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Plant regeneration from protoplasts: a literature review

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INTRODUCTION

Protoplasts can be isolated in large quantities from different plant tissues and organs. They have shown the ability to synthesize new cell walls and to regenerate complete plants when cultured in the appropriate media. Therefore, protoplasts are regarded as totipotent cells, which are well suited for fundamental studies on the one hand, and for genetic manipulation on the other. For crop improvement through genetic manipulation techniques, such as somatic hybridization, micro-injection and electroporation, plant regeneration from protoplasts is of essential significance.

As early as 1892, Af Klercker succeeded in the mechanical isolation of relatively low numbers of protoplasts of *Stratiotes aloïdes* by sectioning plasmolysed tissue. The isolation of protoplasts of *Lycopersicon esculentum*, using cell wall degrading enzymes (Cocking 1960), was a great break-through in protoplast research. The enzymatic treatment enabled the routine isolation of relatively high numbers of protoplasts from almost every organ and tissue of a diversity of plant species. In the period between 1960 and 1970, due to the combined application of cytokinins and auxins, cultured protoplasts showed the ability to form cell walls and to undergo cell divisions leading to colony formation. In 1971, for the first time, plant regeneration from protoplasts was achieved in tobacco (Nagata & Takebe 1971; Takebe *et al.* 1971). Subsequently, regeneration has been described from protoplasts of a great diversity of plant species, especially those that belong to the Solanaceae.

Binding (1985) and Maheshwari *et al.* (1986) have listed about 100 plant species for which successful regeneration procedures have been described. Now this number has increased more than twofold, which justifies the publication of an up-to-date bibliography of all known species. In addition, plant factors and external factors, which proved to be of great importance for successful regeneration, are briefly commented on. In addition, examples of recently developed protoplast culture techniques are described. Finally, some problems that hamper the use of protoplasts in genetic manipulation studies are discussed and attention is given to some techniques that can be used to provide more knowledge on fundamental aspects of protoplast regeneration.

PLANT SPECIES

From protoplasts of 212 higher plant species, representing 96 genera of 31 families, cell colonies and calli have been developed, which regenerated into embryo-like structures, embryoids or shoots (Table 1). Normally, when cultured on appropriate media, these structures are able to develop into plants. Plant species that did not yield embryoids or shoots from protoplast-derived colonies, or that regenerated into roots only, are not included in the list. The species are grouped according to their systematic taxa in alphabetical order. In general, the first publications are presented in which the regeneration procedure for a given plant species is described. From 1970 until 1987, the yearly number of new plant species, for which details on the regeneration from protoplasts have been published, is presented in Fig. 1. Obviously, during the last few years, these numbers have rapidly increased and for the first 9 months of 1988 28 new plant species can be added (not included in Fig. 1).

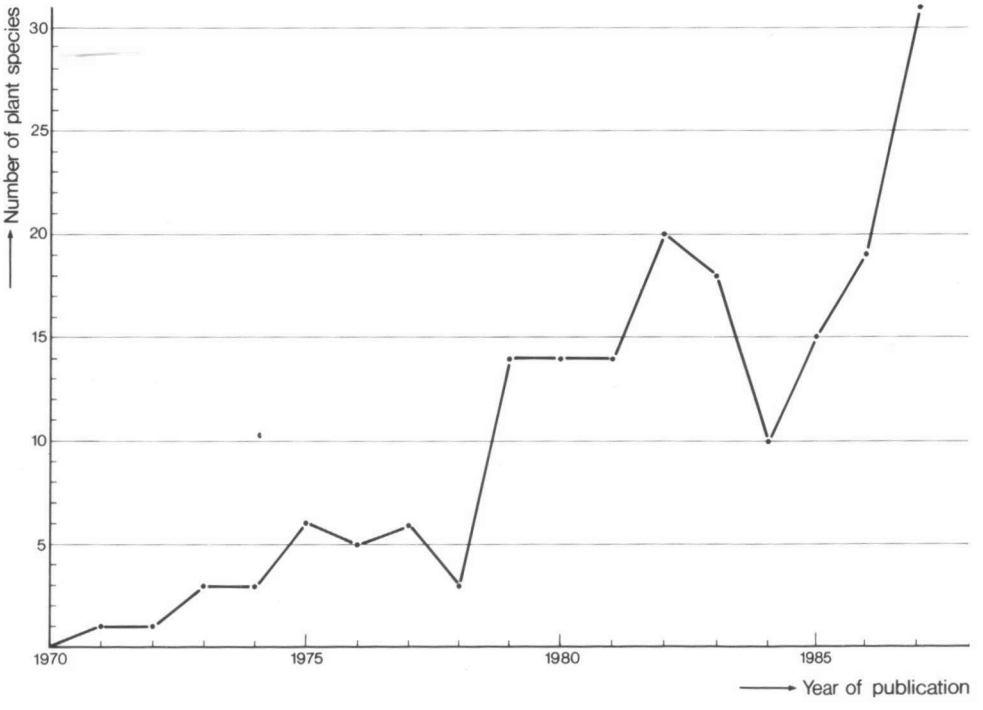

Fig. 1. The number of higher plant species for which regeneration from protoplasts has been published yearly between 1970 and 1987.

Plant species of the Solanaceae appear to be very responsive. Until now, 67 species (±30% of all listed species) of this family have demonstrated regeneration potential. Within the Solanaceae, including commercially important genera like *Lycopersicon, Nicotiana, Petunia* and *Solanum*, plant regeneration is almost exclusively achieved by adventitious shoot formation.

During the last 5 years, substantial progress has been made concerning the regeneration from protoplasts of monocotyledonous and dicotyledonous species, representing several Gramineae, Papilionaceae and woody crops, that were considered to be recalcitrant in

Table 1. (a) List of abbreviations used in Table 1(b)

Donor tissue	Culture technique	Regenerant development
C = callus	B = bead type	C = callus, cell colony
Co = cotyledon, hypocotyl	D = droplet (20–200 µl)	E = somatic embryo, embryo-like structure
L = leaf, shoot apex	L = liquid medium	P = plant(let)
R = root, root apex	LoS = liquid over solid medium	R = root
Sc = suspension cells	Ls = liquid shake	S = shoot
St = stem, internode	N = nurse, feeder	
	R = reservoir medium	a = albino
e = embryogenic	S = solid medium	
v = *in vivo*	SoS = solid over solid medium	
? = unknown	St = streak plating	
	s = semi	
	? = unknown	

tissue culture until a few years ago. This progress is substantially due to the use of embryogenic cell suspension or seedling tissue, instead of leaf tissue as donor sources for protoplast isolation.

Species that belong to the Gramineae mostly regenerated into green shoots and plantlets via embryoid formation. Green corn, fescue, rice and sugarcane plants have been successfully established in the soil, however, some species regenerated into albino shoots and plantlets, e.g. *Bromus inermis, Festuca arundinacea, Hordeum vulgare, Lolium perenne* and *Lolium multiflorum, Panicum miliaceum* and *Poa pratensis.* No successful regeneration has been realized for important cereal food crops such as oat, rye and sorghum.

With respect to the Papilionaceae, to which several important food crops also belong, different species of *Glycine* (amongst them soybean, *G. max*), *Lotus, Medicago, Pisum* and *Trifolium* have recently shown a regeneration capacity. Leguminous species of the genera *Arachis, Phaseolus* and *Vicia*, however, are not amenable, so far, for plant regeneration from protoplasts.

For several woody plant species, representing various families, regeneration procedures have been developed, e.g. *Actinidia chinensis* (Actinidiaceae), *Manihot esculenta* (Euphorbiaceae), *Liriodendron tulipifera* (Magnoliaceae), *Populus* (Salicaceae), *Santalum album* (Santalaceae), *Ulmus* (Ulmaceae) and different species of the Rosaceae (*Malus, Prunus, Pyrus*) and the Rutaceae (*Citrus, Microcitrus*). Quite recently, regeneration procedures have been developed for two woody plant species that belong to the Gymnospermae, namely *Picea glauca* and *Pinus taeda*, however, many important woody plant species, such as forest trees (Betulaceae, Fagaceae, most of the Gymnospermae and tropical tree species), fruit trees and shrubs (banana, cocoa, coconut, coffee, date, grape and tea) and oil palm, still remain recalcitrant or have not yet been subjected to protoplast regeneration studies.

PROTOPLAST REGENERATION

Several factors affect the isolation, culture and regeneration from protoplasts, namely the genotype, the donor tissue and its pre-treatment, the enzyme treatment for protoplast

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

Taxon	Donor tissue	Culture technique	Regenerant development	Reference
Gymnospermae				
Pinaceae				
Picea glauca	eSc	B	E	Attree *et al.* (1987)
Pinus taeda	eSc	S	E→P	Gupta & Durzan (1987)
Angiospermae				
Monocotyledonae				
Amaryllidaceae				
Hemerocallis cv.	Sc	Ls	C→P	Fitter & Krikorian (1981)
Araceae				
Pinellia ternat	vL	Ls	C→E→P	Wu *et al.* (1986)
Gramineae				
Bromus inermis	Sc	D	E→Pa	Kao *et al.* (1973)
Festuca arundinacea	eSc	L	C→E→P, Pa	Dalton (1988a,b)
Hordeum vulgare	eSc	L;S	C→Pa	Lührs & Lörz (1988)
Lolium perenne	eSc	L	C→Pa	Creemers-Molenaar *et al.* (1988),
	Sc	L;B	C→E→Pa	Dalton (1988a,b)
multiflorum	eSc	L	C→S→Pa	Dalton (1988b)
Oryza sativa	Sc	S	C→E→P	Abdullah *et al.* (1986),
	C	L	C→S→P	Coulibaly & Demarly (1986),
	Sc	L	C→P	Fujimura *et al.* (1985),
	C	B	P	Hayashi *et al.* (1986),
	C;Sc	B;BN	C→E→P	Kyozuka *et al.* (1987),
	Sc	B;LoS	C→E→P	Thompson *et al.* (1986),
	Sc	L	C→P	Toriyama *et al.* (1986),
	Sc	L	C→S→P	Yamada *et al.* (1986)
Panicum maximum	eSc	L	C→E→P	Lu *et al.* (1981)
miliaceum	Sc	D	C→E→P, Pa	Heyser (1984)
Pennisetum americanum	eSc	D	C→E→P	Vasil & Vasil (1980)
purpureum	eSc	D;S	E→P	Vasil *et al.* (1983)
Poa pratensis	Sc	D	C→Sa	Van der Valk & Zaal (1988)
Polypogon fugax	?	?	C→P	Li *et al.* (1986)
Saccharum officinarum	Sc	B;D;L	C→S→P	Chen *et al.* (1988)
	eSc	sS	C→E→P	Srinivasan & Vasil (1985)
Triticum aestivum	eSc	L	C→E, S→P	Harris *et al.* (1988)
Zea mays	eC	L	C→E→P	Cai *et al.* (1988),
	eSc	DN	C→E→P	Kamo *et al.* (1987),
	eSc	N	C→E→P	Rhodes *et al.* (1988),
	eSc	L;S	C→E	Vasil & Vasil (1987)
Liliaceae				
Asparagus officinalis	vSt	L	C→S	Bui-Dang-Ha & Mackenzie (1973)
Haworthia magnifica	Sc	L	C→S→P	Sun *et al.* (1987)
Dicotyledonae				
Actinidiaceae				
Actinidia chinensis	C	L	C→S→P	Cai (1988)
Asclepiadaceae				
Asclepias syriaca	Co	L	C→S→P	Singh (1984)
Pergularia pallida	C	L	C→S→P	Bapat *et al.* (1986)
Tylophora indica	C	L	C→E→P	Mhatre *et al.* (1984)
Chenopodiaceae				
Beta vulgaris	?	?	E→P	Steen *et al.* (1986)
Chenopodium glaucum	?	?	C→P	Li *et al.* (1986)
Compositae				
Brachycome dichromosomatica	C	L	C→S→P	Hahne & Hoffmann (1986)
Chrysanthemum morifolium	L	N	C→P	Otsuka (1986), Otsuka *et al.* (1985)

Table 1. (b) (Continued)

Taxon	Donor tissue	Culture technique	Regenerant development	Reference
Cichorium endivia	L	L	C→S→P	Binding *et al.* (1980, 1981)
intybus	L	L	C→S→P	Binding *et al.* (1981)
Crepis capillaris	C	L	C→S→P	Hahne & Hoffmann (1986)
Dimorphotheca aurantiaca	vL;vSt;Co;C	B	C→S→P	Al-Atabee & Power (1987, 1988)
Gaillardia grandiflora	L	L	C→S→P	Binding *et al.* (1980, 1981)
Helianthus annuus	L	L	C→S→P	Binding *et al.* (1980, 1981)
Lactuca saligna	L	B	C→S→P	Brown *et al.* (1987)
sativa	vL;L;Co;R	L;LoS	C→S→P	Berry *et al.* (1982),
	vL	S	C→S→P	Engler & Grogan (1982/1983)
Petasites japonicus	L	L	C→S→P	Yabe *et al.* (1986)
Rudbeckia hirta	vL	L;B	C→S→P	Al-Atabee & Power (1987, 1988)
laciniata	vL;vSt;Co;C	B	C→S→P	Al-Atabee & Power (1988)
purpurea	vL;vSt;Co;C	B	C→S→P	Al-Atabee & Power (1988)
Senecio jacobaea	L	L	C→S→P	Binding *et al.* (1981)
silvaticus	L	L	C→S→P	Binding *et al.* (1981)
vernalis	L	L	C→S→P	Binding *et al.* (1981, 1982)
viscosus	L	L	C→S→P	Binding *et al.* (1981)
vulgaris	L	L	C→S→P	Binding & Nehls (1980), Binding *et al.* (1981)
Convolvulaceae				
Ipomoea batatas	L,St	L	C→S→P	Sihachakr & Ducreux (1987)
Cruciferae				
Arabidopsis thaliana	L	L	C→S→P	Binding *et al.* (1981),
	Sc	L	C→S→P	Xuan & Menczel (1980)
Biscutella laevigata	?	St	C→S→P	Binding *et al.* (1988)
Brassica alboglabra	L;Co;R;St	L	C→S→P	Pua (1987)
campestris	Co	L	C→S→P	Glimelius (1984)
carinata	Co	L	C→S→P	Chuong *et al.* (1987b)
juncea	L	L	C→E, S→P	Chatterjee *et al.* (1985)
napus	L	D	C→S→P	Kartha *et al.* (1974),
	vL;L	L	C→S→P	Thomas *et al.* (1976),
	R	LoS	C→S→P	Xu *et al.* (1982b, 1985)
nigra	St	LoS	C→S→P	Chuong *et al.* (1987a),
	Sc	L	C→E	Klimaszewska & Keller (1986)
oleracea	Co	L	C→S→P	Vatsya & Bhaskaran (1982),
	R	LoS	C→S→P	Xu *et al.* (1982b, 1985)
Eruca sativa	L	L	C→E, S→P	Sikdar *et al.* (1987)
Sinapis alba	L	L	C→S→P	Binding *et al.* (1982)
arvensis	L	L	C→S→P	Binding *et al.* (1982)
Cucurbitaceae				
Cucumis melo	L	L	C→E, S→P	Moreno *et al.* (1986)
sativus	Co	B;D	C→E→P	Colijn-Hooymans *et al.* (1988),
	L	B	C→E→P	Orczyk & Malepszy (1985)
Euphorbiaceae				
Manihot esculenta	vL	L	C→S→P	Shahin & Shepard (1980)
Gentianaceae				
Gentiana scabra	?	?	C→P	Li *et al.* (1986)
Geraniaceae				
Pelargonium aridum	Sc	L	C→S→P	Yarrow *et al.* (1987)
× *hortorum*	L	L	C→S→P	Kameya (1975),
	Sc	L	C→S→P	Yarrow *et al.* (1987)
peltatum	Sc	L	C→S→P	Yarrow *et al.* (1987)

Table 1. (b) (Continued)

Taxon	Donor tissue	Culture technique	Regenerant development	Reference
Hypericaceae				
Hypericum montbretii	?	St	C→S→P	Binding *et al.* (1988)
perforatum	?	St	C→S→P	Binding *et al.* (1988)
Labiatae				
Majorana hortensis	L	L	C→S→P	Binding *et al.* (1982)
Linaceae				
Linum alpinum	?	St	C→S→P	Binding *et al.* (1988),
	L;Co	B	C→E, S→P	Ling & Binding (1987)
amurense	?	St	C→S→P	Binding *et al.* (1988),
	L	B	C→S→P	Ling & Binding (1987)
hologynum	?	St	C→S→P	Binding *et al.* (1988),
	L	B	C→S→P	Ling & Binding (1987)
lewesii	Sc;L	L;LoS	C→E, S→P	Barakat & Cocking (1985)
perenne	?	St	C→S→P	Binding *et al.* (1988),
	L	B	C→S→P	Ling & Binding (1987)
salsoloïdes	?	St	C→S→P	Binding *et al.* (1988),
	L	B	C→S→P	Ling & Binding (1987)
strictum	Sc;L	L;LoS	C→E, S→P	Barakat & Cocking (1985)
usitatissimum	Co;R	L;LoS	C→S→P	Barakat & Cocking (1983),
	L	L	C→S→P	Binding *et al.* (1981,1982),
	L	B	C→S→P	Ling & Binding (1987)
Magnoliaceae				
Liriodendron tulipifera	eSc	D;N	C→E→P	Merkle & Sommer (1987)
Moraceae				
Broussonetia kazinoki	L	L	C→S→P	Oka & Ohyama (1985)
Papilionaceae				
Clianthus formosus	L	L	C→S→P	Binding *et al.* (1983)
Crotalaria juncea	Co	L	C→E, S→P	Rao *et al.* (1982, 1985)
Dolichos biflorus	Sc	D	C→E	Sinha & Das (1986)
Glycine canescens	Co	?	P	Davey & Power (1988),
	Co	B	C→S→P	Hammatt *et al.* (1987),
	Co	R	C→S→P	Newell & Luu (1985)
clandestina	Co	?	P	Davey & Power (1988),
	Co	B	C→S→P	Hammatt *et al.* (1987)
max	Co	L	C→S→P	Wei & Xu (1988)
soja	Sc	D	C→E	Gamborg *et al.* (1983)
tabacina	Sc	D	C→E	Gamborg *et al.* (1983)
Hedysarum coronarium	Co	L	C→S→P	Arcioni *et al.* (1985b)
Lotus corniculatus	Co;R	L	C→S→P	Ahuja *et al.* (1983a)
tenuis	R	L	C→S→P	Piccirilli *et al.* (1988)
Medicago arborea	L	?	C→S→P	Arcioni *et al.* (1985a),
	L;R	L	C→S→P	Mariotti *et al.* (1984)
coerulea	L;Sc	LoS	C→E→P	Arcioni *et al.* (1982)
difalcata	Co	D;L;N	C→E→P	Gilmour *et al.* (1987)
falcata	Co	D;L;N	C→E→P	Gilmour *et al.* (1987)
hemicycla	Co	D;L;N	C→E→P	Gilmour *et al.* (1987)
glutinosa	L;Sc	LoS	C→E→P	Arcioni *et al.* (1982),
	Co	D;L;N	C→E→P	Gilmour *et al.* (1987)
sativa	vL	D	C→E→P	Johnson *et al.* (1981),
	vL	D	E→P	Kao & Michayluk (1980),
	Co;R	L	C→E→P	Lu *et al.* (1982b),
	C;Sc	L	C→E→P	Mezentsev (1981),
	vL	D;LoS	C→E, S→P	Dos Santos *et al.* (1980),
	R	L	C→E→P	Xu *et al.* (1982a)
varia	Co	D;L;N	C→E→P	Gilmour *et al.* (1987)
Onobrychis viciifolia	vL	L;LoS	C→S→P	Ahuja *et al.* (1983b)
Pisum sativum	vL;Co	L;B	C→S	Puonti-Kaerlas & Eriksson (1988)

Table 1. (b) (Continued)

Taxon	Donor tissue	Culture technique	Regenerant development	Reference
Pithecellobium dulce	vL	L	C→S→P	Saxena & Gill (1987)
Psophocarpus tetragonolobus	C	L	C→S→P	Wilson *et al.* (1985),
	Sc	L	C→P	Zakri (1984)
Stylosanthes guyanensis	Sc	L	C→S→P	Meijer & Steinbiss (1983)
Trifolium hybridum	L;R	D;L	C→S→P	Webb *et al.* (1984, 1986)
pratense	Co;L	?	C→E→P	Davey & Power (1988)
repens	vL	L;LoS	C→S→P	Ahuja *et al.* (1983b),
	Sc	D;L	C→S→P	Gresshoff (1980)
rubens	vL;Sc	L	C→E→P	Grosser & Collins (1984)
Trigonella corniculata	L	L	C→E→P	Lu *et al.* (1982a),
	L;Sc	L	C→E→P	Dos Santos *et al.* (1983)
foenum-graecum	vL	D	C→S	Shekhawat & Galston (1983a)
Vigna aconitifolia	vL	D	C→E→P	Shekhawat & Galston (1983b)
mungo	L	D	C→E	Sinha *et al.* (1983)
sinensis	vL	L	C→E, S	Davey *et al.* (1974)
Ranunculaceae				
Nigella arvensis	L	L	C→S→P	Binding *et al.* (1980, 1981)
sativa	Sc	L	C→S→P	Jha & Roy (1982)
Ranunculus sceleratus	L	?	C→E→P	Dorion & Bigot (1985),
	vL	L	C→E→P	Dorion *et al.* (1975, 1984)
Resedaceae				
Reseda alba	?	St	C→S→P	Binding *et al.* (1988)
lutea	?	St	C→S→P	Binding *et al.* (1988)
luteola	L	L;St	C→S→P	Binding *et al.* (1981, 1988)
odorata	?	St	C→S→P	Binding *et al.* (1988)
phyteuma	?	St	C→S→P	Binding *et al.* (1988)
Rosaceae				
Fragaria ananassa	L	L	C→S→P	Binding *et al.* (1982)
Malus × *domestica*	C;Sc	L;sS	C→E	Kouider *et al.* (1984)
Prunus avium × *pseudocerasus*	L;Sc	?	C→R→P	Davey & Power (1988),
	L;Sc	Ls	C→S→P	Ochatt *et al.* (1987, 1988)
cerasus	L;Sc	?	C→R→P	Davey & Power (1988),
	L	B;L;sS	C→R→P	Ochatt & Power (1988)
Pyrus communis	L;Sc	?	C→R→P	Davey & Power (1988),
	vL;L	L;sS	C→S→P	Ochatt & Caso (1986)
Rutaceae				
Citrus aurantium	C	sS	C→E→P	Vardi & Spiegel-Roy (1982)
limon	C	sS	C→E→P	Vardi & Spiegel-Roy (1982)
paradisi	C	sS	C→E→P	Vardi & Spiegel-Roy (1982)
reticulata	C	sS	C→E→P	Vardi & Spiegel-Roy (1982)
sinensis	C	L	E→P	Kobayashi *et al.* (1985),
	C	S;sS	C→E→P	Vardi & Spiegel-Roy (1982), Vardi *et al.* (1975)
Microcitrus australis × *australasica*	C	sS	C→E→P	Vardi *et al.* (1986)
Salicaceae				
Populus alba × *grandidentata*	L	L	C→S→P	Russell & McCown (1986, 1988)
nigra × *trichocarpa*	L	L	C→S→P	Russell & McCown (1988)
tremula	L	L	C→S→P	Russell & McCown (1988)
Santalaceae				
Santalum album	C	L	C→E→P	Bapat *et al.* (1985),
	Sc	L	C→E→P	Rao & Ozias-Akins (1985)
Scrophulariaceae				
Antirrhinum majus	vL	L	C→E	Poirier-Hamon *et al.* (1974)

Table 1. (b) (Continued)

Taxon	Donor tissue	Culture technique	Regenerant development	Reference
Digitalis lanata	vL	L	C→R, S→P	Li (1981)
obscura	L	L;LoS	C→S→P	Brisa & Segura (1987)
Nemesia strumosa	vL	L	C→S→P	Hess & Leipoldt (1979)
Rehmannia glutinosa	L	LoS	C→S→P	Xu & Davey (1983)
Solanaceae				
Atropa belladonna	Sc	L	C→E→P	Gosch *et al.* (1975)
Browallia viscosa	Sc	LoS	C→S→P	Power & Berry (1979)
Capsicum annuum	L	D	C→S→P	Saxena *et al.* (1981b),
	L	B	C→S→P	Diaz *et al.* (1988)
Datura innoxia	L	L	C→S→P	Schieder (1975, 1977)
metel	L	L	C→S→P	Schieder (1977)
meteloïdes	L	L	C→S→P	Schieder (1977)
Hyoscyamus muticus	L;Sc	L	C→S→P	Lörz *et al.* (1979),
	L	L	C→S→P	Wernicke & Thomas (1980), Wernicke *et al.* (1979)
Lycopersicon chilense	Sc	L	C→S→P	Hassanpour-Estahbanati & Demarly (1986)
esculentum	Co	L	C→S→P	Koblitz & Koblitz (1982a,b, 1983),
	C	S	C→S→P	Morgan & Cocking (1982)
peruvianum	vL	L	C→S→P	Mühlbach (1980),
	vL	L;S	C→E, S→P	Zapata & Sink (1981), Zapata *et al.* (1977)
pennellii	L;Sc	L	C→S→P	Tan *et al.* (1987)
pimpinellifolium	Co	LoS	C→S→P	Imanishi & Suto (1987)
Nicotiana acuminata	vL	L	C→S→P	Bourgin *et al.* (1979)
alata	vL	L;S	C→S→P	Bourgin & Missonier (1978), Bourgin *et al.* (1979),
	L	L	C→S→P	Passiatore & Sink (1981)
bonariensis	L	L	C→S→P	Borkird & Sink (1983)
debneyi	L	S	C→S→P	Piven (1981),
	vL	L;S	C→S→P	Scowcroft & Larkin (1980),
	vL	L	C→S→P	Shakurov (1982)
forgetiana	L	L	C→S→P	Passiatore & Sink (1981)
glauca	vL	L	C→S→P	Bourgin *et al.* (1979)
langsdorfii	vL	L	C→S→P	Bourgin *et al.* (1979),
	vL	N	C→S→P	Evans (1979)
longiflora	vL	L	C→S→P	Bourgin *et al.* (1979)
megalosiphon	vL	L	C→S→P	Shakurov (1982)
nesophila	vL	N	C→S→P	Evans (1979)
occidentalis	vL	L	C→S→P	Shakurov (1982)
otophora	vL	L;S	C→S→P	Banks & Evans (1976),
	vL	L	C→S→P	Bourgin *et al.* (1979)
paniculata	vL	L	C→S→P	Bourgin *et al.* (1979)
plumbaginifolia	vL	L	C→S→P	Bourgin *et al.* (1979),
	L	L	C→S→P	Gill *et al.* (1978)
repanda	vL	N	C→S→P	Evans (1979)
rustica	L	LoS	C→S→P	Gill *et al.* (1979),
	vL	L	C→S→P	Shakurov (1982)
sanderae	L	L	C→S→P	Passiatore & Sink (1981)
stocktonii	vL	N	C→S→P	Evans (1979)
suaveolens	vL	L	C→S→P	Bourgin *et al.* (1979),
	vL	L	C→S→P	Shakurov (1982)
sylvestris	vL	L;S	C→S→P	Banks & Evans (1976),
	vL	L	C→S→P	Bourgin *et al.* (1976, 1979),
	L	L	C→S→P	Nagy & Maliga (1976)

Table 1. (b) (Continued)

Taxon	Donor tissue	Culture technique	Regenerant development	Reference
tabacum	vL	S	C→S→P	Nagata & Takebe (1971), Takebe *et al.* (1971)
velutina	vL	L	C→S→P	Shakurov (1982)
Nierembergia sp.	?	?	P	Power & Chapman (1983)
Petunia alpicola	C;Sc	L	C→S→P	Ford-Logan & Sink (1988)
axillaris	vL	LoS	C→S→P	Power *et al.* (1976)
hybrida	vL;L	L	C→S→P	Binding (1974a,b),
	vL	L	C→S→P	Durand *et al.* (1973),
	vL	S	C→S→P	Frearson *et al.* (1973)
inflata	vL	LoS	C→S→P	Power *et al.* (1976)
parodii	vL	L;LoS	C→S→P	Hayward & Power (1975)
parviflora	vL	L;LoS;S	C→S→P	Sink & Power (1977)
violaceae	vL	LoS	C→S→P	Power *et al.* (1976)
Salpiglossis sinuata	C	L	C→S→P	Boyes & Sink (1981), Boyes *et al.* (1980)
Solanum aviculare	Sc	L	C→S→P	Gleddie *et al.* (1985)
brevidens	vL	L	C→S→P	Barsby & Shepard (1983),
	L	SoS	C→S→P	Haberlach *et al.* (1985),
	L	L	C→S→P	Nelson *et al.* (1983)
cardiophyllum	L	L;SoS	C→S→P	Hunt & Helgeson (personal communication)
chacoense	L	L;S	C→S→P	Butenko & Kuchko (1979)
dulcamara	L	L	C→S→P	Binding & Mordhorst (1984), Binding & Nehls (1977), Binding *et al.* (1980, 1981)
etuberosum	vL	LoS	C→S→P	Barsby & Shepard (1983),
	vL	LoS	C→S→P	Haberlach *et al.* (1985)
fernandezianum	vL	LoS	C→S→P	Barsby & Shepard (1983)
insanum	Co	?	S→P	Nishio *et al.* (1987)
khasianum	vL	L	C→S→P	Kowalczyk *et al.* (1983)
laciniatum	L;St	L	C→S→P	Serraf *et al.* (1988)
luteum	L	L	C→S→P	Binding *et al.* (1980, 1981)
lycopersicoïdes	Sc	L	C→S→P	Handley & Sink (1985),
	L;Sc	L	C→S→P	Tan *et al.* (1987)
mammosum	Sc	D;L;sS	C→S→P	Kumar *et al.* (1983)
melongena	vL	L	C→S→P	Bhatt & Fassuliotis (1981),
	Sc	L	C→S→P	Gleddie *et al.* (1982),
	L	SoS	C→S→P	Guri & Izhar (1984),
	vL	L	C→S→P	Jia & Potrykus (1981),
	L	L	C→S→P	Saxena *et al.* (1981a, 1987)
nigrum	vL;L	L	C→S→P	Binding *et al.* (1980, 1981),
	vL;L	L	C→S→P	Nehls (1978)
pennellii	L	SoS	C→S→P	Haberlach *et al.* (1985),
	vL	L	C→S→P	Hassanpour-Estahbanati & Demarly (1985)
phureja	L;Sc	L	C→S→P	Schumann & Koblitz (1983), Schumann *et al.* (1980)
pinnatisectum	L	L	C→S→P	Sidorov *et al.* (1984)
polyadenium	L	L;SoS	C→S→P	Hunt & Helgeson (personal communication)
torvum	vL	L;R	C→S→P	Guri *et al.* (1987)
tuberosum	L	L	C→S→P	Binding *et al.* (1978),
	L	L	C→S→P	Bokelmann & Roest (1983),
	R	D	C→S→P	Laine & Ducreux (1987),
	vL	LoS	C→S→P	Shepard & Totten (1977)
uporo	L	D	C→S→P	Li & Constabel (1984)

Table 1. (b) (Continued)

Taxon	Donor tissue	Culture technique	Regenerant development	Reference
verrucosum	L	L	C→S→P	Tan *et al.* (1987)
xanthocarpum	L	D	C→S→P	Saxena *et al.* (1982)
Ulmaceae				
Ulmus × '*Pioneer*'	C	L	C→S→P	Sticklen *et al.* (1986)
Umbelliferae				
Daucus carota	Sc	D	E→P	Grambow *et al.* (1972),
	vR	Ls	C→E→P	Kameya & Uchimiya (1972)
Foeniculum vulgare	Sc	S	E→P	Miura & Tabata (1986)
Levisticum officinale	vL	L	C→E→P	Jia *et al.* (1988)
Ligusticum wallichii	Co	L	C→E→P	Li & Chen (1986)

isolation, the method of protoplast culture, the culture medium, and the physical environment. These factors will be discussed here briefly. For an extensive literature review, the reader is referred to Maheshwari *et al.* (1986).

For most genotypes of various plant species, regeneration procedures have been developed by trial and error. Therefore, during the last decade, Binding and co-workers have attempted to develop generally applicable protocols. Regeneration capacities of isolated protoplasts of 77 dicotyledonous plant species were checked under similar experimental conditions (Binding *et al.* 1981). Cell divisions were obtained in 54 species, giving rise to continuously proliferating callus in 35 species. In 21 of these species regeneration of shoots or plants was obtained. From these investigations the following parameters were found to be appropriate in various species for successful regeneration: shoot tip regions of aseptic shoot cultures as protoplast sources, high plating densities of isolated protoplasts, V-KM as protoplast culture medium (see the section on 'The culture medium') and prolonged culture on V-KM agar media, and repeated dilutions of the regenerated cell colonies. Recently, Binding *et al.* (1988) reported on another regeneration procedure called 'streak plating'. Protoplasts were cultured in streaks of locally high densities. This technique proved to be advantageous for the culture and regeneration of many plant species. The results of Binding and co-workers provided more knowledge with respect to factors that play decisive and important roles in the isolation, culture and regeneration of protoplasts.

A different successful approach was followed by Cocking and co-workers, who achieved plant regeneration from protoplasts in a variety of plant species by establishing species-specific culture conditions.

Plant factors

The genotype. Plant regeneration from protoplasts greatly varies from species to species. In many cases, e.g. tomato, success was limited to a specific genotype under special conditions (Morgan & Cocking 1982). Restoration of the regeneration potential via somatic hybridization in *Nicotiana* (Maliga *et al.* 1977) and *Lycopersicon* (Adams & Quiros 1985; Koornneef *et al.* 1987; Wijbrandi *et al.* 1988) suggests that the regeneration capacity is a dominant trait, which can be used as a selectable marker in protoplast fusion experiments.

The donor tissue. An increase in the regeneration capacity was observed when protoplasts were isolated from plants that were pre-treated under specific culture conditions (day-length, light intensity, temperature and relative humidity) in growth chambers (Shepard &

Totten 1977; Kao & Michayluk 1980; Y. Chupeau, personal communication). Binding (1974b) investigated the effect of the pre-treatment of aseptic shoot cultures of *Petunia hybrida*: the yield and plating efficiency of protoplasts were markedly affected by the composition of the medium, the light intensity and temperature during the period of shoot culture prior to isolation.

Totipotency is not limited to a certain specialized type of plant cells, since plants have been regenerated from protoplasts derived from all kinds of tissues and organs. Nevertheless, the donor tissue used for protoplast isolation has been found to affect greatly the subsequent culture and regeneration of protoplasts. Meristematic cells from shoot tip regions of aseptic grown plantlets have been proven to be a good source of protoplasts with a high regeneration capacity in a great number of plant species (Binding *et al.* 1981). In addition, aseptic shoot cultures are increasingly used and have been demonstrated to be a good source, e.g. *Capsicum annuum* (Saxena *et al.* 1981b), *Broussonetia kazinoki* (Oka & Ohyama 1985) and *Solanum tuberosum* (Bokelmann & Roest 1983).

Aseptic shoot cultures and shoot tips are recommended as a source of protoplasts for several reasons.

(1) The plant material can be easily cloned and prepared which results in a reproducible high yield of aseptic protoplasts.
(2) The donor tissue is grown under controlled conditions, ensuring a relatively large physiological uniformity and providing fairly consistent results after protoplast culture.
(3) The protoplasts are isolated from meristematic or incompletely differentiated tissues, and characterized by an easily inducible and sustained mitotic activity and a high degree of totipotency.

In Dicotyledonae juvenile mesophyll cells of (the apical region of) aseptic shoots, in particular, are used as protoplast sources whereas, in Monocotyledonae, and woody crops, embryogenic callus or cell suspension cultures, derived from immature embryos, are frequently used. Vasil & Vasil (1980) suggested that the use of embryogenic cultures can overcome the recalcitrance of protoplasts derived from monocotyledons, as demonstrated in *Pennisetum americanum*. Apart from some mitotic activity, so far no regeneration has been observed in mesophyll protoplasts of monocotyledonous plant species. Protoplasts isolated from embryogenic cell suspension cultures of two woody plant species, both belonging to the Gymnospermae, namely *Picea glauca* (Attree *et al.* 1987) and *Pinus taeda* (Gupta & Durzan 1987), regenerated into somatic embryoids and plantlets. In *Picea glauca* protoplasts from a non-embryogenic source divided in culture but they never resulted in organogenesis or embryogenesis. It is generally known, however, that during prolonged culture periods suspension cells often lose their morphogenic potential, due to the occurrence of (epi)-genetic instability. As a consequence, new cell suspensions have to be initiated at regular intervals to keep a useful source for isolation of protoplasts with regeneration capacity.

Roots have also proved to be a good source for protoplast isolation. For example, protoplasts isolated from radicles of 1–3 day-old seeds, that were germinated under aseptic conditions, showed a high division potential and callus formation in most of the 12 plant species tested. From these calli, plantlets were regenerated via shoot formation in *Brassica napus, Brassica oleracea* and *Medicago sativa* (Xu *et al.* 1982a,b, 1985). Plant regeneration was also achieved from root apical protoplasts of *Solanum tuberosum* (Laine & Ducreux 1987). Although root protoplasts are generally isolated at relatively low

quantities, they can provide an additional experimental system which may be of particular importance in species from which it is normally difficult to isolate and/or to culture mesophyll or cell suspension protoplasts.

In species of the Papilionaceae, cotyledonary tissue has been demonstrated to be a good source for isolation and subsequent regeneration of protoplasts.

External factors

The culture medium. An important step in protoplast regeneration appeared to be the development of the highly enriched 8p culture medium (Kao & Michayluk 1975). The medium particularly enhanced the development from cultured protoplasts of a meristematic type of cells, which are able to regenerate shoots, as was recently demonstrated in *Capsicum annuum* (Diaz *et al.* 1988). The V-KM medium, composed of the macro elements of the V-47 medium (Binding 1974b) and the other nutrients of the 8p medium, was developed by Binding and co-workers and proved to be suitable for a variety of plant species (Binding *et al.* 1981), including potato (Bokelmann & Roest 1983).

The physical environment. Chilling freshly isolated mesophyll protoplasts of tomato enhanced the plating efficiency by more than twofold (Mühlbach & Thiele 1981). In addition, heat shock treatment increased cell division activity of rice protoplasts (Thompson *et al.* 1987). Recently, electrostimulation was also used to enhance protoplast division and plant regeneration. Electroporation-mediated improvement of cell divisions, colony formation and regeneration capacity was observed in species of the genera *Glycine, Prunus, Pyrus* and *Solanum* (Rech *et al.* 1987, 1988; Ochatt *et al.* 1988).

Culture techniques. Agarose has been proved to be an important supplement of the protoplast culture medium. Apart from avoiding agglutination of protoplasts, plating in agarose stimulates colony formation from protoplasts of a wide range of species (Shillito *et al.* 1983). Agarose was also found to be beneficial for division of protoplasts of *Oryza sativa* (Thompson *et al.* 1986) and wild *Medicago* species, as compared to culture in a liquid medium (Gilmour *et al.* 1987). Using media solidified with agarose, a stimulating effect was also observed for protoplasts of *Prunus avium* × *pseudocerasus* (Ochatt *et al.* 1987), *Prunus cerasus* (Ochatt & Power 1988), *Pyrus communis* and other deciduous woody species (Ochatt & Caso 1986).

Protoplasts embedded in agarose are most often cultured in combination with a liquid culture medium. Protoplasts were cultured in agarose droplets, e.g. *Liriodendron tulipifera* (Merkle & Sommer 1987), or in agarose beads. This latter bead-type culture technique improved the plating efficiencies in *Lycopersicon esculentum* and *Crepis capillaris*, and enabled sustained proliferation of protoplasts of *Brassica rapa* and *Petunia hybrida* (Shillito *et al.* 1983). This culture technique was also successfully employed for protoplast regeneration of *Picea glauca* (Attree *et al.* 1987), *Cucumis sativus* (Orczyk & Malepszy 1985; Colijn-Hooymans *et al.* 1988), *Lactuca saligna* (Brown *et al.* 1987), *Dimorphotheca* and *Rudbeckia* (Al-Atabee & Power 1987), *Oryza sativa* (Hayashi *et al.* 1986; Kyozuka *et al.* 1987), *Prunus cerasus* (Ochatt & Power 1988) and *Capsicum annuum* (Diaz *et al.* 1988).

Other techniques employed deal with leaf protoplasts of *Populus*, which were cultured in contact with polyester screen discs floated in a liquid medium (Russell & McCown 1986). This study represents the first report of reproducible plant regeneration from protoplasts of non-seedling origin of a tree species. Floatation of protoplasts at the air/

liquid interphase proved to be effective in inducing sustained divisions, callus development and ultimate plant regeneration of *Brassica carinata* (Chuong *et al.* 1987b). Apart from the conditioning medium, which was successfully employed for protoplasts of *Liriodendron tulipifera* (Merkle & Sommer 1987), feeder-layer techniques have also proved to be advantageous for the efficient plating of protoplasts at high and low densities. Galun and co-workers (Raveh *et al.* 1973) were the first to use X-irradiated protoplasts of tobacco as feeder layers to support division of cultured tobacco protoplasts plated at relatively low densities. Recent examples of the successful use of feeder layers as nurse cultures for cultured protoplasts concern *Petunia hybrida* (Shneyour *et al.* 1984), different *Medicago* species (Gilmour *et al.* 1987) and *Oryza sativa* (Kyozuka *et al.* 1987). In addition, somatic hybrid plants were regenerated through the feeder-layer technique from heterokaryons cultured at relatively low densities, which were obtained after electrofusion of different *Solanum* species (Puite *et al.* 1986).

Finally, a microculture technique has been developed, which enabled the culture and regeneration of individual protoplasts of *Nicotiana tabacum* (Koop & Schweiger 1985) and *Brassica napus* (Schweiger *et al.* 1987) in microdroplets in microchambers. This culture technique has been used to develop a sophisticated and automized set-up for handling single protoplasts (Koop & Spangenberg 1988).

CONCLUSIONS AND PERSPECTIVES

Due to several new approaches concerning plant factors, such as the plant genotype and donor tissue, and external factors such as the culture medium, the physical environment and the appliciation of new culture techniques, considerable progress has been made during the last decade in the field of protoplast regeneration. Until now protoplasts from 212 higher plant species, including several important food crops, belonging to 96 genera of 31 families, have been regenerated to plant(let)s, or in some cases to embryo-like structures, embryoids or shoots. In principle, these plant species are accessible for genetic manipulation. Plant regeneration has been reported from protoplasts of several species of the Gramineae, Papilionaceae and of woody plants, which were considered to be recalcitrant. From the data presented in Table 1 and Fig. 1, a further increase in the number of plant species during the forthcoming years is expected. Despite this progress, two major problems still remain and hamper the use of protoplasts for genetic manipulation.

(1) Several economically important crops are not amenable, so far, for plant regeneration from protoplasts. In addition, in some monocotyledons protoplast regeneration resulted in the development of albino plantlets only.
(2) A high degree of genetic variation, so-called somaclonal variation, was observed frequently among the regenerants derived from cultured protoplasts, as reviewed for potato by Sree Ramulu (1986). Although somaclonal variation might be of interest for crop improvement (Scowcroft & Larkin 1982), it is undesirable for genetic manipulation directed towards the addition of desired genetic characters.

Most protoplast regeneration procedures have been developed by an empirical approach. The diversity of protocols clearly demonstrates that we are still far from understanding the fundamental processes of protoplast regeneration. Comparative studies, using experimental parameters like donor tissue, culture medium and plating density, have been helpful to improve the knowledge of the potentials and requirements of protoplasts in culture and have proved to be important for achieving regeneration of many

plant species (Binding *et al.* 1981). However, a real break-through concerning plant regeneration from protoplasts of recalcitrant species can only be realized when the basic conditions and processes leading to organogenic activities are elucidated.

Protoplasts of higher plants in culture exhibit a great heterogeneity with respect to the genotype, the morphology, physiological characteristics and morphogenic capacities. This heterogeneity of protoplasts in relation to response to culture conditions is a drawback in experimental manipulation of higher plant protoplasts (Koop & Spangenberg 1988). Using the microculture technique, developed by Koop & Schweiger (1985), it is now possible to perform experiments with single protoplasts, the tissue origin and experimental treatment of which, prior to culture, are precisely known. The individual culture of protoplasts under controlled conditions represents a powerful tool for fundamental studies on the physiology of different cell types and cell cycle stages, biochemical, biophysical, cytological and morphological properties, the analysis of differentiation programmes and the genetic manipulation of protoplasts (Schweiger *et al.* 1987). To realize a break-through concerning plant regeneration from protoplasts of other recalcitrant species, more fundamental knowledge is required on the basic factors that govern the different steps in the regeneration process from protoplast to plant.

In spite of this fact, the recent progress achieved in the field of protoplast regeneration is an important step forward and allows genetic manipulation of many plant species via several approaches such as complete and partial genome transfer after protoplast fusion (Negrutiu *et al.* 1989), direct (Toriyama *et al.* 1988) and microcell-mediated (Sree Ramulu *et al.* 1988) gene transfer, and micro-injection (Verhoeven & Blaas 1988).

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NOTE ADDED IN PROOF

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

Taxon	Donor tissue	Culture technique	Regenerant development	Reference
Monocotyledonae				
Gramineae				
Dactylis glomerata	eSc	S	C→E→P	Horn *et al.* (1988)
Triticum aestivum	eC	N	C→E→Pa	Hayashi & Shimamoto (1988)
Dicotyledonae				
Rutaceae				
Citrus mitis	eSc	LoS	C→E→P	Sim *et al.* (1988)

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