserved protoplasts of maize (Shillito *et al.* 1989; Zhang *et al.* 1990), *Atropa belladonna, Datura innoxia* and *Nicotiana tabacum* (Bajaj 1988).

Morphogenesis

Cellular aspects. Morphogenesis is the result of organized cell growth, including determination and coordination of the plane and time of cell division, and the degree of cell enlargement. Research at the cellular level on the induction of competence to morphogenesis in callus cultures is often hampered by the presence of numerous unorganized growing cells. Nevertheless, it has been found that morphogenesis in a callus generally starts with the development of polarity within a single, mostly highly vacuolated cell, which subsequently becomes meristematic. In tobacco this process appeared to be linked to an enlargement of the nucleolus and an increased RNA and protein synthesis (Thorpe & Murashige 1970). These cells also showed an accumulation of starch in the plastids (Ross & Thorpe 1973). In *Petunia hybrida*, the formation of preprophase bands was a clear indication for the initiation of organized cell growth. These bands had been preferentially found in small protoplast-derived callus cells with high cytoplasmic content (Traas *et al.* 1990).

Cell heterogeneity in a single population of protoplasts was observed at the level of the regeneration process: in *Nicotiana plumbaginifolia* some leaf protoplasts developed into calli that formed shoots, whereas other leaf protoplasts directly regenerated into roots (Gilissen *et al.* 1991).

Development of regenerants. The regeneration process in most species generally proceeds through two phases: (i) the development of the cell aggregates into a callus; and (ii) the

induction of one or more callus cells (after a few weeks to several months) to undergo morphogenesis. Morphogenesis from protoplast-derived calli can take place via somatic embryogenesis, i.e. the regeneration of somatic embryos, and via organogenesis, i.e. the regeneration of shoots and bulblets.

In some plant species, direct somatic embryogenesis from protoplasts has also been observed (see Table 1 in Roest & Gilssen 1989, and Table 1 in this paper). In this case, the intermediate callus stage is absent. Apparently, these species are able to maintain their cellular polarity, or to restore it very rapidly. Direct somatic embryogenesis was extensively studied in leaf mesophyll protoplasts of *Medicago sativa* (Dijak & Brown 1987; Song *et al.* 1990). Also, protoplasts isolated from seedling roots of alfalfa cv. Adriana showed plant regeneration via direct somatic embryogenesis. Remarkably, in the same cultivar protoplasts from leaf and from hypocotyl-derived cell suspension cultures initially formed an intermediate callus before somatic embryogenesis occurred (Pezzotti *et al.* 1984).

In some plant species, like pea (*Pisum sativum*), regeneration from protoplasts occurred both via organogenesis and somatic embryogenesis. The latter was achieved by the application of strong auxins and an increased osmolality of the culture medium (Lehminger-Mertens & Jacobsen 1989).

In a number of species, only incomplete regeneration or abnormal (albino or sterile) plants were obtained. However, in some of these species, e.g. *Picea glauca* (Attree *et al.* 1989) and apple (*Malus* × *domestica*) (Patat-Ochatt *et al.* 1988), complete plantlets can now be regenerated using improved regeneration methods. In addition, for various graminaceous plant species, regeneration procedures have been developed which lead to green and fertile (instead of albino and sterile) plantlets, as in *Hordeum vulgare* (Yan *et al.* 1990; Jähne *et al.* 1991a,b), *Lolium perenne* (Creemers-Molenaar *et al.* 1989), Indica rice (*Oryza sativa*) (Datta *et al.* 1990a), *Triticum aestivum* (He *et al.* 1992) and *Zea mays* (Prioli & Söndahl 1989; Shillito *et al.* 1989; Morocz *et al.* 1990). Fertile plants have also been regenerated from protoplasts of sunflower (*Helianthus annuus*) (Burrus *et al.* 1991).

Somaclonal variation. While micropropagation through adventitious shoot formation from explants normally does not lead to excessive somaclonal variation, regeneration from protoplasts is mostly associated with a high frequency of somaclonal variation. To satisfy both the need for stability and the exploitation of somaclonal variation, it would be beneficial if the level of genetic instability could be controlled. The degree of somaclonal variation is affected by various factors, i.e. the genotype, the ploidy level of the source material, the protoplast donor source, the tissue culture procedure and the medium composition (Sree Ramulu 1987; Karp 1991). However, morphological and cytological analyses of populations of regenerants from protoplasts derived from leaves and cotyledons of *Lotus corniculatus* exhibited similar frequencies of variation (Webb & Watson 1991). In general, the longer the *in-vitro* culture phase, the higher the degree of genetic instability (Karp 1991). It was suggested that a procedure of rapid regeneration via (direct) somatic embryogenesis, without a prolonged intervening callus stage, would largely avoid the problem of genetic instability. However, no clear relationship could be demonstrated until now between somatic embryogenesis and reduced genetic instability (Karp 1991).

CONCLUSIONS AND PERSPECTIVES

The attractiveness of protoplasts for application in plant breeding is based on the combination of two important aspects: (i) their amenability to various genetic manipulation techniques, e.g. somatic hybridization, cybridization, direct DNA transfer and

micro-injection; and (ii) the potential for plant regeneration enabling the production of a new genotype from each successfully manipulated protoplast.

During the last 3 years, considerable progress has been made in the field of protoplast regeneration. This can be attributed to the use of appropriate genotypes and donor tissues, improved pretreatment conditions of explants and newly developed culture techniques. Up till now, regeneration from protoplasts has been obtained in 320 plant species, including many important agricultural and horticultural crops and woody plant species.

In general, the research activities carried out in the field of plant regeneration from protoplasts were mainly directed towards the optimization of the culture conditions for large populations of protoplasts, and mostly based on 'trial and error'. Therefore, less fundamental knowledge has so far been gained on plant, cellular and external factors, which act at the level of the individual protoplast during the regeneration process. Recently, however, various techniques have become available for fundamental studies on individual protoplasts for their competence to cell division and morphogenesis, e.g. the microscopic device connected with the cell finder system (Verhoeven *et al.* 1990), the computerized hydraulic system (Eigel & Koop 1989), the confocal laser scanning microscope, in combination with various fluorescent staining techniques (Haugland 1989) and flow cytometry. In addition, techniques which were developed for the (direct) regeneration from individually cultured protoplasts, selected subpopulations of protoplasts and cryopreserved protoplasts, are important achievements for both fundamental studies and genetic manipulation of protoplasts.

With respect to genetic manipulation of protoplasts, various procedures have recently been developed: e.g. somatic hybridization by microfusion of defined protoplast and cytoplast pairs (Spangenberg *et al.* 1990; 1991), partial genome transfer via microprotoplasts (Verhoeven *et al.* 1991), transfer of cell organelles, like chloroplasts by microfusion (Eigel *et al.* 1991) and mitochondria by micro-injection (Verhoeven & Blaas 1992), transformation via micro-injection (Schnorf *et al.* 1991), via PEG or electroporation (Potrykus 1990), via liposomes (Spörlein & Koop 1991) and via mild sonication (Joersbo & Brunstedt 1990).

Plant species belonging to the Brassicaceae and Solanaceae proved to be highly responsive in regeneration and genetic manipulation studies. Plants have been obtained after symmetric somatic hybridization in *Brassica* (Glimelius *et al.* 1991) and potato (Helgeson 1989) and after asymmetric hybridization in *Brassica* (Sundberg & Glimelius 1991) and tomato (Derks 1992). In cereals and other monocotyledonous plant species most efforts were directed towards the production of transgenic plants via direct gene transfer (Potrykus 1990; Vasil 1990). In graminaceous species, using PEG treatment or electroporation of protoplasts for DNA uptake, transformed plants were obtained in *Agrostis alba* (Asano *et al.* 1991), orchard grass (*Dactylis glomerata*) (Horn *et al.* 1988), rice (*Oryza sativa*) (Datta *et al.* 1990b; Toriyama *et al.* 1988) and corn (*Zea mays*) (Rhodes *et al.* 1988).

In spite of all these achievements, the routine use of protoplasts in genetic manipulation of important monocotyledonous and dicotyledonous crops might take several years for the following reasons.

1. In order to achieve plant regeneration from protoplasts in recalcitrant species or genotypes, significant fundamental advances have still to be made in our understanding of the phenomenon of totipotency of the plant cell.

2. The genetic analysis of agronomic and horticultural characters is highly complex and up till now only very few important genes have been identified and isolated (Snape *et al.* 1990). Moreover, many of these characters are polygenically controlled, which complicates considerably their identification and isolation, and thus manipulation by transformation technologies. In addition, the expression and maintenance of introduced genes depend on many, less understood aspects, e.g. the integration site of the genes in the host genome.
3. Extensive laboratory and field experiments have to be carried out with genetically manipulated plants in various growing seasons, especially if several progenies have to be tested or if woody plant species are involved.
4. Substantial time is required for regulatory approval, crop certification and public acceptance of the transgenic plants.

Nevertheless, it is expected that in the future the application of protoplasts in genetic manipulation will become an important complement to plant breeding.

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REFERENCES

Acuna, J.R. & Pena, M. de (1991): Plant regeneration from protoplasts of embryogenic cell suspensions of *Coffea arabica* L. cv. caturra. *Plant Cell Rep.* **10:** 345–348.

Adachi, T., Yamaguchi, A., Miike, Y. & Hoffmann, F. (1989): Plant regeneration from protoplasts of common buckwheat (*Fagopyrum esculentum*). *Plant Cell Rep.* **8:** 247–250.

Akashi, R. & Adachi, T. (1992): Plant regeneration from suspension cultured-derived protoplasts of apomictic dallisgrass (*Paspalum dilatatum* Poir.). *Plant Sci.* **82:** 219–225.

Arya, S., Liu, J.R. & Eriksson, T. (1991): Plant regeneration from protoplasts of *Panax ginseng* (C.A. Meyer) through somatic embryogenesis. *Plant Cell Rep.* **10:** 277–281.

Asano, Y., Otsuki, Y. & Ugaki, M. (1991): Electroporation-mediated and silicon carbide fiber-mediated DNA delivery in *Agrostis alba* L. (Redtop). *Plant Sci.* **79:** 247–252.

Asano, Y. & Sugiura, K. (1990): Plant regeneration from suspension culture-derived protoplasts of *Agrostis alba* L. (Redtop). *Plant Sci.* **72:** 267–273.

Asao, H., Tanigawa, M., Arai, S. & Kobatake, H. (1989): Plant regeneration from mesophyll protoplasts of the eggplant and its wild species. *Bull. Nara. Agricult. Exp. Station* **20:** 73–78.

Attree, S.M., Dunstan, D.I. & Fowke, L.C. (1989): Plantlet regeneration from embryogenic protoplasts of white spruce (*Picea glauca*). *Biotechnology* **7:** 1060–1062.

Bajaj, Y.P.S. (1988): Regeneration of plants from frozen (−196°C) protoplasts of *Atropa belladonna* L., *Datura innoxia* Mill and *Nicotiana tabacum* L. *Indian J. Exp. Biol.* **26:** 289–292.

Baset, A., Finch, R.P. & Cocking, E.C. (1991): Plant regeneration from protoplasts of wild rice (*Oryza rufipogon* Griff.). *Plant Cell Rep.* **10:** 200–203.

Bergounioux, C., Perennes, C., Brown, S.C., Sarda, C. & Gadal, P. (1988): Relation between protoplast division, cell-cycle stage and nuclear chromatin structure. *Protoplasma* **142:** 127–136.

Bergounioux, C., Perennes, C., Miege, C. & Gadal, P. (1986): The effect of male sterility on protoplast division in *Petunia hybrida*. Cell cycle comparison by flow cytometry. *Protoplasma* **130:** 138–144.

Biedinger, U. & Schnabl, H. (1991): Ethane production as an indicator of the regeneration potential of electrically fused sunflower protoplasts (*Helianthus annuus* L.). *J. Plant Physiol.* **138:** 417–420.

Bonfils, A.-C., Gleddie, S., Webb, J. & Keller, W. (1991): *Capsella bursa-pastoris* cell suspension and protoplast cultures. *Physiol. Plant.* **82:** A10.

Burrus, M., Chanabe, C., Alibert, G. & Bidney, D. (1991): Regeneration of fertile plants from protoplasts of sunflower (*Helianthus annuus* L.). *Plant Cell Rep.* **10:** 161–166.

Cai, Q.-G., Qian, Y.-Q., Ke, S.-Q. & He, Z.-C. (1991): Preliminary analysis of regenerated plants derived from protoplasts of *Actinidia deliciosa*. *Physiol. Plant.* **82:** A12.

Cardi, T., Puite, K.J. & Sree Ramulu, K. (1990): Plant regeneration from mesophyll protoplasts of *Solanum commersonii* Dun. *Plant Sci.* **70:** 215–221.

Chanabe, C., Burrus, M., Bidney, D. & Alibert, G. (1991): Studies on plant regeneration from protoplasts in the genus *Helianthus*. *Plant Cell Rep.* **9:** 635–638.

Chand, P.K. (1991): Electro-stimulation of growth and differentiation of callus and protoplasts of the medicinal species *Solanum viarum* Dunal. In: *Abstracts Int. Symp. Plant Biotechnology and its Contribution to Plant Development, Multiplication and Improvement*. Geneva.

Chand, P.K., Ochatt, S.J., Rech, E.L., Power, J.B. & Davey, M.R. (1988): Electroporation stimulates plant regeneration from protoplasts of the woody medicinal species *Solanum dulcamara* L. *J. Exp. Bot.* **39:** 1267–1274.

Chen, Z.-X., Li, S.-J., Yue, J.-X., Jiao, G.-L., Liu, S.-X., She, J.-M., Wu, J.-Y. & Wang, H.-B. (1989): Plantlet regeneration from protoplasts isolated from an embryogenic suspension culture of cotton (*Gossypium hirsutum* L.). *Acta Bot. Sin.* **31:** 966–969.

Cheng, J. & Veilleux, R.E. (1991): Genetic analysis of protoplast culturability in *Solanum phureja*. *Plant Sci.* **75:** 257–265.

Creemers-Molenaar, J., Eeuwijk, F.A. van & Krens, F.A. (1992): Culture optimization for perennial ryegrass protoplasts. *J. Plant Physiol.* **139:** 303–308.

Creemers-Molenaar, J., Valk, P. van der, Loeffen, J.P.M. & Zaal, M.A.C.M. (1989): Plant regeneration from suspension cultures and protoplasts of *Lolium perenne* L. *Plant Sci.* **63:** 167–176.

Creemers-Molenaar, J. & Oort, Y. van (1990): Antioxidants influence the plating efficiency and microcallus growth of protoplasts in *Lolium perenne* L. In: Nijkamp, H.J.J., Plas, L.H.W. van der & Aartrijk, J. van (eds.): *Progress in Plant Cellular and Molecular Biology*: 44–49. Kluwer Academic Publishers, Dordrecht.

Criqui, M.-C., Durr, A., Genschik, P., Jamet, E., Marbach, J., Parmentier, Y., Plesse, B. & Fleck, J. (1991): In search of genes involved in the re-entry of mesophyll cells into the cell cycle. *Physiol. Plant.* **82:** A35.

D'Amato, F. (1952): Polyploidy in the differentiation and function of tissues and cells in plants. A critical examination of the literature. *Caryologia* **4:** 311–358.

Datta, S.K., Datta, K. & Potrykus, I. (1990a): Fertile indica rice plants regenerated from protoplasts isolated from microspore derived cell suspensions. *Plant Cell Rep.* **9:** 253–256.

Datta, S.K., Peterhans, A., Datta, K. & Potrykus, I. (1990b): Genetically engineered fertile indica-rice recovered from protoplasts. *Biotechnology* **8:** 736–740.

Davies, P.A., Larkin, P.J. & Tanner, G.J. (1989): Enhanced protoplast division by media ultrafiltration. *Plant Sci.* **60:** 237–244.

Derks, F.H.M. (1992): Organelle transfer by protoplast fusion in *Solanaceae*. *Thesis Free University Amsterdam*, 120 pp. Ponsen & Looyen, Wageningen.

Derksen, J., Wilms, F.H.A. & Pierson, E.S. (1990): The plant cytoskeleton: its significance in plant development. *Acta Bot. Neerl.* **39:** 1–18.

Dijak, M. & Brown, D.C.W. (1987): Patterns of direct and indirect embryogenesis from mesophyll protoplasts of *Medicago sativa*. *Plant Cell, Tiss. Organ Cult.* **9:** 121–130.

Dijak, M. & Simmonds, D.H. (1988): Microtubule organization during early direct embryogenesis from mesophyll protoplasts of *Medicago sativa* L. *Plant Sci.* **58:** 183–191.

Dixon, R., Dalkin, K., Fahrendorf, T., Gowri, G., Harrison, M., Loake, G., Maxwell, C., Ni, W., Paiva, N. & Yu, L. (1991): Expression and manipulation of genes encoding enzymes of phenylpropanoid biosynthesis in alfalfa. In: Hallick, R.B. (ed.): *Molecular Biology of Plant Growth and Development*: Abstract Nr. 152. ISPMB Congress, Tucson.

Dong, J.-J. & Xia, Z.-A. (1989): Plantlet regeneration from *Setaria italica* protoplasts. *Plant Physiol. Commun.* **2:** 56–57.

Dons, J.J.M. & Colijn-Hooymans, C.M. (1989): Agarose plating of protoplasts and its applications. In: Bajaj, Y.P.S. (ed.): *Biotechnology in Agriculture and Forestry*, Vol. 8: *Plant Protoplasts and Genetic Engineering*: 50–62. Springer-Verlag, Berlin.

Dorion, N., Danthu, P., Ohki, S., Preneux, C., Godin, B. & Bigot, C. (1991): Plant regeneration from leaf protoplasts of common elm (*Ulmus campestris* Mill.). *C. R. Acad. Sci.* **313:** 467–473.

Dudits, D., Bögre, L. & Györgyey, J. (1991): Molecular and cellular approaches to the analysis of plant embryo development from somatic cells *in vitro*. *J. Cell Sci.* **99:** 473–482.

Dunbar, K.B. & Stephens, C.T. (1991): Plant regeneration from callus-derived protoplasts of *Pelargonium* × *domesticum*. *Plant Cell Rep.* **10:** 417–420.

Dupuis, J.M., Péan, M. & Chagvardieff, P. (1990): Plant donor tissue and isolation procedure effect on early formation of embryoids from protoplasts of *Helianthus annuus* L. *Plant Cell, Tiss. Organ Cult.* **22:** 183–189.

Eigel, L. & Koop, H.-U. (1989): Nurse culture of individual cells: regeneration of colonies from single protoplasts of *Nicotiana tabacum, Brassica napus* and *Hordeum vulgare*. *J. Plant Physiol.* **134:** 577–581.

Eigel, L., Oelmüller, R. & Koop, H.-U. (1991): Transfer of defined numbers of chloroplasts into albino protoplasts using an improved subprotoplast/protoplast microfusion procedure: Transfer of only two chloroplasts leads to variegated progeny. *Mol. Gen. Genet.* **227:** 446–451.

Elshihy, O.M. & Evans, P.K. (1986): Protoplast isolation, culture and plant regeneration of cotton (*Gossypium barbadense* L.). In: Somers, D.A., Gengenbach, B.G., Biesboer, D.D., Hackett, W.P. & Green, C.E. (eds.): *Abstracts VIth IAPTC Congress*: 52. University of Minnesota, Minneapolis.

Firoozabady, E. (1986): The effects of cell cycle parameters on cell wall regeneration and cell division of cotton protoplasts (*Gossypium hirsutum* L.). *J. Exp. Bot.* **37:** 1211–1217.

Fowke, L.C., Attree, S.M., Wang, H. & Dunstan, D.I. (1990): Microtubule organization and cell division in embryogenic protoplast cultures of white spruce (*Picea glauca*). *Protoplasma* **158:** 86–94.

Ge, K.-L., Wang, Y.-Z., Yuan, X.-M., Huang, P.-M., Yang, J.-S., Nie, Z.-P., Testa, D. & Lee, N. (1989): Plantlet regeneration from protoplasts isolated from mesophyll cells of Adsuki bean. (*Phaseolus angularis*, Wight). *Plant Sci.* **63:** 209–216.

Gigot, C., Kopp, M., Schmitt, C. & Milne, R.G. (1975): Subcellular changes during isolation and culture of tobacco mesophyll protoplasts. *Protoplasma* **84:** 31–41.

Gilissen, L.J.W., Bronsema, F.B.F., Vries-Uijtewaal, E. de, Staveren, M.J. van, Kieft, H. & Schel, J.H.N. (1991). Structure and physiology of the development of roots from microcalli of leaf protoplasts of Ri-transformed and wild type *Nicotiana plumbaginifolia*. *Acta Bot. Neerl.* **40:** 225–235.

Glimelius, K., Fahlesson, J., Landgren, M., Sjödin, C. & Sundberg, E. (1991): Gene transfer via somatic hybridization in plants. *Tibtech.* **9:** 24–30.

Gupta, P.K., Dandekar, A.M. & Durzan, D.J. (1988): Somatic proembryo formation and transient expression of a luciferase gene in Douglas fir and Loblolly pine protoplasts. *Plant Sci.* **58:** 85–92.

Gupta, P.K., Pullman, G.S., Kreitinger, M.E. & Timmis, R. (1990): High frequency plantlet regeneration via somatic embryogenesis from protoplasts of Norway spruce (*Picea abies*) embryonal cells. In: *Abstracts VIIth IAPTC Congress*: 103. Amsterdam.

Hahne, B., Lörz, H. & Hahne, G. (1990): Oat mesophyll protoplasts: their response to various feeder cultures. *Plant Cell Rep.* **8:** 590–593.

Hammatt, N., Jones, B. & Davey, M.R. (1989): Plant regeneration from seedling explants and cotyledon protoplasts of *Glycine argyrea* Tind. *In Vitro Cell. Dev. Biol.* **25:** 669–672.

Haugland, R.P. (1989): *Handbook of Fluorescent Probes and Research Chemicals*. Molecular Probes Inc., Eugene.

He, D.G., Yang, Y.M. & Scott, K.J. (1992): Plant regeneration from protoplasts of wheat (*Triticum aestivum* cv. Hartog). *Plant Cell Rep.* **11:** 16–19.

Helgeson, J.P. (1989): Somatic hybridization of wild *Solanum* species with potato: a potential source of diversity for breeders. In: Louwes, K.M., Toussaint, H.A.J.M. & Dellaert, L.M.W. (eds.): *Parental Line Breeding and Selection in Potato Breeding*, 87–94. PUDOC, Wageningen.

Holme, I., Andersen, B., Knudsen, E., Vangsgård, K. & Nielsen, K.A. (1991): Peroxidase and esterase isozymes expressed upon protoplast isolation and micro callus formation in barley (*Hordeum vulgare* L.). *Physiol. Plant.* **82:** A6.

Horn, M.E., Shillito, R.D., Conger, B.V. & Harms, C.T. (1988): Transgenic plants of orchardgrass (*Dactylis glomerata* L.) from protoplasts. *Plant Cell Rep.* **7:** 469–472.

Huang, H.-C. & Chen, C.-C. (1988): Genome multiplication in cultured protoplasts of two *Nicotiana* species. *J. Hered.* **79:** 28–32.

Hunt, G.J. & Helgeson, J.P. (1989): A medium and simplified procedure for growing single cells from *Solanum* species. *Plant Sci.* **60:** 251–257.

Isa, T., Ogasawara, T. & Kaneko, H. (1990): Regeneration of saffron protoplasts immobilized in Ca-alginate beads. *Jap. J. Breed.* **40:** 153–157.

Ishii, S. (1988): Factors influencing protoplast viability of suspension-cultured rice cells during isolation process. *Plant Physiol.* **88:** 26–29.

Ito, K., Doi, K., Tatemichi, Y. & Shibata, M. (1990): Plant regeneration form protoplasts of *Eucalyptus*. In: *Abstracts VIIth IAPTC Congress*: 19. Amsterdam.

Izhar, S. & Power, J.B. (1977): Genetical studies with petunia leaf protoplasts. I. Genetic variation to specific growth hormones and possible genetic control on stages of protoplast development in culture. *Plant Sci.* **8:** 375–383.

Jähne, A., Lazzeri, P.A., Jäger-Gussen, M. & Lörz, H. (1991a): Plant regeneration from embryogenic cell suspensions derived from anther cultures of barley (*Hordeum vulgare* L.). *Theor. Appl. Genet.* **82:** 74–80.

Jähne, A., Lazzeri, P.A. & Lörz, H. (1991b): Regeneration of fertile plants from protoplasts derived from embryogenic cell suspensions of barley (*Hordeum vulgare* L.). *Plant Cell Rep.* **10:** 1–6.

Jing, Z.-P. & Wang, Z.-Z. (1991): Plant regeneration from leaf callus protoplasts of *Caladium bicolor* Vent. *Physiol. Plant.* **82:** A17.

Joersbo, M. & Brunstedt, J. (1990): Direct gene transfer to plant protoplasts by mild sonication. *Plant Cell Rep.* **9:** 207–210.

Jones, H., Karp, A. & Jones, M.G.K. (1989): Isolation, culture, and regeneration of plants from potato protoplasts. *Plant Cell Rep.* **8:** 307–311.

Kanchanapoom, M. & Kanchanapoom, K. (1991): Isolation, culture and regeneration of *Theobroma* protoplasts. *Physiol. Plant.* **82:** A14.

Karp, A. (1991): On the current understanding of somaclonal variation. In: Miflin, B.J. (ed.): *Oxford Surveys of Plant Molecular and Cell Biology:* **7:** 1–58. Oxford University Press, Oxford.

Kitamura, Y., Morikawa, T. & Miura, H. (1989): Isolation and culture of protoplasts from cell suspension cultures of *Duboisia myoporoides* with subsequent plant regeneration. *Plant Sci.* **60:** 245–250.

Klimaszewska, K. (1989): Recovery of somatic embryos and plantlets from protoplast cultures of *Larix* × *eurolepis*. *Plant Cell Rep.* **8:** 440–444.

Koonen, F. & Jacobsen, H.-J. (1991): Electrophoretic separation and characterization of pea protoplasts. *Physiol. Plant.* **82:** A3.

Koornneef, M., Hanhart, C.J. & Martinelli, L. (1987): A genetic analysis of cell culture traits in tomato. *Theor. Appl. Genet.* **74:** 633–641.

Krasnyanski, S., Polgár, Z., Németh, G. & Menczel, L. (1992): Plant regeneration from callus and protoplast cultures of *Helianthus giganteus* L. *Plant Cell Rep.* **11:** 7–10.

Krens, F.A., Jamar, D., Rouwendal, G.J.A. & Hall, R.D. (1990): Transfer of cytoplasm from new *Beta* CMS sources to sugar beet by asymmetric fusion. 1. Shoot regeneration from mesophyll protoplasts and characterization of regenerated plants. *Theor. Appl. Genet.* **79:** 390–396.

Kumar, A. & Tandon, P. (1991): Morphogenesis in cultured protoplasts of *Pinus kesiya* Royle ex Gord. *Physiol. Plant.* **82:** A12.

Kunitake, H., Kagami, H. & Mii, M. (1991a): Somatic embryogenesis and plant regeneration from protoplasts of 'Satsuma' mandarin (*Citrus unshiu* Marc.). *Scientia Hort.* **47:** 27–33.

Kunitake, H., Kagami, H. & Mii, M. (1991b): Plant regeneration from protoplasts of Satsuma mandarin (*Citrus unshiu* Marc.) via somatic embryogenesis. *Jpn Agr. Res. Quarterly* **24:** 287–291.

Kunitake, H., Kanno, K. & Mii, M. (1990): Plant regeneration from protoplasts of prairie gentian (*Eustoma grandiflorum*). *Jap. J. Breed.* **40:** (suppl. 1): 216–217.

Kunitake, H. & Mii, M. (1990a): Plant regeneration from cell culture-derived protoplasts of statice (*Limonium perezii* Hubbard). *Plant Sci.* **70:** 115–119.

Kunitake, H. & Mii, M. (1990b): Induction of embryogenic calli and protoplast culture of rugosa rose (*Rosa rugosa*). *Jap. J. Breed.* **40:** (suppl. 1): 214–215.

Laine, E. & David, A. (1990): Somatic embryogenesis in immature embryos and protoplasts of *Pinus caribaea*. *Plant Sci.* **69:** 215–224.

Lang, H. & Kohlenbach, H.W. (1989): Cell differentiation in protoplast cultures from embryogenic callus of *Abies alba* L. *Plant Cell Rep.* **8:** 120–123.

Larkin, P.J., Davies, P.A. & Tanner, G.J. (1988): Nurse culture of low numbers of *Medicago* and *Nicotiana* protoplasts using calcium alginate beads. *Plant Sci.* **58:** 203–210.

Lee, J.S., Lee, S.K., Jang, S.S. & Lee, J.J. (1987): Plantlet regeneration from callus protoplasts of *Populus nigra*. *Res. Rep. Inst. For. Gen. Korea* **23:** 143–148.

Lehminger-Mertens, R. & Jacobsen, H.-J. (1989): Plant regeneration from pea protoplasts via somatic embryogenesis. *Plant Cell Rep.* **8:** 379–382.

Li, X.-H. (1991): Advance in research of protoplast regeneration of major crops and economic plants. *Physiol. Plant.* **82:** A7.

Li, Z.-Y., Tanner, G.J. & Larkin, P.J. (1990): Callus regeneration from *Trifolium subterraneum* protoplasts and enhanced protoplast division by low-voltage treatment and nurse cells. *Plant Cell, Tiss. Organ Cult.* **21:** 67–73.

Lin, M.-Y., Lin, C.-Y. & Chen, Y.-M. (1991): Biochemical studies on the enhancement of suspension cells and protoplasts proliferation in rice (*Oryza sativa* L.) by heat shock treatment. *Bot. Bull. Acad. Sin.* **32:** 205–214.

Lin, W. (1985): The binding of anion transport inhibitors on the plasmalemma isolated from corn root protoplasts. In: Pilet, P.E. (ed.): *The Physiological Properties of Plant Protoplasts*: 68–75. Springer-Verlag, Berlin.

Ling, H.Q. & Binding, H. (1992): Improvement of plant regeneration from *Linum* protoplasts by the induction of somatic embryogenesis. *J. Plant Physiol.* **139:** 422–426.

Ling, J.-T., Nito, N. & Iwamasa, M. (1989): Plant regeneration from protoplasts of calamondin (*Citrus madurensis* Lour.). *Scientia Hort.* **40:** 325–333.

Ling, J.-T., Nito, N., Iwamasa, M. & Kunitake, H. (1990): Plant regeneration from protoplasts isolated from embryogenic callus of satsuma. *Hort. Sci.* **25:** 970–972.

Liu, C.-M. & Xu, Z.-H. (1988): Plant regeneration and transformation of mesophyll protoplasts of *Nicotiana glutinosa* L. *Acta Biol. Exp. Sin.* **21:** 273–279.

Liu, Q.-C., Kokubu, T. & Sato, M. (1991): Plant regeneration from *Ipomoea triloba* L. protoplasts. *Jpn J. Breed.* **41:** 103–108.

Lu, D.Y., Chen, Y.M., Wang, C. & Chen, Y. (1986): Plant regeneration from protoplasts and explants of *Lotus corniculatus, Coronilla varia* and *Brassica juncea*. In: Somers, D.A., Gengenbach, B.G., Biesboer, D.D., Hackett, W.P. & Green, C.E. (eds.): *Abstracts VIth IAPTC Congress*: 53. University of Minnesota, Minneapolis.

Magnien, E., Dalschaert, X. & Faraoni-Sciamanna, P. (1982): Transmission of a cytological heterogeneity from the leaf to the protoplasts in culture. *Plant Sci.* **25:** 291–303.

Maheshwari, S.C., Gill, R., Maheshwari, N. & Gharyal, P.K. (1986): Isolation and regeneration of protoplasts from higher plants. In: Reinert, J. & Binding, H. (eds.): *Results and Problems in Cell Differentiation*, Vol. 12: *Differentiation of Protoplasts and of Transformed Plant Cells*: 3–36. Springer-Verlag, Berlin.

Malaure, R.S., Davey, M.R. & Power, J.B. (1990): Plant regeneration from protoplasts of *Felicia* and *Brachycome*. *Plant Cell Rep.* **9:** 109–112.

Manders, G., d'Utra Vaz, F.B., Dos Santos, A.V.P., Davey, M.R. & Power,. J.B. (1991): Plant regeneration from mesophyll protoplasts of passionfruit (*Passiflora edulis* var flavicarpa). *Physiol. Plant.* **82:** A16.

Masuda, K., Kudo-Shiratori, A. & Inoue, M. (1989): Callus formation and plant regeneration from rice protoplasts purified by density gradient centrifugation. *Plant Sci.* **62:** 237–246.

Matthews, D., Mottley, J., Horan, I. & Roberts, A.V. (1991): A protoplast to plant system in roses. *Plant Cell, Tisue Organ Culture* **24:** 173–180.

Meijer, E.G.M., Iren, F. van, Schrijnemakers, E., Hensgens, L.A.M., Zijderveld, M. van & Schilperoort, R.A. (1991): Retention of the capacity to produce plants from protoplasts in cryopreserved cell lines of rice (*Oryza sativa* L.). *Plant Cell Rep.* **10:** 171–174.

Meyer, Y., Grosset, J., Chartier, Y. & Brugidou, C. (1991): Pathogenesis related proteins are expressed in tobacco mesophyll protoplasts. *Physiol. Plant.* **82:** A37.

Mii, M., Kadowaki, S., Ohashi, H., Nemoto, K. & Yamada, K. (1990): Plant regeneration from protoplasts of *Primula malacoides*. *Jap. J. Breed.* **40:** (suppl. 1): 16–17.

Mii, M., Yuzawa, Y. & Godo, T. (1991a): Plant regeneration from protoplasts in *Lilium formolongi* cv. Azusa. *Jap. J. Breed.* **41:** (suppl. 1): 56–57.

Mii, M., Zou, Y.-M., Sugiyama, T., Yanagihara, S. & Iizuka, M. (1991b): High-frequency callus formation from protoplasts of *Vitis labruscana* Baily and *Vitis thunbergii* Sieb. et Zucc. by embedding in gellan gum. *Scientia Hort.* **46:** 253–260.

Möllers, C., Zhang, S. & Wenzel, G. (1992): The influence of silver thiosulfate on potato protoplast cultures. *Plant Breed.* **108:** 12–18.

Montagno, T.J., Jourdan, P.S. & Berry, S.Z. (1991): Plant regeneration from leaf protoplasts of *Lycopersicon hirsutum* f. *hirsutum*. *Plant Cell Rep.* **9:** 680–683.

Mórocz, S., Donn, G., Németh, J. & Dudits, D. (1990): An improved system to obtain fertile regenerants via maize protoplasts isolated from a highly embryogenic suspension culture. *Theor. Appl. Genet.* **80:** 721–726.

Morris, P. (1985): Membrane transport in protoplasts. In: Pilet, P.E. (ed.): *The Physiological Properties of Plant Protoplasts*: 54–67. Springer-Verlag, Berlin.

Nagata, T. & Yamaki, T. (1973): Electron microscopy of isolated tobacco mesophyll protoplasts cultured *in vitro*. *J. Plant Physiol.* **70:** 452–459.

Nakano, M. & Mii, M. (1992): Protoplast culture and plant regeneration of several species in the genus *Dianthus*. *Plant Cell Rep.* **11:** 225–228.

Nayak, P. & Sen, S.K. (1991): Plant regeneration through somatic embryogenesis from suspension culture-derived protoplasts of *Paspalum scrobiculatum* L. *Plant Cell Rep.* **10:** 362–365.

Negaard, K. & Hoffmann, F. (1989): A simple plant and tissue culture growth chamber for the regeneration of tobacco mesophyll protoplasts. *Biotechniques* **7:** 808,810–812.

Ochatt, S.J. (1991a): Requirements for plant regeneration from protoplasts of the shrubby ornamental honeysuckle, *Lonicera nitida* cv. Maigrun. *Plant Cell, Tiss. Organ Cult.* **25:** 161–167.

Ochatt, S.J. (1991b): Strategies for plant regeneration from mesophyll protoplasts of the recalcitrant fruit and farmwoodland species *Prunus avium* L. (sweet/wild cherry), *Rosaceae*. *J. Plant Physiol.* **139:** 155–160.

Ochatt, S.J. (1992): The development of protoplast-to-tree systems for *Prunus cerasifera* and *Prunus spinosa*. *Plant Sci.* **81:** 253–259.

Ochatt, S.J., Escandon, A.S. & Martinez, A.J. (1989): Isolation, culture, and plant regeneration of protoplasts of two shrubby *Oxalis* species from South America. *J. Exp. Bot.* **40:** 493–496.

Oliviera, M.M. & Pais, M.S.S. (1991): Plant regeneration from protoplasts of long-term callus cultures of *Actinidia deliciosa* var. *deliciosa* cv. Hayward (Kiwifruit). *Plant Cell Rep.* **9:** 643–646.

Oliviera, M.M. & Pais, M.S.S. (1992): Somatic embryogenesis in leaves and leaf-derived protoplasts of *Actinidia deliciosa* var. *deliciosa* cv. Hayward (Kiwifruit). *Plant Cell Rep.* **11:** 314–317.

Otani, M., Kunitake, H. & Mii, M. (1989): Plant regeneration from cyclamen protoplasts. *Jpn. J. Breed.* **39:** (suppl. 2): 22–23.

Park, Y.G., Choi, M.S. & Kim, J.H. (1990): Plant regeneration of *Populus glandulosa* from mesophyll protoplasts. *Korean J. Plant Tiss. Cult.* **17:** 189–199.

Park, Y.G. & Lee, S.G. (1990): Plantlet regeneration from protoplasts of *Ailanthus altissima* Swingle. In: *Abstracts VIIth IAPTC Congress*: 123. Amsterdam.

Park, Y.G. & Son, S.H. (1988): Culture and regeneration of *Populus alba* × *glandulosa* leaf protoplasts isolated from *in vitro* cultured explant. *J. Korean For. Soc.* **77:** 208–215.

Park, Y.G. & Son, S.H. (1989): Plant regeneration from protoplasts of *Populus nigra* × *P. maximowiczii* leaf mesophyll cultured *in vitro*. In: Iyama, S. & Takeda, G. (eds.): *Breeding Research: the Key of the Survival of the Earth: Proc. 6th Int. SABRAO Congress*: 849–852. Tsukuba.

Park, Y.G. & Son, S.H. (1992): *In vitro* shoot regeneration from leaf mesophyll protoplasts of hybrid poplar (*Populus nigra* × *P. maximowiczii*). *Plant Cell Rep.* **11:** 2–6.

Patat-Ochatt, E.M., Ochatt, S.J. & Power, J.B. (1988): Plant regeneration from protoplasts of apple rootstocks and scion varieties (*Malus* × *domestica* Borkh.). *J. Plant Physiol.* **133:** 460–465.

Perl, A., Aviv, D. & Galun, E. (1988): Ethylene and *in vitro* culture of potato: suppression of ethylene generation vastly improves protoplast yield, plating efficiency and transient expression of an alien gene. *Plant Cell Rep.* **7:** 403–406.

Pezzotti, M., Arcioni, S. & Mariotti, D. (1984): Plant regeneration from mesophyll, root and cell suspension protoplasts of *Medicago sativa* cv. Adriana. *Genet. Agr.* **38:** 195–208.

Pillai, V., Davey, M.R. & Power, J.B. (1990): Plant regeneration from mesophyll protoplasts of *Centaurea cyanus*, *Senecio* × *hybridus* and *Callistephus chinensis*. *Plant Cell Rep.* **9:** 402–405.

Potrykus, I. (1990): Gene transfer to cereals: an assessment. *Biotechnology* **8:** 535–542.

Prioli, L.M. & Söndahl, M.R. (1989): Plant regeneration and recovery of fertile plants from protoplasts of maize (*Zea mays* L.). *Biotechnology* **7:** 589–594.

Pupilli, F., Arcioni, S., Damiani, F. & Pezzotti, M. (1990): Plant regeneration from callus and protoplast cultures of *Lotus pedunculatus* Cav. *Plant Cell, Tiss. Organ Cult.* **23:** 193–199.

Ratushnyak, Y.I., Piven, N.M. & Rudas, V.A. (1989): Protoplast culture and plant regeneration in *Lycium barbarum* L. *Plant Cell, Tiss. Organ Cult.* **17:** 183–190.

Ratushnyak, Y.I. Rudas, V.A. & Piven, N.M. (1990): Regeneration of *Lycium barbarum* L. plants from leaf tissue, callus culture and callus protoplasts. *Plant Cell Rep.* **9:** 84–87.

Rethmeier, N.O.M., Jansen, C.E., Snel, E.A.M., Nijkamp, H.J.J. & Hille, J. (1991): Improvement of regeneration of *Lycopersicon pennellii* protoplasts by decreasing ethylene production. *Plant Cell Rep.* **9:** 539–543.

Rhodes, C.A., Pierce, D.A., Mettler, I.J., Mascarenhas, D. & Detmer, J.J. (1988): Genetically transformed maize plants from protoplasts. *Science* **240:** 204–207.

Roest, S. & Gilissen, L.J.W. (1989): Plant regeneration from protoplasts: a literature review. *Acta Bot. Neerl.* **38:** 1–23.

Ross, M.K. & Thorpe, T.A. (1973): Physiological gradients and shoot initiation in tobacco callus cultures. *Plant Cell Physiol.* **14:** 473–480.

Saha, T. & Sen, S.K. (1992): Somatic embryogenesis in protoplast derived calli of cultivated jute, *Corchorus capsularis* L. *Plant Cell Rep.* **10:** 633–636.

Sakurai, M. & Kawachi, J. (1990): Plant regeneration from protoplasts of *Pogostemon cablin*. In: *Abstracts VIIth IAPTC Congress*: 35. Amsterdam.

Sasamoto, H. & Hosoi, Y. (1990): Effects of electric fusion on the calli formation and regeneration from the protoplasts of *Quercus* and two *Populus* species. In: *Abstracts VIIth IAPTC Congress:* 36. Amsterdam.

Sauvadet, M.-A., Brochard, P. & Boccon-Gibod, J. (1990): A protoplast-to-plant system in chrysanthemum: differential responses among several commercial clones. *Plant Cell Rep.* **8:** 692–695.

Schäfer-Menuhr, A. (1989): Isolation und Kultur von Lupinenprotoplasten. IV. Regeneration von Pflanzen aus Protoplasten. *Landbauforschung Völkenrode* **39:** 133–136.

Schäffler, E. & Koop, H.-U. (1990): Single cell nurse culture of tobacco protoplasts: physiological analysis of conditioning factors. *J. Plant Physiol.* **137:** 95–101.

Schnorf, M., Neuhaus-Url, G., Galli, A., Iida, S., Potrykus, I. & Neuhaus, G. (1991): An improved approach for transformation of plant cells by microinjection: molecular and genetic analysis. *Transgen. Res.* **1:** 23–30.

Schöpke, C., Müller, L.E. & Kohlenbach, H.-W. (1987): Somatic embryogenesis and regeneration of plantlets in protoplast cultures from somatic embryos of coffee (*Coffea canephora* P. *ex.* Fr.). *Plant Cell, Tiss. Organ Cult.* **8:** 243–248.

Schöpke, C., Müller, L.E. & Kohlenbach, H.-W. (1988): Coffee protoplasts: isolation, culture and plantlet regeneration. *Assoc. Sci. Int. Café* **12:** 426–432.

She, J.-M., Wu, J.-Y., Wang, H.-B., Zhou, H.-Y., Chen, Z.-X., Li, S.-J. & Yue, J.-X. (1989): Somatic embryogenesis and plant regeneration from protoplast culture of cotton. *Jiangsu J. Agricult. Sci.* **5:** 63–68.

Shillito, R.D., Carswell, G.K., Johnson, C.M., DiMaio, J.J. & Harms, C.T. (1989): Regeneration of fertile plants from protoplasts of elite inbred maize. *Biotechnology* **7:** 581–587.

Sidikou-Seyni, R., Rambaud, C., Dubois, J. & Vasseur, J. (1992): Somatic embryogenesis and plant regeneration from protoplasts of *Cichorium intybus* L. × *Cichorium endivia* L. *Plant Cell, Tiss. Organ Cult.* **29:** 83–91.

Sikdar, S.R., Sengupta, S., Das, S. & Sen, S.K. (1990): Plant regeneration from mesophyll protoplasts of *Diplotaxis muralis*, a wild crucifer. *Plant Cell Rep.* **8:** 722–725.

Simmonds, D.H. (1991): Microtubules in cultured plant protoplasts. *Acta Bot. Neerl.* **40:** 183–195.

Smidsród, O. & Skjåk-Bræk, G. (1990): Alginate as immobilization matrix for cells. *Tibtech.* **8:** 71–78.

Snape, J.W., Law, C.N., Worland, A.J. & Parker, B.B. (1990): Targeting genes for genetic manipulation in crop species. In: Gustafson, J.P. (ed.): *Gene Manipulation in Plant Improvement* II: 55–76. Plenum Press, New York.

Song, J.-S., Sorensen, E.L. & Liang, G.H. (1990): Direct embryogenesis from single mesophyll protoplasts in alfalfa (*Medicago sativa* L.). *Plant Cell Rep.* **9:** 21–25.

Spangenberg, G., Freydl, E., Osusky, M., Nagel, J. & Potrykus, I. (1991): Organelle transfer by microfusion of defined protoplast-cytoplast pairs. *Theor. Appl. Genet.* **81:** 477–486.

Spangenberg, G., Osusky, M., Oliveira, M.M., Freydl, E., Nagel, J., Pais, M.S. & Potrykus, I. (1990): Somatic hybridization by microfusion of defined protoplast pairs in *Nicotiana*: morphological, genetic, and molecular characterization. *Theor. Appl. Genet.* **80:** 577–587.

Spörlein, B. & Koop, H.-U. (1991): Lipofectin: direct gene transfer to higher plants using cationic liposomes. *Theor. Appl. Genet.* **83:** 1–5.

Sree Ramulu, K. (1987): Genetic instability during plant regeneration in potato: origin and implications. *Plant Physiol. (Life Sci. Adv.)* **6:** 211–218.

Suga, R., Takagi, Y. & Okutsu, Y. (1990): Protoplast culture of sweet potato and its related wild species—plant regeneration from leaf protoplasts-derived callus of *Ipomoea trifida*. *Jap. J. Breed.* **40:** (suppl. 1): 78–79.

Sundberg, E. & Glimelius, K. (1991): Production of cybrid plants within *Brassicaceae* by fusing protoplasts and plasmolytically induced cytoplasts. *Plant Sci.* **79:** 205–216.

Tabaeizadeh, Z., Bunisset-Bergounioux, C. & Perennes, C. (1984): Environmental growth conditions of protoplast source plants: effect on subsequent protoplast division in two tomato species. *Physiol. Vég.* **22:** 223–229.

Tao, R., Tamura, M., Yonemori, K. & Sugiura, A. (1991): Plant regeneration from callus protoplasts of adult Japanese persimmon (*Diospyros kaki* L.). *Plant Sci.* **79:** 119–125.

Tautorus, T.E., Attree, S.M., Fowke, L.C. & Dunstan, D.I. (1990): Somatic embryogenesis from immature and mature zygotic embryos, and embryo regeneration from protoplasts in black spruce (*Picea mariana* Mill.). *Plant Sci.* **67:** 115–124.

Tegeder, M., Kuch, A., Pickardt, T., Kohn, H. & Schieder, O. (1991): Protoplast technology in *Vicia*. *Physiol. Plant.* **82:** A10.

Thorpe, T.A. & Murashige, T. (1970): Some histochemical changes underlying shoot initiation in tobacco callus cultures. *Can. J. Bot.* **48:** 277–285.

Toriyama, K., Arimoto, Y., Uchimiya, H. & Hinata, K. (1988): Transgenic rice plants after direct gene transfer into protoplasts. *Biotechnology* **6:** 1072–1074.

Traas, J.A., Renaudin, J.P. & Teyssendier de la Serve, B. (1990): Changes in microtubular organization mark the transition to organized growth during organogenesis in *Petunia hybrida*. *Plant Sci.* **68:** 249–256.

Trémouillaux-Guiller, J. & Chénieux, J.-C. (1991): Somatic embryogenesis from leaf protoplasts of *Rauvolfia vomitoria* shoot cultures. *Plant Cell Rep.* **10:** 102–105.

Tusa, N., Grosser, J.W. & Gmitter, F.G. (1990): Plant regeneration of 'Valencia' sweet orange, 'Femminello' Lemon, and the interspecific somatic hybrid following protoplast fusion. *J. Am. Soc. Hort. Sci.* **115:** 1043–1046.

Uijtewaal, B.A. (1987): Ploidy variability in greenhouse cultured and *in vitro* propagated potato monohaploids ($2n = x = 12$) as determined by flow cytometry. *Plant Cell Rep.* **6:** 252–255.

Uijtewaal, B.A., Posthumus, E., Suurs, L.C.J.M., Jacobsen, E. & Hermsen, J.G.Th. (1987): Relative performance of monohaploid potato clones and their diploid parents at plant level and after protoplast isolation and subsequent fusion. *Theor. Appl. Genet.* **74:** 451–458.

Vasil, I.K. (1990): The realities and challenges of plant biotechnology. *Biotechnology* **8:** 296–301.

Verhoeven, H.A. & Blaas, J. (1992): Direct cell to cell transfer of organelles by microinjection. *Plant Cell Rep.* **10:** 613–616.

Verhoeven, H.A., Sree Ramulu, K., Gilissen, L.J.W., Famelaer, I., Dijkhuis, P. & Blaas, J. (1991): Partial genome transfer through micronuclei in plants. *Acta Bot. Neerl.* **40:** 97–113.

Verhoeven, H.A., Wissink, M. & Blaas, J. (1990): A computerized microscope stage for application in plant cell and protoplast research. In: *Abstracts VIIth IAPTC Congress*: 41. Amsterdam.

Vieira, M.L.C., Jones, B., Cocking, E.C. & Davey, M.R. (1990): Plant regeneration from protoplasts isolated from seedling cotyledons of *Stylosanthes guianensis*, *S. macrocephala* and *S. scabra*. *Plant Cell Rep.* **9:** 289–292.

Von Aderkas, P. (1992): Embryogenesis from protoplasts of haploid European larch. *Can. J. For. Res.* **22:** 397–402.

Walters, T.W. & Earle, E.D. (1990): A simple, versatile feeder layer system for *Brassica oleracea* protoplast culture. *Plant Cell Rep.* **9:** 316–319.

Wan, X.-S., Wang, F.-D. & Xia, Z.-A. (1988): Studies on culture conditions and plant regeneration of

Apium graveolens protoplasts. *Plant Physiol. Commun.* **5:** 41–44.

Wang, G.-Y., Xia, Z.-A. & Wang, L.-F. (1986): Regenerated plantlets from cultured mesophyll protoplasts of onion (*Allium cepa* L.). *Acta Biol. Exp. Sin.* **19:** 409–413.

Webb, K.J. & Watson, E.J. (1991): *Lotus corniculatus* L.: morphological and cytological analysis of regenerants from three sources of tissue and selected progeny. *Plant Cell, Tissue Organ Culture* **25:** 27–33.

Wei, Z.-M. & Xu, Z.-H. (1990): Regeneration of fertile plants from embryogenic suspension culture protoplasts of *Sorghum vulgare*. *Plant Cell Rep.* **9:** 51–53.

Wei, Z.-M., Xu, Z.-H., Xu, N. & Huang, M.-R. (1991): Mesophyll protoplasts culture and plant regeneration of oriental planetree (*Platanus orientalis*). *Physiol. Plant.* **82:** A13.

Xia, G.-M., Li, Z.-Y., Guo, G.-Q. & Chen, H.-M. (1992): Direct somatic embryogenesis and plant regeneration from protoplasts of *Bupleurum scorzonerifolium* Willd. *Plant Cell Rep.* **11:** 155–158.

Xia, Z.-A. (1990): Tissue culture and plant regeneration from *Chrysanthemum coronarium* protoplasts. *Plant Physiol. Commun.* **1:** 27–29.

Xu, Y.-S., Pehu, E., Malone, R. & Jones, M.G.K. (1991): Plant regeneration from protoplasts of *Solanum* species with potential agricultural value (*S. hjertingii, S. polyadenium, S. capsicibaccatum*). *Plant Cell Rep.* **9:** 520–522.

Yan, Q.-S., Zhang, X.-Q. & Shi, J.-B. (1990): Regeneration of green plants from protoplasts of barley (*Hordeum vulgare* L., cv. Yong 257). In: *Abstracts VIIth IAPTC Congress*: 42. Amsterdam.

Yasuda, T., Tahara, M., Uchida, N. & Yamaguchi, T. (1986): Somatic embryogenesis from coffee callus and protoplast. In: Somers, D.A., Gengenbach, B.G., Biesboer, D.D., Hackett, W.P. & Green, C.E. (eds.): *Abstracts VIth IAPTC Congress*: 137. University of Minnesota, Minneapolis.

Zaghmout, O.M.-F. & Torello, W.A. (1990): Isolation and culture of protoplasts from embryogenic suspension cultures of red fescue (*Festuca rubra* L.). *Plant Cell Rep.* **9:** 340–343.

Zhan, X.-C., Jones, D.A. & Kerr, A. (1989): *In vitro* plantlet formation in *Linum marginale* from cotyledons, hypocotyls, leaves, roots and protoplasts. *Aust. J. Plant Physiol.* **16:** 315–320.

Zhang, S.-B., Jian, L.-C., Kuo, C.-S., Qu, G.-P. & Qian, Y.-Q. (1990): Buds and roots differentiation of maize (*Zea mays* L.) protoplasts after cryopreservation. *Acta Biol. Exp. Sin.* **23:** 117–121.

Zhao, G.-L., Luo, X.-M. & Liu, Y.-Z. (1991): Plant regeneration of protoplasts of *Trifolium lupinaster* L. from cell suspension cultures. *Acta. Bot. Sin.* **33:** 547–551.

Zhou, C. (1989): Pollen protoplast culture leading to embryogenic divisions in *Hemerocallis fulva*. *Acta Bot. Sin.* **31:** 409–413.

NOTE ADDED IN PROOF

During the publication of the manuscript, new data have been published on regeneration from protoplasts of the following species:

Taxon	Donor tissue	Culture technique	Regenerant development	Reference
MONOCOTYLEDONAE				
Gramineae				
Agrostis palustris	eSc	L	C→S→P	Terakawa *et al.* (1992)
DICOTYLEDONAE				
Caricaceae				
Carica papaya × *cauliflora*	Sc	L	E→P	Chen & Chen (1992)
Compositae				
Senecio fuchsii	Sh	S	C→S→P	Binding *et al.* (1992)
Cruciferae				
Brassica rapa	Co	L	C→S	Hegazi & Matsubara (1992)
Moricandia arvensis	L	L	C→S→P	Murata & Mathias (1992)
Raphanus sativus	Co	L	C→S→P	Hegazi & Matsubara (1992)
Euphorbiaceae				
Euphorbia pulcherrima	Sh	S	C→S	Binding *et al.* (1992)
Linaceae				
Linum catharticum	Sh	S	C→S	Binding *et al.* (1992)
Solanaceae				
Cyphomandra betacea	Sh	S	C→S→P	Binding *et al.* (1992)

REFERENCES

Binding, H., Görschen, E., Hassanein, A.M., Ling, H.Q., Mordhorst, G., Puck, G., Rudnick, J., Wang, G.R. & Truberg, B. (1992): Plant development from protoplasts of members of Bryophyta, Pteridophyta and Spermatophyta under identical conditions. *Physiol. Plant.* **85:** 295–300.

Chen, M.H. & Chen, C.C. (1992): Plant regeneration from *Carica* protoplasts. *Plant Cell Rep.* **11:** 404–407.

Hegazi, H.H. & Matsubara, S. (1992): Callus formation and plant regeneration from protoplasts derived from cotyledons and hypocotyls of radish (*Raphanus sativus* L.) and other cruciferous plants. *J. Japan. Soc. Hort. Sci.* **61:** 63–68.

Murata, T. & Mathias, R.J. (1992): Plant regeneration from mesophyll protoplasts of *Moricandia arvensis*. *Plant Cell Rep.* **11:** 408–411.

Terakawa, T., Sato, T. & Koike, M. (1992): Plant regeneration from protoplasts isolated from embryogenic suspension cultures of creeping bentgrass (*Agrostis palustris* Huds.). *Plant Cell Rep.* **11:** 457–461.