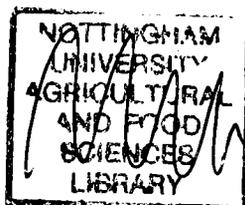


RESPIRATORY GAS CARRIERS IN  
PLANT CULTURE SYSTEMS

by

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## CONTENTS

<b>ACKNOWLEDGEMENTS</b>	<b>i</b>
<b>TABLE OF CONTENTS</b>	<b>ii</b>
<b>ABSTRACT</b>	<b>ix</b>
<b>ABBREVIATIONS</b>	<b>xi</b>

### **CHAPTER 1. General Introduction**

1.1	Principles and current limitations of plant tissue culture	1
1.1.1	Bioreactors for the large-scale <b>cultivation</b> of plant cells	8
1.2	Effects of the gaseous atmosphere on <i>in vitro</i> plant growth and development	14
1.2.1	Beneficial effects of oxygen enrichment of the culture environment	18
1.2.2	Beneficial effects of carbon dioxide enrichment of the environment	19
1.3	Comparison of plant and animal cell culture systems	22
1.4	Perfluorochemicals (PFCs) and cell biotechnology	24
1.4.1	Introduction	24
1.4.2	Properties of PFC liquids	24
1.4.3	Biomedical applications of PFCs	26
1.4.4	Applications of PFCs to cell biotechnology	29
1.4.4.1	Biocompatibility of PFCs in fermenter systems	30
1.4.4.2	Animal cell culture	32
1.4.4.3	PFCs and carbon dioxide	32
1.4.4.4	PFCs and physiological oxygen monitoring	33
1.4.4.5	Culture of cells at PFC-aqueous interfaces	34
1.4.4.6	Fluorocarbon polymers in cell cultures	36
1.5	Haemoglobin-based oxygen carriers and cell biotechnology	36
1.5.1	Recent history	36
1.5.2	Haemoglobin in cell biotechnology	39
1.6	Thesis objectives	40

## **CHAPTER 2. Biocompatibility and Exploitation of PFC Liquids in Plant Protoplast Systems**

2.1	Introduction	42
2.2	Materials and Methods	46
2.2.1	Cassava	46
2.2.1.1	Plant material and establishment of cassava shoot cultures	46
2.2.1.2	Isolation of cassava leaf protoplasts	46
2.2.1.3	Measurement of cassava protoplast yield and viability	47
2.2.1.4	Culture of cassava leaf protoplasts	48
2.2.1.5	Cell wall determination of cultured cassava protoplasts	48
2.2.1.6	Measurement of cassava protoplast growth	48
2.2.1.7	Plant regeneration assessments of cassava protoplast-derived tissues	49
2.2.2	Petunia	49
2.2.2.1	Initiation and maintenance of petunia cell suspensions	49
2.2.2.2	Isolation of cell suspension protoplasts	49
2.2.2.3	Culture of petunia cell suspension-derived protoplasts	50
2.2.2.4	Measurement of petunia protoplast growth	51
2.2.2.5	Fluorocarbon re-cycling and re-use	52
2.2.2.6	Measurement of dissolved oxygen concentration	52
2.2.2.7	Assessment of petunia protoplast-derived cell colony growth by image analysis	52
2.2.3	Assessment of cassava leaf protoplasts cultured with various gas manipulation treatments	53
2.2.4	Statistical analyses	53
2.3	Results	54
2.3.1	Cassava protoplast culture and plant regeneration assessments	54
2.3.1.1	Effect of medium supplementation with glass rods on mitotic division of cultured cassava protoplasts	54
2.3.1.2	Evaluation of plant regeneration potential from cassava protoplast-derived tissues	56
2.3.2	Culture of petunia protoplasts with PFC and <i>Pluronic</i> ® F-68	56
2.3.2.1	Petunia protoplast viability and division	56
2.3.2.2	Changes in oxygen concentration	56

2.3.2.3	Growth of petunia protoplasts with perfluorodecalin	57
2.3.2.4	Growth of petunia protoplasts with perfluorodecalin and <i>Pluronic</i> <sup>®</sup> F-68	60
2.3.2.5	Image analysis of petunia protoplast-derived cell colony growth	60
2.3.3	Effects of various gas-manipulation treatments on mitotic division of cultured cassava protoplasts	63
2.4	Discussion	65

### **CHAPTER 3. Biocompatibility of Haemoglobin-Based Respiratory Gas Carriers in Plant Protoplast Systems**

3.1	Introduction	71
3.2	Materials and Methods	72
3.2.1	Culture of petunia suspension cells on medium supplemented with <i>Erythrogen</i> <sup>™</sup>	72
3.2.2	Isolation and culture of petunia protoplasts	73
3.2.3	Supplementation of protoplast culture medium with <i>Erythrogen</i> <sup>™</sup> alone or with oxygen or carbon monoxide	73
3.2.4	Supplementation of protoplast culture medium with <i>Pluronic</i> <sup>®</sup> F-68	74
3.2.5	Measurement of protoplast viability, growth and biomass	74
3.2.6	Measurement of pH and osmotic pressure in culture medium	75
3.2.7	Comparison of media supplementation with <i>Erythrogen</i> <sup>™</sup> and/or PFC	75
3.2.7.1	Initiation and maintenance of passion fruit cell suspensions	75
3.2.7.2	Isolation of protoplasts from cell suspensions of passion fruit and petunia	75
3.2.7.3	Culture of passion fruit and petunia protoplasts with <i>Erythrogen</i> <sup>™</sup> and/or PFC	76
3.2.7.4	Measurement of IPE	76
3.2.8	Medium supplementation with fresh and stored <i>Erythrogen</i> <sup>™</sup> solutions	76
3.2.9	Statistical analyses	76

3.3	Results	77
3.3.1	Growth of petunia suspension cells on medium supplemented with <i>Erythro</i> <sup>TM</sup>	77
3.3.2	Effect of medium supplementation with <i>Erythro</i> <sup>TM</sup> on mitotic division of petunia protoplasts	78
3.3.3	Evaluation of medium supplementation with <i>Pluronic</i> ® F-68 on culture of petunia protoplasts	79
3.3.4	Assessment of medium supplementation with <i>Erythro</i> <sup>TM</sup> on pH and osmotic shifts	81
3.3.5	Growth of petunia protoplast-derived cell colonies	82
3.3.6	Evaluation of media supplementation with <i>Erythro</i> <sup>TM</sup> and/or PFC on culture of passion fruit and petunia protoplasts	83
3.3.7	Effect of <i>Erythro</i> <sup>TM</sup> efficacy after long-term storage	86
3.4	Discussion	86

**CHAPTER 4. *Pluronic*® F-68-Mediated Post-thaw Recovery of Cryopreserved Plant Cells**

4.1	Introduction	90
4.1.1	Cryopreservation of plant cell suspension cultures	90
4.1.2	Physical and chemical parameters for stimulating post-thaw recovery of cells	91
4.1.3	Novel post-thaw treatments to enhance oxygen delivery to thawed cells	92
4.1.3.1	Supplementation of culture medium with the surfactant <i>Pluronic</i> ® F-68	92
4.2	Materials and Methods	95
4.2.1	Rice and <i>Lolium</i>	95
4.2.1.1	Plant materials and preparation of rice and <i>Lolium</i> cell suspensions	95
4.2.1.2	Cryoprotection and freezing of rice and <i>Lolium</i> suspension cells	95
4.2.1.3	Post-thaw recovery of rice and <i>Lolium</i> suspension cells	96
4.2.14	Measurement of post-thaw viability and biomass	97
4.2.2	<i>Moricandia</i>	97
4.2.2.1	Plant materials and establishment of <i>Moricandia</i> shoot cultures	97

4.2.2.2	Callus initiation and generation of <i>Moricandia</i> cell suspensions	98
4.2.2.3	Growth of <i>Moricandia</i> cell suspensions	98
4.2.2.4	Preparation of <i>Moricandia</i> cells for cryopreservation	99
4.2.2.5	Post-thaw recovery of <i>Moricandia</i> cryopreserved cells	99
4.2.2.6	Re-initiation of <i>Moricandia</i> cell suspensions from cryopreserved cells and isolation of protoplasts	100
4.3	Statistical analyses	101
4.4	Results	101
4.4.1	Cryopreservation of rice and <i>Lolium</i> cells	101
4.4.1.1	Post-thaw viability of rice and <i>Lolium</i> cells	101
4.4.1.2	Post-thaw viability of rice and <i>Lolium</i> cells using TTC buffer supplemented with <i>Pluronic</i> <sup>®</sup> F-68	103
4.4.1.3	Post-thaw growth of rice and <i>Lolium</i> cells	103
4.4.1.4	Supplementation of the cryoprotectant mixture with <i>Pluronic</i> <sup>®</sup> F-68 prior to cryopreservation of rice and <i>Lolium</i> cells	106
4.4.2	Cryopreservation of <i>Moricandia</i> cells	106
4.4.2.1	Comparison of cryoprotectant effectiveness for cryopreservation of <i>Moricandia</i> cells	106
4.4.2.2	Effect of <i>Pluronic</i> <sup>®</sup> F-68 on recovery of cryopreserved <i>Moricandia</i> cells	107
4.4.2.3	Re-establishment of <i>Moricandia</i> cell suspensions and subsequent isolation of suspension-derived protoplasts	109
4.5	Discussion	110

## **CHAPTER 5. PFC-Mediated Post-thaw Recovery of Cryopreserved Rice Cells**

5.1	Introduction	114
5.1.1	Oxygen free radicals and plant antioxidant systems	114
5.1.2	Respiratory imbalances during post-thaw recovery of cryopreserved cells	116
5.2	Materials and Methods	118
5.2.1	Plant materials and preparation of cell suspensions	118
5.2.2	Cryopreservation and post-thaw recovery	118
5.2.3	Measurement of post-thaw viability and biomass	120

5.2.4	Re-initiation of cell suspension cultures	120
5.2.5	Isolation and culture of protoplasts from re-established cell suspension cultures	120
5.2.6	Plant regeneration from protoplast-derived callus	121
5.2.7	Cytological analysis of protoplast-derived plants	122
5.3	Statistical analyses	123
5.4	Results	123
5.4.1	Assessment of PFC medium supplementation on post-thaw cell viability	123
5.4.2	Supplementation of medium with PFC and/or <i>Pluronic</i> ® F-68 on post-thaw cell viability	124
5.4.3	Post-thaw growth of cryopreserved cells recovered in the presence of PFC and/or <i>Pluronic</i> ® F-68	126
5.4.4	Re-establishment of cell suspension cultures	127
5.4.5	Assessment of protoplast viability, yield, plating efficiency and plant regeneration	128
5.4.6	Cytological analysis of regenerated plants	130
5.5	Discussion	130

## **CHAPTER 6. General Discussion**

6.1	Introduction	134
6.2	Protoplast culture systems	134
6.3	Synergistic effects of PFCs and surfactants as protoplast culture medium supplements	136
6.4	Image analysis assessments of cell growth	136
6.5	Comparison of PFCs with other physical and chemical options for enhancing oxygen supply	137
6.6	<i>Pluronic</i> ® F-68 and plant cell cryopreservation	138
6.7	PFCs and post-thaw recovery of cryopreserved rice cells	139
6.8	Synergistic effects of PFCs and surfactants on post-thaw recovery of cryopreserved rice cells	140
6.9	Concluding remarks	142

## **APPENDICES**

1	Enzyme formulation	144
II	Media formulations	145
III	Publications	154
<b>REFERENCES</b>		155

## ABSTRACT

A crucial pre-requisite in genetically manipulating higher plants involves systems for culturing plant protoplasts and cells under static conditions with an adequate oxygen supply. This is especially the case for cells from cryopreservation, where respiratory perturbations are known to occur during early post-thaw recovery. Therefore, studies were undertaken to assess the potential, and actual, beneficial effects involving culture of cells at an interface between inert, oxygen-gassed perfluorocarbon (PFC) liquid overlaid with liquid or semi-solidified media supplemented with or without the non-ionic surfactant, *Pluronic*<sup>®</sup> F-68. Assessments were also made to compare the efficacy of PFC supplementation with other physical (medium implanted with glass rods to increase the surface area available for gaseous exchange) and chemical (haemoglobin; Hb) options, both alone and in combination, for gaseous manipulation of plant protoplast cultures.

Investigations involving novel PFC-mediated oxygen delivery to cultured protoplasts were carried out on a broad range of plant species, which included *Petunia hybrida* (a herbaceous species) and *Passiflora giberti* (a woody species), as model systems, together with cassava (*Manihot esculenta*) a relatively recalcitrant species in tissue culture. Studies revealed enhanced protoplast initial plating efficiencies (IPEs) as measured by increased mitotic division, thereby demonstrating no short-term detrimental effects of exposure to PFC. Similarly, supplementation of culture media with Hb, at 1:50 (v/v), increased the mean IPEs of both *Petunia* and *Passiflora* protoplasts over that of untreated controls. Additionally, supplementation of aqueous medium with 0.01% (w/v) *Pluronic*<sup>®</sup> F-68 not only lowered interfacial tension, but further enhanced mitotic activity over that stimulated by both oxygenated PFC and Hb.

In the context of cryopreservation, media supplementation with *Pluronic*<sup>®</sup> F-68, at 0.01-1.0% (w/v), significantly improved the post-thaw viability and growth of embryogenic suspension cells of the rice (*Oryza sativa* L.) cultivars Taipei 309 and Tarom, together with non-embryogenic cells of *Lolium multiflorum* and *Moricandia arvensis*. Moreover, a more pronounced synergistic effect in terms of viability and growth was observed for Taipei 309 cells when 0.01% (w/v) *Pluronic*<sup>®</sup> F-68 was evaluated in conjunction with oxygenated PFC. Plants regenerated from such cryopreserved cells were morphologically normal with expected chromosome complements ( $2n = 2x = 24$ ), thus confirming the long-term biocompatibility of PFCs, with no adverse effect up on cellular totipotency.

These results indicate, for the first time, that both oxygenated PFC and Hb provide options for enhancing cellular oxygen supply to cultured eukaryotic cells *in vitro*. However, the recoverability and, hence, recyclability of PFCs make them a commercially more attractive option, despite the high initial investment cost. Overall, **PFC-facilitated** improvements in cell culture technology will have increasingly important biotechnological implications in the context of plant micropropagation, somatic hybridisation, transgenic plant production and commercial exploitation of these technologies.

## ABBREVIATIONS

approx.	approximately
ADP	adenosine diphosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
BAP	6-benzylaminopurine
<i>ca.</i>	circa (Latin; about)
CFCs	chlorofluorocarbons
CIAT	International Centre for Tropical Agriculture, Cali, Colombia
cm	centimetre
cm <sup>2</sup>	squared centimetre
Co.	company
CPPU	N-(2-chloro-4-pyridyl)-N'-phenylurea
cv.	cultivar(s)
d	day(s)
diam.	diameter
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dpi	dots per inch
eg-	exempli gratia (Latin; for example)
Ed.	Editor(s)
EDTA	ethylene diamine tetra acetic acid
<i>et al.</i>	<i>et alia</i> (Latin; and others)
FDA	fluorescein diacetate
FPE	final plating efficiency
FTBA	perfluorotributylamine
FTPA	perfluorotripropylamine
f. wt.	fresh weight
Fig.	figure(s)
g f wt.	gramme fresh weight (of tissue)
g	gram
<i>g</i>	relative centrifugal force
h	hour(s)
Hb	haemoglobin
HLB	hydrophobic-hydrophilic balance
IPE	initial plating efficiency

kJ	kilojoules
l	litre(s)
Ltd.	Limited Company
m	metre
M	molar
MES	2- [morpholino]ethanesulphonic acid
mg	milligramme(s)
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
mOsmol kg <sup>-1</sup>	milliosmole per kilogramme
MS	Murashige and Skoog (1962) basal medium
n	number of observations
NAA	<b>a-naphthaleneacetic acid</b>
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
nm	nanometre
NMR	nuclear magnetic resonance
NPR	net photosynthetic rate
P	probability
PCV	packed cell volume
<i>per se</i>	Latin; as such
PFC	perfluorocarbon(s)
pH	hydrogen potential; logarithm of the reciprocal of hydrogen ion concentration, i.e. $\log_{10}(1/[H^+])$ or $-\log_{10}[H^+]$
POE	polyoxyethylene
POP	polyoxypropylene
PPP	pentose phosphate pathway
PTFE	<b>polytetrafluoroethylene</b>
r.p.m.	revolutions per minute
SCV	settled cell volume
s.d.	standard deviation
s.e.m.	standard error of the mean
SOD	superoxide dismutase
spp.	species
STP	standard temperature and pressure
TBA	thiobarbituric acid
TCA	tricarboxylic acid pathway

TTC	triphenyl tetrazolium chloride
UK	United Kingdom
UM	Uchimiya and Murashige (1974) medium
USA	United States of America
UV	ultra-violet (light)
v/v	volume to volume
w/v	weight to volume
%	percent
°C	degrees Celsius
<	less than
=	equals
$\mu$ l	microlitre
$\mu$ m	micrometre(s)
$\mu$ M	micromoles
2,4-D	dichlorophenoxyacetic acid
2,3-DPG	diphosphoglycerate

### **1.1 Principles and current limitations of plant tissue culture**

Plant tissue culture comprises a set of *in vitro* techniques, pre and post culture methods and strategies that collectively have been exploited to create genetic variability from which crop species can be improved (Brown and Thorpe, 1995). Tissue culture approaches to crop improvement cover a wide range of protocols involving the growth, under aseptic conditions, of axenic (organism-free) whole plant organs such as shoots or embryos or alternatively the culture of dedifferentiated calli or single cells and protoplasts as suspensions. Additionally, specialised tissue culture techniques and the availability of gametic cells allow for the production of haploid (and dihaploid) plants, rescue of immature or F1 hybrid embryos, transformation for the production of transgenic plants, somatic hybridisation based predominantly on interspecific protoplast fusion and the production of synthetic seeds using encapsulated somatic embryos (Martin *et al.*, 1998). The induction and sustainability of plant regeneration (totipotency) is the overriding consideration behind such applied technologies. Despite many recent and significant advances in genetic manipulation methodologies and outcomes, particularly in such areas of transgenic plant research and allied molecular biological and recombinant DNA techniques, there still remains a number of practical constraints at the tissue culture underpin technology level.

Micropropagation, although arguably the least sophisticated technique, remains the widest applied use of plant tissue culture technology. Over the last three decades it has become possible to regenerate plants from a wide range of explants/specialised cells of plant species including monocotyledonous species which were until recently generally considered recalcitrant to the cultural strategies that had worked efficiently with dicotyledonous species (Maheshwari *et al.*, 1995; Buiteveld *et al.*, 1998). The

obvious (practical) exploitation of micropropagation (starting with organised tissue/organ explants) include rapid multiplication of elite stock whereby plants can be maintained pathogen-free (Kozai *et al.*, 1997) along with the creation of gene banks for the storage of endangered plant germplasm (Martin *et al.*, 1998).

There are three recognised strategies currently employed for micropropagation: (1) enhancing axillary shoot-bud development; (2) production of adventitious shoots; and (3) the induction of somatic embryogenesis often from an intermediate callus/cell phase. In the latter process, somatic cells develop through a series of characteristic and discrete embryogenic stages (reflecting zygotic embryo developmental pathways) to give whole plants but without gametic cell fusion (De Klerk *et al.*, 1997; Dey *et al.*, 1998). This is in contrast to the alternative pathway, organogenesis, whereby somatic cells under appropriate synergistic media hormonal conditions (cytokinin/auxin ratios) generate shoots and/or adventive roots in a sequential way. Axillary-bud proliferation for most species produces the least number of cloned individuals and is simply regulated by the number of axillary buds introduced into culture. However, this approach has become the most important and widely practiced (commercially applied) propagation method since genetic and hence phenotypic stability can usually be preserved (Karp, 1995).

In the case of the organogenic and somatic embryogenic developmental pathways, differentiated **structures** arise either directly from the input explant or via a (preferably short-lived) callus phase. The latter often leading to genetic variation, collectively termed somaclonal variation (Karp, 1995; Collin and Edwards, 1998). The genetic and phenotypic alterations associated with this poorly understood phenomenon include changes in ploidy status and chromosomal rearrangements, such as loss of whole chromosomes, chromosome arms and chromosome segments and changes at the DNA level, such as transposition and deletion of DNA sequences. Whilst this in the context of generating genetic novelty offers prospects for the

recovery of regenerants with desirable (now or reinforced) traits such as alterations in foliage pigmentation (Franzone *et al.*, 1996), plant vigour and size, leaf shape and flower morphology and floral amendments (Nikova *et al.*, 1997), there remains the possibility of concomitant biochemical changes, such as loss of secondary product biosynthesis (Sevon *et al.*, 1997) or changes in characteristics such as disease resistance/tolerance and overall fertility. For example, Claxton *et al.* (1998) found that 40% of a population of 833 tissue culture-regenerated *Rorippa nasturtium-aquaticum* (watercress) plants were susceptible, as measured by the number of diseased roots, to the fungal pathogen *Spongospora subterranea* f. sp. *nasturtii*. In contrast, only 33% of 500 control cuttings of *R. nasturtium-aquaticum* were found to be susceptible to disease, as measured using the same disease index. The detection of somaclonal variants is relatively simple when these alterations are associated with phenotypic changes. Phenotypic assessment though is not always reliable since, a regular phenotype is not a guarantee of no changes at the genotype level.

Furthermore, a given genotype may be inherently stable, but phenotypic changes may occur, that are temporal in nature and thus are not transmitted through the sexual cycle to the next (seed) generation. Clearly these issues are of less relevance to vegetatively propagated crops and ornamentals. These developmental changes are frequently epigenetic (Lambe *et al.*, 1997). Under certain conditions or after prolonged periods of culture some callus cells acquire an ability to grow in the continued absence of auxin and/or cytokinin. In most cases, this process of habituation is reversible and the callus retains totipotency albeit for a finite period of time. For example, Hoffman and Hoffmannstays (1994) reported that carrot (*Daucus carota* L.) protoplasts isolated from an habituated cell suspension could divide and regenerate to plants without the presence of exogenously supplied growth regulators. A further phenotypic feature observed in micropropagated plants is a tendency to produce fasciated (multi-crowned) regenerants (Tang and Knap, 1998). This phenomenon is often cultivar-specific and can be moderated through the

manipulation of the growth regulators during multiplication. The persistence of an altered growth habit *ex vitro*, sometimes through one or two vegetative propagation-generations again suggests that a transient physiological change has occurred during culture rather than a persistent, stable genetic change.

Somaclonal variation occurs more frequently via dedifferentiated culture such as callus and suspension cultures and is typically less common in cultures where organised plant structures, such as pre-existing meristems are maintained. Factors that may influence the occurrence of such genetic variation have been attributed to source of explant and pre-existing variability, micropropagation cycle, duration of culture period and types and concentrations of growth regulators used in particular synthetic auxins such as 2,4-dichlorophenoxyacetic acid [2,4-D; Bregitzer *et al*, (1995)]. However, such genetic variation can be circumvented by the use of cryopreservation (see Chapters Four and Five of this Thesis). Under conditions of storage in liquid nitrogen (-196 °C), there is complete cessation of all cellular metabolism. Consequently, no cell division, cell degeneration, or genetic changes can take place with time (Grout, 1995). Additionally, cryopreservation negates the need to re-initiate and characterise new cell lines and also provides a constant supply of totipotent, genetically-uniform cells. Moreover, there is no possibility of loss due to bacterial or fungal contamination and significant savings can be made in terms of labour input, culture media, culture vessels and space in controlled-environment growth chambers.

Genetic variation seen in clonal regenerants can also be attributed to the natural variation already present within the donor plant. These somatic mutations will be passed on and perhaps accentuated through any intermediate callus phase and may ultimately be expressed in the regenerant phenotype. Furthermore, since adventitious buds can arise from single cells within the donor parent, any somatic mutations present will be passed directly and rapidly to the apical meristem of the

adventitious bud and thus will be incorporated in all cells of the regenerated plant (Collin and Edwards, 1998).

Apart from the emergence of somaclonal variation within cell cultures another frequent problem is that of lack (or decline) in expression of totipotency (Höxtermann, 1997). The recovery of plants from *in vitro* cultures is most readily achieved using organised input tissues which retain developmental competence. However, with dedifferentiated tissues of some species/genotypes, such as barley [*Hordeum vulgare* L.; Bregitzer *et al*, (1995)] and rice [*Oryza sativa* L.; Utomo *et al*, (1996)] and many monocotyledonous species there remains an inability to express totipotency which obviously precludes plant regeneration potential. This may also be true for such cultures maintained in a dedifferentiated state on culture medium often containing high physiological levels of auxin leading to a gradual loss in the ability to re-differentiate over progressive sub-cultures (Kubalaková *et al*, 1996). In many species, most notably the aforementioned group, *in vitro* culture of cells/tissues in a dedifferentiated state is accompanied by a progression from compact and nodular meristematic callus to friable, vitrified callus concomitant with a reduction of emergent meristematic zones. Furthermore, the relative proportion of such friable **calli/tissues** increases with progressive sub-culture and such tissues are only capable of rhizogenesis at best (Lambe *et al*, 1997). The apparent (permanent) loss of totipotency has been correlated to irreversible genetic alterations, such as single gene mutations, chromosome breakages and ploidy changes. However, Lambe *et al* (1997) have speculated that the progressive loss of totipotency during callus culture may be due, in part, to progressive methylation of genes (gene silencing) specifically relevant to cell differentiation during dedifferentiated cell division. This leads to the continuous elimination (by preferential selection pressure) of cells capable of sustained differentiation. Furthermore, there exists specific developmental pathways of regeneration for species within a family. Organogenesis is the characteristic pathway for members of the *Solanaceae*, such as sweet pepper

[*Capsicum annuum* L.; Franck-Duchenne *et al*, (1998)]. In contrast, somatic embryogenesis is the preferred route for members of the *Umbelliferae*, such as carrot [*Daucus carota* L.; McCabe *et al*, (1997)]. Additionally, some species within a genus can be regenerated from somatic tissues according to standard protocols, whilst other taxonomically closely related species may be unresponsive (Litz and Gray, 1992). For example, Bencheikh and Gallais (1996) found that somatic embryogenesis occurred in lines of pea (*Pisum sativum* L.), whereas lines of *Pisum arvense* L. (wild-type pea) were unresponsive. Moreover, regeneration may be limited to a number of cultivars within a species. In a separate study, Vandoome *et al* (1995) evaluated 16 cultivars of pea and found that for example Stehgolt, Maro and Progreta (commercial cultivars) showed the highest tendency to form somatic embryos, whilst Alaska, Rondo and Ascona were non-competent in the context of embryo production.

Hyperhydricity or vitrification is an additional complication for certain species (and tissue types) when maintained *in vitro*. This physiological disfunction results in excessive hydration of tissues, low lignification of cells and hence reduced mechanical strength of tissue culture-generated regenerants (Ueno *et al*, 1998). Such plants are glossy and translucent in appearance and are usually difficult to proliferate *ex vitro* and can not be successfully established during acclimation. Vitrification is causally related to several culture/environmental régimes, notably excess cytokinin levels in medium (Lu, 1993), high levels of ammonium ions (Brand, 1993), form and type of gelling agents employed (Zimmerman *et al.*, 1995) and for certain genera ethylene production (Righetti, 1996). Use of increased agar concentration as gelling agent may alleviate this condition by reducing water availability and the uptake of cytokinins; this may though have an adverse effect on growth rate (Ghashghaie *et al*, 1991). Raising the cytokinin concentration restored multiplication rates of *in vitro* cultured globe artichoke [*Cynara scolymus*'], Debergh, (1983)]. Ventilation of the culture vessel can similarly be influential on culture

development, since it permits diffusion of water vapour and ethylene from the immediate culture atmosphere (Armstrong *et al.*, 1997). Production of this latter gaseous by-product occurs *in vitro* as a result of tissue wounding during explanting or on sub-culture, together with the cellular metabolism of methionine. Ethylene is a well known plant hormone that inhibits growth, differentiation and in some cases senescence of *in vitro* cultured plants, cells and tissues at concentrations as low as  $0.01 \mu\text{l l}^{-1}$  (Kumar *et al.*, 1998). Morphogenic responses of cultured cells of several *Brassica* species have been shown to be highly sensitive to ethylene (Pua, 1993), whilst many monocotyledonous species are less affected by the gas. Similarly, Lakshmanan *et al.* (1997) found that shoot bud regeneration was delayed, but not totally inhibited by ethylene produced from cultured leaf explants of mangosteen (*Garcinia mangostana* L.). Generally, such differences in morphogenic responses between monocotyledonous and dicotyledonous plant species can, in part, be attributed to the fact that ethylene plays a key role in the fruit ripening process of many dicotyledonous plant species and cells of these species will be responsive to its hormonal effect. In contrast, ethylene is not essential for such a process in many monocotyledonous species and thus cells of the latter have not been conditioned to be responsive. Moreover, ethylene can interact with other *in vitro* plant hormones acting, either synergistically or inhibitory, on the critical threshold values responsible for determining morphogenic responses.

The establishment of *in vitro* plant cultures *per se* involves the physical removal of tissues from the donor and following establishment (*in vitro*) there is mounting evidence that the requisite transition from autotrophic to heterotrophic [sugar-containing (carbon source)-based medium] metabolism, together with the induction of major developmental pathways caused by altered (compared to endogenous levels) hormonal stress, may be expected to promote oxidative stress within the tissues (Benson and Roubelakis-Angelakis, 1994). Indeed, the high physiological concentrations of auxins that are essential for the induction and maintenance of

dedifferentiated cells, callus and isolated protoplasts can increase cellular uptake of oxygen (Chkanikov *et al*, 1990), this in turn leads to the activation of an alternative respiratory pathway (Sluse and Jarmuszkiewicz, 1998) such as the mitochondrial respiratory pathway that branches at the ubiquone pool (Fig. 1.1) and consists of an alternative oxidase encoded by the nuclear gene *Aox1*. This pathway does not permit oxidative phosphorylation of adenosine diphosphate (ADP) and is thus not energy conserving. Alternative oxidase activity is influenced by stress stimuli such as cold, oxidative stress, pathogen attack (in whole plants) and by factors constricting flow through the cytochrome pathway (Fig. 1.1) of respiration (Vanlerberghe and McIntosh, 1997).

### **1.1.1 Bioreactors for the large-scale cultivation of plant cells**

The biotechnological exploitation of plant cell cultures for the production of useful phytochemicals has been studied intensively over the past 30 years (Toivonen, 1993). Such cell and tissue cultures have been used for the synthesis and production of pharmaceuticals [for example, paclitaxel in cell cultures of *Taxus yunnanensis*, Pandey, (1998)], insecticides [production of the quinone, plumbagin in cell suspension cultures of *Drosophyllum lusitanicum*; Nalalka *et al*, (1996)], flavours [alkylcysteinesulphoxides in tissue cultures of *Allium* species; Mellouki *et al*, (1996)] and pigments [alkannin production in cell suspension cultures of *Alkanna tinctoria*, Urbanek *et al*, (1996)]. However, most often plant cell cultures fail to produce, qualitatively or quantitatively, the desired substances found in the intact parent plant from which they were established (Domenburg and Knorr, 1996). Additionally, problems associated with cultural instabilities and the lack of a basic knowledge of the key biosynthetic pathways and their genetic base responsible for the production of plant (secondary) metabolites have severely limited progress. Consequently, efforts have been directed towards increasing product yields based on the selection for high-producing cell lines. For example, Zhao *et al*. (1998) observed that calli of *Saussurea medusa* could be distinguished into two distinct cell types,

one being a faint yellow in colour and the other red; the former was identified by spectrophotometric analysis to produce 2.5 and 3.9 times more flavanoid and jaceosidin respectively than the latter line. In addition, medium optimization and culture conditions have important roles in the large-scale culture of plant cells. In this respect, Huang and Chen (1998) recently demonstrated that 3,4-dihydroxyphenylalanine production in cell suspension cultures of *Stizolobium hassjoo* could be increased four-fold by simply altering the concentrations of the carbon and nitrogen sources, inorganic nutrients, trace elements and organic supplements in the basal culture medium.

Plant cells have been grown in a wide variety of bioreactors. Initially it was considered that conventional stirred tank reactors would be unsuitable for the growth of plant cells due to the high shear forces produced. Consequently, approaches were re-directed at air-driven reactors with comparatively low levels of cell shear. Sterile air is forced up through the cell suspension thereby mixing the cells and maintaining them as a suspension. In most batch cell suspension and organ cultures, maximum secondary product accumulation was observed typically to occur towards the end of the exponential growth phase. For example, cocoa (*Theobroma cacao*) cell suspension cultures were found to produce caffeine and theobromine concomitant with log phase growth (Gumey *et al*, 1992). Similarly, the highest rate of thiophene production in hairy root (transformed) cultures of *Tagetes patula* occurred during late exponential growth, when fresh weight increase (biomass) of the root cultures were maximal (Arroo *et al*, 1995).

Many secondary metabolites are produced by cell cultures that are not undergoing mitosis. These secondary plant products show non-associated growth product kinetics and thus optimization of product formation is dependent on extending the stationary phase of growth responsible for product biosynthesis (Domenborg and Knorr, 1996). However, in order to achieve maximum production, it is necessary to

accumulate a large initial biomass followed by a period in which growth is inhibited. For example, in the case of shikonin production by *Lithospermum erythrorhizon* cultures, a two-stage batch process was used, whereby cells were cultured in a liquid medium favouring rapid cell growth, followed by a second stage of nutrient-limitation coupled with a high sucrose concentration to promote secondary metabolism (Su, 1995). This finding led to the concept of immobilization whereby cells could be maintained continuously for a period of several weeks under limiting growth conditions (Collin and Edwards, 1998). Cells were enclosed in an inert material, usually encapsulated as calcium alginate beads, or grown within the spaces of porous foam blocks. Cell-to-cell contact is maintained, throughout, in a culture medium, usually with reduced phosphate to limit adenosine triphosphate (ATP) biosynthesis and thus cell growth (Su, 1995), reduced nitrate and growth regulator levels, together with a high sucrose concentration. The medium was circulated throughout the bioreactor so that secondary products released into the medium can be removed, thereby avoiding any feedback inhibition. Bioreactors of this type can be of the **airlift** configuration or simply be a flat-bed where the cells are positionally fixed and medium circulated through the bed.

In spite of all the positive advantages immobilization offers there still remain problems with low productivity (Choi *et al.*, 1995), poor secretion of the accumulated product into the surrounding medium (Domenburg and Knorr, 1995) and genetic instability of the cells (Zhong *et al.*, 1995). To date, the technological feasibilities of large-scale plant cell culture remains confined to shikonin production from cell suspensions of *L. erythrorhizon*, berberine from cell cultures of *Coptis japonica* and ginseng together with saponin from suspensions of *Panax ginseng* (Domenburg and Knorr, 1995; Su, 1995). However, these latter processes utilised freely suspended cells in conventionally stirred tank reactors and it is still generally accepted that no immobilized plant cell processes are yet, commercially viable (Collin and Edwards, 1998).

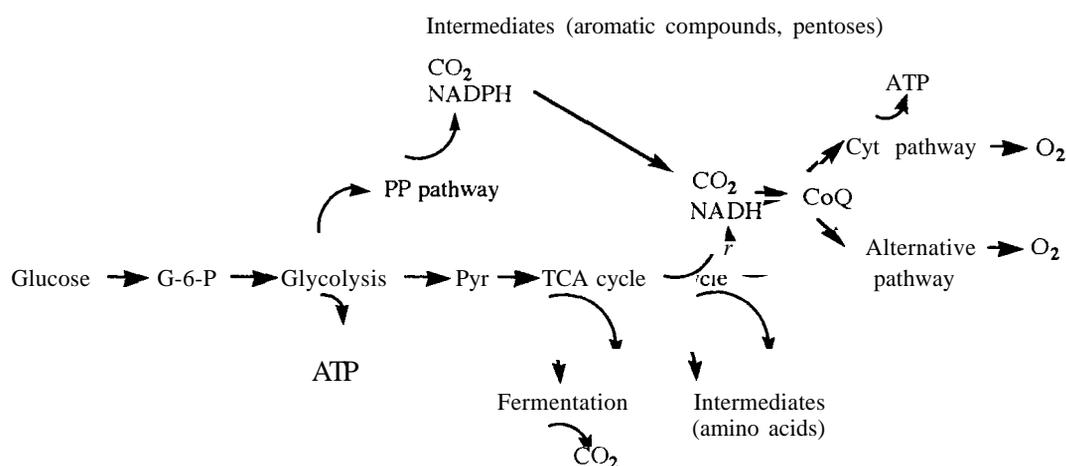
Differentiated tissues such as roots, shoots and somatic embryos exhibit an increased capacity for secondary metabolite production and are inherently more genetically stable than dedifferentiated cells. In this regard, large-scale hairy root culture technologies have recently been developed (Wysokinska and Chmiel, 1997). In contrast to plant cell suspension cultures, hairy root cultures do not require the addition of phytohormones for growth and are often characterised by high biosynthetic capacity. Furthermore, novel moieties, such as the indole alkaloid anthraserpine, in *Catharanthus trichophyllus* have been found in transformed roots and do not occur in the corresponding intact plant (Wysokinska and Chmiel, 1997).

Hairy roots are heterotrophic, so oxygen plays a critical role in their respiratory metabolism. Respiration in living cells is the oxidation of organic substrates usually glucose by specific metabolic pathways. Such substrates are partially oxidized to carbon dioxide concomitant with the release of free energy in the form of oxidative phosphorylation of ADP. There are two pathways available for the respiratory oxidation of glucose: glycolysis (Plaxton, 1996) and the pentose phosphate pathway [PPP; Mamushina *et al*, (1997)] (Fig 1.1). In the former case, glucose is enzymatically converted to pyruvate concomitant with substrate phosphorylation leading to ATP synthesis. Pyruvate is decarboxylated and the remaining acetyl moiety linked with coenzyme A to produce acetyl-CoA which enters the tricarboxylic acid cycle [TCA; Ernes and Neuhaus, (1997)] resulting in progressive oxidation of **substrates** to yield carbon dioxide and pairs of hydrogen atoms which, ultimately, react with oxygen to produce water. The combined actions of glycolysis and TCA result in the complete oxidation of glucose.

In contrast, the PPP does not result in the complete oxidation of glucose and is used by plant cells to produce the pentose sugar, ribulose-5-phosphate, which can be used for the biosynthesis of nucleic acids. This oxidative decarboxylation produces carbon dioxide and pairs of hydrogen atoms which are accepted by the coenzyme

nicotinamide adenine dinucleotide phosphate (NADP) to provide cellular reducing power in the form of NADPH. Thus, the end fate of glucose-6-phosphate (the substrate starting point of both glycolysis and PPP) is dependent on the cellular requirements of ATP, NADP/NADPH and intermediate products of both pathways, although glycolysis is the predominant pathway. However, both pathways ultimately merge to enter the cytochrome system or the alternative oxidase pathway (see Section 1.1).

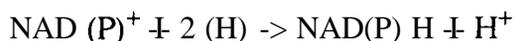
**Fig. 1.1 Biochemical pathways leading to electron transport routes in higher plant respiration<sup>1</sup>**



<sup>1</sup>Modified from van der Plas (1984). ATP, adenosine triphosphate; CO<sub>2</sub>, carbon dioxide; CoQ, coenzyme Q; Cyt, cytochrome; G-6-P, glucose-6-phosphate; NADH, nicotinamide adenine dinucleotide (reduced form); NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); O<sub>2</sub>, oxygen; PP, pentose phosphate; Pyr, pyruvate; TCA, tricarboxylic acid cycle.

The cytochrome system consists of a cytochrome chain (cytochromes a, b and c) along which electrons are passed to molecular oxygen which acts as a terminal acceptor to form water. Cytochromes are protein molecules with a haem prosthetic group, each having an iron atom at its centre. As electrons pass along the cytochrome chain, the iron is reduced to the ferrous state (Fe<sup>2+</sup>) and reoxidized to the ferric state (Fe<sup>3+</sup>) by receiving an electron from the preceding member of the chain. Concomitantly, the

pairs of hydrogen atoms derived from the TCA cycle, except for the succinate oxidation step of the cycle, are used to reduce the coenzymes NAD or NADP according to the reaction:



Oxidized coenzyme      Reduced coenzyme

Upon reduction the coenzymes takes up one hydrogen atom and one electron from the remaining hydrogen atom to leave a free proton ( $\text{H}^+$ ). Subsequently, the electrons are passed from the coenzymes to molecular oxygen via the cytochrome chain. Such passage of electrons along the chain provides the energy to facilitate the oxidative phosphorylation of ADP to form ATP and thus provide cellular energy. The resultant charged oxygen atom is then able to react with the free hydrogen proton and the proton derived from the reoxidation of the reduced coenzyme, to form water.

Provision of an adequate oxygen supply to cells (*in vitro* or *in vivo*) inside hairy root clumps can be impeded by external boundary layers and is exacerbated by the high external oxygen critical levels of 90-100% air saturation required for the growth of hairy roots (Yu and Doran, 1994). Kim and Yoo (1993) investigated the effects of agitation and aeration on the high density growth of carrot hairy roots and found that high hydrodynamic stress inhibited root growth, whilst a high volumetric oxygen transfer resulted in higher specific growth rate. Oxygen supply has also been shown to affect differentiation of embryogenic cell suspensions. High dissolved oxygen concentrations, similar to 60% air saturation, favoured undifferentiated biomass production of transformed *Eschscholtzia californica* cell suspensions at the expense of somatic embryo differentiation. In contrast, too low a dissolved oxygen concentration (similar to 5-10%) inhibited biomass and somatic embryo production (Archambault *et al*, 1994). Similarly, Jay *et al*. (1992) reported that a dissolved oxygen concentration of 10% inhibited carrot somatic embryo production by up to

75% compared to cultures maintained with a dissolved oxygen concentration of 100%.

Novel approaches to supply cultured hairy roots with a sustainable oxygen supply have thus demanded novel approaches in bioreactor concept and design to provide for optimal root growth and product extraction. These have involved growing transformed roots suspended above porous membrane tubing, as a supplementary aeration device, within the bioreactor (Kanokwaree and Doran, 1998). These workers reported that the use of combined air sparging and membrane tubing aeration in a gas-driven bioreactor increased biomass levels of *Atropa belladonna* hairy roots by 32-65% compared to sparging only with air or oxygen-enriched air delivered at the same total gas flow rate ( $0.6 \text{ l}^{-1} \text{ min}$ ).

## **1.2 Effects of the gaseous atmosphere on *in vitro* plant growth and development**

*In vitro* cultures are routinely maintained in semi-sealed vessels, such as glass jars and Petri dishes, which unintentionally restricts gaseous exchange between the culture vessel atmosphere and the external atmosphere. Growth and development of plants and tissues is not only dependent on medium composition but is affected by the gaseous environment within the vessel, which may differ **significantly** in terms of carbon dioxide and oxygen levels from the outside air, allowing accumulation of gaseous components such as carbon dioxide (and ethylene) produced by the **plants** and ongoing metabolism of the culture media components (Blazková *et al*, 1989). A culture medium giving optimal results in one type of culture vessel (and sealing) may fail to do so in another vessel. This indicates that the culture medium, the gas phase composition and the plants/tissues interact with each other in a complex and often poorly understood manner to form more or less favourable conditions for growth and differentiation. Factors that influence the accumulation of gases include the type of tissue (photosynthetic or non-photosynthetic) and amount of viable tissue biomass present, since any increase in tissue fresh weight will lead to increased levels of

photosynthesis and respiratory gaseous products. Additionally, the physical sealing properties of the container, composition of the culture medium, together with aspects of the macroclimate such as temperature and light intensity and quality, the latter two being particularly important in the photosynthetic rates of chlorophyllous tissues grown under photoautotrophic conditions, will all contribute to the composition of the gaseous environment. Therefore, manipulation or optimization of plant growth and development may be possible by controlling the gaseous composition in tissue culture vessels (Buddendorf-Joosten and Woltering, 1994). However, such manipulations are limited in that they do not permit regulation of the composition of the gaseous atmosphere within the culture vessel and hence do not provide a constant gaseous environment for plant growth and development. Culture chambers, with increased atmospheric carbon dioxide, have been used in the micropropagation of cultured shoots (Section 1.2.2), but this requires a gas-tight (sealed) environment. Alternatively gas mixtures, of known **composition**, can be passed through linked culture vessels, but this often introduces contaminants and can reduce humidity (Wardrop *et al*, 1997). The use of perfluorochemical (PFC) media supplements for controlled respiratory gas delivery to *in vitro* cultures provides a better option in avoiding such difficulties and will be discussed more fully in Section 1.4.

The Earth's atmosphere contains about 21% (v/v) oxygen and this is more than adequate to sustain plant respiration at as high a rate as other (limiting) factors will permit. However, this is not the case for *in vitro* tissue cultures where chlorophyllous (shoots, explants, plantlets) and **non-chlorophyllous** (calli, somatic embryos, hairy roots) tissues can be grown under heterotrophic, photoautotrophic (use of a sugar-free medium) or a combination of these conditions, photomixotrophy. For example, shoot cultures of cauliflower [*Brassica oleracea*, Kanechi *et al*, (1998)], gardenia [*Gardenia jasminoides* Ellis; Serret *et al*, (1996)] potato [*Solanum tuberosum* L.; Kozai *et al*, (1995)] and cell suspension cultures of mesembryanthemum [*Mesembryanthemum crystallinum*; Willenbrink and

Husemann, (1995)] have all been grown successfully under photoautotrophic conditions. In contrast, callus cultures of *Coleonema album* (Reil and Berger, 1997), *Digitalis lanata* (Hausmann *et al.*, 1997), *Petroselinum crispum* (Reil and Berger, 1996) and shoot cultures of tobacco [*Nicotiana tabacum* L.; Ticha *et al.*, (1998)] have demonstrated a preference for growth under photomixotrophic conditions.

In chlorophyllous cell/tissue culture, carbon dioxide concentrations within the culture vessel are altered due to respiration and photosynthesis. During the dark period, when photosynthesis is switched off, carbon dioxide concentrations increase due to respiratory metabolism of sugar, using the glycolytic and TCA pathways (Fig. 1.1). However, in the illuminated photoperiod, respiration can also proceed via a light dependent pathway which utilises the fixed carbon products of photosynthesis as substrates (Gillon and Griffiths, 1997). Indeed, under normal atmospheric concentrations of oxygen (21% v/v) and carbon dioxide (0.03% v/v), plants can lose up to half of their photosynthate via this pathway. The biological significance of this pathway is still unclear, but it is generally thought that it serves to protect the chloroplast pigments against photo-oxidation under conditions of high temperature and light intensity in which, more energy may be absorbed than can be used in carbon dioxide fixation. In contrast, under *in vitro* conditions, this respiratory pathway is suppressed by carbon dioxide concentrations much above 0.1% (v/v), which can be carried over from the preceding dark period, thus allowing photosynthesis to operate more efficiently. Moreover, photosynthesis proceeds at much higher rates than respiration, and thus the overall carbon dioxide concentrations within culture vessels decrease during the photoperiod. For example, Serret *et al.* (1997) reported that in the dark period, carbon dioxide concentrations within culture tubes containing axenic shoot cultures of gardenia (*Gardenia jasminoides*) accumulated to levels of *ca.* 0.09% (v/v) in loosely-closed tubes, whilst in tightly-closed tubes, the carbon dioxide accumulated to concentrations of 0.3-

0.8% (v/v). However, this carbon dioxide concentration was found to decrease to 0.01% (v/v) within 4 h of the initiation of the light period.

Despite the decrease in carbon dioxide concentration that occurs during the illuminated photoperiod, an overall increase in carbon dioxide concentration, over time, is often observed, to levels (< 1% v/v) that are generally considered toxic to plants maintained *in vivo*. Concomitantly, with such increases in carbon dioxide concentration within the vessels, there is usually an expected decrease in oxygen levels (Buddenddorf-Joosten and Woltering, 1994). Jackson *et al.* (1991) reported that oxygen concentrations in the gas phase of culture vessels, containing explants of *Ficus lyrata*, decreased to approximately 4% (v/v) in association with an increase of carbon dioxide to 30% (v/v). Furthermore, these workers proposed that such oxygen shortage was likely to have imposed anaerobic respiration within explant tissues, leading to fermentative metabolism in which carbonaceous sources are utilised as electron acceptors, resulting in lactic acid and ethanol accumulation. Similarly, changes in the gaseous composition of the vessel headspace were also observed in four cultivars of cherry (*Prunus avium*) over a 30 day culture period (Righetti and Pacini, 1992). In the case of two cultivars Victoria and Casavecchia, a large oxygen decrease was accompanied by increases in both carbon dioxide *and* ethylene to 19% (v/v) and 5  $\mu\text{l l}^{-1}$ , respectively. Such plants displayed leaf yellowing and tissue softening which was **correlated** to photosynthetic incapability and respiratory stress as measured by acetaldehyde and ethanol production by the tissues. However, with Bigarreau Moreau and Bigarreau Burlat, there was a slight increase in oxygen concentration to 22-24% (v/v) and a decrease in carbon dioxide. This suggested that the function **and** efficacy of the metabolic pathways differed between genotypes within the same species.

### **1.2.1 Beneficial effects of oxygen enrichment of the culture environment**

Oxygen availability during *in vitro* culture of plant cells and tissues is known to be a growth limiting factor in a number of cells/tissues and several species, such as root tips of maize [*Zea mays* L.; Saglio *et al.*, (1984)] and rape protoplasts [*Brassica napus* L.; Brandt, (1991)], particularly when such cells and tissues are cultured statically in liquid medium. The growth of non-photosynthetic cells and tissues under aerobic conditions is dependent on the provision of an adequate supply of oxygen for the production of ATP through oxidative phosphorylation (Van der Plas and Wagner, 1986). Growth and respiration of dedifferentiated tissues may be restricted due to the lack of gaseous exchange within the cell mass and has been demonstrated in a number of species including *Catharanthus roseus* (Tate and Payne, 1991), wheat [*Triticum aestivum* L. cultivar Banks; Adkins, (1992)] and rice [*Oryza sativa* L. cultivars IR42, Khao Dawk Mali 105, FR13A and Kurkaruppan; Adkins *et al.*, (1993)]. In the latter study, manipulation of the culture atmosphere using sterile air was shown to increase cell biomass of all four rice cultivars by 60% in comparison to cells exposed to an enriched carbon dioxide (8% v/v) atmosphere. Similarly, Van der Plas and Wagner (1986) reported that this problem of impaired oxygen diffusion to cells could be overcome by culturing (potato) calli of cultivar Bintje in a 70% (v/v) oxygen atmosphere. This approach of the use of an enriched atmosphere has also been exploited to improve somatic embryo production in alfalfa [*Medicago sativa* L. clone RA-3; Stuart *et al.*, (1987)] and in wheat (Carman, 1988).

Studies have also demonstrated that the mitotic activity of protoplasts of rice cultivar Taipei 309, tomato (*Lycopersicon esculentum* Mill. cultivar Santa Clara) and jute (*Corchorus olitorius* variety D154) could be enhanced by culture in an oxygen-enriched atmosphere (d'Utra Vaz *et al.*, 1992). Additionally, plant regeneration frequencies of rice protoplast-derived colonies could also be improved by exposure to a high oxygen atmosphere. Shoot regeneration efficiency of rice suspension cells was also improved threefold, when cells were cultured in a bioreactor with a 40%

(v/v) oxygen atmosphere in comparison to cultures supplied with air alone (Okamoto, 1996).

Recently, attention has focused on the regulation of oxygen supply to cultured cells and tissues of species that produce commercially important secondary metabolites. For example, production of the alkaloid, ajmalicine, was more than five times higher in cell suspensions of *Catharanthus roseus* cultured with a dissolved oxygen concentration of 85% (v/v) air saturation as opposed to cultures grown with an oxygen concentration of 15% (v/v) (Schlatmann *et al.*, 1994). A more pronounced effect of oxygen regulation on biosynthetic pathways was observed in high density root cultures of *Duboisia myoporoides* that produced both nicotine and tropane alkaloids. *Duboisia* roots cultured in air produced equal amounts of both alkaloids. However, when roots were cultured in pure oxygen, the metabolic flux to tropane alkaloids increased, whilst that to nicotine alkaloids simultaneously decreased (Yukimune *et al.*, 1994). The tropane alkaloids hyoscyamine and scopolamine are important compounds that are used as spasmolytics and anaesthetics, whereas nicotine is toxic and can be considered an undesirable by-product. Therefore, suppressing nicotine production is highly desirable in the context of industrial production and downstream processing of tropane alkaloids.

### **1.2.2 Beneficial effects of carbon dioxide enrichment of the culture environment**

Recent research has revealed that cauliflower shoot cultures [*Brassica oleracea*, Kanechi *et al.*, (1998)], tobacco [*Nicotiana tabacum* L.; Ticha, (1996)] and regenerating rice calli (Seko and Nishimura, 1996) had outstanding photosynthetic ability and sometimes grew better under photoautotrophic than hetero- or photomixotrophic conditions when the culture environment was properly controlled to maximise photosynthesis (Kozai *et al.*, 1997). Carbon dioxide levels in the gaseous environment of such *in vitro* grown plants decreased sharply during the photoperiod and thus may have reached the compensation point within a few hours,

thereby limiting the net photosynthetic rate (NPR). Thus, carbon dioxide enrichment under relatively high light intensities ( $150\text{-}200\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ ) can be used to promote photosynthesis and thus growth of photoautotrophically cultured plants *in vitro*.

Carbon dioxide enrichment of *in vitro* plant cultures can be mediated by placing culture vessels in a growth chamber with an elevated carbon dioxide concentration (Ticha, 1996). This approach relies on passive diffusion of gas into the vessels and results in an increased carbon dioxide concentration in the culture atmosphere. An alternative method requires flushing gas mixtures (of known composition) through the culture vessels in a continuous flow system of forced ventilation (DolcetSanjuan *et al.*, 1997) or by injection of gas directly into the vessel at regular intervals (Infante *et al.*, 1989). The beneficial effects of carbon dioxide enrichment on plant cell cultures are shown in Table 1.1.

A further advantage of carbon dioxide enrichment during culture is that it serves to promote net photosynthesis and prepare the plant for *ex vitro* acclimation (Kanechi *et al.*, 1998). In many cases this can improve plant growth and survival after transfer from *in vitro* culture to soil, since it circumvents the stress exerted when plants are deprived of sucrose, which was previously available. Furthermore, carbon dioxide enrichment may significantly shorten the acclimation period (Desjardins *et al.*, 1990). For example, Laforge *et al.* (1991) reported that *in vitro* treatment with a carbon dioxide enriched atmosphere enhanced the performance of raspberry (*Rubus idaeus* L.) micropropagated plantlets and reduced the acclimation period by 2 weeks.

**Table 1.1 Beneficial effects of carbon dioxide enrichment  
on *in vitro* plant growth**

Species	Response	Reference
<i>Actinidia deliciosa</i>	Increased NPR <sup>1</sup>	Infante <i>et al.</i> (1989)
<i>Brassica oleracea</i>	Increased NPR <sup>1</sup> , Promoted acclimation	Kanechi <i>et al.</i> (1998)
<i>Chrysanthemum</i> (unspecified species)	Increased growth (dry weight)	Cuello <i>et al.</i> (1992)
<i>Dianthus caryophyllus</i>	Increased growth	Solarova and Pospisilova (1997)
<i>Eucalyptus camaldulensis</i>	Increased <b>growth</b> , Increased NPR <sup>1</sup> , Promoted <b>acclimation</b>	Kirdmanee <i>et al.</i> (1995)
<i>Nicotiana tabacum</i>	Increased growth (dry weight)	Ticha (19%) Solarova and Pospisilova (1997)
<i>Pistacia vera</i> , <i>P. attartica</i> <b>x inttegerrima</b>	Increased <b>growth</b> , Promoted <b>acclimation</b>	Parfitt and Almehdi (1994)
<i>Rosa hybrida</i>	Increased chlorophyll <b>content</b> , Inhibition of senescence	Woltering (1990)
<i>Solanum tuberosum</i>	Increased growth (fresh weight)	Buddendorf-Joosten and Woltering (19%)
<i>Vitis vinifera</i>	Increased <b>growth</b> , Promotion of adult morphology	Fomieux and Bessis (1993)

<sup>1</sup>NPR: Net photosynthetic rate

Additionally, a carbon dioxide enriched atmosphere may serve to inhibit *in vitro* ethylene production and accumulation within culture vessels. This gaseous by-product is known to adversely affect growth and development in many plant species (Kevers *et al.*, 1992). For example, with shoot cultures of *Ficus lyrata*, ethylene accumulation was accompanied by a decrease in leaf area and increased callus formation at the expense of shoot proliferation (Buddendorf-Joosten and Woltering,

1994). In contrast, in some *in vitro* cultures, where carbon dioxide enrichment has beneficial effects on plant growth, ethylene concentrations can also increase without any detrimental effect on culture development (Figueira and Janick, 1994).

### **1.3 Comparison of plant and animal cell culture systems**

Animal cell cultures are becoming increasingly useful as tools for the production of highly valued biological products, such as viral vaccines, hormones, growth factors, enzymes and monoclonal antibodies (Wu, 1995). The range of cultured animal cells extends to fibroblasts, endocrine cells (e.g. pancreatic and pituitary cells), melanocytes, and many different types of tumour cells. As in cultured plant cell systems, cells can be cultured as free cell suspensions or interactively with a matrix. Culture is dependent on a number of factors, such as nutritional requirements like selenium or calcium ions, hormones and cell density, all of which can affect cell differentiation and cell proliferation, often adversely (Freshney, 1992). Additionally, dissolved oxygen tension and oxygen uptake rates are critical parameters in such cultures (Palomares and Ramirez, 1996).

Unlike cultured plant cells, animal cells lack the presence of a protective cell wall and are generally more sensitive to fluid mechanical stresses (Namdev and Dunlop, 1995). Interestingly, the latter workers demonstrated that plant protoplasts behaved analogously to animal cells when sheared under defined conditions. Although **animal** cells can be cultured statically as supported monolayers, thereby eliminating fluid mechanical stresses, cell suspension growth in mechanically agitated and aerated bioreactors is more suitable for large-scale production. Sparging is the most convenient method of supplying oxygen to the culture medium in large scale animal cell bioreactors, but unfortunately this can damage cells through cell-bubble interactions and bubble rupture at the free air/liquid surface (Michaels *et al*, 1996). In both animal and plant suspension cultures, sparging can often lead to foaming which necessitates the need for the addition of antifoaming agents. Such additives

can reduce surface tension and act as an additional barrier to gaseous transfer by concentration of molecules at the gas-liquid interface. In animal cell systems, surface active polymers, such as *Pluronic*<sup>®</sup> F68 have been used to stabilise surface foams, thereby reducing the exposure of cells to damaging bubble rupture and coalescence (Zhang *et al*, 1992; Nienow *et al.*, 1996). Cells are further protected against shear stress and bubble damage by hydrophobic binding of the surface active molecules to the cell membranes conferring increased membrane stability (Wu, 1996). In contrast, plant cell systems are more tolerant of such stresses and this negates the need for the addition of surfactants to the culture medium in this context. Therefore, the use of spargers is only limited to animal cells that are unaffected by the presence of bubbles or the addition of surfactants (Moreira *et al*, 1995).

Although most plant and animal cells have lower oxygen requirements compared with microbial systems, oxygen demand increases significantly with scale-up and higher cell densities. In animal cell systems, monoclonal antibody production rate has been found to be a strong function of the viable cell density, increasing with raised cell density (Banik and Heath, 1995). Similarly, in large-scale plant cell suspension cultures, maximum production of secondary metabolites occurs towards the end of the exponential growth phase, which also **correlates** with the highest cell density.

Immobilization of plant and animal cells for use in bioreactors avoids the problem of shear stress and enables cells to be cultured at high densities. In addition, exchange of culture medium is simplified and extracellular products are easily recovered on a continuous basis (Tyler *et al*, 1995). However, cells are dependent on diffusion of nutrients and transport limitations can cause nutrient concentrations and oxygen transfer to decrease towards the centre of the immobilized cell layer (Riley *et al*, 1997).

In order to overcome such oxygen limitations in animal cell culture, efforts have focused on the use of media supplements that are capable of acting as oxygen carriers. These novel approaches have utilised perfluorocarbon and haemoglobin-based technologies and are discussed in Sections 1.4 and 1.5, respectively.

## **1.4 Perfluorochemicals (PFCs) and cell biotechnology**

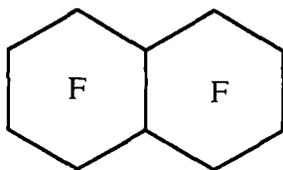
### **1.4.1 Introduction**

Perfluorochemical liquids have properties, especially high gas solubility, which make these compounds extremely useful in medicine and biotechnology. Such properties are potentially particularly relevant to plant tissue culture since limited respiratory and photosynthetic gas supply can adversely effect plant cell and tissue growth during culture. PFC-facilitated improvements in cell culture technology will have increasingly important biotechnological implications in the context of plant micropropagation, somatic hybridisation, transgenic plant production and commercial exploitation of these technologies.

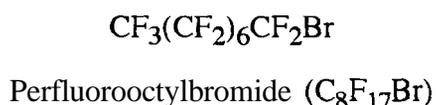
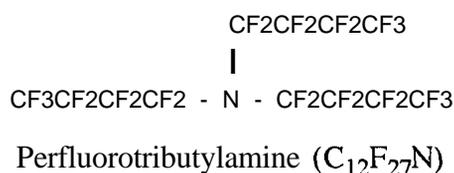
### **1.4.2 Properties of PFC liquids**

PFCs are linear, cyclic or polycyclic hydrocarbons in which hydrogen atoms have been replaced, in general, with fluorine. Some commonly used compounds, such as perfluorotripropylamine (FTPA) or perfluorooctyl bromide (perflubron), also contain residual nitrogen or bromine atoms. PFCs are available as a wide range of molecular configurations as shown in Fig. 1.2 and have exceptional chemical and thermal stability, endowed by the strength of the carbon-fluorine bond (*ca.* 480 kJ mol<sup>-1</sup>). This carbon-fluorine interaction results in the intramolecular carbon atoms being sterically protected by the surrounding water excluding fluorine atoms (Riess and Le Blanc, 1982). Hence, the hydrophobic nature of PFC liquids renders them immiscible and insoluble in aqueous systems. Consequentially, they should not be confused with volatile chlorofluorocarbons (CFCs) that can release highly-reactive chlorine atoms into the stratosphere and which damage the Earth's ozone layer.

**Fig. 1.2 Examples of the chemical structure of PFCs**



Perfluorodecalin ( $C_{10}F_{18}$ )



PFCs were first produced during the Second World War in the search for materials that were resistant to attack by the highly reactive uranium isotopes being produced for the first atomic bomb (Lowe, 1997). The perfluorocarbon manufacturing process can be performed through a number of industrial methodologies. Fluorination of hydrocarbons was originally carried out by either an electrochemical fluorination reaction (Simons *et al*, 1949) or by treating vapourised hydrocarbons with high valency metal fluorides, such as cobalt trifluoride. Today, this latter process has been further refined and involves introducing hydrocarbons and fluorine gas simultaneously into a fluidised bed reactor filled with cobalt fluoride (May, 1997). The resulting PFCs are invariably mixtures of unpredictable composition, due to incomplete fluorination, and require a purification step to remove all traces of hydrocarbons, hydrofluorocarbons and hydrogen fluoride.

**Table 1.2 Solubility of respiratory gases in PFC liquids.**

<u>Respiratory Gas</u>	<u>Solubility<sup>1</sup></u>
N <sub>2</sub>	24 - 32
O <sub>2</sub>	35-44
CO <sub>2</sub>	123-203

<sup>1</sup>values are mmol l<sup>-1</sup> at Standard Temperature (0°C) and Pressure (1.013 bar) (STP).

An unique attribute of PFC liquids is that they dissolve large volumes of respiratory gases (Table 1.2), gas solubility increasing linearly with partial pressure approximating to Henry's Law. The gas solubilities in PFC liquids decrease CO<sub>2</sub> » O<sub>2</sub> > CO > N<sub>2</sub> > H<sub>2</sub> > He, in accord with the decrease in molecular volume of the solute. Gas molecules are thought to occupy 'molecular cavities' within liquid PFCs without chemical reactions being involved.

### **1.4.3 Biomedical applications of PFCs**

The ability of PFC liquids to dissolve respiratory gases has inevitably attracted the interest from clinicians and biotechnologists over the past three decades. This included detailed assessments of PFCs for improving gaseous supply *in vivo*. One of the early and classical biological studies with PFCs was the report of Clark and Gollan (1966), whereby mice could survive for extended periods by "breathing" when immersed in an oxygenated PFC liquid. Subsequently, wide-ranging research has investigated the role of PFCs and their emulsions, as respiratory gas-carrying fluids for intravascular and respiratory applications (Lowe, 1997; Scott *et al*, 1997; Frietsch *et al*, 1998; Sedova *et al*, 1998). PFC emulsions with small droplet diameters (*ca.* 0.2 μm diameter or less) were essential for intravascular use, because PFC liquids themselves are immiscible with aqueous systems, including blood. Furthermore, when PFCs are used as pure chemicals, for intravascular injection,

these dense water-immiscible liquids may cause circulatory abnormalities and embolisms (Waschke *et al*, 1997).

PFC emulsion formulations for intravascular use contain a number of additional compounds, primarily a PFC, but up to 90% (w/v) and a surfactant, such as the polyoxyethylene-polyoxypropylene co-polymer, Pluronic F68 (poloxamer 188), lecithins or a fluorinated surfactant (Riess, 1994; Riess and Weers, 1996; Krafft and Riess, 1998) which are emulsified in an isotonic solution. Emulsification can be performed by ultrasonication which can lead to PFC degradation and fluoride ion release (Riess and Le Blanc, 1982) or by the more favoured process involving high pressure homogenisation (Riess and Le Blanc, 1982). Recent progress in emulsification techniques have incorporated egg yolk phospholipids as a replacement for surfactants, which were unstable and had adverse side effects when used as injectable oxygen carriers (Krafft and Riess, 1998). Consequently, it is not surprising that PFC emulsions have been described as blood substitutes, red cell substitutes (Goodnough *et al*, 1998; Fratantoni, 1999; Winslow, 1999) and oxygen-carrying macromolecules (Minato and Nose, 1992; Nose, 1998). Blood substitutes are also convenient for on-site rescue of trauma victims, support during transfer to hospitals and providing necessary transfusions to patients who refuse blood for religious reasons (May, 1997).

Furthermore, PFCs are not metabolized by the body and are excreted via the lungs, urine and faeces. They are minimally absorbed by the lungs when inhaled and are almost exclusively excreted by the lungs, either primarily or after passage through the reticuloendothelial system, through exhalation (Sakas *et al.*, 1996). The body retention time and rate of excretion is primarily dependent on the molecular weight of the specific PFC. However, for artificial oxygen carriers to become clinically acceptable, a number of criteria must be fulfilled and these are summarised in Table 1.3.

**Table 1.3 Desirable characteristics of PFCs for biomedical application<sup>1</sup>**

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•	Efficacy under physiological conditions
•	Sterility and no disease transmission
•	No toxicity and adverse dmg reactions
•	No immunogenicity
•	Physiological pH, osmolarity and oncontic pressure
•	Sufficient intravasular retention time
•	Prolonged shelf life and ease of storage
•	Universal compatibility
•	<u>Reasonable costs</u>

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<sup>1</sup>Modified from Waschke *et al* (1997)

PFCs are not only confined in their use as red blood cell substitutes and have been exploited in many other medical fields (Table 1.4). For example, PFC based emulsions have been used in the perfusion and preservation of isolated tissues and organs, prior to transplantation (Voiglio *et al*, 1996). In cancer therapy, PFC emulsions have been employed to overcome hypoxia, which can protect cancer cells, within tumours, to the cytotoxic effects of radiotherapy and some chemotherapies (Teicher, 1995). PFCs can also replace eye fluid during surgery since they are injectable, immiscible in water and are optically clear (Ong *et al*, 1993). PFCs are a diverse (liquids, waxes, solids) group of compounds which have properties that make them extremely relevant to cutting edge research and development in medicine and cell biotechnology.

**Table 1.4 Applications of PFCs in biomedical sciences<sup>1</sup>**

<u>Perfluorochemical</u>	<u>Primary application</u>	<u>Reference</u>
<b>Perfluorodichlorooctane</b>	Blood substitute	Rosoff <i>et al.</i> (1998)
Perfluorotributylamine	Myocardial protection against tissue ischemia	<b>Scheule <i>et al.</i></b> (1997)
<b>Fluosol</b>	Tumour treatment	Campbell <i>et al.</i> (1990) <b>Evans <i>et al.</i></b> (1993)
Perfluorooctylbromide	Ventilatory support	Leonard (1998)
<b>Perfluorooctane</b>	<b>Vitreoretinal</b> surgery	<b>Moreira <i>et al.</i></b> (1997)
<b>Perfluorophenanthrene</b>	<b>Vitreoretinal</b> surgery	<b>Wilson <i>et al.</i></b> (1992)
Perfluoroperhydrophenanthrene	Vitreoretinal surgery	Batman and <b>Cekic</b> (1998)
Perfluorodecalin	Vitreous humour substitute during surgery	MalchiodiAlbedi <i>et al.</i> (1998)
<b>Fluosol</b>	Organ transplantation	Frietsch <i>et al.</i> (1998)
Perfluoroalkylethene	Contrast agent in radiographic diagnosis	Sanchez <i>et al.</i> (1994)
Perfluorooctylbromide	<b>Immunohistochemical</b> labelling of cultured dorsal root <b>ganglion cells</b>	<b>Meller <i>et al.</i></b> (1998)
<u>Perfluorooctylbromide</u>	Blood substitute	<b>Habler <i>et al.</i></b> (1998)

<sup>1</sup>Modified from Sakas *et al.* (1996)

#### **1.4.4 Applications of PFCs to cell biotechnology**

PFCs are valuable in both animal and microbial cell culture systems for regulating gas supply, leading to improved growth (King *et al.*, 1989; Van Sonsbeek *et al.*, 1993). In addition, such use of PFCs can also decrease mechanical damage to cells caused by conventional aeration through sparging or stirring. This is significant, not only in basic research, but, more importantly, in those commercially-orientated laboratories which utilise large-volume fermentation vessels. The principal benefits of employing PFC liquids in cell culture systems are given in Table 1.5.

**Table 1.5 Benefits of PFCs in cell culture systems**

- 
- Chemically and biologically inert
  - Ease of sterihsation
  - Recoverable and recycleable
  - Respiratory gas solubility
  - Scavengers of gaseous cellular products
  - Interface and physical support for cell division
- 

#### **1.4.4.1 Biocompatibility of PFCs in fermenter systems**

PFCs have been exploited in microbial culture systems to increase oxygen transfer under fermentation conditions (King *et al.*, 1989). In one study employing a bioreactor, PFC liquid (FC-40; 3M Company, USA) coalescing at the bottom of the culture vessel was re-cycled through an external oxygenater and returned to the container (Van Sonsbeek *et al.*, 1993). More recentiy, Jia *et al.* (1997) demonstrated that the addition of 3% (v/v) PFC to the fermentation broth of cultured yeast cells, *Saccharomyces cerevisiae* increased cell growth by 20% above that of fermentation in the absence of the oxygen vector. PFCs have also been emulsified into droplets, (ca. 20-50  $\mu\text{m}$  in diameter), by mechanical agitation within the fermenter, thus increasing the surface area for gaseous exchange (McMillan and Wang, 1987). PFC emulsions of increasing disperse phase fraction, have also been utilised to increase the oxygen transfer coefficient in bioreactor cultures of *Escherichia coli* (Ju *et al.*, 1991; Martin, 1995).

Recentiy, Elibol and Mavituna (1995; 1997) described the use of perfluorodecalin  
⑤  
(*Flutec* PP6; *Fl* Chemicals Ltd., Preston, UK) as an oxygen carrier in cultures of *Streptomyces coelicolor*, a fungus synthesising the antibiotic, actinorhodin. Significantly, biomass and actinorhodin production were both enhanced when the PFC was added up to 50% (v/v) to shake-flask cultures. Emulsifying the PFC with the polyoxyethylene-polyoxypropylene co-polymer surfactant, *Pluronic*® F-68

(poloxamer 188), also resulted in a significant increase in antibiotic production, without affecting microbial growth. In a similar study with the carbon dioxide-fixing microbe, *Alcaligenes eutrophus*, Yamamoto *et al* (1994) used a mass transfer model to evaluate growth in the presence of perfluorotributylamine (FTBA). This study demonstrated a linear relationship between the volume of added PFC to a stirred bioreactor containing a fixed volume of inoculated liquid culture medium and the diameter of the aqueous medium droplets under agitated conditions (400 r.p.m.). As the volume of PFC was increased, there was a concomitant decrease in the size of the aqueous droplets, thereby increasing the PFC/medium interfacial area available for oxygen transfer. Additionally, the size of the gas bubbles was also found to decrease with increased PFC addition, furthering the gaseous exchange rate between the gas bubbles and the PFC phase. The beneficial effect of these increased oxygen transfer rates was reflected by an enhanced microbial growth rate.

Novel applications of PFC emulsions include the use of human immunoglobulin G (IgG) immobilized poly(vinyl alcohol)-stabilized liquid perfluorocarbon droplets to selectively identify and remove subpopulations of cells from a heterogeneous culture (McCreath and Chase, 1996). This affinity emulsion was exploited to rapidly adsorb *Staphylococcus aureus* cells from a mixed suspension culture of *Saccharomyces cerevisiae* and *S. aureus* cells. Such an approach offers an alternative to conventional immunological methods which utilise antibodies or other recognition molecules immobilized to magnetic polystyrene beads.

A further and innovative use of PFC emulsions involves supplementation for cultures of *Pseudomonas denitrificans*. This bacterium reduces nitrogen oxides to nitrogen, the former are gaseous industrial waste products, responsible for acid rain and stratospheric ozone damage (Turick and Bulmer, 1998). In this study, nitrous oxide reduction and nitrogen production increased 2-3 fold when bacteria were cultured in

the presence of a 10% (v/v) PFC emulsion, concomitant with increases in bacterial growth rates.

#### **1.4.4.2 Animal cell cultures**

Several studies have utilised PFCs to facilitate oxygen supply to cultured animal cells. For example, Cho and Wang (1988) used perfluoro-(methyldecalin) (*Flutec*<sup>®</sup> PP11; F2 Chemicals Ltd.) to oxygenate mouse hybridoma cells, whilst Ju and Armiger (Ju and Armiger, 1992) employed emulsified *Fluorinert*<sup>®</sup> FC-40 (3M Company Ltd.) to enhance oxygen supply to monoclonal antibody-producing hybridoma cells cultured in surface-aerated, rotating tubes. In the latter paper, it was reported that an increase in the density of viable cells **occurred** when the culture medium was supplemented with 10% (v/v) of emulsion. In bioreactor cultures of the same cells, there was also evidence for protection of cells against mechanical damage by emulsion. Unfortunately, the surfactant used to prepare the emulsions was not specified, making it difficult to determine the specific component responsible for such cell protection. However, several commonly-used surfactants, particularly *Pluronic*<sup>®</sup> F-68, are known to protect insect and mammalian cells in culture, probably through the interaction of such compounds with components of cytoplasmic membranes (Lowe *et al.*, 1993). It is feasible that similar, surfactant-mediated cytoprotection operated in the study of Ju and Armiger (1992).

#### **1.4.4.3 PFCs and carbon dioxide**

PFCs have been used to supply carbon dioxide to the gangrene-causing bacterium, *Clostridium perfringens* (Ceschlin *et al.*, 1985). This suggested possible applications for PFCs in anaerobic, as well as aerobic, culture systems. The chemical and biological inertness of PFC liquids, coupled with their high gas solubility (Table 1.2), have made them ideal compounds for evaluating gas transfer dynamics in cultures of obligate anaerobic organisms. In contrast, PFC emulsions have also been employed to facilitate removal of carbon dioxide from the fermentation broth of the

strict anaerobic bacterium, *Clostridium acetobutylicum* (Percheron *et al*, 1995). This latter study utilised the anaerobic bacterium, as a model system, since anaerobic conditions produce significant levels of carbon dioxide partial pressure without being disturbed by oxygen supply. Such use of PFC emulsions could now be applied to aerobic fermentations, since carbon dioxide accumulation in these systems can cause enzyme inhibition and cell lysis.

Recently, perfluorodecalin (*Flutec*<sup>®</sup> PP6; F2 Chemicals Ltd.) has been used to regulate the supply of carbon dioxide to axenic shoots of *Rosa chinensis* (Wardrop *et al*, 1997b). The culture of shoots in semi-solid medium overlaying carbon dioxide-gassed PFC increased shoot biomass and root production. The growth of adventitious roots induced on cultured rose shoots was often inhibited by the production of phenolic compounds, but, interestingly, PFC treatment reduced significantly the accumulation of these compounds. Furthermore, PFCs have been employed in biological systems as scavengers of toxic gaseous by-products, such as ethylene (Lowe *et al*, 1998) and reactive oxygen species (Bekyarova *et al.*, 1996; Rossman *et al*, 1997).

#### **1.4.4.4 PFCs and physiological oxygen monitoring**

Perfluorinated alkyl amines, such as FTPA, can be detected readily by <sup>19</sup>F nuclear magnetic resonance (NMR) spectroscopy and their <sup>19</sup>F relaxation rates ( $1/T_1$ ) vary linearly with oxygen concentration (Thomas *et al*, 1994; Shukla *et al*, 1995). McGovern *et al.* (1993) assessed the oxygen status of tumour cells immobilised in a gel matrix incorporating FTPA and compared their findings with theoretical predictions. They concluded that PFCs were valuable, not only as oxygen carriers, but also as physiological oxygen sensors.

#### 1.4.4.5 Culture of cells at PFC-aqueous interfaces

Mammalian cells, including human foreskin fibroblasts, hepatocytes, retinoblastomas and malignant melanoma (A375) cells have been cultured for extended periods at the interface formed between PFC liquid and aqueous medium (Keese and Giaever, 1983; Nabih *et al*, 1989; Shnyra *et al*, 1990; Terada *et al*, 1992, Mathy-Hartert *et al*, 1997). Such an interface provides support for anchorage-dependent cell growth, additional to any alterations in oxygen supply. Recently, this approach has been extended to the culture of murine hybridoma cells which synthesise IgG antibody (Lowe *et al*, unpublished results, 1996).

In some two-phase, medium-PFC systems, the cultured cells did not grow directly on the PFC, but on a layer of protein that formed spontaneously at the interface that formed between the two fractions. The protein forms a rigid substrate to withstand the forces exerted by the cytoskeletons of the spreading cells. The formation of such a protein monolayer is believed to be stimulated by residual hydrogen-containing impurities present in commercial grade PFCs. The production of a protein monolayer suitable for cell growth can also be induced by the addition of small quantities (*ca.*  $2 \mu\text{g m}^{-1}$ ) of pentafluoro-benzoyl chloride to alumina-purified PFCs (Keese and Giaever, 1983, 1991). Similarly, Rappaport *et al* (1996) reported that anchorage-dependent HeLa cells could be cultured as monolayers, with more than 19 layers of cells, when overlaid onto oxygen-saturated perfluorodecalin. This represented a significant improvement on the conventional use of gas permeable membranes and hollow fibres perfused with oxygenated medium. In the latter cases, cells could only be grown as monolayers to a depth of 2-3 cell layers.

Sanfilippo *et al.* (1988) reported that both oncogene-transformed and non-transformed mammalian fibroblast cells proliferated on a PFC-aqueous interface, provided that appropriate growth regulators were included in the culture medium. These authors emphasised that the two-phase culture system may be superior to

conventional approaches, such as the culture of cells in soft-agar media [0.3% (w/v)] for monitoring the growth of anchorage-independent cells because of the ease of cell plating, the ability to recover cells and secreted products from the PFC-aqueous interface and upper aqueous phase, respectively. An additional advantage of this two-phase system is the shorter growth period required (3-4 days) compared with 10-14 days for routine soft-agar assays.

An extensive investigation of the morphology and proliferation of dermal skin fibroblasts, cultured at interfaces formed between various PFC liquids and aqueous culture medium, was subsequently performed by Sparrow and co-workers (Sparrow *et al*, 1990). Again, cell growth was significantly improved in the presence of commercial grade PFCs, compared with alumina-purified preparations. While PFCs containing trace impurities may be preferable as support/gas regulating systems for cultured animal cells, paradoxically, such materials may be unsuitable for *in vivo* use.

Moussy *et al.* (1990) used the fluorocarbon, FC721 (3M Company Ltd.), to study the factors affecting the force of detachment of human umbilical cord endothelial cells from various substrates. Subsequently, Keese and Giaever (1991) extended this work to evaluate the relationships between cell spreading and substrate mechanics by utilising FTBA-aqueous culture medium interfaces coated with protein films. In a more comprehensive investigation, Ando *et al* (1991) reported that endothelial cells grew more readily on PFC-medium interfaces coated with collagen or fibrinogen, rather than laminin, fibrinectin or gelatin. These studies emphasised the usefulness of **PFC-supplemented** aqueous systems for studying aspects of cell-matrix interactions.

#### **1.4.4.6 Fluorocarbon polymers in cell cultures**

Several studies have demonstrated that fluorocarbon polymers have beneficial effects in both animal and plant cell culture systems. Schneider *et al* (1995) described the use of polytetrafluoroethylene (PTFE; *Teflon*<sup>TM</sup>) membranes, which possess high gas permeability, for oxygenating animal hybridoma cells. These membranes reduced significantly, the mass transfer surface (surface area for bubble-free oxygen diffusion to culture media), compared to aeration with silicone tubing. In related experiments, Fukui and Tanaka (1995) reported that culture vessels made from fluorocarbon polymer films (*Neoflon* PFA<sup>®</sup>, Daikin Industries, Japan), also possessing high gas permeability, could be used to culture cells of *Nicotiana tabacum* and *Lithospermum erythrorhizon* cells without the use of potentially damaging agitation.

### **1.5 Haemoglobin-based oxygen carriers and cell biotechnology**

#### **1.5.1 Recent history**

Haemoglobin (Hb) exists widely in organisms ranging from prokaryotes to eukaryotes. It has been found in a number of plant species, most notably dicotyledonous leguminous plants, such as cowpea [*Vigna unguiculata*; Arredondo-Peter *et al.*, (1997)], broad bean [*Vicia faba*], Fruhling *et al*, (1997)] and the actinorhizal plant *Alnus glutinosa* (Suharjo and Tjepkema, 1995) engaged in symbiotic relationships with nitrogen-fixing bacteria. In legumes, the symbiotic Hbs function by facilitating the flow of oxygen necessary for the **energy-generating** respiration by the nitrogen-fixing bacteria, yet at sufficiently low concentrations that do not inactivate the oxygen-intolerant nitrogen reducing enzyme complexes (Nie and Hill, 1997). Plant haemoglobins have a common genetic origin with animal and microbial haemoglobins (Appleby, 1992). This was confirmed when protein and gene structure studies demonstrated conclusively that this Hb, now referred to as leghaemoglobin, was indeed a member of the broader animal haemoglobin family and is one of the most-cited examples of horizontal gene transmission, possibly via a viral vector, some 1500 million years ago (Appleby *et al*, 1990).

Native human Hb consists of a protein component (globin) comprising four polypeptide chains. This tetrameric molecule is composed of two dimers of  $\alpha$  and  $\beta$ -subunits. An  $\alpha$  subunit binds strongly to a  $\beta$  subunit, creating a dimer; the two  $\alpha$ - $\beta$  dimers then bind weakly to each other resulting in the tetramer. Each sub-unit is associated with a porphyrin derivative (heme) containing one atom of ferrous iron ( $\text{Fe}^{2+}$ ). Each iron atom can rapidly and reversibly bind with one oxygen molecule, but the reaction is oxygenation rather than oxidation, since the redox status of the iron remains unchanged (Azhakanandam *et al*, 1997), each molecule of Hb can thus bind to four molecules of oxygen.

When Hb is removed from red blood cells, there is a tendency for the Hb molecule to dissociate into di- and monomer subunits. Additionally, the ferrous atom becomes susceptible to oxidation and a proportion of the haemoglobin undergoes conversion to the ferric state. This conversion results in the formation of a small quantity of methaemoglobin, a form of Hb that does not bind oxygen and also has a reduced solubility in water. The same problems can arise with Hb. To be effective as an oxygen carrier, Hb must contain 23 diphosphoglycerate (2,3-DPG), which is only present in red blood cells (Nucci and Abuchowski, 1998). This compound acts under conditions of low oxygen tensions to decrease the oxygen affinity of the Hb molecule by cross linking the two  $\beta$  chains of the tetramer, thereby allowing the Hb molecule to release its oxygen. Such loss of 2,3-DPG from extracellular Hb preparations results in Hb acquiring a much higher oxygen affinity and the oxygen remains firmly bound to the Hb molecule. In comparison to intracellular Hb, the  $P_{50}$  (partial pressure of oxygen resulting in a 50% Hb saturation) of free Hb is reduced from 26-28 to 12-15 mmHg. Another reason for the increased oxygen affinity of acellular Hb is the pH-dependency of oxygen binding [Bohr effect; Schubert, (1996)] since the pH inside the red blood cell is lower than in plasma. This reduced  $P_{50}$  impairs the release of adequate amounts of oxygen to tissues (Waschke *et al*, 1997).

For example, at a  $P_{50}$  of 10 mmHg less than 10% of the bound oxygen is released from the Hb molecule (Scott *et al*, 1997).

To overcome such difficulties associated with the use of free Hb, as in the context of animal cell culture and for this Thesis, plant cell culture as related to media supplementation, a number of chemical modifications have been found to be effective, these include cross linking alpha-alpha, beta-beta or alpha-beta chains to decrease the oxygen affinity of the Hb molecule (Jones *et al.*, 1996); polymerization of Hb molecules with glutaraldehyde (Nishide *et al*, 1997); or conjugation to macromolecules such as polyethyleneglycol (Alayash and Cashion, 1995). The latter two approaches also serve to produce polymeric haemoglobins which do not dissociate into dimers and monomers.

A further modification specifically for use as a blood substitute involves the microencapsulation of Hb in liposomes which results in the formation of artificial red blood cells (Tsuchida, 1994; Sakai *et al*, 1996). The advantages of such a system are that the Hb remains tetrameric, does not dissociate into dimers and monomers as is the case for free Hb, and is partially protected from the external environment. However, encapsulated Hb is difficult to remove from the mammalian circulatory system *and* adverse side effects and immune responses may be induced by the use of liposomal membranes.

Outdated human blood units, and animal and genetically engineered recombinant DNA technologies provide sources of Hb solutions for clinical investigation. Bovine haemoglobin offers several advantages over human blood as a source of Hb in that the oxygen affinity of bovine Hb is not governed by 2,3-DPG but by chloride ions which are found in abundance in human plasma. This would also make these Hbs a better option for evaluation in plant systems (see this Thesis) since there is no

shortage of such ions in plant cells. Thus, in the case of extracellular bovine Hb, chemical modifications only have to serve to prevent dissociation of the tetramer.

### **1.5.2 Haemoglobin in cell biotechnology**

In comparison with PFC technologies, haemoglobin-based methodologies have received little attention in the context of use as an artificial oxygen carrier in animal, microbial and certainly (see this Thesis) plant cell culture systems. However, there are a few reports that have demonstrated that **Hb-supplementation** of culture media can be beneficial to animal cell growth. For example, Shi *et al.* (1998) reported that monoclonal antibody production was increased when hybridoma cells were cultured in a bioreactor containing culture medium supplemented with four different Hb preparations. Interestingly, in this study it was found that the inclusion of one of the commercially available Hb preparations was superior to the use of PFC in respect to protein production. In a separate study using cultured (unspecified) insect cells it was found that cell growth, over a 100h culture period, could be increased by 100% in medium containing  $6\text{g l}^{-1}$  Hb in comparison to cells grown in medium lacking Hb (details given in European Patent Office, Rijswijk, The Netherlands; Patent US94, 03116).

In some microbial cell cultures, such as *Pseudomonas fragi* (Champomier-Verges *et al.*, 1996) and *Vibrio anguillarum* (Mazoy and Lemos, 1996), supplementation of the agar media with heme compounds such as hemin and Hb have been shown to beneficially affect bacterial viability (Daniel *et al.*, 1998). Such compounds have been primarily incorporated as alternative sources of metabolic iron. Indeed, globin derived from Hb contains all the essential amino acids for bacterial growth and has been used to replace the usual protein source such as tryptone in agar culture media (Hazarika, 1994). However, their use as respiratory gas carriers in microbial cell culture was impaired by the proteolytic activity of the bacteria. Therefore, it was not surprising that microbial cell culture systems were employed based on PFC

technologies as alternative respiratory gas carriers since these compounds cannot be metabolized by the bacteria.

## 1.6 Thesis objectives

The ultimate focus of this Thesis was to evaluate and exploit, in the context of improved tissue culture competence for a range of plant species, the gas solubility characteristics primarily of PFC liquids for technology options where oxygen availability is actually or (potentially) a growth-limiting factor. This will include an assessment of a diverse range of both monocotyledonous and dicotyledonous genotypes, both over short-term and longer-term culture periods to confirm no possible detrimental effects of PFC on cell genetic status. Against this backdrop such proving this technology will be applicable to plant genetic manipulation *per se*. Such PFC-facilitated improvements in regulation of respiratory gas supply to cultured cells, tissues and organs will have increasingly important biotechnological implication in the context of plant micropropagation, somatic hybridisation and transgenic plant production.

The specific objectives of the studies presented in this Thesis are summarised as follows:

- 1) To assess the biocompatibility of PFC liquids, both alone and in conjunction with surfactants, as media supplements, by investigating the effects of exposure to PFC for **plant** protoplast technology options. Both these supplementation options will be assessed, since they have been successfully exploited, both alone or in combination, in a number of animal cell culture and fermenter systems. This study will also include assessments of cell viability, protoplast cell wall regeneration and mitotic index of protoplast-derived cells isolated from both leaves and cell suspensions. The species of choice will focus on *Petunia hybrida* as a convenient model system and *Manihot esculenta* as a relatively recalcitrant genotype in tissue culture.

- 2) To assess PFC liquids as media supplements in comparison with other respiratory-gas modification options for cultured plant protoplasts and their cellular derivatives. This will also address the appropriateness of these compounds as physical supports for the interfacial growth of plant cells.
- 3) To investigate the potential use of haemoglobin as an alternative (to PFC) respiratory gas carrier for plant cell culture and to compare the efficacies of both haemoglobin and PFC as discrete media supplements.
- 4) To exploit the gas solubility properties of PFC liquids, both alone and in combination with surfactants, for improving the **post-thaw** recovery of cryopreserved plant cells. The latter technology provides a constant supply of competent cells for which some crucial areas of plant biotechnology are totally dependent. This will be potentially particularly beneficial in reducing the time frame for the re-establishment of cell suspension cultures and hence, the return and availability of cells for biotechnology programmes. Such studies will also evaluate both non-embryogenic cells of *Morinda arvensis* and embryogenic cells of *Oryza sativa*, these being a representative of both dicotyledonous and monocotyledonous plant species, respectively. In the context of cryopreservation, PFCs will also be assessed for recovered cell biomass considerations and more crucially plant regeneration as a measure of the efficacy of the process.

The results presented in this Thesis are based on a number of publications which have been included in the Appendices for cross reference.

## CHAPTER TWO: BIOCOMPATIBILITY AND EXPLOITATION OF PFC LIQUIDS IN PLANT PROTOPLAST CULTURE SYSTEMS

### 2.1 Introduction

Cassava (*Manihot esculenta* Crantz.), a perennial shrub of the family *Euphorbiaceae*, is cultivated throughout the lowland tropics. It is an important source of calories, ranking fourth following rice, maize and sugarcane. In contrast to other tropical crops, cassava has suffered from a lack of fundamental research at the tissue culture level. However, genetic manipulation technologies should permit the improvement of cassava with respect to useful agronomic traits via genetic recombination involving nominally sexually-incompatible genotypes (Mathews *et al*, 1993). One key pre-requisite for the application of such technologies is the development of an efficient protoplast-to-plant system and, for somatic hybridisation, the subsequent establishment of an effective selection strategy for the recovery of hybrid products. The recalcitrance of cassava as an actively dividing protoplast system *in vitro* is well documented (Byrne, 1984), and there is, to date, only one, non-reproducible report on plant regeneration from cassava protoplasts (Shahin and Shepard, 1980). Until a reproducible protoplast culture and shoot regeneration protocol has been established, the true potential of protoplast fusion and/or transformation via direct DNA delivery into **protoplasts** for the genetic improvement of cassava cannot be realised.

Preliminary studies using cassava leaf protoplasts cultured in liquid medium, overlaying a basal **agar-solidified** medium, demonstrated that sustained mitotic division only occurred at the periphery of the Petri dishes (Anthony *et al*, 1994a). Here the depth of the liquid medium was at its minimum due to meniscus effects and it is probable that this allowed for greater gaseous exchange between the cultured protoplasts and the atmosphere within the Petri dish. Previous studies have also demonstrated that the depth of the liquid culture medium within Petri dishes

containing statically cultured protoplasts can significantly influence the initial plating efficiency (IPE). For example, Brandt (1991) using hypocotyl-derived protoplasts of rape (*Brassica napus* L. cv. Omega) found that the IPE could be increased over 20-fold by decreasing the amount of liquid protoplast suspension plated per Petri dish from 2 mm in depth to 1 mm. Thus a study was undertaken to increase the number of menisci within Petri dishes, containing cultured cassava leaf protoplasts, by insertion of short glass rods embedded perpendicularly within, and **protruding** from, the agarose layer.

Oxygen deprivation is known to reduce plant cell growth through inhibitory effects on the production of respiratory ATP (Van der Plas and Wagner, 1986). In contrast, high oxygen availability can preferentially enhance the growth of non-embryogenic callus cells from plants such as wheat (Carman, 1988). Additionally, studies have shown that the growth of tomato, jute and rice protoplasts can be improved by culture in oxygen-enriched atmospheres (d'Utra Vaz *et al.*, 1992). The IPE of the protoplasts assessed were increased from 0% to  $23 \pm 1.3\%$  in tomato, from  $10 \pm 0.7\%$  to  $45 \pm 1.9\%$  in jute and from  $0.71 \pm 0.09\%$  to  $1.46 \pm 0.01\%$  in rice. Plant regeneration was also significantly increased in rice protoplast-derived calli which had been exposed to an oxygen-enriched environment from 2% of the calli exhibiting shoot regeneration to 14%. Similarly, the earlier study of Brandt (1991) also demonstrated that the IPE of cultured rape protoplasts could be further increased by 125% over the optimised reduced volume of protoplast suspension plated, by increasing the oxygen concentration of the gas phase within the dish, from normal atmospheric conditions of 20% to 30% (v/v). There is thus a strong case for investigating the growth and differentiation of plant protoplasts, cells and tissues when cultured in the presence of media supplements which, in principle, can facilitate improved oxygen transfer.

PFCs are inert, organic compounds which can dissolve substantial volumes of respiratory gases and have been used for enhancing oxygen supply both *in vivo* and *in vitro* (King *et al*, 1989; Lowe, 1992, 1993). PFCs have been used, for example, in fermenter studies (Junker *et al*, 1990; Ju *et al*, 1991) or hybridoma cell cultures (Ju and Armiger, 1992) to increase oxygen transfer rates. Mammalian cells, including fibroblasts and retinoblastoma cells, have also been cultured successfully at the interface formed between PFC liquid and aqueous media (Giaever and Keese, 1983; Nabih *et al*, 1989). Since there appears to have been no corresponding assessment of the beneficial effects of using PFCs in plant cell cultures, the present study was performed to investigate the growth of isolated protoplasts in such a culture system, using cell suspension-derived protoplasts of albino *Petunia hybrida* as a convenient model for these assessments. This approach could have significant biotechnological application, not only in the context of plant genetic manipulation where the efficacy of protoplast conversion to plants is paramount for successful somatic hybridisation and transgenic plant production, but also in the production of phytochemicals from cultured plant cells and tissues.

In the present study perfluorodecalin was selected as the PFC of choice as a plant cell culture supplement (Anthony *et al*, 1994a, b, 1995b; Lowe *et al*, 1995) since the biocompatibility of this PFC has been widely assessed for use in both mammalian and microbial cell systems. The perfluorodecalin liquids PP5 and PP6 used in this project were provided by F2 Chemicals Ltd., Preston, UK and are hereafter known as *Flutec*<sup>®</sup> fluids. The latter are well suited to plant cell culture and have a boiling point of 142°C which enables sterilization to be performed by autoclaving. Moreover the gas solubility of these PFCs (24.4 ml of oxygen gas per 100 g of liquid) is such that problems of volatility are avoided, since as the gas solubility of the PFC increases, the boiling point decreases and the vapour pressure increases. For this reason other *Flutec*<sup>®</sup> liquids with higher gas solubilities and

hence lower boiling points were unsuitable for use in plant cell culture due to their volatility.

The growth of cells in semi-solid media is commonly assessed simply by visual comparison of areas of coverage of culture plates. Whilst this approach may be adequate if major variations exist between individual treatments, its power of discrimination is inadequate for cultures which show relatively little difference. Under these circumstances, therefore, it is valuable to develop techniques providing a non-invasive and hence, non-destructive, quantitative assessment of cell growth within the confines of a culture vessel.

Computer-based image analysis techniques are extremely valuable in making such measurements but they do not yet appear to have been widely employed for this purpose. This approach enables accurate measurement, not only of total area covered, but also of more sophisticated variables, such as individual cell colony number, colony area and fractal structure. Image analysis has been used, for example, to characterise the growth of *Streptomyces tendae* (Reichl *et al.*, 1992), to assess the growth of mammalian cells on microcarriers (Pons *et al.*, 1992), and to follow the growth of plant somatic embryos cultured in a continuous loop bioreactor (Harrell *et al.*, 1992). However, there appear to be no reports to date of the use of this approach to assess growth characteristics and morphology of plant protoplast-derived cell colonies on agar-solidified media. Therefore, in the present experiments, image analysis has been used to assess the growth of protoplast-derived cell colonies (of *P. hybrida* as a convenient model) in the presence of oxygenated perfluorocarbon and *Pluronic*<sup>®</sup> F-68, either or both of which can promote cell growth (Lowe *et al.*, 1993; Anthony *et al.*, 1994a, b).

## 2.2 Materials and Methods

### 2.2.1 Cassava

#### 2.2.1.1 Plant material and establishment of cassava shoot cultures

Axenic shoot cultures of *Manihot esculenta* Crantz. cv. M. Thai 8 were supplied by the International Centre for Tropical Agriculture (CIAT), Cali, Colombia. Shoots of ca. 2.0 cm in height were routinely maintained on 50 ml aliquots of MS medium (Murashige and Skoog, 1962) lacking growth regulators and made semi-solid with 0.8% (w/v) agar (Difco-Bacto, Detroit, USA; 3 explants per 175 cm<sup>3</sup> glass jar, Beatson Clarke and Co. Ltd., Rotherham, UK). Cultures were maintained under a 12 h photoperiod (19.5  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , daylight fluorescent tubes, Cool Light; Thorn EMI Ltd., Hayes, UK) at 28  $\pm$  2°C and sub-cultured at 28-35 d intervals.

#### 2.2.1.2 Isolation of cassava leaf protoplasts

The second and third fully expanded leaves (from the apex) of axenic shoot cultures, taken 21 d after sub-culture, were used as a source of protoplasts. Leaves were sliced transversely into 1.0 mm strips and plasmolysed by immersion for 1 h in 10 ml of modified CPW salts solution (Frearson *et al*, 1973) containing 1.48 g l<sup>-1</sup> CaCl<sub>2</sub>·2H<sub>2</sub>O supplemented with 9% (w/v) mannitol (CPW9M solution; Appendix II), pH 5.8. The plasmolysis solution was replaced with an enzyme mixture (10 ml of enzyme solution; g.f.wt<sup>1</sup> of tissue) which consisted of 1.0% (w/v) Hemicellulase (Sigma, Poole, UK), 0.4% (w/v) Cellulase RS (Yakult Honsha Co., Nishinimiya Hyogo, Japan), 0.1% (w/v) Pectolyase Y23 (Seishim Pharmaceutical, Tokyo, Japan) and 5.0 mM 2-[N-morpholino]ethane sulfonic acid (MES) in CPW9M solution, pH 5.8 (designated ENZC1). The enzyme solution was **pre-filtered** through a 0.2  $\mu\text{m}$  pore size filter nitrocellulose 47 mm diam. membrane filter (Whatman, Maidstone, UK) to remove insoluble impurities prior to filter-sterilisation through a sterile microbial filter (0.2  $\mu\text{m}$  pore size, 30 mm diam.; Minisart NML, Sartorius AG, Göttingen, Germany). The enzyme solution was stored in aliquots of 30 ml at -20°C and was defrosted at room temperature before use. Incubation was carried out in the

dark ( $25 \pm 2^\circ\text{C}$ ) on a horizontal shaker (40 r.p.m.) for 16 h in 9.0 cm diam. Petri dishes.

The digested tissues were filtered through a  $30\ \mu\text{m}$  pore size nylon sieve (Wilson Sieves, Nottingham, UK) into a 9 cm Petri dish to remove undigested cells. The protoplast-enzyme mixture was transferred, using a Pasteur pipette, into 16 ml **screw-capped** round bottom glass centrifuge tubes (Coming Ltd., Stone, UK) and pelleted by centrifugation at  $80 \times g$  for 7 min in a bench centrifuge (Centaur 1, MSE, R.W. Jennings Co. Ltd., Nottingham, UK). The protoplast pellet was washed twice in CPW9M solution by repeated resuspension and centrifugation and resuspended in a known volume of CPW9M solution for counting and viability assessments.

### **2.2.1.3 Measurement of cassava protoplast yield and viability**

Protoplasts suspended in CPW9M solution were counted using a modified Fuchs-Rosenthal double-chambered haemocytometer with a depth of 0.2 mm (Scientific Laboratory Supplies Ltd., Nottingham, UK) using a Nikon Diaphot TMD inverted microscope. Protoplast yield was calculated and expressed as the number of protoplasts g f wt. of leaf tissue.

Protoplast viability was determined by uptake and cleavage of fluorescein diacetate (FDA), as described previously (Widholm, 1972). An FDA stock solution ( $100\ \mu\text{l}$  of  $3\ \text{mg}\ \text{ml}^{-1}$  FDA in acetone) was added to 16 ml of CPW9M solution, prior to viability assessments. An aliquot ( $200\ \mu\text{l}$ ) of this solution was mixed with an equal volume of the protoplast suspension and incubated for 2-3 min at room temperature. Protoplasts were examined under ultra-violet (UV) illumination using a Nikon Diaphot TMD inverted microscope with high pressure mercury vapour lamp HBO 100 W/2, a **B1 FITC** exciter filter IF 420-485 nm, dichromic mirror DM510 and eyepiece absorption filter 570. Viable protoplasts labelled with FDA were visible under UV light and exhibited yellow-green fluorescence.

#### **2.2.1.4 Culture of cassava leaf protoplasts**

Protoplasts were cultured in the dark ( $28 \pm 2^\circ\text{C}$ ) at a density of  $4.0 \times 10^5 \text{ ml}^{-1}$  in 1.0 ml aliquots of liquid, MS medium lacking  $\text{NH}_4\text{NO}_3$ , but supplemented with 3% (w/v) sucrose,  $2.12 \text{ g l}^{-1} \text{ NaNO}_3$ ,  $2.0 \text{ mg l}^{-1}$   $\alpha$ -naphthaleneacetic acid (NAA),  $0.5 \text{ mg l}^{-1}$  6-benzyladenine (BAP) and 9% (w/v) mannitol, pH 5.8 (designated MSP19M-N medium). One ml aliquots of this liquid medium, with protoplasts, was laid over 1.0 ml volumes of B5 medium (designated 1B5CNK; Misawa *et al.*, 1982) made semi-solid with 0.6% (w/v) Sea Plaque agarose (FMC BioProducts, Rockland, USA), pH 5.8, in 3.5 cm Petri dishes (A/S Nunc, Kamstmp, Denmark). Four autoclaved glass rods (each 6.0 mm diam. x 8.0 mm length) were placed end-on equidistantly within each Petri dish prior to the addition of the semi-solid 1B5CNK medium layer, with the immobilised rods protruding from the agarose medium (Anthony *et al.*, 1995a, b). Control protoplasts were cultured under identical conditions, but in dishes without glass rods.

#### **2.2.1.5 Cell wall determination of cultured cassava protoplasts**

Cell wall regeneration was determined after 2-3 d of culture by use of a commercial fluorescent brightener (Sigma). An aliquot of stock solution ( $100 \mu\text{l}$  of  $10 \text{ mg ml}^{-1}$  in CPW9M solution) was mixed with an equal volume of the protoplast suspension. The protoplasts were examined under UV illumination after 2-3 min incubation at room temperature using a Nikon Diaphot TMD inverted microscope with high pressure mercury vapour lamp HBO 100 W/2, fitted with a 350 nm exciter filter and a GG4+2E barrier filter. The presence of a cell wall was indicated by an intense blue fluorescence.

#### **2.2.1.6 Measurement of cassava protoplast growth**

Growth responses were assessed in terms of initial (IPE; day 25) and final (FPE; day 50) plating efficiencies. IPE was defined as the number of protoplast-derived cells

which had undergone at least one mitotic division; FPE as the number of protoplast-derived cell colonies.

### **2.2.1.7 Plant regeneration assessments of cassava protoplast-derived tissues**

Protoplast-derived cell colonies ( $n = 1(X)$ ) were transferred after 50 d to MS medium lacking  $\text{NaNO}_3$  and mannitol, but supplemented with 3.0% (w/v) sucrose,  $1.65 \text{ g l}^{-1}$   $\text{NH}_4\text{NO}_3$ ,  $2.0 \text{ mg l}^{-1}$  NAA and  $0.5 \text{ mg l}^{-1}$  BAP (designated MSP1) and made semi-solid with 0.4% (w/v) agarose (Sigma Type 1) for further proliferation (20 ml per 9 cm Petri dish). Cultures were maintained under the same growth conditions as for axenic shoots (Section 2.2.1.1). One-month-old protoplast-derived calli (*ca.* 1.0 cm in diam.) were transferred to MS medium of the same composition as MSP1, but with NAA reduced to  $0.2 \text{ mg l}^{-1}$  and  $1.2 \text{ mg l}^{-1}$  N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU) replacing BAP. At this stage, protoplast-derived calli were placed on the surface of 10 ml aliquots of medium contained in 5.0 cm Petri dishes (4 calli/dish) and cultured under the same conditions as described in Section 2.2.1.1.

## **2.2.2 Petunia**

### **2.2.2.1 Initiation and maintenance of petunia cell suspensions**

Cell suspensions of *Petunia hybrida* cv. Comanche were provided by Dr. J.B. Power, University of Nottingham, Nottingham, UK. Cells were maintained in liquid UM medium (Uchimiya and Murashige, 1974; Appendix II) in 250 ml Erlenmeyer flasks with shaking (90 r.p.m.) at  $24^\circ\text{C} \pm 2^\circ\text{C}$  in the light under Cool Light fluorescent tubes (Thorn EMI Ltd.;  $25 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ; 16 h photoperiod). Suspensions were routinely sub-cultured every 7 d by transferring 0.5 ml of settled cells to 50 ml aliquots of fresh medium.

### **2.2.2.2 Isolation of petunia cell suspension protoplasts**

Protoplasts were isolated enzymatically from cell suspensions taken 3-4 d after sub-culture. Cells were harvested using a nylon sieve of  $64 \mu\text{m}$  pore size and a pre-

weighed aliquot of 1g f. wt. of cells was incubated in 20 ml of enzyme solution as described in Section 2.2.1.2. The enzyme solution (designated ENZ223) consisted of 2% (w/v) Rhozyme HP 150 (Genencor, Reading, UK), 2% (w/v) Meicelase (Meiji Seika Kaisha Ltd., Tokyo, Japan), 0.03% (w/v) Macerozyme R10 (Yakult Honsha Co. Ltd.) and modified CPW salts solution (Section 2.2.1.2) supplemented with 13% (w/v) mannitol (CPW13M solution; Appendix II), pH 5.8. The enzyme solution was filter sterilized and stored as in Section 2.2.1.2.

Following incubation, the protoplasts suspension was filtered through a 64  $\mu\text{m}$  pore size nylon sieve into a 9 cm Petri dish to remove undigested cells. The protoplast-enzyme mixture was transferred into 16 ml glass centrifuge tubes and pelleted by centrifugation at 80 x g for 7 min in a bench centrifuge. The protoplast pellets were washed twice in CPW13M solution as described in Section 2.2.1.2, prior to counting and viability assessments.

### **2.2.2.3 Culture of petunia cell suspension-derived protoplasts**

Protoplasts of albino *P. hybrida* were cultured in the dark ( $24 \pm 2^\circ\text{C}$ ) at a plating density of  $2.0 \times 10^5 \text{ ml}^{-1}$  in 2.0 ml aliquots of liquid KM8P culture medium based on the formulation of Kao and Michayluk (1975), as modified by Gilmour *et al.* (1989), and supplemented with 0.1 mg  $\text{l}^{-1}$  2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg  $\text{l}^{-1}$  zeatin (Appendix II). The medium containing protoplasts was placed in 30 ml screw-capped glass bottles, either alone, or over 6.0 ml of sterile perfluorodecalin (*Flutec*<sup>®</sup> PP5; Plate 2.2a, b). The perfluorodecalin was saturated with 100% oxygen by bubbling for 15 min (10 mbar) before use; in some controls, the perfluorodecalin was left unsaturated. It was necessary to maintain a PFC liquid layer at least 5 mm deep in the culture vessels, in order to obtain a stable interface for culture purposes.

In some treatments, the aqueous culture medium was supplemented with 0.01% (w/v) of a commercial-grade of the non-ionic, co-polymer surfactant, *Pluronic*<sup>®</sup> F-68

(BASF, Wyandotte, USA). This concentration of Pluronic was selected on the basis of previous studies of growth-stimulating effects of this compound on *S. dulcamara* protoplasts (Kumar *et al*, 1992; Lowe *et al*, 1993). It was sufficient to reduce the perfluorodecalin-water interfacial tension by approximately 40%, so as to facilitate maximum contact of protoplasts with the interface. Interfacial tensions between perfluorodecalin and *Pluronic*<sup>®</sup> F-68 solutions were measured using a conventional Wilhelmy Plate apparatus attached to Cahn microbalance; it was necessary to use a plate made from PTFE (15 x 15 x 0.8 mm) since the conventional glass or platinum plates are not wetted by the perfluorocarbon liquid.

The experimental design consisted of a 2 x 2 x 2 matrix experiment (total 8 groups).

The treatment groups were:-

- (a) with and without oxygen bubbling for 15 min at 10 m bar;
- (b) with and without supplementation of KM8P medium with 0.01% (w/v) *Pluronic*<sup>®</sup> F-68.
- (c) with and without perfluorodecalin.

At 10 and 17 d, the spent KM8P medium (0.25 ml) was replaced with an equal volume of KM8 medium [of reduced osmotic pressure and prepared to the formulation of medium 8 of Kao and Michayluk (1975) with the modifications of Gilmour *et al*, (1989); Appendix II]. The total population of cell colonies was transferred, on day 40, to the surface of 10 ml of agar-solidified (0.6% w/v; Sigma) UM medium in 5.5 cm Petri dishes. Cultures were incubated in the dark at  $25 \pm 2^\circ\text{C}$ .

#### **2.2.2.4 Measurement of petunia protoplast growth**

Protoplast growth, as determined by IPE was assessed after 10 d. A minimum of 500 protoplasts per treatment were counted; each treatment was replicated 5 times.

### **2.2.2.5 Fluorocarbon re-cycling and re-use**

Perfluorodecalin was recovered for re-use by centrifugation of the culture medium (2000 x g for 15 min). The PFC pellet was shaken with alkaline permanganate (1.0 g l<sup>-1</sup> in 1M sodium hydroxide) and repeatedly rinsed (minimum 5 times) with distilled, deionised water prior to re-use and re-sterilisation by autoclaving (121°C, 20 min).

### **2.2.2.6 Measurement of dissolved oxygen concentration**

Oxygen concentrations in perfluorodecalin or culture medium were measured using a Jenway 9015 Dissolved Oxygen Meter with a POM102 probe (Scientific Laboratory Supplies Ltd.). The instrument was calibrated to measure oxygen concentration in ml/l using oxygen-saturated perfluorodecalin or aqueous medium as standards. The saturation solubilities of oxygen in these media are 423 ml/l and 25 ml/l respectively at STP (Riess and Le Blanc, 1982). The overall coefficient of variation was 5.2%.

### **2.2.2.7 Assessment of petunia protoplast-derived cell colony growth by image analysis**

On day 68 post protoplast isolation, the Petri dishes were photographed onto Fujichrome 64T colour film (Fuji Photo Film Co. Ltd., Minato-ku, Tokyo, Japan) using a Nikon 601 automatic 35 mm camera with a 55 mm macro-lens (1 sec exposure at f16); the dishes were illuminated by bounced lights. The film was processed (E6 method) into slides and printed onto Fuji reversal printing paper. Prints were scanned using an Apple document scanner connected to an Apple Macintosh IIfx computer. The colour images were imported into the image analysis package (NIH Image, Version 1.44) at a resolution of 150 dpi. Protoplast-derived colonies were selected by thresholding. This identified two populations; those showing discrete colony formation and growth, and those in which colony formation was insignificant. The latter were visible only as single pixels on the image, and were eliminated from the thresholded picture using a single cycle of erosion-dilation. This technique (Serra, 1972) erases single point objects whilst maintaining the size

of all larger selected areas, representing the actively dividing protoplast-derived colonies. The main source of error in this process is the selection of the thresholding limits identifying the colonies. The mean variation between such readings was assessed by making multiple, separate measurements.

### **2.2.3 Assessment of cassava leaf protoplasts cultured with various respiratory gas-manipulation treatments**

Cassava cv. M. Thai 8 leaf protoplasts were isolated and cultured as in Sections 2.2.1.2 and 2.2.1.4, respectively. All dishes (with and without glass rods) were transferred into 7.0 cm height sealed glass chambers containing a 1.0 cm thick layer of 0.8% (w/v) aqueous agar solution (d'Utra Vaz *et al*, 1992). For some treatments, the chamber was gassed for 1 min with 100% gaseous oxygen (10 mbar). In a further series of treatments, 0.75 ml of oxygenated (10 mbar, 15 min) perfluorodecalin (*Flutec*<sup>®</sup> PP5) was introduced into the Petri dishes which settled beneath the aqueous culture medium containing the protoplasts. This produced a three-layer culture system viz. agar, perfluorodecalin and aqueous phase. Each treatment was replicated 3 times.

For all treatments, protoplasts were cultured for up to 25 d under the conditions described in Section 2.2.1.4. The IPE was assessed by light microscopy and a minimum of 100 protoplasts were counted per Petri dish.

### **2.2.4 Statistical analyses**

Means and either standard deviations (s.d.) or standard errors (s.e.m.) were used throughout, with statistical significance between mean values assessed using a non-parametric Moods median test to determine significant differences and Mann Whitney *U*-tests to identify significant treatments when analysing protoplast plating efficiency (Snedecor and Cochran, 1989). A probability of  $P < 0.05$  was considered significant.

## 2.3 Results

### 2.3.1 Cassava protoplast culture and plant regeneration assessments

#### 2.3.1.1 Effect of medium supplementation with glass rods on mitotic division of cultured cassava protoplasts

Juvenile leaves, taken from axenic shoot cultures, provided high protoplast yields of  $1.95 \times 10^7$  g.f.wt(Plate 2.1a). Protoplasts were 10-45  $\mu\text{m}$  in diam. with peripherally-aligned chloroplasts, and had a mean viability of  $85 \pm 2\%$ . Cell wall formation commenced after 1-2 d of culture, with a concomitant increase in the volume and cytoplasmic content of the protoplasts. During the first 25 d of culture, a 6-fold increase ( $P < 0.05$ ) in the number of protoplasts entering their first mitotic division, as assessed by IPE, occurred in dishes with glass rods (Plate 2.1b, c). In contrast, only  $1.5 \pm 0.4\%$  of the protoplasts had undergone mitosis in the control cultures (Table 2.1). In the latter, cell division occurred only where freshly-isolated protoplasts, suspended in the liquid phase, were in contact with the meniscus formed where the culture medium touched the sides of the Petri dishes. In control cultures, mitosis was not sustained and significant numbers of cell colonies were not formed (Plate 2.1f). However, when glass rods were introduced into the two-phase culture system, the extent of the liquid meniscus was increased and cell colonies developed in close association with the glass rods. Protoplasts cultured with glass rods proliferated into dense colony aggregates by day 40 (Plate 2.1f, g).

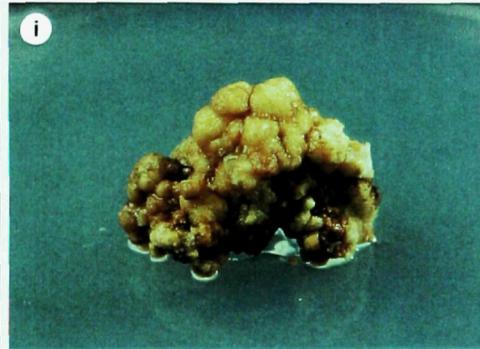
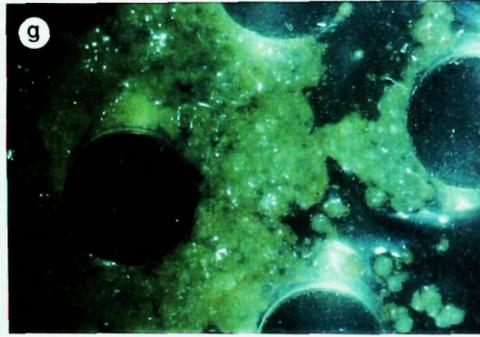
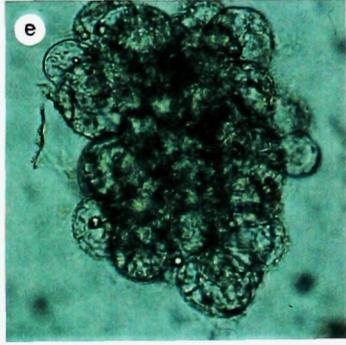
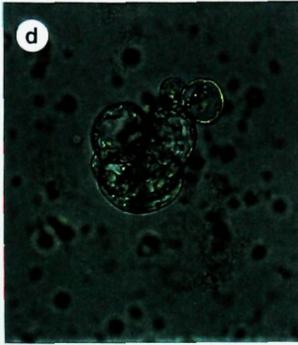
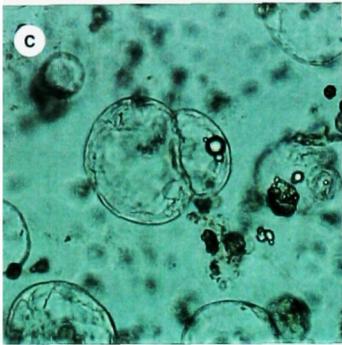
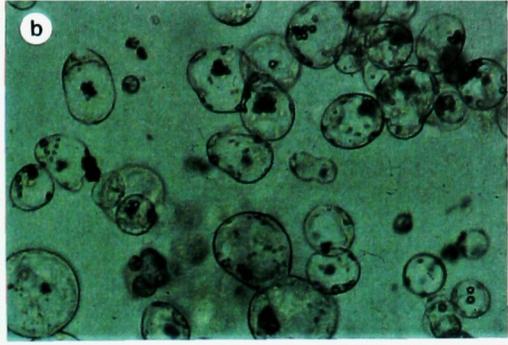
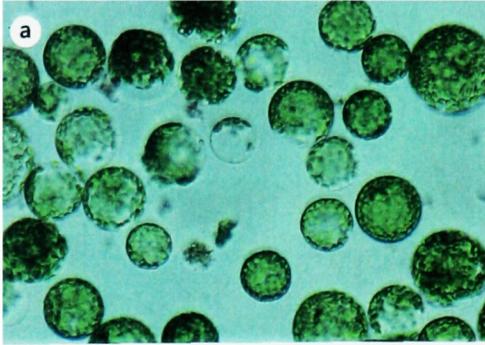
**Table 2.1 Mean initial (IPE) and final (FPE) plating efficiencies of cassava (*M. esculenta* cv. M. Thai 8) leaf protoplasts following culture for 50 d in the presence of glass rods compared with controls lacking glass rods.**

Treatment	IPE (%) (25 d)	FPE (%) (50 d)
Control (no glass rods)	$1.5 \pm 0.4$	No colonies
Glass rods	$8.9 \pm 0.7^*$	$1.0 \pm 0.2$

Values are mean  $\pm$  s.e.m. (n = 5); \* $P < 0.05$

**Plate 2.1 Culture of cassava (*Manihot esculenta* cv. M. Thai 8) leaf protoplasts and plant regeneration assessments of protoplast-derived tissues**

(a) Freshly isolated cassava leaf protoplasts in CPW9M solution (x200), (b) Leaf protoplasts of cassava cultured in the dark, 15 d after isolation in liquid MSP19M-N medium (x200), (c, d) Protoplasts undergoing first mitotic division in liquid MSP19M-N medium after 20 d and repeated mitosis after 30 d culture in the dark (x100, x200 respectively), (e) Micro-colony (35 d old) derived from leaf protoplasts of cassava (x 100), (f) Petri dishes in which cassava leaf protoplasts (40 d old) were cultured in the light: (left) liquid medium over agarose-solidified medium (control), (right) liquid over agarose-solidified medium with glass rods (gr) protruding from the underlying agarose layer (x0.85), (g) higher magnification of (f) showing glass rods surrounded by densely packed protoplast-derived cell colonies (x2.8), (h) Protoplast-derived callus of cassava, (120 d old after protoplast isolation) cultured on MSPI medium and maintained in the light undergoing rhizogenesis (x3), (i) Cassava protoplast-derived callus (125 d old after protoplast isolation), cultured in the light on MS medium containing CPPU showing typical nodular appearance (x2.8).



### **2.3.1.2 Evaluation of plant regeneration potential from cassava protoplast-derived tissues**

Proliferating multicellular colonies, when transferred to semi-solid MSPl medium after 50 d of culture, developed rapidly to produce friable callus (2-3 cm diam.) by day 90. Such callus typically developed 1-2 roots per tissue portion after a further 21 d of culture (Plate 2.1h). Calli transferred to semi-solid MS medium containing CPPU, developed a nodular appearance within 30 d (Plate 2.1i). Such nodular callus, when sectioned, displayed an epidermal layer and distinguishable cortex and vascular tissues, with an internal organisation similar to that of cassava tubers. Meristematic regions were absent.

## **2.3.2 Culture of petunia protoplasts with PFC and *Pluronic*® F-68**

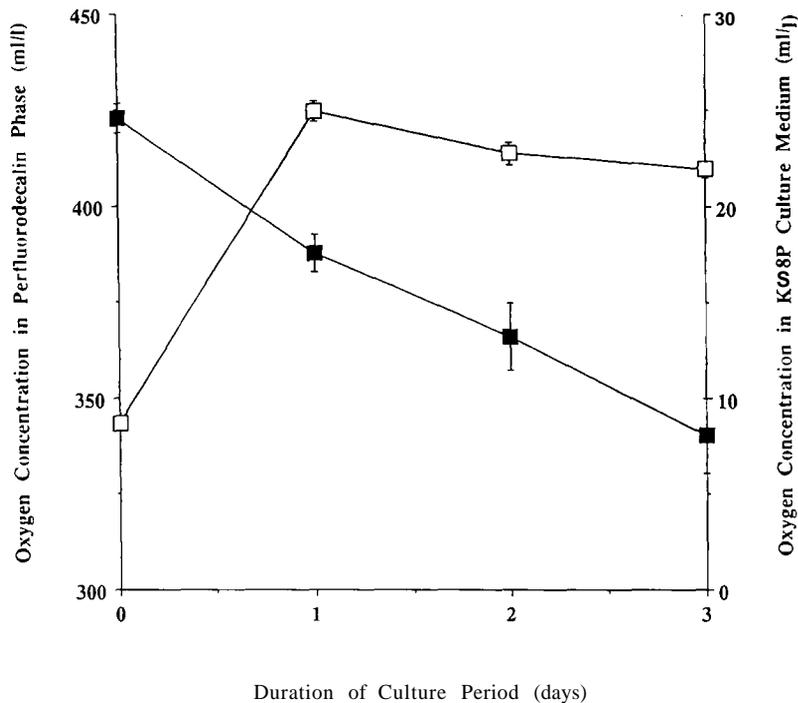
### **2.3.2.1 Petunia protoplast viability and division**

The mean viability of protoplasts was  $95 \pm 1\%$  ( $n = 3$ ) immediately following enzymatic isolation. Protoplasts divided normally at the interface that formed between perfluorodecalin and the aqueous culture medium (Plate 2.2c, d), with no significant loss in viability over the culture period. However, in some cases, there was a tendency for protoplasts to aggregate concomitantly with cell wall regeneration, but this was minimalised in media supplemented with 0.01% (w/v) *Pluronic*® F-68.

### **2.3.2.2 Changes in oxygen concentration**

The mean ( $\pm$  s.d.) oxygen concentration in oxygenated perfluorodecalin at the start of the experiment was  $423 \pm 4$  ml/l, while that of the aqueous phase was  $8.7 \pm 0.1$  ml/l (Fig. 2.1). The mean oxygen concentration in the aqueous medium increased to a maximum of  $25.0 \pm 0.6$  ml/l after 24 h, then declined slowly to  $22.0 \pm 0.4$  ml/l after 3 d. There was a concurrent decrease in the mean concentration of oxygen in the underlying perfluorodecalin phase, to  $340 \pm 10$  ml/l after 3 d (Fig. 2.1).

**Fig. 2.1** Changes in mean ( $\pm$  s.d.) oxygen concentrations (ml/l) in liquid KM8P culture medium (open symbols) overlaying oxygenated perfluorodecalin (solid symbols) during a 3 d culture period at 25°C.

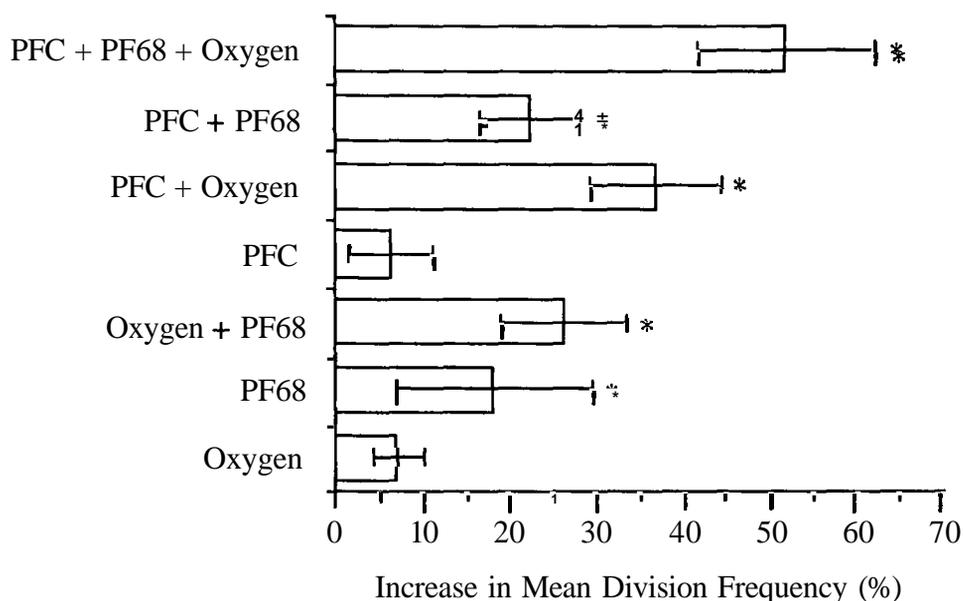


The oxygen measurements, both in the perfluorodecalin and culture medium phases, were restricted to the first 3 d of culture since the protoplasts of *P. hybrida* will have regenerated a new cell wall and entered first mitotic division during this period. No significant difference was observed in the oxygen concentrations in a parallel series of experiments lacking the *Pluronic*<sup>®</sup> F-68 medium component.

### 2.3.2.3 Growth of petunia protoplasts with perfluorodecalin.

The growth of protoplasts was significantly enhanced when they were cultured at the interface between aqueous KM8P medium and oxygenated perfluorodecalin. For example, the mean IPE after 10 d was  $9.7 \pm 0.7$  % ( $n = 5$ ) compared to  $7.1 \pm 0.4$ % for control protoplasts cultured in unsupplemented medium ( $P < 0.05$ ). In contrast, there were no significant changes in protoplast growth when oxygen alone, or non-oxygenated perfluorodecalin were used in separate control experiments. Figure 2.2

shows the percentage increase in mean division frequency over the controls for the various treatment groups.



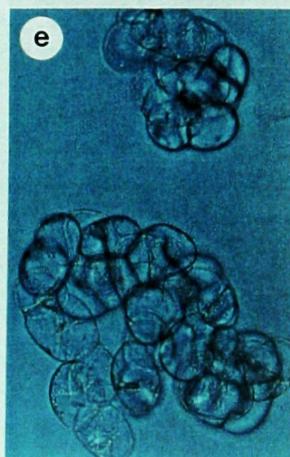
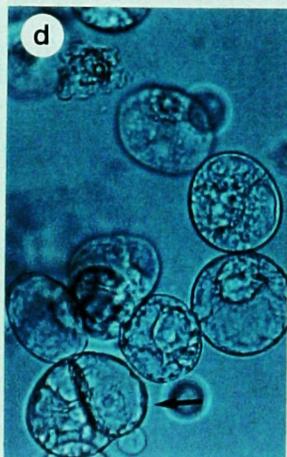
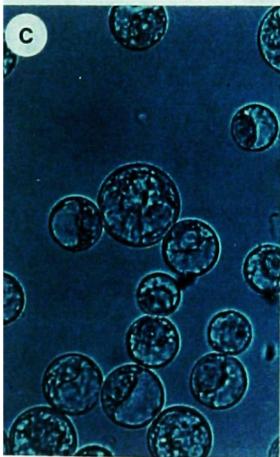
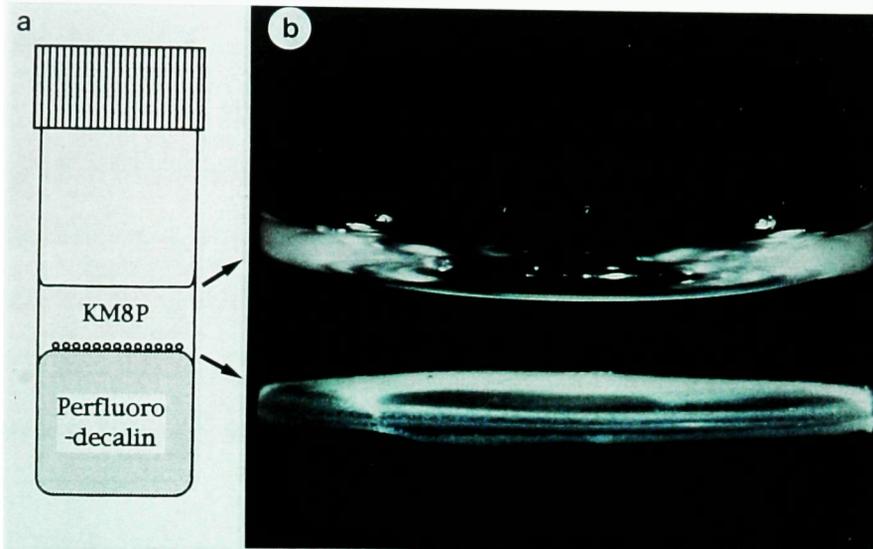
**Fig. 2.2 Mean division frequency (% increase over control) of *P. hybrida* protoplasts after 10 d of culture under the following conditions (top to bottom):**

<b>PFC + PF68 + Oxygen</b>	In KM8P medium + 0.01% (w/v) <i>Pluronic</i> <sup>(F)</sup> F-68 overlaying oxygenated perfluorodecalin
<b>PFC + PF68</b>	In KM8P medium + 0.01% (w/v) <i>Pluronic</i> <sup>(R)</sup> F-68 overlaying perfluorodecalin
<b>PFC + Oxygen</b>	In KM8P medium overlaying oxygenated perfluorodecalin
<b>PFC</b>	In KM8P medium overlaying perfluorodecalin
<b>Oxygen + PF68</b>	In oxygenated KM8P medium + 0.01% (w/v) <i>Pluronic</i> <sup>(R)</sup> F-68
<b>PF68</b>	In KM8P medium + 0.01% (w/v) <i>Pluronic</i> <sup>(R)</sup> F-68
<b>Oxygen</b>	In oxygenated KM8P medium

Values are mean  $\pm$  s.d. (n = 5). \*P < 0.05.

**Plate 2.2 Culture of *Petunia hybrida* protoplasts at a PFC/aqueous medium interface**

(a) Schematic diagram of the culture system, (b) Suspension cell-derived protoplasts of albino *P. hybrida* cv. Comanche maintained in the dark at the interface formed between oxygenated perfluorodecalin and liquid KM8P culture medium (x3), (c) Freshly isolated *P. hybrida* protoplasts in CPW13M solution prior to culture at the PFC/KM8P media interfaces (x200), (d) Protoplast-derived cells, some of which are dividing (arrow), harvested from the interface after 10 d of culture in the dark (x200), (e) Protoplast-derived cell micro-colonies of *P. hybrida* after 40 d culture under dark conditions in liquid KM8P/KM8 medium overlaying oxygenated perfluorodecalin; such micro-colonies could be converted back to discrete calli and eventually cell suspension cultures (x100).



#### **2.3.2.4 Growth of petunia protoplasts with perfluorodecalin and *Pluronic*<sup>®</sup> F-68**

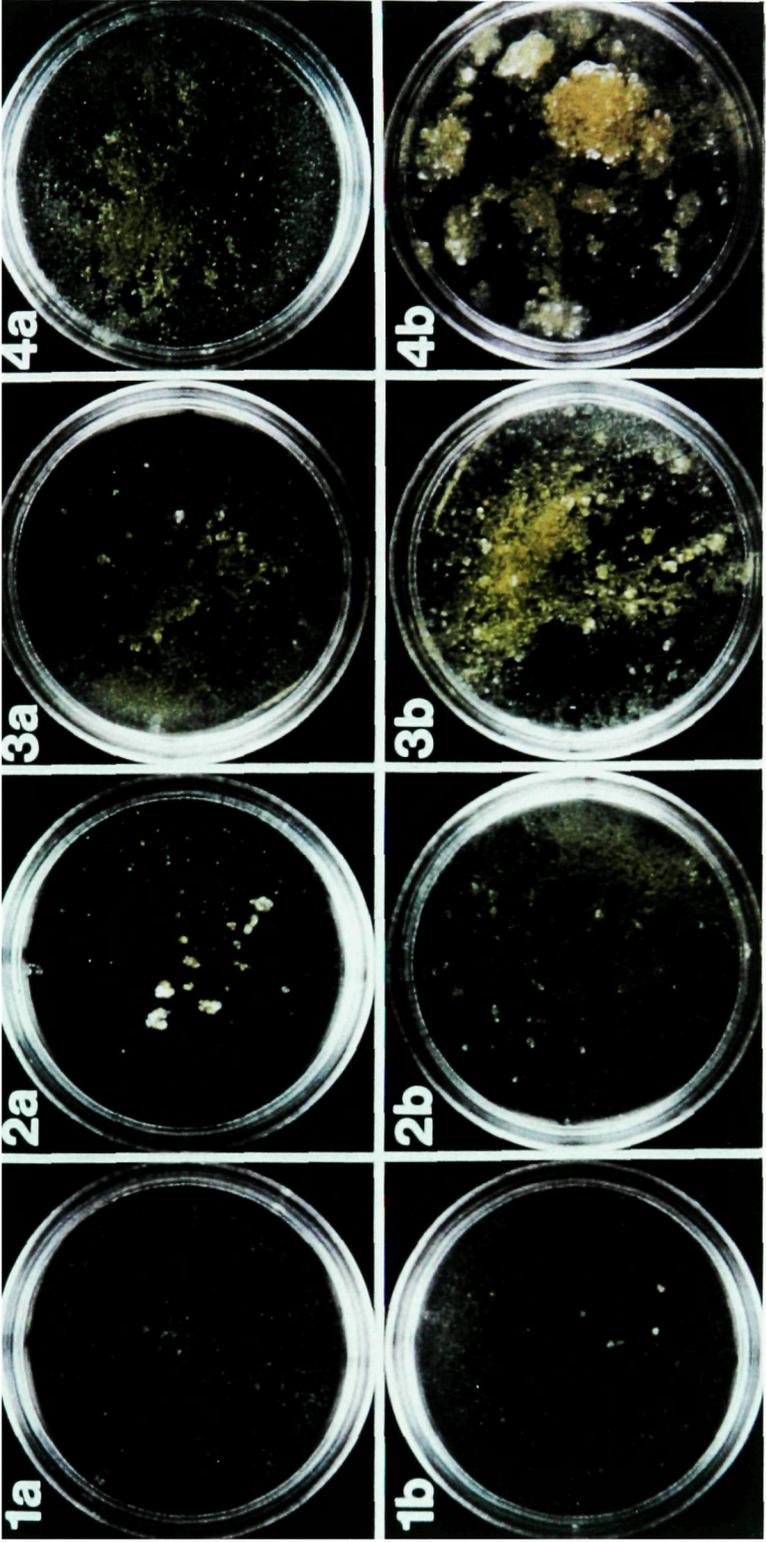
Supplementation of the aqueous culture medium with 0.01% (w/v) *Pluronic*<sup>®</sup> F-68 increased protoplast growth, such that the mean division frequency after 10 d ( $8.4 \pm 1.0\%$ ) was significantly greater ( $P < 0.05$ ) than control (Fig. 2.2). In contrast, no further increase in growth over controls occurred when the *Pluronic*<sup>®</sup> F-68-supplemented culture media were either gassed with oxygen alone or layered over non-gassed perfluorodecalin. Addition of 0.01% (w/v) *Pluronic*<sup>®</sup> F-68 to KM8P medium overlaying oxygenated perfluorodecalin produced the greatest increase in mean protoplast growth after 10 d ( $10.8 \pm 1.1\%$ ; Fig. 2.2). This led to normal protoplast-derived cell colony formation by 40 d, thereby confirming that repeated mitotic division in protoplasts was not impaired by culture in the presence of both oxygenated perfluorodecalin and surfactant (Plate 2.2e). Samples of protoplast-derived colonies could be returned by pipetting to the growth conditions/culture medium as routinely used for the original cell suspension, thereby completing the cultural cycle.

#### **2.3.2.5 Image analysis of petunia protoplast-derived cell colony growth**

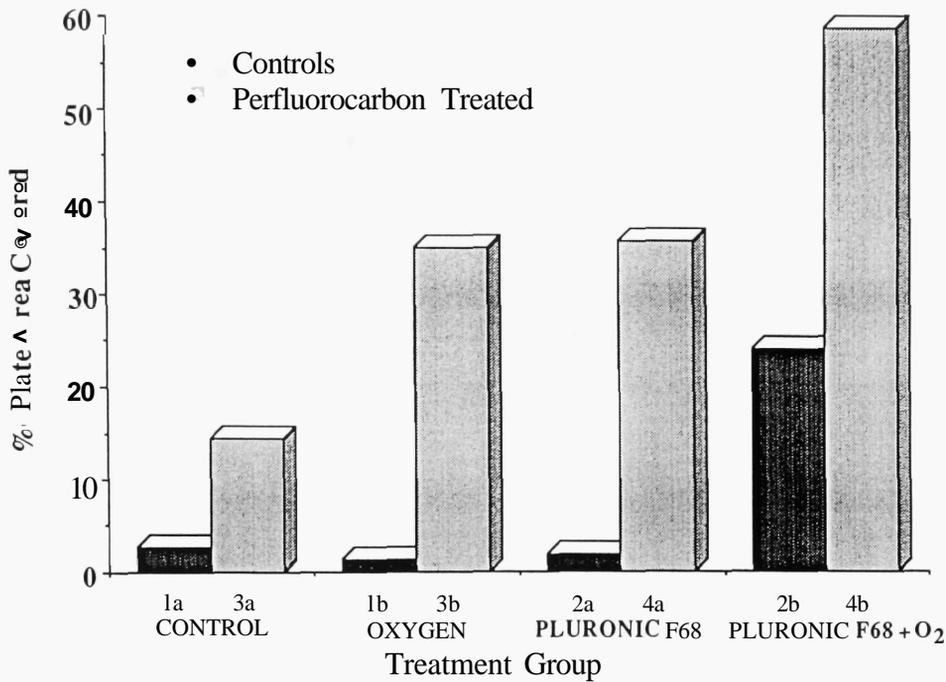
Plate 2.3; Figs. 1-4 show typical protoplast-derived colony formation after 40 d of culture with the various treatments. A naked eye evaluation of plates showed major differences in protoplast growth, with untreated controls characteristically showing typically small, non-proliferated colonies. In contrast, the treated protoplast cultures show extensive regions of cell growth and colony formation, the latter leading, in some cases, to intergrowth of discrete cell colonies somewhat equivalent to artificial tissue production.

**Plate 2.3 Culture and development of *Petunia hybrida* cell suspension protoplast-derived colonies from PFC/aqueous media interfaces and with *Pluronic*® F-68**

Protoplast-derived cell colonies of *P. hybrida* cultured in the light on agar-solidified UM medium showing progressive mitotic activity leading to higher micro-colony throughput; development recorded 68 d after protoplast isolation. Protoplasts were initially cultured in the dark for 40 d under the following conditions prior to transfer to UM medium at day 40: (1a) KM8P/KM8 (3:1 v/v) liquid medium, (1b) Oxygenated (100% oxygen; 10 mbar, 15 min) KM8P/KM8 liquid medium, (2a) KM8P/KM8 liquid medium + 0.01% (w/v) *Pluronic*® F-68, (2b) Oxygenated KM8P/KM8 liquid medium + 0.01% (w/v) *Pluronic*® F-68, (3a) KM8P/KM8 liquid medium overlaying non-oxygenated perfluorodecalin, (3b) KM8P/KM8 liquid medium overlaying oxygenated (100% oxygen; 10 mbar, 15 min) perfluorodecalin, (4a) KM8P/KM8 liquid medium + 0.01% (w/v) *Pluronic*® F-68 overlaying non-oxygenated perfluorodecalin, (4b) KM8P/KM8 liquid medium + 0.01% (w/v) *Pluronic*® F-68 overlaying oxygenated perfluorodecalin. (1a-4b inclusive, x0.9).

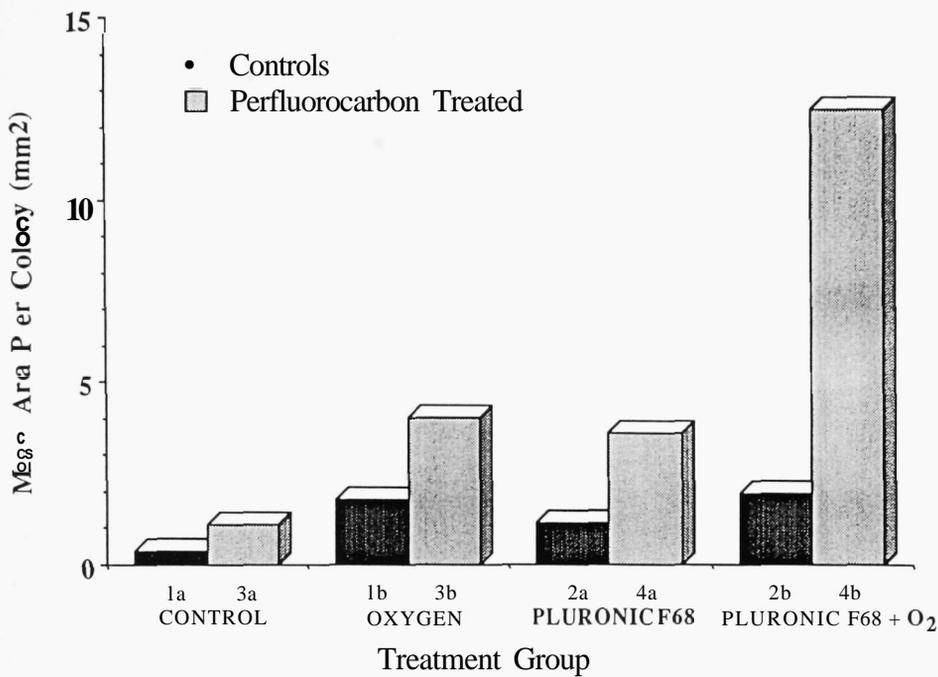


**Fig. 2.3 Percentage of plate area covered by *P. hybrida* protoplast-derived cell colonies**



The percentage area covered (Fig. 2.3) and the mean area per colony (Fig. 2.4) varied from  $2.8 \pm 0.9\%$  and  $0.36 \pm 0.11 \text{ mm}^2$  respectively for untreated controls to  $58.5 \pm 2.1\%$  and  $12.5 \pm 0.5 \text{ mm}^2$  for colonies derived from protoplasts which had previously been cultured on oxygenated perfluorodecalin in the presence of 0.01% (w/v) *Pluronic*<sup>®</sup> F-68. The percentage coverage of the plate for each PFC-treated group was significantly greater than that for the corresponding group not treated with PFC ( $P < 0.001$  for all groups). In addition, oxygenation increased the percentage coverage significantly ( $P < 0.001$ ) for all treatment groups except for oxygenation of aqueous culture medium alone. It is also noteworthy that colony area tended to remain below approx.  $4 \text{ mm}^2$  in all treatments, with the exception of the groups receiving PFC, oxygen and *Pluronic*<sup>®</sup> F-68. These colonies had a mean area of  $12.5 \text{ mm}^2$ , an increase of 35-fold over the untreated controls ( $P < 0.001$ ).

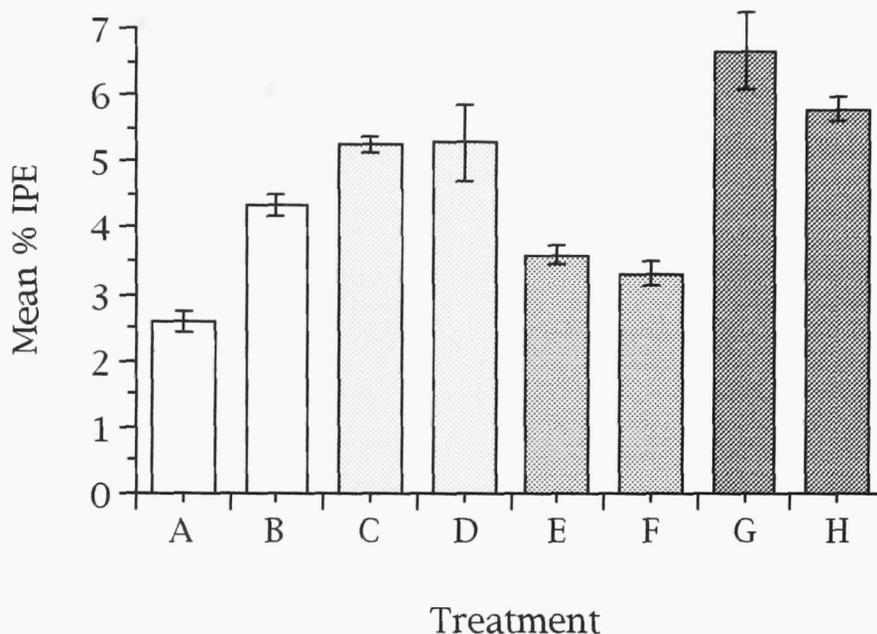
**Fig. 2.4 Mean area (mm<sup>2</sup>) per colony of *P. hybrida* protoplast-derived cell colonies**



### 2.3.3 Effect of various respiratory gas-manipulation treatments on mitotic division of cultured cassava protoplasts

The yield of cassava leaf protoplasts was  $6.9 \pm 0.9 \times 10^6$  g f. wt., with a mean viability of  $74 \pm 4\%$ . During the first 25 d of culture, a 68% increase ( $P < 0.05$ ) in the mean number of protoplasts entering their first mitotic division, as assessed by IPE, occurred in dishes with glass rods compared to their untreated controls under a normal gaseous atmosphere (Fig. 2.5, treatments A, B). Culture of protoplasts under an oxygen-enriched atmosphere, but without glass rods, increased their IPE 2-fold ( $P < 0.05$ ) after 25 d, when compared to protoplasts cultured in the absence of glass rods under normal atmosphere conditions (Fig. 2.5 treatments A, C). There was no further elevation (or suppression) of IPE when protoplasts were cultured under an increased oxygen atmosphere and in the presence of rods (Fig. 2.5, treatments C, D).

**Fig. 2.5 Mean IPE of cassava leaf protoplasts after 25 d of culture in response to respiratory gas-manipulation treatments.**



Protoplasts cultured in: A) medium without glass rods in a normal atmosphere (control), B) with glass rods in a normal atmosphere, C) without glass rods but with an oxygen-enriched atmosphere, D) with glass rods and an oxygen-enriched atmosphere, E) without glass rods and with non-oxygenated PFC, F) with glass rods and non-oxygenated PFC, G) without glass rods and with oxygenated PFC or H) with glass rods and oxygenated PFC. Vertical bars represent s.e.m. (n = 3 throughout).

The mean IPE values following culture of protoplasts with oxygenated perfluorodecalin interjacent layer, either without or with glass rods, were significantly greater ( $P < 0.05$ ) than those of the respective control cultures (Fig. 2.5, treatments A, G; B, H, respectively). Indeed, the mean IPE of protoplasts cultured with oxygenated perfluorodecalin in the presence of glass rods ( $5.8 \pm 0.2\%$ ; n = 3) was over 2-fold greater than in the controls ( $P < 0.05$ ) lacking glass rods (Fig. 2.5, treatments H, A). There were no significant differences between IPEs of cassava

protoplasts when cultured under an increased oxygen atmosphere, or with oxygenated perfluorodecalin, irrespective of the presence or absence of glass rods (Fig. 2.5, treatments C, G; D, H, respectively). In addition, the mean IPEs of protoplasts following culture with non-oxygenated perfluorodecalin, with or without glass rods, were significantly different ( $P < 0.05$ ) from their respective controls; an increase in IPE was observed in the absence of rods (Fig. 2.5, treatments, E, A), whereas a reduced IPE occurred in the presence of rods (Fig. 2.5, treatments F, B). Interestingly, protoplast aggregation or the accumulation of phenolic products did not occur in cultures containing glass rods and oxygenated PFC.

## 2.4 Discussion

The present experiments demonstrate that high yields of cassava protoplasts can be routinely obtained from leaves of cultured shoots. These isolated protoplasts can be induced to undergo sustained division to form multicellular colonies when partially embedded glass rods are included in the two-phase culture system. Such use of glass rods, to increase the number of liquid : glass/plastic contact points, provides a simple method for ensuring a sustainable plating efficiency of cassava leaf protoplasts which, hitherto, has not been obtained with other approaches (Byrne, 1984).

The novel system described here probably facilitates a greater gaseous exchange between the liquid phase, containing the **protoplasts**, and the immediate atmosphere above the medium in the dish. In this regard, previous studies have shown that oxygen-enriched atmospheres also enhance the plating efficiency of cultured protoplasts of rape (Brandt, 1991), jute, rice and tomato (d'Utra Vaz *et al*, 1992). Consequently, it will be of interest, in future work, to assess whether combinations of these **physical** and chemical approaches to gaseous manipulation can enhance mitotic division and cell colony formation in other recalcitrant protoplast systems, including protoplasts which have been modified genetically. In cassava, such studies should also involve the use of a broader range of growth regulators in the medium

ultimately to promote plant regeneration from protoplast-derived tissues. Additionally, it may be feasible to regenerate plants directly from protoplasts through somatic embryogenesis without an intervening callus phase. In this respect, embryogenic cell suspensions may be useful source material for the isolation of protoplasts.

To date, studies have shown adenine-type cytokinins to be ineffective growth regulators for inducing differentiation of cassava protoplast-derived callus (Anthony *et al.*, unpublished). In the present study, the substituted pyridyl phenylurea compound, CPPU, was evaluated, since this synthetic cytokinin is effective at very low concentrations on a wide range of species (Fellman *et al.*, 1987) and induces somatic embryogenesis in grape (Matsuta and Hirabayashi, 1989). The induction, by CPPU, of nodular and tuber-like structures in protoplast-derived callus of cassava, indicates that the use of this phenylurea derivative may, ultimately, result in the differentiation of shoots in the cv. M. Thai 8 tested in these experiments and in other cassava genotypes previously considered to be recalcitrant to differentiation when exposed to adenine-type cytokinins. A focus of future studies to further improve protoplast growth and differentiation in this biotechnologically-important plant will be to optimise not only gas supply, but also growth regulator-induced differentiation.

The results of this study also demonstrate, for the first time, that plant protoplasts will grow normally at the interface between perfluorodecalin and an aqueous culture medium. Thus, the technology previously employed in animal cell cultures (Giaever and Keese, 1983; Nabih *et al.*, 1989) can be successfully adapted for plant systems. The finding that division and colony formation from protoplast-derived cells of *P. hybrida* were not affected by oxygen or perfluorodecalin alone, but only by their subsequent combination, demonstrates the dual efficacy of PFCs for both oxygen storage and effective delivery to cells.

Furthermore, the present study also shows that supplementation with *Pluronic*<sup>®</sup> F-68 of the aqueous culture medium overlaying the perfluorodecalin liquid reduces aggregation of protoplasts associated with the onset of cell wall regeneration, probably through surface tension effects. This is consistent with previous findings that *Pluronic*<sup>®</sup> F-68 reduced clumping in insect cell cultures (Murhammer and Goochee, 1990). This is additional to any growth-enhancing effects of this compound, as reported previously for cultured *Solanum dulcamara* protoplasts (Kumar *et al.*, 1992; Lowe *et al.*, 1993).

The finding that *Pluronic*<sup>®</sup> F-68 further enhanced the growth of protoplasts over the increase promoted by oxygenated perfluorodecalin, suggests that these agents promote protoplast division through separate mechanisms. While there is speculation that Pluronics stimulate growth by enhancing cellular uptake of nutrients and/or growth regulators (Lowe *et al.*, 1993), PFCs most probably act by facilitating oxygen transfer directly into individual cells. This is supported by the present finding that the perfluorodecalin phase acted as an oxygen reservoir, progressively transferring the gas into the aqueous medium and, hence, to the protoplasts. However, the possibility that *Pluronic*<sup>®</sup> F-68 can itself facilitate cellular oxygen uptake cannot be discounted. Alternatively, the likelihood that the PFC may have facilitated protoplast and cell division by removing some unspecified growth regulator from the aqueous medium also cannot be discounted. In this context, it has been suggested previously (King *et al.*, 1989) that one possible application of PFCs in cell culture systems could be as scavengers of toxic gaseous by-products of cell metabolism.

The image analysis technique employed in this investigation provided a precise and convenient method for assessing protoplast responses to perfluorodecalin and *Pluronic*<sup>®</sup> F-68. This approach should be valuable in the quantitative assessment of cell growth in a wide range of prokaryotic and eukaryotic cell culture systems.

Image analysis technology will provide an important adjunct to measuring and discriminating accurately protoplast (and cell) growth responses. Traditionally, these have involved assessments of IPE and FPE (Latif *et al.*, 1993) based on sampling after several days and weeks of culture **respectively**. Image analysis provides, particularly for FPE determinations, an alternative option. This eliminates the inevitable task of identifying discrete colonies which, more often than not, have grown together, making the value of FPE assessments somewhat problematical.

The use of PFC as an oxygenating agent is especially advantageous in static cultures of protoplasts in which conventional aeration through agitation (e.g. stirring) cannot be used because of the tendency to promote cell damage. Further advantages of using PFCs as a culture medium support include their inertness, ease of sterilisation by autoclaving, and **re-cycleability** (King *et al.*, 1989), which offsets the relatively high initial cost of these materials. PFCs are especially useful in illuminated cultures, since they have refractive indices similar to water (Riess and Le Blanc, 1982). One improvement of the present system would be to "recharge" the perfluorodecalin with oxygen during culture, thereby maximising the potential for oxygen delivery to growing protoplasts. In addition to facilitating oxygen transfer, it has been emphasised previously (King *et al.*, 1989) that **PFC-supplemented** systems could be valuable for supplying other gases (e.g. carbon dioxide) to anaerobic cultures.

The present experiments further demonstrate that the use of oxygenated perfluorodecalin for enhancing oxygenation of cultured cassava protoplasts is superior to the inclusion, in semi-solid medium, of partially-embedded glass rods, which increase the surface area for gaseous exchange, thus enhancing protoplast division frequency. The present approach extends previous preliminary work which demonstrated that protoplasts of cassava would undergo sustained division when cultured in the presence of glass rods (Anthony *et al.*, 1995a).

The present finding that the increase in division frequency of cassava protoplasts could not be further enhanced by the inclusion of glass rods in cultures supplemented with oxygenated perfluorodecalin suggests that there was no synergism between these oxygenation options. This contrasts with previous work using protoplasts of *P. hybrida* where supplementation of aqueous medium overlaying oxygenated perfluorodecalin with 0.01% (w/v) of the co-polymer surfactant, *Pluronic*® F-68, promoted a further increase in division frequency (Anthony *et al*, 1994a). An extension of the present investigation with cultured cassava protoplasts would be to assess the beneficial effects of supplementing culture medium with *Pluronic*® F-68, which may stimulate protoplast growth by enhancing the uptake of nutrients and/or protect against mechanical damage (Lowe *et al*, 1993).

Woody plants, such as cassava, are generally considered to have relatively poor protoplast plating efficiencies (Ochatt and Power, 1992). The use of oxygenated perfluorodecalin alone, as shown in the present investigation, may go some way towards the enhanced throughput of protoplast-derived multicellular colonies and, in turn, whole plant regeneration.

Previous studies on the culture of protoplasts from woody plants have emphasised that one frequently encountered problem is that of phenolic oxidation caused by aggregation of cells, especially during the initial stages of culture (Ochatt, 1990; Ochatt and Power, 1992). While some success has been achieved in overcoming this problem through the culture of protoplasts as monolayers on polyester discs within the aqueous medium (Russell and McCown, 1986, 1988), the use of oxygenated perfluorodecalin, as in the present study, to prevent protoplast clumping and phenolic oxidation, provides an alternative option. Further studies are needed to evaluate this approach using protoplasts and cells from other woody plant species.

The use of oxygenated PFC in plant cell cultures could provide a handling strategy for culturing small numbers of protoplasts (e.g. flow-sorted heterokaryons) in media droplets on liquid-PFC interfaces. A more efficient protoplast-to-plant system would have implications for the transformation efficacy of protoplasts using direct DNA delivery technologies. In the context of somatic hybridization, one of the crucial stages is the conversion of heterokaryons to somatic hybrid cells. Thus, an improved plating efficiency, as indicated by the present use of oxygenated PFC, is likely to lead to a greater throughput of putative hybrid material and will have profound implications for plant cell biotechnology and genetic manipulation.

## CHAPTER THREE: BIOCOMPATABILITY OF HAEMOGLOBIN-BASED RESPIRATORY GAS CARRIERS IN PLANT PROTOPLAST CULTURE

### 3.1 Introduction

Isolated plant protoplasts are the source material for the genetic manipulation of plants by interspecific and intergeneric somatic hybridisation through protoplast fusion, transformation by direct DNA uptake and the exposure of somaclonal variation (Blackball *et al.*, 1994; Dixon 1994). One crucial requirement for successful protoplast culture and subsequent plant regeneration from protoplast-derived tissues is the provision of an adequate and sustainable oxygen supply. Previous approaches to enhance respiratory gas exchange for cultured protoplasts have included the use of oxygen-enriched atmospheres (d'Utra Vaz *et al.*, 1992), inert, respiratory gas-dissolving PFC liquids (Anthony *et al.*, 1994a, b, 1995b; Lowe 1997, 1998) and liquid-over-agar media-matrices implanted with glass rods to increase the surface area and gaseous exchange of the liquid culture phase (Anthony *et al.*, 1995a).

An additional and novel approach to further facilitate oxygen supply, which has not been evaluated previously for plant cell systems, is the supplementation of aqueous culture media with a commercial haemoglobin (Hb) solution (*Erythrogen*<sup>TM</sup>). Previous research has focused on the assessment of native and, to a greater extent, chemically-modified haemoglobins as vehicles for respiratory gas transport in intact animals and isolated, perfused organs (Zuck and Riess, 1994; Tsuchida, 1995; Winslow *et al.*, 1995). The most widely studied molecules are of human or bovine origins, although recombinant haemoglobins, expressed in *Escherichia coli* (Hoffman *et al.*, 1990) and transgenic mice (Behringer *et al.*, 1989) and pigs (Swanson *et al.*, 1992), are now available. Haemoglobins liberated from animal erythrocytes are normally treated with pyridoxal phosphate or glutaraldehyde to

reduce their oxygen binding to acceptable physiological levels (Zuck and Riess 1994; Tsuchida, 1995). One advantage of using haemoglobin as a respiratory gas-carrying culture medium supplement compared with PFC liquids is that the latter are immiscible in aqueous systems (King *et al.*, 1989; Anthony *et al.*, 1994a; Lowe *et al.*, 1995). However, paradoxically, this enables PFCs to be recovered readily and to be recycled.

The present investigation has employed cell suspension-derived protoplasts of albino *P. hybrida* cv. Comanche, as a model system to assess the effects on mitotic division of protoplasts and their cellular derivatives of improved gas manipulation through supplementation of the culture medium with a commercially-available, stabilised bovine haemoglobin solution, *Erythrogen*<sup>TM</sup>. The effects of haemoglobin were also evaluated alongside the co-polymer surfactant, *Pluronic*<sup>®</sup> F-68, which is commonly employed as a cytoprotectant for cultured animal cells (Goldblum *et al.*, 1990; Papoutsakis, 1991; Handa-Corrigan *et al.*, 1992). This surfactant has been shown previously to promote growth in cultured plant cells, tissues and organs (Lowe *et al.*, 1993, 1994).

Furthermore, to date, the relative merits of Hb compared to PFCs for stimulating the mitotic division of plant protoplasts and protoplast-derived cells have not been assessed. Consequently, the present investigation has also made a direct comparison of these options, alone and in combination, using cell suspension-derived protoplasts of *Passiflora giberti* and *Petunia* as model systems.

## **3.2 Materials and Methods**

### **3.2.1 Culture of petunia suspension cells on medium supplemented with *Erythrogen*<sup>TM</sup>**

Suspension cells of *P. hybrida* cv. Comanche were harvested, 5 d after sub-culture, onto a 45  $\mu\text{m}$  pore size nylon mesh. Aliquots of 0.5 g f.wt. of cells were placed onto

a 5.5 cm diam. Whatman No. 1 filter paper disk (Whatman International Ltd., Maidstone, UK) overlaying 20 ml aliquots of agar-solidified UM medium contained in 9 cm Petri dishes. In some treatments the UM medium was supplemented with *Erythrogen*<sup>TM</sup> (Biorelease Corporation, Salem, USA, obtained from TCS Biologicals, Botolph Claydon, UK), a stabilised bovine haemoglobin solution (103 g l<sup>-1</sup>, pH 7.42), to final concentrations of 1:50, 1:100 or 1:500 (v/v). Following addition of Hb solution at 1:50 (v/v), the resultant pH of the culture medium was found to have increased from 5.8 to 6.1. Therefore, the pH of the UM medium used for other treatments was adjusted, prior to sterilization, to take account of this pH shift. Petri dishes were maintained in the dark at 25 ± 2°C for 15 d, prior to growth assessments.

### **3.2.2 Isolation and culture of petunia protoplasts**

Protoplasts of *P. hybrida* were isolated enzymatically from a well characterised and established cell suspension, as described in Section 2.2.2.2. Protoplasts were cultured in the dark (25 ± 2°C) at a final plating density of 2.0 x 10<sup>5</sup> ml<sup>-1</sup> in 1.5 ml aliquots of liquid KM8P culture medium. The latter medium, containing protoplasts, was dispensed into 3.5 cm diam. Petri dishes (Nunc). All experimental treatments were replicated 5 times.

### **3.2.3 Supplementation of protoplast culture medium with *Erythrogen*<sup>TM</sup> alone or with oxygen or carbon monoxide**

*Erythrogen*<sup>TM</sup> was added to KM8P medium to final concentrations of 1:50, 1:100 or 1:5(X) (v/v). For the former concentration, a further series of replicates were established whereby the medium was gassed with 1(X)% oxygen (10 mbar, 15 min) or 100% carbon monoxide (P/No: 850203; Phase Separations, Deeside, UK; 20 s), immediately prior to the addition of protoplasts.

### 3.2.4 Supplementation of protoplast culture medium with *Pluronic*® F-68

In an additional assessment using non-gassed medium containing 1:50 (v/v) *Erythro*gen™, the aqueous culture medium was also supplemented with 0.01, 0.1 or 1.0% (w/v) of *Pluronic*® F-68 (Sigma). These concentrations of Pluronic were based on those shown previously to stimulate the growth in culture of cell suspension-derived protoplasts of *S. dulcamara* (Kumar *et al*, 1992). All experiments using *Erythro*gen™, alone or in conjunction with other treatments, utilised protoplasts of the same population, in turn isolated from the same homogenous cell suspensions. Dishes were sealed with Nescofilm (Bando Chemical, Kobe, Japan) and maintained under static conditions.

For all treatments, including controls, spent KM8P medium (0.25 ml per dish) was removed after 9 and 15 d and replaced, on both occasions, with an equal volume of KM8 medium lacking *Erythro*gen™. After 45 d of culture, the total population of protoplast-derived cell colonies from one randomly selected replicate 3.5 cm Petri dish was transferred to the surface of 6 ml of agar-solidified (0.6% w/v) UM medium contained in 5.5 cm diam. Petri dishes. Cell colonies/microcalli were further incubated in the dark at  $25 \pm 2^\circ\text{C}$  for 35 d in order to confirm no adverse influence of the treatments on long-term cell viability.

### 3.2.5 Measurement of protoplast viability, growth and biomass

The viability of freshly isolated protoplasts was determined by uptake and cleavage of FDA, prior to counting as described in Section 2.2.1.3. Protoplast growth and mitotic activity was assessed after 9 d, by the IPE, as defined in Section 2.2.1.6. A minimum of 100 protoplasts per Petri dish were scored; each treatment was repeated 5 times. The **biomass** of protoplast-derived cells was recorded as fresh weight after 80 d of culture with the various treatments.

### **3.2.6 Measurement of pH and osmotic pressure in culture medium**

The pH of the culture medium was measured using a Kent EIL7020 pH meter (Kent Industrial Measurements Ltd., Chertsey, UK) and was adjusted, where appropriate, to pH 6.1 using 1.0 M NaOH. The osmotic pressure of culture medium for all treatments was measured using a Wescor 5500 vapour pressure osmometer (Wescor Inc, Logan, Utah, USA) since it was essential to assess whether there were any shifts in pH and/or osmotic pressure associated with the inclusion of *Erythrogen*<sup>TM</sup> in the medium.

### **3.2.7 Comparison of media supplementation with *Erythrogen*<sup>TM</sup> and/or PFC**

#### **3.2.7.1 Initiation and maintenance of passion fruit cell suspensions**

Cell suspensions of *Passiflora giberti* initiated from leaf-derived embryogenic calli were supplied by Dr W. Otoni, Universidade Federal de Viçosa, Viçosa, Brasil. Suspensions were maintained in modified AA2 medium (Miiller and Grafe, 1978; Appendix II) in 250 ml Erlenmeyer flasks with shaking (90 r.p.m.) at  $24 \pm 2^\circ\text{C}$  in the dark. Cell suspensions were sub-cultured routinely every 7 d by transferring 5 ml SCV to 40 ml aliquots of fresh medium.

#### **3.2.7.2 Isolation of protoplasts from cell suspensions of passion fruit and petunia**

Protoplasts were isolated from embryogenic cell suspensions of *P. giberti*, taken 5 d after sub-culture, using an enzyme mixture (20 ml enzyme solution  $\text{g}^{-1}$  fresh weight of tissue) which consisted of 1.0% (w/v) Cellulase RS, 0.1% (w/v) Pectolyase Y23 and 5 mM MES in CPW13M solution, pH 5.8 (designated ENZ101; Appendix I). Incubation was carried out as described in Section 2.2.1.2. The digested tissues were filtered through a 30  $\mu\text{m}$  pore size nylon sieve and the filtrate centrifuged at  $80 \times g$  for 10 min. The protoplast pellet was washed twice in CPW13M solution by repeated resuspension and centrifugation, prior to counting as described in Section 2.2.1.3. Protoplasts of *P. hybrida* were isolated as described in Section 2.2.2.2.

### **3.2.7.3 Culture of passion fruit and petunia protoplasts with *Erythro*<sup>TM</sup> and/or PFC**

Protoplasts of *P. giberti* were cultured, for 6 d, at a density of  $1.0 \times 10^5 \text{ ml}^{-1}$  in liquid KPR medium (Thompson *et al.*, 1986; Appendix II) and those of *P. hybrida*, for 10 d, at a density of  $2.0 \times 10^5 \text{ ml}^{-1}$  in liquid KM8P medium. Protoplast suspensions (2 ml aliquots) were placed in 30 ml glass bottles, either alone, or overlaying 6 ml aliquots of oxygenated (10 mbar, 15 min) perfluorodecalin (*Flutec*<sup>®</sup> PP6). In a further series of treatments, the media were supplemented with 1:50 (v/v) of *Erythro*<sup>TM</sup> solution. This concentration of Hb was selected on the basis of previous studies, which demonstrated the stimulatory effects of this molecule on the mitotic division of cell suspension-derived protoplasts of *P. hybrida* (Anthony *et al.*, 1997a, b). Protoplasts were cultured at  $25 \pm 2^\circ\text{C}$  in the dark.

### **3.2.7.4 Measurement of IPE**

After 6 d (*Passiflora*) or 10 d (*Petunia*) of culture with oxygenated PFC or *Erythro*<sup>TM</sup>, the IPE of protoplasts was measured as described in Section 2.2.1.3. The differential time scale for measurement of IPE between the two culture systems reflected the inherent differences in mitotic cycle times of *Passiflora* and *Petunia*.

### **3.2.8 Medium supplementation with fresh and stored *Erythro*<sup>TM</sup> solutions**

In the present experiments, the Hb solution was evaluated within 3 months of purchase. In a separate assessment, protoplasts of *P. hybrida* were cultured in liquid KM8P medium supplemented with 1:50 (v/v) of *Erythro*<sup>TM</sup> that had been stored for *ca* 30 months at 4°C prior to use (designated stored-Hb). Protoplasts were isolated and cultured as described in Sections 2.2.2.2 and 3.2.2, respectively.

### **3.2.9 Statistical analyses**

Means and standard errors (s.e.m.) were used throughout, with statistical significance between mean values assessed using a non-parametric Moods median test to

determine significant differences and Mann Whitney *U*-tests to identify significant treatments when analysing protoplast plating efficiency. A conventional parametric one-way ANOVA was used to assess pH and osmotic pressure shifts (Snedecor and Cochran, 1989). A probability of  $P < 0.05$  was considered significant.

### 3.3 Results

#### 3.3.1 Growth of petunia suspension cells on medium supplemented with *Erythrogen*<sup>TM</sup>

The addition of Hb to agar-solidified UM medium resulted in a change of medium colouration from pale yellow to red. Interestingly, when suspension cells of *P. hybrida* (Plate 3.1a) were placed onto filter paper disks, overlaying Hb supplemented medium, such cells also exhibited a colour change from white to red after 3-4 d of culture (Plate 3.1b). This did not appear to inhibit cell growth and after a further 7 d of culture actively growing cells had resumed normal colouration. Indeed, the mean fresh weights of cells after 15 d culture on medium supplemented with 1:50 ( $6.71 \pm 0.21$  g;  $n = 10$ ) and 1:100 (v/v) of *Erythrogen*<sup>TM</sup> ( $6.92 \pm 0.25$  g;  $n = 9$ ) were significantly ( $P < 0.05$ ) greater than the mean fresh weight of control cells cultured in the absence of *Erythrogen*<sup>TM</sup> ( $5.33 \pm 0.22$  g,  $n = 10$ ; Table 3.1).

**Table 3.1 Mean fresh weight and dry weight of *Petunia* suspension cells after 15 d culture on medium with *Erythrogen*<sup>TM</sup>**

<i>Concentration of Erythrogen</i> <sup>TM</sup> (v/v)	f wt. (g)	dry wt. (g)
0 (control)	$5.33 \pm 0.22$	$0.15 \pm 0.007$
1:50	$6.71 \pm 0.21^*$	$0.19 \pm 0.004^*$
1: 100	$6.92 \pm 0.25^*$	$0.18 \pm 0.006^*$
<u>1:500</u>	<u><math>5.32 \pm 0.22</math></u>	<u><math>0.14 \pm 0.008</math></u>

Values are mean  $\pm$  s.e.m.;  $n = 9-10$  throughout. \* $P < 0.05$  compared to control mean value.

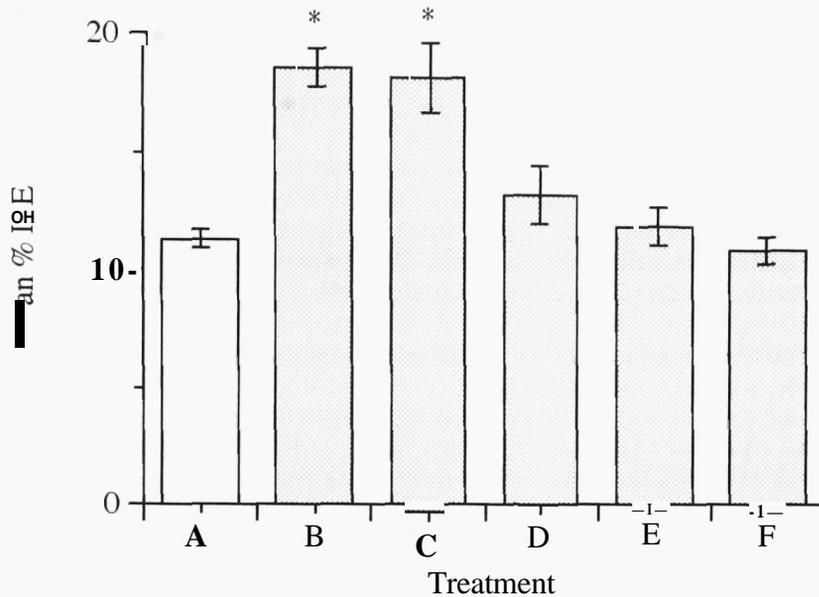
However, there was no significant difference between the former treatments. Similarly, the mean dry weights of such cells were also significantly ( $P < 0.05$ ) greater than cells cultured on medium lacking *Erythrogen*<sup>TM</sup> (Table 3.1). In addition there was no significant difference between the mean dry weights of cells cultured on medium with 1:50 and 1:100 (v/v) of *Erythrogen*<sup>TM</sup> (Table 3.1).

### **3.3.2 Effect of medium supplementation with *Erythrogen*<sup>TM</sup> on mitotic division of cultured petunia protoplasts**

The yield of petunia protoplasts was  $6.24 \pm 0.45 \times 10^5$  g.f.wt of suspension cells, with a mean viability of  $94 \pm 1\%$  ( $n = 3$ ). Culture of protoplasts in KM8P medium supplemented with 1:50 (v/v) of *Erythrogen*<sup>TM</sup> stimulated protoplast division (Plate 3.1c), with an increase in mean IPE of 64% ( $P < 0.05$ ) over control, after 9 d (Fig. 3.1). In contrast, the mean IPE values after 9 d in medium supplemented with 1:100 or 1:500 (v/v) of *Erythrogen*<sup>TM</sup> were not significantly different to control (Fig. 3.1).

For cultures supplemented with 1:50 (v/v) *Erythrogen*<sup>TM</sup> and gassed with oxygen, the mean ( $\pm$  s.e.m.) IPE ( $18.1 \pm 1.5\%$ ,  $n = 5$ ) was significantly ( $P < 0.05$ ) greater than control ( $11.3 \pm 0.4\%$ ,  $n = 5$ ), but was not significantly different from cultures containing the same, but ungasped, quantity of *Erythrogen*<sup>TM</sup> ( $18.5 \pm 0.8\%$ ,  $n = 5$ ; Fig. 3.1). Similarly, for cultures containing 1:50 (v/v) *Erythrogen*<sup>TM</sup>, but gassed with carbon monoxide, the mean IPE after 9 d ( $13.1 \pm 1.2\%$ ,  $n = 5$ ) was also not significantly different to control (Fig. 3.1).

**Fig. 3.1 Mean IPE of *P. hybrida* cell suspension-derived protoplasts after 9 d of culture in medium supplemented with *Erythrogen*<sup>TM</sup>**



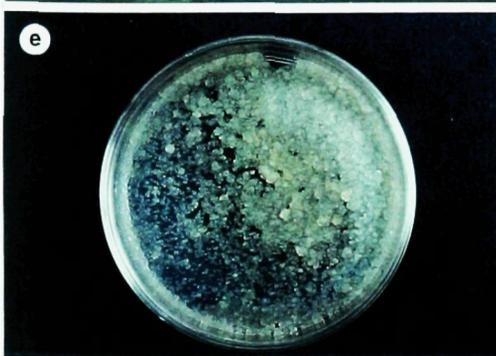
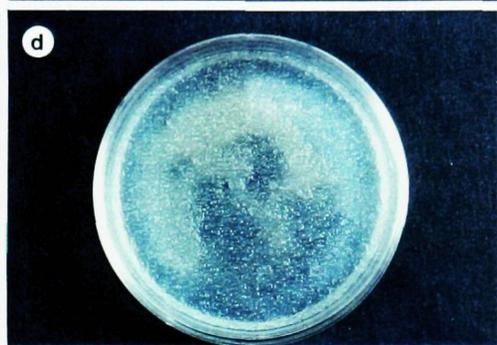
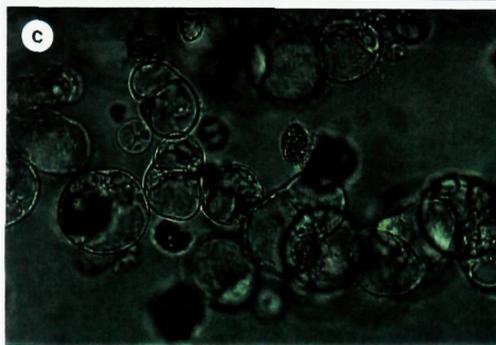
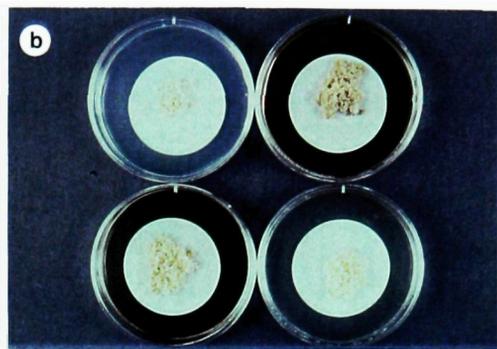
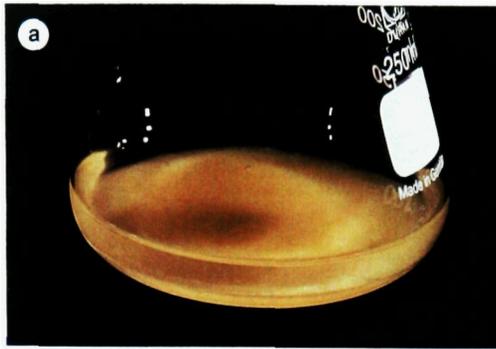
A) medium alone (control), B) *Erythrogen*<sup>TM</sup> (1:50 v/v), C) *Erythrogen*<sup>TM</sup> (1:50 v/v) with 100% oxygen, D) *Erythrogen*<sup>TM</sup> (1:50 v/v) with 100% carbon monoxide, E) *Erythrogen*<sup>TM</sup> (1:100 v/v) and F) *Erythrogen*<sup>TM</sup> (1:500 v/v). Vertical bars represent s.e.m. (n = 5 throughout). \*P < 0.05 compared to control mean value.

### 3.3.3 Evaluation of medium supplementation with *Pluronic*<sup>®</sup> F-68 on culture of petunia protoplasts

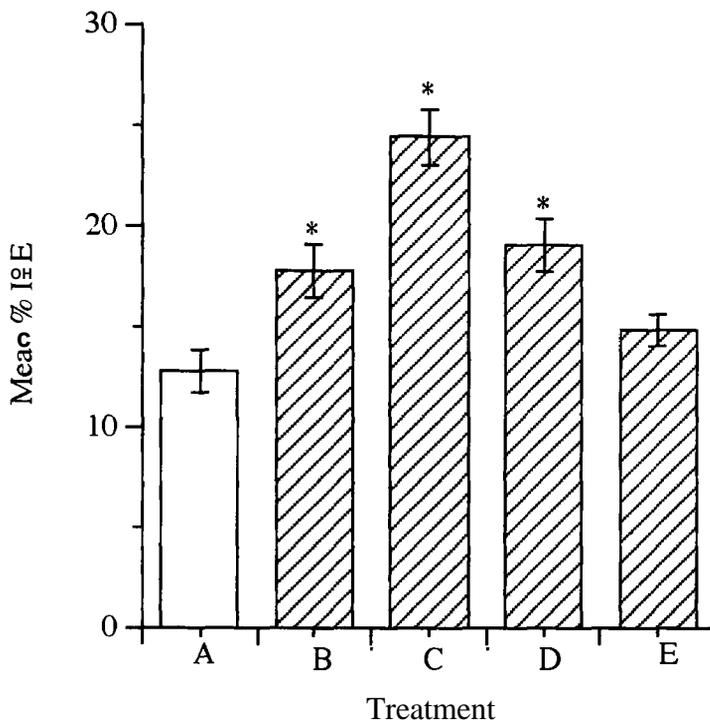
The addition of 0.01% (w/v) *Pluronic*<sup>®</sup> F-68 to cultures containing 1:50 (v/v) of *Erythrogen*<sup>TM</sup> further increased the mean IPE after 9 d of culture by 38% (Fig. 3.2). In contrast, there was no additional beneficial effect of supplementing culture medium with 0.1% or 1.0% *Pluronic*<sup>®</sup> F-68 where the mean IPE after 9 d ( $19.1 \pm 1.3\%$  and  $14.8 \pm 0.8\%$ , respectively n = 5) was not significantly different to control ( $12.7 \pm 2.5\%$ , n = 5; Fig. 3.2).

**Plate 3.1 Culture of cell suspension-derived *Petunia hybrida* protoplasts and/or cell suspension cultures in media supplemented with *Erythro*<sup>TM</sup>**

(a) Cell suspension of *P. hybrida* cv. Comanche in UM medium, 7 d after routine sub-culture (xO.62), (b) Suspension culture-derived cells of *P. hybrida* showing increased mitosis and biomass on 5.5 cm diam. filter paper disks after 5 d culture on (top left) UM agar-solidified medium alone, (top right) UM medium supplemented with 1:50 (v/v) *Erythro*<sup>TM</sup>, (bottom left) UM medium supplemented with 1:100 (v/v) *Erythro*<sup>TM</sup>, (bottom right) UM medium supplemented with 1:500 (v/v) *Erythro*<sup>TM</sup> (xO.24), (c) *P. hybrida* protoplasts undergoing first mitotic division after 7 d of culture in liquid KM8P medium supplemented with 1:50 (v/v) *Erythro*<sup>TM</sup> (x100), (d, e, f, g, h) Petri dishes containing *P. hybrida* protoplast-derived cell colonies showing increased mitosis and micro-colony formation on agar-solidified UM medium (80 d old at time of assessment). Protoplasts were initially cultured for 45 d under the following conditions prior to transfer to UM medium at day 45: (d) KM8P/KM8 (3:1 v/v) liquid medium, (e) KM8P/KM8 (3:1 v/v) liquid medium + 1:50 (v/v) *Erythro*<sup>TM</sup>, (f) KM8P/KM8 (3:1 v/v) liquid medium + 1:50 (v/v) *Erythro*<sup>TM</sup> + 0.01% (w/v) *Pluronic*<sup>®</sup> F-68, (g) KM8P/KM8 (3:1 v/v) liquid medium + 1:50 (v/v) *Erythro*<sup>TM</sup> + 0.1% (w/v) *Pluronic*<sup>®</sup> F-68, (h) KM8P/KM8 (3:1 v/v) liquid medium + 1:50 (v/v) *Erythro*<sup>TM</sup> + 1.0% (w/v) *Pluronic*<sup>®</sup> F-68. (d-h inclusive, xO.78).



**Fig. 3.2 Mean IPE of *P. hybrida* cell suspension-derived protoplasts after 9 d of culture in medium supplemented with *Erythrogen*<sup>TM</sup> and *Pluronic*® F-68**



A) medium alone (control), B) *Erythrogen*<sup>TM</sup> (1:50 v/v), C) *Erythrogen*<sup>TM</sup> (1:50 v/v) with 0.01% (w/v) *Pluronic*® F-68, D) *Erythrogen*<sup>TM</sup> (1:50 v/v) with 0.1% (w/v) *Pluronic*® F-68, E) *Erythrogen*<sup>TM</sup> (1:50 v/v) with 1.0% (w/v) *Pluronic*® F-68. Vertical bars represent s.e.m. (n = 5 throughout). \*P < 0.05 compared to control mean value.

### 3.3.4 Assessment of medium supplementation with *Erythrogen*<sup>TM</sup> on pH and osmotic pressure shifts

Supplementation of culture medium with 1:50 and 1:100 (v/v) *Erythrogen*<sup>TM</sup>, had a significant effect on pH but not osmotic pressure (Table 3.2). Moreover, gassing of culture medium containing 1:50 (v/v) *Erythrogen*<sup>TM</sup> with oxygen or carbon monoxide had no significant effect on mean pH (6.1 throughout) or osmotic pressure ( $764 \pm 7.5$  mOsmol kg<sup>-1</sup> and  $774 \pm 3.1$  mOsmol kg<sup>-1</sup>, respectively), compared to ungasped medium with *Erythrogen*<sup>TM</sup> ( $756 \pm 8.4$  mOsmol kg<sup>-1</sup>). Furthermore, light

microscopy revealed no marked morphological or cytological alterations of protoplasts or protoplast-derived cells in response to any of the culture treatments.

**Table 3.2 Effect of medium supplementation with *Erythrogen*<sup>TM</sup> on pH and osmotic pressure**

Concentration of <i>Erythrogen</i> <sup>TM</sup> (v/v)	pH	osmoticum (mOsmol kg <sup>-1</sup> )
0 (control)	5.73 ± 0.07	736 ± 3.8
1:50	6.13 ± 0.03*	727 ± 1.0
1: 100	6.03 ± 0.03*	724 ± 3.0
1:500	5.73 ± 0.07	736 ± 2.0

Values are mean ± s.e.m.; n = 3 throughout. \*P < 0.05 compared to control mean value.

### 3.3.5 Growth of petunia protoplast-derived cell colonies

Culture of protoplast-derived cells for 45 d with 1:50 (v/v) ungasped *Erythrogen*<sup>TM</sup> produced a 2-fold increase (P < 0.01) in biomass, as reflected by mean fresh weight after a further 35 d of culture on medium lacking *Erythrogen*<sup>TM</sup> (1.9 ± 0.1 g, n = 5, Table 3.3; Plate 3.1e) compared to control (1.0 ± 0.1 g, n = 5, Table 3.3; Plate 3.1d). Culture of protoplast-derived cells for the same period in the presence of 1:50 (v/v) *Erythrogen*<sup>TM</sup> and 0.01% (w/v) *Pluronic*<sup>®</sup> F-68 produced a further significant (P < 0.01) increase in mean fresh weight (2.6 ± 0.1 g, n = 5, Table 3.3; Plate 3.1f) in comparison to control (Table 3.3) and all other treatments. In contrast, there was no additional beneficial effect of supplementing medium containing 1:50 (v/v) *Erythrogen*<sup>TM</sup> with 0.1 or 1.0% (w/v) *Pluronic*<sup>®</sup> F-68 (Table 3.3; Plate 3.1g, h)

**Table 3.3 Mean fresh weight (g) of *P. hybrida* protoplast-derived colonies cultured with 1: 50 (v/v) *Erythroge*<sup>TM</sup> and *Pluronic*<sup>®</sup> F-68**

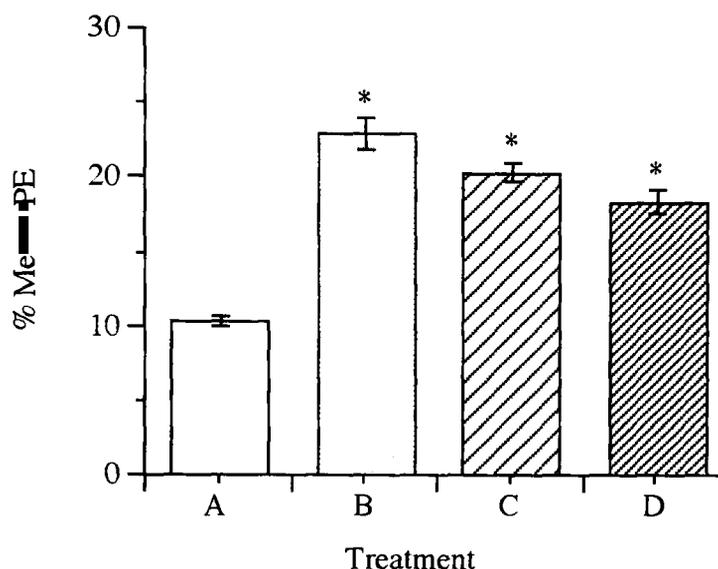
<u>Treatment</u>	<u>Mean f. wt. (g)</u>
control (medium alone)	1.0 ± 0.11
control ( <i>Erythroge</i> <sup>TM</sup> alone)	1.9 ± 0.12*
<i>Pluronic</i> <sup>®</sup> F-68 0.01 % (w/v)	2.6 ± 0.11 *
<i>Pluronic</i> <sup>®</sup> F-68 0.1 % (w/v)	1.2 ± 0.17
<u><i>Pluronic</i><sup>®</sup> F-68 1.0% (w/v)</u>	<u>1.0±0.11</u>

Values are mean ± s.e.m.; n = 5 throughout. \*P < 0.05 compared to control (medium alone) mean value.

### **3.3.6 Evaluation of media supplementation with *Erythroge*<sup>TM</sup> and/or PFC on culture of passion fruit and petunia protoplasts**

The yields of *P. giberti* and *P. hybrida* protoplasts were  $5.83 \pm 0.23 \times 10^6$  g. f. wt and  $2.81 \pm 0.15 \times 10^6$  g. f. wt (n = 3 throughout), with mean viabilities of  $78 \pm 3\%$  (n = 3) and  $80 \pm 2\%$  (n = 3), respectively. The mean IPE of *P. giberti* protoplasts with oxygenated PFC ( $20.3 \pm 0.6\%$ ; n = 5) was significantly (P < 0.05) greater than control ( $10.4 \pm 0.3\%$ , n = 5; Fig. 3.3). A similar, but more pronounced, increase (P < 0.05) in mean IPE of *Passiflora* protoplasts was observed when the medium was supplemented solely with *Erythroge*<sup>TM</sup> ( $22.9 \pm 1.0\%$ , n = 5; Fig 3.3).

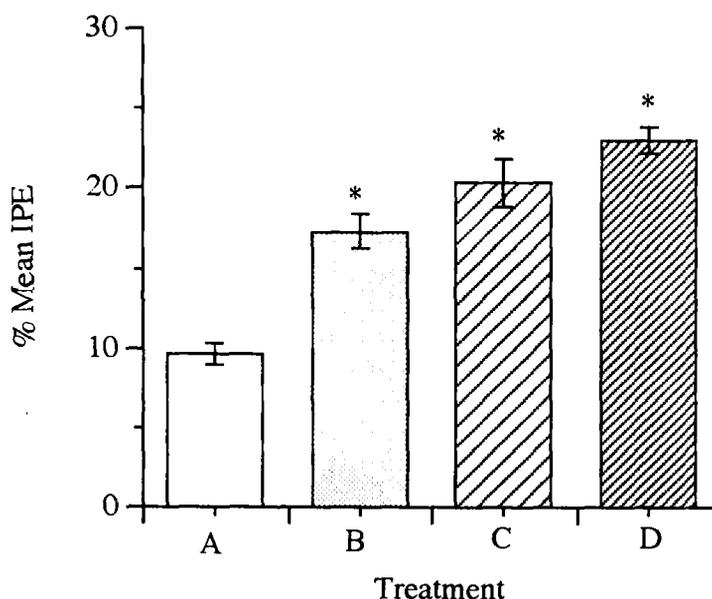
**Fig. 3.3 Mean IPE of *P. giberti* protoplasts cultured for 6 d in medium supplemented with oxygenated PFC and *Erythrogen*<sup>TM</sup>**



A) medium alone (control), B) medium supplemented with 1:50 (v/v) *Erythrogen*<sup>TM</sup>, C) medium overlaying oxygenated PFC or D) medium supplemented with 1:50 (v/v) *Erythrogen*<sup>TM</sup> and overlaying oxygenated PFC. Vertical bars represent s.e.m. (n = 5 throughout). \*P < 0.05 compared to control mean value.

There was no significant difference in mean IPE values for *Passiflora* between the oxygenated PFC and *Erythrogen*<sup>TM</sup> treatments (Fig. 3.3). When the latter two treatments were combined, the mean IPE ( $18.3 \pm 0.8\%$ ; n = 5) was also significantly (P < 0.05) greater than that in untreated controls (Fig. 3.3). Interestingly, there was no significant difference between the mean IPE of the combined treatments and that of protoplasts cultured in the presence of oxygenated PFC alone. However, the mean IPE of protoplasts cultured with oxygenated PFC and *Erythrogen*<sup>TM</sup> in combination was 20% lower (P < 0.05) than that with *Erythrogen*<sup>TM</sup> alone (Fig. 3.3).

**Fig. 3.4 Mean IPE of *P. hybrida* protoplasts cultured for 10 d in medium supplemented with oxygenated PFC and *Erythro*gen™**



A) medium alone (control), B) medium supplemented with 1:50 (v/v) *Erythro*gen™, C) medium overlaying oxygenated PFC or D) medium supplemented with 1:50 (v/v) *Erythro*gen™ and overlaying oxygenated PFC. Vertical bars represent s.e.m. (n = 5 throughout). \*P < 0.05 compared to control mean value.

The mean IPE of *P. hybrida* protoplasts cultured with oxygenated PFC after 10 d of culture ( $20.3 \pm 1.5\%$ ; n = 5 throughout) was significantly ( $P < 0.05$ ) greater than control ( $9.6 \pm 0.6\%$ ; Fig 3.4). In addition, the mean IPE of *Petunia* protoplasts cultured in medium containing *Erythro*gen™ was  $17.3 \pm 1.1\%$  compared to  $9.6 \pm 0.6\%$  in control ( $P < 0.05$ ). As for *Passiflora*, no significant difference in IPE was observed between the oxygenated PFC and *Erythro*gen™ treatments, despite the time-scale difference in the IPE assessments. When the PFC and Hb treatments were combined, the mean IPE ( $23.0 \pm 0.9\%$ ; n = 5) was also significantly ( $P < 0.05$ ) greater than that in untreated controls or with *Erythro*gen™ alone (Fig 3.4). This contrasted with *Passiflora*, where Hb promoted the greatest increase in IPE. However, as with *P. giberti*, there was no significant difference between the mean

IPE of protoplasts cultured with oxygenated PFC and *Erythro*gen™, in combination, and with oxygenated PFC alone.

### 3.3.7 Effect of *Erythro*gen™ efficacy after long-term storage

In a separate experiment comparing the effects of medium supplementation with fresh- or stored-Hb, the mean IPE of *Petunia* protoplasts after 10 d, in the former case ( $26.7 \pm 1.5\%$ ;  $n = 5$  throughout) was significantly greater than with the stored-Hb ( $18.4 \pm 0.9\%$ ). However, the results for both of these treatments were significantly greater ( $P < 0.05$ ) than for the untreated control ( $11.5 \pm 1.0\%$ ; Table 3.4).

**Table 3.4 Mean IPE of *P. hybrida* cell suspension-derived protoplasts cultured for 10 d in medium supplemented with 1:50 (v/v) of fresh- or stored-Hb.**

Treatment	Mean % IPE of protoplasts (10 d)
Control	$11.5 \pm 1.0$
Fresh-Hb	$26.7 \pm 1.5^*$
<u>Stored-Hb</u>	<u><math>18.4 \pm 0.9^*</math></u>

Values are mean  $\pm$  s.e.m.;  $n = 5$  throughout. \* $P < 0.05$  compared to control mean value.

## 3.4 Discussion

The results of these experiments demonstrate, for the first time, that supplementation of medium with *Erythro*gen™ at 1:50 and 1:100 (v/v) dilutions, optimally promoted mitotic division of *P. hybrida* suspension-derived cells over a 15 d culture period, as measured by increases in both mean cell fresh weight and dry weight. Similarly, supplementation of medium with 1:50 (v/v) *Erythro*gen™ enhanced mitotic division in protoplasts of *P. hybrida* over a 9 d culture period. *Pluronic*® F-68 at 0.01%

(w/v) had a synergistic effect by further enhancing protoplast division. This study shows that *Erythroge*<sup>TM</sup>, with or without *Pluronic*<sup>®</sup> F-68, provides a further option for enhancing the growth of cultured plant protoplasts and protoplast-derived cells under static and, perhaps, agitated conditions. This may be especially valuable for facilitating the *in vitro* biosynthesis of phytochemicals, since related studies using cultured animal cells have shown *Erythroge*<sup>TM</sup> not only to stimulate cell division, but also to promote the production of recombinant protein (Goffe *et al*, 1994; Shi *et al*, 1998). This technology could be readily scaled-up to fermenter systems and may be effective in stimulating growth of cultured plant tissues and organs, such as *Agrobacterium rhizogenes*-transformed roots, which are now employed extensively as a source of economically-important plant products (Toivonen, 1993).

The finding that *Pluronic*<sup>®</sup> F-68 further enhanced the growth of *Petunia* protoplasts over that induced by *Erythroge*<sup>TM</sup> alone, suggests that these media supplements stimulate protoplast division through separate mechanisms. *Erythroge*<sup>TM</sup> is believed to "trap" oxygen from air/medium interfaces and to facilitate delivery of this gas directly to cells, concomitantly improving growth (Goffe *et al*, 1994). In addition to alterations in oxygen supply *per se*, it is clearly possible that growth-enhancement produced by supplementing medium with *Erythroge*<sup>TM</sup> may be part-driven by, for example, subtle changes in iron availability to cells, mild buffering of medium pH or the uptake of amino acids from hydrolysed haemoglobin. Clearly, these possibilities, individually or collectively, could be the focus of future studies with eukaryotic cells.

The range of concentrations of *Pluronic*<sup>®</sup> F-68 used in the present study were selected on the basis of previous work, which demonstrated the growth-stimulating effects, in culture, of this agent on isolated protoplasts of *Solanum dulcamara* (Kumar *et al*, 1992). Interestingly, the optimum concentration of 0.01% (w/v) of the surfactant in the present experiments was identical to that found to act synergistically

with the oxygenated PFC liquid, *Flutec*<sup>®</sup> PP5 , in promoting mitosis in *P. hybrida* protoplasts in a static, 2-phase culture system (Anthony *et al*, 1994a).

Studies using animal cells have shown that *Pluronic*<sup>®</sup> F-68 is adsorbed onto cytoplasmic membranes, conferring increased resistance to mechanical damage (Goldblum *et al*, 1990; Handa-Corrigan *et al*, 1992; Lowe *et al*, 1993, 1994). However, the possibility that *Pluronic*<sup>®</sup> F-68 also promotes the uptake of nutrients, growth regulators or oxygen into cultured protoplasts cannot be discounted and should form a basis for future studies.

This study further demonstrates that both oxygenated PFC and *Erythrogen*<sup>™</sup> provide options for enhancing mitotic division, as reflected by increased IPE, of *Passiflora* protoplasts and protoplast-derived cells *in vitro*. Interestingly, the study revealed some variations in the effectiveness of the separate or combined treatments. For *P. giberti*, *Erythrogen*<sup>™</sup> promoted the greatest increase in IPE, whilst for *P. hybrida*, the combined oxygenated PFC and Hb treatments were most effective. Whilst further studies are required, these experiments suggest that the effectiveness of these options for manipulating respiratory gas supply *in vitro* may be species specific. However, the possibility also exists, at least for *Passiflora*, that a combined treatment of Hb and PFC served to suppress mitotic division, through possible oxygen toxicity effects. It may well be that for *Petunia*, such a threshold was not reached.

Furthermore, it will be interesting to determine whether haemoglobin, in combination with *Pluronic*<sup>®</sup> F-68 in aqueous culture medium overlaying oxygenated PFC, will promote protoplast growth over and above the stimulation already achieved by chemical and physical parameters (Anthony *et al*, 1994a, 1995b, 1997a). Future studies should evaluate these strategies in other plant culture systems and determine whether they can be more broadly applied to cultured eukaryotic cells in general. Clearly, such studies should incorporate more detailed assessments,

including measurements of protein biosynthesis, of changes associated with cell growth. This will identify the most effective system for sustaining division of protoplast-derived cells prior to plant regeneration, which will be crucial in maximising plant throughput, especially of genetically-manipulated material.

A further important objective of this study was to assess whether there was any change in the efficacy of the Hb solution following long-term (>1 year) storage at 4°C prior to incorporation into culture medium. The results demonstrate that, whilst there was some diminution in the effectiveness of stored *Erythrogen*<sup>TM</sup> to stimulate mitotic of cultured *Petunia* protoplasts, there remained, nevertheless, a significant benefit in supplementing culture medium with the stored preparation. In contrast, for PFCs which are chemically inert (Lowe *et al*, 1998), their ability to facilitate respiratory gas supply in cell culture systems is undiminished by long-term storage. Thus, the recoverability and recycleability of PFCs, may make them a commercially more attractive option compared to haemoglobin solutions, despite a relatively high investment cost of PFC liquids.

## CHAPTER FOUR: *PLURONIC*® F-68-MEDIATED POST-THAW RECOVERY OF CRYOPRESERVED PLANT CELLS

### 4.1 Introduction

#### 4.1.1 Cryopreservation of plant cell suspension cultures

The storage of cells at ultra-low temperatures in liquid nitrogen is a procedure which has assumed an increasingly important role in underpinning many aspects of biotechnology. De-differentiated plant cells maintained as suspension cultures in liquid medium have been successfully cryopreserved and recovered in a range of agronomically-important crops, including asparagus [*Asparagus officinalis* L.; Nishizawa *et al.*, (1992)], banana [*Musa sapientum* L.; Panis *et al.*, (1990)], barley [*Hordeum vulgare* L.; Fretz *et al.*, (1992)], bramble [*Rubus fruticosus* L.; Lett and Schmitt, (1992)], grape [*Vitis vinifera* L.; Dussert *et al.*, (1991)], millet [*Setaria italica* L.; Lu and Sun, (1991)], orange [*Citrus deliciosa* L.; Aguilar *et al.*, (1993)], pine [*Pinus caribea* L.; Laine *et al.*, (1992)], rice [*Oryza sativa* L.; Meijer *et al.*, (1991)], mbber [*Hevea brasiliensis* L.; Veisseire *et al.*, (1993)] and soybean [*Glycine max* (L.) Men-; Luo and Widholm, (1997)].

Embryogenic cells cultured as suspensions in liquid medium are used routinely as source materials for the isolation of totipotent protoplasts. The latter have been exploited extensively in plant genetic manipulation, particularly in the case of cereals such as rice (Blackball *et al.*, 1994). Such embryogenic cell cultures also provide an alternative to immature zygotic embryo-derived tissues for the production of fertile, **transgenic** rice plants following gene delivery by biolistics (Sivamani *et al.*, 1996). Additionally, embryogenic suspension-cultured cells are also amenable to **Agrobacterium-mediated** transformation (Hiei *et al.*, 1994). In general, the establishment and maintenance of embryogenic cell suspensions of rice, particularly those of **Indica** rices, presents technical difficulties, with the morphogenic

competence of suspensions usually declining with successive sub-culture over prolonged periods (Abe and Futsuhara, 1991). Such changes are often accompanied by the production of morphologically abnormal, infertile plants (Rajasekaran, 1996), particularly from cells of long-established suspensions. Such loss of totipotency is not unique to rice suspension cultures.

Cryopreservation is now exploited as a routine procedure for the long-term storage of biological materials at ultra-low temperatures (Grout, 1995). This technique circumvents the loss of totipotency in plant systems and eliminates or reduces genetic perturbations, together with alterations in secondary product biosynthesis in some cell systems, frequently associated with extended culture of suspensions at physiologically normal temperatures (usually about 25°C). Cryopreservation also negates the labour-intensive requirement to re-initiate periodically and to characterise, in terms of their growth and totipotency, new cell lines. Indeed, storage at ultra-low temperature provides a readily available and constant supply of morphogenetically competent cells (Lynch *et al*, 1994).

#### **4.1.2 Physical and chemical parameters for stimulating post-thaw recovery of cells**

Early cryopreservation studies with rice cell suspensions involved assessments of the freezing and thawing conditions that permit the successful recovery of viable cells and their subsequent growth in culture (Lynch *et al*, 1994). Previously, Meijer *et al* (1991) reported that supporting post-thawed rice cells on filter paper disks, as opposed to placing cells directly on the surface of the culture medium employed during cell recovery, resulted in significant increases in cell regrowth. Subsequently, Lynch *et al*. (1994) also found that the duration of post-thaw culture prior to the transfer to new medium of filter disks supporting the thawed cells was an important consideration, with the correct timing of the transfer decreasing the time required for

the re-establishment of new cell suspensions. Additionally, these workers noted that the recovery of rice cells from freezing in 9 cm diam. Petri dishes was superior compared to their culture in 5.5 cm diam. vessels. Presumably, in the larger dishes, the greater volume of culture medium assisted the rehydration of cells and the diffusion away from the cells of the potentially toxic cryoprotectant, dimethyl sulphoxide (DMSO), together with any oxidation products from the cells. Interestingly, the inclusion of activated charcoal in the cell recovery medium has been demonstrated to have beneficial effects on the post-thaw recovery of suspension cultured cells of grape (Dussert *et al*, 1991), presumably through the absorption of toxic cellular waste products.

#### **4.1.3 Novel post-thaw treatments to enhance oxygen delivery to thawed cells**

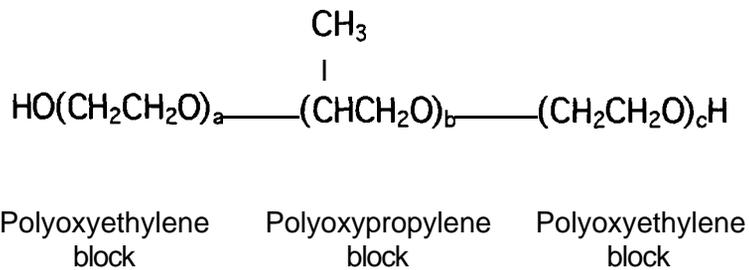
Novel treatments which focus specifically on enhancing oxygen delivery to cells, are important in maximising the post-thaw recovery of plant cells. In recent years, several approaches have been evaluated, including the use of surfactants (Anthony *et al*, 1996, 1997c; Craig *et al*, 1997), perfluorochemical liquids (Anthony *et al*, 1997c) and modified haemoglobin (Azhakanandam *et al*, 1998) as supplements to the culture medium used during the post-thaw recovery phase of cell growth. Cell suspensions of rice have been exploited as a "model" system in several of these investigations, because of the importance of rice as a major cereal crop.

##### **4.1.3.1 Supplementation of culture medium with the surfactant Pluronic® F-68**

The non-ionic, polyoxyethylene (POE)-polyoxypropylene (POP) surfactant, *Pluronic*® F-68 (Fig 4.1), has been employed extensively as a low cost, non-toxic, cell-protecting agent in both animal (Papoutsakis, 1991; Handa-Corrigan *et al*, 1992) and plant (Lowe *et al*, 1993, 1994) culture systems. *Pluronic*® F-68 acts as a foam-stabilising agent in agitated/aerated cultures and is believed to protect cells against fluid-mechanical damage (Handa-Corrigan *et al*, 1989). *Pluronic*® F-68 is

adsorbed onto cell surfaces and studies with both cultured insect cells (Murhammer and Goochee, 1988, 1990; Goldblum *et al*, 1990) and mammalian hybridomas (Zhang *et al*, 1992) have demonstrated that interaction of the surfactant with cytoplasmic membranes can increase the resistance of cells to shear forces, albeit over differing time periods depending on the cell type.

**Fig. 4.1 General chemical structure of *Pluronic*® F-68<sup>1</sup>**



<sup>1</sup>Lowe *et al* (1993). Average values of a, b, and c = 75, 30, and 75, respectively. Approx. molecular weight = 8350

The cytoprotectant properties of *Pluronic*® F-68 make this compound a strong and obvious candidate for use in plant cell cryopreservation protocols. Early studies showed that the Pluronics could prevent haemolysis of human red blood cells in response to freeze-thawing procedures (Glauser and Talbot, 1956), and subsequent studies demonstrated that *Pluronic*® F-68 was an effective cryoprotectant of cultured Chinese Hamster cells (Ashwood-Smith *et al*, 1973). Surprisingly, there has been no evaluation of the effects of using *Pluronic*® F-68 with plant cells, either as a cryoprotectant *per se* or post-thaw cytoprotecting agent, despite the increasing interest in cryopreservation for conserving agronomically-important and endangered plant germplasms (Benson, 1994). Consequently, the present investigation has employed embryogenic cells of Japonica rices, together with non-embryogenic cells of *Lolium multiflorum* (Italian ryegrass), to evaluate the potential beneficial effects of *Pluronic*® F-68, either as a **cell-protectant** during freezing or as a specific culture

medium supplement, in enhancing post-thaw cellular growth following short-term (30 d, *Oryza sativa* cv. Tarom, *Lolium*) or long-term (3 years, *O. sativa* cv. Taipei 309) cryopreservation.

In a further separate assessment cryopreservation protocols were evaluated for non-embryogenic cell suspension cultures of *Moricandia arvensis* (L.) DC. ( $2n=2x=28$ ), within the sub-tribe *Moricandiinae* of the *Brassicaceae*. This dicotyledonous plant species is a wild relative of crop Brassicas, with an intermediate C3 and C4 photosynthetic/photorespiratory system (Holaday *et al*, 1981) giving an efficient recapture of photorespiration-released carbon dioxide. This accounts for a low carbon dioxide compensation point for this plant (McVetty *et al*, 1989). Cell suspensions were initiated as a source of protoplasts for somatic hybridisation and cybrid production with *Brassica napus*, to incorporate, ultimately, the C3-C4 metabolic pathway into oilseed rape. To this end, such suspension cultures required the development of long-term preservation protocols and subsequent post-thaw handling strategies. Cryopreservation for the *Brassicaceae* is restricted to isolated microspores (Chen and Beversdorf 1992a, 1992b) and microspore-derived embryos (Bajaj, 1983; Uragami *et al*, 1993) of *Brassica napus*, together with cell suspensions of *Arabidopsis thaliana* (Ford, 1990; Ribeiro *et al*, 1996). Therefore, an assessment was carried out to develop a cryopreservation protocol for *Moricarulia* suspension cells since, to date, no freeze-thaw handling strategy exists. A further extension of the work was to determine any beneficial growth effects of supplementing the post-thaw recovery medium with *Pluronic*<sup>®</sup> F-68. These cells being representative of a dicotyledonous plant species.

## 4.2 Materials and Methods

### 4.2.1 Rice and *Lolium*

#### 4.2.1.1 Plant materials and preparation of rice and *Lolium* cell suspensions

Cell suspensions of *O. sativa* L. cv. Taipei 309 and *O. sativa* L. cv. Tarom were initiated from embryogenic calli derived from mature seed scutella (Finch *et al.*, 1991). Cell suspensions of *Lolium multiflorum* were supplied by Dr E. Guiderdoni, IRAT/CIRAD, Montpellier, France. Cell suspensions were maintained in AA2 medium [cv. Taipei 309, Abdullah *et al.*, (1986); Appendix II], R2 medium [cv. Tarom, Ohira *et al.*, (1973); Appendix II] and N6 medium [*Lolium*, Chu *et al.*, (1975); Appendix II] in 100 ml Erlenmeyer flasks with shaking (120 r.p.m., 2.5 cm throw) at  $28 \pm 1^\circ\text{C}$  in the dark. Rice cell suspensions (both cvs.) were sub-cultured routinely every 7 d by transferring 1 ml of settled cells with 7 ml of spent medium to 22 ml aliquots of fresh medium; *Lolium* cell suspensions were similarly sub-cultured by transfer of settled cells (3 ml) and 7 ml of spent medium to 40 ml aliquots of fresh medium. Prior to cryopreservation, cells were cultured for 3-4 d in their respective liquid medium supplemented with  $60.0 \text{ g l}^{-1}$  mannitol.

#### 4.2.1.2 Cryoprotection and freezing of rice and *Lolium* suspension cells

The cryopreservation protocol was based on that of Meijer *et al.* (1991). Cells were harvested onto a nylon mesh (45  $\mu\text{m}$  pore size) and placed into 2  $\text{cm}^3$  polypropylene vials (Sarstedt, Leicester, UK) with approximately 0.2 g fresh weight of cells per vial. Approximately 0.75 ml of a cryoprotectant mixture [ $46.0 \text{ g l}^{-1}$  glycerol,  $39.0 \text{ g l}^{-1}$  DMSO,  $342.0 \text{ g l}^{-1}$  sucrose,  $5.0 \text{ g l}^{-1}$  proline] was added to each vial. The cryoprotectant mixture was prepared in the liquid culture medium appropriate for each species and the pH adjusted to 5.8, prior to filter sterilization (0.2  $\mu\text{m}$  pore size; Sartorius). All cryoprotectants were of Analar grade except DMSO, which was spectroscopically pure.

Cells were cryoprotected for 1 h on iced water, vials containing the cells were transferred to aluminium canes, and the cells were frozen at a controlled rate ( $-1^{\circ}\text{C min}^{-1}$ ) from 0 to  $-35^{\circ}\text{C}$  and held at this temperature for 35 min in a programmable freezer (Planer Cryo 10 Series, Planer Biomed, Sunbury-on-Thames, UK), prior to storage in liquid nitrogen at  $-196^{\circ}\text{C}$ . Cells of cv. Tarom and *Lolium* were stored for 30 d, whereas those of cv. Taipei 309 were cryopreserved for 3 years.

#### **4.2.1.3 Post-thaw recovery of cryopreserved rice and *Lolium* suspension cells**

Cells of both species (two cvs. for rice) were thawed by plunging the vials into sterile water at  $45^{\circ}\text{C}$ ; excess cryoprotectants were removed under axenic conditions from the cells using a disposable Pasteur pipette. The cells were placed onto two superimposed 5.5 cm diam. Whatman No. 1 filter paper discs overlying 20 ml aliquots of the appropriate culture medium. The latter was semi-solidified with 0.4% (w/v) SeaKem Le agarose (FMC BioProducts). In some treatments, the medium was supplemented with 0.01, 0.1 or 0.2% (w/v) of *Pluronic*<sup>®</sup> F-68 (Sigma). These concentrations of Pluronic were selected on the basis of previous studies demonstrating the stimulatory effects of this compound on the growth in culture of protoplasts isolated from cell suspensions of *Solanum dulcamara* (Kumar *et al.*, 1992). In an additional assessment, the cryoprotectant mixture for both species was also supplemented with the aforementioned concentrations of *Pluronic*<sup>®</sup> F-68 and cells cryopreserved for 14d in liquid nitrogen.

Cells were cultured for all treatments in the dark for 3 d at  $28 \pm 1^{\circ}\text{C}$  prior to transfer of the upper filter disk containing the cells to the respective fresh media. Cells were cultured for a further 24 h prior to viability assessments and, where appropriate, for a further 26 d, under the same conditions, for biomass determinations. Each experiment was replicated up to 20 times, except where surfactant was incorporated into the cryoprotectant, where  $n = 7$ .

#### 4.2.1.4 Measurement of post-thaw viability and biomass

The post-thaw viability and metabolic capacity of cells were assessed by the reduction of triphenyl tetrazolium chloride (TTC; Steponkus and Lamphear, 1967). Three millilitre aliquots of TTC buffer, consisting of 0.6% (w/v) TTC in 0.05M  $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$  buffer containing 0.05% (v/v) Tween 80 (Sigma, pH 7.4), was added to 50 mg of cells contained in 16.0 ml screw-capped centrifuge tubes. Cells were incubated at  $28 \pm 1^\circ\text{C}$  for 16-18 h in the dark, washed once with distilled water, and extracted with 7.0 ml of 95% (v/v) ethanol in a boiling water bath for 5 min. Supernatants were cooled, and each was adjusted to 10.0 ml with 95% (v/v) ethanol. The absorbance was measured at 490 nm using a Lambdabio spectrophotometer (Perkin Elmer Ltd., Beaconsfield, UK). The same protocol was also employed for unfrozen cells 4d following sub-culture. In some assessments, using both cv. Taipei 309 and *Lolium* cells, the incubation buffer was supplemented with 0.01 or 0.1% (w/v) *Pluronic*<sup>®</sup> F-68, respectively, as a control for any effects of the surfactant on TTC reduction. The fresh weight of thawed cells was recorded to determine biomass (Lynch *et al.*, 1994).

### 4.2.2 *Moricandia*

#### 4.2.2.1 Plant material and establishment of *Moricandia* shoot cultures

Seeds of *Moricandia arvensis* (L.) DC were provided by R.J. Mathias. Seeds were surface sterilised with 15 % (v/v) "Domestos" bleach solution (Lever Industrial Ltd., Runcom, UK.) for 15 min, followed by three washes with sterile tap water. Seeds were germinated in 175 ml capacity glass jars (10 seeds per jar) each containing 50 ml of MS semi-solid medium with 30 g l<sup>-1</sup> sucrose and 0.8 % (w/v) agar, but lacking growth regulators (designated MSO). These, and all subsequent cultures used in this study, were maintained under a 16 h photoperiod ( $21.45 \mu\text{mol m}^{-2} \text{s}^{-1}$ , Cool-White fluorescent tubes; Thorn EMI Ltd.) at  $22 \pm 2^\circ\text{C}$ , unless otherwise stated. Seed-

derived shoots were maintained by transfer, every 28 d, of shoot tips (upper 3 nodes) to fresh medium (4 shoots/jar).

#### **4.2.2.2 Callus initiation and generation of *Moricandia* cell suspensions**

Callus initiation and cell suspension establishment was carried out following modification of published procedures (Toriyama *et al*, 1987). Stem internodes of cultured shoots (28 d after sub-culture) were cut into 0.5 cm lengths and placed longitudinally in 9 cm Petri dishes each containing 25 ml of MS-based medium (designated MSD1; Appendix II) with  $1.0 \text{ mg l}^{-1}$  2,4-D and semi-solidified with 0.4% (w/v) Sea Kem Le agarose. Callus resulting from stem sections was used to produce cell suspensions, the latter being initiated by transferring approx. 0.8 g f wt. portions of rapidly growing, friable 4-6 week-old callus to 8 ml aliquots of liquid MSD1 medium in 50 ml Erlenmeyer flasks, on a rotary shaker (90 r.p.m.). After 7-10 d, the contents of each flask were transferred to 100 ml flasks to which 10 ml of liquid MSD1 medium was added. Those cultures surviving 7-10 d of culture had an additional 10 ml of medium added and became stocks for the subsequent initiation of fast-growing cell suspensions in 250 ml flasks. This was effected by transfer of 1.5 ml packed cell volume (PCV) of cells, with 8.5 ml of partially-spent medium, to a 250 ml flask containing 30 ml of fresh medium (40 ml total culture volume). Thereafter, cell suspensions were sub-cultured every 7 d by transfer of 1.5 ml PCV, as already described.

#### **4.2.2.3 Growth of *Moricandia* cell suspensions**

A known volume of cells (1.5 ml PCV) in 8.5 ml of partially-spent medium was transferred to a 250 ml flask, with a graduated side-arm, the flask containing 30 ml of fresh medium. Cell growth was measured daily, over 15 d, by allowing 10 ml of suspension to settle into the graduated side-arm. The settled cell volume (SCV) was expressed as the volume (ml) of cells in 10 ml of culture. Growth curves were

plotted as a mean of 3 separate assessments; 5 readings were recorded at each sampling time. The morphology and viability as measured by FDA staining (see Section 2.2.1.3 for details) of 3-4 month-old (established) suspensions was assessed every 7 d.

#### **4.2.2.4 Preparation of *Moricandia* cells for cryopreservation**

Cell suspensions were partially dehydrated, 7 d after transfer and prior to cryopreservation, by replacing spent MSDI medium with fresh MSDI medium supplemented with 6 % (w/v) mannitol. The suspensions were cultured for a further 7 d, after which suspensions were sieved (45  $\mu\text{m}$ ) and the spent medium discarded. Cells (0.25 ml PCV) were transferred to 2,0  $\text{cm}^3$  capacity polypropylene vials and freshly prepared filter-sterilised cryoprotectant added (0.75 ml). Two cryoprotectant mixtures, cryoprotectant A (46.0  $\text{g l}^{-1}$  glycerol, 39.0  $\text{g l}^{-1}$  DMSO, 342.0  $\text{g l}^{-1}$  sucrose) and cryoprotectant B (92.0  $\text{g l}^{-1}$  glycerol, 78.0  $\text{g l}^{-1}$  DMSO, 342.0  $\text{g l}^{-1}$  sucrose) as modified from Lynch *et al* (1994), both based on MSDI medium, were assessed. Chilled vials were loaded onto aluminium canes and frozen as described in Section 4.2.1.2, prior to storage in liquid nitrogen.

#### **4.2.2.5 Post-thaw recovery of *Moricandia* cryopreserved cells**

Cells cryopreserved for a minimum of 7 d were recovered from liquid nitrogen by plunging the vials into sterile water at 45°C (10 min). Cells were transferred onto two layers of sterile filter paper (No.1, 5.5 cm; Whatman) overlaying 25 ml aliquots of MSDI medium semi-solidified with 0.8% (w/v) agar, contained in 9 cm Petri dishes. MSDI medium contained *Pluronic*® F-68, at 0, 0.01, 0.1 or 1.0 % (w/v). The contents of one vial were placed in each Petri dish. Cells were cultured for 3 d (dark, 22  $\pm$  2°C), prior to viability assessments, using the TTC assay, as described in Section 4.2.1.4. The remaining cells were transferred, attached to the uppermost filter paper, to fresh MSDI medium with *Pluronic*® F-68, as appropriate. Cells were

sub-cultured every 28 d. Callus f wt. was recorded 28 d and 70 d post-thawing for a minimum of 10 replicate plates for each treatment.

#### **4.2.2.6 Re-initiation of *Moricandia* cell suspensions from cryopreserved cells and isolation of protoplasts**

Callus from *M. arvensis* cells cultured for 80-100 d post-thawing, was used to re-initiate cell suspensions in MSDI liquid medium. Re-initiated suspensions were maintained alongside non-cryopreserved suspensions of the same cell line. Growth curves were plotted for re-established and control suspensions.

Protoplasts were isolated from suspension cells 4 d after sub-culture and 125-130 d after re-initiation of suspensions following modified protocols (Glimelius, 1984). Cells were harvested on a 100  $\mu\text{m}$  mesh nylon sieve and the cells (10-12 ml SCV) incubated (14-16 h;  $22 \pm 2^\circ\text{C}$ ) in a 250 ml flask containing 40 ml of enzyme solution. The latter consisted of 0.03 % (w/v) Macerozyme R10, 2.0 % (w/v) Meicelase (Meiji Seika Kaisha Ltd., Tokyo, Japan) and 2.0 % (w/v) Rhozyme HP150 (Röhms and Haas Co., Philadelphia, USA) in CPW13M solution at pH 5.7. Digestion was carried out in the dark on a rotary shaker (40 r.p.m.).

After incubation, the cmde digests were passed through a 100  $\mu\text{m}$  nylon mesh to remove undigested cell colonies and spontaneous fusion bodies, and the filtrate collected in 9 cm diam. Petri dishes. W5 salts solution (Menczel *et al*, 1981), at approx. one-third volume of the original enzyme-protoplast mixture, was added to the protoplast suspension prior to transfer to 16 ml screw-capped tubes and centrifuging (7 min; 100 x g). The enzyme supernatant was removed, the protoplast pellets resuspended in W5 salts solution (16 ml) and centrifuged. Protoplast pellets were resuspended in W5 solution (2 ml) and purified by layering onto a 21 % (w/v) sucrose solution in 16 ml screw-capped tubes and centrifuging (5 min; 150 x g).

Intact protoplasts forming a layer at the W5 solution-21 % (w/v) sucrose solution interface, were collected by Pasteur pipette and transferred to 16 ml tubes. Protoplasts were washed twice by resuspension in W5 solution with centrifugation (7 min; 100 x g) and finally resuspended in W5 solution prior to yield and viability assessments as described in Section 2.2.1.3. A minimum of 500 cells were counted per culture.

### **4.3 Statistical analyses**

Means and standard errors (s.e.m.) were used throughout; statistical significance between mean values was assessed using a conventional one-way ANOVA and identified using the Tukey-HSD test (Snedecor and Cochran, 1989). A probability of  $P < 0.05$  was considered significant.

## **4.4 Results**

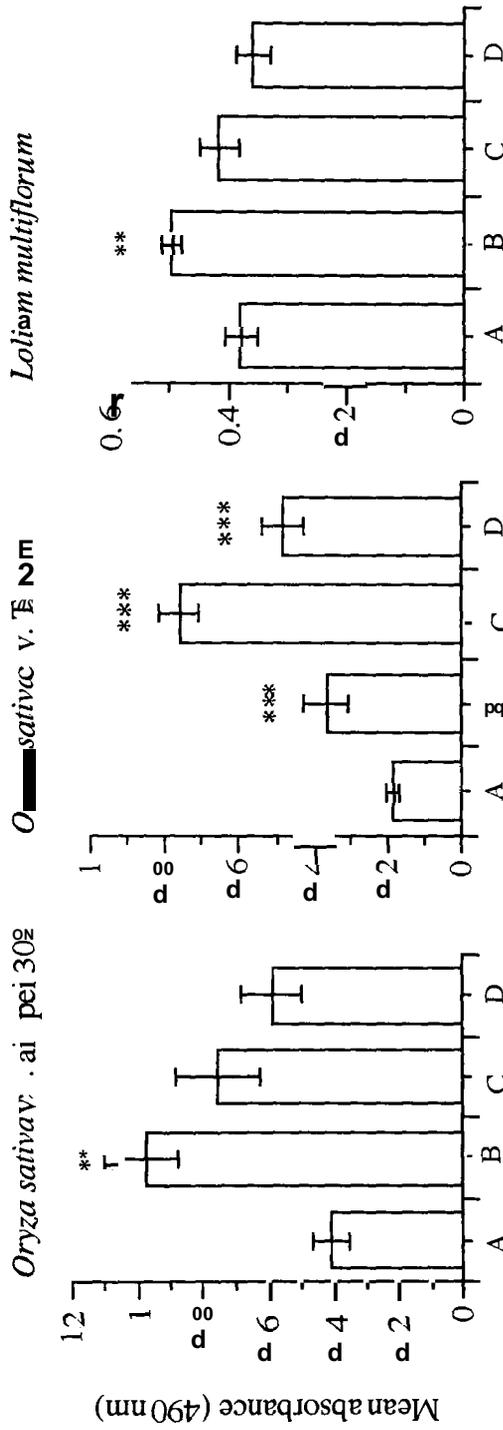
### **4.4.1 Cryopreservation of rice and *Lolium* cells**

#### **4.4.1.1 Post-thaw viability of cryopreserved rice and *Lolium* cells**

The mean absorbance of unfrozen cells, as measured by TTC reduction, was  $1.60 \pm 0.11\%$  ( $n = 10$ ) for *O. sativa* cv. Tarom and  $0.54 \pm 0.04\%$  ( $n = 10$ ) for *Lolium*. In the case of *O. sativa* cv. Taipei 309, the mean cell absorbance, prior to long-term (3 year) cryopreservation, was  $0.96 \pm 0.05\%$  ( $n = 10$ ).

Supplementation of culture medium for the cv. Taipei 309 with *Pluronic*® F-68 at 0.01% (w/v) significantly increased the mean post-thaw cell absorbance following TTC reduction to more than 2-fold greater ( $P < 0.05$ ) than that in untreated controls (Fig. 4.2). A similar, but less pronounced effect also occurred with 0.1% (w/v) surfactant (Fig. 4.2). In contrast, there was no corresponding increase in absorbance in these rice cells when 0.2% (w/v) Pluronic was incorporated into the culture medium.

Fig. 4.1A **is or base, following TTC reduction, by plant cells post-thawed in the presence of Pluronic® F-68**



Concentration of Pluronic® F-68 in cell recovery medium

A) 0%, B) 0.01%, C) 1%, D) 2% (w/v). Values are means of 20 observations; vertical bars represent s.e.m.s. \*\*P < 0.01, \*\*\*P < 0.001 compared to mean control (0% Pluronic).

In the case of *O. sativa* cv. Tarom, supplementation of medium with *Pluronic*® F-68 had a consistently greater stimulatory effect on the mean post-thaw cell absorbance compared with that of untreated controls (Fig. 4.2). The most pronounced increase occurred with 0.1% (w/v) surfactant, which promoted a 4-fold increase ( $P < 0.05$ ) in absorbance over the control. Supplementation of medium for Tarom cells with 0.01% (w/v) or 0.2% (w/v) *Pluronic* increased the mean cell absorbances by 2-fold ( $P < 0.05$ ) and 3-fold ( $P < 0.05$ ), respectively (Fig. 4.2).

A significant ( $P < 0.05$ ) increase in mean post-thaw cell absorbance was also recorded when *Pluronic*® F-68 at 0.01% (w/v) was added to the culture medium for *Lolium* cells, although the mean maximum increase over control of 31% (Fig. 4.2) was markedly lower than that observed when the same concentration of surfactant was used with cells of *O. sativa* cv. Taipei 309 (140%) or cv. Tarom (95%).

#### **4.4.1.2 Post-thaw viability of cryopreserved rice and *Lolium* cells using TTC buffer supplemented with *Pluronic*® F-68**

No significant difference was observed, using post-thawed cv. Taipei 309 cells, between the mean cell absorbance in the TTC assay ( $0.62 \pm 0.05$ ,  $n = 15$ ) and in the same assay using buffer supplemented with 0.01% (w/v) *Pluronic*® F-68 ( $0.61 \pm 0.05$ ,  $n = 15$ ). Comparable results were also obtained with post-thawed *Lolium* cells using 0.1% (w/v) surfactant (control,  $0.41 \pm 0.03$ ,  $n = 15$ ; *Pluronic*  $0.39 \pm 0.04$ ,  $n = 5$ ).

#### **4.4.1.3 Post-thaw growth of cryopreserved rice and *Lolium* cells**

Addition of *Pluronic*® F-68 to culture medium also fostered an increase in biomass, as measured by cell fresh weight following 30 d of post-thaw culture, in both *O. sativa* cv. Taipei 309 and *Lolium*, with significant ( $P < 0.05$ ,  $P < 0.01$ ) increases in the presence of 0.01% (w/v) of the surfactant in both cases (Table 4.1, 4.2). The

greatest increase occurred with cv. Taipei 309 supplemented with 0.01% (w/v) of *Pluronic*® F-68, where the mean fresh weight after 30 d was 32% greater than the corresponding mean control value (Table 4.1; Plate 4.1).

**Table 4.1 Mean fresh weight (g) of cryopreserved cells of *O. sativa* cv. Taipei 309 recovered for 30 d with 0.01-0.2% (w/v) *Pluronic*® F-68 in the culture medium.**

<u>Treatment</u>	<u>Day 0</u>	<u>Day 30</u>
Control (0.0% Pluronic)	0.2	2.07 ± 0.28
Pluronic 0.01%	0.2	2.74 ± 0.13*
0.1%	0.2	2.46 ± 0.11
<u>0.2%</u>	<u>0.2</u>	<u>1.98 ± 0.16</u>

Values are mean ± s.e.m., n = 11 throughout, \*P < 0.05 compared to control (0.0% Pluronic)

**Table 4.2 Mean fresh weight (g) of cryopreserved cells of *L. multiflorum* recovered for 30 d with 0.01-0.2% (w/v) *Pluronic*® F-68 in the culture medium.**

<u>Treatment</u>	<u>Day 0</u>	<u>Day 30</u>
Control (0.0% Pluronic)	0.2	3.49 ± 0.18
Pluronic 0.01%	0.2	4.50 ± 0.20*
0.1%	0.2	3.29 ± 0.21
<u>0.2%</u>	<u>0.2</u>	<u>3.32 ± 0.19</u>

Values are mean ± s.e.m., n = 14 throughout, \*P < 0.05 compared to control (0.0% Pluronic)

**Plate 4.1** *Pluronic*<sup>®</sup> F-68-mediated post-thaw recovery of cryopreserved rice cells (*O. sativa* cv. Taipei 309)



Petri dishes containing post-thawed rice cells of known initial post-thaw fresh weight on 5.5 cm diam. filter paper disks showing increased biomass production (25 d old post-thaw at time of assessment). Cells were initially cultured for 3 d in the dark under the following conditions: (top left) AA2 medium alone (control), (top right) AA2 medium supplemented with 0.01% (w/v) *Pluronic*<sup>®</sup> F-68 (bottom left) AA2 medium supplemented with 0.1% (w/v) *Pluronic*<sup>®</sup> F-68, (bottom right) AA2 medium supplemented with 0.2% (w/v) *Pluronic*<sup>®</sup> F-68. The filter paper disks containing cells were transferred after 3 d to fresh AA2 medium, lacking *Pluronic*<sup>®</sup> F-68 and cultured for a further 27 d in the dark, prior to biomass determinations at day 30 (xO.44).

#### 4.4.1.4 Supplementation of the cryoprotectant mixture with *Pluronic*® F-68 prior to cryopreservation of rice and *Lolium* cells

The addition of 0.01-0.2% (w/v) of *Pluronic*® F-68 to the cryoprotectant mixture, both before and during freezing, did not affect the post-thaw viability of cells from either rice cultivar or *Lolium*. For example, the mean absorbance of *Lolium* cells at 4 d following thawing in the presence of 0.01% (w/v) Pluronic ( $0.74 \pm 0.04$ ,  $n = 7$ ) was not significantly different to control lacking surfactant ( $0.70 \pm 0.04$ ,  $n = 7$ ). A similar pattern was observed with both rice cvs. (Table 4.3).

**Table 4.3 Mean post-thaw absorbance of cells of *O. sativa* cvs. Taipei 309 and Tarom and *L. multiflorum* cryopreserved with 0.01-0.2% (w/v) *Pluronic*® F-68 in the cryoprotectant mixture**

Treatment	Absorbance (490 nm)		
	<i>O. sativa</i> cv. Taipei 309	<i>O. sativa</i> cv. Tarom	<i>L. multiflorum</i>
0% (control)	$0.68 \pm 0.02$	$0.89 \pm 0.02$	$0.70 \pm 0.10$
0.01%	$0.67 \pm 0.07$	$0.94 \pm 0.21$	$0.74 \pm 0.11$
0.1%	$0.74 \pm 0.06$	$1.00 \pm 0.20$	$0.73 \pm 0.17$
0.2%	$0.74 \pm 0.04$	$0.93 \pm 0.17$	$0.76 \pm 0.18$

Values are means  $\pm$  s.e.m.,  $n = 7$  throughout.

#### 4.4.2 Cryopreservation of *Moricandia* cells

##### 4.4.2.1 Comparison of cryoprotectant effectiveness for cryopreservation of *Moricandia* cells

The viability of unfrozen cells, as determined by mean cell absorbance following TTC reduction, was  $19.0 \pm 0.8$ , whereas the viability, of partially dehydrated, non-cryopreserved cells at day 3 was  $13.2 \pm 0.5$  ( $n = 10$  throughout). In the case of frozen cells, there was no significant difference at 3d post-recovery, in the mean

absorbance of cells cryopreserved with cryoprotectant A ( $3.5 \pm 0.4$ ;  $n = 10$ ) or cryoprotectant B ( $2.8 \pm 0.2$ ;  $n = 10$ ). All cells were orange-brown in colour following 3d post-thaw culture.

There was no significant difference in the mean cell viability and the mean f. wt. of cells at 28 d post-thawing of cells treated with cryoprotectants A or B (Table 4.4). However, the mean f. wt. of cells treated with cryoprotectant B, after 70 d of culture was 12 % greater ( $P < 0.05$ ) than that of cells treated with cryoprotectant A (Table 4.4). There was also a significant ( $P < 0.05$ ) increase in f wt. of cells at 70 d compared to 28 d with cryoprotectant B. In addition, cells treated with cryoprotectant B were friable and chlorophyllous in appearance by 70 d of post-thaw culture, compared to those cryopreserved with cryoprotectant A. The latter remained similar in appearance and colour to freshly thawed cells.

**Table 4.4 Effect of cryoprotectant on viability and fresh weight (mg) of *Moricandia* cells 28 and 70 d post-thawing**

Treatment	28 d post-thawing		70 d post-thawing
	<u>Cell viability</u>	<u>Callus</u>	<u>f. wt.</u>
	(Absorbance)	(mg)	(mg)
Cryoprotectant A	$1.08 \pm 0.09$	$120.4 \pm 15.3$	$139.1 \pm 6.9$
Cryoprotectant B	$1.45 \pm 0.21$	$129.2 \pm 8.2$	$156.4 \pm 4.6^*$

Values are mean  $\pm$  s.e.m. ( $n = 10$  throughout), \*  $P < 0.05$  compared to corresponding mean value for Cryoprotectant A.

#### 4.4.2.2 Effect of *Pluronic*® F-68 on recovery of cryopreserved *Moricandia* cells

Cell viability was comparable at all concentrations of Pluronic tested, except at 0.1% (w/v) after 3 d of culture when viability was increased significantly (Table 4.5).

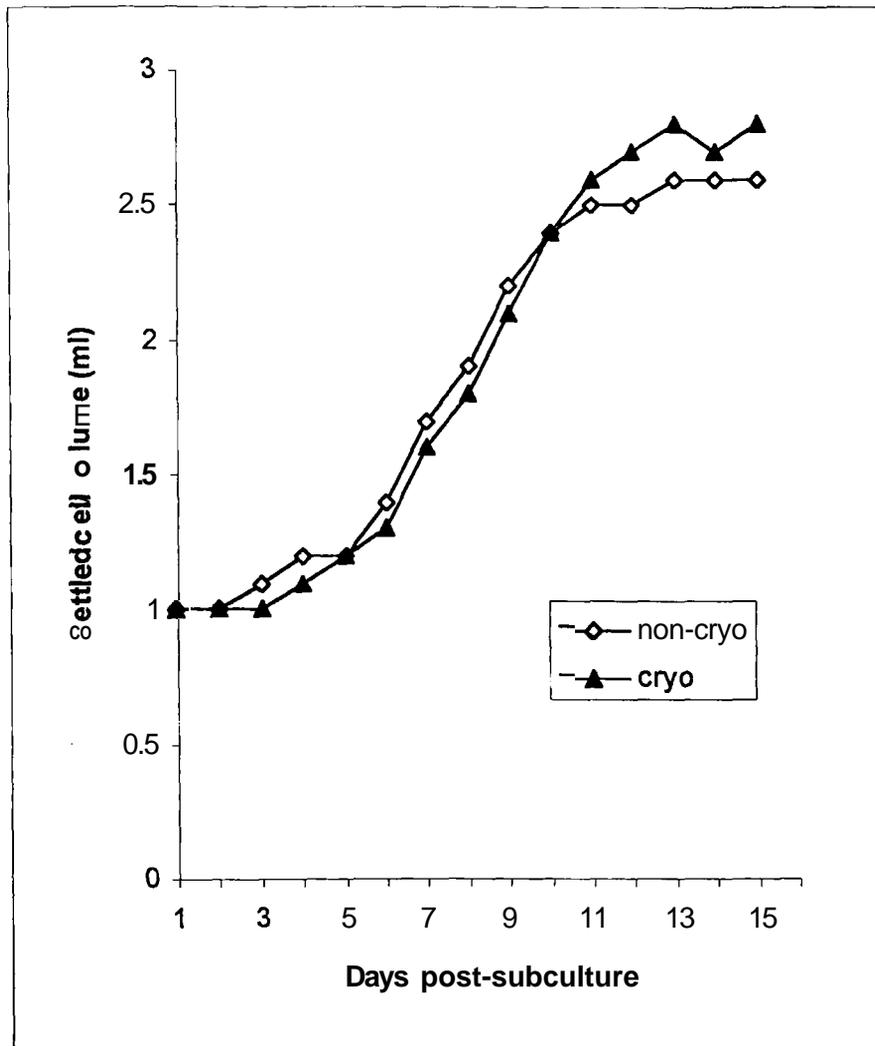
Supplementation of the culture medium with *Pluronic*® F-68 increased biomass, as measured by mean cell f. wt. following 28 d of post-thaw culture, with significant increases at all concentrations of *Pluronic*® F-68 used, compared to the untreated control (Table 4.5). The greatest increase of 76% over control was recorded when 0.01% (w/v) *Pluronic*® F-68 was added to the medium. Similar, less pronounced increases were observed with cells exposed to 0.1 or 1.0% (w/v) *Pluronic*® F-68, where the mean f. wts. were 19% and 7% respectively, above that for cells recovered in the absence of *Pluronic*® F-68. A sustained, 89% increase in mean biomass was recorded for cells thawed in the presence of 0.01% (w/v) *Pluronic*® F-68 compared to control lacking Pluronic, as measured after 70 d of culture. In contrast, there were no significant differences in biomass, after 70 d between cells recovered on medium supplemented with 0.1% or 1.0% (w/v) *Pluronic*® F-68 and cells thawed in the absence of *Pluronic*® F-68 (Table 4.5).

**Table 4.5 Effect of *Pluronic*® F-68 0-1.0% (w/v) on viability and fresh weight of *Moricandia* cells 3 and 28 d post-thawing using cryoprotectant B**

Treatment	Cell viability (3 d) (Absorbance)	Cell viability (28 d) (Absorbance)	Callus f. wt. (28 d) (mg)	Callus f.wt. (70 d) (mg)
0 (Control)	1.39 ± 0.21	2.1 ± 0.3	97.2 ± 1.3	99.6 ± 4.6
0.01	1.95 ± 0.45	2.2 ± 0.1	171.1 ± 1.0***	188.0 ± 6.0***
0.1	3.00 ± 0.31**	1.7 ± 0.2	115.4 ± 1.2***	127.4 ± 8.4
1.0	0.99 ± 0.11	3.1 ± 1.1	104.2 ± 1.2***	117.6 ± 7.0

Values are mean ± s.e.m. (n = 10 throughout), \*\* P < 0.01, \*\*\* P < 0.001 compared to mean value for control treatments lacking surfactant.

**Fig. 4.3** Growth curves of suspension cultures of *Moricandia arvensis*, in MSDI medium, initiated from cryopreserved (cryo) and non-cryopreserved (non-cryo) cells.



#### 4.4.2.3 Re-establishment of *Moricandia* cell suspensions and subsequent isolation of suspension-derived protoplasts

Cells of the suspensions re-established from cryopreserved cells were morphologically similar to cells of the original line. The growth pattern of suspensions re-initiated from cryopreserved cells was very similar to that of the non-cryopreserved control as shown in Fig. 4.3. In addition, the mean viability ( $58.3 \pm 2.7 \%$ ) and yield ( $5.7 \pm 0.6 \times 10^6$ ) of protoplasts from re-established suspensions

were not significantly different ( $P < 0.05$ ) to the corresponding values for control, non-cryopreserved suspensions of the same line ( $55.2 \pm 4.4\%$  and  $6.2 \pm 0.8 \times 10^6$ , respectively).

#### 4.5 Discussion

The results of these experiments demonstrate that supplementation of the culture medium with *Pluronic*® F-68 increases the post-thaw metabolism and growth of cryopreserved rice and *Lolium* cells. While it is generally accepted that reduction of TTC can be used as an indicator of cell viability and metabolism (Benson *et al*, 1992), the present study also includes data on biomass increases to support the conclusion of the growth-enhancing effects of *Pluronic*® F-68. It is probable that the present observations reflect a combination of increased cell survival post-thawing coupled, perhaps, with a growth-stimulating effect of the surfactant in plant culture systems, as reported previously (Lowe *et al*, 1993). Assessment of TTC reduction was made 4 days post-thaw since previous related work indicated that, at this time, the TTC assay provides optimal information on cell metabolic capacity (Lynch *et al*, 1994).

The results of the present experiments also demonstrate that *Moricandia* cell suspensions can be cryopreserved and that cryoprotectant mixture B, consisting of DMSO, glycerol and sucrose, each at 1.0 M, was optimal for successful post-thaw recovery. This study extends the earlier investigation which demonstrated that supplementation of medium with *Pluronic*® F-68 enhanced post-thaw growth of rice and *Lolium* suspension cells. The observation that such use of *Pluronic*® F-68 with *Moricandia* enhances post-thaw division leading to increased biomass production, implies that this relatively low-cost strategy can be readily extended to plant systems in general. The *M. arvensis* cell suspensions recovered from cryogenic storage maintained growth characteristics and levels of protoplast yield and viability

comparable to the original, unfrozen cultures. However, *M. arvensis* cells were slow to recover their normal growth rates after thawing, compared to cryopreserved cells of rice where growth can be readily re-established (Lynch *et al*, 1994). This may be due to the nature of the original non-embryogenic *Moricandia* cell suspensions, since other workers (Lynch *et al*, 1994) observed differential responses of embryogenic and non-embryogenic rice cells to cryopreservation, with embryogenic cells exhibiting significantly faster growth rates than non-embryogenic cells after thawing.

An important finding from this investigation was that the optimum concentration of surfactant, which increased cell growth, differed between the two rice cvs. and between *O. sativa* cv. Tarom and *Lolium*. The present observations, showing that there are both species- and cultivar-specific responses to *Pluronic*® F-68, are consistent with previous observations using *Chrysanthemum morifolium*, in which the optimum concentration of surfactant that stimulated adventive shoot regeneration from cultured leaf explants differed by an order of magnitude between cvs. (Khehra *et al*, 1995). Differences in tissue and organ responsiveness to the growth-promoting effects of *Pluronic*® F-68 have also been observed in *Solanum dulcamara* (Kumar *et al*, 1992), *Corchorus capsularis* (Khatun *et al*, 1993), *Hypericum perforatum* (Bmtovská *et al*, 1994) and *Populus* spp. (Jordan-Costache *et al*, 1995). For example, culture of *C. capsularis* cotyledons with attached petioles in the presence of up to 0.5% (w/v) *Pluronic*® F-68 increased shoot production, with no further stimulation at higher concentrations (Khatun *et al*, 1993). In contrast, maximum growth of transformed roots of *Solanum dulcamara* occurred with 0.01% (w/v) *Pluronic*® F-68 and that of leaf-derived callus with 0.1% (w/v) of the surfactant (Khatun *et al*, 1993). The present approach should be precision tuned for individual species to optimise the concentration of *Pluronic*® F-68 eliciting maximum recovery following cryopreservation.

Studies using animal cells have shown that *Pluronic*<sup>®</sup> F-68 adsorbs onto cytoplasmic membranes, conferring increased resistance to mechanical damage (Handa-Corrigan *et al*, 1992; Lowe *et al.*, 1993, 1994). The Pluronic polyols have hydrophobic POP cores, which are believed to become embedded in the phospholipid membranes of cells, leaving their hydrophilic, POE tails outside. This would have the effect of reducing the interfacial tension of the cells and sterically hindering adhesive interaction between molecules on the cell surfaces. Such reduction in adhesive interaction would prevent cell-to-cell contact and, thus, further reduce mechanical damage.

*Pluronic*<sup>®</sup> F-68 may also promote the increased uptake of nutrients, growth regulators or oxygen into cells during the post-thaw period. Indeed, related experiments using animal cells cultured under static conditions have shown that concentrations of *Pluronic*<sup>®</sup> F-68 comparable to those used in the present investigation, stimulated both 2-deoxyglucose uptake and cellular amino acid incorporation (Cawrse *et al*, 1991). In addition, related evidence from studies with yeast has demonstrated that *Pluronic*<sup>®</sup> F-68, at concentrations comparable to those used here, enhanced the uptake of FDA and antibiotics (King *et al*, 1993; Bassetti and Tramper, 1995). Any increase in nutrient uptake promoted by Pluronic would be expected to alter metabolic flux, allowing biochemical pathways to operate more efficiently, particularly under the stress conditions of early post-thaw recovery. In this regard, previous work has shown respiratory impairment occurs during the early post-thaw period (Cella *et al*, 1982, Benson *et al*, 1992), and it is possible that *Pluronic*<sup>®</sup> F-68 assists in overcoming such perturbations. The adsorption of Pluronic molecules onto the cytoplasmic membranes of post-thawed plant cells may also reduce cellular damage which is known to occur during rehydration when the DMSO cryoprotectant is removed progressively from the system (Benson and Withers, 1987).

Further studies are required to determine the mechanism(s) by which surfactants, such as *Pluronic*® F-68, can facilitate post-thaw survival and growth of plant cells. One focus of such work should be to investigate the effects of *Pluronic*® F-68 on respiratory gas dynamics, since related work has provided evidence that this compound can alter oxygen transport in agitated, sparged bioreactors (Murhammer and Pfalzgraf, 1992). There is also speculation that *Pluronic*® F-68 may influence the release of carbon dioxide from cells (Lowe *et al.*, 1993) or inhibit ethylene production in a manner comparable to that promoted by Triton X-100 (Sauerbrey *et al.*, 1988). Interestingly, experiments have demonstrated that inert, oxygen-dissolving perfluorochemical liquids, emulsified with *Pluronic*® F-68, were effective in prolonging the fertilising capability of turkey spermatozoa stored at lowered temperature (Thurston *et al.*, 1993). Whilst the specific effects of *Pluronic* were not evaluated, it is possible that the surfactant may have contributed to cell survival, either through cytoprotection or, perhaps, by enhancing oxygen and/or nutrient uptake. This earlier work of Thurston *et al.* (1993), together with the results reported in the present study, indicate that the mechanism(s) whereby *Pluronic*® F-68 facilitates survival of both animal and plant cells warrants further detailed study.

The present data strongly suggest that *Pluronic*® F-68, which is relatively inexpensive and commercially available, could be incorporated routinely into post-thaw culture media, to increase plant cell recovery and growth during the post-thaw handling procedures. It will be interesting to extend these studies by evaluating the effects of media supplementation with surfactants with different physico-chemical properties to elucidate the underlying mechanism for their beneficial effects. Such experiments should take account of previous investigations demonstrating that growth enhancement by surfactants in cultured jute tissues was related to the hydrophobic-hydrophilic balance (HLB) of the compound (Khatun *et al.*, 1993).

## CHAPTER FIVE: PFC-MEDIATED POST-THAW RECOVERY OF CRYOPRESERVED RICE CELLS

### 5.1 Introduction

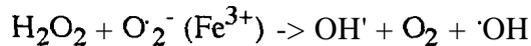
#### 5.1.1 Oxygen free radicals and plant antioxidant systems

Whilst the provision of an adequate oxygen supply is essential for cellular respiratory metabolism (see Section 1.1), the reductive environment of the cellular milieu provides ample opportunities for oxygen to undergo unscheduled reduction leading to the production of reactive intermediates (Davies, 1995). Reduced oxygen species, such as the superoxide and hydroxyl radicals have unpaired electrons in their orbital shells, and it is this instability that causes them to be reactive species as they attempt to restore normal electron pairing. In this respect, such radicals can react with other molecules to produce another new radical. For example, polyunsaturated fatty acids which are important constituents of cell membranes are particularly susceptible to interaction with hydroxyl radicals and this, in turn, leads to the production of lipid peroxy radicals and lipid hydroperoxides that are also reactive (Barclay and McKersie, 1994). In plant systems, as well as being produced as a result of normal cellular metabolism, oxygen free radicals are produced in response to a number of environmental stresses, such as chilling (Shen *et al*, 1997), *in vitro* culture (Benson *et al*, 1997), pollution, (Hippeli and Elstner, 1996), and fungal infection (von-Tiedemann, 1997). However, plants have evolved a wide range of mechanisms to contend with this problem which comprises of a variety of antioxidant molecules such as glutathione (Vanacker *et al*, 1998), ascorbic acid (vitamin C; Wheeler *et al*, 1998) and  $\alpha$ -tocopherol (vitamin E; Navarilzzo *et al*, 1997) together with detoxifying enzyme systems (Gille and Sigler, 1995).

Superoxide ( $O_2^-$ ) is the first radical generated by the reduction of molecular oxygen. Under certain conditions of physiological pH, this molecule will slowly dismutate to

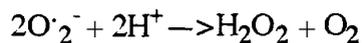
produce hydrogen peroxide, not itself a radical, but a weak oxidizing agent which can react catalytically with transition metal ions (**Haber-Weiss** reaction now called the Fenton reaction; Fig. 5.1) to liberate the highly reactive hydroxyl radical ( $\cdot\text{OH}$ ; Fuller *et al.*, 1988).

**Fig. 5.1 Summary of the Haber-Weiss (Fenton) reaction**



This dismutation of the superoxide radical can also be brought about more rapidly by the action of a group of enzymes known as superoxide dismutases (SOD; Fig. 5.2). The latter are mainly located within the chloroplasts of photosynthetic tissues, since these sites are the most susceptible to toxic radicals, whereas in non-chlorophyllous tissues they are distributed throughout the cytoplasm and mitochondrial membranes. Thus, SOD is an extremely important cellular defence enzyme, since it directly controls the availability of substrates for the Fenton reaction by removal of the superoxide radical.

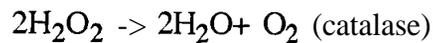
**Fig. 5.2 Reaction catalysed by SOD**



The resultant hydrogen peroxide can be cytotoxic if allowed to accumulate and can re-enter the Fenton reaction to produce hydroxyl radicals. To this end, plants have evolved a catalase enzyme system to dissipate any potential hydrogen peroxide-mediated hydroxyl formation (Fig. 5.3). However, not all of the hydrogen peroxide is metabolised by catalase and plants also contain a second detoxifying enzyme system consisting of several different peroxidases. Unlike SOD and catalase, peroxidases require a substrate for the catalysis of hydrogen peroxide. The nature of the plant substrates still remains unclear but a general scheme has been elucidated from work with spinach chloroplasts and is shown in Fig. 5.3. Moreover,

peroxidases have other functions in plant cells, for example to promote the lignification of plant cell walls utilising free radicals to mediate crosslinking of phenolic groups.

**Fig. 5.3 Reaction catalysed by catalase and peroxidase**



In spite of the presence of such antioxidant enzymes and compounds in plant tissues they are not completely effective in preventing oxidative damage to cells (Davies, 1995).

**5.1.2 Respiratory imbalances during post-thaw recovery of cryopreserved cells**

Cryopreservation comprises several distinct stages, of which freezing is one. Whilst the successful and reproducible recovery of frozen cells depends upon the pre-freezing, cryogenic and post-freezing conditions, the transition of tissues from ultra-low temperature (-196°C) in liquid nitrogen to physiologically normal temperatures and oxygen tensions, induces respiratory imbalances which stimulate the production of toxic oxygen radicals (Fuller *et al*, 1988). In this respect, physiological investigations of cryopreserved rice cells have already demonstrated respiratory impairment associated with post-thaw recovery (Cella *et al*, 1982). Furthermore, in the case of barley, it was observed that pre-treatment of embryogenic cell suspensions for 3 d with high concentrations (29-87 mM) of the antioxidant vitamin C prior to freezing, had a positive influence on post-thaw oxidative stress and lipid peroxidation through the scavenging of oxygen-free radicals (Fretz and Lörz, 1995). It is well known that the presence of transition metals, particularly iron, in the early stages of post-thaw cell recovery can catalyse the production of active oxygen intermediates which react with the polyunsaturated fatty acids of tissue membranes leading to lipid peroxidation (Benson *et al*, 1992). These latter workers investigated

supplementation of the post-thaw recovery medium of rice cells with the iron chelating dmg, desferrioxamine, which had been used previously to reduce oxidative stress in mammalian tissues subjected to low temperatures (Benson *et al*, 1995). However, although positive and beneficial effects were observed in terms of cell viability and cellular regrowth of rice cells, there was no significant reduction in lipid peroxidation as assessed by thiobarbituric acid (TBA) production (Benson *et al*, 1992).

A novel approach for enhancing oxygen supply to post-thaw cryopreserved cells is the use of chemically-inert, oxygen-carrying PFC liquids. These compounds dissolve substantial volumes of key respiratory gases and have been studied in, for example, emulsified form as vehicles for oxygen transport *in vivo* (Lowe, 1994; Lowe *et al*, 1995). PFC liquids have also been used to routinely facilitate oxygen supply to cultured plant protoplasts and protoplast-derived cells at normal temperatures (25°C) using standard tissue culture practices (Anthony *et al*, 1994a; Lowe *et al.*, 1995a, b, 1997). In one investigation, supplementation of an aqueous culture medium overlaying the PFC with a low concentration (*ca.* 0.01% w/v) of the non-ionic, polyoxyethylene (POP) - polyoxypropylene (POE) surfactant, *Pluronic*<sup>®</sup> F-68 further enhanced mitotic division of protoplast-derived cells at the PFC-medium interface (Anthony *et al*, 1994a; Lowe *et al.*, 1995a).

*Pluronic*<sup>®</sup> F-68 has been widely used as a cytoprotectant and growth-promoting additive to animal cell and microbial cultures (Lowe *et al*, 1993, 1994). It has also been evaluated as a cryoprotectant for Chinese hamster cells (Ashwood-Smith *et al*, 1973), but there have been relatively few comparable studies with plant cells. Recently, Anthony *et al* (1996) reported that the incorporation of *Pluronic*<sup>®</sup> F-68 into the recovery medium enhanced the growth of cells of both rice (*Oryza sativa*) and *Lolium multiflorum*. It was speculated, from these experiments, that the beneficial effects *Pluronic*<sup>®</sup> F-68 were primarily mediated through changes in

oxygen flux. Therefore, a strong case can be made for evaluating the post-thaw growth of plant cells cultured in the presence of oxygen-gassed PFC liquid, in combination with *Pluronic*<sup>®</sup> F-68. An objective of the study was to extend earlier findings that mitotic division of cultured protoplasts and protoplast-derived cells can be enhanced by elevated oxygen atmospheres (d'Utra Vaz *et al*, 1992).

In the present investigation, embryogenic (totipotent) suspension cells of rice were used to assess the potential beneficial effects of oxygenated perfluorodecalin, alone and in combination with *Pluronic*<sup>®</sup> F-68, on post-thaw growth following long-term (*ca.* 3 years) cryopreservation.

## **5.2 Materials and methods**

### **5.2.1 Plant materials and preparation of cell suspensions**

Embryogenic calli were induced from mature seed scutella of the Japonica rice, *O. sativa* L. cv. Taipei 309 (Finch *et al*, 1991) and such tissues were pooled and used to initiate cell suspensions. The latter were maintained as described in Section 4.2.1.1. Suspensions were split into two populations. One population was maintained by regular sub-culture for a minimum period of 3 years; the other population was cryopreserved. In the latter case, cells were cultured, immediately before cryopreservation, for 3-4 d in liquid AA2 medium supplemented with 60.0 g l<sup>-1</sup> mannitol.

### **5.2.2 Cryopreservation and post-thaw recovery**

The cryopreservation procedure was based on that of Lynch *et al* (1994). Cells were pooled and harvested from mannitol-supplemented AA2 medium onto a nylon mesh (45 *µm* pore size) before being frozen as described in Section 4.2.1.2. Vials were stored for 3 years in liquid nitrogen at -196°C.

Cells were thawed by plunging the frozen vials into sterile water at 45°C; excess cryoprotectant was removed, under axenic conditions, from the cells using a Pasteur pipette. The cells from individual vials were placed onto the top of two superimposed 2.5-cm diam. Whatman No. 1 filter paper disks overlaying 5.0 ml aliquots of AA2 medium made semi-solid with 0.4% (w/v) Sea Kem Le agarose contained in 100 ml capacity screw-capped glass jars (Beatson Clarke and Co. Ltd.) (Treatment A). In some treatments, the AA2 culture medium was supplemented with 0.01% (w/v) *Pluronic*® F-68 (Sigma; Treatment B), this concentration of Pluronic being selected based on previous studies which confirmed the beneficial effects of this compound on post-thaw growth of cells of *O. sativa* and *L. multiflorum* (Anthony *et al*, 1996). In a further series of treatments, the cells were placed on semi-solidified AA2 medium overlaying 20.0 ml aliquots of oxygenated (10 mbar, 15 min) perfluorodecalin (*Flutec*® PP6; Treatment C). Additionally, the AA2 medium overlaying oxygenated perfluorodecalin, was supplemented with 0.01% (w/v) *Pluronic*® F-68 (Treatment D).

Cells of all treatments were cultured in the dark for 3 d at  $28 \pm 1^\circ\text{C}$  prior to transfer of the upper filter disk, supporting the cells, to fresh medium lacking *Pluronic*® F-68 and/or overlaying ungasped perfluorodecalin, as appropriate. Cells were cultured for a further 24 h prior to viability assessments, followed by another 26 d, under the same conditions, before biomass determinations. Each of the treatments consisted of 20 replicates of cells of the same cell line taken from 20 individual vials.

In order to assess the feasibility of recovering cryopreserved cells on a smaller scale, the post cryopreservation cell handling protocol was further modified, using 24 well Petri dishes (Costar Ltd., High Wycombe, UK). Each well contained 2.0 ml aliquots of oxygenated perfluorodecalin and 1.0 ml of semi-solidified AA2 medium, with the filter papers (1.3 cm diam.; Whatman No.1), overlaid with 50 mg fresh weight of cells. The 50 mg cell aliquots used were each taken from 20 individual vials.

### **5.2.3 Measurement of post-thaw viability and biomass**

The reduction of TTC was used to assess the post-thaw viability of cells based on a modification of the method of Steponkus and Lamphear (1967) as described in Section 4.2.1.4. Biomass was recorded by determining the fresh weight of thawed cells (Lynch *et al.*, 1994).

### **5.2.4 Re-initiation of cell suspension cultures**

Five cell suspensions were re-established from each of the treatments by removing the cells from the surface of 5 randomly selected filter disks, after 30 d of post-thaw culture, and placing into 22 ml aliquots of AA2 liquid medium in 100 ml Erlenmeyer flasks. Suspensions were sub-cultured thereafter every 7 d for 28 d by removing all the spent medium and replacing with the equivalent volume of fresh AA2 liquid medium. Subsequently, suspensions were maintained as described earlier in Section 4.2.1.1.

### **5.2.5 Isolation and culture of protoplasts from re-established cell suspension cultures**

After 8 passages, cell suspensions were used for protoplasts isolations as described by Jain *et al.* (1995). Cells were harvested onto a nylon mesh (45  $\mu$ m pore size) and a pre-weighed aliquot of 1 g f wt. of cells was incubated in 20 ml of enzyme solution as described in Section 2.2.1.2. The enzyme solution (designated ENZ101; Appendix I) which consisted of 1% (w/v) Cellulase RS, 0.1% Pectolyase Y23 and 5mM MES in CPW13M solution, pH 5.8, was filter sterilized and frozen as described in Section 2.2.1.2. The latter was thawed and diluted with CPW13M (1:3), prior to overnight incubation.

Following digestion, the protoplasts were washed, counted and the viabilities assessed as described in Sections 2.2.1.2 and 2.2.1.3, respectively. Protoplasts were suspended in liquid KPR medium (Abdullah *et al.*, 1986; Appendix II) and their

density adjusted to give an overall plating density of  $5.0 \times 10^5$  protoplasts  $\text{ml}^{-1}$ . Protoplasts were spread uniformly in single 200  $\mu\text{l}$  aliquots over the surface of 47 mm diam., 0.2  $\mu\text{m}$  pore size nitrocellulose nitrate filter membranes (Whatman International Ltd.) using a sterile plastic loop (Bibby-Sterilin Ltd.) to cover approx. 90% of the surface area of each membrane. Prior to spreading of the protoplasts on the membranes, the latter were placed on the surface of 20 ml aliquots of KPR medium, semi-solidified with 0.8% (w/v) Sea Plaque agarose and containing suspension cells of *Lolium multiflorum* as a nurse system in 9 cm diam. Petri dishes. Cell suspension cultures of *L. multiflorum* were maintained as described in Section 4.2.1.1. The nurse cells were harvested 3 d after sub-culture and spread at a density of 5 ml PCV in 100 ml aliquots of semi-solidified KPR medium. Individual membranes were placed on the surface of the medium, after a further 24 h, prior to dispensing the protoplasts. Cultures were incubated in the dark at  $28 \pm 2^\circ$  for 14 d. Thereafter, the membranes containing protoplast-derived cells were transferred to 20 ml aliquots of LS2.5 medium (Linsmaier and Skooge, 1965; Appendix II) made semi-solid with 0.4% (w/v) Sea Kem Le agarose contained in 9 cm Petri dishes. Cultures were maintained in the dark as described earlier for a further 14 d.

### **5.2.6 Plant regeneration from protoplast-derived callus**

Protoplast-derived colonies were transferred from the membranes to 20 ml aliquots of MSKN medium, the latter being modified from the formulation of Jain *et al* (1995), and contained in 9 cm Petri dishes (25 colonies/dish). Modified MSKN medium contained maltose [5% (w/v)] and was semi-solidified with 1% (w/v) Sea Kem Le agarose. Cell colonies were cultured in the dark at  $26 \pm 1^\circ\text{C}$  for 10 d, prior to transfer to the same medium, but with the agarose concentration reduced to 0.4% (w/v). Cultures were transferred to the light ( $55 \mu\text{mol m}^{-2} \text{s}^{-1}$ , 16 h photoperiod; Cool White fluorescent tubes) and regenerating shoots (75 per treatment) were detached from individual calli after 40 d and transferred to 175 ml capacity screw-capped glass jars, each containing 50 ml of MS based medium supplemented with

1.5 mg l<sup>-1</sup> NAA, 5% (w/v) sucrose (designated MSN1.5 medium) and semi-solidified with 0.2% (w/v) Phytigel (Sigma). Cultures were maintained under a 16 h photoperiod, as described previously.

### 5.2.7 Cytological analysis of protoplast-derived plants

Cytogenetic studies were based on the chromosome spreading technique developed by Andras *et al.* (1999). Actively growing roots (1 cm in length) were harvested from protoplast-derived *in vitro* rice plants (Treatments A-D), after 21 d of transfer to MSN1.5 medium (Section 5.2.6) and immersed in 1  $\mu$ m trifluralin (Dow AgroSciences Ltd., Hitchin, UK) (1 mM stock solution in DMSO diluted to 1  $\mu$ m in reverse-osmosis water) for 4 h at room temperature. Subsequently roots were fixed in ethanol: glacial acetic acid [3:1 (v/v)] and stored at 4°C for 16 h. Fixed roots were washed 3 times in reverse-osmosis water. Root tips (50; approx. 1 mm in length) were excised and transferred to 40  $\mu$ l aliquots of reverse-osmosis water contained in 3.5 cm Petri dishes. The water was removed and replaced with an equal volume of 1N HCl whereupon root tips were incubated in a humid chamber at 37°C for 15 min.

Following incubation, the HCl was removed and root tips equilibrated (1 min) in an equal volume of TE buffer (10 mM Tris-HCl, 1 mM EDTA; Sigma), pH 8.0. The latter was replaced with initially 10-20  $\mu$ l of an enzyme mixture [4 % (w/v) Cellulase R10, 1% (w/v) Pectolyase Y23] in citrate buffer (0.01M citric acid, 0.01M sodium citrate; pH 4.6) and the root tips gently macerated with a brass rod prior to the enzyme solution being increased to a final volume of 200  $\mu$ l. The material was incubated in a humid chamber at 37°C for 1 h, prior to transfer to a 1.5 ml capacity Eppendorf tube (Scientific Laboratory Supplies Ltd.). Incubation was terminated by the addition of 200  $\mu$ l of reverse-osmosis water and the digested cells were centrifuged (300 x g, 10 min) in a MSE Micro Centaur centrifuge (Jennings Co. Ltd.). The supernatant was discarded and the pellet resuspended in 400  $\mu$ l of reverse-osmosis water, prior to centrifugation and resuspension in 10  $\mu$ l of reverse-

osmosis water. The latter was replaced with 400  $\mu\text{l}$  of freshly prepared methanol: glacial acetic acid [4:1 (v/v)] and centrifuged as described earlier. The pellet was resuspended in 50  $\mu\text{l}$  of the methanol: glacial acetic acid mixture whereupon aliquots (8  $\mu\text{l}$ ) of the resulting suspension were dropped onto individual microscope slides from a height of 10-15 cm and allowed to air dry.

Chromosomes were stained with 15  $\mu\text{l}$  of La Cour acetic orcein solution (R.A. Lambe Ltd., London, UK; Yong, 1997) and a cover slip applied which was sealed with mbbber solution (Dunlop Adhesives, Birmingham, UK), prior to examination on a Nikon Microphot SA microscope.

### **5.3 Statistical analyses**

Means and standard errors (s.e.m.) were used throughout; statistical significance between mean values was assessed, *as* appropriate using a conventional one-way ANOVA coupled with a Tukey-HSD post test and Student's *t* test (Snedecor and Cochran, 1989). A probability of  $P < 0.05$  was considered significant.

### **5.4 Results**

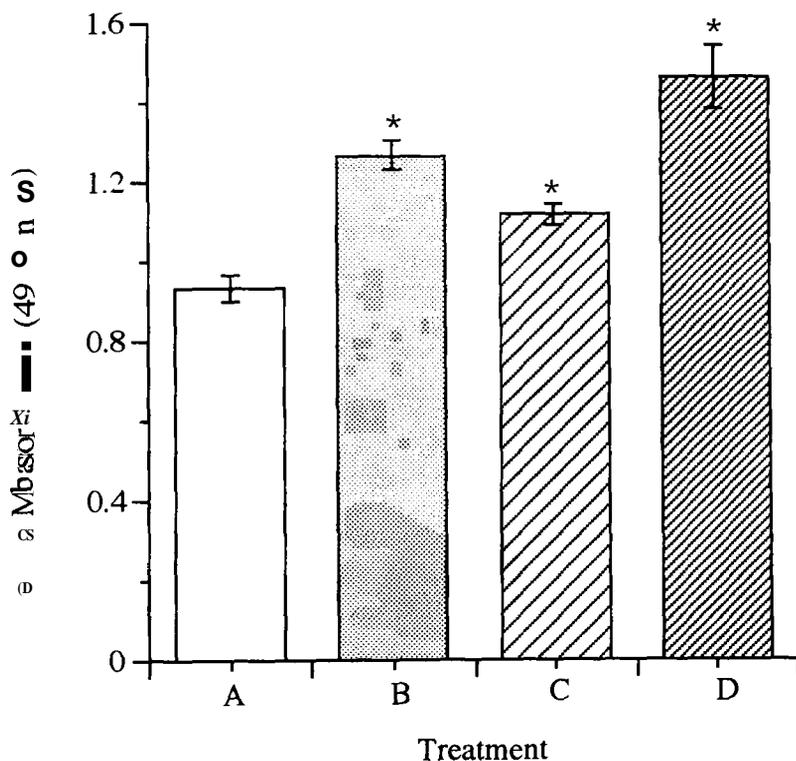
#### **5.4.1 Assessment of PFC medium supplementation on post-thaw cell viability**

The mean absorbance, as an indicator of cell viability, of unfrozen suspension cells was  $0.96 \pm 0.05$  ( $n = 10$ ) prior to long-term (3 year) cryopreservation. The mean absorbance of cryopreserved cells following recovery in the presence of oxygenated perfluorodecalin (Treatment C;  $0.45 \pm 0.07$ ;  $n = 20$ ; Plate 5.1a) was significantly ( $P < 0.05$ ) greater than for the mean of the (control) treatment which lacked perfluorodecalin (Treatment A;  $0.35 \pm 0.08$ ;  $n = 20$ ; Plate 5.1a). Similar differences in post-thaw viabilities were also observed ( $P < 0.05$ ) in the reduced-scale series of experiments when cells were recovered using 24-well Petri dishes (control:  $1.17 \pm 0.06$ ,  $n = 20$ ; oxygenated PFC:  $1.45 \pm 0.07$ ,  $n = 20$ ;  $P < 0.05$ ).

### 5.4.2 Supplementation of medium with PFC and/or *Pluronic*<sup>®</sup> F-68 on post-thaw cell viability

Fig. 5.4 shows the mean absorbance when cryopreserved rice cells were recovered in the presence of 0.01% (w/v) *Pluronic*<sup>®</sup> F-68, oxygenated perfluorodecalin or oxygenated PFC combined with *Pluronic*<sup>®</sup> F-68. The mean absorbance of cells recovered in the presence of 0.01% (w/v) *Pluronic*<sup>®</sup> F-68 (Treatment B;  $1.27 \pm 0.04$ ,  $n = 20$ ) or oxygenated PFC (Treatment C;  $1.13 \pm 0.03$ ,  $n = 20$ ) was significantly greater ( $P < 0.05$ ) than on semi-solid AA2 medium alone (Treatment A;  $0.93 \pm 0.03$ ,  $n = 20$ ; Fig. 1).

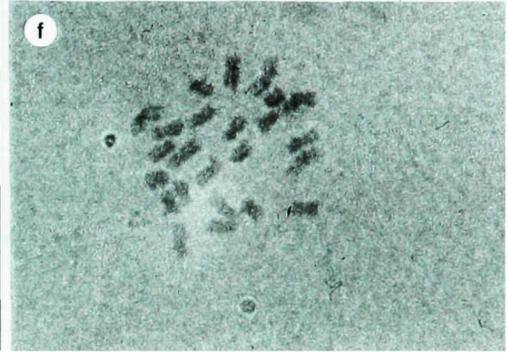
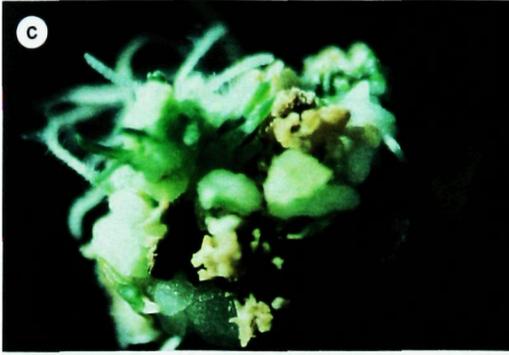
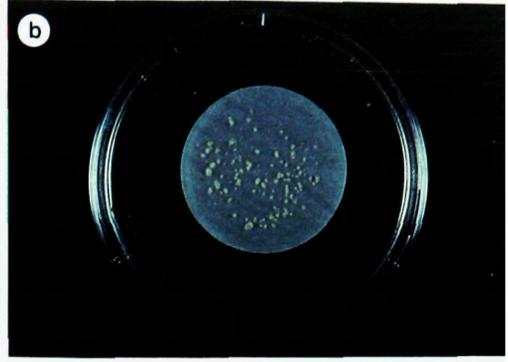
**Fig. 5.4 Mean absorbance of cryopreserved rice cells recovered for 4 d in the presence of various medium supplements**



(A) medium alone (control), (B) medium with *Pluronic*<sup>®</sup> F-68 (0.01% w/v), (C) medium underlaid with oxygenated PFC, and (D) a combination of treatments (B) and (C). Vertical bars represent s.e.m. ( $n = 20$  throughout). \* $P < 0.05$ .

**Plate 5.1 PFC and/or *Pluronic*® F-68-mediated post-thaw recovery of cryopreserved (*O. sativa* cv. Taipei 309) rice cells**

(a) Post-thawed rice cells (15 d old post-thaw at time of assessment). Cells were post-thaw cultured for 3 d in the dark under the following conditions: (left) AA2 medium alone (control), (right) AA2 medium overlying oxygenated (100% oxygen; 10 mbar, 15 min) perfluorodecalin. Cells were subsequently transferred to fresh AA2 medium and AA2 medium overlaying ungasged perfluorodecalin, respectively and cultured in the dark for a further 27 d prior to biomass determinations (x0.66), (b) Petri dish containing protoplast-derived cell colonies (21 d old) originally isolated from cryopreserved rice cells that were post-thaw cultured on AA2 medium overlying oxygenated perfluorodecalin (x0.5), (c) Protoplast-derived callus on MSKN medium undergoing embryogenesis, 50 d after protoplast isolation (x6.24), (d) Shoot proliferation and extension from protoplast-derived callus, 60 d after protoplast isolation (x4), (e) Rooted, protoplast-derived rice plants (100 d old after protoplast isolation) from cryopreserved cells that were cultured post-thaw under the following conditions: (left to right) AA2 medium alone, AA2 medium supplemented with 0.01% (w/v) *Pluronic*® F-68, AA2 medium overlaying oxygenated perfluorodecalin and a combination of the latter two media supplements (x0.27), (f) Somatic (root) chromosome spread of root tips stained with acetic orcein taken from a protoplast-derived rice plant regenerated from cryopreserved cells that were cultured **post-thaw** in the presence of oxygenated perfluorodecalin; chromosomes are consistent with the normal diploid status of rice with  $2n = 2x = 24$  (x1340).

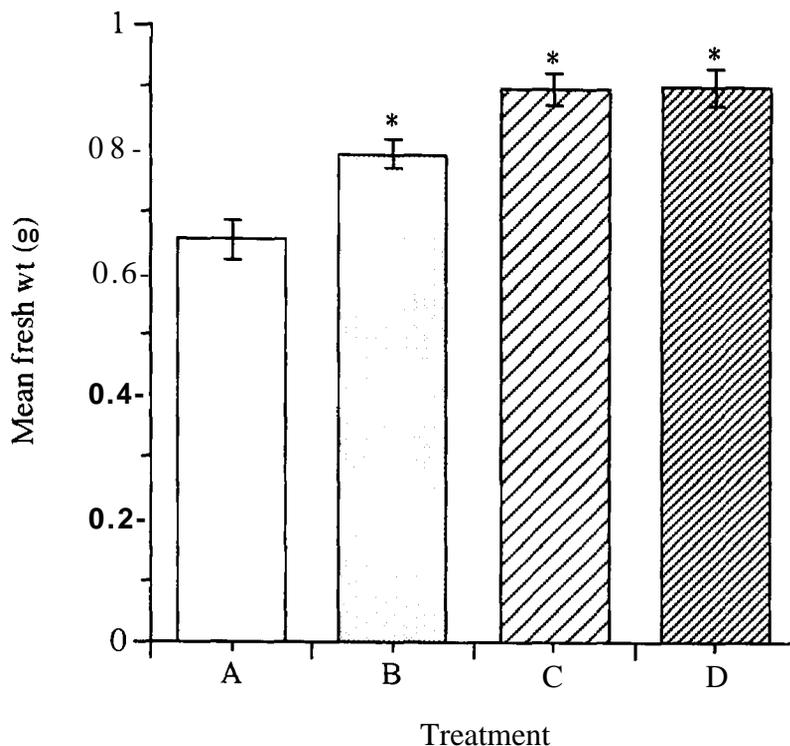


A further synergistic increase ( $P < 0.05$ ) in mean absorbance was also observed when *Pluronic*<sup>®</sup> F-68 and perfluorodecalin were used in combination (Treatment D;  $1.46 \pm 0.08$ ,  $n = 20$ ), compared to all other treatments. No significant differences were observed between Treatment B (0.01% (w/v) *Pluronic*<sup>®</sup> F-68) and Treatment C (oxygenated perfluorodecalin).

### **5.4.3 Post-thaw growth of cryopreserved cells recovered in the presence of PFC and/or *Pluronic*<sup>®</sup> F-68**

The recovery of cells with 0.01% (w/v) *Pluronic*<sup>®</sup> F-68 (Treatment B) or oxygenated perfluorodecalin (Treatment C) promoted sustained mitotic division, since biomass, measured as increase in fresh weight at 30 d post-thaw, was elevated by 21% and 38% ( $P < 0.05$ ), respectively, compared to the biomass of cells in unsupplemented medium (Treatment A; Fig. 5.5). When PFC and *Pluronic*<sup>®</sup> F-68 treatments were combined (Treatment D), a significant ( $P < 0.05$ ) increase in biomass was also observed, that was greater than for Treatment B, but not significantly different to that found for Treatment C (Fig. 5.5).

**Fig. 5.5 Mean fresh weight (g) of cryopreserved rice cells recovered after 30 d in the presence of various medium supplements**



(A) medium alone (control), (B) medium with *Pluronic*® F-68 (0.01% w/v), (C) medium underlaid with oxygenated PFC, and (D) a combination of treatments (B) and (C). Vertical bars represent s.e.m. (n = 20 throughout). \*P < 0.05.

#### **5.4.4 Re-establishment of cell suspension cultures**

Cell suspensions (n = 5), re-initiated simultaneously from cells recovered from all treatments, ultimately exhibited growth rates after 35 d of culture comparable to those of unfrozen suspensions maintained by regular sub-culture every 7 d for 3 years, this time being equivalent to the cryopreservation period.

#### **5.4.5 Assessment of protoplast viability, yield, plating efficiency and plant regeneration**

There were no significant differences, in terms of yields, viabilities, plating efficiencies (Plate 5.1b) and plant regeneration frequencies (Table 5.1; Plate 5.1c, d), of protoplasts isolated from cryopreserved cells exposed to the experimental treatments A, B, C, and D. Additionally, there were no significant differences in terms of protoplast yield ( $4.0 \times 10^6 \text{ g}^{-1} \text{ f.wt}$ ), mean protoplast viability ( $89 \pm 2\%$ ,  $n = 3$ ) and the mean number of protoplast-derived calli regenerating shoots ( $44 \pm 4\%$ ,  $n = 100$ ) between cells subjected to cryopreservation followed by treatments A-D and unfrozen suspension cells assessed prior to their 3 year cryopreservation procedure. Protoplast isolation was not attempted from suspensions maintained continuously for 3 years without cryopreservation, since the suspensions had lost their totipotency, as expected by the end of this culture period.

Table 5.1P Latency efficiency and plant regeneration from protoplasts of rice cells regenerated from cryopreservation in the presence of (A) methylamine (control), (B) medium with *Puronic*® F68 (0.01% w/v) (C) medium and related with oxygenated PFC, and (D) inhibition of treatments (B) and (C).

Treatment	Protoplast yield <sup>a</sup> (g. f. wt. x 10 <sup>6</sup> )	Protoplast viability <sup>b</sup> (%)	Planting efficiency <sup>c</sup> (%) after 30d	% of colonies regenerated plants <sup>d</sup> after 70d
A	3.0 ± 0.0	75.06 ± 3.1	12.5 ± 0.03	38.7 ± 2.7
B	4.97 ± 0.85	76.55 ± 2.9	1.29 ± 0.05	41.3 ± 4.8
C	5.89 ± 0.85	79.86 ± 1.2	1.07 ± 0.04	40.0 ± 2.3
D	5.07 ± 0.09	76.89 ± 2.3	1.27 ± 0.06	37.3 ± 2.7

Values represent mean ± s.e. <sup>a</sup> n = 3, <sup>b</sup> n = 8, <sup>c</sup> n = 2, <sup>d</sup> n = 75.

#### 5.4.6 Cytological analysis of regenerated plants

Plants regenerated from cryopreserved cells that were post-thaw cultured with Treatments A-D were morphologically normal (Plate 5.1e), with expected diploid chromosome complements ( $2n = 2x = 24$ ; Plate 5.1f).

### 5.5 Discussion

The present study utilised the same cell suspension for all treatments in order to eliminate any somaclonal effects. Lack of somaclonal variation was confirmed by there being no significant differences in terms of plant regeneration potential of cryopreserved cells and unfrozen cells of the same suspension as assessed 3 years earlier.

These experiments show that the post-thaw viability and growth of cryopreserved rice cells *per se* is increased in the presence of oxygenated perfluorodecalin either alone, or in combination with *Pluronic*® F-68. Additionally, the culture of rice protoplasts and of their cell derivatives at the interface between oxygen-gassed PFC overlaid with liquid or agarose-solidified culture medium also enhances mitotic division and, in totipotent systems, stimulates shoot formation (Wardrop *et al.*, 1997). This confirms a genuine growth enhancement of this treatment, rather than a marginal effect on cells recovered from cryopreservation. PFC is believed to act as a reservoir for oxygen which diffuses into the aqueous medium/cell phase during initial culture. This is supported by changes in oxygen tension in the medium (Anthony *et al.*, 1994a). It is probable, therefore, that the increased and sustainable post-thaw growth of rice cells was also due to an enhanced oxygen supply provided by the PFC. Indirect evidence for the diffusion of oxygen from the PFC to aqueous culture medium, comes from related observations with suspension-derived protoplasts of *Salpiglossis sinuata*, in which an increase in intracellular superoxide dismutase (SOD) occurred after 3 days culture (Lowe *et al.*, 1997). This, in turn, was consistent with studies using *Mycobacterium* spp., in which SOD was elevated

during culture of the bacterium in perfluorodecalin-supplemented medium (Popkova *et al*, 1988). Lipid peroxidation and protein degradation can occur in the early stages of post-thaw recovery (Fuller *et al*, 1988; Benson *et al*, 1995). Increased SOD biosynthesis associated with culture of protoplasts and protoplast-derived cells with oxygenated PFC may protect cells not only against a supplemented oxygen supply, but also against oxygen radicals generated by impaired oxygen flux during thawing. Further studies should determine the early time-course of changes in SOD and other oxygen-sensitive enzymes during cell recovery.

The present study has extended earlier investigations which demonstrated that culture medium containing *Pluronic*® F-68 increased post-thaw growth of cryopreserved rice cells (Anthony *et al*, 1996). *Pluronic*® F-68 adsorbs onto cell membranes, increasing resistance to mechanical damage (Handa-Corrigan *et al*, 1992; Lowe *et al*, 1993, 1994). Pluronics have hydrophobic POP cores, which are believed to become incorporated into membranes, leaving their hydrophilic, POE tails outside the cell. This would lower the interfacial tension by sterically hindering adhesive interactions between molecules on cell surfaces and, in turn, minimise cell-to-cell contacts, thereby reducing mechanical disruption. However, it is possible that the responses to Pluronic could differ between plant cells with intact walls, and animal cells or plant protoplasts, which have naked plasma membranes, the latter albeit for a finite period of time when in culture.

As discussed previously (Anthony *et al*, 1996), *Pluronic*® F-68 may increase the uptake of nutrients, growth regulators or respiratory gases into cells during post-thawing. Studies with animal cells have demonstrated that *Pluronic*® F-68 at concentrations comparable to those used in the present investigation, stimulated 2-deoxyglucose uptake and incorporation of amino acids (Cawrse *et al*, 1991). Changes in nutrient uptake, promoted by Pluronic, would be expected to alter metabolic flux, allowing biochemical pathways to operate more efficiently,

especially under the stress of initial post-thaw recovery (Anthony *et al*, 1996). Additional studies are required, since the composition of the culture medium may influence the growth of cells during the recovery phase. For example, uptake of ammonium ions is known to inhibit the growth of thawed rice cells (Kuriyama *et al*, 1989), although in the present study, the cell recovery medium lacked ammonium ions. Adsorption of Pluronic molecules onto post-thawed plant cells may also reduce cellular damage which can occur during rehydration when the DMSO cryoprotectant is removed progressively from the system (Benson and Withers, 1987) and thus help to preserve, in the short term, a stable cell:medium density equilibrium crucial to the re-establishment of maximal mitotic activity.

Interestingly, whilst Pluronic stimulated post-thaw growth of cryopreserved rice cells, there was no further growth enhancement when the surfactant was added to the culture medium overlying oxygenated PFC. This contrasted with earlier observations using protoplasts of *P. hybrida*, where Pluronic acted in an additive manner with oxygenated perfluorodecalin to increase the plating efficiency by 57% above control (Anthony *et al*, 1994a). Such differences may relate to the fact that the latter study involved protoplasts of cell suspension-derived cells that had not been cryopreserved. Nevertheless, these results demonstrate that, in terms of simply promoting post-thaw growth of rice cells based on biomass assessments, oxygenated perfluorodecalin alone is superior for inclusion in cryopreservation protocols compared to *Pluronic*<sup>®</sup> F-68, either alone or in combination with PFC.

PFCs may also be useful as gas delivery vehicles for multicellular explants in the context of cryopreservation. For example, the conversion of cryopreserved apical meristems to intact plants may well be facilitated by oxygen-gassed PFC, to promote initial post-cryopreservation survival, and by carbon dioxide-gassed PFC to stimulate rooting and *ex vitro* acclimation (Wardrop *et al*, 1997). Such applications should take account of related studies in which PFCs and their emulsions facilitated survival

of fish semen during low temperature storage (McNiven *et al*, 1993) and promoted hypothermic preservation of mammalian organs, (Umshihara *et al*, 1994).

The present results indicate that oxygenated PFC, together with *Pluronic*® F-68 should be incorporated routinely into post-thaw culture media and handling strategies, to maximise plant cell viability and recovery. A further advantage of using PFCs in such systems is that they are easily recoverable and recyclable, thereby providing a cost effective underpin to germplasm storage technologies (Lowe *et al*, 1997). Moreover, the present demonstration that PFCs can be exploited in small-scale culture systems, makes their routine use economically feasible.

## CHAPTER SIX: GENERAL DISCUSSION

### 6.1 Introduction

Oxygen deprivation can reduce the growth of plant cells *in vitro* through inhibition of the production of respiratory ATP (Van der Plas and Wagner, 1986). Conversely, high oxygen availability preferentially enhances the growth of plant cells, particularly non-embryogenic callus cells of wheat (Carman, 1988). Mitotic division of plant protoplasts isolated enzymatically from cells and tissues of several species, can be enhanced by culture in an oxygen-enriched atmosphere (d'Utra Vaz *et al.*, 1992). Similarly, the production of commercially-important secondary metabolites can be increased when plant cell suspensions are exposed to high dissolved oxygen concentrations (Schlatmann *et al.*, 1994). Furthermore, gassing of bioreactors with > 40% (v/v) oxygen increases the shoot regeneration efficiency of rice (*Oryza sativa*) cells cultured in bioreactors (Okamoto *et al.*, 1996). A novel approach for enhancing oxygen supply to cultured plant cells is the use of chemically-inert, perfluorochemical (PFC) liquids. PFCs are linear, cyclic or bicyclic hydrocarbons in which most or all hydrogen atoms have been replaced with fluorine. PFC liquids typically have specific gravities about twice that of water (Lowe *et al.*, 1998). One of the most significant attributes of such PFC liquids is that they dissolve substantial volumes of oxygen and other respiratory gases and have been studied in, for example, emulsified form as vehicles for oxygen transport *in vivo* (Lowe *et al.*, 1994, 1995). In principle, PFCs offer a more convenient approach to facilitate respiratory gas transfer in plant cell cultures than direct gassing or sparging (Lowe *et al.*, 1997) and it is this property that has been exploited in the present studies.

### 6.2 Protoplast culture systems

Plant protoplasts, of various species including those of petunia (*Petunia hybrida*), cassava (*Manihot esculenta*), passion fruit (*Passiflora giberti*) have been successfully cultured for extended periods at an interface formed between

oxygenated perfluorodecalin ( $C_{10}F_{18}$ ) and the appropriate aqueous culture medium. In petunia, which has been studied extensively as a model system (Anthony *et al*, 1994a), protoplasts synthesised new cell walls, divided normally, and had a mean viability exceeding 92% at the end of the culture period. Their mean plating efficiency (ability to produce cell colonies) was elevated by 37% following culture at such a perfluorodecalin/medium interface. In comparison, growth was similar to that in control (medium alone) when protoplasts were cultured in medium overlaying non-gassed perfluorodecalin, or in oxygenated medium not overlaying perfluorodecalin. Whilst these latter studies did not demonstrate plant regeneration from PFC-treated protoplast-derived cells, since the protoplast systems employed were either originally non-totipotent (*M. esculenta*, *P. hybrida*) or had lost totipotency (*P. giberti*) at the time of assessment. However, subsequent related studies have shown that phenotypically normal, fertile rice (*O. sativa* cv. Taipei 309) plants could be regenerated from protoplasts cultured for up to 34 days with oxygenated PFC, confirming that there were no adverse effects of this novel culture régime on the expression of totipotency (Wardrop *et al*, 1996). Moreover, these workers found that culture of rice protoplasts with oxygenated PFC actually led to an increase in plant regeneration frequency by 12% over untreated controls cultured in the absence of PFC.

Whilst little attention has focused on the molecular responses of cells cultured in the presence of oxygenated PFC, Wardrop *et al.* (1997a) reported significant increases in intracellular SOD activity in protoplasts of *Salpiglossis sinuata* after 3 days of culture in aqueous medium overlaying a commercial grade of oxygenated perfluorodecalin (*Flutec*® PP6), compared to protoplasts cultured with non-oxygenated PFC. These authors speculated that such an increase in SOD would help to protect cells from oxidative damage arising from free radicals generated through prolonged exposure to the increased oxygen supplied by the PFC.

### **6.3 Synergistic effects of PFCs and surfactants as protoplast culture medium supplements**

A further refinement of the PFC-aqueous medium system exploited in the present studies was to supplement the aqueous component with low concentrations (typically 0.01% w/v) of the non-ionic surfactant, *Pluronic*<sup>®</sup> F-68. The surfactant lowered the interfacial tension and also possibly acted as a cell protecting/growth-enhancing agent (Lowe *et al*, 1993), especially as the protoplasts synthesised new cell walls and entered mitotic division. The concentration of *Pluronic*<sup>®</sup> F-68 selected for these experiments was based on earlier studies of the growth-stimulating effects of this compound on *Solanum dulcamara* protoplasts (Kumar *et al*, 1992; Lowe *et al*, 1993).

The addition of *Pluronic*<sup>®</sup> F-68 to the aqueous culture medium was sufficient to reduce the PFC-water interfacial tension by approximately 40%, thus facilitating maximum contact of protoplasts with the interface. Such use of *Pluronic*<sup>®</sup> F-68 would also minimise any protein-stripping effects of the PFC surface indicated in previous investigations with animal cells (Ju *et al*, 1991). Significantly, the mean plating efficiency of petunia protoplasts at the end of the culture period was increased by 52% in the presence of oxygenated PFC overlaid by aqueous culture medium supplemented with *Pluronic*<sup>®</sup> F-68 at 0.01% (w/v). Future studies should evaluate the extent to which other, related non-ionic surfactants such as Tween and Triton X100, may have similar beneficial effects to *Pluronic*<sup>®</sup> F-68.

### **6.4 Image analysis assessments of cell growth**

Computer-based image analysis techniques have been extensively employed for non-invasive/non-destructive assessments of the growth of cells within culture vessels. This approach enables accurate measurement, not only of the total area occupied by cells, but also of more specific parameters, such as the number of individual cell colonies, colony area and fractal structure. Image analysis has been used in the

present studies to assess the growth of cell suspension-derived protoplasts of *P. hybrida*, again as a convenient model, following culture at a PFC/aqueous medium interface. The mean area of protoplast-derived cell colonies after 68 days of growth was increased 35-fold in the presence of oxygenated perfluorodecalin and aqueous culture media supplemented with 0.01% (w/v) *Pluronic*® F-68 (Anthony *et al*, 1994b). Image analysis has also been used to monitor shoot regeneration from rice protoplasts following their culture in the presence of oxygenated PFC (Wardrop *et al*, 1996). A further extension of this technology could also be readily exploited to monitor the growth of protoplast-derived cells following PFC-regulated delivery of other gases, such as carbon dioxide, to cultured cells, tissues and organs (Lowe *et al*, 1997).

## **6.5 Comparison of PFCs with other physical and chemical options for enhancing oxygen supply**

Whilst PFC liquids and surfactants can improve culture conditions for prokaryotic or eukaryotic cells, their benefits must be evaluated alongside other physical or chemical options for regulating respiratory gases. In this respect, Anthony *et al* (1995b) demonstrated that glass rods inserted vertically into agarose-solidified culture medium enhanced the division of cassava leaf mesophyll protoplasts plated in an overlying liquid layer. The glass rods and their associated menisci were believed to increase the surface area for gaseous exchange. Interestingly, these studies also demonstrated that the use of an underlying oxygenated perfluorodecalin (*Flutec*® PP5) layer was, in fact, superior to the use of glass rods for enhancing the plating efficiency of cassava leaf protoplasts. In a separate assessment, the mitotic division of petunia protoplasts was enhanced significantly by supplementing aqueous culture medium with 1:50 (v/v) of a commercial stabilised bovine haemoglobin preparation, *Erythrogen*™ (Anthony *et al* 1997b). This result was consistent with findings from, related studies by Azhakanandam *et al* (1997) which similarly demonstrated that culture media supplementation with 1:50 (v/v) *Erythrogen*™ promoted mitotic

division, cell colony formation and, importantly, plant regeneration from rice cv. Taipei 309 protoplasts. Additionally, protoplast studies have also focused on the extent to which haemoglobin, as a respiratory gas carrier, may act synergistically with PFCs for gas delivery to cell cultures (Anthony *et al*, 1997a). An interesting finding from this work was that the effectiveness of these separate or combined treatments may be species-specific. In the case of *P. giberti* protoplasts, *Erythrogen*<sup>TM</sup> promoted the greatest increase in mean plating efficiency, whereas for *P. hybrida* protoplasts, a combination of oxygen-saturated PFC and *Erythrogen*<sup>TM</sup> was the most effective. Future studies should evaluate the beneficial effects of these technologies on other recalcitrant protoplast systems.

### **6.6 Pluronic® F-68 and plant cell cryopreservation**

Early studies showed that supplementation of medium with Pluronics could prevent haemolysis of human red blood cells in response to freeze-thawing procedures (Glaser and Talbot, 1956). Subsequent investigations demonstrated that *Pluronic*® F-68 was an effective cryoprotectant for cultured Chinese Hamster cells (Ashwood-Smith *et al*, 1973). Surprisingly, the effects had not been evaluated of using *Pluronic*® F-68 with plant cells, either as a cryoprotectant *per se*, or as a post-thaw cytoprotecting agent, despite the increasing interest in cryopreservation for conserving agronomically-important and endangered plant germplasms (Benson, 1994). Consequently, the present studies demonstrated that media supplementation with *Pluronic*® F-68 significantly enhanced post-thaw cellular growth following cryopreservation of both embryogenic and non-embryogenic suspension-cultured cells of the Japonica rices *Oryza sativa* cvs. Taipei 309 and Tarom, together with non-embryogenic cells of *Lolium multiflorum* (Anthony *et al*, 1996) and *Moricandia arvensis* (Craig *et al*, 1997). It is noteworthy that there was no measurable beneficial effect of adding *Pluronic*® F-68 to the cryoprotectant before freezing of rice and *Lolium* cells.

An important finding from the present studies was that the optimum concentration of *Pluronic*® F-68, which increased cell growth, differed between the two rice cultivars and between the rice cv. Tarom and the non-embryogenic cells of *Lolium*. Consequently, there were both species and cultivar-specific responses to *Pluronic*® F-68. These responses of cryopreserved cells were consistent with previous observations using cells and tissues not exposed to ultra-low temperatures, as in *Chrysanthemum morifolium*, in which the optimum concentration of surfactant which stimulated adventive shoot regeneration from cultured leaf explants differed by an order of magnitude between cultivars (Khehra *et al*, 1995). Indeed, differences in the responsiveness of tissues and organs to the growth-promoting effects of *Pluronic*® F-68 have also been observed in *Solanum dulcamara* (Kumar *et al*, 1992), *Corchorus capsularis* (Khatun *et al*, 1993), *Hypericum perforatum* (Brutovská *et al.*,1994) and *Populus* spp. (Iordan-Costache *et al.*,1995).

In plant cells, *Pluronic*® F-68 may promote the increased uptake of nutrients, growth regulators or oxygen into cells during the post-thaw culture period. Any increase in nutrient uptake promoted by Pluronic would be expected to alter metabolic flux, allowing biochemical pathways to operate more efficiently, particularly under the stress conditions of early post-thaw recovery. The adsorption of Pluronic molecules onto the cytoplasmic membranes of post-thawed plant cells may also have reduced any cellular damage which is known to occur during rehydration when the DMSO in the cryoprotectant is removed progressively from the system (Benson *et al*, 1987). However, further studies are needed to determine the precise mechanism of action of *Pluronic*® F-68 as a cytoprotectant.

### **6.7 PFCs and post-thaw recovery of cryopreserved rice cells**

Previous work using cryopreserved rice cells, as a model system, focused on the optimisation of the post-thaw conditions for the successful recovery of suspension cells from long-term storage in liquid nitrogen (Lynch *et al*, 1994). However, these

studies did not address the basic requirement of providing thawed cells with an adequate supply of oxygen, particularly during the early and crucial stages of post-thaw recovery. It is at this critical stage that oxidative stress conditions arise leading to the production of toxic oxygen radicals, which are well known to cause lipid peroxidation of the cells (Fuller *et al*, 1988; Benson *et al*, 1995). This, in turn, can lead to rancidification of adjacent cells which, in some cases, may result in unsuccessful recovery of cells from cryopreservation, through insufficient viable cells being available to enter sustained mitotic division.

Embryogenic suspension cells of rice cv. Taipei 309 were used to assess the potential beneficial effects of oxygenated perfluorodecalin (*Flutec*® PP6) on the post-thaw viability, following long-term cryopreservation in liquid nitrogen. Mean cell viability, as assessed by TTC reduction at 4 days after thawing was increased by 20% over the control by oxygenated perfluorodecalin in 100 ml glass jars, and, similarly, by 24% when the cells were recovered on a smaller scale in 24-well Petri dishes. Related studies using protoplasts isolated from unfrozen cell suspensions of the rice cv. Taipei 309 showed that mitotic division was enhanced during culture of the protoplasts at the interface between oxygen-gassed PFC overlaid with liquid or agarose-solidified culture medium. Related work has showed that shoot formation was also stimulated in this totipotent cell system (Wardrop *et al*, 1996). Overall, these results confirmed a genuine growth enhancement of PFC treatment, rather than a marginal effect on cells recovered from cryopreservation.

### **6.8 Synergistic effects of PFCs and surfactants on post-thaw recovery of cryopreserved rice cells**

Interestingly, experiments with animal cells have demonstrated that PFC liquids, emulsified with *Pluronic*® F-68, were effective in prolonging the fertilising capability of turkey spermatozoa stored at 4°C (Thurston *et al*, 1993). In assessing any synergistic effect of PFC and surfactants in plant systems, PFC both alone and in

combination with 0.01 (w/v) *Pluronic*® F-68 was used to supplement the medium used for the recovery of cryopreserved rice cells of the cv. Taipei 309. The results of such experiments did, indeed, demonstrate a synergistic effect of such compounds. For example, a 21% increase in post-thaw viability over controls was observed with oxygenated perfluorodecalin alone, with a 36% increase in post-thaw viability when cells were exposed to medium supplemented with 0.01% (w/v) *Pluronic*® F-68 alone. However, a more pronounced, synergistic increase in viability of up to 57% over control occurred with rice cells recovered in the presence of both oxygenated perfluorodecalin and 0.01% (w/v) *Pluronic*® F-68 (Anthony *et al*, 1996). Similarly, perfluorodecalin and *Pluronic*® F-68 treatments, either alone or in combination, also promoted an increase in biomass, when measured as fresh weight gain 30 days after thawing of rice cells, to a maximum of 38% above the control value in the case of PFC alone and when oxygen-gassed perfluorodecalin was used in combination with *Pluronic*® F-68. Interestingly, when the PFC and *Pluronic*® F-68 treatments were combined, the increase in biomass was greater than that of cells recovered in the presence of *Pluronic*® F-68 alone, but not significantly different from that with PFC alone. In addition, there were no significant differences, in terms of yields, viabilities, plating efficiencies and plant regeneration frequencies of protoplasts isolated from cryopreserved cells exposed to the various experimental treatments. Similarly, there were no significant differences in terms of protoplast yield, viability and the mean number of protoplast-derived calli regenerating shoots between cells subjected to cryopreservation followed by the various treatments and unfrozen suspension cells assessed prior to their 3 year cryopreservation procedure. Moreover, plants regenerated from cryopreserved rice cells were morphologically normal, with expected diploid chromosome complements ( $2n = 2x = 24$ ) thus demonstrating no long-term detrimental effects of exposure to PFC.

To date, the results with plant cells indicate that the use of oxygenated PFC underlying culture medium, combined with the inclusion of a surfactant, such as

*Pluronic* F-68 in the medium itself, may be useful if incorporated routinely into post-thaw culture media and handling strategies, to maximise plant cell viability during culture after recovery from cryopreservation. However, once cells have recovered from the freezing process, there is no additional advantage of including both oxygen-gassed PFC and surfactant in the culture medium, since the presence of oxygenated PFC alone is adequate to maximise biomass production, at least in rice cells. The use of PFC and surfactant options may be especially relevant in post-thaw handling strategies for cells of those plant species which, normally, respond poorly to conventional **recovery** procedures following storage at ultra-low temperatures. Moreover, the fact that PFCs can be exploited in small-scale culture systems, makes their routine use economically feasible.

PFCs may also be useful as gas delivery vehicles for multicellular explants in the context of cryopreservation. For example, the conversion following thawing of cryopreserved apical meristems to intact plants may be facilitated by oxygen-gassed PFC, to promote initial post-cryopreservation survival, followed by exposure of meristem-derived shoots to carbon dioxide-gassed PFC to stimulate rooting and *ex vitro* acclimation (Wardrop *et al*, 1997b). Such applications should take account of related studies in animal systems, in which PFCs and their emulsions facilitated survival of fish semen during low temperature storage (McNiven *et al*, 1993) and promoted hypothermic preservation of mammalian organs (Umshihara *et al*, 1994).

## **6.9 Concluding remarks**

PFCs clearly have multi-faceted applications in both prokaryotic and eukaryotic cell culture systems. Their principal physical property is to facilitate respiratory gas supply which, in turn, regulates biomass production and allied yields of commercially-important cellular secondary products. The maintenance of protoplasts at the interfacial boundary between PFC liquids and aqueous culture media is convenient for regulating gas supply and provides a novel support option

from which cells can be readily aspirated. The chemical inertness of PFCs has the advantage that such compounds can be readily recovered and hence, re-cycled, thus negating a relatively high initial investment cost. Thus, PFC-facilitated improvements that have been demonstrated in both animal and microbial cell culture technologies can clearly be extended to many fields of plant biotechnology and will complement their progressive exploitation in medicine.

## APPENDICES

### APPENDIX I - Enzyme formulations

#### Composition of Enzyme ENZC1

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Component	Concentration (mg l <sup>-1</sup> )
Hemicellulase	10000
Cellulase RS	4000
Pectolyase Y23	1000
MES	11000
pH 5.8	

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ENZC1 was made up in CPW9M solution and filter sterilized by passing through a 0.2  $\mu$ m pore size membrane.

#### Composition of Enzyme ENZ223

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Component	Concentration (mg l <sup>-1</sup> )
Rhozyme	20000
Meicelase	20000
Macerozyme R10	300
pH 5.8	

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ENZ223 was made up in CPW13M solution and filter sterilized by passing through a 0.2  $\mu$ m pore size membrane.

#### Composition of Enzyme ENZ101

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Component	Concentration (mg l <sup>-1</sup> )
Cellulase RS	10000
Pectolyase Y23	1000
MES	11000
pH 5.8	

---

ENZ101 was made up in CPW13M solution and filter sterilized by passing through a 0.2  $\mu$ m pore size membrane.

## APPENDIX II - Media formulations

### Composition of MS medium (Murashige and Skoog, 1962)

Component	Concentration (mg l <sup>-1</sup> )
CaCl <sub>2</sub> ·2H <sub>2</sub> O	440
NH <sub>4</sub> NO <sub>3</sub>	1650
KNO <sub>3</sub>	1900
KI	0.83
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.025
KH <sub>2</sub> PO <sub>4</sub>	170
H <sub>3</sub> BO <sub>3</sub>	6.2
NaMoO <sub>4</sub>	0.25
MgSO <sub>4</sub> ·7H <sub>2</sub> O	370
MnSO <sub>4</sub> ·H <sub>2</sub> O	22.3
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.025
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	8.6
FeSO <sub>4</sub> ·7H <sub>2</sub> O	27.85
Na <sub>2</sub> EDTA	37.25
Glycine	2.0
Inositol	100
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Sucrose	30000
pH 5.8	

The above components, with the exception of sucrose, are contained in the dried preparation marketed by Sigma, UK. Medium was made up in reverse osmosis water and autoclaved at 121°C for 20 min.

### Composition of MSKN medium

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Component	Concentration (mg l <sup>-1</sup> )
The following additions were made to MS medium	
Kinetin	2.0
NAA	0.5
pH 5.8	

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Medium was made up in reverse osmosis water and autoclaved at 121°C for 20 min.

### Composition of MSP1 medium

---

Component	Concentration (mg l <sup>-1</sup> )
The following additions were made to MS medium	
NAA	2.0
BAP	0.5
pH 5.8	

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Medium was made up in reverse osmosis water and autoclaved at 121°C for 20 min.

### Composition of MSP19M-N medium

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Component	Concentration (mg l <sup>-1</sup> )
The following additions were made to MS medium lacking NH <sub>4</sub> NO <sub>3</sub>	
NaNO <sub>3</sub>	2120
NAA	2.0
BAP	0.5
Mannitol	90000
pH 5.8	

---

Medium was made up in reverse osmosis water and autoclaved at 121°C for 20 min.

### Composition of MSDI medium

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Component	Concentration (mg l <sup>-1</sup> )
The following additions were made to MS medium	
2,4-D	1.0
pH 5.8	

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Medium was made up in reverse osmosis water and autoclaved at 121°C for 20 min.

### Composition of LS 2.5 medium (Linsmaier and Skoog, 1965)

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Component	Concentration (mg l <sup>-1</sup> )
CaCl <sub>2</sub> ·2H <sub>2</sub> O	440
NH <sub>4</sub> NO <sub>3</sub>	1650
KNO <sub>3</sub>	1900
KI	0.83
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.025
KH <sub>2</sub> PO <sub>4</sub>	170
H <sub>3</sub> BO <sub>3</sub>	6.2
NaMoO <sub>4</sub>	0.25
MgSO <sub>4</sub> ·7H <sub>2</sub> O	370
MnSO <sub>4</sub> ·H <sub>2</sub> O	22.3
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.025
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	8.6
FeSO <sub>4</sub> ·7H <sub>2</sub> O	27.85
Na <sub>2</sub> EDTA	37.25
Inositol	100
Thiamine HCl	1.0
Sucrose	30000
2,4-D	2.5
pH 5.8	

---

Medium was made up in reverse osmosis water and autoclaved at 121°C for 20 min.

### Composition of N6 medium (Chu *et al.*, 1975)

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Component	Concentration (mg l <sup>-1</sup> )
CaCl <sub>2</sub> .2H <sub>2</sub> O	166
(NH <sub>4</sub> )SO <sub>4</sub>	463
KNO <sub>3</sub>	2830
KH <sub>2</sub> PO <sub>4</sub>	400
H <sub>3</sub> BO <sub>3</sub>	1.6
MgSO <sub>4</sub> .7H <sub>2</sub> O	185
ZnSO <sub>4</sub> .7H <sub>2</sub> O	1.5
MnSO <sub>4</sub> .4H <sub>2</sub> O	3.3
FeSO <sub>4</sub> .7H <sub>2</sub> O	24.9
Na <sub>2</sub> EDTA	26.1
Glycine	2.0
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	1.0
2,4-D	2.0
Sucrose	30000
pH 5.8	

---

Medium was made up in reverse osmosis water and autoclaved at 121°C for 20 min.

### Composition of UM medium (Uchimiya and Murashige, 1974)

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Component	Concentration (mg l <sup>-1</sup> )
The following additions were made to MS medium	
2,4-D	2.0
Kinetin	0.25
Thiamine HCl	9.91
Pyridoxine HCl	9.5
Nicotinic acid	4.5
Casein hydrolysate	2000
pH 5.8	

---

Medium was made up in reverse osmosis water and autoclaved at 121°C for 20 min.

**Composition of AA2 medium<sup>1</sup> (Müller and Grafe, 1978) and R2 medium (Ohira *et al.*, 1973)**

Component	Concentration (mg l <sup>-1</sup> )	
	AA2 medium <sup>2</sup>	R2 medium <sup>3</sup>
CaCl <sub>2</sub> ·2H <sub>2</sub> O	440	150
KH <sub>2</sub> PO <sub>4</sub>	170	170
MgSO <sub>4</sub> ·7H <sub>2</sub> O	370	270
KCl	2940	
NaH <sub>2</sub> PO <sub>4</sub> ·2H <sub>2</sub> O		310
KNO <sub>3</sub>		4040
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>		330
KI	0.83	
H <sub>3</sub> BO <sub>3</sub>	6.2	0.5
MnSO <sub>4</sub> ·4H <sub>2</sub> O	22.3	0.5
NaMoO <sub>4</sub> ·2 H <sub>2</sub> O	0.25	0.05
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	8.6	0.5
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.025	0.05
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.025	
FeSO <sub>4</sub> ·7H <sub>2</sub> O	27.85	
Na <sub>2</sub> EDTA	37.25	
NaFeEDTA		2.5
Myo-inositol	100	too
Nicotinic acid	0.5	
Pyridoxine HCl	0.1	0.5
Thiamine HCl	0.5	0.1
Glycine	75	2.0
L-Glutamine	877	
L-Aspartic acid	266	
L-Arginine	228	
L-Proline		560
2,4-D	2.0	1.0
Gibberellic acid	0.1	
Kinetin	0.2	
Sucrose	20000	
Maltose		30000
pH	5.8	5.8

<sup>1</sup>Modified AA2 medium contains 100 mg l<sup>-1</sup> myo-inositol, 0.5 mg l<sup>-1</sup> thiamine HCl, 7.5 mg l<sup>-1</sup> L-glycine and 2.0 mg l<sup>-1</sup> 2,4-D.

<sup>2</sup>AA2 medium was made up in reverse osmosis water and filter sterilized by passing through a 0.2 *µm* pore size membrane.

<sup>3</sup>R2 medium was made up in reverse osmosis water and autoclaved at 121°C for 20 min.

### Composition of CPW salts solution<sup>1</sup> (Frearson *et al.*,1973)

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Component	Concentration (mg l <sup>-1</sup> )
KH <sub>2</sub> PO <sub>4</sub>	27.2
KNO <sub>3</sub>	101
CaCl <sub>2</sub> .2H <sub>2</sub> O	440
MgSO <sub>4</sub> .7H <sub>2</sub> O	246
KI	0.16
CuSO <sub>4</sub> .5H <sub>2</sub> O	0.025

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<sup>1</sup>Modified CPW salts solution contains 1480 mg l<sup>-1</sup> CaCl<sub>2</sub>.2H<sub>2</sub>O

To prepare: CPW9M add 9% (w/v) mannitol

CPW13M add 13% (w/v) mannitol

CPW21S add 21% (w/v) sucrose

For all CPW solutions; pH 5.8. Solutions were made up in reverse osmosis water and autoclaved at 121°C for 20 min.

### Composition of W5 solution (Menczel *et al.*, 1981)

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Component	Concentration (mg l <sup>-1</sup> )
NaCl	9000
CaCl <sub>2</sub> .2H <sub>2</sub> O	18370
KCl	373
Glucose	901
pH 5.6	

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W5 solution was made up in reverse osmosis water and autoclaved at 121°C for 20 min.

**Composition of 1B5CNK medium (Misawa *et al*, 1982)**

<b>Component</b>	<b>Concentration (mg Y<sup>-1</sup>)</b>
CaCl <sub>2</sub> ·2H <sub>2</sub> O	150
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	134
KNO <sub>3</sub>	2500
KI	0.75
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.025
NaH <sub>2</sub> PO <sub>4</sub> ·2H <sub>2</sub> O	150
H <sub>3</sub> BO <sub>3</sub>	3.0
NaMoO <sub>4</sub>	0.25
MgSO <sub>4</sub> ·7H <sub>2</sub> O	250
MnSO <sub>4</sub> ·4H <sub>2</sub> O	10
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.025
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	2.0
FeSO <sub>4</sub> ·7H <sub>2</sub> O	27.85
Na <sub>2</sub> EDTA	37.25
Myo-inositol	100
Nicotinic acid	1.0
Pyridoxine HCl	1.0
Thiamine HCl	10.0
Casamino acids	2000
Kinetin	0.25
NAA	1.0
2,4-D	1.0
Sucrose	20000
Glucose	61250
pH 5.8	

Medium was made up in reverse osmosis water and autoclaved at 121°C for 20 min.

Composition of KM8P and KM8 **media**<sup>1</sup> (Kao and Michaluk, 1975) and K8 medium (Kao, 1977)

Component	Concentration (mg l <sup>-1</sup> )		
	KM8P	KM8	K8P
NH <sub>4</sub> NO <sub>3</sub>	600	600	600
KNO <sub>3</sub>	1900	1900	1900
CaCl <sub>2</sub> .2H <sub>2</sub> O	600	600	600
MgSO <sub>4</sub> .7H <sub>2</sub> O	300	300	300
KH <sub>2</sub> PO <sub>4</sub>	170	170	170
Sequestrene 330 Fe	28	28	28
KI	0.75	0.75	0.75
H <sub>3</sub> BO <sub>3</sub>	3.0	3.0	3.0
MnSO <sub>4</sub> .4H <sub>2</sub> O	10.0	10.0	10.0
ZnSO <sub>4</sub> .7H <sub>2</sub> O	2.0	2.0	2.0
NaMoO <sub>4</sub> .2 H <sub>2</sub> O	0.25	0.25	0.25
CuSO <sub>4</sub> .5H <sub>2</sub> O	0.025	0.025	0.025
CoCl <sub>2</sub> .6H <sub>2</sub> O	0.025	0.025	0.025
Myo-inositol	too	100	too
Nicotinamide	1.0	1.0	1.0
Pyridoxine HCl	1.0	1.0	1.0
Thiamine HCl	1.0	1.0	1.0
D-Ca Pantothenate	1.0	1.0	0.5
Folic acid	0.4	0.4	0.2
Abscisic acid	0.02	0.02	0.01
Biotin	0.01	0.01	0.005
Choline chloride	1.0	1.0	0.5
Riboflavin	0.2	0.2	0.1
Ascorbic acid	2.0	2.0	1.0
Vitamin A	0.01	0.01	0.005
<b>Vitamin D<sub>3</sub></b>	0.01	0.01	0.005
<b>Vitamin B<sub>12</sub></b>	0.02	0.02	0.01
Na pyruvate	20	20	5
Citric acid	40	40	10
Malic acid	40	40	10
Fumaric acid	40	40	10
Fructose	250	250	125
Ribose	250	250	125
Xylose	250	250	125
Mannose	250	250	125
Rhamnose	250	250	125
Cellobiose	250	250	125
Sorbitol	250	250	125
Mannitol	250	250	125
Vitamin free			
Casamino acids	250	250	125
Coconut milk	<b>20 ml l<sup>-1</sup></b>	<b>20 ml l<sup>-1</sup></b>	<b>10 ml l<sup>-1</sup></b>
2,4-D	0.2	0.1	0.2
Zeatin	0.5	0.2	0.5
NAA	1.0	1.0	1.0
Sucrose	250	20000	250
Glucose	100000	10000	100000
pH	5.8	5.8	5.8

<sup>1</sup>Modified KM8P and KM8 media (Gilmour *et al*, 1989) based on the formulation of Kao and Michaluk, 1975

Media were made up in reverse osmosis water and filter sterilized by passing through a 0.2 *µm* pore size membrane.

**Composition of KPR medium (Abdullah *et al*, 1986)**

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<b>Component</b>	<b>Concentration (mg l<sup>-1</sup>)</b>
The following additions were made to KP8 medium	
2,4-D	0.3
pH 5.8	

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Medium was made up in reverse osmosis water and filter sterilized by passing through a 0.2 *µm* pore size membrane.

## **APPENDIX III - Publications**

## Synergistic enhancement of protoplast growth by oxygenated perfluorocarbon and Pluronic F-68

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**Summary.** Cell suspension-derived protoplasts of albino *Petunia hybrida* were grown for 10 d at the interface between aqueous culture medium (KM8P) and an oxygenated (10 mbar for 15 min) perfluorocarbon liquid, perfluorodecalin. Protoplasts synthesised new cell walls and divided normally at the perfluorodecalin/culture medium interface, with a mean viability after 10 d of > 92.0%. The mean plating efficiency of protoplasts was elevated by 37% ( $P < 0.05$ ) following culture at the perfluorodecalin/medium interface, but was unaltered by perfluorodecalin or oxygen separately. The mean plating efficiency of protoplasts cultured at the interface was further increased to a maximum of 52% above control, in the presence of oxygenated perfluorodecalin and KM8P medium supplemented with the non-ionic, co-polymer surfactant, Pluronic F-68 at 0.01% (w/v). These findings demonstrate the effectiveness of oxygenated perfluorodecalin for promoting protoplast growth, by facilitating oxygen delivery. The finding that Pluronic F-68 further increased the plating efficiency of protoplasts cultured at the perfluorocarbon/aqueous interface suggests that these agents improve growth through separate, but cumulative, mechanisms.

**Key words:** *Petunia hybrida*, protoplasts, oxygen delivery, perfluorochemicals, Pluronic F-68, surfactant, cell division.

**Abbreviations:** ATP, adenosine triphosphate; PFCs, perfluorochemicals; STP, standard temperature and pressure.

### Introduction

Oxygen deprivation is known to reduce plant cell growth through inhibitory effects on the production of respiratory ATP (Van der Plas and Wagner 1986). In contrast, high oxygen availability can preferentially enhance the growth of non-embryogenic callus cells from plants such as wheat (Carman 1987). Additionally, recent studies have shown that the growth of tomato, rice and jute protoplasts can be improved by culture in oxygen-enriched atmospheres (d'Utra

Vaz *et al.* 1992). There is thus a strong case for investigating the growth and differentiation of plant protoplasts, cells and tissues when cultured in the presence of media supplements which, in principle, can facilitate improved oxygen transfer.

PFCs are inert, organic compounds which can dissolve substantial volumes of respiratory gases and have been used for enhancing oxygen supply both *in vivo* and *in vitro* (King *et al.* 1989; Lowe 1992, 1993). PFCs have been used, for example, in fermenter studies (Junker *et al.* 1990; Ju *et al.* 1991) or hybridoma cell cultures (Ju and Armiger 1992) to increase oxygen transfer rates. Mammalian cells, including fibroblasts and retinoblastoma cells, have also been cultured successfully at the interface formed between PFC liquid and aqueous media (Giaever and Keese 1983; Nabih *et al.* 1989). Since there appears to have been no corresponding assessment of the beneficial effects of using PFCs in plant cell cultures, the present study was performed to investigate the growth of isolated protoplasts in such a culture system, using cell suspension-derived protoplasts of albino *Petunia hybrida* as a convenient model for these assessments. This approach could have significant biotechnological application, not only in the context of plant genetic manipulation where the efficacy of protoplast conversion to plants is paramount for successful somatic hybridization and transgenic plant production, but also in the production of phytochemicals from cultured plant cells and tissues.

### Materials and methods

**Protoplast isolation and culture systems.** Protoplasts of albino *Petunia hybrida* cv. Comanche were isolated enzymatically from cell suspensions (Power *et al.* 1990) and were cultured in the dark (25°C) at a final plating density of  $2.0 \times 10^5/\text{ml}$  in 2.0 ml aliquots of liquid KM8P culture medium (Gilmour *et al.* 1989). The medium containing protoplasts was placed in 30 ml screw-capped glass bottles, either

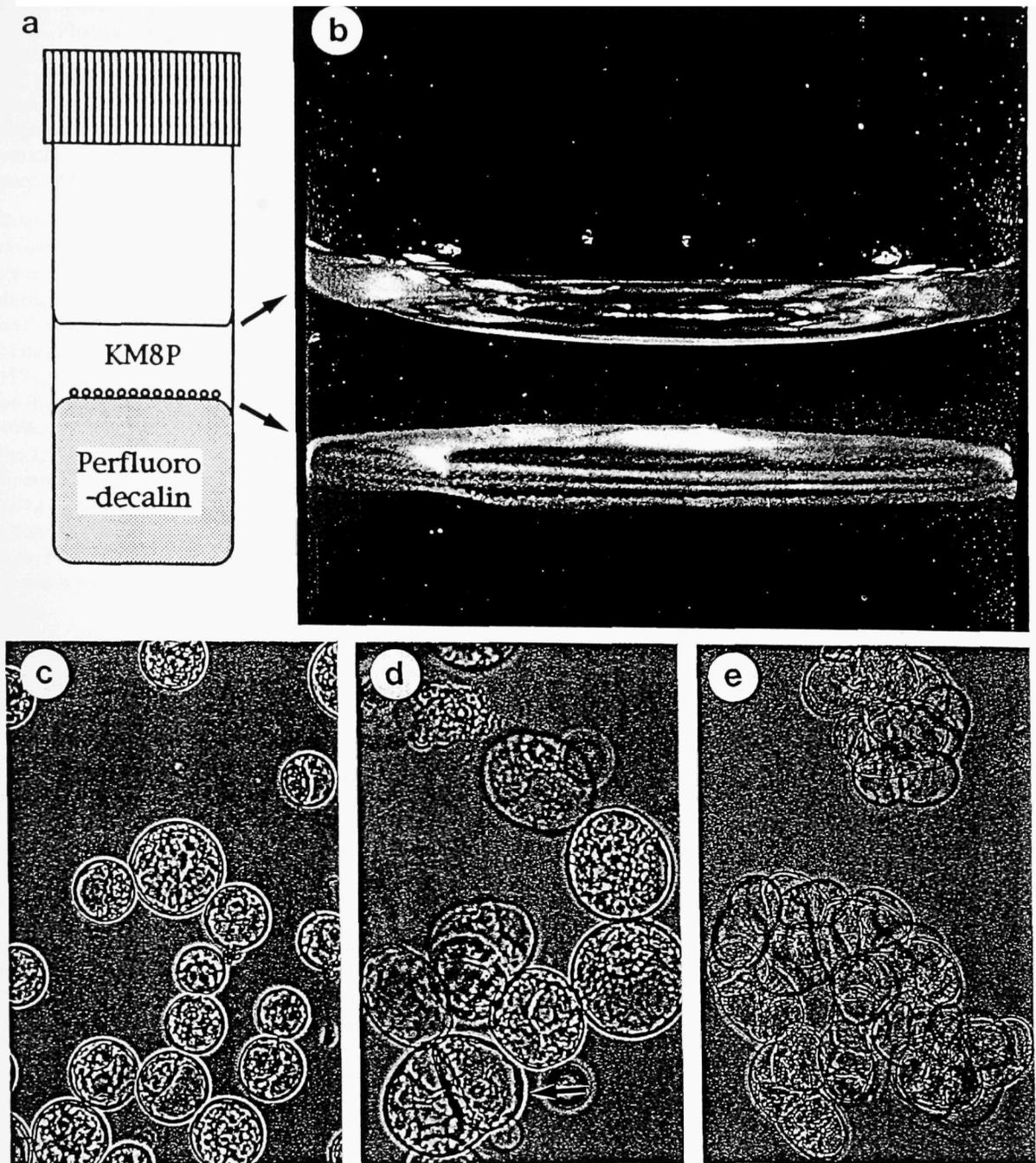


Figure 1. (a) Schematic drawing of the culture system; (b) Suspension cell-derived protoplasts of albino *P. hybrida* cv. Comanche growing at the interface formed between oxygenated perfluorodecalin and KM8P culture medium (width of bottle = 27 mm); (c) Freshly isolated *P. hybrida* protoplasts (x 100); (d) Protoplast-derived cells, some of which are dividing (arrow), harvested from the interface after 10 d of culture (x 100); (e) Protoplast-derived cell colonies of *P. hybrida* after 64 d of culture in KM8P medium overlying oxygenated perfluorodecalin (x 50).

alone, or over 6.0 ml of sterile **perfluorodecalin** (Flutec PP5; BNFL Fluorochemicals Ltd. Preston, U.K.; Fig. 1a,b). The perfluorodecalin was saturated with 100% oxygen by bubbling for 15 min. (10 mbar) before use; in some controls, the perfluorodecalin was left unsaturated. It was necessary to maintain a PFC liquid layer at least 5 mm deep in the culture vessels, in order to obtain a stable interface for culture purposes.

In some treatments, the aqueous culture medium was supplemented with 0.01% (w/v) of a commercial-grade of the non-ionic, co-polymer surfactant, Pluronic F-68 (BASF, Wyandotte, U.S.A.). This concentration of Pluronic was selected on the basis of previous studies of growth-stimulating effects of this compound on *S. dulcamara* protoplasts (Kumar *et al.* 1992; Lowe *et al.* 1993). It was sufficient to reduce the perfluorodecalin-water interfacial tension by approximately 40%, so as to facilitate maximum contact of protoplasts with the interface (Fig. 2). Interfacial tensions between perfluorodecalin and Pluronic F-68 solutions were measured using a conventional Wilhelmy Plate apparatus attached to Cahn microbalance; it was necessary to use a plate made from PTFE (15 x 15 x 0.8 mm) since the conventional glass or platinum plates are not wetted by the perfluorocarbon liquid.

**Measurement of protoplast viability.** Protoplast viability was determined by uptake of fluorescein diacetate, as described previously (Widholm 1972).

**Measurement of protoplast growth.** Protoplast growth, as determined by the division frequency (measured as the number of protoplasts that had regenerated a new cell wall and had undergone at least first mitotic division), was assessed after 10 d. A minimum of 500 protoplasts per treatment were counted; each experiment was repeated 5 times.

**Measurement of dissolved oxygen concentration.** Oxygen concentrations in perfluorodecalin or culture medium were measured using a Jenway 9015 Dissolved Oxygen Meter with a POM102 probe (Scientific Laboratory Supplies Ltd., Nottingham, U.K.). The instrument was calibrated to measure oxygen concentration in ml/l using oxygen-saturated perfluorodecalin or aqueous medium as standards. The saturation solubilities of oxygen in these media are 423 ml/l and 25 ml/l respectively at STP (Riess and Le Blanc, 1982). The overall coefficient of variation was 5.2%.

**Fluorocarbon re-cycling and re-use.** Perfluorodecalin was recovered for re-use by centrifugation of the culture medium (2000 g; 15 min). The PFC pellet was shaken with alkaline permanganate (1.0 gA in 1M sodium hydroxide) and repeatedly rinsed (minimum 5 times) with distilled, deionised water prior to re-use and re-sterilisation by autoclaving (121°C, 20 min).

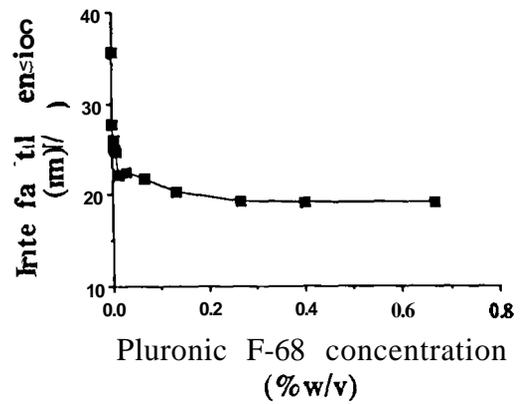


Figure 2. Interfacial tension (mN/m) of a perfluorodecalin-water interface as a function of concentration of Pluronic F-68 (% w/v) in the aqueous phase.

**Statistical methods.** Statistical analyses were performed according to Snedecor and Cochran (1989). Means and either standard deviations (s.d.) or standard errors (s.e.m.) were used throughout and statistical significance between mean values was assessed using a conventional Student's *t*-test. A probability of  $P < 0.05$  was considered significant.

## Results

**Changes in oxygen concentration.** The mean ( $\pm$  s.e.m.) oxygen concentration in oxygenated perfluorodecalin at the start of the experiment was  $423 \pm 4$  ml/l, while that of the aqueous phase was  $8.7 \pm 0.1$  ml/l (Fig. 3). The mean oxygen concentration in the aqueous medium increased to a maximum of  $25.0 \pm 0.6$  ml/l after 24 h, then declined slowly to  $22.0 \pm 0.4$  ml/l after 3 d. There was a concurrent decrease in the mean concentration of oxygen in the underlying perfluorodecalin phase, to  $340 \pm 10$  ml/l after 3 d (Fig. 3). The oxygen measurements, both in the perfluorodecalin and culture medium phases, were restricted to the first 3 d of culture since the protoplasts of *P. hybrida*

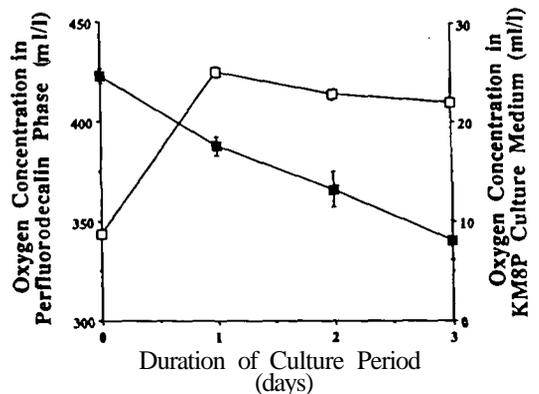


Figure 3. Changes in mean ( $\pm$  s.d.) oxygen concentrations (ml/l) in liquid KM8P culture medium (open symbols) overlaying oxygenated perfluorodecalin (solid symbols)

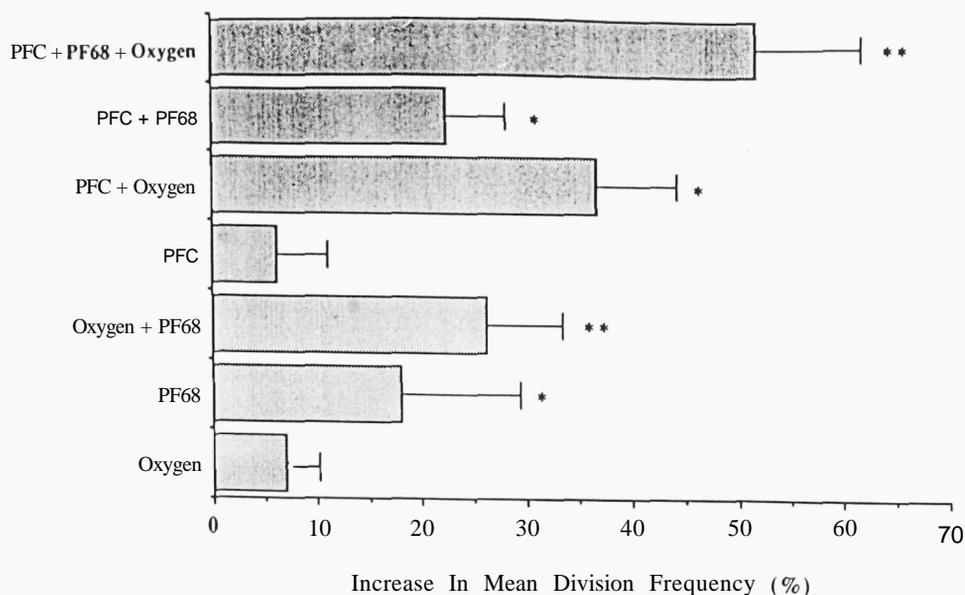


Figure 4. Mean division frequency (% increase over control) of *P. hybrida* protoplasts after 10 d of culture under the following conditions (top to bottom):

PFC + PF68 + Oxygen	In KM8P medium + 0.01% (w/v) Pluronic F-68 overlaying oxygenated perfluorodecalin
PFC + PF68	In KM8P medium + 0.01% (w/v) Pluronic F-68 overlaying perfluorodecalin
PFC + Oxygen	In KM8P medium overlaying oxygenated perfluorodecalin
PFC	In KM8P medium overlaying perfluorodecalin
Oxygen + PF68	In oxygenated KM8P medium + 0.01% (w/v) Pluronic F-68
PF68	In KM8P medium + 0.01% (w/v) Pluronic F-68
Oxygen	In oxygenated KM8P medium

will have regenerated a new cell wall and entered first mitotic division during this period. No significant difference was observed in the oxygen concentrations in a parallel series of experiments lacking the Pluronic F-68 medium component.

**Protoplast viability and division.** The mean viability of protoplasts was  $95 \pm 1\%$  ( $n = 3$ ) immediately following enzymatic isolation. Protoplasts divided normally at the interface that formed between perfluorodecalin and the aqueous culture medium (Fig. 1c,d), with no significant loss in viability over the culture period. However, in some cases, there was a tendency for protoplasts to aggregate concomitantly with cell wall regeneration, but this was minimalised in media with 0.01% (w/v) Pluronic F-68.

**Growth of protoplasts with perfluorodecalin.** The growth of protoplasts was significantly enhanced when they were cultured at the interface between aqueous KM8P medium and oxygenated perfluorodecalin. For example, the mean division frequency (plating efficiency) after 10 d was  $9.7 \pm 0.7\%$  ( $n = 5$ ) compared to  $7.1 \pm 0.4\%$  for control protoplasts cultured in unsupplemented medium ( $P < 0.05$ ). In contrast, there were no significant changes in protoplast growth when oxygen alone, or non-oxygenated perfluorodecalin were

used in separate control experiments. Figure 4 shows the percentage increase in mean division frequency over the controls for the various treatment groups.

**Growth of protoplasts with perfluorodecalin and Pluronic F-68.** Supplementation of the aqueous culture medium with 0.01% (w/v) Pluronic F-68 increased protoplast growth, such that the mean division frequency after 10 d ( $8.4 \pm 1.0\%$ ) was significantly greater ( $P < 0.05$ ) than control (Fig. 4). In contrast, no further increase in growth over controls occurred when the Pluronic F-68-supplemented culture media were either gassed with oxygen alone or layered over non-gassed perfluorodecalin. Addition of 0.01% (w/v) Pluronic F-68 to KM8P medium overlaying oxygenated perfluorodecalin produced the greatest increase in mean protoplast growth after 10 d ( $10.8 \pm 1.1\%$ ; Fig. 4). This led to normal protoplast-derived cell colony formation by 64d, thereby confirming that repeated mitotic division in protoplasts was not impaired by culture in the presence of both oxygenated perfluorodecalin and surfactant (Fig. 1e). Samples of protoplast-derived colonies could be returned by pipetting to the growth conditions/culture medium as routinely used for the original cell suspension, thereby completing the cultural cycle.

## Discussion

The present **experiments** demonstrate, for the first time, that plant protoplasts **will** grow normally at the interface between perfluorodecalin and an aqueous culture medium. Thus, the technology previously employed in animal cell cultures (Giaever and Keese, 1983; Nabih *et al.* 1989) can be successfully adapted for plant systems. The finding that division and colony formation from protoplast-derived cells of *P. hybrida* were not affected by oxygen or perfluorodecalin **alone, but only by their subsequent combination, demonstrates** the dual efficacy of PFCs for both oxygen storage and effective delivery to cells.

The present study also shows that supplementation with Pluronic F-68 of the aqueous culture medium overlaying the **perfluorodecalin** liquid reduces aggregation of protoplasts associated with the onset of cell wall regeneration, probably through surface tension effects. This is consistent with previous findings that Pluronic F-68 reduced clumping in insect cell cultures (Murhammer and Goochee 1990). This is additional to any growth-enhancing effects of this compound, as reported previously for cultured *Solanum dulcamara* protoplasts (Kumar *et al.* 1992; Lowe *et al.* 1993).

The finding that Pluronic F-68 further enhanced the growth of protoplasts over the increase promoted by oxygenated perfluorodecalin, suggests that these agents promote protoplast division through separate mechanisms. While there is speculation that Pluronics stimulate growth by enhancing cellular uptake of nutrients and/or growth regulators (Lowe *et al.* 1993), PFCs most probably act by facilitating oxygen transfer directly into individual cells. This is supported by the present finding that the perfluorodecalin phase acted as an oxygen reservoir, progressively transferring the gas into the aqueous medium and, hence, to the protoplasts. However, the possibility that Pluronic F-68 can itself facilitate cellular oxygen uptake cannot be discounted.

The use of PFC as an oxygenating agent is especially advantageous in static cultures of protoplasts in which conventional aeration through agitation (e.g. stirring) cannot be used because of the tendency to promote cell damage. Further advantages of using PFCs as a culture **medium** support include their inertness, ease of sterilisation by autoclaving, and re-cycleability (King *et al.* 1989), which offsets the relatively high initial cost of these materials. PFCs are especially useful in illuminated cultures, since they have refractive indices similar to water (Riess and Le Blanc, 1982). One improvement of the present system would be to "recharge" the perfluorodecalin with oxygen during culture, thereby maximising the potential for oxygen delivery to growing protoplasts. In addition to facilitating oxygen transfer, it has been proposed (King *et al.* 1989) that PFC-supplemented systems could be valuable for supplying other gases (e.g. carbon dioxide) to anaerobic cultures.

The present technology could provide a handling strategy for

culturing small numbers of protoplasts (e.g. flow-sorted heterokaryons) in media droplets on a liquid-PFC interface. A more efficient protoplast-to-plant system will have implications for the transformation **efficacy** of protoplasts using direct DNA delivery systems. In the context of somatic hybridization, one of the crucial stages is the conversion of heterokaryons to somatic hybrid cells. Thus, an improved plating efficiency, as indicated by the present approach, is likely to lead to a greater throughput of putative hybrid material and will have profound implications for plant cell biotechnology and genetic manipulation.

## Acknowledgements

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# Image analysis assessments of perfluorocarbon- and surfactant-enhanced protoplast division

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**Key words:** cell culture, image analysis, *Petunia hybrida*, protoplast, perfluorocarbon, surfactant

## Abstract

Image analysis has been used to assess the growth of cell suspension-derived protoplasts of *Petunia hybrida* cv. Comanche at an interface between aqueous culture medium (KM8P), supplemented with 0.01% (w/v) Pluronic F-68, and oxygenated (10 mbar; 10 min) perfluorodecalin. Protoplasts synthesised a new cell wall and entered normal mitotic division which was sustainable to the cell colony/callus stage. This process was accentuated by the collective and additive effects of oxygen, perfluorodecalin and surfactant media supplements. The mean area ( $\text{mm}^2$ ) of protoplast-derived cell colonies after 68 days of growth was increased 35 fold over control (media alone) in the presence of these combined treatments. The new cultural regime, leading to improved cell throughput from protoplasts, is discussed primarily in relation to the role of perfluorodecalin as a gas carrier and possible effects of Pluronic F-68 in stimulating cellular uptake of nutrients and/or growth regulators. Image analysis provides a novel and accurate approach to quantifying cell growth responses.

**Abbreviations:** dpi - dots per inch, FPE - final plating efficiency, IPE - initial plating efficiency, KM - Kao & Michayluk (1975), PFC - Perfluorocarbon, UM - Uchimiya & Murashige (1974)

## Introduction

Perfluorocarbon (PFCs) liquids can dissolve substantial volumes of respiratory gases, which makes them attractive for regulating gas supply in cell culture systems. This approach has been used to improve oxygen supply in microbial and animal cell cultures (King et al. 1989; Rols & Goma 1989; Junker et al. 1990; Ju et al. 1991; Ju & Armiger 1992). In addition, recent experiments have shown that plant protoplast growth is enhanced at the interface between oxygenated perfluorodecalin and aqueous medium supplemented with the non-ionic surfactant, Pluronic F-68 (Anthony et al. 1994). Such use of PFCs to facilitate cellular gas exchange, coupled with surfactant supplements which may enhance nutrient uptake (Lowe et al. 1993), could have profound implications in plant cell biotechnology. One pre-requisite to wider applications of this novel technology, is the assessment of the beneficial effects

of such agents, not only on mitotic division of single cells (or protoplasts), but also on their multi-cellular derivatives.

The growth of cells in semi-solid media is commonly assessed simply by visual comparison of areas of coverage of culture plates. Whilst this approach may be adequate if major variations exist between individual treatments, its power of discrimination is inadequate for cultures which show relatively little difference. Under these circumstances, therefore, it is valuable to develop techniques providing a non-invasive and hence, non-destructive, quantitative assessment of cell growth within the confines of a culture vessel.

Computer-based image analysis techniques are extremely valuable in making such measurements but they do not yet appear to have been widely employed for this purpose. This approach enables accurate measurement, not only of total area covered, but also of more sophisticated variables, such as individual

cell colony number, colony area and fractal structure. Image analysis has been used, for example, to characterise the growth of *Streptomyces tendae* (Reichl et al. 1992), to assess the growth of mammalian cells on microcarriers (Pons et al. 1992), and to follow the growth of plant somatic embryos cultured in a continuous loop bioreactor (Harrell et al. 1992). However, there appear to be no reports to date of the use of this approach to assess growth characteristics and morphology of plant protoplast-derived cell colonies on agar-solidified media. Therefore, in the present experiments, image analysis has been used to assess the growth of protoplast-derived cell colonies (of *Petunia hybrida* as a convenient model) in the presence of oxygenated perfluorocarbon and Pluronic F-68, either or both of which can promote cell growth (Lowe et al. 1993; Anthony et al. 1994).

## Materials and methods

### *Protoplast isolation, culture systems and experimental design*

Protoplasts of albino *Petunia hybrida* cv. Comanche were isolated enzymatically from cell suspensions (Power et al. 1990) and were cultured in the dark (25°C) at a plating density of  $2.0 \times 10^5 \text{ ml}^{-1}$  in 2.0 ml aliquots of liquid KM8P culture medium [the protoplast culture medium of Kao & Michayluk (1975) as modified by Gilmour et al. (1989)]. Protoplast viability was assessed by cleavage of fluorescein diacetate (Widholm 1972). The medium containing protoplasts was placed in 30 ml screw-capped glass bottles, either alone, or over 6.0 ml of sterile perfluorodecalin (Flutec®PP5; BNFL Fluorochemicals Ltd, Preston, UK). It was necessary to maintain a PFC liquid layer at least 5 mm deep in the culture vessels, in order to obtain a stable interface with the aqueous medium for culture evaluation purposes.

The experimental design consisted of a 2 x 2 x 2 matrix experiment (total 8 groups). The treatment groups were:-

- with and without oxygen bubbling for 15 min at 10 m bar;
- with and without supplementation of KM8P medium with 0.01 % (w/v) of commercial-grade Pluronic F-68 (BASF, Wyandotte, U.S.A.). This concentration of surfactant was sufficient to reduce the perfluorodecalin-water interfacial tension by approximately 40%, so as to facilitate maximum

contact of protoplasts with the interface (Anthony et al. 1994);

- with and without perfluorodecalin.

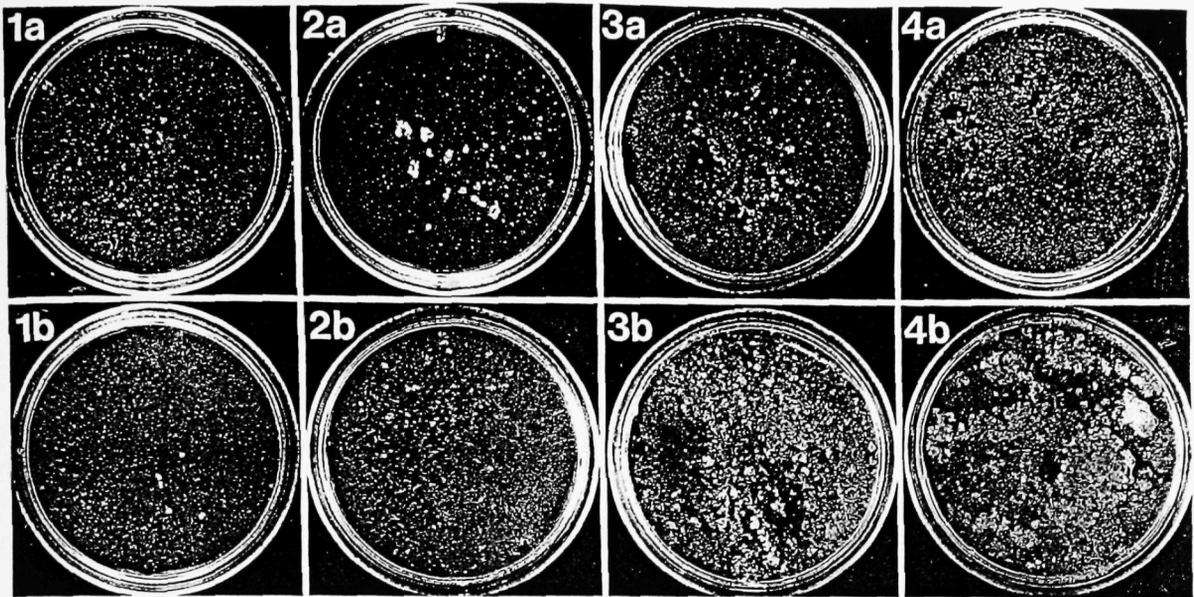
At 10 and 17 days, spent KMBP medium (0.25 ml) was replaced with an equal volume of KM8 medium [of reduced osmotic pressure and prepared to the formulation of medium 8 of Kao & Michayluk (1975) with the modifications of Gilmour et al. (1989)]. The total population of cell colonies was transferred, on day 40, to the surface of 10.0 ml of agar-solidified (0.6% w/v; Sigma) UM medium (Uchimiya & Murashige 1974) in 5.5 cm diameter Petri dishes. Cultures were incubated in the dark at  $25 \pm 2^\circ\text{C}$ .

### *Assessment of protoplast-derived cell colony growth by image analysis*

On day 68 post protoplast isolation, the Petri dishes were photographed onto Fujichrome 64T colour film using a Nikon 601 automatic 35 mm camera with a 55 mm macro-lens (1 s exposure at f16); the dishes were illuminated by bounced lights. The film was processed (E6 method) into slides and printed onto Fuji reversal printing paper (Fuji, Japan). Prints were scanned using an Apple document scanner connected to an Apple Macintosh IIfx computer. The colour images were imported into the image analysis package (NIH Image, Version 1.44) at a resolution of 150 dpi. Protoplast-derived colonies were selected by thresholding. This identified two populations; those showing discrete colony formation and growth, and those in which colony formation was insignificant. The latter were visible only as single pixels on the image, and were eliminated from the thresholded picture using a single cycle of erosion-dilation. This technique (Serra 1972) erases single point objects whilst maintaining the size of all larger selected areas, representing the actively dividing protoplast-derived colonies. The main source of error in this process is the selection of the thresholding limits identifying the colonies. The mean variation between such readings was assessed by making multiple, separate measurements.

### *Statistical methods*

Statistical analyses were performed according to Snedecor & Cochran (1989). Means and standard deviations (s.d.) were used throughout and statistical significance between mean values was assessed using a conventional Student's t-test. A probability of  $p < 0.05$  was considered significant.



Figs. 1-4. Protoplast-derived cell colonies of *P. hybrida* after 68 days of culture under the following conditions: (1 a) KM8P/KM8 medium, (1 b) Oxygenated KM8P/KM8 medium, (2 a) KM8P/KM8 medium + 0.01 % (w/v) Pluronic F-68, (2 b) Oxygenated KM8P/KM8 medium + 0.01 % (w/v) Pluronic F-68, (3 a) KM8P/KM8 medium overlaying perfluorodecalin (3 b) KM8P/KM8 medium overlaying oxygenated perfluorodecalin, (4 a) KM8P/KM8 medium + 0.01% (w/v) Pluronic F-68 overlaying perfluorodecalin, (4 b) KM8P/KM8 medium + 0.01% (w/v) Pluronic F-68 overlaying oxygenated perfluorodecalin. (Petri dish diameter = 5.5 cm).

## Results

### *Protoplast viability and division*

Immediately following enzymatic isolation, the mean viability of protoplasts was  $95 \pm 1\%$  ( $n = 3$ ). Protoplasts divided normally at the interface that formed between perfluorodecalin and the aqueous culture medium, with no significant loss in viability over the culture period. However, in some cases, there was a tendency for protoplasts to aggregate concomitantly with cell wall regeneration, but this was minimalised in media supplemented with 0.01% (w/v) Pluronic F-68.

### *Protoplast-derived cell colony growth*

Figs 1-4 show typical protoplast-derived colony formation after 68 d of culture with the various treatments. A naked eye evaluation of plates showed major differences in protoplast growth, with untreated controls characteristically showing typically small, non-proliferated colonies. In contrast, the treated protoplast cultures show extensive regions of cell growth and colony formation, the latter leading, in some cas-

es, to intergrowth of discrete cell colonies somewhat equivalent to artificial tissue production.

The percentage area covered (Fig. 5a) and the mean area per colony (Fig. 5b) varied from  $2.8 \pm 0.9\%$  and  $0.36 \pm 0.11 \text{ mm}^2$  respectively for untreated controls to  $58.5 \pm 2.1\%$  and  $12.5 \pm 0.5 \text{ mm}^2$  for colonies derived from protoplasts which had previously been cultured on oxygenated perfluorodecalin in the presence of 0.01% Pluronic F-68. The percentage coverage of the plate for each PFC-treated group was significantly greater than that for the corresponding group not treated with PFC ( $p < 0.001$  for all groups). In addition, oxygenation increased the percentage coverage significantly ( $p < 0.001$ ) for all treatment groups except for oxygenation of aqueous culture medium alone. It is also noteworthy that colony area tended to remain below approximately  $4 \text{ mm}^2$  in all treatments, with the exception of the groups receiving PFC, oxygen and Pluronic F-68. These colonies had a mean area of  $12.5 \text{ mm}^2$ , an increase of 35 fold over the untreated controls ( $p < 0.001$ ).

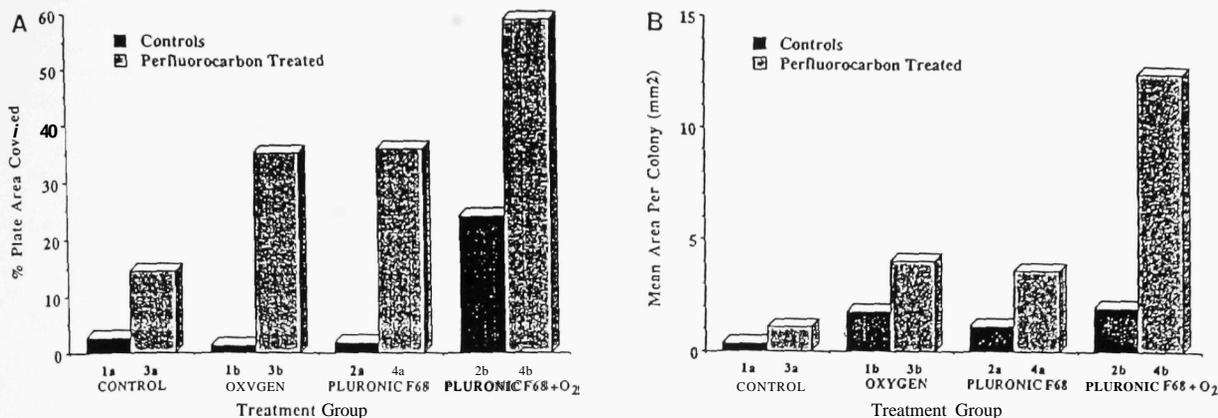


Fig. 5. Image analysis assessments of Figs 1-4:- (A) Percentage of plate area covered by *P. hybrida* protoplast-derived cell colonies. (B) Mean area (mm<sup>2</sup>) per colony of *P. hybrida* protoplast-derived cell colonies.

## Discussion and conclusions

These results demonstrate a clear, beneficial effect of supplementing protoplast cultures with oxygenated perfluorodecalin and Pluronic F-68. This study further shows, for the first time, that the subsequent growth of protoplast-derived cell colonies of *P. hybrida* and, by implication, other plant species, can be significantly enhanced in the presence of these agents. The present experiments provide further evidence of a synergistic effect of perfluorodecalin and Pluronic F-68 in enhancing plant protoplast division leading to more rapid cell proliferation. There is speculation that PFCs most probably act by facilitating oxygen transfer directly to individual cells, whilst Pluronic F-68 may enhance cellular uptake of nutrients and/or growth regulators through effects on membrane permeability (Lowe et al. 1993; Anthony et al. 1994). Alternatively, the possibility that the PFC may have facilitated protoplast and cell division by removing some unspecified growth regulator from the aqueous medium cannot be discounted. In this context, it has been suggested previously (King et al. 1989) that one possible application of PFCs in cell culture systems could be as scavengers of toxic gaseous by-products of cell metabolism.

The image analysis technique employed in this investigation provided a precise and convenient method for assessing protoplast responses to perfluorodecalin and Pluronic F-68. This approach should be valuable in the quantitative assessment of cell growth in a wide range of prokaryotic and eukaryotic cell culture systems. Image analysis technology will provide an important adjunct to measuring and discriminating accurately protoplast (and cell) growth responses. Tra-

ditionally, these have involved assessments of IPE and FPE (Latif et al. 1993) based on sampling after several days and weeks of culture respectively. Image analysis provides, particularly for FPE determinations, an alternative option. This eliminates the inevitable task of identifying discrete colonies which, more often than not, have grown together, making the value of FPE assessments somewhat problematical.

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Research Note

## An improved protocol for the culture of cassava leaf protoplasts

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**Key words:** *Manihot esculenta*, two-phase culture system, glass rods, plating efficiency

### Abstract

Viable protoplasts (yield  $> 1.9 \times 10^7 \text{ g}^{-1}$  fresh weight; mean viability  $85 \pm 2\%$ ,  $n = 5$ ) were isolated from leaves of axenic shoot cultures of *Manihot esculenta* Crantz. cv. M. Thai 8. Protoplasts were cultured for up to 50 days in liquid, ammonium-free MS medium, overlying agarose-solidified B5 medium with short glass rods embedded perpendicularly within, and protruding from, the agarose layer. Control protoplasts were cultured identically, but without glass rods. Sustained protoplast division was observed only in the presence of glass rods, where the initial plating efficiency was almost 6-fold greater than control ( $p < 0.05$ ). The mean final plating efficiency of treated cultures was  $1.0 \pm 0.2\%$  while, in contrast, significant colony formation was not observed in controls.

**Abbreviations:** BA - 6-benzyladenine, CPPU - N-(2-chloro-4-pyridyl)-N'-phenylurea, MES - 2[N-morpholino]ethane sulphonic acid, MS - Murashige & Skoog (1962), NAA -  $\alpha$ -naphthaleneacetic acid, IPE - initial plating efficiency, FPE - final plating efficiency

Cassava (*Manihot esculenta* Crantz.), a perennial shrub of the family *Euphorbiaceae*, is cultivated throughout the lowland tropics. It is an important source of calories, ranking fourth following rice, maize and sugarcane. In contrast to other tropical crops, cassava has suffered from a lack of fundamental research at the tissue culture level. However, genetic manipulation technologies should permit the improvement of cassava with respect to useful agronomic traits via genetic recombination involving nominally sexually-incompatible genotypes (Mathews *et al.* 1993). One key pre-requisite for the application of such technologies is the development of an efficient protoplast-to-plant system and, for somatic hybridisation, the subsequent establishment of an effective selection strategy for the recovery of hybrid products. The recalcitrance of cassava as an actively dividing protoplast system *in vitro* is well documented (Byrne 1984), and there is, to date, only one, non-reproducible report on plant regeneration from cassava protoplasts (Shahin & Shepard 1980). Until a reproducible protoplast culture and

shoot regeneration protocol has been established, the true potential of protoplast fusion and/or transformation via direct DNA delivery into protoplasts for the genetic improvement of cassava cannot be realised. This paper describes a protocol for inducing sustained division of cassava leaf protoplasts using a novel, simple, glass rod-medium system.

Axenic shoot cultures of *Manihot esculenta* cv. M. Thai 8 were supplied by the International Centre for Tropical Agriculture (CIAT), Cali, Colombia. Shoots of *ca.* 2.0 cm in height were routinely maintained on 50 ml aliquots of MS medium with 87.6 mM sucrose, lacking growth regulators and made semi-solid with 0.8% (w/v) agar (Difco-Bacto, USA; 3 explants per 175  $\text{cm}^3$  glass jar). Cultures were kept under a 12 h photoperiod ( $19.5 \mu\text{mol m}^{-2} \text{ s}^{-1}$ , daylight fluorescent tubes, Coolight; Thorn EMI Ltd., UK) at  $28 \pm 2 \text{ }^\circ\text{C}$  and sub-cultured at 28-35 day intervals.

The second and third fully expanded leaves (from the apex) of axenic shoot cultures were used as a source of protoplasts. Leaves were sliced transversely into 1.0

Table 1. Mean initial (IPE) and final (FPE) plating efficiencies of cassava (*M. esculenta* cv. M. Thai 8) leaf protoplasts following culture for 50 days in the presence of glass rods compared with controls lacking glass rods.

	IPE (%) (25 days)	FPE (%) (50 days)
Control (no glass rods)	1.5 ± 0.4	No colonies
Glass rods	8.9 ± 0.7*	1.0 ± 0.2

Values are mean ± s.e.m. (n = 5); \*p < 0.05

mm strips and plasmolysed by immersion for 1 h in 10 ml CPW salts solution (Frearson *et al.* 1973) containing 0.5 M mannitol (designated CPW9M), pH 5.8. The plasmolysis solution was replaced with an enzyme mixture (10 ml enzyme solution g<sup>-1</sup> fresh weight of tissue) which consisted of 1.0% (w/v) Hemicellulase (Sigma, UK), 0.4% (w/v) Cellulase RS (Yakult Honsha Co., Japan), 0.1% (w/v) Pectolyase Y23 (Seishim Pharmaceutical, Japan) and 5.0 mM MES in CPW9M solution, pH 5.8. Incubation was carried out in the dark (25 ± 2 °C) on a shaker (40 rpm) for 16 h in 9.0 cm diameter Petri dishes. The digested tissues were filtered through a nylon sieve (30 µm pore size; Wilson Sieves, UK) and the filtrate centrifuged (80 x g; 10 min). The protoplast pellet was washed twice in CPW9M solution by repeated resuspension and centrifugation. Protoplast viability was determined by uptake of fluorescein diacetate and the diameter of isolated protoplasts was measured by light microscopy using a stage micrometer.

Protoplasts were cultured in the dark (28 ± 2 °C) at a density of 4.0 x 10<sup>5</sup> ml<sup>-1</sup> in 1.0 ml aliquots of liquid, MS medium lacking NH<sub>4</sub>NO<sub>3</sub>, but supplemented with 87.6 mM sucrose, 25 mM NaNO<sub>3</sub>, 10.7 µM NAA, 2.22 µM BA and 0.5 M mannitol, pH 5.8 (designated MSP19M-N medium). One ml aliquots of this liquid medium, with protoplasts, was laid over 1.0 ml volumes of B5 medium (designated 1B5CNK; Misawa *et al.* 1982) made semi-solid with 0.6% (w/v) Sea Plaque agarose (FMC Corporation, USA), pH 5.8, in 3.5 cm Petri dishes (Nunc, Denmark). Four autoclaved glass rods (each 6.0 mm diameter x 8.0 mm length) were placed end-on equidistantly within each Petri dish prior to the addition of the semi-solid 1B5CNK medium layer, with the immobilised rods protruding from the agarose medium. Control protoplasts were cultured under identical conditions, but in dishes without glass rods.

Cell wall regeneration was determined after 3 days, by mixing equal volumes (50 µl) of 0.1% (w/v) Calcofluor White (Sigma) in CPW9M solution with cultured protoplasts suspended in MSPI9M-N medium and viewing under UV illumination. Growth responses were assessed in terms of initial (IPE; day 25) and final (FPE; day 50) plating efficiencies. IPE was defined as the number of protoplast-derived cells which had undergone at least one mitotic division; FPE as the number of protoplast-derived cell colonies. The latter (n = 100) were transferred after 50 days to MS medium lacking NaNO<sub>3</sub> and mannitol, but supplemented with 87.6 mM sucrose, 20.6 mM NH<sub>4</sub>NO<sub>3</sub>, 10.7 µM NAA and 2.22 µM BA (designated MSP1) and made semi-solid with 0.4% (w/v) agarose (Sigma Type 1) for further proliferation (20 ml per 9 cm Petri dish). Cultures were maintained under the same growth conditions as for axenic shoots. One-month-old protoplast-derived calli (*ca.* 1.0 cm in diameter) were transferred to MS medium of the same composition as MSP1, but with NAA reduced to 0.107 µM and 4.8 µM CPPU replacing BA. At this stage, protoplast-derived calli were placed on the surface of 10 ml aliquots of medium contained in 5.0 cm Petri dishes (4 calli/dish) and cultured as above.

Means and standard errors (s.e.m.) were used throughout; statistical significance between mean values was assessed using a conventional Student's *t*-test. A probability of *p* < 0.05 was considered significant.

Juvenile leaves, taken from axenic shoot cultures, provided high protoplast yields of 1.95 x 10<sup>7</sup> g<sup>-1</sup> fresh weight. Protoplasts were 10-25 µm in diameter with peripherally-aligned chloroplasts, and had a mean viability of 85 ± 2%. Cell wall formation commenced after 1-2 days of culture, with a concomitant increase in the volume and cytoplasmic content of the protoplasts. During the first 25 days of culture, a 6-fold increase (*p* < 0.05) in the number of protoplasts entering their first mitotic division, as assessed by IPE, occurred in dishes with glass rods. In contrast, only 1.5 ± 0.4% of the protoplasts had undergone mitosis in the control cultures (Table 1). In the latter, cell division occurred only where freshly-isolated protoplasts, suspended in the liquid phase, were in contact with the meniscus formed where the culture medium touched the sides of the Petri dishes. In control cultures, mitosis was not sustained and significant numbers of cell colonies were not formed (Fig. 1 A). However, when glass rods were introduced into the two-phase culture system, the extent of the liquid meniscus was increased and cell

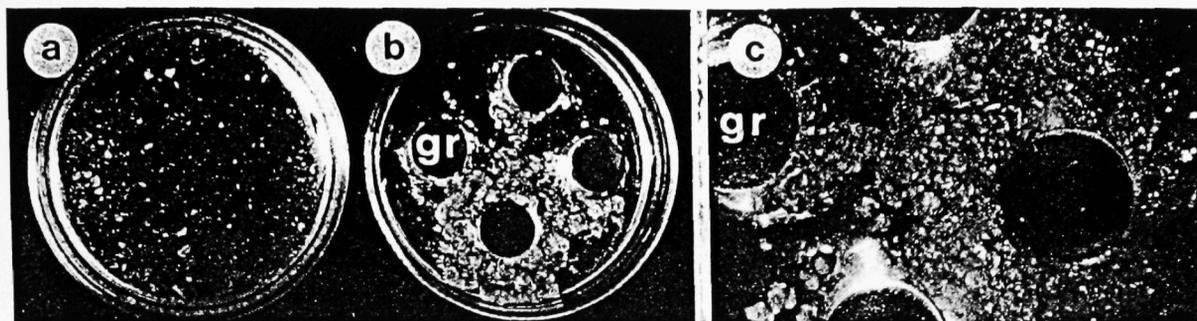


Fig. 1. Petri dishes (3.5 cm diameter) in which leaf protoplasts of *M. esculenta* cv M. Thai 8 were cultured for 40 days in (a) liquid medium over agarose-solidified medium (control), (b) liquid over agarose-solidified medium with glass rods (gr) protruding from the underlying agarose layer, and (c) higher magnification of part of (b) showing glass rods surrounded by protoplast-derived cell colonies.

colonies developed in close association with the glass rods. Protoplasts cultured with glass rods proliferated into dense colony aggregates by day 40 (Figs. 1B, 1C).

Proliferating multicellular colonies, when transferred to semi-solid MSPI medium after 50 days of culture, developed rapidly to produce friable callus (2–3 cm diameter) by day 90. Such callus typically developed 1–2 roots per tissue portion after a further 21 days of culture. Calli transferred to semi-solid MS medium containing CPPU, developed a nodular appearance within 30 days. Such nodular callus, when sectioned, displayed an epidermal layer and distinguishable cortex and vascular tissues, with an internal organisation similar to that of cassava tubers. Meristematic regions were absent.

The present experiments demonstrate that high yields of cassava protoplasts can be routinely obtained from leaves of cultured shoots. These isolated protoplasts can be induced to undergo sustained division to form multicellular colonies when partially embedded glass rods are included in the two-phase culture system. Such use of glass rods, to increase the number of liquid:glass/plastic contact points, provides a simple method for ensuring a sustainable plating efficiency of cassava leaf protoplasts which, hitherto, has not been obtained with other approaches (Byrne 1984).

The novel system described here probably facilitates a greater gaseous exchange between the liquid phase, containing the protoplasts, and the immediate atmosphere above the medium in the dish. In this regard, previous studies have shown that oxygen-enriched atmospheres also enhance the plating efficiency of cultured protoplasts of jute, rice and tomato

(d'Utra Vaz *et al.* 1992). Furthermore, the division of protoplasts from cell suspensions of albino *Petunia hybrida* is stimulated by culture of the protoplasts at the interface between oxygenated perfluorocarbon and an overlying aqueous culture medium (Anthony *et al.* 1994). Consequently, it will be of interest, in future work, to assess whether combinations of these physical and chemical approaches to gaseous manipulation can further enhance protoplast division and cell colony formation in cassava, including protoplasts which have been modified genetically. In cassava, such studies should also involve the use of a broader range of growth regulators in the medium ultimately to promote plant regeneration from protoplast-derived tissues. Additionally, it may be feasible to regenerate plants directly from protoplasts through somatic embryogenesis without an intervening callus phase. In this respect, embryogenic cell suspensions may be useful source material for the isolation of protoplasts.

To date, studies have shown adenine-type cytokinins to be ineffective growth regulators for inducing differentiation of protoplast-derived callus (Anthony *et al.* unpublished). In the present study, the substituted pyridyl phenylurea compound, CPPU, was evaluated, since this synthetic cytokinin is effective at very low concentrations on a wide range of species (Fellman *et al.* 1987) and induces somatic embryogenesis in grape (Matsuta & Hirabayashi 1989). The induction, by CPPU, of nodular and tuber-like structures in protoplast-derived callus of cassava, indicates that the use of this phenylurea derivative may, ultimately, result in the differentiation of shoots in the cv. M. Thai 8 tested in these experiments and in other cassava genotypes previously considered to be recalcitrant to differen-

tiation when exposed to adenine-type cytokinins. A focus of future studies to further improve protoplast growth and differentiation in this biotechnologically-important plant will be to optimise not only gas supply, but also growth regulator-induced differentiation.

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STRATEGIES FOR PROMOTING DIVISION OF CULTURED PLANT PROTOPLASTS:  
BENEFICIAL EFFECTS OF OXYGENATED PERFLUOROCARBON

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SUMMARY

Assessments have been made of physical and chemical options, alone and in combination, for gaseous manipulation of cassava (*Manihot esculenta* Crantz.) leaf protoplast cultures. Protoplasts were cultured for 25 d in liquid medium at an initial plating density of  $4 \times 10^5 \text{ ml}^{-1}$  overlying (1) agar-solidified medium (control), (2) agar medium under an oxygen-enriched (10 mbar, 1 min) atmosphere, (3) agar medium supplemented with perfluorodecalin (Flutec® PP5), or (4) agar medium supplemented with oxygenated (10 mbar, 15 min) perfluorodecalin. Similar experimental treatments were also set up, with glass rods embedded in the agar medium. The mean initial plating efficiency (IPE) of protoplasts following culture with oxygenated perfluorodecalin without glass rods ( $6.7 \pm 0.6\%$ ;  $n = 3$ ) was over **2-fold** greater ( $P < 0.05$ ) than that of control cultures ( $2.6 \pm 0.2\%$ ;  $n = 3$ ). The mean IPE of protoplasts cultured with oxygenated perfluorodecalin in the presence of glass rods ( $5.8 \pm 0.2\%$ ;  $n = 3$ ) was also over **2-fold** greater ( $P < 0.05$ ) than controls. There was no significant difference between the IPE of protoplasts cultured under an increased oxygen atmosphere or with oxygenated perfluorodecalin, irrespective of the presence of glass rods.

INTRODUCTION

A key **pre-requisite** for the application of plant cell culture biotechnology to plant improvement through genetic engineering is, ultimately, the development of efficient protoplast-to-plant regeneration systems, based on a maximisation of sustainable mitotic division. Previous approaches for enhancing oxygen supply to cultured protoplasts include the use of oxygen-enriched atmospheres (d'Utra Vaz *et al.*, 1992), or **liquid-over-agar** medium implanted with glass rods to increase the surface area of the liquid layer to promote respiratory gas exchange (Anthony *et al.*, 1995). A further and novel innovation has been the use of oxygenated perfluorocarbon (PFC) liquid to facilitate respiratory gas transfer to plant protoplasts and cells cultured under static conditions (Anthony *et al.*, 1994a,b). PFCs are inert, organic compounds which can dissolve large volumes of respiratory gases. They have been used for enhancing oxygen supply both *in vivo* and *in vitro* (King *et al.* 1989; Lowe, 1994; Lowe *et al.*, 1995). The extent to which PFCs *per se* will be of routine use in plant cell biotechnology will depend upon their ability to facilitate improved and sustainable conversion of protoplasts to **cells**, whose mitotic activity and totipotency leads, ultimately, to plant regeneration. The relative **efficiency** of PFCs must be compared with other options, such as the use of oxygen-enriched atmospheres (d'Utra Vaz *et al.*, 1992), or increased **medium** surface area to promote gaseous exchange.

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The present investigation employed mesophyll protoplasts of cassava (*Manihot esculenta* Crantz.) to assess the effects of improved gas manipulation methods on the mitotic division of protoplast-derived cells. Attention was focused on cassava, since protoplasts of this species, like those of several other major crop plants, are still regarded as relatively recalcitrant to culture when compared to well resolved, ubiquitous plant models, such as tobacco.

## MATERIALS AND METHODS

### Plant material and establishment of cultures

Axenic shoots (Stamp & Henshaw, 1987) of *Manihot esculenta* Crantz. cv. M. Thai 8 [supplied by the International Centre for Tropical Agriculture (CIAT), Cali, Colombia] were cultured (3 explants per 175 cm<sup>3</sup> glass jar) on 50 ml aliquots of MS medium (Murashige & Skoog, 1962) lacking growth regulators and made semi-solid with 0.8% (w/v) agar (Difco-Bacto, Detroit, Michigan, USA), pH 5.8. Cultures were maintained under a 12 h photoperiod (19.5 μmol m<sup>-2</sup> s<sup>-1</sup>, Coolight daylight fluorescent tubes; Thorn EMI Ltd., Ruislip, UK) at 28 ± 2°C and sub-cultured every 28-35 d by transferring 2.0 cm apical sections to fresh medium.

### Protoplast isolation

Second and third fully expanded leaves (from the apex) of axenic cultured shoots were used as a source of protoplasts (1.0 g f.wt. per isolation). Leaves were sliced transversely into 1.0 mm strips and plasmolysed, by immersion for 1 h, in 10 ml CPW salts solution (Frearson *et al.*, 1973) containing 0.5 M mannitol (designated CPW9M solution), pH 5.8, in 9.0 cm diameter Petri dishes. The plasmolysis solution was replaced with an enzyme mixture (10 ml enzyme solution g<sup>-1</sup> f. wt. of tissue) which consisted of 1.0% (w/v) Hemicellulase (Sigma, UK), 0.4% (w/v) Cellulase RS (Yakult Honsha Co., Nishinomiya, Japan), 0.1% (w/v) Pectolyase Y23 (Seishim Pharmaceutical, Tokyo, Japan) and 5.0 mM 2-2-N-[mopholino] ethane sulphonic acid in CPW9M solution, pH 5.8. Incubation was in the dark (25 ± 2°C) on a rotary shaker (40 r.p.m.) for 16 h in 9.0 cm diameter Petri dishes sealed with Nescofilm (Bando Chemical Ind., Kobe, Japan). The digested tissues were passed through a nylon sieve (30 μm pore size; Wilson Sieves, Nottingham, UK) and the filtrate centrifuged (80 x g; 10 min). The protoplast pellet was washed twice in CPW9M solution by repeated resuspension and centrifugation. Protoplast viability was determined by uptake and cleavage of fluorescein diacetate (Widholm, 1972), prior to counting.

### Culture of protoplasts

Protoplasts were cultured in the dark (28 ± 2°C) at a final plating density of 4.0 X 10<sup>5</sup> ml<sup>-1</sup> in 0.75 ml aliquots of liquid MS medium which lacked NH<sub>4</sub>NO<sub>3</sub>, but which was supplemented with 87.6 mM sucrose, 25 mM NaNO<sub>3</sub>, 10.7 μM α-naphthaleneacetic acid, 2.22 μM 6-benzyladenine and 0.5 M mannitol, pH 5.8 (designated MSP19M-N medium). Aliquots (0.75 ml) of protoplast suspension were laid over 0.75 ml volumes of B5 medium (designated 1B5CNK; Misawa *et al.*, 1982) semi-solidified with 0.6% (w/v) Sea Plaque agarose (FMC Corporation, Rockland, USA), pH 5.8, in the bottom halves of 3.5 cm Petri dishes (Nunc, Kamstrup, Denmark) (treatment A, control). The Petri dish lids were not used. All dishes were transferred into 7.0 cm high glass chambers with lids. Each chamber contained a 1.0 cm thick layer of 0.8% (w/v) aqueous agar solution (d'Utra Vaz *et al.*, 1992). Some chambers were gassed (flushed) for 1 min with 100% gaseous oxygen (10 mbar) prior to sealing to retain an oxygen-enriched atmosphere

(treatment C). A further modification was the addition of 10 ml aliquots of non-oxygenated or oxygenated (10 mbar, 15 min) perfluorodecalin (*Flutec*® PP5, BNFL Fluorochemicals Ltd., Preston, UK) into the Petri dishes (treatments E, G, respectively). This produced a triple, discontinuous layered culture system (agar medium, overlaid by perfluorodecalin and aqueous medium phases). Experimental treatments were also established with the inclusion of four autoclaved glass rods (each 6.0 mm diameter x 8.0 mm length) placed end-on equidistantly within each Petri dish prior to the addition of the agar-solidified IB5CNK medium layer. The immobilised rods protruded from the agarose medium (Anthony *et al.*, 1995) (treatments B, D, F, H). Each treatment, with and without glass rods, was replicated 3 times.

Protoplasts were cultured for up to 25 d under the above conditions. All treatments utilised protoplasts from the same protoplast population. The IPE, defined as the number of protoplast-derived cells that had undergone at least one mitotic division, was assessed by light microscopy; a minimum of 100 protoplasts were scored per Petri dish.

### Statistical analyses

Means and standard errors (s.e.m.) were used throughout; statistical significance between mean values was assessed using a conventional Student's *t*-test (Snedecor & Cochran, 1989). A probability of  $P < 0.05$  was considered significant.

## RESULTS

The yield of cassava leaf protoplasts was  $6.9 \pm 0.9 \times 10^6$  g.f.wt, with a mean viability of  $74 \pm 4\%$ . During the first 25 d of culture, a 68% increase ( $P < 0.001$ ) in the mean number of protoplasts entering their first mitotic division, as assessed by IPE, occurred in dishes with glass rods compared to their untreated controls under a normal gaseous atmosphere (Fig. 1, treatments A, B). Culture of protoplasts under an oxygen enriched-atmosphere, but without glass rods, increased their IPE 2-fold ( $P < 0.05$ ) after 25 d, when compared to protoplasts cultured in the absence of glass rods under normal atmosphere conditions (Fig. 1, treatments A, C). There was no further elevation (or suppression) of IPE when protoplasts were cultured under an increased oxygen atmosphere and in the presence of rods (Fig. 1, treatments C, D). The mean IPE values following culture of protoplasts with the oxygenated perfluorodecalin interjacent layer, either without or with glass rods, were **significantly** greater ( $P < 0.05$ ) than those of the respective control cultures (Fig. 1, treatments A, G; B, H, respectively). Indeed, the mean IPE of protoplasts cultured with oxygenated perfluorodecalin in the presence of glass rods ( $5.8 \pm 0.2\%$ ;  $n = 3$ ) was over 2-fold greater than in the controls ( $P < 0.05$ ) lacking glass rods (Fig. 1, treatments H, A). There were no significant differences between IPEs of cassava protoplasts when cultured under an increased oxygen atmosphere, or with **oxygenated** perfluorodecalin, irrespective of the presence or absence of glass rods (Fig. 1, treatments, C, G; D, H, respectively). In addition, the mean IPEs of protoplasts following culture with non-oxygenated perfluorodecalin, with or without glass rods, were significantly different ( $P < 0.05$ ) from their respective controls; an increase in IPE was observed in the absence of rods (Fig. 1, treatments E, A), whereas a reduced IPE occurred in the presence of rods (Fig. 1, treatments F, B). Interestingly, protoplast aggregation or the accumulation of phenolic products did not occur in cultures containing glass rods and oxygenated PFC.

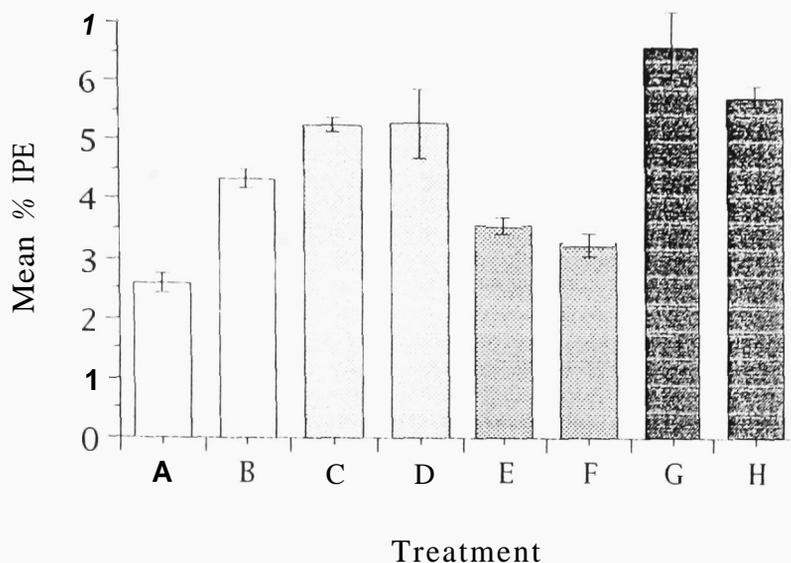


Figure 1. Mean initial plating efficiency (IPE) of cassava leaf protoplasts after 25 d of culture in response to gas-manipulation treatments. Protoplasts cultured in: A) medium without glass rods in a normal atmosphere (control), B) with glass rods in a normal atmosphere, C) without glass rods but with an oxygen-enriched atmosphere, D) with glass rods and an oxygen-enriched atmosphere, E) without glass rods and with non-oxygenated PFC, F) with glass rods and non-oxygenated PFC, G) without glass rods and with oxygenated PFC or H) with glass rods and oxygenated PFC. Vertical bars represent s.e.m. (n = 3 throughout).

## DISCUSSION

Oxygenated perfluorodecalin enhanced the mitotic activity of cassava leaf protoplasts, presumably by facilitating oxygen delivery. This approach was simpler and superior to the inclusion of partially-embedded glass rods to increase the surface area for gaseous exchange. Previously, it was demonstrated that protoplasts of cassava would undergo sustained division only when cultured in the presence of glass rods (Anthony *et al.*, 1995). The fact that the increase in division frequency of cassava protoplasts could not be further enhanced by the inclusion of glass rods in cultures supplemented with oxygenated perfluorodecalin, suggests that there was no synergism between these oxygenation options. Earlier work, using protoplasts of *Petunia hybrida*, showed that supplementation of aqueous medium with 0.01% (w/v) of the co-polymer surfactant, *Pluronic*<sup>®</sup> F-68, overlaying oxygenated perfluorodecalin, promoted a further increase in division frequency (Anthony *et al.*, 1994a). Assessment of the effect of *Pluronic*<sup>®</sup> F-68, would be a logical extension to the present investigation using cassava. The finding that mitotic activity was also increased in cassava by non-oxygenated perfluorodecalin, was probably due to an improved physical environment, whereby the protoplasts were subjected to lower interfacial tensions.

The use of gassed perfluorodecalin as a "reservoir" of oxygen was also superior to the use of oxygen-enriched atmospheres, which have previously been demonstrated to enhance division of protoplasts of tomato, rice and jute (d'Utra

Vaz *et al.*, 1992). Arguably, the use of perfluorodecalin is a more convenient and less hazardous option for oxygen delivery than the use of oxygen atmospheres. Supplementation of aqueous culture medium with oxygenated perfluorodecalin liquid, which is readily recoverable, sterilisable and, hence, recycleable (Anthony *et al.*, 1994a; Lowe *et al.*, 1995), clearly provides a convenient approach for facilitating respiratory gas supply not only to protoplasts of cassava, but also to those of other plant species. Despite the high initial cost of the PFC liquid, this approach should have wide applications in plant cell biotechnology. As emphasised previously (Anthony *et al.*, 1994a), a particular advantage of the use of PFC as an oxygenating agent is in static cultures of protoplasts (or cells) where conventional aeration through agitation (e.g. stirring) cannot be employed because of the tendency to cause cell damage.

Woody plants, such as cassava, are generally considered to have relatively poor protoplast plating efficiencies (Ochatt & Power, 1990). The use of oxygenated perfluorodecalin alone, as shown in the present investigation, may go some way towards the enhanced throughput of protoplast-derived multicellular colonies and, in turn, whole plant regeneration.

A difficulty frequently encountered during the early stages of protoplast culture, particularly of woody species, is that of phenolic Oxidation accentuated by the aggregation of protoplast-derived cells (Ochatt, 1989; Ochatt & Power, 1990). While some success has been achieved in overcoming this fundamental problem through the culture of protoplasts, for example as monolayers on polyester discs interspersed within the aqueous medium (Russell & McCown, 1986, 1988), the use of oxygenated perfluorodecalin, as in the present study, to prevent protoplast clumping and phenolic oxidation, provides an additional, convenient approach. One further refinement of the present system would be to "recharge" the PFC with oxygen during culture, since previous studies have demonstrated that oxygen-saturated PFC becomes depleted of oxygen after 3 days during culture of *P. hybrida* protoplasts (Anthony *et al.*, 1994a). This would maximise and extend oxygen delivery to protoplast-derived cells.

Oxygenated PFC layers, droplets or emulsions, as media adjuncts, will now provide a baseline for the culture of limited numbers or individual protoplasts (e.g. flow-sorted heterokaryons or micromanipulated cells), since such supplementation enhances division potential. Likewise, the availability of efficient protoplast-to-plant systems has implications in terms of their transformation efficacy via protoplasts using direct DNA delivery technologies (Davey *et al.*, 1989). Additionally, in the context of somatic hybridisation, the conversion of heterokaryons (intergeneric and interspecific protoplast fusion products) to somatic hybrid cells will be facilitated by improved plating efficiencies.

#### ACKNOWLEDGEMENTS

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## Pluronic F-68 Increases the Post-thaw Growth of Cryopreserved Plant Cells

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The beneficial effects of supplementing culture media with Pluronic F-68 (0.01-0.2% w/v), on post-thaw growth following cryopreservation of suspension-cultured cells of *Oryza sativa* cvs. Taipei 309 and Tarom (*Japonica rices*), together with *Lolium multiflorum* (Italian ryegrass), were studied. The mean absorbance, following triphenyltetrazolium chloride (TTC) reduction, of Taipei 309 cells 4 days after thawing was increased twofold over control by 0.01% (w/v) Pluronic F-68 ( $P < 0.01$ ) and almost twofold by 0.1% (w/v) surfactant. A more pronounced, fourfold increase ( $P < 0.001$ ) in post-thaw absorbance occurred with Tarom cells exposed to 0.1% (w/v) Pluronic F-68; cell absorbance was elevated threefold with 0.2% (w/v) Pluronic ( $P < 0.001$ ) and twofold ( $P < 0.001$ ) by 0.01% (w/v) surfactant. TTC reduction by *Lolium* cells was elevated 31% ( $P < 0.01$ ) with 0.01% (w/v) surfactant. Pluronic F-68 (0.01% w/v) also promoted sustained mitotic activity since biomass, measured 30 days post-thawing, was increased to a maximum of 32% above control ( $P < 0.05$ ), in both Taipei 309 and *Lolium*. There was no measurable beneficial effect of adding Pluronic F-68 to the cryoprotectant before freezing. These results, which demonstrate a marked cytoprotection of Pluronic F-68 on plant cells recovered from cryostorage, suggest that this surfactant is a useful additive agent in post-thaw handling strategies. © 1996 Academic Press, Inc.

The nonionic polyoxyethylene (POE)-polyoxypropylene (POP) surfactant Pluronic F-68 (Poloxamer 188) has been employed extensively as a low-cost, nontoxic, cell-protecting agent in both animal (14, 27) and plant (19, 20) culture systems. Pluronic F-68 acts as a foam-stabilizing agent in agitated/aerated cultures and is believed to protect cells against fluid-mechanical damage (13). Pluronic F-68 is adsorbed onto cell surfaces, and studies with both cultured insect cells (24, 23, 12) and mammalian hybridomas (32) have demonstrated that interaction of the surfactant with cytoplasmic membranes can increase the resistance of cells to shear forces, albeit over differing time periods depending on the cell type.

The cytoprotectant properties of Pluronic F-68 make this compound a strong and obvious candidate for use in plant cell cryopreservation protocols. Early studies showed that the Pluronics could prevent hemolysis of human red blood cells in response to freeze-thawing procedures

(11), and subsequent studies demonstrated that Pluronic F-68 was an effective cryoprotectant of cultured Chinese hamster cells (2). Surprisingly, there has been no evaluation of the effects of using Pluronic F-68 with plant cells, either as a cryoprotectant per se or a post-thaw cytoprotecting agent, despite the increasing interest in cryopreservation for conserving agronomically important and endangered plant germplasms (3). Consequently, the present investigation has employed embryogenic cells of *Japonica rices*, together with nonembryogenic cells of *Lolium*, to evaluate the potential beneficial effects of Pluronic F-68, either as a cell protectant during freezing or as a specific culture medium supplement, in enhancing post-thaw cellular growth following short-term (30 days, *Oryza sativa* cv. Tarom, *Lolium*) or long-term (3 years, *O. sativa* cv. Taipei 309) cryopreservation.

### MATERIALS AND METHODS

#### *Plant Materials and Preparation of Cell Suspensions*

Cell suspensions of *O. sativa* L. cv. Taipei 309 and *O. sativa* L. cv. Tarom were initiated

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from embryogenic calli derived from mature seed scutella (10). Cell suspensions of *Lolium multiflorum* were supplied by Dr. E. Guiderdoni, IRAT/CIRAD, Montpellier, France. Cell suspensions were maintained in AA2 medium [cv. Taipei 309 (1), R2 medium (cv. Tarom) (26), and N6 medium (*Lolium*) (9)] in 100-ml Erlenmeyer flasks with shaking (120 rpm, 2.5-cm throw) at  $28 \pm 1^\circ\text{C}$  in the dark. Rice cell suspensions (both varieties) were subcultured routinely every 7 days by transferring 1 ml of settled cells with 7 ml of spent medium to 22-ml aliquots of fresh medium; *Lolium* cell suspensions were similarly subcultured by transfer of settled cells (3 ml) and 7 ml of spent medium to 40-ml aliquots of fresh medium. Prior to cryopreservation, cells were cultured for 3-4 days in their respective liquid medium supplemented with 60.0 g liter<sup>-1</sup> mannitol.

#### *Cryopreservation and Post-thaw Recovery*

The cryopreservation protocol was based on that of Meijer *et al.* (22). Cells were harvested onto a nylon mesh (45- $\mu\text{m}$  pore size) and placed into 2-cm<sup>3</sup> polypropylene vials (Sarstedt, Leicester, UK) with approximately 0.2 g fresh weight of cells per vial. Approximately 0.75 ml of a cryoprotectant mixture [46.0 g liter<sup>-1</sup> glycerol, 39.0 g liter<sup>-1</sup> dimethyl sulfoxide ( $\text{Me}_2\text{SO}$ ), 342.0 g liter<sup>-1</sup> sucrose, 5.0 g liter<sup>-1</sup> proline] was added to each vial. The cryoprotectant mixture was prepared in the liquid culture medium appropriate for each species and the pH adjusted to 5.8, prior to filter sterilization (0.2- $\mu\text{m}$  pore size; Sartorius, Gottingen, Germany). All cryoprotectants were of Analar grade except  $\text{Me}_2\text{SO}$ , which was spectroscopically pure.

Cells were cryoprotected for 1 h on iced water, vials containing the cells were transferred to aluminum canes, and the cells were frozen at a controlled rate ( $-1^\circ\text{C min}^{-1}$ ) from 0 to  $-35^\circ\text{C}$  and held at this temperature for 35 min in a programmable freezer (Planer Cryo 10 Series, Planer Biomed, Sunbury-on-Thames, UK), prior to storage in liquid nitrogen at  $-196^\circ\text{C}$ . Cells of cv. Tarom and *Lolium*

were stored for 30 days, whereas those of cv. Taipei 309 were cryopreserved for 3 years.

Cells of both species (two cultivars for rice) were thawed by plunging the vials into sterile water at  $45^\circ\text{C}$ ; excess cryoprotectant was removed under axenic conditions from the cells using a disposable Pasteur pipet. The cells were placed onto two superimposed 5.5-cm-diameter Whatman No. 1 filter paper disks overlying 20-ml aliquots of the appropriate culture medium. The latter was semisolidified with 0.4% (w/v) Sea Kem Le agarose (FMC Corp., Rockland, ME). In some treatments, the medium was supplemented with 0.01, O.I. or 0.2% (w/v) Pluronic F-68 (Sigma, Poole, UK). These concentrations of Pluronic were selected on the basis of previous studies demonstrating the stimulatory effects of this compound on the growth in culture of protoplasts isolated from cell suspensions of *Solanum dulcamara* (18). In an additional assessment, the cryoprotectant mixture for both species was also supplemented with the aforementioned concentrations of Pluronic F-68 and cells cryopreserved for 14 days in liquid nitrogen.

Cells were cultured for all treatments in the dark for 3 days at  $28 \pm 1^\circ\text{C}$  prior to transfer of the upper filter disk containing the cells to the respective fresh media. Cells were cultured for a further 24 h prior to viability assessments and, where appropriate, for a further 26 days, under the same conditions, for biomass determinations. Each experiment was replicated up to 20 times, except where surfactant was incorporated into the cryoprotectant, where  $n = 1$ .

#### *Measurement of Post-thaw Viability and Biomass*

The post-thaw viability and metabolic capacity of cells were assessed by the reduction of triphenyltetrazolium chloride (TTC) (30). Three-milliliter aliquots of TTC buffer, consisting of 0.6% (w/v) TTC in 0.05 M  $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$  buffer containing 0.05% (v/v) Tween 80 (Sigma, pH 7.4), was added to 50 mg of cells contained in 16.0-ml screw-capped centrifuge tubes (Coming, Coming, NY). Cells were incubated at  $28 \pm 1^\circ\text{C}$  for

16-18 h in the dark, washed once with distilled water, and extracted with 7.0 ml of 95% (v/v) ethanol in a boiling water bath for 5 min. Supernatants were cooled, and each was adjusted to 10.0 ml with 95% (v/v) ethanol. The absorbance was measured at 490 nm. The same protocol was also employed for unfrozen cells 4 days following subculture.

In some assessments, using both cv. Taipei 309 and *Lolium* cells, the incubation buffer was supplemented with 0.01 or 0.1% (w/v) Pluronic F-68, respectively, as a control for any effects of the surfactant on TTC reduction. The fresh weight of thawed cells was recorded to determine biomass (21).

### Statistical Analyses

Means and standard errors (SEM) were used throughout; statistical significance between mean values was assessed using a conventional analysis of variance (ANOVA) and Student's *t* test, as appropriate (29). A probability *P* less than 0.05 was considered significant.

### RESULTS

The mean absorbance of unfrozen cells, as measured by TTC reduction, was  $1.60 \pm 0.11\%$  ( $n = 10$ ) for *O. sativa* cv. Tarom and  $0.54 \pm 0.04\%$  ( $n = 10$ ) for *Lolium*. In the case of *O. sativa* cv. Taipei 309, the mean cell absorbance, prior to long-term (3-year) cryopreservation, was  $0.96 \pm 0.05\%$  ( $n = 10$ ).

Supplementation of culture medium for the cv. Taipei 309 with Pluronic F-68 at 0.01% (w/v) significantly increased the mean post-thaw cell absorbance following TTC reduction to more than twofold greater ( $P < 0.01$ ) than that in untreated controls (Fig. 1). A similar, but less pronounced effect also occurred with 0.1% (w/v) surfactant (Fig. 1). In contrast, there was no corresponding increase in absorbance in these rice cells when 0.2% (w/v) Pluronic was incorporated into the culture medium.

In the case of *O. sativa* cv. Tarom, supplementation of medium with Pluronic F-68 had a consistently greater stimulatory effect on the

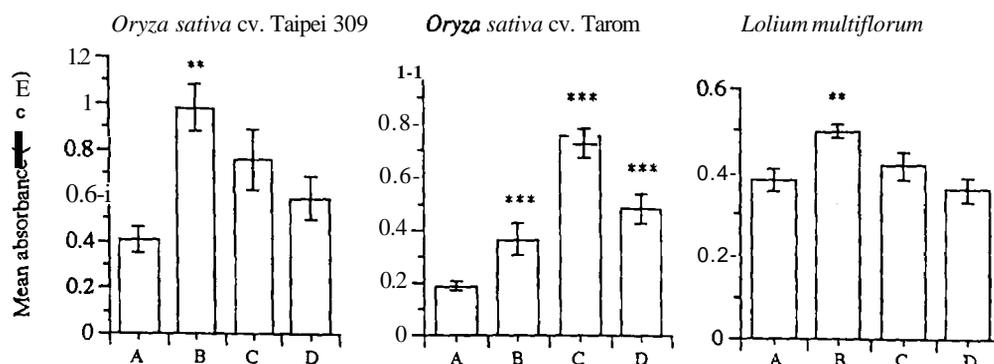
mean post-thaw cell absorbance compared with that of untreated controls (Fig. 1). The most pronounced increase occurred with 0.1% (w/v) surfactant, which promoted a fourfold increase ( $P < 0.001$ ) in absorbance over the control. Supplementation of medium for Tarom cells with 0.01% (w/v) or 0.2% (w/v) Pluronic increased the mean cell absorbances by twofold ( $P < 0.001$ ) and threefold ( $P < 0.001$ ), respectively (Fig. 1).

A significant ( $P < 0.01$ ) increase in mean post-thaw cell absorbance was also recorded when Pluronic F-68 at 0.01% (w/v) was added to the culture medium for *Lolium* cells, although the mean maximum increase over control of 31% (Fig. 1) was markedly lower than that observed when the same concentration of surfactant was used with cells of *O. sativa* cv. Taipei 309 (140%) or cv. Tarom (95%).

No significant difference was observed, using post-thawed cv. Taipei 309 cells, between the mean cell absorbance in the TTC assay ( $0.62 \pm 0.05$ ,  $n = 15$ ) and in the same assay using buffer supplemented with 0.01% (w/v) Pluronic F-68 ( $0.61 \pm 0.05$ ,  $n = 15$ ). Comparable results were also obtained with post-thawed *Lolium* cells using 0.1% (w/v) surfactant (control,  $0.41 \pm 0.03$ ,  $n = 15$ ; Pluronic,  $0.39 \pm 0.04$ ,  $n = 5$ ).

Addition of Pluronic F-68 to culture medium also fostered an increase in biomass, as measured by cell fresh weight following 30 days of post-thaw culture, in both *O. sativa* cv. Taipei 309 and *Lolium*, with significant ( $P < 0.05$ ,  $P < 0.01$ ) increases in the presence of 0.01% (w/v) surfactant in both cases (Table 1). The greatest increase occurred with cv. Taipei 309 supplemented with 0.01% (w/v) Pluronic F-68, where the mean fresh weight after 30 days was 32% greater than the corresponding mean control value (Table 1).

The addition of 0.01-0.2% (w/v) Pluronic F-68 to the cryoprotectant mixture, both before and during freezing, did not affect the post-thaw viability of cells from either rice cultivar or *Lolium*. For example, the mean absorbance of *Lolium* cells 4 days following thawing in the presence of 0.01% (w/v) Pluro-



Concentration of Pluronic® F-68 in cell recovery medium

FIG. 1. Absorbance, following TTC reduction, by plant cells post-thawed in the presence of Pluronic F-68: (A) 0%, (B) 0.01%, (C) 0.1%, (D) 0.2% (w/v). Values are means of 20 observations; vertical bars represent SEMs. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with mean control (0% Pluronic).

nic ( $0.74 \pm 0.04$ ,  $n = 7$ ) was not significantly different from that of control lacking surfactant ( $0.70 \pm 0.04$ ,  $n = 7$ ). A similar pattern was observed with both rice cultivars (data

not shown). Cells recovered of both rice cultivars and *Lolium* had a dark, wet appearance, during the first few days of culture, following their initial recovery from cryopreservation. By day 10 of culture, however, cell color and friability were the same as for unfrozen controls.

TABLE 1

Mean Fresh Weight of Cryopreserved Cells of *Oryza sativa* cv. Taipei 309 and *Lolium multiflorum* Recovered after 30 Days with 0.01–0.2% (w/v) Pluronic F-68 in the Culture Medium

Treatment	Mean fresh weight on day 30 (g)
<i>Oryza sativa</i> cv. Taipei 309	
Control (0% Pluronic)	2.07 ± 0.28
Pluronic	
0.01%	2.74 ± 0.13*
0.1%	2.46 ± 0.11
0.2%	1.98 ± 0.16
<i>Lolium multiflorum</i>	
Control (0% Pluronic)	3.49 ± 0.18
Pluronic	
0.01%	4.50 ± 0.20**
0.1%	3.29 ± 0.21
0.2%	3.32 ± 0.19

Note. Values are means ± sem,  $n = 11-14$  throughout. For all treatments, ca. 0.2 g of cells was used as starting material (Day 0).

\* $P < 0.05$ , \*\* $P < 0.01$  compared with control (0% Pluronic).

Cell suspensions ( $n = 9$ ) reinitiated from recovered cells of *O. sativa* cv. Tarom in the presence of 0.01% (w/v) Pluronic F-68 showed no difference (after 28 days) in mean absorbance following TTC reduction compared with control ( $1.04 \pm 0.16$  and  $0.87 \pm 0.60$ , respectively). Additionally, their overall growth rates were comparable to those of controls and they were routinely subcultured in accord with previously established protocols. Interestingly, these cell lines gave viable protoplast yields approximately twice those of controls (data not shown).

#### DISCUSSION

The results of these experiments demonstrate that supplementation of the culture medium with Pluronic F-68 increases the post-thaw metabolism and growth of cryopreserved rice and *Lolium* cells. While it is generally accepted that reduction of TTC can be used as an indicator of cell viability and metabo-

lism (5), the present study also includes data on biomass increases to support the conclusion of growth-enhancing effects of Pluronic F-68. It is probable that the present observations reflect a combination of increased cell survival post-thawing coupled, perhaps, with a growth-stimulating effect of the surfactant in plant culture systems, as reported previously (19). Assessment of TTC reduction was made 4 days post-thaw since previous related work indicated that, at this time, the TTC assay provides optimal information on cell metabolic capacity (21).

An important finding from this investigation was that the optimum concentration of surfactant, which increased cell growth, differed between the two rice cultivars and between *O. sativa* cv. Tarom and *Lolium*. The present observations, showing that there are both species- and cultivar-specific responses to Pluronic F-68, are consistent with previous observations using *Chrysanthemum morifolium*, in which the optimum concentration of surfactant that stimulated adventive shoot regeneration from cultured leaf explants differed by an order of magnitude between cultivars (17). Differences in tissue and organ responsiveness to the growth-promoting effects of Pluronic F-68 have also been observed in *Solanum dulcamara* (18), *Corchorus capsularis* (16), *Hypericum perforatum* (6), and *Populus* spp. (15).

Studies using animal cells have shown that Pluronic F-68 adsorbs onto cytoplasmic membranes, conferring increased resistance to mechanical damage (14, 19, 20). The Pluronic polyols have hydrophobic POP cores, which are believed to become embedded in the phospholipid membranes of cells, leaving their hydrophilic POE tails outside. This would have the effect of reducing the interfacial tension of the cells and sterically hindering adhesive interaction between molecules on the cell surfaces. Such reduction in adhesive interaction would prevent cell-to-cell contact and, thus, further reduce mechanical damage.

Pluronic F-68 may also promote the increased uptake of nutrients, **growth regulators**,

or oxygen into cells during the post-thaw period. Indeed, related experiments using animal cells cultured under static conditions have shown that concentrations of Pluronic F-68 comparable to those used in the present investigation stimulated both 2-deoxyglucose uptake and cellular amino acid incorporation (7). Any increase in nutrient uptake promoted by Pluronic would be expected to alter metabolic flux, allowing biochemical pathways to operate more efficiently, particularly under the stress conditions of early post-thaw recovery. In this regard, previous work has shown that respiratory impairment occurs during the early post-thaw period (5, 8), and it is possible that Pluronic F-68 assists in overcoming such perturbations. The adsorption of Pluronic molecules onto the cytoplasmic membranes of post-thawed plant cells may also reduce cellular damage which is known to occur during rehydration when the  $\text{Me}_2\text{SO}$  cryoprotectant is removed progressively from the system (4).

Further studies are required to determine the mechanism(s) by which surfactants, such as Pluronic F-68, can **facilitate** post-thaw survival and growth of plant cells. One focus of such work should be to investigate the effects of Pluronic F-68 on respiratory gas dynamics, since related work has provided evidence that this compound can alter oxygen transport in agitated, sparged bioreactors (25). There is also speculation that Pluronic F-68 may influence the release of carbon dioxide from cells (19) or inhibit ethylene production in a manner comparable to that promoted by Triton X-100 (28). Interestingly, experiments have demonstrated that inert, oxygen-dissolving perfluorochemical liquids, emulsified with Pluronic F-68, were effective in prolonging the fertilizing capability of turkey spermatozoa stored at lowered temperature (31). While the specific effects of Pluronic were not evaluated, it is possible that the surfactant may have contributed to cell survival, either through cytoprotection or, perhaps, by enhancing oxygen and/or nutrient uptake. This earlier work of Thurston *et al.* (31), together with the results reported in the present paper, indicate that the

mechanism(s) whereby Pluronic F-68 facilitates survival of both animal and plant cells warrants further detailed study.

The present data imply that Pluronic F-68, which is relatively inexpensive and commercially available, could be incorporated routinely into post-thaw culture media, to increase plant cell recovery and growth during post-thaw handling procedures.

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# Enhanced mitotic division of cultured *Passiflora* and *Petunia* protoplasts by oxygenated perfluorocarbon and haemoglobin

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Cell suspension-derived protoplasts were cultured in (A) liquid medium, (B) medium overlaying oxygenated perfluorodecalin (PFC), (C) medium containing 1:50 (v.v) of haemoglobin solution (*Erythrogen™*), or (D) medium with 1:50 (v.v) *Erythrogen™* overlaying oxygenated PFC. In *Passiflora*, mitotic division of protoplasts was increased ( $P < 0.05$ ) by all treatments, with *Erythrogen™* being the most effective (120% increase over control). For *Petunia*, treatment D induced maximum mitosis (140% over control), whilst *Erythrogen™* alone produced a less pronounced (80% over control) increase.

## Introduction

A key requirement in the genetic manipulation and biotechnological exploitation of higher plants is the culture of protoplasts (wall-less cells) and cells under static conditions with adequate respiratory gas supply. Previous approaches for enhancing  $O_2$  supply to cultured protoplasts include the use of  $O_2$ -enriched atmospheres (d'Utra Vaz *et al.*, 1992) and liquid-over-agar medium implanted with glass rods to increase the surface area of the liquid layer (Anthony *et al.*, 1995). Mitotic division of protoplasts can also be enhanced by culture with  $O_2$ -gassed perfluorocarbons (PFCs), inert, highly fluorinated organic liquids that dissolve large volumes of respiratory gases (Anthony *et al.*, 1994; Lowe *et al.*, 1997).

Related experiments have shown that supplementation of aqueous culture medium with low concentrations (ca. 2.0 g l<sup>-1</sup>) of a commercial bovine haemoglobin (Hb) solution (Anthony *et al.*, 1997) enhances division of cell suspension-derived protoplasts of *Petunia hybrida* *in vitro*. The present study extends these observations with direct comparisons of oxygenated PFC and Hb, alone and in combination, using *Passiflora* (a woody species) and *Petunia* (a herbaceous species) as model protoplast systems.

## Materials and methods

### Plant materials and protoplast culture

Protoplasts were isolated from embryogenic cell suspensions of *Passiflora giberti* using an enzyme mixture

(20 ml enzyme solution g<sup>-1</sup> fresh wt. cells) which consisted of 1.0% (w/v) Cellulase RS (Yakult Honsha Co., Nishinomiya Hyogo, Japan), 0.1% (w/v) Pectolyase Y23 (Seishim Pharmaceutical, Tokyo, Japan) and 5 mM 2-[*N*-morpholino]ethane sulphonic acid in CPW salts solution (Frearson *et al.*, 1973). The latter contained 27.2 mg/l  $KH_2PO_4$ , 101 mg/l  $KNO_3$ , 246 mg/l  $MgCl_2 \cdot 7H_2O$ , 0.16 mg/l KI, 0.025 mg/l  $CuSO_4 \cdot 5H_2O$ , 1480 mg/l  $CaCl_2 \cdot 2H_2O$  and 130 g/l mannitol (CPW13M), pH 5.8. Protoplasts of an albino mutant of *Petunia hybrida* cv. Comanche were isolated from cell suspensions using the same enzyme solution, but at half enzyme strength. Incubation was performed in the dark ( $25 \pm 2^\circ C$ ) on a horizontal rotary shaker (40 rpm) for 16 h in 9.0 cm diam Petri dishes. The digested cells were filtered through 30  $\mu m$  and 64  $\mu m$  pore size nylon sieves (Wilson Sieves, Nottingham, U.K.) for *Passiflora* and *Petunia* protoplasts, respectively. The filtrates were centrifuged (80  $\times$  g; 10 min) and the protoplast pellets washed twice in CPW13M solution by repeated resuspension and centrifugation. Protoplast viability was determined by the uptake and cleavage of fluorescein diacetate (Widholm, 1972), prior to counting and plating.

### Culture of protoplasts with PFC and/or haemoglobin

Protoplasts of *P. giberti* were cultured, for 6 d, at  $1.0 \times 10^5$  ml<sup>-1</sup> in liquid KPR medium (Thompson *et al.*, 1986) and those of *P. hybrida*, for 10 d, at  $2.0 \times 10^5$  ml<sup>-1</sup> in liquid KM8P medium based on the

formulation of Kao and Michayluk (1975), as modified by Gilmour *et al.* (1989). Protoplast suspensions (2 ml aliquots) were placed in 30 ml glass bottles, either alone, or overlaying 6 ml aliquots of oxygenated (10 mbar, 15 min) perfluorodecalin (*Fluorocel*® PP6, BNFL Fluorochemicals, **Springfields**, Preston, U.K.). In some treatments, the media were supplemented with 1:50 (v:v) of the commercial bovine Hb solution, *Erythrogen*™ (Biorelease Corporation, **Salem**, U.S.A.). This concentration of Hb was selected on the basis of previous studies, which demonstrated a stimulatory effect of Hb on mitotic division of cell suspension-derived protoplasts of *P. hybrida* (Anthony *et al.*, 1997). Following the addition of Hb solution, the resultant pH of the medium was 6.1 and, therefore, all comparative assessments utilised media at this value. Protoplasts were cultured at 25 ± 2°C in the dark.

#### Measurement of initial plating efficiency

After 6 d (*Passiflora*) or 10 d (*Petunia*) of culture with oxygenated PFC or *Erythrogen*™, an initial plating efficiency, defined as the percentage of protoplasts originally plated that had regenerated a new cell wall and undergone one or more mitotic divisions, was measured. The differential time scale for measurement of initial plating efficiency between the two culture systems reflected the inherent differences in mitotic cycle times of *Passiflora* and *Petunia*.

#### Statistical analyses

Means and standard errors (s.e.m.) were used throughout, with statistical significance between mean values assessed using a non-parametric Mann-Whitney U test (Snedecor and Cochran, 1989). A probability of  $P < 0.05$  was considered significant.

#### Results

Yields of *P. giberti* and *P. hybrida* protoplasts were  $5.83 \pm 0.23 \times 10^6 \text{ g}^{-1} \text{ f. wt.}$  and  $2.81 \pm 0.15 \times 10^6 \text{ g}^{-1} \text{ f. wt.}$  ( $n = 3$  throughout), with mean viabilities of  $78 \pm 3\%$  ( $n = 3$ ) and  $80 \pm 2\%$  ( $n = 3$ ), respectively. The mean initial plating efficiency of *P. giberti* protoplasts with oxygenated PFC ( $20.3 \pm 0.6\%$ ;  $n = 5$ ) was significantly ( $P < 0.05$ ) greater than control ( $10.4 \pm 0.3\%$ ;  $n = 5$ ; Fig. 1). A similar, but more pronounced, increase ( $P < 0.05$ ) in mean initial plating efficiency of *Passiflora* protoplasts was observed when the medium was supplemented solely with *Erythrogen*™ ( $22.9 \pm 1.0\%$ ;  $n = 5$ ; Fig. 1). There was no significant difference in mean initial plating efficiency values between the oxygenated PFC and *Erythrogen*™ treatments (Fig. 1). When the latter two treatments were combined, the mean initial plating efficiency ( $18.3 \pm 0.8\%$ ;  $n = 5$ ) was also

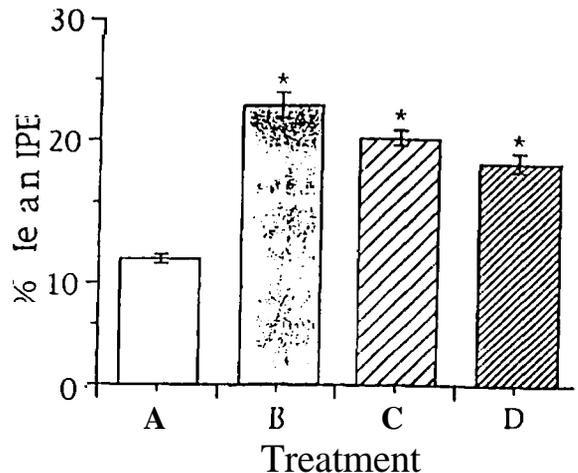


Figure 1 Mean initial plating efficiency (IPE) of *Passiflora giberti* protoplasts cultured for 6 d in A) KM8P medium alone (control), B) medium supplemented with 1:50 (v:v) *Erythrogen*™, C) medium overlaying oxygenated PFC or D) medium supplemented with 1:50 (v:v) *Erythrogen*™ and overlaying oxygenated perfluorocarbon. Vertical bars represent s.e.m. ( $n = 5$  throughout). \* $P < 0.05$  compared to control mean value.

significantly ( $P < 0.05$ ) greater than in untreated controls (Fig. 1). Interestingly, there was no significant difference between the mean initial plating efficiency with the combined treatments and that of protoplasts cultured in the presence of oxygenated PFC alone. However, the mean initial plating efficiency of protoplasts cultured with oxygenated PFC and *Erythrogen*™, in combination, was 20% lower ( $P < 0.05$ ) than with *Erythrogen*™ alone (Fig. 1).

The mean initial plating efficiency of *P. hybrida* protoplasts cultured with oxygenated PFC after 10 d of culture ( $20.3 \pm 1.5\%$ ;  $n = 5$  throughout) was significantly ( $P < 0.05$ ) greater than control ( $9.6 \pm 0.6\%$ ; Fig. 2). In addition, the mean initial plating efficiency of *Petunia* protoplasts cultured in medium containing *Erythrogen*™ was  $17.3 \pm 1.1\%$  compared to  $9.6 \pm 0.6\%$  in control ( $P < 0.05$ ). As for *Passiflora*, no significant difference in mean initial plating efficiency was observed between the oxygenated PFC and *Erythrogen*™ treatments, despite the time-scale difference in the initial plating efficiency assessments. When the PFC and Hb treatments were combined, the mean initial plating efficiency ( $23.0 \pm 0.9\%$ ;  $n = 5$ ) was also significantly ( $P < 0.05$ ) greater than that in untreated controls or with *Erythrogen*™ alone (Fig. 2). This contrasted with *Passiflora*, where Hb promoted the greatest increase

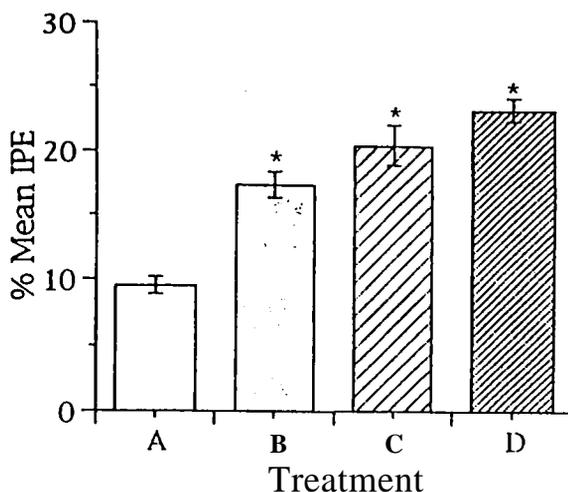


Figure 2 Mean Initial plating efficiency (IPE) of *Petunia hybrida* protoplasts cultured for 10 d in A) KM8P medium alone (control), B) medium supplemented with 1:50 (v:v) *Erythrogen™*, C) medium overlaying oxygenated PFC or D) medium supplemented with 1:50 (v:v) *Erythrogen™* and overlaying oxygenated perfluorocarbon. Vertical bars represent s.e.m. (n = 5 throughout). \*P < 0.05 compared to control mean value.

in mean initial plating efficiency. However, as with *P. giberti*, there was no significant difference between the mean initial plating efficiency with oxygenated PFC and *Erythrogen™*, in combination, and with oxygenated PFC alone.

## Discussion

The results of these experiments demonstrate that oxygenated perfluorodecalin and *Erythrogen™* provide options for enhancing mitotic division, as reflected by increased initial plating efficiency, of both *Passiflora* and *Petunia* protoplasts and their respective protoplast-derived cells *in vitro*. Interestingly, the study revealed some variations in the effectiveness of the separate or combined treatments. In the case of *P. giberti*, *Erythrogen™* promoted the greatest increase in mean initial plating efficiency, whilst for *P. hybrida*, the combined oxygenated PFC and Hb treatment was the most effective. Whilst further studies are required, these experiments suggest that the effectiveness of these options for manipulating respiratory gas supply *in vitro* may be species-specific. However, the possibility also exists, at least for *Passiflora*, that the combined treatment of Hb and PFC served to suppress mitotic division, through possible O<sub>2</sub> toxicity effects. It may well be that for the chosen *Petunia* system, such a threshold was not reached.

Previously, oxygenated PFC has been shown to effectively enhance mitotic division of cultured higher plant protoplasts of several species, including those of *P. hybrida* (Anthony *et al.*, 1994), *Manihot esculenta* (cassava; Anthony *et al.*, 1995) and *Oryza sativa* (rice) (Wardrop *et al.*, 1996). In the latter study, morphologically and cytologically normal plants were regenerated from protoplasts cultured in the presence of oxygenated PFC. Whilst clear evidence of changes in O<sub>2</sub> flux between the PFC phase and the overlying aqueous medium has been obtained, at least during the first 48-72 h of culture (Anthony *et al.*, 1994), it is not inconceivable that such a liquid-liquid interface provides a more optimised physical micro-environment conducive for cell division *per se*. This would be consistent with studies in which mammalian cells were successfully cultured for extended periods at such interfaces (King *et al.*, 1989; Lowe *et al.*, 1997).

The finding that supplementation of culture medium with *Erythrogen™* can stimulate protoplast division was consistent with previous studies using animal cells, in which *Erythrogen™* not only enhanced mitotic division, but also stimulated production of recombinant protein (Goffe *et al.*, 1994). It is speculated that, in the present experiments, *Erythrogen™* captured dissolved O<sub>2</sub> from air-medium interfaces, thereby facilitating delivery of gas to protoplast-derived cells. This hypothesis would support claims from the earlier investigations with animal cells.

Future studies should evaluate these growth-enhancing strategies in other plant culture systems and determine whether they can be applied more broadly to the culture of eukaryotic cells. Whilst Hb is a convenient option for enhancing cellular O<sub>2</sub> supply *in vitro*, currently available commercial formulations have limited shelf-life. Thus, the recoverability and hence, recycleability of PFCs may make them a commercially more attractive and routine option, despite the high initial investment cost.

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## Strategies for promoting division of cultured plant protoplasts: synergistic beneficial effects of haemoglobin (*Erythro*) and Pluronic F-68

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**Abstract** Novel approaches, involving supplementation of aqueous culture medium with haemoglobin solution (*Erythro*), in the presence or absence of the copolymer surfactant, Pluronic F-68, have been evaluated to facilitate cellular oxygen availability to promote mitotic division. Cell-suspension-derived protoplasts of albino *Petunia hybrida* cv. Comanche were cultured for up to 45 days in KM8P medium containing 1:50–1:500 (vol:vol) *Erythro*. The mean initial protoplast plating efficiency after 9 days with 1:50 *Erythro* (18.5%) was significantly greater ( $P < 0.05$ ) than in untreated controls (11.3%). Supplementation of culture medium with 1:50 *Erythro*, together with 0.01% (wt/vol) Pluronic F-68, increased the mean plating efficiency after 9 days (24.4%) by 92% ( $P < 0.05$ ) over the control (12.7%). These treatments also produced increases in biomass of protoplast-derived cells up to 2.5-fold greater than control ( $P < 0.01$ ) over 80 days. Gassing the medium, containing 1:50 *Erythro*, with carbon monoxide abolished the increase in plating efficiency. There was no additional benefit of gassing *Erythro*-supplemented medium with 100% oxygen. The synergistic, beneficial effect of *Erythro* and Pluronic F-68 on protoplast division has implications for plant biotechnology utilising protoplasts.

**Key words** *Petunia hybrida* • Oxygen delivery • *Erythro* • Haemoglobin • pluronic F-68

**Abbreviations** *IPE* Initial protoplast plating efficiency • *PFC* Perfluorochemical

### Introduction

Isolated plant protoplasts are the source material for the genetic manipulation of plants by interspecific and intergeneric somatic hybridisation through protoplast fusion, transformation by direct DNA uptake and the exposure of somaclonal variation (Blackball et al. 1994; Dixon 1994). One crucial requirement for successful protoplast culture and subsequent plant regeneration from protoplast-derived tissues is the provision of an adequate and sustainable oxygen supply. Previous approaches to enhance respiratory gas exchange for cultured protoplasts have included the use of oxygen-enriched atmospheres (d'Utra Vaz et al. 1992), inert, respiratory gas-dissolving perfluorochemical (PFC) liquids (Anthony et al. 1994a, b, 1995b) and liquid-over-agar media matrices implanted with glass rods to increase the surface area and gaseous exchange of the liquid culture phase (Anthony et al. 1995a).

An additional and novel approach to further facilitate oxygen supply, which has not been evaluated previously for plant cell systems, is the supplementation of aqueous culture media with a commercial haemoglobin solution (*Erythro*). Previous research has focused on the assessment of native and, to a greater extent, chemically modified haemoglobins as vehicles for respiratory gas transport in intact animals and isolated, perfused organs (Zuck and Riess 1994; Tsuchida 1995; Winslow et al. 1995). The most widely studied molecules are of human or bovine origin, although recombinant haemoglobins, expressed in *Escherichia coli* (Hoffman et al. 1990), transgenic mice (Behringer et al. 1989) and pigs (Swanson et al. 1992), are now available. Haemoglobins liberated from animal erythrocytes are normally treated with pyridoxal phosphate or glutaraldehyde to reduce their oxygen binding to acceptable physiological levels (Zuck and Riess 1994; Tsuchida 1995). One advantage of using haemoglobin as a respiratory-gas-carrying culture medium supplement compared with PFC liquids is that the latter are immiscible in aqueous systems (King et al. 1989; Anthony et al. 1994a; Lowe et al. 1995). However,

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paradoxically, this enables PFCs to be recovered readily and to be recycled.

The present investigation has employed cell-suspension-derived protoplasts of albino *Petunia hybrida* cv. Comanche, as a model system to assess the effects on mitotic division of protoplasts and their cellular derivatives of improved gas manipulation through supplementation of the culture medium with a commercially available, stabilised bovine haemoglobin solution, *Erythro*gen. The effects of haemoglobin were also evaluated alongside the copolymer surfactant, Pluronic F-68, which is commonly employed as a cytoprotectant for cultured animal cells (Goldblum et al. 1990; Papoutsakis 1991; Handa-Corrigan et al. 1992). This surfactant has been shown previously to promote growth in cultured plant cells, tissues and organs (Lowe et al. 1993, 1994).

## Materials and methods

### Isolation and culture of protoplasts

Protoplasts of an albino accession of *Petunia hybrida* cv. Comanche were isolated enzymatically from a well-characterised and established cell suspension, as reported previously (Power et al. 1990). Protoplasts were cultured in the dark ( $25\pm 2^\circ\text{C}$ ) at a final plating density of  $2.0\times 10^5$  ml<sup>-1</sup> in 1.5-ml aliquots of liquid KM8P culture medium (osmotic pressure 755 mmol kg<sup>-1</sup>) based on the formulation of Kao and Michayluk (1975), as modified by Gilmour et al. (1989), and supplemented with 0.1 mg l<sup>-1</sup> 2,4-dichlorophenoxyacetic acid and 0.2 mg l<sup>-1</sup> zeatin. The latter medium, containing protoplasts, was dispensed into 3.5-cm-diameter plastic Petri dishes (Nunc, Roskilde, Denmark). All experimental treatments were replicated five times.

### Supplementation of medium with haemoglobin alone or with oxygen or carbon monoxide

*Erythro*gen (Biorelease Corporation, Salem, Mass., obtained from TCS Biologicals, Botolph Claydon, UK), a stabilised bovine haemoglobin solution (103 g l<sup>-1</sup>, pH 7.42), was added to KM8P medium to final concentrations of 1:50, 1:100 or 1:500 (vol:vol). For the former concentration, a further series of replicates were established whereby the medium was gassed with 100% oxygen (10 mbar, 15 min) or 100% carbon monoxide (No: 850203; Phase Separations, Deeside, UK; 20 s), immediately prior to the addition of protoplasts.

### Supplementation of medium with haemoglobin and Pluronic F-68

In an additional assessment using non-gassed medium containing 1:50 (vol: vol) *Erythro*gen, the aqueous culture medium was also supplemented with 0.01%, 0.1% or 1.0% (wt/vol) of Pluronic F-68 (Sigma, Poole, UK). These concentrations of Pluronic were based on those shown previously to stimulate the growth in culture of cell suspension-derived protoplasts of *Solanum dulcamara* (Kumar et al. 1992). All experiments using *Erythro*gen, alone or in conjunction with other treatments, utilised protoplasts of the same population, in turn isolated from the same homogenous cell suspensions. Dishes were sealed with Nescofilm (Bando Chemical, Kobe, Japan) and maintained under static conditions.

For all treatments, including controls, spent KM8P medium (0.25 ml per dish) was removed after 9 and 15 days and replaced, on both occasions, with an equal volume of KM8 medium of reduced osmotic pressure (220 mmol kg<sup>-1</sup>), based on the formulation of medium 8 of Kao and Michayluk (1975), as modified by Gilmour et al. (1989). This medium lacked *Erythro*gen. After 45 days of culture,

the total population of protoplast-derived cell colonies from one randomly selected replicate 3.5-cm Petri dish was transferred to the surface of 6 ml of agar-solidified (0.6% wt/vol; Sigma) UM medium (Uchimiya and Murashige 1974) contained in 5.5-cm-diameter Petri dishes. Cell colonies/microcalli were further incubated in the dark at  $25\pm 2^\circ\text{C}$  for 35 days in order to confirm no adverse influence of the treatments on long-term cell viability.

### Measurement of protoplast viability, growth and biomass

The viability of freshly isolated protoplasts was determined by uptake and cleavage of fluorescein diacetate (Widholm 1972), prior to counting. Protoplast growth and mitotic activity was assessed after 9 days, by the initial plating efficiency (IPE), defined as the percentage of protoplasts originally plated that had regenerated a new cell wall and undergone at least their first mitotic division. A minimum of 100 protoplasts per Petri dish were scored; each treatment was repeated five times. The biomass of protoplast-derived cells was recorded as fresh weight after 80 days of culture with the various treatments.

### Measurement of pH and osmotic pressure in the culture medium

The pH of the culture medium was measured using a Kent EIL7020 pH meter and was adjusted, where appropriate, to pH 6.1 using 1.0 M NaOH. The osmotic pressure of the culture medium for all treatments was measured using a Wescor 5500 vapour pressure osmometer, since it was essential to assess whether there were any shifts in pH and/or osmotic pressure associated with the inclusion of *Erythro*gen in the medium.

### Statistical analyses

Means and standard errors (SE) were used throughout; statistical significance between mean values was assessed using a parametric Moods median test and a Mann-Whitney U-test (Snedecor and Cochran 1989). A probability of  $P<0.05$  was considered significant.

## Results

The yield of *Petunia* protoplasts was  $6.24\pm 0.45\times 10^5$  per gram fresh weight of suspension cells, with a mean viability of  $94\pm 1\%$  ( $n=3$ ). Culture of protoplasts in KM8P medium supplemented with 1:50 (vol:vol) *Erythro*gen stimulated protoplast division, with an increase in mean IPE of 64% ( $P<0.05$ ) over the control after 9 days (Fig. 1). In contrast, the mean IPE values after 9 days in medium supplemented with 1:100 or 1:500 (vol:vol) of *Erythro*gen were not significantly different to the control (Fig. 1).

For cultures supplemented with 1:50 (vol: vol) *Erythro*gen and gassed with oxygen, the mean ( $\pm$ SE) IPE ( $18.1\pm 1.5\%$ ,  $n=5$ ) was significantly ( $P<0.05$ ) greater than the control ( $11.3\pm 0.4\%$ ,  $n=5$ ), but was not significantly different from cultures containing the same, but un-gassed, quantity of *Erythro*gen ( $18.5\pm 0.8\%$ ,  $n=5$ ; Fig. 1). Similarly, for cultures containing 1:50 (vol: vol) *Erythro*gen, but gassed with carbon monoxide, the mean IPE after 9 days ( $13.1\pm 1.2\%$ ,  $n=5$ ) was also not significantly different to the control (Fig. 1).

The addition of 0.01% (wt/vol) Pluronic F-68 to cultures containing 1:50 (vol:vol) *Erythro*gen further increased the mean IPE after 9 days of culture by 38%

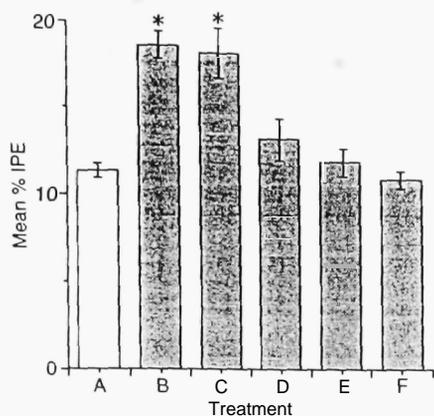


Fig. 1 Mean IPE of *Petunia* cell-suspension-derived protoplasts after 9 days of culture in medium alone (control; A), *Erythrogen* (1:50 vol:vol; B), *Erythrogen* (1:50) with 100% oxygen (C), *Erythrogen* (1:50) with 100% carbon monoxide (D), *Erythrogen* (1:100; E) and *Erythrogen* (1:500; F). Vertical bars represent SE ( $n=5$  throughout); \* $P<0.05$  compared to control mean value

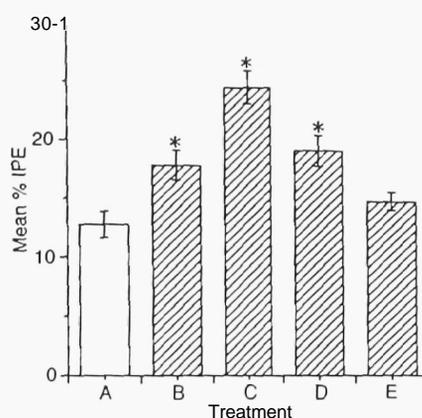


Fig. 2 Mean IPE of *Petunia* cell-suspension-derived protoplasts after 9 days of culture in medium alone (control; A), *Erythrogen* (1:50 vol:vol; B), *Erythrogen* (1:50) with 0.01% Pluronic F-68 (C) *Erythrogen* (1:50) with 0.1% Pluronic F-68 (D) and *Erythrogen* (1:50) with 1.0% (wt/vol) Pluronic F-68 (E). Vertical bars represent SE ( $n=5$  throughout); \* $P<0.05$  compared to control mean value

(Fig. 2). In contrast, there was no additional beneficial effect of supplementing culture medium with 0.1% or 1.0% Pluronic F-68 where the mean IPE after 9 days ( $19.1 \pm 1.3\%$  and  $14.8 \pm 0.8\%$ , respectively,  $n=5$ ) was not significantly different to the control ( $12.7 \pm 2.5\%$ ,  $n=5$ ; Fig. 2). Supplementation of culture medium with *Erythrogen*, had no significant effects on pH or osmotic pressure. Similarly, gassing the culture medium containing 1:50 (vol:vol) *Erythrogen* with oxygen or carbon monoxide had no significant effect on mean pH ( $6.1$  throughout) or osmotic pressure ( $764 \pm 7.5$  mmol kg<sup>-1</sup> and  $774 \pm 3.1$  mmol kg<sup>-1</sup>, respectively), compared to ungasged medium with *Erythrogen* ( $756 \pm 8.4$  mmol kg<sup>-1</sup>). Furthermore, light microscopy revealed no marked morphological or cytological alterations of protoplasts or protoplast-derived cells in response to any of the culture treatments.

Culture of protoplast-derived cells for 80 days with 1:50 (vol:vol) ungasged *Erythrogen* produced a two-fold increase ( $P<0.01$ ) in biomass, as reflected by mean fresh weight ( $1.9 \pm 0.1$  g,  $n=5$ ) compared to the control ( $1.0 \pm 0.1$  g,  $n=5$ ). Culture of protoplast-derived cells for the same period in the presence of 1:50 (vol:vol) *Erythrogen* and 0.01% (wt/vol) Pluronic F-68 produced a further significant ( $P<0.01$ ) increase in mean fresh weight ( $2.6 \pm 0.1$  g,  $n=5$ ).

## Discussion

The results of these experiments demonstrate, for the first time, that supplementation of medium with *Erythrogen* at a 1:50 (vol:vol) dilution, optimally promoted mitotic division in protoplasts of *P. hybrida* over a 9-day culture period. Pluronic F-68 at 0.01% (wt/vol) had a synergistic effect by further enhancing protoplast division. This study

shows that *Erythrogen*, with or without Pluronic F-68, provides a further option for enhancing the growth of cultured plant protoplasts and protoplast-derived cells under static and, perhaps, agitated conditions. This may be especially valuable for facilitating the *in vitro* biosynthesis of phytochemicals, since related studies using cultured animal cells have shown *Erythrogen* not only to stimulate cell division, but also to promote the production of recombinant protein (Goffe et al. 1994). This technology could be readily scaled up to fermenter systems and may be effective in stimulating growth of cultured plant tissues and organs, such as *Agrobacterium rhizogenes-transformed* roots, which are now employed extensively as a source of economically important plant products (Toivonen 1993).

The finding that Pluronic F-68 further enhanced the growth of *Petunia* protoplasts over that induced by *Erythrogen* alone suggests that these media supplements stimulate protoplast division through separate mechanisms. *Erythrogen* is believed to "trap" oxygen from air/medium interfaces and to facilitate delivery of this gas directly to cells, concomitantly improving growth (Goffe et al. 1994). The range of concentrations of Pluronic F-68 used in the present study were selected on the basis of previous work, which demonstrated the growth-stimulating effects, in culture, of this agent on isolated protoplasts of *5. dulcamara* (Kumar et al. 1992). Interestingly, the optimum concentration of 0.01% (wt/vol) of the surfactant in the present experiments was identical to that found to act synergistically with the oxygenated PFC liquid, Flutec PP5 (BNFL Fluorochemicals, UK), in promoting mitosis in *P. hybrida* protoplasts in a static, two-phase culture system (Anthony et al. 1994a).

Studies using animal cells have shown that Pluronic F-68 is adsorbed onto cytoplasmic membranes, conferring increased resistance to mechanical damage (Goldblum et al. 1990; Handa-Corrigan et al. 1992; Lowe et al. 1993,

1994). However, the possibility that Pluronic F-68 also promotes the uptake of nutrients, growth regulators or oxygen into cultured protoplasts cannot be discounted and should form a basis for future studies. Furthermore, it will be interesting to determine whether haemoglobin, in combination with Pluronic F-68 in aqueous culture medium overlaying oxygenated PFC, will promote protoplast growth over and above the stimulation already achieved by chemical and physical parameters (Anthony et al. 1994a, b, 1995a, b). Clearly, such studies should incorporate more detailed assessments, including measurements of protein biosynthesis, of changes associated with cell growth. This will identify the most effective system for sustaining division of protoplast-derived cells prior to plant regeneration, which will be crucial in maximising plant throughput, especially of genetically manipulated material.

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## Synergistic Enhancement of the Postthaw Growth of Cryopreserved Rice Cells by Oxygenated Perfluorocarbon and Pluronic F-68

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The beneficial effects were assessed of supplementing culture medium with oxygenated perfluorocarbon, both alone and in combination with 0.01 (w/v) Pluronic F-68, on the postthaw viability, following cryopreservation, of suspension cultured cells of the Japonica rice, *Oryza sativa* cv. Taipei 309. The mean viability, as assessed by triphenyl tetrazolium chloride reduction, of cells at 4 days after thawing was increased 20% over control by oxygenated perfluorodecalin ( $P < 0.05$ ) in 100-ml glass jars and similarly by 24% ( $P < 0.01$ ) when recovered on a smaller scale in 24-well petri dishes. In a separate assessment, a 21% increase above control treatments ( $P < 0.05$ ) in postthaw viability was also observed with oxygenated perfluorodecalin. A similar, 36% increase ( $P < 0.05$ ) in postthaw viability occurred with cells exposed to 0.01% (w/v) Pluronic F-68 alone. A more pronounced, synergistic increase in viability, up to 57% over control ( $P < 0.05$ ), occurred with cells recovered in the presence of both oxygenated perfluorodecalin and 0.01% (w/v) Pluronic F-68. No significant difference was observed between Pluronic F-68 and oxygenated perfluorodecalin treatments. Both the perfluorodecalin and the Pluronic F-68 treatments alone and in combination also promoted an increase in biomass, measured as fresh weight gain 30 days after thawing, to a maximum of 38% above control ( $P < 0.05$ ). These results demonstrate the marked cytoprotectant effects of oxygenated perfluorodecalin and Pluronic F-68, both alone and/or in combination, for plant cells recovered from cryostorage. Such options offer alternative postthaw handling strategies to cells of those plant species which, normally, respond poorly to conventional recovery procedures. © 1997 Academic Press

**Key Words:** cryopreservation; oxygenated perfluorocarbon; Pluronic F-68; plant cell culture; *Oryza sativa*: postthaw growth recovery.

Cryopreservation is a routine procedure for the stable, long-term storage of biological tissues at ultralow temperatures (12). This technique circumvents the loss, in plant systems, of totipotency and genetic perturbations and alterations in secondary product biosynthesis normally associated with extended culture of plant cell suspensions at physiologically normal temperatures (usually about 25°C). Cryopreservation comprises several distinct stages, of which freezing is one. While the successful and reproducible recovery of frozen cells depends upon prefreeze, cryogenic, and post-freeze conditions, the transition of tissues between ultralow and physiologically normal

temperatures and oxygen tensions induces respiratory imbalances which stimulate the production of toxic oxygen radicals (11). In this respect, physiological investigations of cryopreserved rice cells have already demonstrated respiratory impairment associated with post-thaw recovery (8).

A novel approach for enhancing oxygen supply to postthaw cryopreserved cells is the use of chemically inert, oxygen-carrying perfluorochemical (PFC) liquids. These compounds dissolve substantial volumes of key respiratory gases and have been studied in, for example, emulsified form as vehicles for oxygen transport *in vivo* (16, 20). PFC liquids have also been used to routinely facilitate oxygen supply to cultured plant protoplasts and protoplast-derived cells at normal temperatures (25°C) using Standard tissue culture practices (2, 19, 21). In one investigation,

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dium overlaying the PFC with a low concentration (ca. 0.01% w/v) of the nonionic, polyoxyethylene (POP)-polyoxypropylene (POE) surfactant Pluronic F-68 (Poloxamer 188) further enhanced mitotic division of protoplast-derived cells at the PFC-medium interface (2, 19).

Pluronic F-68 has been widely used as a cytoprotectant and growth-promoting additive to animal cell and microbial cultures (17, 18). It has also been evaluated as a cryoprotectant for Chinese hamster cells (4), but there have been relatively few comparable studies with plant cells. Recently, Anthony *et al.* (3) reported that the incorporation of Pluronic F-68 into the recovery medium enhanced the growth of cells of both rice (*Oryza sativa*) and *Lolium multiflorum*. It was speculated, from these experiments, that the beneficial effects of Pluronic F-68 were primarily mediated through changes in oxygen flux. Therefore, a strong case can be made for evaluating the postthaw growth of plant cells cultured in the presence of oxygen-gassed PFC liquid, in combination with Pluronic F-68. An objective of the study was to extend earlier findings that mitotic division of cultured protoplasts and protoplast-derived cells can be enhanced by elevated oxygen atmospheres (9).

In the present investigation, embryogenic (totipotent) suspension cells of rice were used to assess the potential beneficial effects of oxygenated perfluorodecalin, alone and in combination with Pluronic F-68, on postthaw growth following long-term (ca. 3 years) cryopreservation.

#### MATERIALS AND METHODS

##### *Plant Materials and Preparation of Cell Suspensions*

Embryogenic calli were induced from mature seed scutella of the Japonica rice, *O. sativa* L. cv. Taipei 309 (10), and such tissues were pooled and used to initiate cell suspensions. The latter were maintained in 29-ml aliquots of AA2 liquid medium (1) in 100-ml Erlenmeyer flasks with shaking (120 rpm, 2.5-cm throw) at  $28 \pm 1^\circ\text{C}$  in the dark. Cell sus-

pensions were subcultured every 7 days by transferring 1 ml of settled cells, in 7 ml of spent medium, to 22-ml aliquots of fresh medium. Suspensions were split into two populations. One population was maintained by regular subculture for a minimum period of 3 years; the other population was cryopreserved. In the latter case, cells were cultured, immediately before cryopreservation, for 3-4 days in liquid AA2 medium supplemented with  $60.0 \text{ g liter}^{-1}$  mannitol.

##### *Cryopreservation and Postthaw Recovery*

The cryopreservation procedure was based on that of Lynch *et al.* (22). Cells were pooled and harvested from mannitol-supplemented AA2 medium onto a nylon mesh (45- $\mu\text{m}$  pore size) before being placed into 2-cm<sup>3</sup> polypropylene vials (Sarstedt, Leicester, UK; 0.2 g fresh weight of cells per vial). To each vial was added ca. 0.75 ml of a cryoprotectant mixture composed of AA2 liquid medium containing 46.0 g liter<sup>-1</sup> glycerol, 39.0 g liter<sup>-1</sup> dimethyl sulfoxide ( $\text{Me}_2\text{SO}$ ), 342.0 g liter<sup>-1</sup> sucrose, and 5.0 g liter<sup>-1</sup> proline, at pH 5.8. The cryoprotectant mixture was filter sterilized (0.2- $\mu\text{m}$  pore size; Sartorius, Göttingen, Germany) prior to use. All cryoprotectant components were of Analar grade except  $\text{Me}_2\text{SO}$ , which was spectroscopically pure.

Vials containing cells in cryoprotectant were incubated for 1 h on iced water. Subsequently, the vials with their contents were transferred to aluminium canes, cooled from 0 to  $-35^\circ\text{C}$  at  $-1^\circ\text{C min}^{-1}$ , and held at this temperature for 35 min in a programmable freezer (Planer Cryo 10 Series, Planer Biomed, Sunbury-on-Thames, UK). Vials were stored for 3 years in liquid nitrogen at  $-196^\circ\text{C}$ .

Cells were thawed by plunging the frozen vials into sterile water at  $45^\circ\text{C}$ ; excess cryoprotectant was removed, under axenic conditions, from the cells using a Pasteur pipet. The cells from individual vials were placed onto the top of two superimposed 2.5-cm-diameter Whatman No. 1 filter paper disks overlaying 5.0-ml aliquots of AA2 medium made semisolid

with 0.4% (w/v) SeaKem LE agarose (FMC Corporation, Rockland, ME, U.S.A.) contained in 100-ml capacity screw-capped glass jars (Beatson Clark and Co. Ltd., Rotherham, UK) (Treatment A). In some treatments, the AA2 culture medium was supplemented with 0.01% (w/v) Pluronic F-68 (Sigma, Poole, UK; Treatment B), this concentration of Pluronic being selected based on previous studies which confirmed the beneficial effects of this compound on postthaw growth of cells of *O. sativa* and *L. multiflorum* (3). In a further series of treatments, the cells were placed on semisolidified AA2 medium overlaying 20.0-ml aliquots of oxygenated (10 mbar, 15 min) perfluorodecalin (Flutec PP6; BNFL Fluorochemicals Ltd., Preston, UK; Treatment C). Additionally, the AA2 medium overlaying oxygenated perfluorodecalin was supplemented with 0.01% (w/v) Pluronic F-68 (Treatment D).

Cells of all treatments were cultured in the dark for 3 days at  $28 \pm 1^\circ\text{C}$  prior to transfer of the upper filter disk, supporting the cells, to fresh medium lacking Pluronic F-68 and/or overlaying ungassed perfluorodecalin, as appropriate. Cells were cultured for an additional 24 h prior to viability assessments, followed by another 26 days, under the same conditions, before biomass determinations. Each of the treatments consisted of 20 replicates of cells of the same cell line taken from 20 individual vials.

In order to assess the feasibility of recovering cryopreserved cells on a smaller scale, the postcryopreservation cell handling protocol was further modified, using 24-well petri dishes (Costar Ltd., High Wycombe, UK). Each well contained 2.0-ml aliquots of oxygenated perfluorodecalin and 1.0 ml of semisolidified AA2 medium, with the filter papers (1.3-cm-diameter; Whatman No. 1), overlaid with 50 mg fresh weight of cells. The 50-mg cell aliquots used were each taken from 20 individual vials.

#### *Measurement of Postthaw Viability and Biomass*

The reduction of triphenyl tetrazolium chloride (TTC) was used to assess the postthaw

viability of cells based on a modification of the method of Steponkus and Lamphear (26). The wetting agent used in the original assay was replaced with 0.05% (v/v) Tween 80 (Sigma) and the absorbance was read spectrophotometrically at 490 nm and not at 530 nm. Biomass was recorded by determining the fresh weight of thawed cells (22).

#### *Reinitiation of Cell Suspensions and Subsequent Isolation of Protoplasts*

Five cell suspensions were reestablished from each of the treatments by removing the cells from the surface of five randomly selected filter disks, after 30 days of postthaw culture, and placing into 22-ml aliquots of AA2 liquid medium in 100-ml Erlenmeyer flasks. Suspensions were subcultured thereafter every 7 days for 28 days by removing all the spent medium and replacing with the equivalent volume of fresh AA2 liquid medium. Subsequently, suspensions were maintained as described earlier. After eight passages, protoplasts were isolated enzymatically and cultured in the presence of *L. multiflorum* nurse cells, as described by Jain *et al.* (14). Protoplast-derived colonies were transferred from the membranes to 20-ml aliquots of MSKN medium, the latter being modified from the formulation of Jain *et al.* (14), and contained in 9-cm-diameter petri dishes (25 colonies/dish). Modified MSKN medium contained maltose [5% (w/v)] and was semisolidified with 1% (w/v) SeaKem LE agarose. Cell colonies were cultured in the dark at  $26 \pm 1^\circ\text{C}$  for 10 days, prior to transfer to the same medium, but with the agarose concentration reduced to 0.4% (w/v). Cultures were transferred to the light ( $55 \mu\text{E m}^{-2} \text{s}^{-1}$ ; 16 h photoperiod; Cool White fluorescent tubes; Thorn EMI Ltd., Hayes, UK) and regenerating shoots (75 per treatment) were detached from individual calli after 40 days and transferred to 175-ml capacity screw-capped glass jars, each containing 50 ml of MS-based medium supplemented with 15 mg liter<sup>-1</sup> a-naphthaleneacetic acid, 5% (w/v) sucrose, and semisolidified with 0.2% (w/v) Phytigel (Sigma).

Cultures were maintained under a 16-h photoperiod, as described previously.

### Statistical Analyses

Means and standard errors (SEM) were used throughout. Statistical significance between mean values was assessed, as appropriate, using a conventional ANOVA and Student's *t* test (25). A probability of *P* less than 0.05 was considered significant.

### RESULTS

The mean absorbance, as an indicator of cell viability, of unfrozen suspension cells was  $0.96 \pm 0.05$  ( $n = 10$ ) prior to long-term (3-year) cryopreservation. The mean absorbance of cryopreserved cells following recovery in the presence of oxygenated perfluorodecalin (Treatment C;  $0.45 \pm 0.07$ ;  $n = 20$ ) was significantly ( $P < 0.05$ ) greater than for the mean of the (control) treatment which lacked perfluorodecalin (Treatment A;  $0.35 \pm 0.08$ ;  $n = 20$ ). Similar differences in postthaw viabilities were also observed ( $P < 0.05$ ) in the reduced-scale series of experiments when cells were recovered using 24-well petri dishes (control:  $1.17 \pm 0.06$ ,  $n = 20$ ; oxygenated PFC:  $1.45 \pm 0.07$ ,  $n = 20$ ;  $P < 0.05$ ).

Figure 1 shows the mean absorbance when cryopreserved rice cells were recovered in the presence of 0.01% (w/v) Pluronic F-68, oxygenated perfluorodecalin, or oxygenated PFC combined with Pluronic F-68. The mean absorbance of cells recovered in the presence of 0.01% (w/v) Pluronic F-68 (Treatment B;  $1.27 \pm 0.04$ ,  $n = 20$ ) or oxygenated PFC (Treatment C;  $1.13 \pm 0.03$ ,  $n = 20$ ) was significantly greater ( $P < 0.05$ ) than on semisolid AA2 medium alone (Treatment A;  $0.93 \pm 0.03$ ,  $n = 20$ ; Fig. 1). A further synergistic increase ( $P < 0.05$ ) in mean absorbance was also observed when Pluronic F-68 and perfluorodecalin were used in combination (Treatment D;  $1.46 \pm 0.08$ ,  $n = 20$ ), compared to all other treatments. No significant differences were observed between Treatment B (0.01% (w/v) Pluronic F-68) and Treatment C (oxygenated perfluorodecalin).

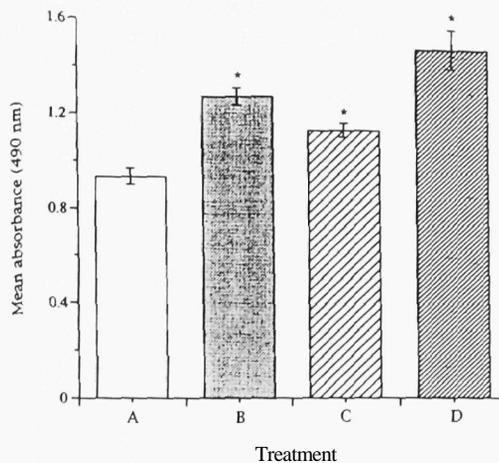


FIG. 1. Mean viability of cryopreserved rice cells recovered for 4 days in the presence of (A) medium alone (control), (B) medium with Pluronic F-68 (0.01% w/v), (C) medium underlaid with oxygenated PFC, and (D) a combination of Treatments (B) and (C). Vertical bars represent SEM ( $n = 20$  throughout). \* $P < 0.05$ .

The recovery of cells with 0.01% (w/v) Pluronic F-68 (Treatment B) or oxygenated perfluorodecalin (Treatment C) promoted sustained mitotic division, since biomass, measured as increase in fresh weight at 30 days postthaw, was elevated by 21 and 38% ( $P < 0.05$ ), respectively, compared to the biomass of cells in unsupplemented medium (Treatment A; Fig. 2). When PFC and Pluronic F-68 treatments were combined (Treatment D), a significant ( $P < 0.05$ ) increase in biomass was also observed that was greater than for Treatment B, but not significantly different from that found for Treatment C (Fig. 2).

Cell suspensions ( $n = 5$ ), reinitiated simultaneously from cells recovered from all treatments, ultimately exhibited growth rates after 35 days of culture comparable to those of unfrozen suspensions maintained by regular subculture every 7 days for 3 years, this time being equivalent to the cryopreservation period.

There were no significant differences, in terms of yields, viabilities, plating efficiencies and plant regeneration frequencies (Table 1), of protoplasts isolated from cryopreserved

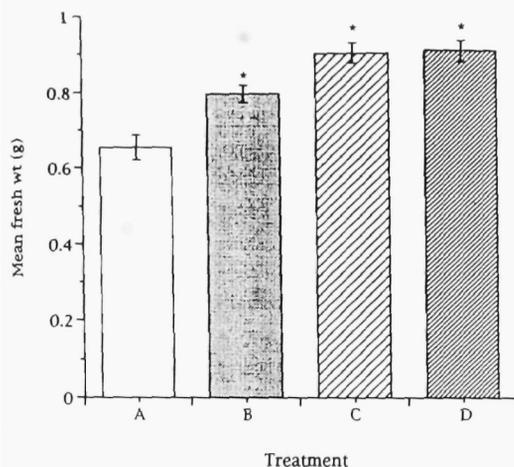


FIG. 2. Mean fresh weight (g) of cryopreserved rice cells recovered after 30 days in the presence of (A) medium alone (control), (B) medium with Pluronic F-68 (0.01% w/v), (C) medium underlaid with oxygenated PFC, and (D) a combination of Treatments (B) and (C). Vertical bars represent SEM ( $n = 20$  throughout). \* $P < 0.05$ .

cells exposed to the experimental Treatments A, B, C, and D. Additionally, there were no significant differences in terms of protoplast yield ( $4.0 \times 10^6$  g<sup>-1</sup> f.wt.), mean protoplast viability ( $89 \pm 2\%$ ,  $n = 3$ ) and the mean number of protoplast-derived calli regenerating

shoots ( $44 \pm 4\%$ ,  $n = 100$ ) between cells subjected to cryopreservation followed by Treatments A-D and unfrozen suspension cells assessed prior to their 3-year cryopreservation procedure. Protoplast isolation was not attempted from suspensions maintained continuously for 3 years without cryopreservation, since the suspensions had lost their totipotency, as expected by the end of this culture period. Plants regenerated from cryopreserved cells were morphologically normal, with expected diploid chromosome complements ( $2n = 2x = 24$ ).

#### DISCUSSION

The present study utilized the same cell suspension for all treatments in order to eliminate any somaclonal effects. Lack of somaclonal variation was confirmed by there being no significant differences in terms of plant regeneration potential of cryopreserved cells and unfrozen cells of the same suspension as assessed 3 years earlier.

These experiments show that the postthaw viability and growth of cryopreserved rice cells per se is increased in the presence of oxygenated perfluorodecalin either alone or in combination with Pluronic F-68. Additionally, the culture of rice protoplasts and of their cell

TABLE 1

Plating Efficiencies and Plant Regeneration from Protoplasts of Rice Cell Suspensions Recovered from Cryopreservation in the Presence of (A) Medium Alone (Control), (B) Medium with Pluronic F-68 (0.01% w/v), (C) Medium Underlaid with Oxygenated PFC, and (D) a Combination of Treatments (B) and (C).

Treatment	Protoplast yield <sup>a</sup> (g fwt. $\times 10^6$ )	Protoplast viability <sup>b</sup> (%)	Protoplast plating efficiency <sup>c</sup> (%) after 30 days	% of colonies regenerating plants <sup>d</sup> after 70 days
Control	$3.80 \pm 0.80$	$75.06 \pm 3.1$	$1.25 \pm 0.03$	$38.7 \pm 2.7$
Pluronic F-68	$4.27 \pm 0.85$	$76.55 \pm 2.9$	$1.29 \pm 0.05$	$41.3 \pm 4.8$
PFC	$5.32 \pm 0.85$	$79.86 \pm 1.2$	$1.27 \pm 0.04$	$40.0 \pm 2.3$
PFC/Pluronic F-68	$5.07 \pm 0.02$	$76.89 \pm 2.3$	$1.27 \pm 0.06$	$37.3 \pm 2.7$

Note. Values represent mean  $\pm$  SEM.

<sup>a</sup>  $n = 3$ .

<sup>b</sup>  $n = 3$ .

<sup>c</sup>  $n = 9$ .

<sup>d</sup>  $n = 15$ .

derivatives at the interface between oxygen-gassed PFC overlaid with liquid or agarose-solidified culture medium also enhances mitotic division and, in totipotent systems, stimulates shoot formation (2, 28). This confirms a genuine growth enhancement of this treatment, rather than a marginal effect on cells recovered from cryopreservation. PFC is believed to act as a reservoir for oxygen which diffuses into the aqueous medium/cell phase during initial culture. This is supported by changes in oxygen tension in the medium (2). It is probable, therefore, that the increased and sustainable postthaw growth of rice cells was also due to an enhanced oxygen supply provided by the PFC. Indirect evidence for the diffusion of oxygen from the PFC to aqueous culture medium comes from related observations with suspension-derived protoplasts of *Salpiglossis sinuata*, in which an increase in intracellular superoxide dismutase (SOD) occurred after 3 days culture (21). This, in turn, was consistent with studies using *Mycobacterium* spp., in which SOD was elevated during culture of the bacterium in perfluorodecalin-supplemented medium (24). Lipid peroxidation and protein degradation can occur in the early stages of postthaw recovery (6, 11). Increased SOD biosynthesis associated with culture of protoplasts and protoplast-derived cells with oxygenated PFC may protect cells not only against a supplemented oxygen supply, but also against oxygen radicals generated by impaired oxygen flux during thawing. Further studies should determine the early time-course of changes in SOD and other oxygen-sensitive enzymes during cell recovery.

The present study has extended earlier investigations which demonstrated that culture medium containing Pluronic F-68 increased postthaw growth of cryopreserved rice cells (3). Pluronic F-68 adsorbs onto cell membranes, increasing resistance to mechanical damage (13, 17, 18). Pluronics have hydrophobic POP cores, which are believed to become incorporated into membranes, leaving their hydrophilic, POE tails outside the cell. This would lower the interfacial tension by

sterically hindering adhesive interactions between molecules on cell surfaces and, in turn, minimize cell-to-cell contacts, thereby reducing mechanical disruption. However, it is possible that the responses to Pluronic could differ between plant cells with intact walls and animal cells or plant protoplasts, which have naked plasma membranes, the latter albeit for a finite period of time when in culture.

As discussed previously (3), Pluronic F-68 may increase the uptake of nutrients, growth regulators, or respiratory gases into cells during postthawing. Studies with animal cells have demonstrated that Pluronic F-68 at concentrations comparable to those used in the present investigation stimulated 2-deoxyglucose uptake and incorporation of amino acids (7). Changes in nutrient uptake, promoted by Pluronic, would be expected to alter metabolic flux, allowing biochemical pathways to operate more efficiently, especially under the stress of initial postthaw recovery (3). Additional studies are required, since the composition of the culture medium may influence the growth of cells during the recovery phase. For example, uptake of ammonium ions is known to inhibit the growth of thawed rice cells (15), although in the present study, the cell recovery medium lacked ammonium ions. Adsorption of Pluronic molecules onto postthawed plant cells may also reduce cellular damage which can occur during rehydration when the  $\text{Me}_2\text{SO}$  cryoprotectant is removed progressively from the system (5) and thus help to preserve, in the short term, a stable cell:medium density equilibrium crucial to the reestablishment of maximal mitotic activity.

Interestingly, while Pluronic stimulated postthaw growth of cryopreserved rice cells, there was no further growth enhancement when the surfactant was added to the culture medium overlaying oxygenated PFC. This contrasted with earlier observations using protoplasts of *Petunia hybrida*, where Pluronic acted synergistically with oxygenated perfluorodecalin to increase the plating efficiency by 57% above control (2). Such differences may relate to the fact that the latter study in-

volved protoplasts of cell suspension-derived cells that had not been cryopreserved. Nevertheless, these results demonstrate that in terms of simply promoting postthaw growth of rice cells based on biomass assessments, oxygenated perfluorodecalin alone is superior for inclusion in cryopreservation protocols compared to Pluronic F-68, either alone or in combination with PFC.

PFCs may also be useful as gas delivery vehicles for multicellular explants in the context of cryopreservation. For example, the conversion of cryopreserved apical meristems to intact plants may well be facilitated by oxygen-gassed PFC, to promote initial postcryopreservation survival, and by carbon dioxide-gassed PFC to stimulate rooting and *ex vitro* acclimation (29). Such applications should take account of related studies in which PFCs and their emulsions facilitated survival of fish semen during low temperature storage (23) and promoted hypothermic preservation of mammalian organs (27).

The present results indicate that oxygenated PFC, together with Pluronic F-68 should be incorporated routinely into postthaw culture media and handling strategies, to maximize plant cell viability and recovery. A further advantage of using PFCs in such systems is that they are easily recoverable and recycleable, thereby providing a cost-effective underpin to germplasm storage technologies (21). Moreover, the present demonstration that PFCs can be exploited in small-scale culture systems makes their routine use economically feasible.

#### ACKNOWLEDGMENT

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CRYOPRESERVATION OF *MORICANDIA ARVENSIS* (L.) DC  
(BRASSICACEAE) CELL SUSPENSIONS: BENEFICIAL EFFECTS OF  
PLURONIC F-68

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### Summary

Cell suspension cultures of *Moricandia arvensis*, a photosynthetic C<sub>3</sub>-C<sub>4</sub> intermediate within the *Brassicaceae*, were recovered from cryogenic storage. Post-thaw cell viability, determined by triphenyltetrazolium chloride reduction and cell re-growth, was influenced by composition of the cryoprotectant and, positively, by incorporation into the post-thaw medium of 0.01% (w/v) of the non-ionic surfactant, Pluronic F-68. The growth characteristics of re-established suspension cultures were similar to those of non-cryopreserved suspensions. Re-established suspensions also released viable protoplasts in yields comparable to the same non-cryopreserved cell suspensions.

### Key words

*Brassicaceae*, cell suspensions, cryopreservation, *Moricandia arvensis*, protoplasts, Pluronic F-68

### Introduction

*Moricandia arvensis* (L.) DC. (2n=2x=28), within the sub-tribe *Moricandiinae* of the *Brassicaceae*, is a wild relative of crop Brassicas, with an intermediate C<sub>3</sub> and C<sub>4</sub> photosynthetic/photorespiratory system (1) giving an efficient recapture of photorespiration-released carbon dioxide. This accounts for a low carbon dioxide compensation point for this plant (2). Cell suspensions were initiated as a source of protoplasts for somatic hybridisation and cybrid production with *Brassica napus*, to incorporate, ultimately, the C<sub>3</sub>-C<sub>4</sub> metabolic pathway into oilseed rape. To this end, suspension cultures require the development of long-term preservation protocols to negate the requirement for periodic re-initiation, and to maximise mitotic competence.

Cryopreservation techniques have been developed and employed for cell suspensions of numerous species (3, 4), with demonstrable plant regeneration post-thaw (5), thus supplying competent target cells and, indirectly, protoplasts for plant genetic manipulation (6, 7). The risk of spontaneous and undesirable genetic change (somaclonal variation) inherent in cultured, dedifferentiated cells is thus minimised. Cryopreservation for the *Brassicaceae* is restricted to isolated microspores (8, 9) and microspore-derived embryos (10, 11) of *Brassica napus*, together with cell suspensions of *Arabidopsis thaliana* (12, 13).

Tissue culture studies using *Solanum dulcamara* (14) and *Corchorus capsularis* (15), have demonstrated that low concentrations of the non-ionic, co-polymer surfactant Pluronic F-68

(Poloxamer 188), stimulate both growth and differentiation of cultured tissues and organs. It has been proposed that such effects of Pluronic F-68 are mediated, in part, through increased plasma membrane permeability to nutrients and/or growth regulators (16). Post-thaw sub-culture regimes have been shown to influence significantly the regrowth of thawed cells (6, 17). In this respect, supplementation of the recovery medium with 0.01 - 0.2% (w/v) Pluronic F-68 enhanced the post-thaw viability of embryogenic cells of the Japonica rice cvs. Taipei 309 and Tarom (18). The possibility exists that this relatively inexpensive, commercially available surfactant could be more widely applicable to freeze-thaw programmes, including non-embryogenic cells, such as those of *Moricandia arvensis*. Therefore, Pluronic F-68 was incorporated into the post-thaw recovery protocols for this species. This paper reports (i) the establishment of a reproducible cryopreservation protocol for *M. arvensis* cell suspensions, and (ii) assesses the effect of incorporation of Pluronic F-68 into the post-thaw cell recovery medium.

## Materials and methods

### *Plant material*

Seeds of *Moricandia arvensis* (L.) DC were provided by R.J. Mathias. Seeds were surface sterilised with 15 % (v/v) "Domestos" bleach solution (Lever Industrial Ltd., Runcom, UK.) for 15 min, followed by three washes with sterile tap water. Seeds were germinated in 175 ml capacity glass jars (Beatson Clarke and Co. Ltd., Rotherham, UK; 10 seeds per jar) each containing 50 ml of semi-solid medium consisting of Murashige and Skoog (MS) salts and vitamins (19) with 30 g l<sup>-1</sup> sucrose and 0.8 % (w/v) agar (Sigma, Poole, UK), but lacking growth regulators (designated MSO). These, and all subsequent cultures used in this study, were maintained under a 16 h light/8 h dark photoperiod (21.45  $\mu\text{Em}^{-2} \text{s}^{-1}$ , Cool-White fluorescent tubes; Thorn EMI Ltd., Hayes, UK) at  $22 \pm 2^\circ\text{C}$ , unless otherwise stated. Seed-derived shoots were maintained by transfer, every 28 days, of shoot tips (upper 3 nodes) to fresh medium (4 shoots/jar).

### *Callus initiation and generation of cell suspensions*

Callus initiation and cell suspension establishment was carried out following modification of published procedures (20). Stem internodes of cultured shoots (28 days after subculture) were cut into 0.5 cm lengths and placed longitudinally in 9 cm Petri dishes (Bibby-Sterilin, Stone, UK) each containing 25 ml of MS-based medium (designated MSD1) with 1.0 mg l<sup>-1</sup> 2,4-dichlorophenoxyacetic acid (2,4-D) and semi-solidified with 0.4% (w/v) Sea Kem Le agarose (FMC Bioproducts, Rockland, ME 04841, USA). Callus resulting from stem sections was used to produce cell suspensions, the latter being initiated by transferring approx. 0.8 g f. wt. portions of rapidly growing, friable 4-6 week-old callus to 8 ml aliquots of liquid MSD1 medium in 50 ml Erlenmeyer flasks, on a rotary shaker (90 rpm). After 7-10 days, the contents of each flask were transferred to 100 ml flasks to which 10 ml of liquid MSD1 medium were added. Those cultures surviving 7-10 days of culture had an additional 10 ml of medium added and became stocks for the subsequent initiation of fast-growing cell suspensions in 250 ml flasks. This was effected by transfer of 1.5 ml packed cell volume (PCV) of cells, with 8.5 ml of partially-spent medium, to a 250 ml flask containing 30 ml of fresh medium (40 ml total culture volume). Thereafter, cell suspensions were subcultured every 7 days by transfer of 1.5 ml PCV, as already described.

### *Growth of cell suspensions*

A known volume of cells (1.5 ml PCV) in 8.5 ml of partially-spent medium was transferred to a 250 ml flask with a graduated side-arm, the flask containing 30 ml of fresh medium. Cell growth was measured daily, over 15 days, by allowing 10 ml of suspension to settle into the

graduated **side-arm**. The settled cell volume (SCV) was expressed as the **volume** (ml) of cells in 10 ml of culture. Growth curves were plotted as a mean of 3 separate assessments; 5 readings were recorded at each sampling time. The morphology and viability [fluorescein diacetate (FDA) staining (21)] of 3-4 month-old (established) suspensions was assessed every 7 days.

#### *Preparation of cells for cryopreservation*

Seven days after transfer and prior to **cryopreservation**, the spent MSD1 medium was replaced with fresh MSD1 medium supplemented with 6 % (w/v) mannitol. The suspensions were cultured for a further 7 days, after which suspensions were sieved (45  $\mu\text{m}$ ) and the spent medium discarded. Cells (0.25 ml PCV) were transferred to 2.0 ml capacity polypropylene vials (Sarstedt Ltd., Leicester, UK) and freshly prepared filter-sterilised cryoprotectant added (0.75 ml). Two cryoprotectant mixtures, **cryoprotectant A** (0.5 M dimethylsulphoxide [DMSO], 0.5 M glycerol, 1.0 M sucrose) and **cryoprotectant B** (DMSO, glycerol and sucrose each at 1.0 M) as modified from [6], both based on MSD1 medium, were assessed. Chilled vials (ice, 1 h), were loaded onto aluminium canes and transferred to a programmable freezer (Cryo 10 Series; Planer Biomed, Sunbury-on-Thames, UK). Cells were cooled at 1  $^{\circ}\text{C min}^{-1}$  to a terminal temperature of  $-35^{\circ}\text{C}$  and held (35 min) at this temperature prior to storage ( $-196^{\circ}\text{C}$ ) in liquid nitrogen. Ice nucleation was allowed to initiate spontaneously.

#### *Post-thaw recovery of cryopreserved cells*

Cells cryopreserved for a minimum of 7 days were recovered from liquid nitrogen by plunging the vials into sterile water at  $45^{\circ}\text{C}$  (10 min). After thawing, excess cryoprotectant was removed and the cells transferred onto two layers of sterile filter paper (No.1, 5.5 cm; Whatman, Maidstone, UK) overlaying 25 ml aliquots of MSD1 medium semi-solidified with 0.8% (w/v) agar, contained in 9 cm Petri dishes. MSD1 medium contained Pluronic F-68 (Sigma) at 0, 0.01, 0.1 or 1.0 % (w/v). The contents of one vial were placed in each Petri dish. Cells were cultured for 3 days (dark,  $22 \pm 2^{\circ}\text{C}$ ). A sample of cells (50 mg f. wt.) was removed from each Petri dish for triphenyltetrazolium chloride (TTC) viability assessment. The remaining cells were transferred, attached to the uppermost filter paper, to fresh MSD1 medium with Pluronic F-68, as appropriate. Cells were sub-cultured every 28 days. Callus f. wt. was recorded 28 days and 70 days post-thawing for a minimum of 10 replicate plates for each treatment.

#### *TTC assay*

Active dehydrogenases in viable cells reduce TTC to a red formazan product which is measured spectrophotometrically. Three ml of 0.6 % (w/v) TTC in 0.05 M  $\text{Na}_2\text{HPO}_4 - \text{KH}_2\text{PO}_4$ , 0.05 % (v/v) Tween 80 (Sigma), pH 7.4, was added to cells harvested 3 and 28 days after thawing (50 mg f. wt.) in 16 ml glass, screw-capped centrifuge tubes (Coming Glass Works, New York, USA) and the cells incubated (dark,  $28^{\circ}\text{C}$ ) for 16-18 h. To each tube was added 95 % ethanol (10 ml) and the tetrazolium salt extracted ( $100^{\circ}\text{C}$ , 10 min). Tube contents were adjusted to 7 ml by addition of 95 % (v/v) ethanol; the absorbance of each supernatant was measured ( $\text{OD}_{490}$ ). **Viability** was expressed as  $\text{OD}_{490} \text{ g}^{-1} \text{ f. wt.}$  Assays were performed on a minimum of 10 samples per treatment in each experiment; each experiment was repeated twice.

#### *Re-initiation of cell suspensions from cryopreserved cells and isolation of protoplasts*

Callus from *M. arvensis* cells cultured for 80-100 days post-thawing, was used to re-initiate cell suspensions in MSD1 liquid medium. Re-initiated suspensions were maintained alongside non-cryopreserved suspensions of the same cell line. Growth curves were plotted for re-established and control suspensions.

Protoplasts were isolated from suspension cells 4 days after sub-culture and 125-130 days after re-initiation of suspensions following modified protocols (22). Cells were harvested on a 100  $\mu\text{m}$  mesh nylon sieve (Wilson Sieves, Hucknall, Nottingham, UK) and the cells (10-12 ml SCV) incubated (14-16 h;  $22 \pm 2^\circ\text{C}$ ) in a 250 ml flask containing 40 ml of enzyme solution. The latter consisted of 0.03 % (w/v) Macerozyme R10 (Yakult Honsha Co. Ltd., Nishinomiya, Japan), 2.0 % (w/v) Meicelase (Meiji Seika Kaisha Ltd., Tokyo, Japan) and 2.0 % (w/v) Rhozyme HP 150 (Rohm and Haas Co., Philadelphia, USA) in CPW salts solution (23) at pH 5.7, in the dark on a rotary shaker (40 rpm).

After incubation, the crude digests were passed through a 100  $\mu\text{m}$  nylon mesh to remove undigested cell colonies and spontaneous fusion bodies, and the filtrate collected in 9 cm diameter Petri dishes. W5 salts solution (24), at approx. one-third volume of the original enzyme-protoplast mixture, was added to the protoplast suspension prior to transfer to 16 ml screw-capped tubes and centrifuging (7 min;  $100 \times g$ ). The enzyme supernatant was removed, the protoplast pellets resuspended in W5 salts solution (16 ml) and centrifuged. Protoplast pellets were resuspended in W5 solution (2 ml) and purified by layering onto a 21 % (w/v) sucrose solution in 16 ml screw-capped tubes and centrifuging (5 min;  $150 \times g$ ). Intact protoplasts forming a layer at the W5 solution-21 % (w/v) sucrose solution interface, were collected by Pasteur pipette and transferred to 16 ml tubes. Protoplasts were washed twice by resuspension in W5 solution with centrifugation (7 min;  $100 \times g$ ) and finally resuspended in W5 solution prior to yield and viability assessments. Protoplast yield was expressed as the total number of protoplasts isolated g<sup>-1</sup> f. wt. of suspension culture. Cell viability was determined using FDA staining (21) and expressed as the percentage of viable cells in a minimum of 500 cells per culture.

#### *Statistical analyses*

Means and standard errors (s.e.m.) were used throughout; statistical significance between mean values was assessed using a conventional analysis of variance (ANOVA) and Student's *t* test, as appropriate (25). A probability of  $P < 0.05$  was considered significant.

#### **Results**

##### *Comparison of cryoprotectant effectiveness*

The viability of unfrozen cells, as determined by mean cell absorbance following TTC reduction, was  $19.0 \pm 0.8$ , whereas the viability, of non-cryopreserved cells pre-cultured in mannitol enriched medium for 3 days was  $13.2 \pm 0.5$  ( $n = 10$  throughout). In the case of frozen cells, there was no significant difference at 3 days post-recovery, in the mean absorbance of cells cryopreserved with cryoprotectant A ( $3.5 \pm 0.4$ ;  $n = 10$ ) or cryoprotectant B ( $2.8 \pm 0.2$ ;  $n = 10$ ). All cells were orange-brown in colour following 3 days post-thaw culture.

There was no significant difference in the mean cell viability and the mean f. wt. of cells at 28 days post-(hawing of cells treated with cryoprotectants A-or B (Table 1). However, the mean f. wt. of cells treated with cryoprotectant B, after 70 days of culture was 12 % greater ( $P < 0.05$ ) than that of cells treated with cryoprotectant A (Table 1). There was also a significant ( $P < 0.05$ ) increase in f. wt. of cells at 70 days compared to 28 days with cryoprotectant B. In addition, cells treated with cryoprotectant B were friable and chlorophyllous in appearance by 70 days of post-thaw culture, compared to those cryopreserved with cryoprotectant A. The latter remained similar in appearance and colour to freshly thawed cells.

*Effect of Pluronic F-68 on recovery of cryopreserved cells*

Cell viability was comparable at all concentrations of Pluronic tested, except at 0.1% (w/v) after 3 days of **culture** when viability was increased significantly (Table 2). Supplementation of the culture medium with Pluronic F-68 increased biomass, as measured by mean cell f. wL following 28 days of post-thaw culture, with significant increases at all concentrations of Pluronic F-68 **used**, compared to the untreated control (Table 2). The greatest increase of 76% over control was recorded when 0.01% (w/v) Pluronic F-68 was added to the medium. Similar, less pronounced increases were observed with cells exposed to 0.1 or 1.0% (w/v) Pluronic F-68, where the mean f. wts. were 19% and 7% respectively, above that for cells recovered in the absence of Pluronic F-68. A sustained, 89% increase in mean biomass was recorded for cells thawed in the presence of 0.01% (w/v) Pluronic F-68 compared to control lacking Pluronic, as measured after 70 days of culture. In contrast, there were no significant differences in biomass, after 70 days between cells recovered on medium supplemented with 0.1% or 1.0% (w/v) Pluronic F-68 and cells thawed in the absence of Pluronic F-68 (data not shown).

Table 1. Effect of cryoprotectant on viability and fresh weight of *Moricandia arvensis* cells 28 and 70 days post-thawing.

Treatment	28 days post-thawing		70 days post-thawing
	Cell viability (Absorbance)	Callus f.wt. (mg)	Callus f.wt (mg)
Cryoprotectant A	1.08 ±0.09	120.4 ±15.3	139.1 ±6.9
Cryoprotectant B	1.45 ±0.21	129.2 ± 8.2	156.4 ± 4.6*

Values are mean ± s.e.m. (n = 10 throughout), \* P < 0.05 compared to corresponding mean value for Cryoprotectant A.

Table 2. Effect of Pluronic F-68 on viability and fresh weight of *Moricandia arvensis* cells (frozen using cryoprotectant B) at 3 and 28 days post-thawing.

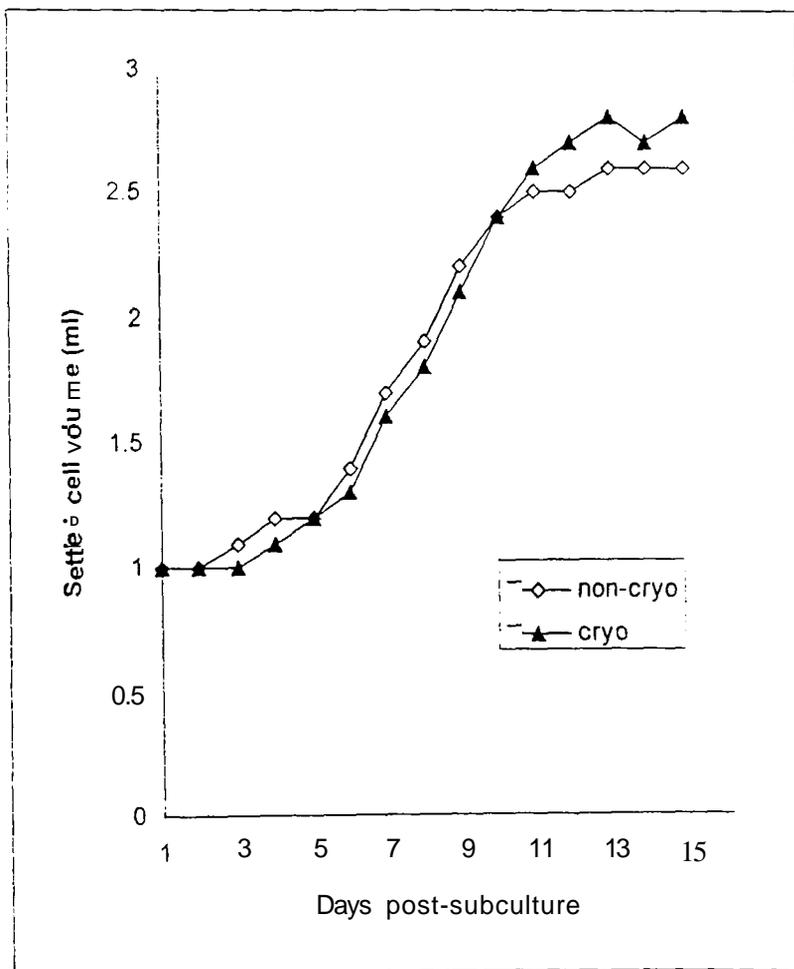
Surfactant conc. (% w/v Pluronic F-68)	Cell viability (3 days) (Absorbance)	Cell viability (28 days) (Absorbance)	Callus f.wt. (28 days) (mg)
0	1.39 ±0.21	2.1 ± 0.3	97.2 ± 13
0.01	1.95 ± 0.45	2.2 ±0.1	171.1 ± 1.0***
0.1	3.00 ±0.31**	1.7 ±0.2	115.4 ±1.2***
1.0	0.99 ± 0.11	3.1 ±1.1	104.2 ± 1.2***

Values are mean ± s.e.m. (n = 10 throughout), \*\* P < 0.01, \*\*\* P < 0.001 compared to mean value for control treatments lacking surfactant.

*Re-establishment of cell suspensions and subsequent isolation of suspension-derived protoplasts*

Cells of the suspensions re-established from cells frozen with cryoprotectant B and recovered under optimal conditions were morphologically similar to cells of the original line. The growth pattern of suspensions re-initiated from cryopreserved cells was not significantly different ( $P > 0.05$ ) from that of the non-cryopreserved control (Fig. 1). In addition, the mean viability ( $58.3 \pm 2.7 \%$ ) and yield ( $5.7 \pm 0.6 \times 10^6$ ) of protoplasts from re-established suspensions were not significantly different ( $P > 0.05$ ) to the corresponding values for control, non-cryopreserved suspensions of the same line ( $55.2 \pm 4.4 \%$  and  $6.2 \pm 0.8 \times 10^6$ , respectively).

Fig. 1. Growth curves of suspension cultures of *Moricandia arvensis*, in MSD1 medium, initiated from cryopreserved (cryo; cryoprotectant B) and non-cryopreserved (non-cryo) cells.



**Discussion**

The results of the present experiments demonstrate that *Moricandia* cell suspensions can be cryopreserved and that cryoprotectant mixture B, consisting of DMSO, glycerol and sucrose, each at 1.0 M, was superior to cryoprotectant mixture A for successful post-thaw recovery. This study extends an earlier investigation (18) which demonstrated that supplementation of medium with

Pluronic F-68 enhanced post-thaw growth of rice suspension cells. The observation that such use of Pluronic F-68 with *Moricandia* enhances post-thaw division leading to increased biomass production, implies that this relatively low-cost strategy can be readily extended to plant systems in general. The *M. arvensis* cell suspensions recovered from cryogenic storage maintained growth characteristics and levels of protoplast yield and viability comparable to the original, unfrozen cultures. However, *M. arvensis* cells were slow to recover their normal growth rates after thawing, compared to cryopreserved cells of rice, where growth can be readily re-established (6). This may be due to the nature of the original non-embryogenic *Moricandia* cell suspensions, since other workers (6) observed differential responses of embryogenic and non-embryogenic rice cells to cryopreservation, with embryogenic cells exhibiting significantly faster growth rates than non-embryogenic cells after thawing. Further studies should examine the effects of initiation of ice crystals by seeding, since previous studies have demonstrated that the regrowth after thawing of cell suspension cultures of *Musa* could be enhanced by manual initiation of ice nucleation (26).

The present investigation was consistent with previous studies using Pluronic F-68 as a growth-enhancing medium supplement, where differences in response were seen not only between cells, tissues and organs from the same plant species, but also between species of different genera (16, 27). For example, culture of *C capsularis* cotyledons with attached petioles in the presence of up to 0.5% (w/v) Pluronic F-68 increased shoot production, with no further stimulation at higher concentrations (15). In contrast, maximum growth of transformed roots of *Solanum dulcamara* occurred with 0.01% (w/v) Pluronic F-68 and that of leaf-derived callus with 0.1% (w/v) of the surfactant (15). The present approach should be precision tuned for individual species to optimise the concentration of Pluronic F-68 eliciting maximum recovery following cryopreservation. Whilst the precise mechanisms underpinning the effects of Pluronic F-68 on the growth of plant protoplasts, cells, tissues and organs have not been determined, the effects may be mediated through increases in cell membrane permeability to nutrients and/or growth regulators (16, 27, 28). Indeed, related evidence from studies with yeast has demonstrated that Pluronic F-68, at concentrations comparable to those used here, enhanced the uptake of FDA and antibiotics (29, 30).

It will be interesting to extend these studies by evaluating the effects of media supplementation with surfactants with different physico-chemical properties to elucidate the underlying mechanism for their beneficial effects. Such experiments should take account of previous investigations demonstrating that growth enhancement by surfactants in cultured jute tissues was related to the hydrophobic-hydrophilic balance (HLB) of the compound (15).

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