BIOTECHNOLOGICAL APPROACHES TO ROSE BREEDING.

by

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Thesis submitted to the University of Nottingham
for the degree of

Doctor of Philosophy,

July 1994.

ACKNOWLEDGEMENTS.

I wish to thank Drs. J. B. Power and M. R. Davey for their help and supervision, S.E.R.C. and David Austin Roses, Albrighton, UK for funding this research and Dr. J. M. Chartier-Hollis for useful discussions. I also wish to thank all of my research colleagues for their help and friendship. My family and friends deserve special thanks for the patience, inspiration, practical help, encouragement and loving support without which I would not have been able to complete my studies. The photographic skills of Brian Case, and the technical assistance provided by Dave Wilson, John Gaskin and Ian Gilder are also gratefully acknowledged.

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ABSTRACT.

The production of new rose cultivars by sexual crossing is problematic and time consuming due to sexual incompatibility, the failure of seeds to germinate, and to a limited gene pool. Biotechnology provides an obvious alternative for the creation of genetic novelty in rose. This thesis focuses on the development of novel approaches, based on embryo rescue, pollen cryopreservation, protoplast and transformation technologies.

A reproducible embryo rescue technique was developed in which embryos were excised and germinated on agar solidified medium containing a basic salt mixture and carbohydrate. The choice of carbohydrate and the growth conditions employed were demonstrated to markedly affect the percentage germination and subsequent plantlet development. This technique was used to greatly increase the production of F_1 hybrid progeny when compared to conventional germination methods.

The failure of sexual crosses between several English rose cultivars was shown to be due to a combination of low pollen viability and to the operation of a pollen-style incompatibility mechanism (probably of the gametophytic self-incompatibility type). Degree of flower opening and method of pollen dehiscence were shown to significantly affect pollen viability.

A technique was developed for the effective cryopreservation of English rose pollen. Using this technique it was possible to store pollen at ultra-low temperatures without any significant loss in viability. Such a technique compared favourably with conventional techniques (refrigeration and freezing) in which a loss in viability over time was demonstrated to occur.

In vitro shoot cultures of English rose were established on MS-based media containing BAP, GA_3 and NAA following the treatment of explants with an antioxidant solution to negate the effects of phenolic oxidation. The production of callus was shown to be genotype dependant and lacked regeneration potential. Rhizogenic responses were observed in leaf discs of two cultivars however shoot regeneration was not observed.

Using a variety of enzyme mixtures it was possible to isolate protoplasts from both *in vitro* leaf material and from cell suspensions. Both mesophyll and cell suspension derived protoplasts were cultured to a microcallus stage. Plating density, growth regulator concentration and the use of antioxidants were all demonstrated to have a significant affect on the protoplast plating efficiency. Rhizogenesis was achieved from mesophyll protoplast-derived calli.

Protoplasts, sometimes labelled with a fluorescent marker, were subjected to both chemical and electrofusion. Using micromanipulation, heterokaryons, formed during electrofusion, were recovered. Such heterokaryons, when cultured, underwent division and formed microcalli which subsequently developed into calli. The hybrid nature of such calli were confirmed by isozyme analysis, determination of ploidy level and RAPD analysis.

The introduction of a plasmid containing a *gus* marker gene into zygotic embryos of English rose was shown to be possible. This was achieved by microprojectile-mediated DNA delivery using a laboratory built electrical discharge device. The efficiency of this technique was influenced by the concentration of microprojectiles and DNA used, and by firing distance and choice of DNA construct.

The relevance of this study and its applications, in the context of rose breeding, are discussed.

ABBREVIATIONS.

ABA	abscisic acid
AC	alternating current
ABAD	English rose cultivar Abraham Darby
AIB	*Agrobacterium*-induction broth
Anon	anonymous
approx.	approximately
B5	Gamborg *et al.* (1968) basal medium
BAP	6-benzylaminopurine
B.C.	before Christ
Bt	*Bacillus thuringiensis* gene
C.	century
^{o}C	degrees Celsius
c.	*circa* (Latin; around)
cat	chloramphenicol acetyltransferase gene
CAT	chloramphenicol acetyltransferase protein
Ca^{2+}	calcium ion
cm	centimetre
CPW	cell and protoplast washing
cv.	cultivar
cvs.	cultivars
d	day
DC	direct current
diam.	diameter
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylene diamine tetra-acetic acid
eg.	*exampli gratia* (Latin; for example)
et al.	*et alia* (Latin; and others)
F_1	first filial generation
FDA	fluorescein diacetate
Fig.	figure
FPE	final plating efficiency
f. wt.	fresh weight
g	gram
GA_3	gibberellic acid
gus	β-glucuronidase gene
GUS	β-glucuronidase protein
Gy	Gray; unit of absorbed radioactive dose
h	hour
HER	English rose cultivar Heritage
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
ie.	*id est* (Latin; for instance)
IEF	isoelectric focusing
IPE	initial plating efficiency
kb	kilobase
kg	kilogramme
KHz	kilohertz
KM	Kao and Michayluk (1975) basal medium
kV	kiloVolt
l	litre
log	logarithmic
LUC	English rose cultivar Lucetta
M	molar

m	metre
mA	milliampere
MES	2-(N-morpholino) ethane sulphonic acid
mg	milligramme
mg l^{-1}	milligramme per litre
MHz	megahertz
min	minute
ml	millilitre
mM	millimolar
mm	milimetre
mm Hg	millimetres of mercury
mOsmol kg^{-1}	milliosmole per kilogramme
MP	English rose cultivar Marie Pavié
MPE	intermediate plating efficiency
MR	English rose cultivar Mary Rose
MS	Murashige and Skoog (1962) basal medium
ms	millisecond
m.w.	molecular weight
nl	nanolitre
nm	nanometre
NAA	naphthalene acetic acid
NOA	2-naphthyloxyacetic acid
npt II	neomycin phosphotransferase II gene
NPT II	neomycin phosphotransferase II protein
OD_{600}	optical density at 600 nm
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
pers. comm.	personal communication
p	page
pp	pages
pH	negative logarithm of hydrogen ion concentration
PVP	polyvinylpyrrolidone
RITC	rhodamine isothiocyanate
rpm	revolutions per minute
s	second
scv	settled cell volume
SDS	sodium dodecyl sulphate
SEM	scanning electron microscope/microscopy
SH	Schenk and Hildebrandt (1972) basal medium
suppl	supplement
TDZ	thidiazuron; 1-Phenyl-3-(1,2,3-thiadiazol-5-yl) urea
TE	Tris EDTA
TIBA	2,3,5-triiodobenzoic acid
Tris	2-amino-2 (hydroxymethyl)-1,3-propanediol
UK	United Kingdom
USA	United States of America
US$	United States of America dollars
UV	ultra violet
V	Volt
var.	variety
v/v	volume to volume ratio
WB	English rose cultivar Wife of Bath
w/v	weight to volume ratio
wt.	weight
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid
z	zeatin

μg	microgramme
$\mu Em^{-2}s^{-1}$	microEinsteins per metre square per second; unit of illumination
μm	micrometre
μM	micromolar
μs	microsecond
2,i-P	2-isopentenyladenine
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
2,4-D	2,4 dichlorophenoxyacetic acid
4-MUG	4-methylumbelliferyl β-D-glucoronic acid
8-HQS	8-hydroxyquinolinol sulphate
<	less than
>	more than
=	equals
%	percent/percentage
[+]	somatic hybrid
x	magnification
xg	acceleration due to gravity
£	pounds sterling

CHAPTER 1.
General Introduction.

1.1 Tissue culture of woody species: an update.

The successful tissue culture of woody species is not a new development. In 1940, Gautheret cultured cambial tissues derived from trees, using newly discovered growth promoting auxins (Gautheret, 1940). Jacquoit (1949) investigated bud differentiation from cambial explants of several tree species, whilst Ball (1950) studied bud differentiation in cultured shoot tips of *Sequoia*. Shortly afterwards, the discovery and synthesis of cytokinins by Miller *et al.* (1955) enabled commercial micropropagation to become possible. *Populus tremuloides* was probably the first woody species to be cultured successfully, with both roots and shoots being regenerated from callus (Mathes, 1964). This concept for micropropagation then extended rapidly to include many other woody species, including ornamentals such as *Rhododendron* (Anderson, 1975).

Micropropagation has become an important technology for the multiplication of several species of woody plants, because it enables the rapid production of large numbers of uniform plants under highly controlled conditions. In the USA alone, the annual production of woody plant propagules for the nursery trade, using *in vitro* techniques, is in excess of 30 million per year, with a market value of approximately US $15 million. However, the market for woody plants is small when compared to other agricultural sectors (eg., the market for hybrid seed maize in the USA is US $1.5 billion per annum; Einset, 1991). The relatively restricted market for woody species, and the fact that many such species are still relatively recalcitrant in tissue culture, has resulted in much attention being focused on simple plant micropropagation, with little or no attention, until recently, being paid to more sophisticated and demanding aspects of tissue culture. The latter include the production of somatic embryogenic calli, the efficient isolation of, and plant

regeneration from, protoplasts and their genetic manipulation via transformation and cell fusion technologies. Recently, plantlets have been recovered from various organ and tissue explants, either via somatic embryogenesis, or by the formation of adventitious shoots, as demonstrated for several timber and fruit tree species (James, 1987; Valobra and James, 1990; Jones, 1992). Since the first example of the successful regeneration of plants from mesophyll protoplasts of paper mulberry *Broussonetia kazinoki* (Oka and Ohyama, 1985), regeneration has been achieved from protoplasts for an increasing number of of woody plant species. These include *Citrus* (Sim *et al.*, 1988), several temperate Rosaceous top-fruit species such as pear (Ochatt and Power, 1988a) and apple (Patat-Ochatt *et al.*, 1988), as well as important forest tree species, including poplar (Russell and McCown, 1988) and conifers, such as *Picea glauca* (Attree *et al.*, 1989).

The regeneration of adventitious shoots through tissue culture often results in the emergence of heritable mutations, known as somaclonal variation (Peschke and Phillips, 1992). However, only a small proportion of such variation will be useful. Somaclonal variation was first detected in a woody species by Lester and Berbee (1977), who observed variations in the height, branching and leaf traits of regenerated poplar plants. The ability of a plant to resist atttack by pests and/or disease is also often changed by somaclonal variation. Prakash and Thielges (1989) detected both enhanced and reduced resistance to the leaf rust pathogen *Melampsora medusae* in regenerated plants of *Populus deltoides* (Eastern cottonwood). Peach plantlets exhibiting enhanced resistance to the bacterial pathogen *Xanthomonas campestris* var. *pruni* have arisen as a result of somaclonal variation (Hammerschlag, 1988), as have apple trees resistant to *Erwinia amylovora* , the causal agent of fireblight (Donovan, 1991). Salt-resistant somaclones have been obtained from explants and protoplasts of the cherry rootstock 'Colt' (Ochatt and Power, 1989) and from protoplasts of *Citrus sinensis* (Ben-Hayyim and Goffer, 1989).

Despite rapid advances in recent years, it is clear that the success of many biotechnological techniques are genotype-dependent. For instance, Roest and Gilissen (1993) reported that the regeneration of protoplasts is very much species dependant, with protoplasts from different species having extremely different requirements for regeneration; some fail to undergo shoot regeneration despite being subjected to a range of treatments that have been shown to be effective for protoplasts of other similar species. Although limited investigation into such effects has been attempted with non-woody species (Koorneef et al., 1987; Cheng and Veilleux,1991), such techniques will generally remain restricted to responsive genotypes until the cause has been accurately determined and, more importantly, can be overcome. Inevitably, this severely limits the number of woody plant species which can benefit from these techniques.

Juvenillity is a major problem with most woody plant species as they have prolonged juvenille periods prior to the production of flowers, fruits and/or marketable quantities of wood. Current methods in woody plant biotechnology almost invariably rely on embryonic or juvenille seedling tissue for experimental material, with mature material failing to respond to most biotechnological approaches (Paton, 1991).

1.2 Tissue culture for genetic engineering of woody species.

1.2.1 Somatic hybridisation.

The availability of a reliable protoplast-to-plant system is an essential prerequisite for the production of somatic hybrids. Such a system will include a method for consistently isolating and culturing protoplasts, an efficient protoplast-to-plant regeneration system, and a method for identifying/selecting any somatic hybrid material that is produced, either prior to or post regeneration. The usefulness of protoplast fusion as a means for the genetic improvement of plants has been reviewed by Pental and Cocking (1985). Woody species are ideal candidates for the application of protoplast technology, since improvement by conventional methods is

restrained by their complex life-cycles. These features make conventional breeding methods lengthy and difficult because desirable genes from one plant cannot be introduced into another by sexual crossing due to natural incompatibility barriers. Where sexual crossing is posssible, the long juvenille phase often means that it is generally many years before it can be determined whether or not the progeny of a particular cross is, in fact, improved when compared to the parent(s).

The relatively low number of protoplast-to-plant systems in woody species is reflected in the low number of somatic hybrid plants that have been produced to date. Species of the family *Rutaceae* provided the first examples of the successful recovery of woody somatic hybrid plants. Ohgawara *et al.* (1985) and Grosser *et al.* (1988) reported the somatic hybridisation of *Citrus sinensis* with *Poncirus trifoliata*, two sexually compatible genotypes. The majority of somatic hybrids produced between woody plants have been between *Citrus* species and their close relatives (Grosser *et al.*, 1990). This success reflects the relative ease with which plants can be regenerated from *Citrus* protoplasts. With the exception of *Citrus*, the only other somatic hybrid that has been produced in woody species, has been that between wild pear (*Pyrus communis*) and 'Colt' cherry (*Prunus avium* x *pseudocerasus*) (Ochatt *et al.*, 1989a). The somatic hydrid that was produced was pentaploid, and researchers have suggested that it may prove useful as a multipurpose rootstock for both pear and cherry scions.

It is clear that until techniques for the regeneration of plants from protoplasts of woody species is improved, the scope for somatic hybridisation will be limited. However, the examples already described clearly illustrate that where a protoplast-to-plant system is available, somatic hybridisation, via protoplast fusion, can play a rôle in the breeding strategies for woody species.

1.2.2 Genetic transformation.

Genetic transformation involves the introduction of foreign genes into the genome of recipient cells, followed by the recovery from those cells, of plants which stably express the foreign gene. Ideally the gene should be be heritable through sexual crossing and/or vegetative propagation. At present, it is usual to introduce into the recipient genome not only a desired foreign gene, but also a suitable marker gene. The latter enables transgenic plants to be descriminated from non-transformed plants. Transformation offers great potential for the improvement of woody plants. However, success in achieving stably transformed plants has been minimal, mainly due, again, to problems associated with the regeneration of plants and their recalcitrance in tisssue culture. The two main methods of transformation that have been attempted with woody species are the use of *Agrobacterium* as a gene vector and/or the direct uptake of foreign DNA into regenerable plant protoplasts.

A comprehensive study of the susceptibility of woody plants to *Agrobacterium* has not been undertaken. De Cleene and De Ley (1976), in an attempt to determine the host range of wild-type strains of *A. tumefaciens*, noted that the bacterium would infect some conifers, such as *Picea abies* and *Pseudotsuga menziesii*, and woody angiosperms such as *Alnus rubra* and *Prunus domestica.* However, since this survey, the number of woody species found to be susceptible to infection by *Agrobacterium* has increased. There are several publications which report the use of agrobacteria to produce transformed tissues of woody plants. These include poplar (Parsons *et al.*, 1986), Loblolly pine (Sederoff *et al.*, 1986), alder (Mackay *et al.*, 1988) as well as the production of the first genetically transformed top-fruit tree species, *Malus pumila* Mill, containing the nopaline synthase (*nos*) and neomycin phosphotransferase (*npt* II) genes, folowing co-cultivation of leaf discs with *A. tumefaciens* (James *et al.*, 1989).

A. rhizogenes has also been used to stimulate the rooting of woody species *in vitro* when the production of roots has proved difficult. Strobel and Nachmias (1985) showed an enhanced rooting of almond by inoculating bare rootstocks with *A. rhizogenes*. The development of roots from the *A. rhizogenes* infected plants was greatly increased when compared to un-inoculated controls and also to plants inoculated with the non-pathogenic species *A. radiobacter*. Increased rooting was also induced in kiwifruit (*Actinidia deliciosa* var. Hayward) following the stable insertion of the *A. rhizogenes rol* genes A, B and C, which are responsible for the induction and maintainance of the hairy root phenotype (Rugini *et al.*, 1991). In the experiments of Rugini *et al.* (1991) transformation was effected by the co-cultivation of leaf discs with *A. rhizogenes* strain LBA 4404. Shoots which were regenerated from leaf-derived callus displayed NPT II activity and had shorter internodes and wrinkled green leaves characteristic of the Ri phenotype. Cuttings taken from the regenerated shoots exhibited an increased ability to undergo rooting, compared with non-transformed, control plants.

The limited susceptibility of many woody plant tissues to infection by *Agrobacterium*, and the inability to regenerate adventitious shoots, even when transformation has been successful, has led to methods for direct gene transfer into protoplasts being adopted in an attempt to produce stably transformed plants. The production of transformed plants by direct gene uptake into protoplasts, mediated either by high voltage electrical pulses (electroporation) or by polyethylene glycol (PEG) treatment, relies on an efficient protoplast-to-plant system being available. An increase in the number of woody species that can be regenerated from protoplasts has consequently led to an increase in the number of species that may be transformed using these techniques. To date, the number of woody plant species that have been transformed using this method remains small, and is restricted to fruit trees, particularly *Citrus sinensis* (Kobayashi and Uchimiya, 1989) and *C. jambhiri* (Vardi *et al.*, 1990). In the latter, the chloramphenicol acetyltransferase (*cat*) gene was

introduced into protoplasts by PEG-mediated uptake. Transgenic plants, expressing NPT II activity, were recovered from the protoplasts, via somatic embryogenesis, and transferred to field conditions.

High-velocity microprojectile delivery of DNA into plant tissues as a method for transformation, has also been attempted for woody species and has been reviewed recently by Davey and Rech (1992). The technique avoids the use of protoplasts and can therefore circumvent problems with plant regeneration which are encountered when other methods are employed. Therefore, this technique may well increase in the future the range of genotypes that can be successfully transformed. For example, using microprojectile delivery, it has been possible to obtain transient expression of the β-glucuronidase (*gus*) gene in somatic embryos and seedlings of white spruce (*Picea glauca*; Ellis *et al.*, 1990) and in cotyledonary cells of Loblolly pine (*Pinus taeda*; Stomp *et al.*, 1990). The first report of the recovery of a transgenic woody plant, using this technique, was that by McCown *et al.* (1991), who bombarded tissues of various *Populus* species with gold particles coated with plasmid DNA. The plasmid contained the *gus* and *npt* II genes as well as the *Bt* gene which codes for the production of the *Bacillus thuringiensis* toxin. The *Bt* gene confers resistance to lepidopteran larvae which predate these species. Following bombardment, shoot production led to the recovery of four transgenic plants carrying all three genes. Of these, two failed to express GUS activity, possibly as a result of methylation of this gene. However, one of the plants exhibited an increased resistance to feeding by two lepidopteran pests.

Continued research into procedures, such as tissue culture and protoplast culture/regeneration, is essential. The only method of transformation that does not depend on any type of regeneration procedure is the direct infection of plants with agrobacteria. All other transformation techniques rely on there being an effective way to regenerate shoots from some type of explant. A large number of

transformation techniques using *Agrobacterium* employ co-cultivation of the bacteria with the explants which subsequently have to undergo shoot regeneration to produce transformed plants. This is the case in the production of transgenic apple, in which leaf discs were co-cultivated with *Agrobacterium* (James *et al.*, 1989) and also with grapevine and plum, where hypocotyl segments were infected with *Agrobacterium* (Mullins *et al.*, 1990; Mante *et al.*, 1990). The use of high velocity microprojectile delivery of DNA relies on there being a method to regenerate plants from bombarded tissues. The transformation of *Picea glauca* using high velocity microprojectile delivery depended on there being a method to regenerate plants from the target callus and embryos (Ellis *et al.*, 1993). Similarly, the transformation of *Populus* was only possible because researchers were able to regenerate plants from nodular callus following bombardment (McCown *et al.*, 1991)

1.2.3 Tissue culture and genetic engineering of woody ornamental species.

Research has concentrated on the development of micropropagation systems for ornamentals which have a high unit value and which are grown in large numbers, *Rhododendron* is such an example (Anderson, 1975). Since the first reports of the micropropagation of woody species, the number of woody ornamental species being propagated in this manner has continued to increase. Jones (1985) indicates that out of a total annual production in the USA of 60.7 million units per year, some 25.5 million units are woody ornamental plants. Developments for many ornamentals has been allied to parallel studies on related non-ornamental species such as *Malus* and *Pyrus*. Several species of these genera have been micropropagated for ornamental use, or for commercial fruit production (Briggs and McCulloch, 1983).

Despite an ever-increasing market demand, with the exception of developmental research into basic micropropagation (where progress has been virtually as rapid as for non-woody species), restricted progress has been made with research into other aspects of *in vitro* culture technology, such as the production of embryogenic callus,

regeneration from leaf discs and genetic engineering of woody ornamental species. In order to overcome natural persistent seed dormancy, embryo rescue has been attempted for several woody ornamental species such as holly (Hu, 1978). This has led to a shortening of the normal breeding cycle and the recovery of novel F_1 hybrids. These studies, have inevitably led to further research into aspects of tissue culture, such as somatic embryogenesis in callus cultures. However, this approach is still regarded by many as a method of improving the *in vitro* propagation systems, rather than as a potential source of regenerating tissue for genetic manipulation and plant improvement. The production of somatic hybrids, by fusion of holly protoplasts has been suggested (Hu, 1989), though, to date, reports of success have not emerged. Similarly, protocols for the isolation of protoplasts from *Rhododendron* have been published (Lloyd and McCown, 1980). Despite protoplast survival being generally low (Smith and McCown, 1983) when compared to herbaceous annuals, Norton and Norton (1989) have suggested that this could form the basis of an improvement strategy for *Rhododendron*, involving somatic hybridisation or genetic transformation. However, they recognise that plant regeneration from callus needs to be resolved before this could become a realistic possibility. A protocol, recently published, describes a method for the regeneration of plants from protoplasts of ornamental honeysuckle (*Lonicera nitida*) (Ochatt, 1991a), with the suggestion that this may be used in future somatic hybridisation studies to generate novel, ornamental accessions. Undoubtedly the number of woody ornamental species suggested as possible targets for somatic hydridisation and genetic transformation using protoplasts will increase as techniques for the culture and regeneration of protoplasts are further developed.

Other tissue culture-based techniques that have been used with ornamental woody species have included anther culture and pollen embryogenesis, both have been used to produce haploid varieties of various Indian ornamental species (Bajaj and Dhanju,

1983). The induction of haploids through pollen culture ofers several advantages as detailed by Radojevic and Kovoor (1986). Specifically:

a) Homozygotes following meiotic segregation could reveal a number of valuable recessive characters which have accumulated and remained unexpressed in natural heterozygous populations.

b) In some cases, the number of androgenetic progeny can be phenomenally large and the time taken to obtain plants is considerably shortened compared to that involving hand-pollination, fruiting, seed set and germination required to produce inbreds by conventional methods.

c) Widespread self-incompatibility is another handicap in trees, which can be overcome through androgenesis.

Despite recent successes in genetically transforming woody plant tissues by the introduction of novel and useful genes (see review Manders *et al.*, 1992), the *in vitro* genetic transformation of woody ornamentals has been described infrequently to date. This is probably because attention has understandably been focused on woody plants that form the basis of plantation crops, such as timber or fruit trees and, in which losses due to pests, pathogens, opportunistic organisms and various environmental factors will have a much greater effect on the producer in terms of income loss.

With an ever-increasing consumer market, the need for breeders/growers of woody ornamental plants to produce genetically novel varieties, with improved characteristics, including resistance to pests and disease, and improved ornamental traits underpins a need to develop more biotechnological approaches. The probablility of a universal method being developed to overcome many of the problems encountered when such techniques are employed with woody species is small, and progress is likely to be made on a species-by-species basis rather than on an extensive range of woody plants.

1.3 Classification and history of Roses.

1.3.1 Introductory remarks.

Roses are grown for a number of reasons. They serve as rootstocks, onto which other species or cultivars are grafted to increase their rate of propagation, they supply the cut-flower market, and are used in the extraction of attar (rose oil). The latter is a highly-prized essential oil used in the cosmetics industry (Lata and Gupta, 1971). The principal use of roses is in ornamental planting. However, it is difficult to assess the total number of plants that are propagated or planted in any one year, partly because many countries do not monitor sales and also because many current, commercially viable varieties are no longer covered by 'patents' or Plant Breeders Rights. As a result, details of the number of plants propagated are not recorded. Short and Roberts (1991) estimated that in excess of 200 million rose bushes are planted annually worldwide, which represents a total retail market of some US $720 million. In the UK, approximately 30 million field grown plants and at least 0.5 million cut flowers are produced each year. Current data (1991/92) suggests that the value of UK sales in the amenity sector alone is in excess of £2.5 million, which represents up to 7% of the total expenditure on plants (Anon, 1992).

1.3.2 Biological and field classification.

The genus *Rosa* includes over 100 species distributed throughout the world (Rehder, 1960). Chromosome numbers in this genus range from 2n=2x=14 to 2n=8x=56 (Darlington and Wylie, 1955). The social history of *Rosa* has been long and complex, and has resulted in an elaborate, but somewhat unclear botanical classification. The system of classification that is generally accepted to be accurate is based on that of Rehder (1949). He divides the genus *Rosa* into four subgenera, namely, *Platyrhodon*, *Hesperhodos*, *Hulthemia* (sometimes referred to as *Simplicifolia*) and *Eurosa*. The subgenus *Eurosa* contains the roses, as we know them today, whilst the other three subgenera are aberrant and have played only a small rôle in the history of cultivated roses. The subgenera *Eurosa* is further divided into the 10 sections: *Banksianae*,

Laevigatae, *Bracteatae*, *Carolinae*, *Cinnamomeae*, *Caninae*, *Gallicanae*, *Pimpinellifoliae*, *Synstylae* and *Chinensis*. Three of these sections are small, and again, have little or no significance in the development of cultivated roses. The remaining seven sections, the plants of which all have adnate stipules, include the subgenera *Chinensis* and *Synstylae*. These can be collectively distinguished by their species possessing exserted styles, which are free in the *Chinensis* and fused in the *Synstylae*. Both are diploid with 14 chromosomes and both subgenera include species highly relevant to the development of modern roses. In some texts, a further section, the *Cassiorhodon*, is sometimes recognised, although it is often regarded as a subsection of the *Cinnamomeae* (Beales, 1992). All ten sections can be further subdivided each containing several different species, which, themselves, are divided into varieties. Each species has a number of varieties, which, numerically, are always increasing.

Just as the botanical method of classifying roses is complex, so also is the field classification. In recent years, three major organisations have been concerned with the formulation and nomenclature of rose classes. These are the American Rose Society, the World Federation of Rose Societies and the British Association of Rose Breeders. Unfortunately, each formulate different lists of rose classes, none of which are universally adopted. Difficulties arise not only at the specific level but also at the varietal level. Each organisation decides into which class a variety should be placed. This is dependant on somewhat arbitary factors, such as usage, growth habit, tradition and sentiment. The American Rose Society has the most comprehensive list, with 56 classes. In this system, the placing of a variety into a particular class is very much influenced by individual opinion, rather than defined criteria. The World Federation of Rose Societies lists 39 classes divided into three groups; modern garden roses, old garden roses and wild roses. The British Association of Rose Breeders has the shortest list, with only 30 classes and this system reflects the botanical classification of Rehder (1949). It is probably the easiest to understand, due to its simplicity and

the fact that it is based on defined criteria for the placement of varieties into classes. Until a comprehensive and universally accepted method for field classification is devised, the continued breeding of roses will inevitably lead to increased complications in the context of classification, as more and more varieties fail to clearly fit into an individual assigned class.

The naming of roses can also lead to complications in classification. It is not uncommon for a single rose variety to be known by different names in different countries, or for the name of a rose variety to change during its lifetime. This was the case with the rose variety known in the UK as 'Peace'. The name was changed several times during its development, and it is known in other countries as 'Gloria Dei' (Germany), 'Madame A. Meilland' (France) and 'Gioia' (Italy) (Krussman, 1981). Most roses bred recently also have commercial synonyms. These are used for trademark purposes and, to some extent, this overcomes the problems that arise when a variety has been differently named across the world. However, this system has not been universally adopted, and often does not apply to varieties that have been in existence for longer periods of time.

1.3.3 The history of the cultivation and breeding of roses.

The early history of the rose is uncertain and must inevitably be a matter of conjecture. Fossil records and the present day distribution would seem to indicate that it has been present in some form since early prehistoric times (Beales, 1992). The precise location in which the genus *Rosa* originated will probably never be determined. The natural geographic spread of roses lies in the Northern hemisphere between 20 and 70 degrees; there are no indigenous roses in the Southern hemisphere. Because of the numerous indigenous species found in Asia, it is likely that the ancestors of the modern garden roses originated in this region. The most important of these species are *Rosa indica* and *R. chinensis*. Forms of these roses may have been cultivated in China around 3000 B.C. It was in the sixteenth and

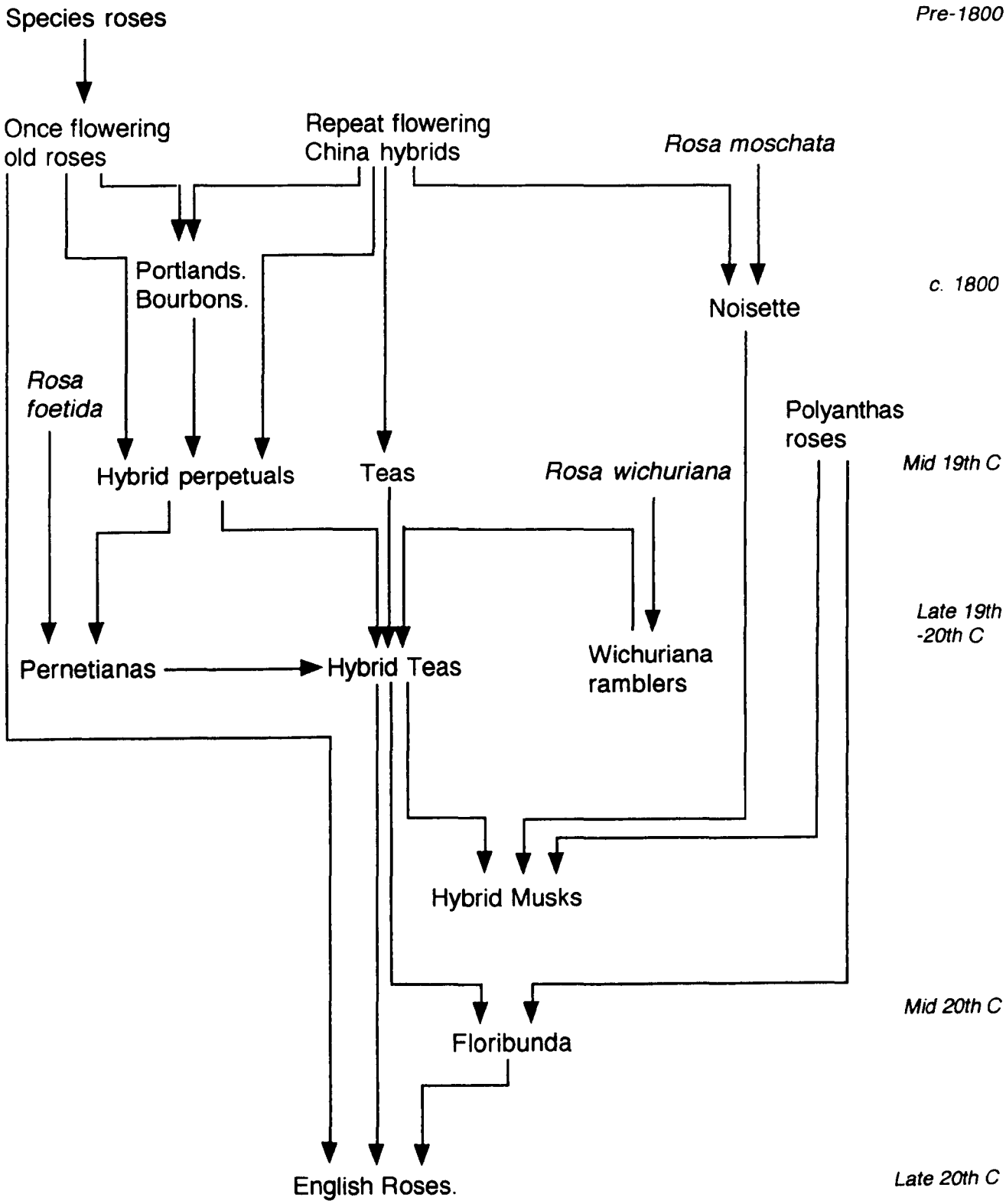
sevententh centuries that man first attempted rose hybridisation. Despite much interest in the laws of heredity at this time, it is unlikely that the first rose breeders were aware of this work. The first hybridisers were probably the French, producing new varieties for the royal courts in and around Paris in the late 1700s (Krussman, 1981). From this time, the breeding of roses became more widespread, especially in the countries of Northern Europe. A simplified geneology illustrating the ancestry of the main groups of roses is shown in Fig 1.1.

In the late ninteenth and early twentieth centuries a new class of roses was developed, namely the Hybrid Tea roses. Hybrid Teas resulted from crosses made between Tea roses and the Hybrid Perpetuals or *Wichuriana* climbing varieties. They were considered a marked improvement on Tea Roses, which were direct descendants of wild roses from China and previously the most popular group of roses. Many of the Tea roses were not hardy and, as a result, did not grow well in Northern Europe. By comparison, Hybrid Tea varieties were much more hardy, and possessed improved floral characteristics. The breeding of Hybrid Tea roses continues to this day, and the group has become the largest worldwide, with over 6000 registered varieties. As a result of progressive crossing, in-breeding and the persuit of fixed breeding patterns, the quality of the Hybrid Tea varieties has degenerated considerably. They have now been replaced, to a great extent with more modern groups of rose particularly the Floribundas, which are a product of crosses between the Hybrid Teas and Polyantha roses (Krussman, 1981) . The Floribundas are a class that consist of generally strong, vigorous plants, of short to medium height with an extensive floral colour range. The blooms tend to occur in large trusses of flat or cup shapped flowers, which usually lack scent.

The 'English Roses', which form the basis of this study, have been developed over the last thirty years, almost exclusively by the rose breeder, David Austin, Albrighton, UK. They have resulted from crosses made between modern Floribundas, Hybrid

Teas and certain climbers and old roses. Due to differences in the shape, size and floral characteristics, these roses have emerged as an individual class. The flowers, of this class, tend to be cupped or flat, usually composed of a large number of petals and, unlike other modern varieties, are highly scented. The floral characteristics tend to be similar to the old rose varieties such as the Gallicas, Centifolias, Damasks and Bourbons, but the growth habit tends towards the modern shrub roses, Floribundas and Hybrid Teas. Despite being highly regarded for their growth habit and floral characteristics, the English Roses have lost some desirable characteristics, such as disease resistance and field hardiness (Beales, 1992), that were present in their ancestors such as the Polyanthas roses of the early nineteenth century. English Roses are generally placed in the section *Chinensis* due to their remontancy (ability for repeat flowering), which most workers regard as being derived from the China hybrids.

Fig 1.1 The geneology of the rose, from pre-1800 to the present day (adapted from Wylie, 1954 and 1955, and from Krussman, 1981) (C=century).



1.4 Breeding objectives.

Unlike many cultivated plants, where there is a general consensus amongst breeders as to the 'ideal plant', there is wide opinion as to what characters are desirable in roses. Most breeders look, initially, towards floral characteristics combined with long, recurrent or remontant flowering throughout the season. Flower colour may also be important. Some breeders produce varieties only for the cut flower market, where red and pink flowers are highly desirable. Some breeders strive for highly scented varieties, as with the English Roses, while others may disregard scent in preference to disease resistance and vigour, as seen in the Floribundas. Bush shape can also be of importance commercially, with shrubby varieties generally having a greater sales volume than climbing or procumbent types.

The English Roses have been developed with the objective of combining the character and growth habit of the Old Roses, with the repeat-flowering habit and wider colour range of the modern roses. Indeed, the English Roses could be described as repeat-flowering Old Roses. The aim has been to develop in them a delicacy of appearance that is lacking in modern roses and to retain the unique features, such as scent, that are associated with the Old Roses (Austin, 1988). In striving for a particular form of flower, it has been difficult for the breeders of English Roses to develop, and in some cases retain, other important qualities, such as disease resistance. Repeat flowering roses, such as the English Rose, tend, by their very nature, to always produce new growth for more flowers. This provides a continual supply of soft, young growth which is more susceptible to disease infection and pest infestation than older, woodier parts of the plant (Austin, 1988). In particular, this is the case with diseases such as Black spot, caused by the ascomycete fungus *Diplocarpon rosae*. The fungus infects roses by penetrating the cuticule of the leaves with germ tubes emitted from conidia that are dispersed by rain splashes. Clearly, new growth will have a thinner cuticle leading to a greater chance of infection (Cook, 1981). As well as concentrating on the production of new varieties with more

interesting flower forms, breeders of English Roses are now focusing attention on the production of varieties that possess not only the desirable growth habits and flower characteristics of existing varieties, but also resistance to pests and disease and tolerance to stress.

1.5 General background to rose biotechnology and genetic engineering.

There are a number of factors which make rose very appropriate for improvement using biotechnological approaches. Traditionally, roses are multiplied asexually by grafting buds onto rootstocks. This is extremely time consuming and labour intensive, and has a significant impact on the product cost. Various tissue culture techniques, such as micropropagation, may decrease propagation time and could virtually eliminate the need for grafting onto rootstocks (Skirvin *et al.*, 1990). The production of new rose varieties by traditional sexual crossing is also time consuming. Many crosses fail to produce plants, either because the crosses are incompatible with no seed set, for reasons that are not clearly defined, or because seed which is produced either fails to germinate or exhibits a very low germination rate. A large number of these difficulties may be overcome by the application of biotechnological techniques to the breeding programme. The possibility of introducing, into existing rose varieties, genes which may confer resistance to pests or diseases to which a large proportion of rose varities are susceptible (Beales, 1992) or tolerance to stress is also desirable.

1.5.1 Micropropagation of rose: its development and commercial uses.

Until the late 1960s, attempts to propagate roses *in vitro* from meristems or shoot tips were unsuccessful (Hollings, 1965). The first report of the *in vitro* growth of rose shoots was that of Jacobs *et al* (1969), who investigated the effects of auxin and cytokinin ratios on the culture of Hybrid Tea Roses. This report concentrated more on the effects of growth regulators than the actual possibility of commercially propagating material *in vitro*. It was followed by several other studies whereby

Elliot (1970) reported on the axenic culture of apical meristems of *Rosa multiflora*. It was this later report that first illustrated that excised shoot apices/meristems could be successfully grown into plantlets *in vitro*, on a defined medium consisting of inorganic salts, sucrose, thiamine, inositol and a cytokinin. This was followed by a number of other publications detailing the micropropagation of many different types of rose, including species roses such as *R. chinensis* and *R nitida* (Rosten and McCown, 1981), New and Old-world roses (two *R. hybrida* cultivars together with *R. canina* L. and *R. damascena*; Khosh-Khui and Sink, 1982) and Floribunda, ground cover and minature roses (Douglas *et al.*, 1989).

Most procedures for the micropropagation of rose use media which typically contain the salts, vitamins and amino acids of Murashige and Skoog (MS; 1962), with sucrose (30-40 g l^{-1}), a cytokinin (most frequently 6-benzylaminopurine; BAP) at 0.5-3.0 mg l^{-1}, an auxin, such as naphthaleneacetic acid (NAA) at 0.004-0.1 mg l^{-1} or indoleacetic acid (IAA) (0.1 mg l^{-1}) and sometimes a low level of gibberellic acid (GA_3) at 0.1 mg l^{-1}. Media are typically in the pH range 5.6-5.8, and generally made semi-solid with agar. Hassegawa (1980) demonstrated that shoot multiplication of *R. hybrida* was less satisfactory if BAP were replaced by 2-isopentyl adenine (2-iP) or kinetin. Bressan *et al.* (1982) found that a temperature of 21°C and a photon flux density (400-700 nm) of 17 $\mu Em^{-2}s^{-1}$ for 12 to 24 h daily was optimal in stimulating shoot multiplication of several cultivars of *R. hybrida*. Comprehensive lists of the media and growth conditions for micropropagation of a large number of different types of roses have been produced, for example, by Skirvin *et al.* (1990) and Short and Roberts (1991).

The multiplication of rose plantlets *in vitro* must inevitably end with the induction of an adventitious root system, followed by *ex vitro* acclimation and transfer to glasshouse/field conditions. Hasegawa (1980) reported for the variety 'Improved Blaze', that root formation occurred 10-13 days after shoots were transferred to basal

media lacking growth regulators. He also showed that both root induction and transplantability were enhanced by the inclusion of NAA (0.03-0.10 mg l^{-1}) or IAA (1.0 mg l^{-1}) in the culture medium, coupled with the MS salt concentration at a quarter or half strength. Bressan *et al.* (1982) found that a temperature of 21°C and a photon flux density of 6.6 $\mu Em^{-2}s^{-1}$ was optimal for root initiation and for subsequent transplantation of tissue culture-derived plants to soil. Mortality on transplantation to compost has been shown to be significantly reduced if plantlets are rooted *in vitro* in Sorbarod® plugs (Baumgartner Papiers SA, Lausanne, Switzerland) (Lloyd *et al.*, 1988; Douglas *et al.*, 1989).

Many other factors that may influence micropropagation of roses have been investigated, such as the node position from which axillary buds, used for initating cultures, are excised from the parent plant (Bressan *et al.*, 1982). Woltering (1990) showed that supplementation by carbon dioxide in the culture vessel leads to improved growth of *Rosa* plantlets. Mekers *et al.* (1984) and Kevers *et al.* (1992) demonstrated that ethylene produced *in vitro* by micropropagated rose plantlets can have a major effect on their growth, and that inhibitors of ethylene biosynthesis reduce leaf chlorosis, abscission shoot tip necrosis and have a beneficial effect on shoot multiplication rate.

Micropropagation has been shown to be a highly effective method of rapidly propagating disease-free, uniform rose plants. The multiplication of rose, based on the subdivision of *in vitro* plantlets into branches, terminal buds and nodal sections, has been shown to give multiplication rates of two- to nine-fold over a four week period (Tweedle *et al.*, 1984). Martin (1985) has shown that, using micropropagation of *R. hybrida*, up to 400 000 plants can be produced annually from an individual rose 'mother plant'.

The fact that micropropagated rose plants are not grafted onto rootstocks alleviates the need to remove suckers arising from rootstocks. Such self-rooted plants also tend to be bushier and produce more flowers. It is generally agreed that these features make micropropagated plants more attractive to the buyer. Micropropagation of roses has considerable implications for rose breeders and growers, as it enables rapid multiplication of newly introduced varieties, along with a greater response to market requirements. This has been reflected in the increasing number of micropropagation facilities that have been created, and the ever increasing number of rose plants that are being produced in this way. Almost all miniature roses sold in the UK, are now produced *in vitro* .

1.5.2 Plant regeneration from cultured rose tissues.

Although the micropropagation of rose shoots derived from axillary buds and meristems is well established, and despite the fact that proliferating callus was initiated from young stem explants of the rose variety Paul's Scarlet as early as 1959 (Tulecke and Nickell, 1959), the ability to regenerate adventitious shoots from initially dedifferentiated rose tissues is a relatively recent development.

Until 1988, the only report that indicated a possibility of aventitious shoot regeneration was that of Hill (1967). He induced callus from stem explants of a Hybrid Tea rose, on an unspecified synthetic medium which included either 0.2 mg l^{-1} kinetin and 2.0 mg l^{-1} NAA, or 2.0 mg l^{-1} kinetin and 0.2 mg l^{-1} NAA. When the resulting callus was transferred to a medium containing 1.0 mg l^{-1} kinetin, shoots were reported to have formed. Lloyd *et al.* (1988) suggested that such shoots may not have been of adventitious origin, but may have been derived from primary meristems that had been retained despite the unorganised proliferation of adjacent cells.

In 1988, Lloyd *et al.* reported the induction of adventitious shoots from excised leaves, roots and callus of *R. persica* x *xanthia*, and on excised leaves of *R. laevigata*

and *R. wichuriana* when cultured at 23°C at a photon flux density of 8.5 $\mu Em^{-2}s^{-1}$ (16h photoperiod) on MS medium containing BAP and NAA. Organogenesis in *R. persica* x *xanthia* declined rapidly over ten weeks, and adventitious shoots were never produced from callus or roots of either *R. laevigata* or *R. wichuriana*. In 1987, Valles and Boxus reported the regeneration of adventitious shoots from *R. hybrida* callus, whilst Ishioka and Tanimoto (1990) described the induction of adventitious buds from Bulgarian rose callus.

In 1990, Arif and Khatamian documented somatic embryogenesis in callus from leaves of the *R. hybrida* variety 'Tifany'. They reported callus production, on medium containing 2,4-D, which initially showed only rhizogenic potential, but which later, upon transfer to half-strength MS based medium containing 1 mg l^{-1} kinetin, produced somatic embryos. Despite the embryos developing chlorophyll, plant regeneration was not reported. De Wit *et al.* (1990) detailed somatic embryogenesis in leaf callus of two cut rose varieties, cultured on media containing 0.1 mg l^{-1} kinetin and 0.05 mg l^{-1} NAA or 0.1 mg l^{-1} 2-naphthyloxyacetic acid (NOA). Embryos produced after 6 to 12 weeks in culture could be germinated, and the resulting plants transferred to the glasshouse where they matured into phenotypically normal plants.

Rout *et al.* (1991) demonstrated the induction of somatic embryos on callus derived from both leaf and stem internode segments of the rose cultivar 'Landora', on a medium containing BAP, NAA, GA_3 and L-proline. However, plants were not produced. The results of this study also indicated that induction of embryogenesis, in callus cultures of this rose variety, was dependent on L-proline in the medium. It was further shown that L-proline was only essential for the induction of somatic embryogenesis during the initial culture passage. Once induced, somatic embryogenesis and secondary somatic embryogenesis were retained without the decline seen in other *Rosa* species and rose varieties.

Noriega and Söndahl (1991) carried out an extensive study of somatic embryogenesis in Hybrid Tea roses. Callus induction and embryogenic response was assessed on a number of different explants including those from pistil, filament, petal, meristem, root and mature leaf. Regeneration was achieved only from filament-derived callus. Globular embryos differentiated in the presence of 2,4-D and zeatin. Periodic subculture onto a high 2,4-D and zeatin containing medium, and stock callus culture maintainence in the presence of NAA and zeatin, enabled embryogenesis to be retained for more than eighteen months. Somatic embryos were periodically isolated from embryogenic tissue and were reported to have undergone differentiation, maturation, germination and plantlet development. Plantlets obtained through this method developed normally under glasshouse conditions, proved to be morphologically true to type upon flowering, and displayed the flower colour of the original genotype.

Recently, Kunitake *et al.* (1993) demonstrated the ability to achieve plant regeneration via somatic embryogenesis from calli initiated from immature seeds (2-3 weeks after anthesis) of *Rosa rugosa* cultured on MS medium without growth regulators. They indicated that the formation of embryogenic callus showed a close similarity to nucellar callus formation by ovule cultures of *Citrus* species, as described by Kochba *et al.* (1978) in that it occurred without the aid of growth regulators. The choice of sugars was essential in determining the success of embryogenic callus initiation and subsequent development of somatic embryos into plantlets.

All the reports of plant regeneration from rose tissues, that are detailed so far, are characterised by having extremely low regeneration frequencies. In the case of adventitious regeneration, the percentage of explants (either calli, excised leaves, leaf discs or roots) that expressed organogenic potential was low. The number of buds/adventitious shoots produced per explant was small, the maximum being

5.0±2.67 from individual explant callus derived from internodal segments of *R. persica* x *xanthia* (Lloyd *et al.*, 1988). Likewise, the reports concerned with embryogenesis in rose, all describe low proportions of the initiated calli expressing embryogenic potential, and/or only a very small proportion of the embryogenic callus was shown to regenerate beyond the primary embryo stage, ie. into whole plants/plantlets. Several authors also reported that the organogenic or embryogenic potential of cultures was lost after a relatively short period of time. Recently, such a loss in embryogenic potential in cultures of *R. hybrida* L. cv Deladel has been attributed to ploidy changes brough about by somaclonal variation (Moyne *et al.*, 1993). Many workers also experienced difficulty in attempting *in vitro* clonal propagation, or transfer of *in vitro* regenerated plantlets to *ex vitro* conditions.

The wide range of growth conditions (environmental and media composition) required to achieve plant regeneration, and the range of different responses that have been observed, would seem to indicate that, in rose, the ability to obtain such regeneration is highly cultivar/species dependent, and that when successful regeneration is achieved it is often at a very low frequency. The results of these studies suggests that material, such as embryogenic callus, is not ideal for use in genetic manipulation, as it usually is difficult to obtain and to maintain, and has a relatively low regeneration potential. Frequently, it loses its ability to regenerate over a period of time.

1.5.3 Ploidy manipulation and mutagenesis.

Interest in the ability to alter chromosome number in rose has arisen, principally, from the desire to sexually cross rose varieties that are incompatible and, where this incompatibility is caused by differences in the genomic complement or where there is persistent sterility. Hybrid Teas and Floribundas are generally tetraploid but occasionally, triploid (Shahare and Shastry, 1963). As sterility of some diploid interspecific hybrids arises through genomic differences, chromosome doubling may

be expected to increase fertility. Thus, the sterile diploid *R. rugosa* x *wichuriana* 'Max Graf' spontaneously produced a tetraploid seedling, *Rosa* x *kordesii*, that was fertile (Wulff, 1951).

Tabaeezadeh and Khosh-Khui (1981) reported the production of haploid callus from anthers of the two tetraploid rose varieties *R .hybrida* L. 'Baccara' and *R. damascena* Mill. However, plant regeneration was not achieved. In 1990, Roberts *et al.* treated plantlets of *R. wichuriana* with colchicine in order to increase the fertility of this diploid species by the induction of tetraploidy. Cytological examination of root tips excised from colchicine-treated plantlets, revealed that a proportion (32.7%) of the root meristematic cells were tetraploid. To date, there have been no reports of regeneration from such tissues, despite the authors suggesting that they anticipated that a satisfactory yield of non-chimeral tetraploid plants could be obtained. Unlike this report, where the efffect of the mutagen was designed to influence chromosome number, mutagens may also be used for the production of plants with novel morphological characteristics.

The exposure of basal segments of *in vitro* derived shoots of several cut-flower rose varieties, to X-ray dosages between 25 and 60 Gy, has been used to produce novel mutants (Walther and Sauer, 1986). Not surprisingly, these workers reported that whilst dose increased, the percentage of mutated plants, and the number of mutations per plant, increased. However, plant survival declined as dose increased, although a small proportion of rose plants survived even the maximum dose. Following radiation treatment, approximately nine months were required to select mutants for rootstock grafting. Several phenotypic changes were induced. Normal rose cultivars, for example, have odd-pinnate leaves with 3-7 or more leaflets, whilst 13% of these treated plants had a changed trifoliate leaf-type, similar to those in *R. laevigata.* Others had leaves similar in appearance to *R. persica*, but with an entire leaf margin. A large variation in plant height was also observed. Some mutants had elongated

internodes, which at flowering resulted in these plants being designated as 'creepers'. Chlorophyll-deficient mutants of the 'viridis'-type (light green coloured leaves) were also induced. The most striking changes were those concerning floral features. In some cases, flower size and the number of petals/flower was altered with some flowers failing to open fully due to increased petal number. The majority of alterations were associated with flower colour, where, besides dark-red floral mutants, a majority showed pink-coloured flowers through to almost pure white petals. Amongst the flower colour mutants, a few mericlinal chimeras were detected, and it is assumed that these were caused by incomplete induced rearrangements. The selection of a limited number of mutants with fragrant flowers (the so called odoratus type) proves that this character can also be induced.

1.5.4 Isolation and culture of protoplasts and subsequent plant regeneration.

The first report of the isolation of rose protoplasts was that of Pearce and Cocking (1973), who obtained protoplasts from actively dividing cell suspension cultures of the rose variety 'Paul's Scarlet'. Although large numbers of protoplasts could be isolated, cultured protoplasts only formed incomplete cell walls, budded and exhibited abnormal cytokinesis. Mitotic division and cell colony formation was not observed.

Krishnamurthy *et al.* (1979) and Strauss and Potrykus (1980) isolated protoplasts from floral bud callus of *Rosa* x *hybrida* variety 'Soraya' and from cell suspension of the variety 'Paul's Scarlet' respectively. Both studies resulted in the formation of microcolonies which were subsequently cultured to callus. However, attempts to regenerate plants were not reported. At that time, Strauss and Potrykus (1980) identified a number of difficulties in culturing rose cell suspension protoplasts. In particular, a low protoplast plating efficiency and a reduced number of protoplast-derived calli. However, they did note, that colony formation was dependent upon frequent subculturing of source callus from which the protoplasts were isolated.

The only report of sucessful regeneration of plants from rose protoplasts is that of Matthews *et al.* (1991). Embryogenic cell suspensions were initiated using callus derived from roots of *R. persica* x *xanthia*. An enzyme mixture consisting of 2.0% cellulase 'Onozuka' R-10, 0.1% petolyase Y-23 and 1.0% hemicellulase was found to be optimal for the isolation of protoplasts. Following isolation, protoplasts were cultured in agarose-solidified KM8P medium (Kao and Michayluk, 1975), containing 2 mg l^{-1} NAA and 1mg l^{-1} BAP. Microcolonies/calli were formed, and were subsequently transferred to SH medium (Schenk and Hildebrandt, 1972) containing 3 mg l^{-1} 2,4-D. Globular and later-stage embryos formed, of which approximately 30% developed into plantlets on transfer to basal SH medium. The authors reported difficulty in maintaining the embryogenic potential of the cell suspension beyond two subcultures, necessitating repeated reinitiation. Difficulties in the rooting of protoplast-derived shoots were also noted, and transfer of protoplast-derived plantlets to glasshouse and/or field conditions was not reported.

1.5.5 The rôle of somatic hybridisation in rose improvement.

Somatic hybridisation has been used as a method for the improvement of a number of woody plants (see section 1.2.1). To date, there are no reports of somatic hybridisation being attempted in rose, though a number of publications concerned with isolation, culture, and regeneration from rose protoplasts clearly and predictably suggest this approach as being a possible method for rose improvement (Strauss and Potrykus, 1980; Matthews *et al.*, 1991).

The possibilities for rose breeding are extensive. Modern garden and cut-flower roses have a narrow genetic base to which only eight species have contributed significantly (Wylie, 1954 and 1955). This leaves potential contributions from more than 100 other wild and cultivated *Rosa* species unexplored. Wright (1965), identified a number of rose species, that had not contributed to the gene pool of the modern

cultivated varieties. These possess desirable characteristics, including 'improved' flower colours, dwarfing and drought tolerance. However, several of these species of rose cannot be crossed sexually (Krussman, 1981). Somatic hybridisation may, therefore, allow such species to be crossed with cultivated species/varieties and, in so doing, facilitate exploitation of a wider gene pool and genetic base. Self-fusion of rose protoplasts leading to homokaryon formation, may also create a more efficient means of doubling the chromosome number, to produce new and fertile teraploids from sterile diploid species/varieties (see also section 1.5.3).

Somatic hybridisation would be beneficial to breeding programmes that include triploid rose varieties. Triploid roses generally originate from crossing of diploid and tetraploid garden roses. Many of todays cultivated triploid varieties have reduced fertility due to meiotic failure. This creates difficulties if the breeder wishes to cross these with other varieties (Krussmann, 1981), which is the case with the Hybrid Rugosas which are mainly diploid or triploid, with just a few tetraploids. The triploids in this group are persistently sterile, forming non-viable pollen, thus excluding them from any futher crossing by conventional means (Wylie,1954).

The improvement of many other classes of rose is also often hampered by barriers of sexual incompatibility. Breeders of English Roses wish, for example, to cross existing tetraploid varieties with diploid Polyanthas varieties. Although Polyanthas roses are distant ancestors of the English Rose, many of their desirable characteristics, such as disease resistance, are no longer expressed in the English Roses, as selection during classical breeding has been mainly for ornamental characteristics leading to a dilution of many other desirable traits.

1.5.6 Transformation technologies as applied to rose.

Although somatic hybridisation is desirable, in that it overcomes natural sexual incompatibility between varieties/species, one serious drawback, for rose is that the

resulting hybrid will express a combination of parental characters resulting in desirable characters being lost or diluted. In order to produce a hybrid that combined the genome of one parent with one or a few desirable characters of a second parent, a procedure of repeated backcrossing over at least 3-4 generations would have to be performed, thereby resulting in the 'dilution'/elimination of the undesirable gene(s) and the retention of the desirable gene(s). As with most woody crops, including rose, this technique is both time consuming and costly due to long generation times. An additional limitation is the phenomenon known as linkage drag, in which selection for a certain allele results in the simultaneous selection of others linked to it. Sometimes, such linked alleles may cause phenotypical effects that may be detrimental to the market value of the plant.

One method of overcoming most of these problems is via transformation, in which a gene of interest is inserted, by one of several techniques, into the genome of a single cell (Dandekar *et al.*, 1993). There are a number of desirable genes that would benefit rose if they could be introduced into new or existing varieties of rose. These include genes for pest and disease resistance, for tolerance to various adverse environmental conditions, as well as genes coding for novel flower colour, shape and absence of thorns. Some of these genes, such as those for pest and disease resistance have already been identified and isolated from other plants. For example, Ellis *et al.* (1993) have isolated the *Bt* gene and introduced it into Picea glauca to confer resistance to Lepidopteran pests, while Broglie *et al.* (1991) have similarly introduced a chitinase gene isolated from bean into tobacco confering an increased resistance to fungal pathogens. Elomaa *et al.* (1993) have introduced antisense cDNA encoding chalcone synthetase into *Gerbera hybrida* to alter flower colour. Such genes could be used in a similar manner in rose. However many genes, such as those for thornlessness, still remain to be identified and isolated.

To date a small number of publications report the genetic transformation of rose. The soil-borne bacteria *Agrobacterium tumefaciens* and *rhizogenes*, are known to infect rose, under natural conditions (DeCleene and DeLey, 1976; DeCleene and DeLey, 1981), but there are only two brief reports of *in vitro Agrobacterium*-mediated transformation of rose (Matthews *et al.*, 1990; Firoozabady *et al.*, 1991). In both publications, transgenic rose plants were produced by the co-cultivating agrobacteria with embryogenic calli. Matthews *et al.* (1990) performed transformation using *A. tumefaciens* carrying a plasmid containing genes for opine production, kanamycin resistance and GUS activity. Firoozabady *et al.* (1991) employed *A. tumefaciens* strain LBA4404 and *A. rhizogenes* strain 15834, both harbouring a vector containing the *npt* II and *gus* genes.

In addition to these reports of *in vitro* genetic transformation of rose, there is also one report of the *in vivo* transformation of rose rootstocks (van der Mark *et al.*, 1990). These workers infected two varieties of rose rootstocks with three wild-type strains of *A. rhizogenes* carrying a number of marker genes. This resulted in the production of adventitious roots carrying the genes of interest. Subsequently the original (non-transformed) root system was removed and the plant was cultivated on its transformed roots. The growth of plants on a transformed root system was the same as that on untransformed control roots. This approach to transformation could be used to introduce a number of different genes and, as a result, would produce rootstocks for grafting, which were resistant to pests or disease. Despite the major rôle that roses play in the horicultural market, and their well documented susceptibility to pests, disease and stress, there are no other reports of transgenic rose. This could be due to the fact that rose species are considerably more recalcitrant to *in vitro* manipulations than most other species and, secondly, the published regeneration protocols are non-reproducible and often exhibit very low plant regeneration frequencies. Rose, transformation systems may well have been developed by commercial organisations but details not published in order to protect intellectual

property rights. In general, these reasons apply to the genetic manipulation of both woody and non-woody floricultural crops and are not specific to rose (Hutchinson *et al.*, 1992).

1.7 Thesis objectives.

To date, the breeding of roses has been very comprehensive and the ability to create new varieties has diminished due to sexual incompatibility between existing varieties, together with difficulties in other aspects of conventional breeding programmes, such as problems of germinating seed derived from many sexual crosses.

Against this background, the objectives of this project are divided into five sections:

1. To examine the nature of the incompatibility mechanism and to investigate the possibility of developing embryo rescue technologies to recover plants resulting from crosses that produce seed which will not normally germinate, or that exhibit very low levels of germination.
2. To investigate the long-term storage of rose pollen to increase scope for the breeder to cross plants which are spatially separated, or which flower at different times.
3. To establish *in vitro* culture of English roses in order to provide source material for genetic manipulation.
4. To bypass incompatibility barriers within roses via cell fusion through the isolation, culture and fusion of protoplasts.
5. To introduce isolated DNA into rose. This could enable existing varieties to be improved by the incorporation of genes, such as those for herbicide tolerance or pest/disease resistance. The use of *Agrobacterium*-mediated genetic transformation was investigated. High velocity microprojectile delivery for the introduction of DNA into embryos and axillary buds was also assessed.

CHAPTER 2.
Sexual hybridisation, embryo rescue and the generation of intervarietal rose hybrids.

2.1 Introduction.

If a rose breeder is to maintain his market share, he must be able to regularly produce novel cultivars. Many years are required to develop a new rose cultivar. Following sexual crossing, several thousand new hybrids may result, but only a small proportion of these (sometimes just one or two) may ultimately prove suitable for the commercial market. Thus, the rose breeder always aims to maximise the number of hybrid plants produced per cross, in order to increase the inherently low probability that any cross will result in a novel cultivar which can, in turn, be registered for sale.

A major obstacle, in many rose breeding programmes, is that seeds (correctly referred to as achenes), which are often produced in very low numbers, exhibit a very low percentage germination (typically less than 20%). Lack of germination is usually attributed to a mechanical restriction of embryo expansion by the presence of a thick pericarp or by dormancy regulated by growth inhibitors within the achene (Jackson and Blundell, 1963). Many attempts have been made to promote germination. These have consisted of methods designed to increase permeability of achenes, such as prolonged soaking in water, scarification, and/or exposure to oxygen (Tincker, 1935). Popcov and Buc (1967) employed an acid treatment and reported some success. More recently, the use of commercially available compost activators was suggested as a method for enhancing microbial degradation of the pericarp and, hence, of increasing the percentage germination of achenes (Cullum *et al.*, 1990). Enzymatic digestion has also been used for one rose cultivar of *R. multiflora*, albeit with limited success, to remove the pericarp and thereby increase germination (Yambe *et al.*, 1992).

During the 1930s and 1940s, a number of researchers reported on the possibility of isolating, and artificially culturing, embryos removed from the seeds of several trees, including peach, cherry, pear and apple (Tukey, 1934). Later, stimulated by this apparent success with deciduous fruit species, attention was focused on the possibility of rescuing embryos from rose achenes (Hardy, 1949; Asen and Larson, 1951). These attempts were poorly defined, and the percentage germination of such rescued embryos was often lower than that observed for untreated achenes (Asen and Larson, 1951). In addition, embryo rescue procedures were often hampered by microbial contaminants, whilst transfer and acclimation of seedling material to glasshouse conditions frequently proved difficult. Because of the many problems encountered with the technique of embryo rescue, further work on the application of embryo rescue in rose breeding programmes was not carried out and the opportunities that may have been gained for rose were never realised.

Advancement and reproducibility in laboratory techniques means that the use of embryo rescue is now possible as a realistic, commercially viable method for increasing hybrid rose plant production. Reproducibility is a key requirement if this technique is to be employed by breeders and to have a generality of approach. The aim of this part of the study was to develop a fully defined protocol which would give consistently high percentage germination for rose embryos, with an associated and acceptable percentage survival of seedlings upon transfer to glasshouse conditions. It was also possible to identify a number of key factors which influenced the success of embryo rescue in rose.

Embryo rescue has been developed as one of many biotechnological methods that can complement traditional breeeding techniques. It is designed to complement traditional breeding when a senario arises in which crossing is difficult but not strictly impossible and where the production of seed was previously the point beyond which the traditional breeding process did not progress. Alongside the failure of achenes to

germinate, rose breeding is often hampered by a failure of seed production due to sexual incompatibility. This chapter also details the results of preliminary investigations into the nature of the sexual incompatibility mechanism, in order to determine whether the failure to set seed could be attributed to reduced pollen viability and/or to post fertilisation pollen tube arrest. This, in turn, will determine whether the application of other biotechnologicl techniques, such as somatic hybridisation and genetic transformation, to rose breeding programmes would be desirable.

2.2 Materials and Methods.

2.2.1 Embryo rescue techniques.

2.2.1.1 Source of material.

Crosses were made between plants of the English rose cvs. Heritage (HER) and Lucetta (LUC), and between cv. Wife of Bath (WB) and an unnamed accesssion coded CHL/MR. All rose accessions were provided by David Austin Roses, Albrighton, WV7 3HB, UK. Stock plants were grown in 20 L plastic pots containing a 1:1 (v/v) mixture of Levington 3M soilless compost (Fisons, Ipswich, UK) and John Innes No3 compost (J. Bentley, Barrow-on-Humber, UK). Plants were maintained in a mesh-tunnel under natural light and temperature conditions and were regularly treated with systemic fungicides and insecticides (these were varied to avoid disease/pest resistance developing). Growmore fertiliser (PBI Ltd, Waltham Cross, Herts, UK) was applied to the surface of the growth substrate every three months. Crossings were carried out in the mesh tunnel in which the plants had been grown. Within three days of the onset of anthesis, pollen was removed from a flower of the male parent using a camel hair paintbrush and placed on the stigma of an emasculated flower of the maternal parent. Pollen was only placed onto the surface of stigmas that were considered to be receptive; it is generally agreed that this is when they are pale in colour and possess secretions to which the pollen adheres (Krüssman, 1981). Following reciprocal crossing, the emasculated/pollinated flowers were enclosed in

greaseproof paper bags in order to prevent rogue fertilisation by airborne or insect vectored pollen. The greaseproof paper bags were removed when cynarrhodium (hip) formation was seen to have been initiated, which was normally 10-14 d post crossing. Rose cynarrhodia were harvested from the plants at the orange/red stage, 35-49 d after pollination. When embryo rescue could not be carried out immediately, collected cynarrhodia were stored in sealed glass jars in the dark at 4°C, for 14 to 28 d.

2.2.1.2 Conventional germination of rose seed.

To enable the efficiency of the embryo rescue technique to be compared with conventional methods of germination, a small proportion of the seeds, produced from each cross, were germinated under glasshouse conditions. Randomly selected achenes, from a total of thirty cynarrhodia, were extracted from the cynarrhodia, and were stratified in sealed, screw-capped glass jars, in the dark at 0°C for 56 days. Following this period, achenes were sown at a depth of approx. 5 mm in 7.5 cm diameter plastic pots each containing a 1:1 (v/v) mix of Levington 3M soilless compost and John Innes No3 compost, and placed in a glasshouse at 25±3°C, with natural daylight supplemented with 25 $\mu Em^{-2}s^{-1}$ of fluorescent illumination, 16 h photoperiod. A total of 50 achenes from the cross HER x LUC, and 30 from the cross WB x CHL/MR were sown (5 achenes per pot). Seventy days after sowing, the percentage of achenes that had germinated was noted.

2.2.1.3 Procedure for excising embryos.

Intact rose hips were washed in running water for at least 1 h, whereupon the cynarrhodia were surface sterilised at room temperature by immersion in 0.1% (w/v) $HgCl_2$ solution for 15 min, followed by three rinses in sterile, distilled water. After surface sterilisation, cynarrhodia were opened under aseptic conditions using forceps and a scalpel, and the achenes removed. Achenes (Plate 2.1a), awaiting dissection, were placed in sterile water to prevent desiccation.

The pericarp was removed by making lateral cuts, with a scalpel, on each side of the achene, running from the micropyle to the basal end. The two resultant sections of the pericarp were separated using fine forceps to reveal the testa which contained the embryo (Plate 2.1a). The thin, papery testa was removed using surgical eye forceps and a mounted hypodermic needle. Excised embryos, with the attached cotyledons intact (Plate 2.1a) were then immediately placed on the appropriate culture medium (Section 2.2.2.1).

2.2.2 Investigations into the requirements for the culture of excised rose embryos.

Due to a shortage of rose achenes being produced from the crosses described in the previous section, it was not possible to carry out an experiment to examine the combined effects of culture medium and environmental conditions (i.e.. temperature and light intensity) on the germination of excised rose embryos. Instead, two separate assessments were undertaken. The first examined the effect of culture medium composition, in particular, of the choice of basal media type and of growth regulator. Whilst the second examined the rôle of temperature and light intensity on the germination and subsequent development of excised embryos cultured on a medium which had been determined previously to be optimal for germination in the first experiment.

2.2.2.1 The effect of culture medium composition on excised rose embryo germination.

In order to determine the optimum media composition for the germination of rose embryos, excised embryos were placed on the surface of 30ml aliquots of agar solidified (0.8% Sigma; pH 5.8) medium (see Table 2.1 and Appendix I) in 175 ml capacity screw-capped glass jars (5 embryos/jar). Media contained one of four carbohydrates and either lacked growth regulators or contained either BAP or IBA. The carbohydrates were chosen because they had been employed in the embryo

rescue of other species (Stafford and Davies, 1979; Kasten and Kunert, 1991) and, likewise, the growth regulators had both been used in other embryo rescue protocols (Kasten and Kunert, 1991), including those for Rosaceous species (Tukey, 1934). They had also been shown to have an effect on breaking the dormancy of intact rose seed (Jackson and Blundell, 1963). A total of 30 embryos per cross were cultured per medium, which were initially maintained in the dark (for 14 days at 22°C), followed by their transfer to a 16 h photoperiod provided by 35 $\mu Em^{-2}s^{-1}$ daylight fluorescent illumination (Thorn Cool light, Thorn EMI Ltd, UK) at the same temperature. After 70 d, the number of embryos that had germinated was recorded and the percentage germination calculated (Table 2.2). Seedlings resulting from germinated excised embryos were subsequently grown as described in Section 2.2.2.3.

In order to determine if the osmotic potential of the media was having any effect (promotory/inhibitory) on embryo germination, the osmotic potential of a 25µl aliquot of a liquid version of each of the media detailed in Table 2.1 was measured using a Wescor 5500 vapour pressure osmometer (Wescor Inc, South Main Street, Logan, Utah, USA).

Table 2.1. Composition of media used to investigate the requirements for the germination and culture of excised rose embryos.

Medium (Thesis code number)	Basal salt/vitamin mixture	Primary carbohydrate ($mg\ l^{-1}$)	Growth regulator ($mg\ l^{-1}$)
1	AL [1]	5.0 glucose	-
2	AL	5.0 glucose	3.0 BAP
3	AL	5.0 glucose	1.5 IBA
4	AL	20.0 sucrose	-
5	AL	20.0 sucrose	3.0 BAP
6	AL	20.0 sucrose	1.5 IBA
7	MS [2]	5.0 glucose	-
8	MS	5.0 glucose	3.0 BAP
9	MS	5.0 glucose	1.5 IBA
10	MS	20.0 sucrose	-
11	MS	20.0 sucrose	3.0 BAP
12	MS	20.0 sucrose	1.5 IBA
13	MS	10.0 fructose	-
14	MS	10.0 maltose	-

[1] Mineral salt mixture defined by Asen and Larson (1951). [2] Basal salts and vitamins of Murashige and Skoog (1962).

2.2.2.2 Effects of light and temperature on the germination of rescued embryos.

Once the optimal medium for the germination of excised rose embryos had been determined (see Results, Section 2.3.2.1), investigations were carried out to optimise

the environmental conditions required for the germination of excised embryos, and to examine if it would be possible to further increase the percentage germination.

Embryos were excised as described in Section 2.2.1.3 and placed on the medium that gave optimal germination under the conditions described in section 2.2.2.1. Twenty embryos (5 embryos/jar; 4 replicates/treatments) were then subjected to one of five different cultural regimes:

i) Placed immediately in a 16 h photoperiod (35 $\mu Em^{-2}s^{-1}$) at 22°C,

ii) Placed in the dark for 28 d at 4°C, followed by transfer to light (35 $\mu Em^{-2}s^{-1}$) at 22°C,

iii) Placed in the dark for 14 d at 25°C, followed by transfer to light (35 $\mu Em^{-2}s^{-1}$) at 25°C,

iv) Placed in the dark for 14 d at 22°C, followed by transfer to light (75 $\mu Em^{-2}s^{-1}$) at 22°C,

v) Placed in the dark for 14 d at 18°C, followed by transfer to the light (35 $\mu Em^{-2}s^{-1}$) at 18°C.

For all the above treatments (except ii), after 70 d culture, the percentage embryo germination was determined. Comparable data was obtained after 98 d for embryos subjected to treatment (ii), since 28 d at 4°C was not regarded as effective growth conditions, but as a pre-culture (equivalent) stratification treatment. The number of germinated embryos that subsequently developed to a stage at which they required transfer to glasshouse conditions (as described in Section 2.2.2.3) was also noted for each treatment used. Embryos germinated under the conditions described in Section 2.2.2.1 were used as comparative controls.

2.2.2.3 Transfer and acclimation of embryo rescue derived seedlings to glasshouse conditions, and the use of Sorbarods®.

When seedlings, produced from the excised, cultured embryos, reached a height of approx. 5.0 cm (Plate 2.1b), they were considered suitable for transfer to glasshouse conditions for a number of reasons. Specifically, i) they had reached a stage equivalent to that at which seedlings in a conventional breeding programme would be transferred from seed trays to pots, ii) they had reached a size at which they could easily be handled, iii) further growth under *in vitro* conditions would be limited by the size of the culture vessel. Seedlings were removed from the culture vessels and any agar-medium adhering to the roots was washed away with distilled water. Seedlings were planted in 7.5 cm diameter plastic pots, containing equal volumes of Levington M3 and John Innes No 3 composts. The seedlings were initially watered with a 0.5 g l^{-1} solution of Benlate fungicide (ICI Chemicals, Farnham, UK) and later watered when required to ensure that the compost remained moist. Prior to their transfer to the glasshouse, each pot was covered with a 20 x 13 cm polythene bag to maximise relative humidity and to prevent desiccation. Fourteen days after transfer to the glasshouse, one corner of the polythene bag was removed, followed 7 d later, by excision of the other corner, to facilitate a gradual acclimatisation to conditions of lower humidity and temperature. Bags were removed by 35 d after transfer to the glasshouse. Plants were maintained under natural daylight at 23 $\pm 2^{o}$C and were transferred to 13 cm diameter pots as they matured, typically after 8 - 10 weeks. Six weeks after transfer to the glasshouse, the number of seedlings that had survived transfer was recorded. Embryo-derived plants were grown to a flowering stage.

In order to minimise the handling of the delicate seedlings, the possibility, of replacing agar-solidified medium with Sorbarod® plugs (Baumgartner Papiers SA, Lausanne, Switzerland) as a physical-supporting medium was investigated. The latter consisted of cylinders made of laminated cellulose in a sleeve of perforated paper which were then soaked in liquid culture medium. Thirty embryos were obtained as

previously described (Section 2.2.1.3). They were placed on the surface of the Sorbarod® plugs, contained within 175 ml capacity glass jars. Sufficient liquid medium (approx. 25 ml) of the type determined to be optimal (see Sections 2.2.2.1 and 2.3.2.1) was added to saturate the plugs, but in order to maximise aeration, no surplus medium was left remaining in the culture vessel. Cultures were maintained under previously determined (optimal) conditions (see Results, Section 2.3.2.2). After 70 d, the number of germinated embryos was noted. When these seedlings had reached approx. 5 cm in height under *in vitro* conditions, they were removed with the attached Sorbarod® and root system and planted directly into pots of potting compost as previously described (Section 2.2.2.3). Seedlings were placed so that the Sorbarod®/root system was located just below the soil surface. Subsequent culture conditions were identical to those previously employed. Seedling survival was noted 6 weeks post-transfer.

2.2.2.4 Investigations into the failure of conventional achene germination.

Possible physical and/or physiological reasons for the failure of achenes to germinate under normal handling conditions were investigated to determine if it would be possible to enhance germination using techniques other than embryo rescue. This was in an attempt to explain why techniques used by breeders to increase germination were frequently ineffective, and also to reveal why the embryo rescue technique used here proved to be so effective in increasing rose seed germination.

Achenes from the cross HER x LUC, were extracted from the cynarrhodia (section 2.2.1.3) and cultured directly on the optimal medium (see Results, Section 2.3.2.1). A number of modifications were made to the original protocol:

a) Only a small proportion of the pericarp and testa were removed (creating a small hole at the micropylar end). Achenes treated in this manner were then placed with the intact basal end in contact with the medium.

b) The pericarp was removed completely, but the testa left intact, before culture of the achenes.

c) The excised embryos were placed on the surface of the medium, with the excised pericarp and testa lying adjacent to them on the suface of the medium in order to determine if water soluble growth inhibitors were present in the pericarp and/or testa.

d) Excised embryos were placed on water (distilled, deionised) agar-solidified (0.8%w/v Sigma) medium lacking growth regulators, carbohydrates, salts and vitamins. It was hoped that this may have shown if such components were necessary for embryo germination/growth.

The primary aim of these treatments was to determine if the testa and/or pericarp were acting as a physical constraint and/or a source of chemical inhibitors to germination. These assessments might also indicate whether the failure of achene germination was associated primarily with deficiencies in embryo nutrition.

2.2.3 Determination of sexual incompatibility in intercrossed English rose cultivars.

In order to investigate failure of seed set, the industrial collaborator (David Austin), had previously identified several intervarietal crosses, which were known to persistently result in little or no seed set.

The crosses identified were those between the English rose cvs. Abraham Darby (ABAD) and Marie Pavié (MP), and between Mary Rose (MR) and MP. The former cross (ABAD x MP) had failed to produce seed in a large number of crosses carried out over several seasons (D. Austin; pers. comm.), whilst < 10% of the latter cross (MR x MP) produced seed, but only in very small quantities. As the results of these two varietal crosses were atypical of crosses in general between other English rose cultivars, rose breeders assume that some form of incompatibility reaction must be occuring.

2.2.3.1 Assessment of pollen viability.

The viability of pollen was assessed by its ability to germinate *in vitro*. Pollen was collected at 09:00 daily over a period of two weeks from open flowers of the three rose cultivars used in this study, which in turn were grown in a mesh tunnel. Pollen was collected using a camel hair brush, placed into 2.0 cm^3 polypropylene vials (Sarstedt Ltd, Boston Road, Leicester) which were sealed and transported to the laboratory. The vials were vortexed for 30 s in order to separate the adhering pollen grains. Samples of pollen were then removed from each vial using a camel hair brush and placed on the surface of semi-solid pollen germination medium (400 mg l^{-1} H_3BO_3, 226.5 mg l^{-1} $CaCl_2.2H_2O$, 150 g l^{-1} sucrose, 4.0 gl $^{-1}$ Sigma agarose Type 1, pH 5.6 prior to autoclaving) in 5 cm Petri dishes (8.0 ml of medium/dish). Dishes were incubated at 23$\pm$2^{o}C under a 12 h photoperiod provided by daylight fluorescent illumination (35 $\mu Em^{-2}s^{-1}$). After 24 h, the pollen was examined microscopically and the percentage viability was determined by counting the number which had germinated from a sample of 100 grains. Germination was considered to have occured when the length of the emergent pollen tube was in excess of the diameter of the pollen grain. Twenty germination tests were carried out on pollen derived from different flowers of each of the three cultivars over a period of one week.

2.2.3.2 Methods of observing of pollen germination and pollen tube growth on and within stylar tissues.

Reciprocal sexual crosses were made between the cvs. ABAD and MP, and between MR and MP. Crosses were carried out as described in Section 2.2.1.1. Five days after crossing, the greaseproof paper bags, preventing rogue crossings, were removed and the pistils excised using fine forceps and a scalpel. The pistils were immediately fixed in a solution which consisted of formalin: acetic acid: absolute alcohol (1:1:8, v:v:v) for 18 h at 4^{o}C. Following fixation, the pistils were placed in 8N NaOH at 23$\pm$2^{o}C (8 h). Pistills were stained by placing and squashing (using a coverslip) in a drop of 0.1% (w/v) aniline blue in 0.1M K_3PO_4 (pH 12.4) on a microscope slide.

The edges of the coverslip were sealed with petroleum jelly, and the slides placed at 4°C for at least 48 h. The extent of pollen tube growth was observed under ultra-violet fluorescent illumination using a Nikon 'Diaphot TMD' inverted microscope with a high pressure mercury vapour lamp HB100 W/2, fitted with a dichroic mirror DM510 and an absorption filter 570. A B1 FITC exciter filter was used for the visualisation of fluorescing pollen grains and tubes. Approximately 20 pistils per cross were examined. The number of pollen grains on the pellicle (stigmatic surface) was noted, and the percentage of these that had produced a visible pollen tube was determined, along with the extent of pollen tube descent. Photographic records were also made.

2.2.4 Statistical analysis.

Where appropriate, the statistical significance of the various treatments was determined using Duncan's multiple regression test (Duncan, 1975).

2.3 Results.

2.3.1 Conventional germination of rose seed.

Seventy days after sowing, 24% (27 of 50) achenes from the cross (HER x LUC) and 33% (10 of 30) of the achenes resulting from the cross (WB x CHL/MR) had germinated. Achenes that had not germinated at this stage were retained, for a further 35 d, under the same conditions, but no further germination occurred.

2.3.2 Recovery and culture of embryos.

2.3.2.1 Effects of culture media composition on the germination of excised rose embryos.

The effects of basal medium composition/growth regulator/carbon source, expressed in terms of percent germination of excised embryos, are presented in Table 2.2. When germination occured (after 5 -10 d culture), the cotyledons enlarged considerably and developed chlorophyll. The plumule unfolded and the radicle

elongated to form a prominent root. Percentage germination of excised embryos ranged from 43.3 - 100.%. Maximum germination was observed in embryos from the cross (HER x LUC) on Medium 1. In general, on the media tested, germination of excised embryos from the cross (CHL/MR x WB) was lower than those from the cross (HER x LUC). In the majority of cases, the percentage germination of excised embryos was greater than that observed for achenes germinated under soil/glasshouse conditions.

No significant differences were observed between the percentage germination values of embryos cultured on media containing MS salts and vitamins as compared to the use of those of Asen and Larson (1951) (Table 2.2). Likewise, the incorporation of 1.5 mg l^{-1} IBA or 3.0 mg l^{-1} 6-BAP into the media had no significant effect on germination. However, due to a shortage of available achenes, sample size was low and statistical significance of incorporating IBA into the media could not be properly determined. When percentage germination levels of embryos cultured either without growth regulators or in the presence of 3.0 mg l^{-1} 6-BAP alone were examined, significant differences ($p<0.05$) were observed for those media containing glucose as compared to sucrose. Significantly greater ($p<0.05$) germination was also observed in embryos on MS based medium with fructose compared to those with maltose.

Measurements of the osmotic potential of the media showed that media containing a monosaccharide carbohydrate source (ie. glucose or fructose) had a mean osmotic potential of 150 ± 24 mOsmol Kg^{-1}, while media containing disaccharide carbohydrates (ie.maltose or sucrose) had a mean osmotic potential of 350 ± 11 mOsmol Kg^{-1}.

Table 2.2 Germination levels of excised embryos on different media.

Medium (Thesis code number)	Percentage germination	
	CHL/MR x WB	HER x LUC
1	83.3 ae	100.0 uy
2	80.0 ae	83.3 uy
3	76.6	73.3
4	60.0 bf	53.6 vz
5	63.3 bf	46.6 vz
6	60.0	46.6
7	93.3 ce	86.6 wy
8	86.6 ce	90.0 wy
9	80.0	86.6
10	63.3 df	46.6 xz
11	56.6 df	43.3 xz
12	53.6	50.0
13	80.0 e	95.0 y
14	50.0 f	60.0 z

For each sexual cross, percent germination values followed by the same suffix are not significantly different at $p<0.05$ (determined using Duncan's multiple regression test (Duncan, 1975).

2.3.2.2 Effects of light and temperature on the germination of excised rose embryos.

The effects of different light and temperature regimes, described in Section 2.2.2.2, on germination and development of excised rose embryos are shown in Table 2.3. Percentage germination ranged from 55 - 95% for the five treatments. The proportion

of germinated embryos reaching plantlet stage, where they were considered suitable for transfer to the glasshouse (as defined in section 2.2.2.3), ranged from 46 - 100%. Maximal germination was observed when the first stage of embryo culture was in the dark. The placing of excised embryos directly into the light had a deleterious effect, thereby reducing the percentage germination. This treatment also had a deleterious effect on the subsequent growth of the germinated embryos, with only 46-53% reaching the viable plantlet stage for transfer to the glasshouse. Many germinated embryos, subjected to this treatment, developed abnormally, producing an extremely twisted, single prominent root, from which lateral roots did not develop. Similar effects were observed when embryos, germinated initialy in the dark, were subjected to relatively high light intensities [75 $\mu Em^{-2}s^{-1}$; Treatment (iv)]. Such plantlets developed only a small number of leaves, a large proportion of which became chlorotic, eventually browned and resulted in death of the developing seedlings. Temperature was also observed to have a significant effect on the germination and development of the excised embryos (Table 2.3). An increase in temperature from 22°C to 25°C resulted in an increase in the percentage germination of the embryos, but the proportion of embryos reaching the transferable plantlet stage was reduced. A decrease in the temperature to 18°C resulted in a lower percentage germination, but the proportion of embryos-derived plantlets that subsequenty were suitable for transfer to glasshouse conditions remained relatively high. Pre-germination chilling at (4°C) did not increase percentage germination beyond that observed at 18°C or 22°C.

Table 2.3 The effect of light and temperature on the percentage germination of excised rose embryos, and attainment of seedlings for transfer to the glasshouse.

	Intervarietal Cross			
	(CHL/MR x WB)		(HER x LUC)	
Culture Treatment*	Embryo Germination (%)	Transferable Seedlings (%)	Embryo Germination (%)	Transferable Seelings (%)
Control	80	100	95	94
(i)	65	53	75	46
(ii)	75	93	85	94
(iii)	85	65	95	63
(iv)	55	100	70	93
(v)	80	87	90	94

* see Section 2.2.2.2.

2.3.2.3 Transfer of seedlings to *ex vitro* (glasshouse) conditions.

Six weeks after being removed from culture, in excess of 80% of seedlings had survived transfer to *ex vitro* conditions. The use of Sorbarods® did not increase percentage survival *ex vitro* of embryo rescue-derived seedlings. The composition of the medium initially used to culture the excised embryos had no effect on their subsequent survival *ex vitro*. Likewise, light and temperature regimes used in the *in vitro* culture of the embryos appeared to have no long-term effect following *ex vitro* transfer.

The majority of seedlings that ultimately did not survive, died several weeks after removal of the polythene bags from the pots. Such seedlings showed typical signs of fungal infection (i.e. a weft of mycelial growth on softer tissues). The most likely cause of this was thought to be the damping-off pathogen *Pythium ultimum* (J. Lucas;

pers. comm.). With both crosses, plants resulting from embryo rescue flowered approximately 84 d after transfer to the glasshouse.

Of particular interest in this study, were a number of intervarietal F_1 hybrids produced by embryo rescue, which upon flowering, exhibited combined floral characteristics of the two parents. This was frequently observed as the recovered progeny displaying the flower shape of one parent with the flower colour of the other (Plate 2.2a - d). Most commercially exploited rose cultivars result from a lengthy development process that includes repeated crossings, combined with the testing of progeny for desirable characteristics such as floral attributes and disease resistance. It is highly unlikely that the hybrid plants produced in this study would be suitable for direct market release but that, as in a conventional situation, further crossing would lead to a cultivar suitable for release to the market.

2.3.3 Investigations into the failure of conventional achene germination.

Incomplete excision of the embryo, where only a proportion of the pericarp and testa were removed or where the testa remained intact [treatments a) and b) in Section 2.2.2.4], resulted in a decreased percentage germination (20% and 26% respectively), as compared to those embryos in which the pericarp and testa had been completely removed (see Table 2.2). A lower percentage germination (30%) was observed when embryos were cultured in the presence of the removed pericarp and testa [Treatment c)]. Fully isolated embryos, cultured on water-agar [treatment d)] also exhibited a reduced percentage germination (37%) compared to those on media containing mineral salts and a carbohydrate source.

2.3.4 Possible causes of sexual incompatibility in English Rose crosses.

2.3.4.1 Assessment of pollen viability.

Percentage pollen viability, as determined on artificial medium (see Section 2.2.3.1), was as follows:

Parent Cultivar	Pollen Viability (%±S.D).
Abraham Darby (ABAD)	23.1 ±7.3
Mary Rose (MR)	22.3 ±9.6
Marie Pavié (MP)	25.9 ±4.5

2.3.4.2 Observation of pollen germination and growth on/within the style.

The percentage pollen germination on the pellicle ranged from 14 - 21% (see Table 2.4). This level of germination was generally comparable to that obtained on the synthetic germination media (see Section 2.3.4.1). In the crosses carried out in this study, pollen tube growth was independent of genotypes and direction of the cross. Extensive pollen tube abortion was observed either on the pellicle or within 3 mm of the pellicle (Plate 2.1d); pollen tubes were also aborted along the entire length of the style. Aborted pollen tubes grew erratically, with malformed and twisted/spiralling tubes (Plate 2.1e) often accompanied by callose deposition, and sometimes terminating in a swollen tip. Between 2 - 8% of descending pollen tubes reached the ovarian tissues (see Table 2.4). However, even at this late stage a number were seen to abort, and it was not possible to accurately determine if any of the pollen tubes had actually entered the nucellus via the micropyle to affect fertilisation.

Table 2.4 Assessment of pollen tube development in the stylar tissues.

Cultivar Female Parent (Pollen Acceptor)	Cultivar Male Parent (Pollen Donor)	Pollen germination (pellicle) (%)	Pollen tubes reaching the micropylar region (%)
Abraham Darby	Marie Pavié	19	5
Marie Pavié	Abraham Darby	14	4
Mary Rose	Marie Pavié	21	2
Marie Pavié	Mary Rose	17	8

Plate 2.1.

a. A= Intact rose achene, resulting from cross HER x LUC, harvested 45 days post pollination, B=Achene with pericarp removed, C=Excised rose embryo of HER x LUC (x 10).

b. Seedling derived from rescued embryo of HER x LUC, ready for transfer to glasshouse conditions (x 2).

c. Embryo rescue derived plant resulting from HER x LUC cross (x 0.15).

d. Abortion of developing Abraham Darby pollen tubes on the pellicle of cv. Marie Pavié observed 5 d post pollination (x 30).

e. Aborting, spiralling Abraham Darby pollen tubes (arrowed) in the stylar canal of cv. Marie Pavié 5 d post pollination (x 40).

Plate 2.1 is blank in original thesis

2.4 Discussion.

2.4.1 Embryo rescue.

The embryo rescue technique described here was efficient, in that embryo germination was doubled and, in some cases gave 100% conversion of embryos to F_1 hybrid progeny. Germination, using both embryo rescue and conventional approaches, varied between the two crosses of English rose used in this study, but embryo rescue allows inherent differences to be maximised. This, in turn, suggests that embryo rescue, as developed here for rose, will firstly increase the total number of plants obtainable from a specific sexual cross, and will inevitably increase the possibility of a given cross giving rise to plants suitable either for direct marketing, or for further refinement in a breeding programme.

The low germination level for excised embryos on water-agar medium suggested that mineral salts and a carbon source are essential if percentage germination is to be enhanced beyond that observed for achenes germinated using conventional methods. This could be attributed to the possibility that some potentially viable embryos lack the ability to utilise and/or metabolise nutrients within the endosperm and hence fail to germinate. In normal seeds, there are amounts of soluble substrates that are normally present in the form of raffinose; these are acted upon by amylolytic and lipolytic enzymes producing proteins initially utilised for axis extension (Wilkins, 1984). However, it is possible that the seeds used in this study lack such a substrate, and that the inclusion of carbohydrates in the artificial medium provides otherwise lacking food reserves for the developing embryo.

The lack of any significant difference between the germination of excised embryos on MS based medium as compared to media containing the salts of Asen and Larson (1951), indicated that the provision of a complex formulation of vitamins and mineral salts, such as provided in the former, is not essential for maximum

germination. However, the commercial availability of MS based medium makes such a formulation a more logical choice for general usage.

Despite the growth regulator BAP having been shown to be implicated in the breaking of dormancy in seeds of *R. arvensis* (Jackson, 1963), there were no significant differences between germination on media with or without 3.0 mg l^{-1} BAP. This may be because BAP is thought to affect the balance between endogenous growth promoters and inhibitors that normally prevent germination from occurring until the pericarp is weakened by decay (Jackson, 1963). By physically removing the pericarp, this balance may be instantly disrupted or negated thus, effectively eliminating any dormancy requirement. Therefore BAP would have no rôle in promoting germination, as removal of the pericarp inevitably tips the balance towards that of promoting embryo/seedling growth. Likewise, the results indicated that IBA also failed to enhance germination, possibly for identical physiological reasons. The high levels of embryo germination observed here would seem to indicate that growth regulators are not essential for obtaining maximal germination of excised rose embryos. Previous reports on embryo rescue in Rosaceous species are restricted to those published in the 1930s, at which time plant growth regulators were not being synthesised commercially. It would, however, appear that it was possible to achieve relatively high levels of embryo germination in Rosaceous species such as sweet cherry (Tukey, 1934), peach, apple and pear (Tukey, 1938) despite synthetic growth regulators not being available. These studies, along with the present one on English rose, contrast with observations of embryo rescue in the genera *Manihot*, *Lupinus* and *Gossypium* , where the presence of one or more growth regulators was required for the efficient germination of excised embryos (Biggs *et al.*, 1986; Kasten and Kunert, 1991; Liu *et al.*, 1992 respectively).

In this study, significant differences were observed in an effective, higher germination level of embryos on those media containing a monosaccharide sugar (glucose or

fructose), as compared to a disaccharide sugar (maltose or sucrose), independent of other media components. This suggested that monosaccharides could be more readily metabolised by the excised rose embryos. This may be due to these rose embryos lacking the relatively more complex pathways which are required to metabolise disaccharides (Stryer, 1988). It may be possible that, at an early embryo stage, these rose cultivars have sorbitol rather than sucrose translocated in the phloem, as is comon in many Rosaceous species (Salisbury and Ross, 1985). As sorbitol is produced by the enzymatic reduction of monosaccharide sugars such as glucose (Salisbury and Ross, 1985), this may also explain why embryo germination/growth is maximal in the presence of such compounds.

The choice of carbohydrate can also affect the osmolarity of the medium. In this study, the maximal germination of excised embryos was observed on medium containing monosaccharides, with a relatively low osmotic potential. Such observations contrast with those of Kasten and Kunert (1991), who noted maximal germination of *Lupinus* embryos in medium with a much higher osmotic potential (400 mOsmol Kg^{-1}). Similar results were obtained by Stafford and Davies (1979), with the culture of immature embryos of *Pisum sativum.* In the former example, it has been suggested that growth of excised embryos of *Lupinus* is stimulated by a culture medium of high osmotic potential (Kasten and Kunert, 1991). Protocols for the embryo rescue of sweet cherry (Tukey, 1934), peach, apple and pear (Tukey, 1938) all employ media containing sucrose. However, there are no previous reports investigating the rôle of carbohydrate type on the germination of embryos from Rosacous species. Indications are that the choice of carbohydrate, for use with embryo rescue in rose, is more dependent on the ability of the embryo to effectively utilise the carbohydrate source rather than on the effects of the carbohydrate on the osmotic potential of the medium.

This study has also shown that light and temperature regimes have a major effect on the germination of isolated rose embryos, and their subsequent development. Many protocols require the placement of rescued embryos directly into light conditions, such as those with *Mannihot esculentum* (Biggs *et al.*, 1986). Such a procedure has been shown, in this study, to be deleterious to both the germination and to the subsequent development of the embryo. The cause of this is unknown. It could be that the light harvesting systems of the plant are undeveloped at this early stage, and that light retards growth and leads to permanent damage, as has been observed in lettuce (Stuart and Jones, 1977). In rose, seeds are usually planted below the surface of a growth substrate such as sand or compost. Initial germination and growth (for the first few days) therefore, takes place in darkness. A germination procedure such as used in the controls and treatments (ii) - (v) therefore mimics the light regime that would normally be encountered in a natural situation, and would hence appear to favour effective germination of embryos and the development of seedlings derived from these. The higher light intensities employed in this study are probably much greater than those which would be encountered in a natural situation, as rose seed germination would typically take place in early spring when maximum light intensities remain relatively low. In this study, deleterious effects are observed when embryos cultured in the dark for two weeks are placed into very high levels of illumination. This again could be caused by the photosystem of the developing plants being permanently damaged by the very high number of photons that would be being received. Such an effect is known as solarisation, in which a light-dependent inhibition of photosynthesis occurs followed by oxygen-dependent bleaching of chloroplast pigments (Salisbury and Ross, 1985). Exposure of plant material to high light intensities can also lead to the evolution of high levels of free radicals which will also lead to permant tissue damage (Stryer, 1988).

Long-term effects of temperature were restricted to those embryos that had been subjected to temperatures of 25°C. At this temperature, the actual percentage of

embryos germinating was greater than at 22°C. However, the seedlings that subsequently developed at the higher temperature were malformed and the percentage of germinated embryos that effectively translocated to plants in the glasshouse was reduced in comparison to those raised at 22°C. When embryos were cultured at 18°C, the percentage germination was again reduced. However, a large proportion of the germinated embryos developed to the stage by which they could be successsfully transferred to the glasshouse. The observed effects may be attributed to the effects of temperature on the plants enzyme systems. All plants have optimal temperatures for germination and growth which are linked to enzymatic reactions within the plant tissues. As temperatures increase the rate of the enzymatic reations will increase up to a point, beyond which the enzymes become denatured as hydrogen bonds in the enzymes become irreversiby broken (Salisbury and Ross, 1985). Such effects are reflected on a whole plant basis, in that exposure to higher temperatures will lead to an increased initial growth rate, which will become reduced as the enzymes become denatured. Such an effect is irreversible. At temperatures lower than the optimal, the action of the enzymes slows but denaturation rarely occurs (Stryer, 1988). This would typically lead to a reduction in plant growth rate. However, the effect of low temperatures can be regarded as reversible, in that when the optimal temperature is restored the enzyme returns to functioning in an optimal manner and normal growth of the plant is resumed. The results with rose, indicated that the optimal temperature for the germination/growth of rose embryos is 22°C and that temperatures above or below this can lead to deleterious effects and plant loss.

Exposing the rescued embryo to low temperatures (4°C) prior to culture at 22°C also failed to enhance germination. This stratification treatment was attempted because for many years low temperatures have been known to contribute to breaking the dormancy of intact rose seeds (Rowley, 1956; Jackson, 1963). Again, this effect would seem to be related to the presence of the pericarp, assisting either in the physical weakening of this barrier to germination, or it may have an effect on the

chemical inhibitors to germination contained within the pericarp. This would appear to be confirmed by the observation that when the pericarp and testa are removed, low temperature stratification treatment has no beneficial effect in increasing percentage germination. Naturally-occuring chemical gemination inhibitors have beeen known to exist for some considerable period of time. The inhibitors may be specific, involved in growth regulation such as abscisic acid (ABA), or non-specific such as phenolic acids (Wilkins, 1984). These compounds inhibit certain enzymes that are associated with germination such as endo-β-mannanase (Halmer *et al.*, 1976). ABA can also affect RNA metabolism (Walbot *et al.*, 1975). The reduction in rose embryo germination in the presence of the excised pericarp/testa strongly suggested that the pericarp and/or testa contained a diffusable substance(s) such as ABA/phenolic acids, which suppressed embryo germination. In the case of intact achenes, this could be transported directly to the embryo, so as to inhibit germination. These results are in general agreement with those of Jackson and Blundell (1963) who showed that aqueous extracts from achenes inhibited the germination of *Rosa arvensis*.

When only part of the pericarp and testa was removed from the embryo, the percentage germination was reduced, confirming that, in rose, the pericarp/testa are not simply acting as a physical barrier to germination, but are implicated in inhibitory growth regulator synthesis. If this were not the case, then it would be expected that a larger percentage of the achenes would germinate simply when an apperture was created in the pericarp/testa. Similar results were obtained when the entire pericarp was removed, but the testa left intact, thus confirming that the inherently low percentage germination of achenes is not solely regulated by the physical presence of the pericarp/testa. These observations further confirm why traditional methods of enhancing germination, such as acid treatment (Popcov and Buc, 1967) and scarification (Tincker, 1935), which thin or partially remove part of the pericarp, are very ineffective when compared to embryo rescue as developed here for English rose.

The general findings in this study indicated that germination observed with achenes of English rose achenes using conventional techniques, is regulated by a combination of factors, including physical restriction by the pericarp and/or the testa and by the presence of germination inhibitors within those tissues. Such observations are in line with studies of other cultivars and species of *Rosa*, such as *R. arvensis* (Jackson and Blundell, 1963). These workers concluded that with *R. arvensis* the physical weakening of the pericarp, by decay, is a prerequisite for germination, and that a function of the germination inhibitors found in the pericarp is to prevent the initiation of embryo germination until this weakening has occurred.

A relatively large proportion of embryo rescue-derived rose hybrids survived transfer to the glasshouse, and subsequently matured to flowering. It was not possible to conclusively determine whether losses due to fungal infection were via opportunistic infections of the plantlets weakened due to physical damage occurring simply as a result of handling during transfer. Losses could also be attributed directly to infection by one or more fungal pathogens (irrespective of any physical damage) . The latter would seem more likely since the use of Sorbarods®, which eliminated handling, did not result in any detectable increase in percentage seedling survival. This contrasts with the reports of Lloyd *et al.* (1988), who showed that Sorbarod® plugs increased the percentage survival of the species hybrid *R. persica* x *xanthia* upon transfer from *in vitro* conditions to the glasshouse. The reduction in mortality was dually attributable to the physical protection given to the root system, and water availability provided by the cellulose matrix. If fungal infections predominate then seedling survival may be enhanced by the use of broad spectrum fungicides such as Triadimenol, other than or in addition to Benomyl, at the transfer stage. Although, approximately 20% of the embryo-derived hybrid plants did not survive, this was not much greater than for seedlings derived from conventionally germinated seed (D. Austin per. comm).

It should now be possible, using the simple procedure developed here, to target specific rose crosses, where the products of such a cross exhibit low (or zero) levels of germination and where F_1 hybrid production is essential, and to employ embryo rescue to produce plants either for direct propagation and sale, or for incorporation into a breeding programme.

2.4.2 Possible causes of sexual incompatibility.

The viability of rose pollen has been shown to be highly variable. Pearson and Harney (1984), for example, compared the viability of fresh pollen (estimated by percent germination) collected from six *Rosa* species (*Rosa spinosissima* L., *R. fedtschenkoana* Regel, *R. pedulina* L., *R. arkansana* Porter, *R. carolina* L., *R. virginiana* Miller), one botanical variety (*R. spinosissima altaica* Rehd.) and three cultivars (Betty Bland, George Will, Prarie Princess). Significant differences were reported between the genotypes, with germination ranging from 1.7 - 69.2%. The pollen viability of the English rose cultivars investigated in this study appeared to be towards the lower end of the range as identified by Pearson and Harney (1984). Pollen viability, as determined by *in vitro* germination assays, has been correlated to fertilisation success in other species (Hicks *et al.*, 1987). Low pollen viability, as observed for English rose, will inevitably reduce the chances of effective fertilisation occurring in the crosses under investigation in this study.

The precise reasons for pollen failing to germinate (both *in vivo* and *in vitro*) have not been determined. Guidin and Arene (1991) identified pH of the stigmatic exudate as having an effect on rose pollen germination *in vivo*. In artificial media, germination of *R. hybrida* was maximal at pH 5.0 (minimal at pH 3.0 and pH 9.0). In this study of English rose, the fact that percentage pollen germination was similar on both the pellicle and on an artificial medium (pH of 5.6) would seem to indicate that pH of the stigmatic exudate is not the key factor regulating pollen germination.

A number of other factors have been identified as influencing rose pollen viability. These have included temperature, hygrometry (Guidin *et al.*, 1991a) and seasonal effects (Guidin *et al.*, 1991b). The small variation detected in pollen viability of pollen for English rose when collected over a four month period (where these factors would vary) , suggested that seasonal factors are not relevant here. Other factors are also known to influence the viability of pollen. Adverse plant growth conditions (including temperature deviations and nutrient deficiencies) during the time of microsporogenesis can lead to pollen being structurally malformed, which prevents its rehydration on the pellicle (Heslop-Harrison, 1992a). In apple, the production of viable pollen has been shown to be reduced due to boron deficiency, though the exact reason why this lead to a reduction in pollen viability was not determined (Heslop-Harrison, 1992a). Viable pollen contains high levels of reserves to provide a source of energy for the growth of the pollen tube. Typically, pollen will contain enlarged endoplasmic reticulum which forms pockets lined with lipid bodies and filled with dictyosome vessels (Jensen and Wetzel, 1992). The mechanisms by which these reserves are produced are presently being investigated in species such as cotton (Jensen and Wetzel, 1992). However, it is possible that if these mechanisms are disrupted then the pollen will be inviable, as it will fail to contain sufficient food reserves to fuel pollen tube development.

Incompatibility occurs because of a failure of the pollen tube to totally penetrate the stigma, or owing to inhibition of pollen tube growth within the stigma and style as a result of a genetically determined interaction between a pollen tube and stigma-style product.

Of the English rose pollen grains that germinate on the pellicle, only a small proportion could actually effect fertilisation, since the growth of the majority was arrested during their passage down the style canal. In their study of pollen tube arrest in *Rhododendron*, Williams *et al.* (1982) identified a total of seven discrete stages at

which pollen tube growth was halted. Stages 1, 2 and 3 were all observed in the rose crosses in this study, namely, failure to pollen to germinate, pollen tube abortion on the pellicle and early abortion of pollen tubes within the stylar canal. It was not possible to determine if other stages of pollen tube arrest, such as failure to enter the ovary or failure to enter the micropyle were also operating.

Specific studies into the mechanism of the sexual incompatibility reaction in rose have not been undertaken. Other studies have demonstrated that pollen tubes growing on the stigma and within the style secrete enzymes which soften the cutin of the stigma and the middle lamella of the cell walls (thereby facilitating pollen tube penetration), and also produce auxin (or a factor promoting auxin synthesis) involved in the initiation of fruit development (Street and Öpik, 1984). It is necessary for the pollen to be supplied with sugars and also cations such as calcium in order for it to achieve sufficient growth to reach the ovarian tissues (Street and Öpik, 1984). There is evidence that all these factors are usually provided by the stigma and style tissues. In addition other factors, such as gibberellins may also be required for effective tube growth (Street and Öpik, 1984). It is possible that in English roses, the failure to produce one or more of these factors in the stylar tissues is also resulting in the arrest of pollen tube growth. However, it is known that the English rose cultivars used in this study can be effectively pollinated by other English rose cultivars. This would seem to suggest that the lack of these factors is not the sole cause of incompatibility and that active incompatibility mechanisms may be operating (either additionally or in isolation) to prevent fertilisation occuring in the crosses in this study.

In sporophytic incompatibility systems, pollen tube arrest occurs on the surface of the stigmatic papillae and is accompanied by deposits of callose both in the tip of the emerging pollen tube and on the papillae surface in contact with the pollen (Mau *et al.*, 1992). Although some callose deposition has been observed in the English rose crosses here, if an incompatibility reaction were occurring at the pellicle, then the

percentage germination of pollen on artificial medium would be expected to be greater. As was is not the case, these results suggested that an incompatibility reaction did not occur at the pellicle level.

Features of the incompatibility reaction observed in the English rose crosses in this study, such as the arrest of pollen tube growth within the stylar canal, resemble those associated with gametophytic self-incompatibility (Mau *et al.*, 1992). This type of incompatibility involves the interaction of a product of the haploid genome of the male gametophyte (carried within the pollen grain) and a product of the diploid tissue of the sporophyte, the pistil (Mau *et al.*, 1992; Thompson and Kirch, 1992). Since materials carried on the surface of pollen grains are dispersed in the liquid, the theory that gametophytic self-incompatibility is operating is further supported by the presence, in these English rose cultivars, of wet stigmata. Such stigmata are associated only with gametophytic self-incompatibility systems in which the pollen genotype itself determines the outcome of the reaction (Heslop-Harrison, 1992b; Thompson and Kirch, 1992). Although the English rose crosses investigated here were between different cultivars, it is likely that the close genetic similarity between those cultivars has lead to a self-incompatibility reaction arising. Because it is difficult to ascertain the exact breeding history of many of the English rose cultivars, including those in this study (D. Austin; pers. comm.), it would prove virtually impossible to illustrate if this fact is correct, especially in the older cultivars. Attempts to mimic the cytological abnormalities seen in incompatible pollen tube reactions, by the treatment of pollen tubes in compatible reactions with chemicals, such as gibberellins, known to affect cell wall formation or carbohydrate metabolism (Street and Öpik, 1984), may give further information on the nature of controls exerted by the pistil on the growth of pollen tubes.

The low initial viability of pollen, combined with the arrest of a large proportion of the pollen tubes that do develop, means that the chances of effective fertilisation

occurring in intervarietal crosses, such as those studied here, are minimal. A number of strategies could be employed to overcome these problems, including pre-germination of pollen prior to its placement on the stigma, the decapitation of the style prior to pollination and application of auxins to the pedicel. However, several of these have been attempted by rose breeders without success (D. Austin; pers. comm.). Somatic hybridisation (protoplast fusion) would therefore appear a more desirable alternative option for the production of many possible English rose hybrids.

2.5 Summary.

i) Embryo rescue has been demonstrated to increase the conversion of embryos to F_1 hybrid progeny, in English rose, when compared to conventional germination methods. The use of complex salt and vitamin formulations was not essential either for effective embryo germination or for the subsequent growth of plantlets. The use of BAP and IBA failed to increase embryo germination. Choice of carbohydrate and growth conditions were found to markedly affect percentage embryo germination and subsequent plantlet development.

ii) *In vitro* germination assays demonstrated that the cultivars of English rose employed in this study have pollen of a low viability when compared to other rose cultivars/species in other studies.

iii) A pollen-style incompatability mechanism was shown to be operating. This resulted in the arrest of pollen tube growth within the stylar tissues. Features of the incompabability reaction indicated that it was probably of the gametophytic self incompatibility type.

Plate 2.2.

a. Flower of parent English rose cv. CHL/MR (x 0.6).

b. Flower of parent English rose cv. Wife of Bath (x 0.6).

c, d. Flowers of plants derived from embryos rescued after crossing CHL/MR with Wife of Bath (x 0.6).


a
b
c
d

CHAPTER 3.
Cryopreservation of rose pollen.

3.1 Introduction.

As discussed in Section 2.1, it is essential for the rose breeder to be able to consistently produce new cultivars in order to maintain market share. Hence, ongoing breeding programmes are necessary, in which the long-term storage of viable pollen would be desirable in order to enable breeding accessions, which flower at different times and/or are spatially separated, to be routinely crossed (Luza and Polito, 1988). This is particularly important with woody species such as apple and cherry, in which late flowering cultivars are often used to pollinate early flowering cultivars (Towill, 1985). The ability to maintain pollen in a viable condition is one way of guaranteeing that pollen will be readily available when stigmatic receptivity of the female parent is maximal (Lee *et al.*, 1985). The long-term storage of pollen would also safeguard against any failure in the production of pollen in subsequent seasons, due to the effects of pests, diseases or adverse environmental conditions. In addition, long-term pollen storage may also be of value in supplementing germplasm preservation strategies (Towill, 1985). Clonal storage would continue to provide the main preservation option, but pollen banks may also be desirable (Harrington, 1970).

Before any pollen storage technique can be assessed, it is essential that an efficient method for determining pollen viability before, during, and after storage, is developed. There are a number of criteria that must be fulfilled if a method of storing pollen over a prolonged period of time is to be effective. Firstly, the pollen must be stored in such a way that its viability and quality is maintained at or near the levels encountered prior to its storage. Secondly, the storage system must be reliable and not subject to mechanical or electrical failure, and should be cost effective and easy to operate.

Conventional techniques for storing pollen have involved collecting the pollen from dehiscing anthers and placing it directly in low temperature storage, either in a refrigerator at 4°C or in a freezer at -20°C. In several cases, this has been accompanied by storage over silica gel (Towill 1985). Fertilisation success and seed set has been related to both pollen viability and quality (Hicks *et al.*, 1987; Struik *et al.*, 1986). Techniques for storing pollen, such as refrigeration or freezing, although widely employed in rose breeding programmes, have been shown to be ineffective, as pollen viability can decline rapidly over a period of just a few months (Khosh-Khui *et al.*, 1976).

An alternative technique is the storage of pollen at ultra-low temperatures, in liquid nitrogen at -196°C. Two of the earliest reports that pollen could survive ultra-low temperatures were those of Knowlton (1922), who observed that *Antirrhinum* pollen germinated after exposure to liquid oxygen (-180°C), and Bredemann *et al.*, (1947) who found *Lupinus* pollen to be viable after three months storage in liquid air (-192°C). Since that time, cryopreservation has been employed for the storage of pollen from a number of plant species (Bowes, 1990), including members of the genus *Prunus* (Parfitt and Almehdi, 1984). In the latter study, *in vivo* germination of pollen recovered from cryogenic storage was demonstrated, although seed set was not reported. In his review of low temperature and freeze-/vacuum-drying preservation of pollen, Towill (1985) indicated that many reports show considerable variation in the viability of pollen tested by germination after cryopreservation. Such investigations often fail to explore parameters to determine whether higher percentages of pollen survival could have been achieved, or to evaluate the possible causes of injury in cryopreserved pollen. A number of factors, such as plant genotype (Towill, 1981; Ganeshan and Alexander 1991) and water content of pollen (Barnabas, 1984), have been suggested as affecting the viability of pollen which was subjected to cryogenic storage. Barnabas and Rajki (1976) showed that drying prior to cryopreservation was

important to aid the storage of maize pollen, but that excessive drying could adversely affect the recovery of viable pollen grains.

In this study, the potential for the cryopreservation of pollen from two cultivars of English rose has been investigated. The ability to store and to maintain the viability of English rose pollen over a prolonged period of time is highly desirable, because the future improvement of English roses will involve crosses between English rose cultivars and their ancestral species/cultivars, many of which flower only once annually. Cryopreservation as a method for the long-term storage of rose pollen is assessed, and compared to other conventional storage techniques at 4°C and -20°C. A number of factors which could affect the efficacy of cryopreservation as a method of storing English rose pollen are also examined, including rate of cooling and pre-cryopreservation drying treatments.

3.2 Materials and Methods.

3.2.1 Assessment of different methods for determining pollen viability.

Pollen was routinely collected, using a camel hair brush, in the early morning (before 08:00), from the semi-open flowers on glasshouse-grown plants of the English rose cv. Heritage. Pollen from 15 flowers was combined and subjected to one of three tests, each test being replicated five times;

i) A drop of acetocarmine solution (Marks, 1952; 4% [w/v] carmine in 50% glacial acetic acid) was placed on a microscope slide and pollen was scattered over the liquid surface. A cover slip was applied and the slide warmed gently over a flame. Slides were examined under bright field using a Nikon "Diaphot TMD" inverted microscope. One hundred grains were examined on each slide, and those which were fully rounded and deeply stained red were deemed viable. Those which showed no staining, or which were only very faintly stained were assumed to be non-viable. The viability of five hundred grains from five different slides was determined, and the

mean number of viable pollen grains was calculated as a percentage of the total pollen population.

ii) A drop of Gram's iodine was placed on a microscope slide and pollen was scattered onto the drop (Jeffries, 1977), which was then covered with a coverslip and after 10 min was examined under the microscope, as detailed in (i). The assessment of viability followed the previous procedure, with pollen which had become deeply stained being regarded as viable, while that lacking stain was assumed to be non-viable.

iii) Samples of pollen were placed on the surface of modified Gudin and Arene's (1991) semi-solid pollen grain germination medium (Chapter 2, Section 2.2.3.1), in 5.5cm Petri dishes (8.0 ml of medium/dish). The dishes were incubated at $23\pm2^{o}C$ under continuous fluorescent illumination (7.0 $\mu Em^{-2}s^{-1}$). After 24 h, the pollen grains were examined microscopically and the percentage viability determined by counting the number of pollen grains which had germinated from a minimum number of 1000 grains. Germination was defined as having occurred when the length of the pollen tube was greater than the diameter of the pollen grain (Plate 3.1a).

3.2.2 Collection and dehiscence of anthers: the effects of dehiscence treatment and degree of flower opening on pre-storage pollen viability.

The English rose cvs. Heritage and The Countryman were used in this study. Anthers were collected between 07:30-08:30, from bushes grown in a mesh tunnel house, from flowers at 3 defined stages of development. Specifically, (i) closed bud, in which the calyx had separated, but the petals remained closed, (ii) semi-open, where the flower bud had reached its final and true varietal colour, and several petals of the outer whorl were slightly detached from the rest of the bud (Guidin *et al.*, 1991a), and (iii) fully open, where expansion of the petals was complete and the anthers and style were exposed. Detached anthers, from individual flowers, were sealed in 2.0 cm^3

polypropylene vials (Starstedt Ltd, Boston Road, Leicester; 12 anthers/vial) and transported to the laboratory. After approximately 1 h the vials were opened and maintained at 25±2°C, with a relative humidity of 30% under a 12 h photoperiod provided by cool daylight fluorescent illumination (7.0 $\mu Em^{-2}s^{-1}$) for 48 h, which caused the anthers to dehisce. Subsequently, half of the vials were vortexed in order to separate the pollen from the anthers, after which the anthers were removed. Using a camel hair brush, small samples of pollen were then removed from each of the vials and subjected to a germination test as detailed in Section 3.2.1. The remaining vials were immediately placed in a dessicator containing silica gel (200 g per 30 vials) for 24 h, under the same temperature and light conditions. Subsequently, each vial was vortexed to separate the pollen from the anthers, which were then removed. To assess viability of collected pollen, small samples were subjected to germination tests as previously described [Section 3.2.1, iii)]. For each treatment the percentage viability was determined from a sample of 100 grains. Mean viability and the standard error of ten replicates were calculated for each treatment. Pollen remaining in the vials, after use for the germination tests, was retained for subsequent cryopreservation.

3.2.3 Cryopreservation of rose pollen.

Vials containing pollen were re-sealed and mounted on aluminium canes prior to cryopreservation. Three methods of cryopreservation were evaluated, each treatment having 10 replicates. Specifically:

i) Rapid freezing, where the samples were plunged directly into liquid nitrogen (-196°C).

ii) Controlled-rate freezing, where the samples were initially cooled to -40°C at the rate of -1°C/min in a controlled-rate freezer (Planer Cryo 10 series, Planer Biomed, Sunbury-on-Thames, Middlesex, TW16 7HD, UK). The samples were then maintained at -40°C for 10 min, prior to plunging into liquid nitrogen.

iii) Controlled-rate freezing, where the samples were initially cooled to -130°C at the rate of -1°C/min in a controlled-rate freezer, maintained at -130°C for 10 min prior to plunging in liquid nitrogen.

All samples were maintained in liquid nitrogen in an XC47/11 low temperature canister storage container (Minnesota Valley Engineering, New Prague, Minnesota 56071, USA) for a minimum period of 48 h. The pollen was thawed by placing the sealed vials in 30 ml of sterile water at +45°C for 2 min, after which the pollen grains were removed and viability was assessed using the *in vitro* germination technique (Chapter 2, Section 2.2.3.1).

3.2.4 Morphological examination of pollen prior to and post-cryopreservation.

Electron microscopy was used to determine the gross morphological characteristics of pollen, collected from semi-open flowers, that had either been dehisced without silica gel, or that had been recovered from 48 h cryopreservation following Method i).

Pollen grains were mounted on 2.5 cm diameter aluminium stubs using double-sided tape (3M Corporation, Massachusetts, USA) and sputter-coated with gold in a nitrogen-argon atmosphere (25mA, 10Pa, 90 sec) using a Poloron E5100 sputter coating unit. Specimens were examined using a JEOL-JSM 840 Scanning Electron Microscope at an accelerating voltage of 20 kV. Images were recorded photographically (see Plate 3.1b, c and d) on Agfa Apex 100 film (Agfa, Levercunsen, Germany). Measurements of the polar length, equatorial axis and surface aperture diameters of 50 grains for each sample, were taken from the photographic records, and mean values calculated. A number of grains showed twisting along the polar axis (Plate 3.1d). A note was made of the number of grains in each sample that exhibited this abnormal morphology.

3.2.5 Use of cryopreserved pollen to effect fertilisation of rose flowers.

The ability of pollen grains, cryopreserved using Method i), to effect fertilisation was assessed and compared with non-cryopreserved pollen. Pollen grains of the cvs. Heritage and The Countryman were used in reciprocal cross pollinations of plants grown under natural conditions in a mesh tunnel. This was achieved by emasculating between five and ten flowers per cross and immediately applying cryopreserved pollen directly from the storage vial onto the stylar surface, using a camel hair brush, such that the whole of the stylar surface was covered with pollen. The flowers were enclosed in a paper bag for one week after pollination to prevent insect cross pollination. For the purposes of comparison, crosses were performed in an identical manner using non-cryopreserved pollen grains, taken directly from flowers. After 6-8 weeks, the formation of cynarrhodia was noted and the number of seeds in each recorded.

3.2.6 Efficacy of pollen cryopreservation in comparison to other techniques.

The efficacy of pollen cryopreservation as a long-term storage method for rose pollen grains, was compared to storage at +4°C and -20°C. In excess of 1000, randomly selected, anthers were collected from flowers of cv. Heritage. These were dehisced for 48 h (without the use of silica), as previously described (see Section 3.2.2), after which the pollen grains were separated from the anthers by vortexing, and were combined so as to minimise any effects of inter-floral variation. Collected, mixed, pollen grains were then subdivided equally into 540 2.0 cm^3 polypropylene vials. Each vial was placed under one of three storage conditions (+4°C or -20°C, in a dessicator containing silica gel (10g/vial) or at -196°C using the rapid freezing method described previously (Section 3.2.3, i). The viability of three randomly selected samples (vials) of pollen from each of the three treatments was assessed by *in vitro* germination (Chapter 2, Section 2.2.3.1) at weekly intervals, over a period of 8 weeks.

3.2.7 Statistical analyses.

Where appropriate, the percent decline (change) in pollen grain viability was calculated based on the pretreatment viability. The statistical significance of the various treatments was determined using Duncan's multiple range test (Duncan, 1975).

3.3 Results.

3.3.1 Methods for assessing pollen viability.

The three tests described in Section 3.2.1 gave the following values for percent viability from the total population of pollen from the cv. Heritage:

Assessment Method	Mean Viability (%)	Standard Error (±)
i) Acetocarmine	23	12
ii) Gram's iodine	18	9
iii) Germination	29	4

Values for percent viability are the means of five replicates.

3.3.2 Effects of dehiscing treatments and degree of flower opening on pre-storage rose pollen viability.

The effects of the various dehiscing treatments and of the degree of flower opening on the pre-storage viability of rose pollen are shown in Tables 3.1 and 3.2. Higher pre-storage viabilities were observed with pollen that had been dehisced over silica gel than that which had not been subjected to further drying. With both cultivars, highest pre-storage viabilities were observed in pollen collected from semi-open flowers.

3.3.3 Cryopreservation.

3.3.3.1 Assessment of different methods of cryopreservation.

The percentage viability of pollen grains of cv. Heritage, recovered from cryogenic storage, was consistently higher than for cv. The Countryman (Table 3.1). However, for both cultivars, no significant difference was observed in the viability of pollen grains recovered after freezing with either Method (i) (direct plunging into liquid nitrogen at -196°C), or with Method (ii) (controlled-rate freezing at -1°C/min to -40°C maintained for 10 min then plunged to -196°C), with the exception of pollen grains from The Countryman, recovered after dehiscence over silica gel and rapidly frozen using Method i). Attempts to cryopreserve pollen using Method (iii) (cooling to -130°C then plunging to -196°C) failed due to the inability of the Controlled-Rate Freezer to complete the required freezing programme before consuming all of the available liquid nitrogen.

3.3.3.2 Effects of dehiscing treatments on post-cryopreservation pollen viability.

The effects of the two dehiscence treatments on the post-cryopreservation pollen viabilities are shown in Table 3.1, and the effect of the degree of flower opening on post-cryopreservation pollen viabilities are shown in Table 3.2. The percentage decrease in pollen grain viability, of both rose cultivars, was significantly higher when anthers were dehisced over silica gel prior to pollen grain cryopreservation (Table 3.1). The percentage decrease in viability, after cryopreservation, was consistently greatest for pollen grains derived from semi-open flowers of both cultivars, but was independent of the dehiscing treatment used.

3.3.4 Morphological examination of pre- and post-cryopreserved pollen using scanning electron microscopy (SEM).

SEM showed that both non-cryopreserved pollen and cryopreserved pollen were observed to have operculate colpi (membrane covering the colpus is thickened, except for a thin area all round which joins the operculum to the colpus margin; Plate 3.1b, c

and d). The opercula were narrow and short, and had the same sculpturing to the rest of the exine i.e. fine, short striae, variable in distribution and length. From these observations the pollen could be classified as trizonocolporate, as defined by Moore *et al.* (1991).

Non-cryopreserved pollen had a mean polar length of 58±6μm, mean equatorial diameter of 28±3μm, and mean surface aperture diameter of 0.25±0.02μm (Plate 3.1b), while cryopreserved pollen had a mean polar length of 55±4μm, mean equatorial diameter of 29±4μm, and mean surface aperture diameter of 0.25±0.03μm (Plate 3.1c). Abnormal morphology (twisting along the polar axis) was observed in 37±4% of the non-cryopreserved pollen, and 34±2% of the cryopreserved pollen (Plate 3.1d).

3.3.5 Cross pollination using cryopreserved pollen.

Pollen grains of both rose cultivars were able to effect fertilisation after recovery from cryogenic storage. The number of seeds produced/cynarrhodium for each reciprocal cross using cryopreserved pollen grains was comparable to that set with fresh pollen. For cv. The Countryman, a mean value of 21.6±1.8 seeds/cynarrhodium was found when flowers were cross fertilised with cryopreserved pollen grains from cv. Heritage, as compared to a value of 21.4±1.2 seeds/cynarrhodium for flowers pollinated with fresh pollen grains. Likewise, when cv. The Countryman was the male parent in crosses with cv. Heritage, a mean value of 21.3±3.6 seeds/cynnarhodium was found with cryopreserved pollen, compared to 22.6±2.6 seeds/cynarrhodium using non-cryopreserved pollen grains.

3.3.6 Comparison of cryopreservation for long-term storage of rose pollen, compared to other methods.

Figure 3.1 shows the effects of storage treatment on pollen grain viability over the eight week test period. All storage treatments, resulted in an initial reduction in

pollen grain viability. This decrease was least in the cryopreserved samples. Over the subsequent weeks, samples held at both +4°C and at -20°C showed a steady and progressive decline in viability, whereas the viability of those samples recovered from storage at -196°C remained constant. The decline in viability was greatest for pollen grains refrigerated at +4°C and, additionally, after six weeks, these samples became overgrown with fungal contaminants.

Table 3.1. Effects of dehiscence and freezing treatments on rose pollen grain viability.

Rose Cultivar	Dehiscence Treatment (Silica gel)	Freezing Method	Pre-Freeze Pollen Viability (%)	Post-Freeze Pollen Viability (%)	Decrease in Pollen Viability (%)
Heritage	+	i	30.6 ab (6.02)	25.1 b (5.60)	17.9 e
Heritage	+	ii	25.3 ab (8.25)	21.7 b (5.31)	14.2 e
Heritage	-	i	27.6 ac (5.40)	24.6 c (5.80)	10.8 d
Heritage	-	ii	26.5 ac (7.30)	23.8 c (6.80)	10.2 d
The Countryman	+	i	17.5 w (6.30)	10.5 (1.25)	40.0 y
The Countryman	+	ii	18.0 w (5.60)	12.2 x (4.00)	32.2 y
The Countryman	-	i	15.4 x (4.40)	14.2 x (2.20)	7.8 zd
The Countryman	-	ii	16.2 wx (2.70)	14.3 x (1.80)	11.7 zd

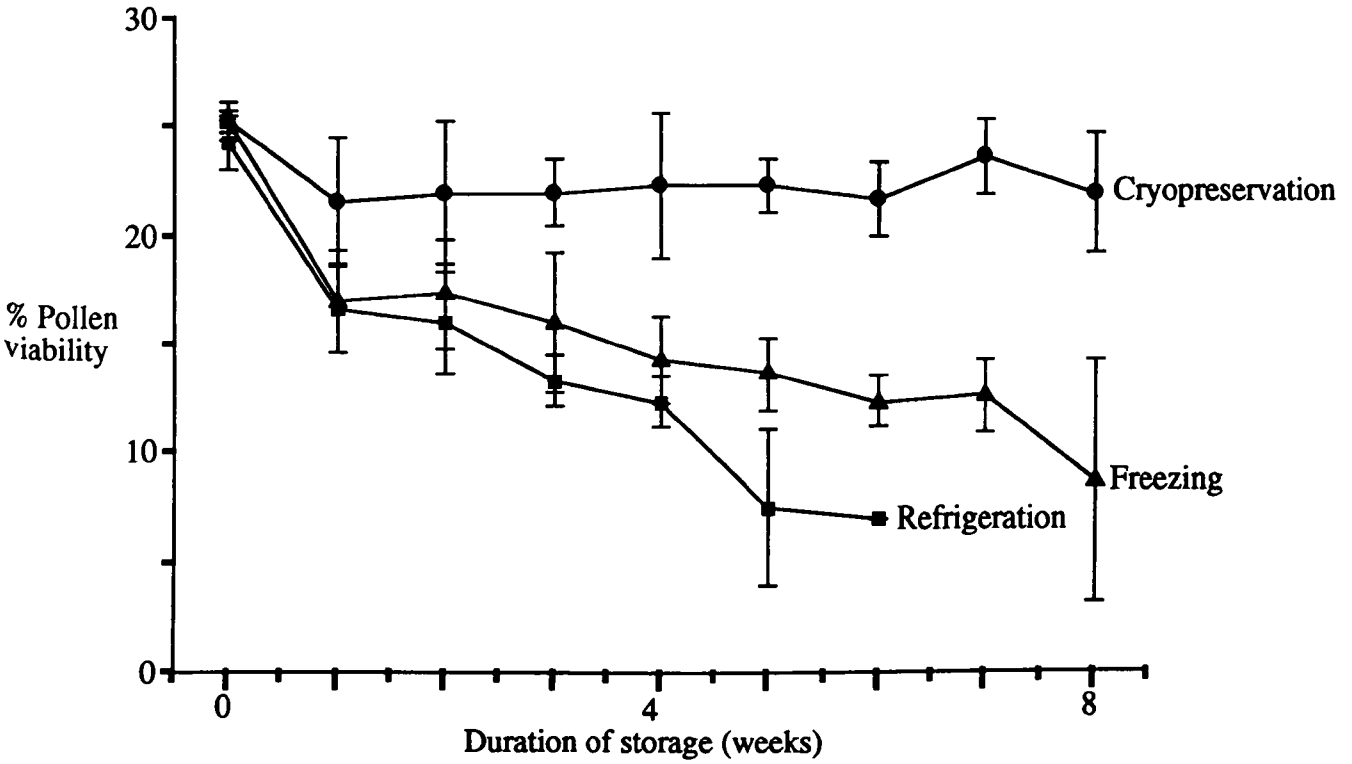
Pollen used was from semi-open flowers. The values for percentage pollen grain viability are the mean of ten replicates. Figures in parenthesis are standard errors. +/-: with or without silica gel treatment. Freezing Methods, i: rapid (direct plunging into liquid nitrogen), ii: controlled (controlled-ratefreezing at -1°C/min to -40°C maintained for 10 min then plunged to -196°C). Values followed by the same suffix are not significantly different at $p<0.05$.

Table 3.2. Effects of the stage of flower development and dehiscence treatment on pre- and post-cryopreservation pollen grain viabilities.

Rose Cultivar	Stage of Flower Opening	Dehiscence Treatment (Silica gel)	Pre-Freeze Pollen Viability (%)	Post-Freeze Pollen Viability[1] (%)	Decrease in Pollen Viability (%)
Heritage	open	-	27.3 a (5.5)	25.5 ab (5.3)	6.5
Heritage	open	+	22.5 b (7.4)	20.3 b (6.3)	9.7
Heritage	semi-open	-	30.8 a (4.2)	26.6 b (5.5)	13.3
Heritage	semi-open	+	30.8 a (4.5)	25.4 b (4.3)	17.5
Heritage	closed	-	19.4 bc (3.2)	17.7 c (3.1)	8.7
Heritage	closed	+	23.5 b (9.7)	21.9 b (5.4)	6.8
The Countryman	open	-	18.6 z (5.0)	15.0 z (3.5)	9.3
The Countryman	open	+	16.6 z (5.3)	15.0 z (1.6)	9.6
The Countryman	semi-open	-	26.4 y (3.4)	20.2 x (5.4)	23.4
The Countryman	semi-open	+	28.0 y (6.1)	22.4 x (3.6)	20.0
The Countryman	closed	-	14.3 w (2.1)	13.1 w (4.8)	8.4
The Countryman	closed	+	19.4 z (4.8)	18.1 z (5.2)	6.7

[1] Using freezing Method i); direct plunging into liquid nitrogen. The values for percentage pollen viability are the mean of ten replicates. Figures in parenthesis are standard errors. +/-: with or without silica gel treatment. Values followed by the same suffix are not significantly different at $p<0.05$.

Figure 3.1 The effects of storage duration and condition on rose pollen grain viability for *Rosa chinensis* cv. Heritage over an eight week period.



Values are means. Bars indicate standard error.

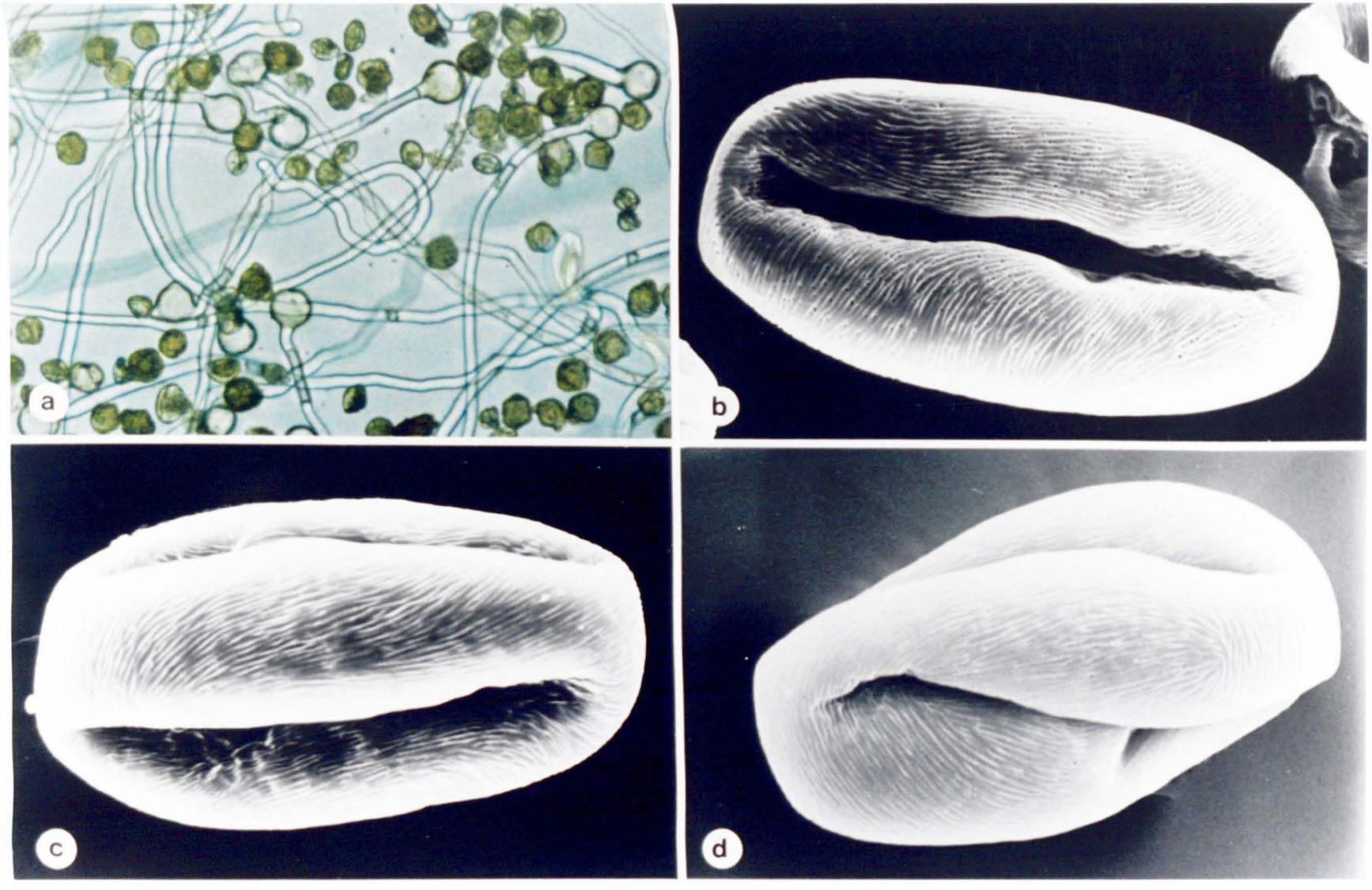
Plate 3.1.

a. Pollen from the English rose cv. The Countryman, recovered from 5 weeks cryopreservation, developing on semi-solid pollen grain germination medium (Section 3.2.1 iii) (x 100).

b. Scanning electron micrograph of freshly dehisced English rose pollen of the cv. The Countryman (x 1600).

c. Scanning electron micrograph of rose pollen of cv. The Countryman after recovery from cryogenic storage (x 1600).

d. Scanning electron micrograph of rose pollen of cv. The Countryman showing twisting of pollen grain following recovery from cryopreservation (x 1600).


a
b
c
d

3.4 Discussion.

3.4.1 Methods for assessing pollen viability.

The three methods of estimating pollen viability investigated in this study gave highly differing results. It is widely accepted that most *in vitro* methods of assessing pollen viability can be unreliable in that *in vitro* tests may indicate pollen has a low viability, and yet the same pollen can yield a high percentage of seed or fruit set when used *in vivo* (Johri and Vasil, 1961). However, the use of *in vivo* germination tests, such as the fluorescence staining technique (Chapter 2; Section 2.2.3.2), are laborious and time consuming, and require large numbers of receptive styles which were not available for rose in sufficient numbers for this particular study. In an investigation, such as that described here, where the results of different storage treatments are being compared, it is important that viability tests give consistent results in order to highlight the differences and/or trends between treatments, even if the data for pollen viability determined by *in vitro* germination varies from the actual percent viability. It is for this reason that any *in vitro* tests of pollen viability must be regarded as indices or estimates of viability, rather than absolute measurements. The two staining methods for assessing pollen viability, studied here, gave results which had very large standard errors. This further supports the observations of Pearson and Harney (1984), who concluded that although the correlation between pollen viability determined by percentage germination and staining with acetocarmine was significant ($r = 0.5$), the corresponding coefficient of determination ($R^2 = 0.25$) suggested that staining with acetocarmine provided only a very rough estimate of viability. The *in vitro* germination test used in this study, produced results with a considerably smaller standard error. This means that if such techniques were to be used to compare the effects of any treatments on the viability of pollen, it would be more likely that observed differences between those treatments would occur as a result of one or both of the treatments rather than as a consequence of the method of determining viability. Hence, the *in vitro* germination test was adopted as the method for assessing pollen viability in other fascets of this study. The use of this test also meant that results

obtained here could be more meaningfully compared with other studies of rose pollen where identical or similar methods have been used for assessing pollen viability (Guidin *et al.*, 1991a and b; Visser *et al.*, 1977).

3.4.2 The long-term storage of rose pollen.

If the long-term storage of pollen grains from cultivated rose is to be of commercial application and value, it is essential, for practical reasons, that there be a minimal loss of viability during storage. This is particularly important in cases where the pollen viability, prior to storage, is already low, such as for the English roses, since a further reduction in pollen viability would significantly reduce the chances of sucessful fertilisation occurring when the pollen is subsequently used in hybridisation.

This study has shown that pollen grains from the two chosen English rose cultivars (and these are probably typical) can be successfully recovered from cryogenic storage, without significant loss in viability. Both the pre- and post-cryopreservation pollen grain viabilities were influenced by the use of silica gel in the anther dehiscence treatments. Since higher pre-cryopreservation viabilities were observed in pollen grain samples obtained from anthers dehisced over silica gel, such a technique would, in itself, be useful for obtaining pollen grains to be used for immediate crossing, in that it would maximise the number of viable pollen grains available to effect fertilisation. However, treatment with silica gel appeared to be detrimental to the viability of pollen grains recovered from cryogenic storage. It has been shown that water content has a major influence on the ability to store pollen grains at ultra-low temperatures (Barnabas, 1984). Reduction in pollen moisture content below a species-specific threshold level prior to exposure to low temperatures is critical in ensuring survival of pollen during cryopreservation (Towill, 1985). Although drying is important to aid pollen grain storage, over-drying can adversely affect the recovery of viable pollen grains (Barnabas and Rajki, 1976). If the moisture content of the rose pollen grains used in this study were already at, or close to, the threshold level, then it

is possible that exposure to silica will result in an over-drying, and hence a reduction in post-thaw viability. This could account for the larger decline in the post-thaw viability of cryopreserved pollen grains from the cv. The Countryman, after dehiscence of anthers over silica gel.

The relatively small quantities of pollen, available for this study, made it impossible to estimate the water content of the pollen either by weighing prior to and after drying. Although the use of techniques such as X-ray diffraction or nuclear magnetic resonance imaging can be used to estimate the water content of such samples (Towill, 1985), such techniques were beyond the scope of this project. Thus, over-drying could not be confirmed as the cause of the reduction in pollen viability.

Although different cryopreservation methods are known to result in different degrees of cell dehydration (Towill, 1985), there was no apparent difference between the two methods employed here for rose pollen cryopreservation. Reports suggest that if cooling is too rapid, vapour-pressure equilibrium between the cell and extracellular environment is not maintained and intracellular ice formation occurs leading to damage of the cells (Streponkus *et al.*, 1985). For this reason, two-step cooling methods, in which the temperature of the material is first reduced, in a controlled manner, to an initial holding temperature (such as -40°C) at which it is then maintained for ten minutes prior to plunging to -196°C, are often used. These protocols are designed to eliminate damage to the cell which can occur if ice crystal formation is induced. The fact that the two-step method of cryopreservation investigated here did not give significantly higher levels of post-cryopreservation pollen viability, would seem to indicate that the level of hydration of the grain was sufficiently low to minimise or eradicate ice crystal formation, and resulting cell damage, when cryopreservation was carried out. Although some research has shown a two-step method is necessary for the cryopreservation of pollen of Narcissus (Bowes, 1990) present observations on rose are typical of most of those relating to

pollen cryopreservation, such as those on Citrus (Ganeshan and Alexander, 1991) where direct plunging into liquid nitrogen was found to be equally effective. These results suggest that it is the pre-cryopreservation pollen viability and quality, rather than the method of cryopreservation, that is the more significant factor in influencing the post-cryopreservation pollen viability.

Currently, there is scant information on the influence of rose flower maturity on pollen grain viability. The results of this study show that the highest pollen grain viability (26.4 - 30.8%) was obtained from semi-open flowers. However, pollen grains collected from such flowers also showed the greatest decrease in viability following cryogenic storage. These results may again reflect the relative moisture content of such samples. In order to minimise loss of viability, it would therefore appear desirable to use pollen grains collected from open or closed flowers, despite the lower initial viability of pollen grains from these samples. In practical terms, collection of pollen grains from closed flowers would be particularly desirable since it would eliminate the risk of the cross-contamination of samples with insect- or wind-borne pollen from other plants and it is also more likely to be free from microbial contaminants.

The examination of pollen grains using the electron microscope showed them to be of the trizonocolporate type. This observation conforms with those made by Moore *et al.*, 1991 of various rose species/cultivars. As pollen generally changes size/shape according to its degree of hydration (Heslop-Harrison, 1979), the observation that cryopreservation caused no significant change in the overall dimensions of pollen grains would seem to suggest that the level of pollen hydration was not affected by cryopreservation. This is important as a change in hydration would probably have caused a reduction in the viability of recovered pollen. The observation that the size of the surface apertures was not altered significantly by cryopreservation is also important, as these have been shown to effect the rehydration of the pollen grain

following dehiscence (Heslop-Harrison, 1979). If the physical structure of the pollen grains had been affected by cryopreservation, resulting in a reduction in the size of the apertures, this may have led to less effective rehydration of the pollen grains on the germination media or stylar surface. This would hence be reflected in a reduction in the post-cryopreservation viability. Another observation which indicates that cryopreservation does not significantly alter the water relations of the pollen grain is that the percentage of grains exhibiting twisting along the polar axis termed harmomegathy (Wodehouse, 1935), which is a response to water loss, is not changed by cryopreservation. If pollen grains were becoming more dehydrated by the cryopreservation treatment an increased percentage of pollen grains would exhibit twisting, and, if conversely, pollen grains were gaining water during cryopreservation, fewer would exhibit such twisting post-cryopreservation.

The ability of rose pollen grains to effect fertilisation does not appear to be adversely influenced by cryopreservation. Save there was a close similarity in the number of seeds produced per cynarrhodium using cryopreserved pollen grains as compared to fresh pollen leading to the assumption that cryopreservation had no adverse effect on the ability of pollen grains to produce competent pollen tubes capable of delivering viable male gametes. This is consistent with the results of Parfitt and Almehdi (1984), who showed that pollen tube development in other Rosaceous species was unaffected by cryopreservation. The maintenance of the ability of pollen, subjected to cryopreservation, to effect fertilisation is supported by the observed similarity in the surface apertures on cryopreserved and non-cryopreserved pollen grains. As such apertures may play a key rôle in the recognition system that occurs on the stylar surface (Horner and Pearson, 1978), any alterations would have reduced the chances of effective fertilisation from occurring.

3.4.3 Efficacy of cryopreservation, for the long term storage of rose pollen, in comparison to other techniques.

Khosh-Khui *et al.*, (1976) concluded that rose pollen grains cannot be maintained in a viable state at 0°C for periods in excess of nine weeks. However, Visser *et al.*, (1977), using pollen grains with an initial viability in excess of 45%, claimed that up to 20% of this pollen could be maintained in a viable state at +1°C and a relative humidity of 20% or less, for periods of up to 40 weeks, although the ability of the pollen to effect fertilisation declined the longer it had been stored. This decline correlated with the reduction in pollen viability observed over the period of storage.

The present study showed, that while it was possible to store pollen for short periods, either at 4°C or at -20°C, there was a significant loss of viability. Such a loss would inevitably be reflected in a reduction in fertilisation/seed-set if such pollen were to be used for hybridisation. This would be critical if the initial pre-storage pollen viability was low. For English rose pollen, with inherently low viability, it was been shown that effective long-term storage can only be achieved at ultra-low temperatures. At such temperatures, cell metabolism is effectively suspended (Mazur, 1984) and should virtually eliminate any time-related biochemical changes (Towill, 1991). Difficulties in maintaining biological samples by cryopreservation, normally relate to the transitions to and from the storage temperature, rather than cryogenic storage for a particular period of time. Since the results with rose pollen indicate that its viability remains stable under cryogenic conditions, it may be assumed that pollen grain viability will be maintained at the observed levels well beyond the eight week period used in this study.

3.5 Summary.

i) *In vitro* germination tests were confirmed as the best option for assessing viability of English rose pollen.

ii) Both the degree of flower opening and method of anther dehiscence were shown to affect the viability of English rose pollen.

iii) Both rapid and controlled-rate freezing were effective for cryopreservation of English rose pollen. The gross morphology of pollen from English roses, and its ability to effect fertilisation, were not affected by cryopreservation.

iv) When compared to more conventional techniques for the long-term storage of English rose pollen, such as storage at +4°C or -20°C, cryopreservation was demonstrated to be more effective.

CHAPTER 4.
Development of tissue culture systems for rose.

4.1 Introduction.

The majority of plant genetic manipulation technologies rely on the establishment of efficient and reliable methods for the *in vitro* culture of plant organs and tissues which can then act as source/target materials. Following such manipulations, tissue culture protocols, such as direct shoot regeneration from leaf discs or via somatic embryogenesis, provide pathways back to whole plants. The availability of *in vitro* cultured plant material is also relevant for the isolation of protoplasts. Due to problems of reproducibility and viability when using *in vivo* grown plants, protoplasts are usually isolated from axenic shoots or plantlets (Roest and Gilissen, 1993). Low reproducibility of protocols for isolation, culture and regeneration of protoplasts for many *in vivo* maintained species is due to physiological variations in donor tissues (Revilla *et al.*, 1987). Additionally, with woody species, for which mesophyll protoplasts are difficult, if not impossible, to obtain from *in vivo* sources, the presence of a thickened cuticle and wall secondary products reduces yields. Conversely, fully expanded leaves from fast growing axenic plants give optimum results in terms of protoplast yield and/or viability (Ochatt and Power, 1992). This is partly due to the fact that the cell wall becomes thinner as the rate of cell wall elongation outstrips cellulose biosynthesis (Roberts and Butt, 1968).

A common problem, encountered during the isolation of protoplasts from woody species, is a reduction in viability attributable to phenolic oxidation (Ochatt and Power, 1992). Although this can be alleviated, to some extent, by various treatments at both the isolation and culture stages, the use of *in vitro*-generated propagules reduces this problem. Tissues grown *in vitro* do not have, for example, the same degree of lignification, which is associated with the production of phenolic compounds (Porter, 1991). *In vitro* micropropagation of shoots therefore, not only

provides a controlled method of plant multiplication, but such shoots also serve as a reliable and contamination-free source of protoplasts.

Callus and cell suspension cultures are alternative sources for protoplasts and if such lines are totipotent, this not only increases the likelihood of regeneration from protoplasts (Roest and Gilissen, 1993), but also provides a basis for transformation via *Agrobacterium* vector systems or microprojectile gene delivery.

There are many difficulties inherent in woody plant tissue culture. Bacterial contamination remains a major problem, especially with explants of field origin (Leifert and Waites, 1990). Effective surface disinfectants and the incorporation of antibiotics such as tetracycline and rifampicin into the culture medium can minimise the extent of contamination (Young *et al.*, 1984). However, many of the antibacterial compounds are bacteriostatic rather than bacteriocidal and can also have deleterious effects on plant cell viability (Bastiaens *et al.*, 1983; Young *et al.*, 1984; Okkels and Pedersen, 1988), although surprisingly, some antibiotics, notably cefotaxime, have been shown to have beneficial effects in terms of promoting the sustained division of *Passiflora* protoplasts (d' Utra Vaz *et al.*, 1993). Oxidative browning and vitrification (Gaspar, 1991) of woody plant cultures is not uncommon. Oxidation is the process whereby the liberation of highly active oxidases convert polyphenols (present at high levels in many woody species) into quinones, which, in turn, are capable of producing brown phenol-protein pigment complexes that inhibit enzyme activity and retard cellular growth. Vitrification manifests itself by the glassy appearance of shoots and is associated with a defective development of the palisade mesophyll tissue due to hyperhydration. Hyperhydration is affected by physical (eg. temperature, gel solidity) and chemical (eg. cytokinins, ammonium ions) factors (Gaspar, 1991), and there is no general strategy for reversal from a vitrified state.

Although there are many reports of the *in vitro* propagation of rose (see Chapter 1; Section 1.5.1), the requirements for effective micropropagation, in terms of medium composition and culture conditions, vary between species and varieties (Skirvin *et al.*, 1990; Short and Roberts, 1991). Likewise, the requirements for the initiation of callus (both embryogenic and non-embryogenic) and cell suspension cultures from rose appear also to be species/cultivar dependant (Skirvin *et al.*, 1990). The ability to regenerate adventitious shoots, from callus or from explants such as leaf discs, is currently limited to a small number of rose cultivars (Chapter 1; Section 1.5.2). In the light of such observations, and because there were no reports on the tissue culture of the English roses, as employed in this study, it was crucial to evaluate tissue culture systems for the specific cultivars under study. This chapter focuses on the establishment and improvement of *in vitro* axenic shoot culture protocols, callus induction and the conversion of callus to cell suspensions. Attempts were also made to initiate embryogenic callus, as well as to regenerate plants from callus and/or leaf tissues, for several of the English rose cultivars.

4.2 Materials and Methods.

4.2.1 Plant material: origin and sources.

The cultivars of English rose used in this part of the study were all developed and supplied by David Austin Roses, Albrighton, UK. Stock plants (not grafted onto rootstocks) were grown in 20 l plastic pots containing a 1:1 (v:v) mixture of Levington 3M soilless compost (Fisons, Ipswich, UK) and John Innes No3 compost (J. Bentley, Barrow-on-Humber, UK). Plants were maintained in a glasshouse at approximately 25$\pm$3°C, with a 16 h photoperiod (25 $\mu Em^{-2}s^{-1}$). Plants were watered twice daily and were treated (every 4 weeks) with fungicides (benomyl and carbendazim) and insecticides (pyrethrum and permethrin); the combination of these was varied to avoid the development of disease/pest resistance. Toprose fertiliser (PBI Ltd, Waltham Cross, Herts, UK) was applied to the surface of the compost every 6 weeks.

4.2.2 Establishment of axenic shoot cultures.

4.2.2.1 Assessment of the efficacy of various surface sterilants.

In order to asess the efficacy of various surface sterilants, it was decided to compare the effectiveness of various compounds in eliminating microbial contaminants on explants of a sample rose cultivar. A total of 150 stem sections (1 cm in length), all of which included one lateral bud, were collected from glasshouse-grown plants of the English rose cultivar Marie Pavié. Fifty explants (in batches of 10) were then subjected to one of three sterilisation procedures. Stem sections were:

i) Placed in 5% (v/v) 'Domestos' solution (Lever Brothers, Kingston-upon-Thames, UK.) for 10 min and then rinsed 5 times with sterile distilled water.

ii) Placed in 10% (v/v) 'Domestos', then as above.

iii) Placed in 0.1% (w/v) mercuric chloride solution with 0.1% Tween-20 (v/v; Sigma Poole, UK) for 10 min followed by 5 rinses in sterile distilled water.

Following the appropriate treatment, explants were placed, (10 explants/jar) onto the surface of 30 ml of semi-solid medium contained within 350 ml screw cap glass jars (Beatson Clark, Rotherham, UK.). The medium was basal MS (Appendix I) with 0.8% (w/v) agar (Type IV; Sigma, Poole, UK). After 3 weeks (25°C), with a 16 h photoperiod (18 $\mu Em^{-2}s^{-1}$), the number of explants that exhibited microbial contamination was noted, along with the number that had become achromatic (bleached) in appearance. All values were calculated as a percentage of the total number of explants. The method of surface sterilisation deemed optimal (lowest level of contamination and achromatic explants, see Results Section 4.3.1.1) was adopted thereafter.

4.2.2.2 Influence of growth regulators on the initiation of axenic shoot cultures.

This investigation was carried out on each of the following seven English rose cvs., namely Abraham Darby, Marie Pavié, Mary Rose, Little White Pet, Heritage, The Countryman and Katharina Zeimet. With each cultivar, lateral shoots with their

leaves removed were cut into approx. 3cm long sections, each with an axillary bud. They were surface sterilised by soaking (10min) in 0.1% (w/v) HgCl with 0.1% (v/v) Tween-20 with 5 rinses in sterile distilled water. Stem sections (trimmed to 1 cm in length), each with an axillary bud, were placed horizontally on the surface of 50 ml of culture medium in 350 ml screw cap glass jars (10 explants/jar, 10 jars/medium type). The medium (prepared as in Appendix I) was MS-based with 0.8% agar, and contained the growth regulator combinations given in Table 4.1. Cultures were maintained in the dark (25°C) for 2 weeks, followed by 4 weeks with a 16h photoperiod (27 $\mu Em^{-2}s^{-1}$) provided by daylight fluorescent illumination (Thorn Cool Light, Thorn EMI Ltd, UK.). The choice of media was partly based upon those that previously had been successfully employed in the *in vitro* culture of several different species/cultivars of rose (see Table 4.1 for references). After 6 weeks, the number of explants that had produced axillary shoots was noted, along with the total number of shoots/explant and the number of explants that were necrotic. Such shoots were transferred to fresh medium, of the same composition, and subcultured, by division, onto fresh medium at 4 weekly intervals. After 4 successive subcultures (22 weeks post-initiation) the multiplication rates for cultures on each medium type was determined, based on the method of Douglas *et al.*, 1989.

Table 4.1. Growth regulator combinations used to investigate the initiation and growth rate of axenic shoot cultures.

Medium Code	Growth Regulator (mg l^{-1})			
	BAP	GA_3	NAA	IAA
RSC 1	1.0	0.1	0.004	-
RSC2	2.0	0.1	0.004	-
RSC3	3.0	0.1	0.004	-
RSC4	10.0	0.1	0.004	-
RSC5	1.0	-	-	0.3
RSC6	2.0	-	-	0.3
RSC7	3.0	-	-	0.3
RSC8	10.0	-	-	0.3
RSC9	0.1	-	-	0.1
RSC10	0.5	-	-	0.1
RSC11	1.0	-	-	0.1
RSC12	0.1	-	-	0.5
RSC13	0.5	-	-	0.5
RSC14	1.0	-	-	0.5

RSC 1 - 4: Lloyd *et al.*, 1988; Horn *et al.*, 1988. RSC 5 - 8: Bressan *et al.*, 1982. RSC 9 - 14: Martin *et al.*, 1981.

4.2.2.3 Prevention of tissue browning.

In order to attempt to eliminate browning of explants due to phenolic oxidation, a number of tissue treatments were investigated at the culture initiation stage with the cvs. Abraham Darby, Heritage and Marie Pavié. Media were dispensed as in Section 4.2.2.1, with the optimal growth regulator régimes (Results, Section 4.3.1.2). As the cv. Heritage had failed to exhibit shoot proliferation in previous studies (Results,

Section 4.3.1.2), this cultivar was cultured on RSC2 medium (Table 4.1). With all three cultivars, shoot explants (as described in Section 4.2.2.2) were surface sterilised (Section 4.2.1.2.) and were then subjected to one of five treatments:

i) Explants were placed onto media supplemented with 1.0% (w/v) polyvinylpyrrolidone (PVP-10; MW 10 000; Sigma, Poole, UK).

ii) Explants were placed onto media containing 0.25% (w/v) acid washed, tissue culture grade activated charcoal (Sigma, Poole, UK).

iii) Prior to placing explants onto the surface of the medium, they were soaked for 12h in a 1.0% (w/v) solution of PVP-10 or (iv) 4.0% (w/v) ascorbic acid and 1.2% (w/v) sodium borate or (v) 0.1% (w/v) solution of 8-hydroxy-quinolinol-sulphate (8-HQS) (Riedel de Haën, Seelze, Germany).

A total of 50 explants per rose cultivar were subjected to each treatment. A further 50 explants were untreated controls. Cultures were maintained in the dark (25°C) for 2 weeks, followed by 2 weeks (also at 25°C) in a 16 h photoperiod (27 $\mu Em^{-2}s^{-1}$) provided by daylight fluorescent tubes. After this period, the number of explants that had browned for each cultivar/treatment was noted and calculated as a percentage of the total number of explants.

4.2.2.4 Rooting and *ex vitro* transfer of rose shoots.

In order to promote rooting, single shoots, with their basal leaves removed, were transferred to 35 ml of medium in 175 ml screw-capped glass jars. The medium used was as described in Section 4.2.2.2, ie. MS medium, but lacking growth regulators (MSO medium), with cultures kept at 25°C [16 h photoperiod (18 $\mu E\ m^{-2}s^{-1}$)] for 2-5 weeks after which root initiation and elongation had occurred. Rooted plantlets from which the agar medium was removed with running water, were transferred to 8 cm diameter pots, each containing a 1:1 (v:v) mixture of Levington 3M soilless compost (Fisons, Ipswich, UK) and Perlite (Silvaperl, Gainsborough, UK). After watering, each pot was covered with a 20 x 13 cm polythene bag. Pots were then maintained

under glasshouse conditions (Section 4.2.1) and the polythene bags were progressively opened over a 3 week period. After 2 months, established plants were transferred into 12.5 cm diameter plastic pots containing a 1:1 (v:v) mixture of Levington 3M soilless compost (Fisons, Ipswich, UK) and John Innes No 3 Compost (J. Bentley, Barrow-on-Humber, UK). Plants were subsequently maintained under the conditions previously described (Section 4.2.1).

4.2.3 Initiation, maintenance and regeneration assessments of callus cultures of English rose.

4.2.3.1 Establishment and maintenance of callus cultures.

Callus induction was investigated with the English rose cvs. Abraham Darby, Marie Pavié, Mary Rose, The Countryman, Katharina Zeimet, Ballerina, Phyllis Bide and Francine Austin. Leaf discs (approx. 0.75 cm diam.) and internodal sections (1.0cm in length) derived from *in vitro* cultures established as described in Section 4.2.2.2, were used as explants, which were subjected to the antioxidant treatment described in Section 4.2.2.3 (iv). In order to place all observations on a comparative basis, a single semi-solid medium, namely MS with 2.0 mg l^{-1} NAA, 0.5 mg l^{-1} BAP and 0.8% (w/v) agar, pH 5.8 (hereafter referred to as MSP1 medium), was employed throughout the study. In addition, media which had been shown in other studies to be effective for the induction of callus in certain rose cultivars, were also used (Table 4.2). All these media contained 30 g l^{-1} sucrose and 0.8% (w/v) agar (Type IV; Sigma), pH 5.8. Five explants of each source were placed onto the surface of 25 ml of medium contained in 9 cm plastic Petri dishes and sealed with Nescofilm. These were incubated at 25°C under either a 16 h photoperiod (18 $\mu Em^{-2}s^{-1}$), or in the dark. Each medium/cultivar experiment included at least 10 replicates and experiments were repeated at least twice under both light and dark conditions. After 30 d, the following callusing responses were determined:

1. Proliferation: this was assessed as the mean area of callus produced, which was measured using the point counting method of Mottley and Keen (1987). Point counts

were made by placing an acetate sheet at random on the base of the Petri dish. The sheet had points marked on its surface, arranged in a square pattern at intervals of 1.0 mm. Only those points with their centres exactly on or inside the edge of each callus area were counted; the areas of individual calli were calculated using the formula $A=N \times D^2$, where A= calculated area, N= number of points counted and D= distance between the points.

2. Texture: this was assessed visually and calli allocated to one of three groups. Specifically, hard, intermediate or friable.

3. Colour: This was assessed using the Royal Horticultural Society Colour Charts (RHS., London/ Flower Council of Holland, Leiden).

Once callus lines had been established, all were maintained by a monthly subculture of approximately 125 mm^3 portions of callus (200 mg f. wt) as inoculum onto 40 ml of the appropriate, semi-solid medium (dispensed into 175 ml screw cap glass jars).

Table 4.2. Media used (in addition to MSP1) in the investigation of rose callus initiation and establishment.

Medium Code	Salts and Vitamins	Growth Regulators (mg l^{-1})						Key Reference
		NAA	IBA	GA_3	BAP	2,4D	kinetin	
CM1	MS [1]	2.0	-	-	-	-	-	Ishioka and Tanimoto, 1990
CM2	MS	-	0.1	0.2	1.0	-	-	Valles and Boxus, 1987
CM3	SH [2]	-	-	-	-	2.0	0.2	Arif and Khatamian, 1990
CM4	SH	-	-	-	-	0.5	0.1	Khosh-Khui and Sink, 1982
CM5	MS	-	-	-	-	2.0	0.25	as above
CM6	SH	-	-	-	-	3.0	-	Matthews *et al.*, 1991
CM7	MS	0.05	-	-	-	-	0.1	de Wit *et al.*, 1990
CM8	MS	0.01	-	-	-	-	0.2	as above
CM9	MS	0.02	-	-	-	-	0.2	as above
CM10	1/2MS[3]	1.0	-	-	0.5	0.5	-	Rout *et al.*, 1991

[1] Basal salts and vitamins of Murashige and Skoog, 1962. [2] Basal salts and vitamins of Schenk and Hildebrandt, 1972. [3] Half strength dilution of basal salts and vitamins of Murashige and Skoog, 1962.

4.2.3.2 Regeneration from callus.

In order to attempt to trigger organogenesis from callus accessions, calli (subcultured at least once but not more than 5 times) were transferred to 20 ml of agar-solidified regeneration medium in 9cm plastic Petri dishes (5 calli/dish) each sealed with Nescofilm. Regeneration medium was based on that of Valles and Boxus (1987), and was of the same basal type as had been used for the initiation of the callus used in this study, but contained 0.1 mg l^{-1} or 0.2 mg l^{-1} IBA, 2.5 or 5.0 mg l^{-1} BAP and with either 0.1 mg l^{-1} GA_3 or GA_3 omitted. In addition, a proportion of calli were transferred to medium of the same basal composition containing 1.0, 2.0 or 3.0 mg l^{-1}

2,4-D as employed by Matthews *et al.* (1991) for the species hybrid *R. persica* x *xanthia*. A total of 50 calli of each cultivar were subjected to each treatment. In all cases, media were prepared as described in Section 4.2.2.2. Cultures were maintained either in the dark or with a 16h photoperiod (18 $\mu Em^{-2}s^{-1}$) at 28°C. Initial assessment of cultures was after 3 weeks in culture and every 2 weeks thereafter.

4.2.4 Establishment and maintenance of cell suspension cultures of English rose.

Leaf-derived calli of cv. Mary Rose and stem derived calli of the cvs. Mary Rose and Phyllis Bide were deemed to be source tissues most suitable for the initiation of cell suspensions, as they were both highly friable and exhibited a high proliferation rate. Friable callus portions (5.0 g f. wt) cultured on solidified medium, were dispersed into 50 ml of liquid medium (either MSP1 or a liquid version of the medium on which they had been maintained), in 250 ml Erlenmeyer flasks, maintained on a rotary shaker (90 rpm). Ten replicate flasks were used per tissue source and for each medium. All were maintained at 28°C either in the dark or under a 16 h photoperiod (20 $\mu Em^{-2}s^{-1}$). After 10 d the callus pieces had disassociated into small cell clusters, whereupon they were filtered through a 500 μm nylon sieve (Wilson Sieves, Long Acre, Hucknall, Nottingham, UK) and the filtrate recovered into a large sterile glass jar. The cell suspension was diluted with an equal volume of fresh medium (of the same type), and dispensed, as 50 ml aliquots, into 250 ml Erlenmeyer flasks. After 14 d cell suspensions were subcultured by transfering 10 ml of suspension into 65 ml of fresh medium contained in 250 ml Erlenmeyer flasks. Following microscopic examination, those cultures which consisted of discrete clusters of near-spherical cells (Plate 4.1e) were maintained, whilst others containing an abundance of elongated filamentous cells (Plate 4.1f) were discarded. After 3 successive subcultures, the growth curves of the cell suspensions were determined, in order to establish the time at which exponential growth occurred. This was generally regarded as the optimal time to subculture cell suspensions and to isolate protoplasts. Measurements were taken every 3 d using the following procedures.

i) 250 ml Erlenmeyer flasks with a graduated side-arm were used and 5 ml of cell suspension were decanted into the side-arm and allowed to settle for at least 30 min. The volume of cellular material was recorded and the settled cell volume (scv) calculated as a percentage of the total volume.

ii) A 1.0 ml aliquot of cell suspension (fully suspended) was removed aseptically and spun at 700 xg; 5 min. The medium was discarded and replaced by 2 ml of 10% (v/v) HCl to which was added 2 ml of 10% (w/v) chromium trioxide in water. This mixture was incubated at room temperature overnight and the cell number then assessed using a modified Fuchs Rosenthal haemocytometer, taking into account the dilution of the sample. At least 10 counts were performed per sample.

4.2.5 Adventitious regeneration from rose tissues.

Explants to be used were randomly selected leaves from five nodes below the apex and excised at the time of routine subculturing of *in vitro* grown shoots of the cvs. Abraham Darby and Marie Pavié. The distal part of the limb (1 mm in length) was discarded. The leaves were scored several times across their midribs using a scalpel, and were placed, abaxial surface down, onto the surface of 10 ml of regeneration medium contained in 5.5 cm Petri dishes (A/S Nunc, Roskilde, Denmark; 5 leaves/dish) sealed with Nescofilm. The regeneration medium was MS basal medium solidified using 0.8% agar (Type IV; Sigma), and containing a mixture of one of the three cytokinins BAP, TDZ or kinetin (0, 1.0, 2.0, 3.0, 4.0 or 5.0 mg l^{-1}) and/or auxins NAA (0, 0.1, 1.0, 3.0 or 5.0 mg l^{-1}), IAA (0, 0.5 or 1.0 mg l^{-1}) or 2,4-D (0, 1.0, 2.0, 3.0, 4.0 or 5.0 mg l^{-1}) giving 207 factorial combinations of growth regulators. All media were prepared as described (Appendix I) and each treatment included 20 replicated explants. Cultures were maintained either in darkness or under a 16 h photoperiod (18 $\mu Em^{-2}s^{-1}$; 25°C). Following initiation, cultures were examined on a weekly basis and the response of explants recorded.

4.3 Results.

4.3.1 Axenic shoot cultures.

4.3.1.1 Efficacy of surface sterilants.

Results are given in Table 4.3, along with the percentage of explants that were suitable for further culture; microbial contamination was minimal following surface sterilisation with 0.1% HgCl solution, as was the extent of achromatism.

Table 4.3. Effects of surface sterilants on *in vivo*-derived explants of English rose cv. Marie Pavié.

Method of Sterilisation	Explants Contaminated (%)	Explants Achromatic (%)	Explants suitable for further culture (%)
5% Domestos	30.0	12.0	66.0
10% Domestos	10.0	30.0	64.0
0.1% HgCl	6.0	2.0	92.0

4.3.1.2 Growth of axenic shoot cultures.

The effects of growth regulators on the growth of *in vitro* shoot cultures of the six English rose varieties are shown in Tables 4.4 and 4.5, with the optimal growth regulator combinations summarized in Table 4.6. The percentage of explants producing shoots ranged from 0 - 89%, whilst the mean number of shoots/explant varied between 1.0 ± 0.1 and 2.4 ± 0.2, and multiplication rates were between 0 and 3.1 ± 0.3. In general, when a high percentage of explants were producing shoots this was accompanied by a relatively high mean number of shoots/explant and also by a high multiplication rate. Conversely, high rates of browning were reflected in a low percentage of explants producing shoots and/or low numbers of shoots/explant and/or

reduced multiplication rates. After 6 weeks, all the explants of the cv. Heritage had browned and failed to produce shoots.

The responses of explants to different media types were highly cultivar specific. The cv. Abraham Darby did not multiply at the highest concentrations of BAP (10.0 mg l^{-1}) or when media contained only BAP and NAA; growth only occured when the concentration of BAP was 1.0 mg l^{-1} (Plate 4.1a, b). The cv. Marie Pavié produced shoots only when BAP, GA_3 and NAA were all present. As with Abraham Darby, multiplication did not occur at the highest concentration of BAP (10.0 mg l^{-1}). Explants of the cv. Mary Rose showed shoot proliferation when BAP, GA_3 and NAA were all present, even with BAP at 10.0 mg l^{-1}. Similar responses were also observed when only BAP and IAA were used, though shoot multiplication only occured at the highest concentration of IAA (0.3 mg l^{-1}). Shoot proliferation in the cv. Little White Pet was restricted to media with BAP concentrations of less than 3.0 mg l^{-1} and although proliferation occurred in the absence of GA_3 and NAA, multiplication only took place when IAA was present at a concentration of 0.1 mg l^{-1}. As with Marie Pavié, shoot multiplication only occurred for cv. The Countryman when BAP, GA_3 and NAA were all present. However, shoot initiation was also recorded with media containing BAP and 0.3 mg l^{-1} IAA, but with no multiplication. The cv. Katharina Zeimet produced shoots on media containing IAA. Proliferation failed to occur when GA_3/NAA was present and, in all cases, proliferation and multiplication only occurred when BAP was also included in the medium.

In all of the successfully micropropagated English rose varieties used as part of this study, rooting was easily achieved, with in excess of 95% of shoots producing roots when subjected to the procedure described in Section 4.2.2.4 (Plate 4.1c). Of those rooted shoots, in excess of 90% survived transfer to the glasshouse.

Table 4.4 Growth of *in vitro* shoot cultures of cvs. Abraham Darby, Marie Pavié and Mary Rose.

	Abraham Darby				Marie Pavié				Mary Rose			
Medium Code[1]	Explants Producing Shoots (%)	Mean No. of Shoots per Explant[2]	Explants Browning (%)	Multiplic-ation Rate[3]	Explants Producing Shoots (%)	Mean No. of Shoots per Explant[2]	Explants Browning (%)	Multiplic-ation Rate[3]	Explants Producing Shoots (%)	Mean No. of Shoots per Explant[2]	Explants Browning (%)	Multiplic-ation Rate[3]
RSC 1	80	2.9±0.2	11	3.1±0.3	70	2.1±0.5	28	2.5±0.3	59	1.5±0.1	27	1.5±0.3
RSC 2	79	1.0±0.1	20	2.7±0.2	84	2.1±0.3	11	2.9±0.2	71	2.4±0.2	16	2.3±0.3
RSC 3	74	1.2±0.3	22	1.5±0.1	59	1.8±0.3	14	1.8±0.5	44	1.3±0.1	29	1.7±0.1
RSC 4	68	1.2±0.1	12	0	30	1.9±0.3	20	0	34	1.3±0.2	33	1.9±0.2
RSC 5	35	1.2±0.3	17	0.5±0.3	0	0	54	0	32	1.8±0.2	28	1.6±0.2
RSC 6	46	1.0±0.2	11	1.0±0.1	0	0	56	0	71	1.7±0.3	13	2.1±0.3
RSC 7	39	1.0±0.3	18	1.2±0.2	0	0	43	0	12	1.1±0.1	64	0
RSC 8	15	1.5±0.2	22	0	0	0	58	0	9	1.0±0.1	72	0
RSC 9	4	1.0±0.3	25	0	0	0	53	0	0	0	74	0
RSC 10	9	1.0±0.0	13	0	0	0	55	0	0	0	87	0
RSC 11	28	1.1±0.1	16	0.4±0.1	0	0	70	0	17	1.2±0.2	59	1.2±0.5
RSC 12	7	1.0±0.1	18	0	0	0	43	0	0	0	86	0
RSC 13	13	1.0±0.3	9	0	0	0	51	0	0	0	94	0
RSC 14	30	1.2±0.2	10	1.0±0.5	0	0	59	0	4	1.2±0.1	65	0

[1] As described in Table 4.1. [2] Data are means of 10 replicates±standard error. [3] Determined using method described by Motley and Keen (1987).

Table 4.5 Growth of in vitro shoot cultures of cvs. Little White Pet, The Countryman and Katherina Zeimet.

	Little White Pet				The Countryman				Katherina Zeimet			
Medium Code[1]	Explants Producing Shoots (%)	Mean No. of Shoots per Explant[2]	Explants Browning (%)	Multiplic-ation Rate[3]	Explants Producing Shoots (%)	Mean No. of Shoots per Explant[2]	Explants Browning (%)	Multiplic-ation Rate[3]	Explants Producing Shoots (%)	Mean No. of Shoots per Explant[2]	Explants Browning (%)	Multiplic-ation Rate[3]
RSC 1	62	1.4±0.2	17	1.4±0.1	73	1.6±0.3	25	1.7±0.1	0	0	41	0
RSC 2	56	1.2±0.1	19	1.1±0.1	79	2.0±0.2	13	2.1±0.1	0	0	54	0
RSC 3	0	0	34	0	68	1.3±0.1	17	1.8±0.1	0	0	76	0
RSC 4	0	0	29	0	89	1.1±0.1	11	1.1±0.2	0	0	62	0
RSC 5	51	1.1±0.4	29	1.0±0.1	44	1.0±0.1	57	0	31	1.1±0.1	37	1.1±0.1
RSC 6	37	1.1±0.1	32	1.1±0.1	27	1.2±0.4	53	0	0	0	90	0
RSC 7	0	0	43	0	14	1.1±0.1	55	0	0	0	94	0
RSC 8	0	0	37	0	9	1.0±0.1	76	0	0	0	100	0
RSC 9	29	1.1±0.1	23	1.5±0.3	0	0	78	0	19	1.5±0.2	23	1.4±0.2
RSC 10	24	1.2±0.1	26	1.0±0.1	0	0	64	0	55	1.8±0.2	39	1.8±0.3
RSC 11	30	1.4±0.1	19	1.3±0.1	0	0	87	0	62	2.2±0.3	35	2.1±0.3
RSC 12	13	1.0±0.1	21	0	0	0	82	0	38	1.0±0.1	22	1.6±0.4
RSC 13	11	1.0±0.0	37	0	0	0	94	0	45	1.4±0.1	12	1.0±0.2
RSC 14	17	1.1±0.1	39	0	0	0	68	0	67	1.9±0.1	17	2.0±0.3

[1, 2, 3] See footnote Table 4.4

Table 4.6 Summary of the optimal growth regulator concentrations required for micropropagation of English rose cultivars.

Cultivar	Growth Regulator (mg l^{-1})				Medium
	BAP	GA_3	NAA	IAA	Code
Abraham Darby	1.0	0.1	0.004	-	RSC1
Marie Pavié	2.0	0.1	0.004	-	RSC2
Mary Rose	2.0	0.1	0.004	-	RSC2
Little White Pet	0.1	-	-	0.1	RSC9
The Countryman	2.0	0.1	0.004	-	RSC2
Katharina Zeimet	1.0	-	-	0.1	RSC11

4.3.1.3 Prevention of tissue browning.

The effects of the various treatments aimed to reduce browning on 3 of the English rose cultivars are shown in Table 4.7. In the untreated controls, explant browning was maximal with cv. Heritage and minimal with cv. Abraham Darby. In all cultivars, a 12 h soak in ascorbic acid and sodium borate solution resulted in the smallest percentage of explants browning, the lowest value being observed with this treatment when applied to the cv. Abraham Darby. Generally, the 12 h soak treatments prior to placing explants into culture had greater effects, in terms of a reduction of browning, than did incorporating identical compounds into the agar-solidified culture media.

Table 4.7 Effects of various treatments on the *in vitro* browning of explants of three English rose cultivars.

Treatment	% Explants Browning		
	Abraham Darby	Heritage	Marie Pavié
Control	12	100	15
a. PVP in medium	13	89	15
b. Charcoal in medium	11	100	11
c. 12 h soak: PVP solution	9	76	10
d. 12 h soak: ascorbic acid and sodium borate solution	4	59	5
e. 12 h soak: 8-HQS solution	12	71	10

4.3.2 Initiation, maintenance and regeneration of callus cultures.

4.3.2.1 Establishment of callus cultures.

There were clear differences in callusing responses between cultivars, explants and media (see Tables 4.8 - 4.15). Callusing was most frequently observed on explants that had been cultured in the dark. In some cultivars, such as Francine Austin, callus production was entirely restricted to explants that had been held under dark conditions. In general, calli produced in the dark were more friable and paler in colour than calli produced in the light on comparable medium. The cvs. Mary Rose (Plate 4.1d), Katharina Zeimet and Phyllis Bide produced calli on the majority of explants and media types and under both dark and light conditions. However, the calli produced did vary in both colour and texture. Other cultivars showed a much more specific response to the growth regulator components of the media. For instance, the cv. Abraham Darby produced calli only on media that contained 2,4-D (except for MSP1), while explants of the cv. Marie Pavié only produced callus on

media containing NAA and either 2,4-D or kinetin. The cv. Ballarina exhibited callus production only on media containing kinetin or low concentrations of BAP (0.5 mg l^{-1}) irrespective of the presence or absence of 2,4-D.

In no cultivar was callus proliferation exclusive to one particular explant type. In the cv. Abraham Darby, leaf discs generally callused more profusely than did internodal sections. This contrasts with the cv. Mary Rose in which callusing was more profuse on internodal sections. In the majority of cultivars, the effect of media type or light/dark culture was independent of explant type.

Table 4.8 Callusing responses of the English rose cv. Mary Rose after 30 d culture.

Medium	Explant	Proliferation [1]		Texture [2]		Colour [3]	
		Light	Dark	Light	Dark	Light	Dark
MSP1	Leaf	7	14	I	F	yg	yw
	Internode	12	19	I	I	yg	yw
CM1	Leaf	15	19	I	I	yg	yw
	Internode	16	22	I	I	yg	yw
CM2	Leaf	11	21	I	F	yg	gg
	Internode	30	29	I	F	yg	gg
CM3	Leaf	13	14	I	F	yg	gg
	Internode	11	16	I	F	yg	gg
CM4	Leaf	4	8	I	F	yg	gg
	Internode	3	8	I	I	yg	yw
CM5	Leaf	3	13	I	I	yg	yw
	Internode	24	29	I	I	yg	gg
CM6	Leaf	17	24	I	I	yg	gg
	Internode	20	27	I	I	yg	yw
CM7	Leaf	14	12	I	F	yg	yw
	Internode	18	25	I	F	yg	yw
CM8	Leaf	22	22	I	I	yg	yw
	Internode	24	29	I	I	yg	gg
CM9	Leaf	7	4	I	I	yg	yw
	Internode	9	12	I	I	yg	yw
CM10	Leaf	22	24	I	F	yg	gg
	Internode	14	29	I	F	yg	gg

[1] Proliferation was assessed as mean callus area (mm^2). [2] Texture: H = hard, I = intermediate, F = friable. [3] Colour: b = greyed-orange (Group 176c), bg = greyed-

brown (Group 199d), by = greyed-orange (Group 164d), g = green (Group 143b), gy = yellow-green (Group 144a), yg = yellow-green (Group 151c), yw = yellow-white (Group 158b), gg = greyed-green (Group 196d).

Table 4.9 Callusing responses of the English rose cv. Phyllis Bide after 30 d culture.

Medium	Explant	Proliferation [1]		Texture [2]		Colour [3]	
		Light	Dark	Light	Dark	Light	Dark
MSP1	Leaf	8	30	I	F	g	yw
	Internode	7	38	I	F	g	yw
CM1	Leaf	12	12	I	I	gy	yw
	Internode	8	25	I	F	gy	yw
CM2	Leaf	4	20	I	F	g	yw
	Internode	5	28	I	F	g	yw
CM3	Leaf	9	26	I	F	yg	ywyw
	Internode	9	22	I	F	g	ywgg
CM4	Leaf	2	18	I	I	yg	yw
	Internode	8	27	I	F	g	yw
CM5	Leaf	6	13	I	I	g	yw
	Internode	14	16	F	I	g	yw
CM6	Leaf	3	33	I	F	gy	yw
	Internode	3	34	I	F	gy	gg
CM7	Leaf	0	25	-	F	-	yw
	Internode	4	28	F	F	gy	yw
CM8	Leaf	9	15	F	F	g	yw
	Internode	6	29	F	F	gy	yw
CM9	Leaf	5	12	I	I	g	yg
	Internode	7	19	I	I	g	yw
CM10	Leaf	2	14	I	I	g	yw
	Internode	4	17	I	I	g	yg

[1, 2, 3] See footnote Table 4.8.

Table 4.10 Callusing responses of the English rose cv. Katharina Zeimet after 30 d culture.

Medium	Explant	Proliferation [1]		Texture [2]		Colour [3]	
		Light	Dark	Light	Dark	Light	Dark
MSP1	Leaf	3	7	H	H	b	by
	Internode	6	12	H	H	b	by
CM1	Leaf	2	6	H	H	b	b
	Internode	5	5	H	H	b	b
CM2	Leaf	2	7	H	H	b	bg
	Internode	7	4	H	H	b	bg
CM3	Leaf	3	5	H	H	b	bg
	Internode	9	13	H	H	b	bg
CM4	Leaf	9	8	H	H	b	by
	Internode	2	4	H	H	b	by
CM5	Leaf	2	11	H	H	b	by
	Internode	4	9	H	H	b	by
CM6	Leaf	10	12	H	H	b	by
	Internode	10	18	H	H	b	by
CM7	Leaf	3	3	H	H	b	bg
	Internode	1	4	H	H	b	bg
CM8	Leaf	5	7	H	H	b	bg
	Internode	4	11	H	H	b	bg
CM9	Leaf	2	0	H	H	b	by
	Internode	6	4	H	H	b	bg
CM10	Leaf	8	9	H	H	b	bg
	Internode	3	6	H	H	b	bg

[1, 2, 3] See footnote Table 4.8.

Table 4.11 Callusing responses of the English rose cv. Ballerina after 30 d culture.

Medium	Explant	Proliferation [1]		Texture [2]		Colour [3]	
		Light	Dark	Light	Dark	Light	Dark
MSP1	Leaf	0	0	-	-	-	-
	Internode	0	0	-	-	-	-
CM1	Leaf	0	0	-	-	-	-
	Internode	0	0	-	-	-	-
CM2	Leaf	0	0	-	-	-	-
	Internode	0	0	-	-	-	-
CM3	Leaf	2	3	H	H	bg	by
	Internode	7	9	H	H	bg	by
CM4	Leaf	2	3	H	I	bg	by
	Internode	2	2	H	H	bg	by
CM5	Leaf	3	6	H	H	bg	by
	Internode	7	9	H	H	bg	by
CM6	Leaf	0	0	-	-	-	-
	Internode	0	0	-	-	-	-
CM7	Leaf	2	2	H	I	by	by
	Internode	8	3	H	I	bg	by
CM8	Leaf	0	9	-	H	-	by
	Internode	3	2	H	H	by	by
CM9	Leaf	5	7	H	H	by	by
	Internode	6	3	H	H	by	by
CM10	Leaf	3	3	H	I	by	by
	Internode	9	3	H	I	by	by

[1, 2, 3] See footnote Table 4.8.

Table 4.12 Callusing responses of the English rose cv. Francine Austin after 30 d culture.

Medium	Explant	Proliferation [1]		Texture [2]		Colour [3]	
		Light	Dark	Light	Dark	Light	Dark
MSP1	Leaf	0	2	-	H	-	b
	Internode	0	4	-	H	-	b
CM1	Leaf	0	4	-	H	-	b
	Internode	0	3	-	H	-	b
CM2	Leaf	0	6	-	H	-	b
	Internode	0	2	-	H	-	b
CM3	Leaf	0	2	-	H	-	b
	Internode	0	8	-	H	-	b
CM4	Leaf	0	0	-	-	-	-
	Internode	0	0	-	-	-	-
CM5	Leaf	0	3	-	H	-	by
	Internode	0	3	-	H	-	bg
CM6	Leaf	0	8	-	H	-	b
	Internode	0	2	-	H	-	bg
CM7	Leaf	0	7	-	H	-	b
	Internode	0	2	-	H	-	b
CM8	Leaf	0	7	-	H	-	b
	Internode	0	4	-	H	-	b
CM9	Leaf	0	4	-	H	-	b
	Internode	0	9	-	H	-	b
CM10	Leaf	0	0	-	-	-	-
	Internode	0	0	-	-	-	-

[1, 2, 3] See footnote Table 4.8.

Table 4.13 Callusing responses of the English rose cv. Abraham Darby after 30 d culture.

Medium	Explant	Proliferation [1]		Texture [2]		Colour [3]	
		Light	Dark	Light	Dark	Light	Dark
MSP1	Leaf	0	20	-	F	-	yg
	Internode	0	28	-	F	-	yw
CM1	Leaf	0	0	-	-	-	-
	Internode	0	6	-	H	-	yw
CM2	Leaf	0	0	-	-	-	-
	Internode	0	0	-	-	-	-
CM3	Leaf	0	0	-	-	-	-
	Internode	0	0	-	-	-	-
CM4	Leaf	0	19	-	F	-	yg
	Internode	0	19	-	F	-	yg
CM5	Leaf	0	35	-	F	-	gg
	Internode	0	22	-	F	-	gg
CM6	Leaf	0	30	-	F	-	yg
	Internode	0	13	-	M	-	yg
CM7	Leaf	0	0	-	-	-	-
	Internode	0	0	-	-	-	-
CM8	Leaf	0	0	-	-	-	-
	Internode	0	0	-	-	-	-
CM9	Leaf	0	0	-	-	-	-
	Internode	0	0	-	-	-	-
CM10	Leaf	0	18	-	M	-	gy
	Internode	0	7	-	I	-	gy

[1, 2, 3] See footnote Table 4.8.

Table 4.14 Callusing responses of the English rose cv. The Countryman after 30 d culture.

Medium	Explant	Proliferation [1]		Texture [2]		Colour [3]	
		Light	Dark	Light	Dark	Light	Dark
MSP1	Leaf	0	0	-	-	-	-
	Internode	0	0	-	-	-	-
CM1	Leaf	0	0	-	-	-	-
	Internode	0	0	-	-	-	-
CM2	Leaf	0	0	-	-	-	-
	Internode	0	0	-	-	-	-
CM3	Leaf	0	23	-	I	-	by
	Internode	0	18	-	I	-	by
CM4	Leaf	0	21	-	H	-	yg
	Internode	0	27	-	H	-	yg
CM5	Leaf	0	14	-	I	-	yw
	Internode	0	19	-	I	-	yw
CM6	Leaf	0	26	-	I	-	yw
	Internode	0	36	-	I	-	yw
CM7	Leaf	0	0	-	-	-	-
	Internode	0	0	-	-	-	-
CM8	Leaf	0	0	-	-	-	-
	Internode	0	0	-	-	-	-
CM9	Leaf	0	0	-	-	-	-
	Internode	0	0	-	-	-	-
CM10	Leaf	0	5	-	H	-	by
	Internode	0	8	-	H	-	bg

[1, 2, 3] See footnote Table 4.8.

Table 4.15 Callusing responses of the English rose cv. Marie Pavié after 30 d culture.

Medium	Explant	Proliferation [1]		Texture [2]		Colour [3]	
		Light	Dark	Light	Dark	Light	Dark
MSP1	Leaf	0	29	-	H	-	yw
	Internode	0	38	-	H	-	yw
CM1	Leaf	0	0	-	-	-	-
	Internode	0	0	-	-	-	-
CM2	Leaf	0	0	-	-	-	-
	Internode	0	0	-	-	-	-
CM3	Leaf	0	0	-	-	-	-
	Internode	0	0	-	-	-	-
CM4	Leaf	0	0	-	-	-	-
	Internode	0	0	-	-	-	-
CM5	Leaf	0	0	-	-	-	-
	Internode	0	0	-	-	-	-
CM6	Leaf	0	18	-	H	-	yg
	Internode	0	22	-	H	-	yg
CM7	Leaf	0	9	-	H	-	gg
	Internode	0	10	-	H	-	gg
CM8	Leaf	0	28	-	H	-	gg
	Internode	0	29	-	H	-	gg
CM9	Leaf	0	31	-	H	-	gg
	Internode	0	36	-	H	-	gg
CM10	Leaf	0	30	-	H	-	gg
	Internode	0	33	-	H	-	gg

[1, 2, 3] See footnote Table 4.8.

4.3.2.2 Regeneration from rose callus.

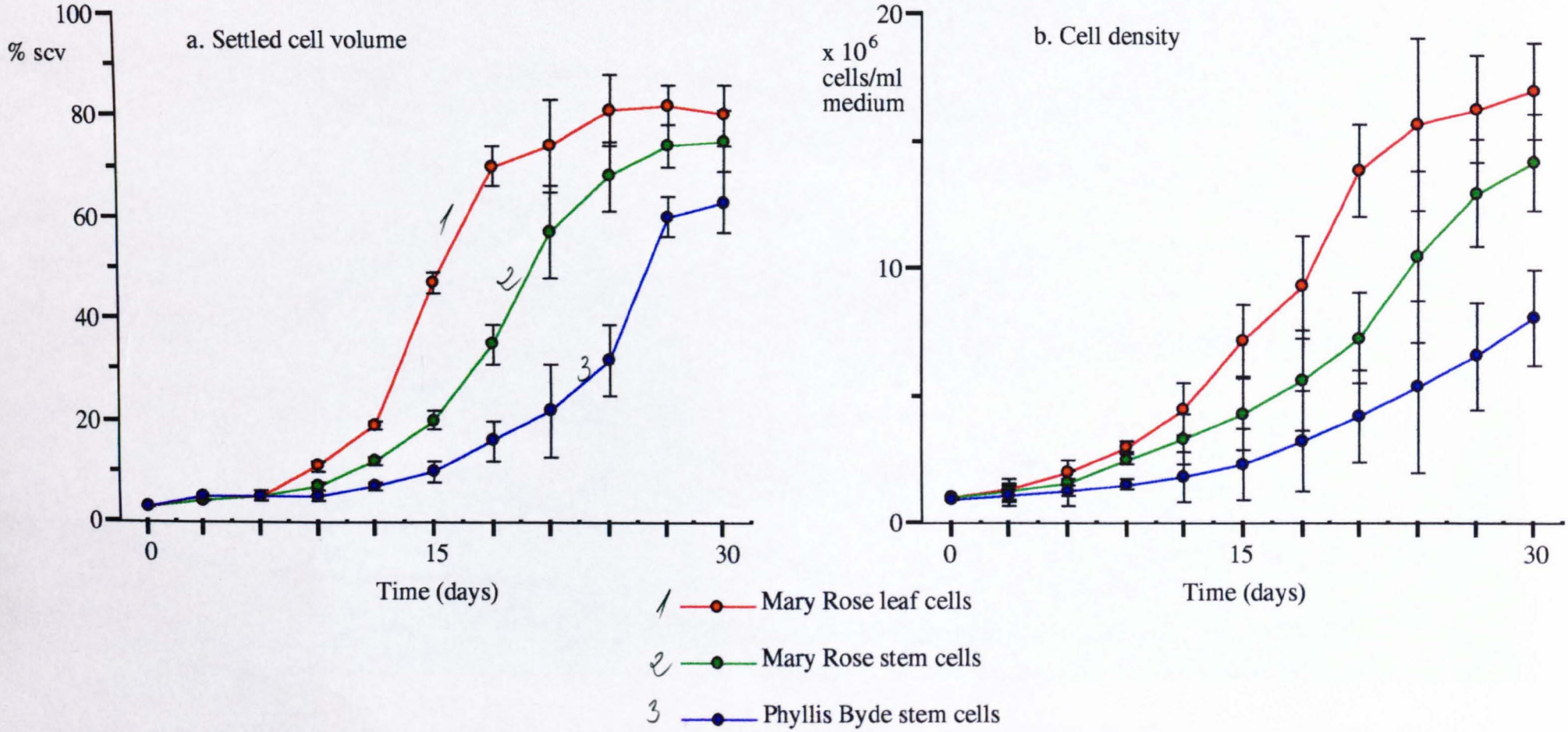
Callus placed on regeneration media was maintained under the conditions described in Section 4.2.3 for up to 6 months. During this time callus that proliferated was subcultured at 4 weekly intervals. After approx. 10 weeks, callus incubated under light conditions tended to brown and failed to exhibit sustained growth. Callus maintained under dark conditions continued to proliferate and retained its characteristic appearance, but at no time was either adventitious shoot regeneration or embryo induction observed.

4.3.3 Initiation and maintainance of cell suspensions.

Leaf and stem callus from the cv. Mary Rose and stem callus from cv. Phyllis Bide were chosen for the initiation of cell suspensions, because these calli were rapidly proliferating, friable and pale in colour. It was only possible to initiate cell suspensions in MSP1 medium. When calli were transfered to liquid counterparts of their initiation media they remained as large cell aggregates, failing to become friable and frequently becoming necrotic. Figure 4.1 shows the growth curves of cell suspension cultures of the cv. Mary Rose (leaf and stem origin) and of the cv. Phyllis Bide (stem origin), as determined by settled cell volume (Fig 4.1a) and by measurements of cell density (Fig 4.1b). Leaf derived cells of the cv. Mary Rose showed a more rapid growth rate when compared to stem-derived cells of the same cultivar. Stem-derived cells of the cv. Phyllis Bide exhibited an even slower rate of growth. Based on this basic data, cell suspensions were subsequently subcultured after 14 d by transfering 10 ml of suspension (containing 2 ml of settled cells) to 70 ml of fresh medium. After 4-6 weeks, microscopic examination revealed that such suspensions contained small aggregates of spherical cells (approximately 20 cells per aggregate) which grew to give aggregates of approximately 100 cells (Plate 4.1e). After 6 - 8 subcultures, cell suspensions were prone to cellular reaggregation, cell elongation and browning, which made them unsuitable for subsequent manipulations,

such as the isolation of protoplasts. For these reasons, cell suspensions for protoplast sources were regularly reinitiated on a short-term basis.

Figure 4.1 Growth of rose cell suspensions.



Values are means of 10 replicates. Bars represent standard errors.

Plate 4.1.

a. *In vitro* shoot production from an *in vivo*-derived internodal explant of the English rose cv. Abraham Darby on RSC 1 medium (x 4.6).

b. *In vitro* shoot multiplication of English rose cv. Abraham Darby on RSC 1 medium after 3 subcultures (x 0.8).

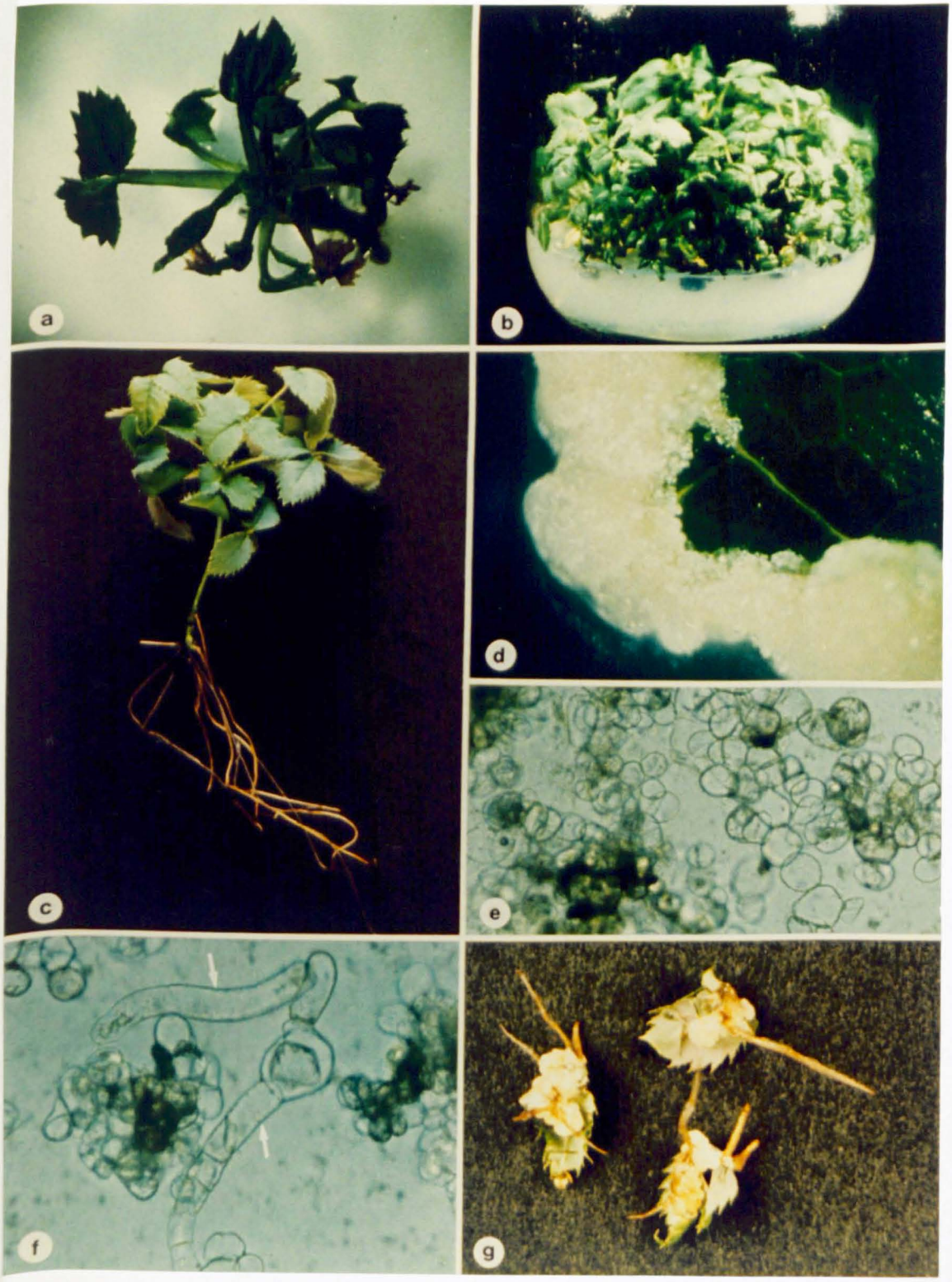
c. *In vitro* derived shoot of English rose cv. Abraham Darby displaying adventitious rooting following the procedure described in Section 4.2.2.4 (x 1.3).

d. Callus, deemed suitable for the initiation of cell suspensions, produced after 30 d culture from the margin of leaf discs of the English rose cv. Mary Rose on CM 2 medium (x 8).

e. Cellular aggregates in cell suspension of cv. Mary Rose after 4 subcultures in MSP1 medium (x 150).

f. Cellular aggregates in cell suspensions of cv. Mary Rose after 8 subcultures. Note elongated cells (arrowed), which ultimately renderd the suspensions unsuitable for further manipulations (x 150).

g. Rhizogenesis from excised *in vitro* leaves of English rose cv. Abraham Darby cultured on medium containing 3.0 mg l^{-1} 2, 4-D (x 2.3).


a
b
c
d
e
f
g

4.3.4 Adventitious regeneration from rose tissues.

After 8 weeks of culture, out of the 207 combinations of growth regulators tested, only those that included 3.0 mg l^{-1} 2,4-D, either without cytokinins or cytokinins at concentrations less than 1.0 mg l^{-1}, produced adventive regeneration. All of the explants (Abraham Darby and Marie Pavié) subjected to this treatment expressed a rhizogenic response, together with a small amount of callus on the margins of the scored leaf sections (see Plate 4.1g). Such roots were subjected to the shoot-regeneration procedure of Ochatt and Power (1988) for *Prunus cerasus* L., in which rhizogenesis (from protoplast-derived calli) was an intermediate step towards shoot bud differentiation. However, plant regeneration from rose root material did not occur.

4.4 Discussion.

4.4.1 Explant sterilisation and browning.

Mercuric chloride was selected as the sterilant for routine use, since it resulted in good control of microbial contamination. Although mercuric chloride presented some problems in its use (it is a potential carcinogen and must be disposed of with caution to avoid environmental damage), it was felt that the use of this compound was justified, since microbial contamination was minimised. It has been shown that reducing the bacterial loading of initial explants to a minimum will result in a decrease in the possibility/occurence of endogenous contaminants at later stages of tisssue culture (Bastiaens, 1983). This is particularly important, as many of the antibiotics that have been used in tissue culture to remove endogenous contaminants of shoots post-initiation of cultures may have a deleterious effect at a subsequent stage (such as during later growth of the shoots or callus initiation) (Bastiaens, 1983). Such effects may be attributed either to the compounds having a direct action on the plant cells (such as the disruption photosynthetic activity) (Bastiaens, 1983), or to the growth regulator-like properties of some antibiotics and their breakdown components (Holford and Newbury, 1992; Debergh *et al.*, 1993). It was therefore felt that the use

of mercuric chloride would reduce the chances of having to resort to the use of antibiotics to eliminate endogenous contaminants. However, it became apparent that a small number of culture lines did contain endogenous microbial contaminants which were observed as 'haloes' of bacterial contamination on, or just below, the medium surface surrounding the shoot bases. The use of mercuric chloride, in a commercial situation for rose micropropagation, is probably not feasible, because the increased costs that would result from its use (the need to purchase fume hoods for handling, and disposal costs) are probably greater than the expenditure that would be saved in reducing losses. This is particularly the case with commercial rose micropropagation companies, who can maintain cultures, albeit in a contaminated condition, as long as the contamination does not result in a reduction in the growth of the cultures (N. Wright, Microprop Roses Ltd, East Leake, UK; pers. comm.), since such cultures are rarely required to be axenic for any subsequent procedures such as protoplast isolation. Additional cultures are usually initiated in order to offset predicted losses due to microbial contamination.

In addition to microbial contamination, the other persistent problem encountered with the establishment of *in vitro* cultures of woody species is that of explant browning. This has been reported in many woody species, incuding apple (Laimer de Câmara Machado *et al.*, 1991), coffee and *Cacao* (Duhem *et al.*, 1988). It leads not only to explant death, but also to necrosis of any tissues, such as shoots or calli, that are attached to the explants. Additionaly, when explants brown, they often also exude phenolic compounds into the culture medium (Preece and Compton, 1991), which can lead to death of adjacent tissues on the surface of the medium, as was found for English roses. Many remedies have been proposed to solve this problem, including the use of ascorbic acid, polyvinylpyrrolidone (PVP) and activated charcoal (Duhem *et al.*, 1988; George and Sherington, 1984; Lê, 1985 respectively). Such treatments have differing success rates with several different plant species. In some of the rose cultivars used in this study, explant browning at the initiation stage was a major

problem. Generally though, when the levels of explant browning were high, the rate of micropropagation, in terms of the percentage of explants producing shoots, number of shoots per explant and multiplication rates were, perhaps not surprisingly, reduced. Agents that were investigated for the reduction of browning had all been used previously for many woody species, except for 8-HQS which has only been reported as being effective in reducing explant browning in apple (Laimer de Câmara Machado *et al.*, 1991). In the present study, the use of a combination of ascorbic acid and sodium borate as a 12h soak was most effective in reducing this problem. Since plants produce a range of phenolic compounds (Preece and Compton, 1991), it may be that multicomponent treatments are going to be more effective in cases such as rose. Protocols were more effective when incorporating a 12 h liquid soak period rather than their use in semi-solidified culture media. The possible reasons for this are twofold. Firstly, in using a soak treatment, explants are subjected to treatment at a much earlier stage and this is especially important when the timing of phenolic oxidation is considered. It is likely that the oxidation of the phenolic compounds begins very shortly after the explant is trimmed and, as such, it is important that the treatment is received early. Secondly, liquid treatments may penetrate readily to where phenolic oxidation is occurring. It should also be noted that compounds, such as PVP and the ascorbic acid - sodium borate mixture, which inhibit the oxidation of phenolics by blocking polyphenoloxidases, appear to be more effective than those, such as activated charcoal, which target the toxins produced once phenolic compounds have been oxidised. In the cv. Heritage, the minimal (percentage) explant browning that could be achieved was 59% with the the ascorbic acid - sodium borate treatment. The fact that explant browning remained high, even with antioxidant treatments, and that there remained great difficulty in establishing cultures even when explant browning was reduced, would seem to suggest that for this group other compounds may be acting as inhibitory agents. It is known, for example, that in addition to polyphenols, plants may contain other inhibitory compounds, such as coumarins, terpenoids and steroids (Muhitch and Fletcher, 1985).

4.4.2 Axenic shoot cultures.

Overall, the results of attempts to establish axenic shoot cultures of English rose were similar to those observed in other rose species/cultivars. With most of the rose cultivars employed in this study, the mean numbers of explants producing shoots were comparable to studies involving cultivars of *R. hybrida* (Skirvin and Chu, 1979; Davies, 1980; Douglas *et al.*, 1989) and other species of rose, including *R. canina* and *R. damascena* (Khosh-Khui and Sink, 1982). To date, there have been no reports on the successful micropropagation of the rose species *R. chinensis*, to which English roses are usually ascribed (Chapter 1; Section 1.3.3). Consequently, comparisons of the protocols established here with similar cultivars are not possible.

Whilst two of the cultivars were propagated on media containing BAP and IAA, the optimal medium for the *in vitro* culture of the majority of the English rose cultivars used in this study consisted of a combination of BAP, GA_3 and NAA. Although BAP and NAA, at similar concentrations to those used here, have commonly been employed in the micropropagation of a number of rose species/cultivars, including *R. rugosa* (Tweedle *et al.*, 1984), *R. canina* (Martin *et al.*, 1981), *R hybrida* cv. Ilseta (Sauer *et al.*, 1985) and *R. hybrida* cv. Forever Yours (Skirvin and Chu, 1979), it is less common for GA_3 to be a critical component. The medium for shoot multiplication of *R. hybrida* cvs. Kings Ransom, Parade and Plentiful (Davies, 1980) contained 0.1 mg l^{-1} GA_3, as did that of Horn *et al.* (1988), which was used for the multiplication of several cultivars of *R. hybrida*, including Alexander, Golden Times and Kardinal. However, Hasegawa (1979) reported that shoot proliferation was inhibited in *R. hybrida* cv. Improved Blaze when GA_3 was used. In a related genus, *Pyrus communis* L. cv. Barlett, GA_3 neither stimulated nor inhibited shoot multiplication (Lane, 1979). Despite these observations, the results of this study would appear to indicate that GA_3 is required for the effective *in vitro* propagation of a number of English rose cultivars. The results also concur with those of Khosh-Khui and Sink (1982) who demonstrated that levels of BAP in excess of 4 mg l^{-1}

suppressed the *in vitro* growth of *R. hybrida*. It is clear that for efficient micropropagation, rose cultivars/species have different requirements in terms of growth regulators, as can be seen in recent reviews and discussions of rose micropropagation (Skirvin *et al.*, 1990; Short and Roberts, 1991) and by the obervations of this study. The reasons why the cultivars in the present study showed such a distinct difference in terms of their growth regulator requirements cannot be determined. However, the difference between cultivars with respect to their responses in culture, is almost certainly genetically determined. The genetic basis underlying the variation in tissue culture response has been studied in several species, including tomato (Frankenberger *et al.*, 1981), maize (Tomes and Smith, 1985) and white spruce (Park *et al.*, 1993), using the analytical techniques of quantitative genetics. To date, attempts to assign genes controlling the tissue culture behaviour to specific chromosome segments has only been reported for wheat (Langridge *et al.*, 1991) and tomato (Koornneef, 1993). The observations in this present study on English rose may simply reflect the diverse and complex origins of many cultivars, and perhaps provide circumstantial evidence for the incorrect classification and assumed origins of at least some of the cultivars ascribed to this group.

The ease with which *in vitro* rooting was achieved for English rose cultivars employed in this study, is consistent with other studies, such as those involving *R. hybrida* (Hasegawa, 1980); Khosh-Khui and Sink, 1982) in which relatively high percentages of explants produced roots even in the absence of added growth regulators. These authors reported that the inclusion of IAA or NAA in the medium further enhances *in vitro* rooting. Despite the relative ease of rooting *in vitro* generated rose shoots, difficulties in transferring such material to *ex vitro* conditions are apparent (Skirvin and Chu, 1979; Douglas *et al.*, 1989). Smith *et al*, (1991) have also reported that the structure of rose roots generated *in vitro* can be anomalous and that the root to shoot ratio on plants, originally rooted *in vitro*, remains abnormally low, even in later *ex vitro* production stages. Lloyd *et al.* (1988) and Roberts *et al.*

(1990) have suggested that the use of cellulose plugs (Sorbarods®) may help in the production of roots with a more normal morphology and may prevent damage of delicate roots during the transfer process, thus leading to an increased survival of plants *ex vitro*. In this study, with English rose, it was felt that the use of Sorbarods® was unnecessary, as a high survival was achieved *ex vitro*. However, on a commercial scale, the use of Sorbarods®, along with misters and humidifiers at the transfer stage, would probably be deemed desirable on commercial grounds.

There are a number of factors, in addition to those investigated in this study, which have been shown to affect the *in vitro* propagation of several rose cultivars. In the present study, attempts were made to optimise factors such as type of explant, temperature and light regime based previous reports, such as those by Jacobs *et al.* (1969), Hasegawa 1980 and Bressan *et al.* (1982). Other workers have demonstrated that other factors to such as the introduction of CO_2 into the culture vessel (Woltering, 1990), use of different types of culture vessels (McClelland and Smith, 1990) and the use of ethylene inhibitors (Kevers *et al.*, 1992) improve the *in vitro* propagation of rose. These were not investigated in the present study as it was felt that the growth of the cultures was more than adequate in terms of providing source materials for further manipulations including protoplast studies.

4.4.3 Callus cultures.

The cultivar-dependent responses that were observed during attempts to initiate English rose callus in this study are typical of those observed in a wide range of rose species/cultivars (Short and Roberts, 1991). Both leaf and internode explants have previously been employed for the initiation of rose callus (de Wit *et al.*, 1990; Ishioka and Tanimoto, 1990), and it would appear that, in some cultivars, one particular explant type, such as stem-derived material, is superior. Such a marked response was not substantiated in this study, although in general leaf discs gave more callus than internodal sections. Most reports on the initiation of rose callus suggest that initiation

is improved in the dark (de Wit *et al.*, 1990); such results were reflected in this study. A large proportion of the media/protocols used for callus induction in this study were based on protocols that had been established for embryogenic callus production, or to enable adventitious regeneration to occur in rose (see references in Table 4.2) or in related species such as *Prunus* (Druart, 1980). In many of these reports, plant regeneration was observed on the same medium on which the calli were initiated/maintained, whilst in other reports (Valles and Boxus, 1987), callus had to be transferred to different media (as described in Section 4.2.3.2) in order to induce embryogenesis/regeneration. However, during this study, neither embryogenic calli nor adventitious regeneration from callus were induced. Despite the fact that rose has been investigated for many years, rose regeneration has only recently been developed, and is particularly cultivar-dependant as found for the English rose cultivars. Some studies have indicated that compounds such as L-proline, when incorporated into the culture medium, may have a rôle to play in promoting embryogenesis in certain cultivars of *R. hybrida* (Rout *et al.*, 1991), whilst investigations by Kunitake *et al.* (1993) have shown that the choice of carbohydrate is critical for somatic embryogenesis in *R. rugosa.* In particular, 0.1M fructose or sucrose was demonstrated to enhance embryo formation, and 0.1M sorbitol to promote subsequent embryo germination and growth.

4.4.5 Cell suspensions.

Cell suspensions were successfully established from friable callus (Section 4.2.3.1) as this has been shown to be the most logical starting material in most systems (Hall, 1991). Of the two methods used to determine growth curves, the measurement of cell number was more accurate as it is an exact measurement as compared to settled cell volume, where the error may be as great as 3%. However, the measurement of cell numbers using a haemocytometer necessitates cell separation using the chromium trioxide technique (Section 4.2.4 ii) before the measurement can be made. Growth curves were similar to those reported for other Rosaceous genera including *Prunus*

(Ochatt, 1989), *Malus* (Manders, 1992) and for cultures of *R. hybrida* cv. Paul's Scarlet (Nash and Dudley, 1972). The reaggregation of cellular material, observed in English roses, has also been noted for other genera such as *Prunus* (Ochatt, 1989).

The reason why cell suspensions could not be sustained on a liquid version of their initiation medium is unclear, but it could be that they have different requirements, such as respiratory gases.

4.4.6 Adventitious regeneration.

One of the few reports of direct adventitious regeneration from leaf explants in rose is that of Lloyd *et al.* (1988), in which shoot formation occurred from excised leaves of *R. persica* x *xanthia*, *R laevigata* and *R. wichuriana* on a medium containing 0.5 mg l^{-1} BAP alone. The use of media containing a high cytokinin to low auxin ratio has been widely employed for the induction of adventitious regeneration in many plant species, including woody species such as pear (Chevreau *et al.*, 1989). In English rose, the use of such media failed to result in adventitious shoot regeneration. Moreover, in recent years, it has been demonstrated that the potent cytokinin TDZ can effectively induced adventitious shoot regeneration in previously recalcitrant woody species [see review by Lu (1993) and Huetteman and Preece (1993)]. TDZ has been successfully used to induce adventitious regeneration in many Rosaceous species, including *Malus domestica* (Sriskandarajah, 1990), *Pyrus communis* (Leblay *et al.*, 1991) and *Rubus idaeus* (Cousineau and Donelly, 1991). However, for English roses, TDZ was also ineffective and, with the exception of limited root production from leaf explants cultured in the presence of 3 mg l^{-1} 2,4-D, regeneration was not observed. Root production, directly from leaf explants, is uncommon in woody species, though it has been reported for some *Prunus* species (Mehra and Mehra, 1974; Hedtrich, 1977). The suppression of shoot regeneration, allied to such rhizogenesis, may be caused by a relatively high level of endogenous auxins, which are sufficient to repress

shoot formation. However, when media are supplemented with highly physiologically active auxin such as 2,4-D, this leads to root production.

It was anticipated that the proven ability to regenerate shoots via tissue culture-generated roots of excised leaf origin may provide an alternative pathway to rose regeneration. In this respect, Ochatt and Power (1988) regenerated plants from protoplasts of *Prunus cerasus* via rhizogenic calli using BAP and low concentrations of NAA. Lloyd *et al.* (1988) also demonstrated that it was possible to induce shoots from excised tissue culture-derived roots of *R. persica* x *xanthia*, with only BAP present. However, for English roses these options were not realised.

4.5 Summary.

i) Phenolic browning of rose explants maintained *in vitro* was reduced with a 12 h treatment, prior to culture, in an antioxidant mixture consisting of 4.0% ascorbic acid and 1.2% sodium borate.

ii) Micropropagation strategies were established for several English rose cultivars using MS-based media, but differential cultivar growth regulator requirements were apparent. A threeway combination of BAP, GA_3 and NAA was required.

iii) Rooting of axenic shoot cultures was readily achieved on semi-solid MS medium which lacked growth regulators, preceding transfer of plantlets to *ex vitro* conditions.

iv) Genotype-dependent callusing responses were observed, but friable, rapidly growing English rose callus was obtained. However, this lacked embryogenic and/or adventitious regeneration potential on the media that were screened.

v) Cell supensions of leaf derived calli for the cv. Mary Rose and from stem derived calli of the cvs. Mary Rose and Phyllis Byde, were established in liquid MSP1 medium.

vi) Rhizogenesis was induced in leaf discs of the cvs. Abraham Darby and Marie Pavié on medium containing 3.0 mg l^{-1} 2,4-D, but indirect shoot regeneration was not possible.

CHAPTER 5.
Isolation, culture and regeneration assessments for English rose protoplasts.

5.1 Introduction.

There are a number of advantages to be gained from developing protoplast-to-plant systems for commercially important species, since such systems may ultimately lead to improvements being introduced into plants which, perhaps, are not readily achievable by other methods. Such examples include the exploitation of protoclonal (somaclonal) variation, creation of novel genotypes via somatic cell fusion, and transformation by direct gene uptake. Studies aimed at the establishment of efficient, dividing protoplast systems for several English rose cultivars was seen as the basis for their subsequent genetic manipulation through cell fusion and transformation.

Leaf mesophyll tissues, suspension cultured cells and callus tissues were assessed as protoplast sources. Despite a published protocol which detailed the isolation, culture and regeneration of protoplasts from embryogenic cell suspensions of the infrequently cultivated rose variety *R. persica* x *xanthia* (see Chapter 1, Section 1.5.4), an investigation into the isolation, culture and regeneration of English rose mesophyll protoplasts was regarded as necessary for a number of reasons. Specifically,

i) The established protocol was for *R. persica* x *xanthia*. The variation in responses to *in vitro* culture, observed with the different cultivars used in this study (see Chapter 4, Sections 4.3.1.2, 4.3.2.1 and 4.4), and in reports for different classes of rose, suggested that the existing protocol was unlikely to be applicable to a wide range of rose cultivars. This was proved to be correct in that it was not possible to establish embryogenic cell lines of English rose (see Chapter 4, Sections 4.3.2.2 and 4.4).

ii) As there were no previous reports on the isolation, culture and regeneration of rose mesophyll protoplasts, there was clear justification for the establishment of this procedure since similar work with other Rosaceous species, such as *Prunus avium* x

pseudocerasus (Ochatt and Power, 1989), *Pyrus communis* (Ochatt and Power, 1988a, b, c) and *Malus domestica* (Patat-Ochatt and Power, 1989) was successful.

iii) Since floricultural crops, such as rose, are vegetatively propagated (in order to maintain varietal fidelity), regeneration systems should be designed to minimise somaclonal variation. This is particularly important in rose, which possesses a large combination of desirable traits, all of which must be retained. The choice of mesophyll tissue as a protoplast source, eliminates the requirement for a callus phase prior to protoplast isolation. This is important, as callus displays high levels of somaclonal variation (Peschke and Phillips, 1992) and has been demonstrated in *R. hybrida* to result in changes in both the ploidy level of cultures (Moyne *et al.*, 1993) and in the phenotype of regenerated plants (Arene *et al.*, 1993).

iv) The utilization of mesophyll protoplasts, when fused with albino (or colourless) cell suspension/callus protoplasts, provides for rapid visual identification of heterokaryons and, when coupled with cell sorting or micromanipulation, could lead to a relatively rapid method of selecting potential fusion products.

v) As mesophyll protoplasts are able to express totipotency more readily than protoplasts isolated from non-embryogenic cell suspensions or callus (Roest and Gilissen, 1993), their unilateral use would be more likely to result in the regeneration of somatic hybrid plants.

Investigations were also carried out into the isolation of cell suspension/callus protoplasts not only as potential fusion partners with mesophyll protoplasts, but also to enable comparisons to be made between the cultural responses of these protoplasts and the hitherto uninvestigated mesophyll protoplasts. Thus, protoplasts were isolated from *in vitro* derived mesophyll tissues of the English rose cvs. Abraham Darby, Marie Pavié and The Countryman, from cell suspension cultures of cvs. Mary Rose and Phyllis Byde and from callus cultures of cvs. Abraham Darby, Mary Rose and Phyllis Byde. Source cultures were initiated and maintained as described previously (Chapter 4, Sections 4.2.2.2, 4.2.4 and 4.2.3 respectively).

Attention was focused on the use of protoplast media with reduced concentrations of, or lacking, ammonium ions since in other woody, Rosaceous genera (*Prunus*, *Pyrus*) this was key to successful protoplast culture. The use of antioxidants in the culture media was also investigated along with their incorporation in enzyme solutions, since beneficial effects are well established for protoplast viability of recalcitrant species, particularly deciduous trees (Ochatt and Power, 1992). The use of antioxidants has resulted in higher yields of viable protoplasts in the Rosaceous woody species *Prunus avium* (Ochatt, 1991b). They have also been shown to be essential for successful culture and regeneration from protoplasts of non-woody species such as *Lolium perenne* (Creemers-Molenaar and Van Oort, 1990) and *Beta vulgaris* (Krens *et al.*, 1990).

In addition to cultural factors, electroenhancement of English rose protoplasts was investigated to promote regeneration. Voltages of 250V to 1000V and a pulse duration of 20 - 40 μs triggered early onset of mitotic division and enhanced plating efficiency in several woody species, including *Solanum dulcamara*, *Pyrus communis* and *Prunus avium* x *pseudocerasus* (Rech *et al.*, 1987) and promoted long-term plant regeneration potential and rooting (Chand *et al.*, 1988b; Ochatt, 1990; Ochatt *et al.*, 1988c).

5.2 Materials and Methods.

5.2.1 Isolation of Protoplasts.

5.2.1.1 Source of mesophyll protoplasts.

Leaves from axenic shoot cultures and of two types of leaf material were used these being either fully expanded leaves (5 x 10 mm) or newly expanded leaves (3 x 6 mm long), collected at subculturing. For all experiments, 1.0 g f.wt. of leaf tissues were incubated in 10 ml aliquots of enzyme solution in 5.5 cm Petri dishes, sealed with Nescofilm.

5.2.1.2 Sources of cell suspension and callus protoplasts.

Suspension cultures (Chapter 4; Section 4.3.3) 14 d after subculture and callus tissues, 20 d post-subculture were used (1.0 g f.wt. callus tissue or 1.0 ml packed cell volume cell suspension per 10 ml of each enzyme solution).

5.2.1.3 Pre-treatments of tissues for protoplast isolation.

In all cases, leaves were cut into strips (approx. 0.5 mm wide), with the central/main veins removed. Callus portions were converted to 3 mm^3 sections, whilst avoiding necrotic tissues.

Tissues (all sources) were either digested directly or plasmolysed, prior to enzyme incubation. In the latter, 1.0 g f.wt. of tissues (1.0 ml packed cell volume) was treated for 1 h, in 10 ml of an osmoticum which consisted of CPW salts solution (Power *et al.*, 1989; see Appendix II) with 13% (w/v) mannitol (CPW 13M).

5.2.1.4 Enzymes, enzyme solutions and incubation conditions.

Enzymes (Appendix III) were dissolved in CPW 13M solution and 5 mM 2-N-morpholinothane sulfonic acid (MES) buffered (pH 5.6 (using KOH or HCl) and were filter sterilised (0.2 μm pore diameter, Sartorius filter), dispensed in 30 ml screw-capped glass bottles, and stored deep-frozen at -20°C until required (see Table 5.1 for compositions).

For rose leaf protoplast isolations, all enzyme solutions were prepared with the addition of several antioxidants [eg. PVP-10; 0.5, 1.0 or 2.0% (w/v), glycine; 0.1, 0.2 or 0.3M, 8-HQS; 0.05, 0.1, 0.2% (w/v)] to negate phenolic oxidation. In order to prevent the multiplication of endogenous bacteria which may have been present in the leaf tissues, the antibiotic cefotaxime ('Claforan'; Roussel Laboratories, Uxbridge, UK) was included in the enzyme solution at 250 $\mu g\ ml^{-1}$.

All tissue incubations were at 25±2°C, with samples taken at hourly intervals (up to 24 h), in order to determine the optimum period of digestion for each cultivar/tissue type. The effect, on protoplast release, of stationary versus agitated culture (40 or 80 rpm), and dark compared to constant illumination (19.5 $\mu Em^{-2}s^{-1}$) were assessed. All experiments were replicated at least three times, each with three individual treatments per parameter.

Table 5.1 Composition of enzyme solutions used for the evaluation of rose protoplast isolation.

Enzyme Code	Enzyme Concentration (%, w/v)						
	DRI	HEM	MAC	MEI	CR 10	C RS	PEC
1	-	-	2.0	-	2.0	-	1.0
2	-	-	-	-	-	1.0	0.1
3	-	-	1.0	-	2.0	-	-
4	-	-	0.1	-	0.5	-	-
5	-	1.0	0.1	-	-	1.0	0.05
6	-	1.0	0.1	-	-	0.7	0.1
7	-	1.0	0.1	-	-	0.4	0.05
8	-	1.0	-	1.0	1.0	-	0.1
9	0.1	-	0.2	-	1.0	-	-
10	-	-	-	-	1.0	-	0.01
11	0.5	-	1.0	-	2.0	-	0.1
12	-	2.0	-	-	2.0	-	0.1
13	-	2.0	-	-	1.0	-	0.1
14	-	1.0	-	-	0.5	-	0.01
15	-	1.0	-	-	0.5	-	0.05
16	-	1.0	-	-	0.5	-	0.1
17	-	1.0	-	-	1.0	-	0.01
18	-	1.0	-	-	1.0	-	0.05
19	-	1.0	-	-	1.0	-	0.1
20	-	1.0	-	-	2.0	-	0.01
21	-	1.0	-	-	2.0	-	0.05
22	-	1.0	-	-	2.0	-	0.1

DRI = Driselase; HEM = Hemicellulase; MAC = Macerozyme R-10; MEI = Meicellase; CR 10 = Cellulase Onozuka R-10; CR S = Cellulase RS, PEC = Pectolyase Y-23. See also Appendix III for details. All mixtures were made up in CPW 13M solution.

5.2.1.5 Protoplast purification.

At the end of the incubation period, the protoplasts and partly digested cells were placed sequentially on two nylon sieves (100 μm and 64 μm pore sizes) whereupon any undigested tissues were rinsed twice with approx. 15 ml of CPW 13M solution (Appendix II). The final filtrate, containing protoplasts, was dispensed (as 10 ml aliquots) into 16 ml screw-capped glass tubes, centrifuged (100 xg, 10 min) and the supernatants discarded.

For the purification of protoplasts, three strategies were investigated:

i) The resuspension of the protoplast pellets in 10 ml of CPW 13M solution and passage through nylon sieves of decreasing pore size (53, 45 and 30 μm respectively).

ii) The pellets were resuspended in 1.5 ml of CPW 13M solution and layered on top of 12 ml of either CPW 21S or CPW 25S solution (Appendix II), in 16 ml screw-capped glass tubes, and centrifuged (100 xg, 10 min). Protoplasts, free of debris, were carefully removed from the interface.

iii) The pellets were resuspended in CPW 13M solution (as in ii), and this, in turn, layered (as 1.5 ml aliquots) on top of Percoll (Sigma, UK)/CPW 13M solution gradient. This consisted of 2.5 ml layers (v/v, from bottom to top) of 30.0, 25.0, 20.0, 15.0, and 10% Percoll, dispensed in 16 ml screw-capped glass centrifuge tubes. Tubes were centrifuged (100 xg, 10 min) and aliquots, carefully removed from each of the interfaces, were then examined microscopically in order to determine which interface gave protoplasts with the minimal amount of debris. Protoplasts were collected from the interface and resuspended in 10 ml of CPW 13M solution. They were washed twice by centrifugation (100 xg, 10 min) and resuspended in CPW 13M solution. Protoplasts were finally resuspended in 10 ml of CPW 13M solution.

5.2.1.6 Determination of the presence of residual cell wall material, protoplast viability, yield and diameter.

5.2.1.6.1 Cell wall.

For assessments of cellulosic cell wall material on the isolated protoplasts, an aliquot (approx. 100 µl) of protoplasts, suspended in CPW 13M solution, was mixed with an equal volume of a solution of Calcofluor white (0.1%, w/v), in CPW 13M solution. Preparations were examined under UV light using a Nikon inverted microscope Diaphot TMD (high pressure mercury vapour lamp HBO 100 w/z) fitted with B1 filter IF 420-485, dichroic mirror DM510 and eyepiece absorption filter 570. Only protoplast preparations that were free of debris were used in all subsequent culture assessments.

5.2.1.6.2 Protoplast viability.

Protoplasts, suspended in CPW 13M solution (100 µl aliquots), were mixed with an equal volume of appropriate medium containing FDA (0.1 ml of a 5.0 mg ml^{-1} stock in acetone per 10 ml of medium) and observed under UV illumination using a Nikon inverted microscope as in Section 5.2.1.6.1. Protoplasts exhibiting a green/yellow fluorescence were regarded as being viable. Results were expressed as the percentage of viable (fluorescing) protoplasts per field, with each count including at least 500 randomly-chosen protoplasts.

5.2.1.6.3 Protoplast yield.

Protoplasts, in CPW 13M solution, were counted using a modified Fuchs Rosenthal haemocytometer. Results were expressed as yield of protoplasts per g f.wt. of starting tissue. All yield assessments were repeated at least 5 times per isolation.

5.2.1.6.4 Protoplast size.

There is a clear relationship between protoplast size and their ability to withstand electric pulses in the context of electrofusion and/or electrostimulation of growth.

Size determination was therefore a prerequisite. The diameter was determined for at least 200 protoplasts of each type/treatment using a calibrated eyepiece graticule fitted to a Nikon inverted microscope (x 40 objective).

5.2.2 Protoplast culture.

5.2.2.1 Choice of culture medium.

Protoplasts of many woody species exhibit a marked decline in viability during culture, irrespective of the culture medium employed (Ochatt, 1989). Thus, a representative sample (5.0 ml) of protoplasts (for all cultivars and tissue sources) was stored in the dark at 4°C suspended in CPW 13M solution, in order to monitor the baseline decline in viability. This was assessed every 48 h, which, in turn, provided a reference point for the optimisation of culture media components for a given cultivar, whilst simultaneously placing all further viability determinations during the culture of protoplasts on a comparative basis.

For all cultivars and sources, protoplasts were cultured in each of eight different formulations of basal media (Table 5.2). Additionally, all MS- and KM-based media, were evaluated with 116 factorial combinations of growth regulators. These consisted of NAA + BAP, NAA + kinetin or BAP + 2,4-D all at concentrations of 0.0, 0.1, 0.5, 1.0, 2.0 or 3.0 mg l^{-1}. In addition, kinetin and 2,4-D were used individually at the same concentrations. Several modifications were also made to a proportion of the KM-based media. Either the final concentration of glucose was reduced from 68.0 to 34.0 g l^{-1} and the mannitol concentration was increased from 0.13 to 65.0 g l^{-1}, or the glucose was replaced with 30 g l^{-1} sucrose and the mannitol concentration increased to 90 g l^{-1}. Half-strength KM medium was also supplemented with a complex osmoticum formulation consisting of 6.0% myo-inositol, 1.0% glucose, 1.0% sorbitol and 0.5% galactose. All MS-based media and modifications contained 9% (w/v) mannitol as an osmoticum at pH 5.8 (adjusted with KOH or HCl). Growth regulators were added prior to autoclaving (Appendix I).

Table 5.2 Basal media employed for the cultural evaluation of a range of English rose protoplast systems.

Basal Inorganic Salts [1]	Organic Components [2]
MS	MS
MS	C
MS - NH_4NO_3 free	MS
MS - NH_4NO_3 free	C
KM	KM
KM	KM - lacking coconut milk
1/2 KM	1/2 KM
SH	SH

[1] MS = Murashige and Skoog (1962); MS - NH_4NO_3 free = MS lacking ammonium ions; KM = Kao and Michayluk (1975); 1/2 KM = KM at half concentration; SH = Schenk and Hildebrandt (1972). [2] C = mixture of organic components (as described by Ochatt and Caso; 1986) used in addition to the MS organic components; MS, KM and SH as in [1]. All culture media were prepared at either single (for liquid culture) or double-strength (for semi-solid culture) and stored in liquid form in the dark, at room temperature, until required.

Protoplasts of rose were extremely prone to phenolic oxidation during culture. Hence, protoplast media were supplemented with one of three antioxidants; PVP-10 (0.5, 1.0 or 2.0%, w/v), 8-HQS (0.05, 0.1 or 0.2% w/v) or glycine (0.1, 0.2 or 0.3M). Additionally, where semi-solid droplet culture was employed (Section 5.2.2.2) the addition of antioxidants only to the liquid medium was investigated. Each treatment was replicated at least five times.

Because of its reported role in stimulating sustained cell division in other woody species (d' Utra Vaz *et al.*, 1993), the antibiotic cefotaxime was also included in the protoplast culture media at concentrations of 0, 50, 100, 250 or 500 $\mu g\ ml^{-1}$.

5.2.2.2 Culture method.

Protoplasts (all sources) were cultured in 3.5 cm plastic Petri dishes in either liquid-layers (4.0 ml aliquots), semi-solid layers (3.5 ml aliquots), 10 (100 μl) semi-solid droplets (beads) surrounded (but not submerged) by 3 ml aliquots of liquid medium, or embedded in alginate layers (see Appendix IV) The later, in turn, were floated in 5 ml aliquots of liquid culture medium.

For all semi-solid media, SeaPlaque agarose (FMC, Rockland, USA), at 0.6% (w/v) was used, with semi-solid medium being prepared by diluting double-strength liquid culture medium with an equal volume of molten (60°C) double-strength agarose (1.2% w/v) in water.

Protoplasts were diluted directly with liquid media, or mixed with semi-solid media, while this was still liquid (approx. 40°C), to give the final plating density required. Droplets were routinely surrounded by liquid medium, of the same composition (but lacking agarose), and in some experiments the liquid medium also contained antioxidants (Section 5.2.2.1).

5.2.2.3 Culture conditions.

Protoplasts were plated at initial densities of 0.1, 0.5, 1.0, 2.5, 5.0 or 10.0 x 10^5 per ml of culture medium and dishes, sealed with Nescofilm, maintained at 20 or 25°C, either in the light (1.8 or 9.0 $\mu Em^{-2}s^{-1}$) or initially in the dark and then into light conditions (1.8 or 9.0 $\mu Em^{-2}s^{-1}$). Liquid cultures were maintained either statically or shaken (40 cycles per min.). In all experiments a minimum of three replicates per

plating density/cultural condition were tested with each experiment being repeated twice.

5.2.2.4 Determination of cell wall regeneration and protoplast plating efficiencies.

The regeneration of cell walls was assessed using Calcofluor White (Section 5.2.1.6.1).

Protoplast plating efficiency was determined, for all systems, at defined stages, the timing of which depended on the cultivar and protoplast source. However, these were always allied to a distinct developmental stage as defined by Ochatt and Power (1992). Specifically,

i) Initial Plating Efficiency (IPE); defined as the percentage of the originally plated protoplasts that had regenerated a cell wall and undergone at least one mitotic division.

ii) Intermediate Plating Efficiency (MPE); defined as the percentage of the originally plated protoplast that had divided to give colonies of at least 10 cells.

iii) Final Plating Efficiency (FPE); defined as the percentage of the originally plated protoplasts that had proliferated to visible microcalli (1 - 2 mm diam.).

5.2.3 Plant regeneration from protoplast-derived calli.

Groups of 20 - 25 protoplast-derived microcalli (each approx. 2.0 mm diam.) were transferred, to 10 ml of semi-solid, osmoticum-free, medium dispensed into 9 cm Petri dishes and sealed with Nescofilm. MS-based media, supplemented with growth regulators at concentrations optimal for callus proliferation, were employed (see Chapter 4, Section 4.3.2.1). All protoplast-derived tissues were maintained either in the dark or under continuous illumination (19.5 $\mu Em^{-2}s^{-1}$) at $25\pm2^{o}C$, and were subcultured every 2 weeks.

Only calli of approx. 200 mg f.wt were capable of continued survival (see Results; Section 5.3.4.1). Therefore, regeneration assessments were confined to calli that had survived for 2 passages on the appropriate callus proliferation medium.

Portions (200 mg f.wt; 75 mm^3 approx.) were separated from protoplast-derived callus tissues and 4 - 5 pieces transferred to 10 ml semi-solid (0.8%, w/w agar) regeneration medium (pH 5.8), the latter being dispensed into 9 cm Petri dishes.

Regeneration media were based on full- or half-strength MS media or full-strength SH medium. MS based media had either the normal concentration of B vitamins, or were supplemented with double this concentration (ie. 0.2 mg l^{-1} thiamine-HCL, 1.0 mg l^{-1} pyridoxine-HCL, 1.0 mg l^{-1} nicotinic acid). All media had the growth regulators NAA, IBA, 2,4-D, BAP, Z or TDZ added at concentrations of 0.0, 0.1, 0.5, 1.0, 2.0 and 3.0 mg l^{-1}. These growth regulators were combined factorially in the following combinations: NAA+BAP, NAA+BAP+Z, IBA+BAP, NAA+TDZ, IBA+TDZ, NAA+BAP+TDZ, 2,4-D+BAP, 2,4-D alone, NAA alone and IBA alone. To these growth regulator combinations, 0.0, 1.0 or 2.0% (w/v) PVP-10, 0.0, 300.0 or 600.0 mg l^{-1} L-proline, 0.0, 1.0 10.0 mg l^{-1} silver thiosulphate, 0.0, 10.0 or 20.0 g l^{-1} 2,3,5-triiodobenzoic acid (TIBA), alone or in conjunction were added.

All media assessments were replicated at least ten times (eg. 40 - 50 calli/medium) with all experiments being repeated at least twice. Results were assessed as the percentage of calli that expressed shoot/root regeneration and the number of regenerated shoots/roots per callus.

When only rhizogenesis occurred, shoot regeneration was subsequently attempted from protoplast-derived roots. Roots were detached from protoplast-derived calli. Intact roots or roots which had been divided into three equally sized lengths (approx. 5 mm), were transferred individually to 3.0 ml aliquots of agar-solidified (0.8%, w/v)

MS-based medium with 0.0, 0.01 or 0.025 mg l^{-1} NAA and 1.0 or 2.0 mg l^{-1} BAP. In addition, PVP-10, silver thiosulphate or TIBA were also used at the concentrations detailed earlier in this section. All cultures were maintained at 25°C, either in the dark or under continuous illumination (19.5 $\mu Em^{-2}s^{-1}$). Each medium was replicated at least ten times and all experiments repeated twice.

5.2.4 Electro-enhancement of the growth of cultured rose protoplasts.

An evaluation was carried out to determine whether the repeated application of electric pulses would promote plating efficiency or shoot/root regeneration for English rose protoplasts. Protoplasts (all sources) were subjected to a square wave discharge DC pulse of 20 or 40 μs duration at 250, 500, 750 and 1000 V cm^{-1}. The electrofusion apparatus, used to apply the electric pulses, was of the design described by Jones *et al.* (1994; see also Chapter 6; Section 6.2.5). Voltages were applied to the protoplasts using 7 parallel nickel-silver plate electrodes with a 2.8 mm separation (Watts and King, 1984). Protoplasts were suspended at a density of 1 x 10^6 ml^{-1} in 0.5mM MES buffer containing 0.5mM $CaCl_2$ and 13% (w/v) mannitol (pH 5.6). Aliquots (0.5 ml) of resuspended protoplasts were placed into the compartments of a 5 x 5 plastic dish (Sterilin, Hounslow, UK). The electrode assembly was first sterilised in 80% (v/v) ethanol solution (1 h) and placed, in turn, into the compartments containing the protoplast suspensions. Following electrical-treatment, protoplasts were cultured using the medium and culture method previously determined to be optimal for culture (see Sections 5.2.2 and 5.3.4). Cell wall regeneration and plating efficiencies were subsequently determined.

In order to evaluate the effects of such treatments, the raw data were subjected to a non-parametric 2-way analysis of variance (Meddis, 1984). This statistical test ranked the data to avoid any incorrect assumptions about the distribution of the data. This enabled a number of specific questions to be addressed:

i) Does the FPE (of each sample) increase significantly with increasing pulse duration?

ii) Does the FPE (of each sample) increase significantly as the applied voltage increases?

iii) Does the difference between FPE values of protoplast samples, treated at different voltages, increase with increased pulse duration?

iv) Was the overall effect of the electrotreatment of English rose protoplasts, enhancing either their plating efficiencies or regeneration potential or both?

5.3 Results.

5.3.1 Isolation of mesophyll protoplasts: General observations.

Following comparisons for newly-expanded leaves and fully-expanded leaves taken from *in vitro* plantlets, it was found that the largest yields of protoplasts, coupled with highest viability, were obtained, for all cultivars, when newly expanded leaves were used as source material (Plate 5.1a). Relatively older fully-expanded leaves, resulted in the production of partially degraded cells and negligible numbers of protoplasts.

In all cultivars, mesophyll protoplast release was enhanced when leaves were incubated in enzyme solutions with continuous shaking at 40 rpm, but at a higher speed (80 rpm) protoplast release and viability were both reduced. Protoplast viability was also reduced with incubation in the light. The benefits of 1.0% PVP-10 were clearly evident, since in its absence, both protoplast yield and viability were reduced, for all cultivars. The use of PVP-10 below 1.0% had no positive effect nor did concentrations above 1.0%. Neither glycine or 8-HQS, in the enzyme solution, had any beneficial effect. Protoplast diameters ranged from 22 - 30 μm , with a mean diameter of 24 μm and all protoplasts failed to float on CPW 21S solution.

5.3.1.1 Mesophyll protoplasts of the cv. Abraham Darby.

Optimum yields and viabilities were from sections from newly-expanded leaves pre-plasmolysed for 1 h, in CPW 13M solution (Table 5.3), and incubated (shaking 40 rpm) for 17 h in Enzyme Solution 5 supplemented with 1.0% PVP-10 (Yield; 1.87 x 10^6 protoplasts/ g f.wt., viability; 87%). Serial sieving failed to produce debris-free preparations, but protoplasts were successfully purified by floatation on CPW 25S solution. The use of Percol gradients resulted in reduced yield and viability (the latter from 87 to 22%).

5.3.1.2 Mesophyll protoplasts of the cv. Marie Pavié.

Optimum protoplast release was from newly-expanded leaves, chopped, plasmolysed for 1 h in CPW 13M solution prior to incubation for 17 h in the dark (shaking 40 rpm) in Enzyme Solution 6 with 1.0% PVP-10 (Table 5.4). Again, floatation on CPW 25S solution was effective, together with Percoll gradients, whereby protoplasts accumulated at the 25% Percoll interface. however, viability was reduced when Percoll was employed.

5.3.1.3 Mesophyll protoplasts of the cv. The Countryman.

Again, newly expanded leaves were the best source tissue, which were pre-plasmolysed (1 h), in CPW 13M solution and incubated (17 h; shaking 40 rpm) in Enzyme Solution 5 with 1.0% PVP-10. As with the other cultivars, a plasmolysis pre-treatment increased yield and viability with debris-free protoplast preparations obtained with CPW 25S solution.

Table 5.3 Effect of pre-plasmolysis treatment on yield and viability of protoplasts from newly-expanded leaves of cv. Abraham Darby.

Enzyme Solution	Normal		Pre-plasmolysis	
	Yield x 10^6*	% Viability	Yield x 10^6*	% Viability
1	0.15±0.03	24±4	0.21±0.05	31±
2	0.06±0.02	9±1	0.10±0.03	12±1
3	0.19±0.03	15±4	0.30±0.03	27±3
4	0.28±0.04	21±4	0.41±0.03	30±3
5	**0.76±0.10**	**59±3**	**1.87±0.10**	**87±5**
6	0.62±0.11	61±4	1.64±0.11	79±4
7	0.58±0.09	44±4	1.30±0.09	60±4
8	0.05±0.005	18±3	0.29±0.01	29±1
9	0.05±0.005	13±2	0.27±0.01	20±2
10	0.61±0.09	12±2	0.31±0.05	24±3
11	0.29±0.06	19±2	0.30±0.01	22±2
12	0.48±0.15	28±3	0.62±0.04	40±3
13	0.39±0.01	31±3	0.60±0.03	43±3
14	0.25±0.04	24±4	0.41±0.05	49±4
15	0.33±0.02	27±4	0.59±0.03	54±4
16	0.40±0.01	34±3	0.66±0.05	50±3
17	0.35±0.01	30±1	0.54±0.03	48±4
18	0.21±0.004	45±2	0.50±0.02	55±4
19	0.30±0.02	29±3	0.71±0.04	42±3
20	0.35±0.01	20±2	0.61±0.04	38±3
21	0.11±0.007	21±4	0.40±0.03	39±2
22	0.29±0.003	26±3	0.54±0.02	44±4

* Number of protoplasts per g f. wt. Data are means ±standard error. All enzyme solutions contained 1.0% PVP-10. Enzyme incubations were carried out in the dark with shaking (40 rpm).

Table 5.4 Effect of pre-plasmolysis treatment on yield and viability of protoplasts from newly-expanded leaves of cv. Marie Pavié.

Enzyme Solution	Normal		Pre-plasmolysis	
	Yieldx 10^{6*}	% Viability	Yield x 10^{6*}	% Viability
1	0.11±0.09	26±3	0.19±0.10	29±5
2	0.10±0.07	21±2	0.15±0.08	25±3
3	0.09±0.01	18±1	0.10±0.05	17±2
4	0.14±0.05	9±2	0.19±0.05	12±2
5	0.69±0.20	53±4	1.38±0.10	68±5
6	**0.78±0.31**	**66±3**	**1.55±0.1**	**75±4**
7	0.63±0.22	55±3	1.21±0.1	69±5
8	0.03±0.01	14±1	0.09±0.03	25±4
9	0.01±0.005	11±1	0.02±0.003	19±2
10	0.44±0.13	17±2	0.56±0.09	26±3
11	0.39±0.11	13±2	0.49±0.05	21±3
12	0.37±0.09	15±3	0.44±0.05	22±1
13	0.41±0.20	18±3	0.53±0.04	27±2
14	0.27±0.17	21±3	0.31±0.04	32±2
15	0.35±0.18	16±3	0.29±0.5	30±3
16	0.22±0.11	11±2	0.34±0.04	19±3
17	0.29±0.12	13±2	0.31±0.05	25±1
18	0.15±0.06	8±1	0.20±0.06	12±1
19	0.19±0.08	12±1	0.21±0.04	19±3
20	0.26±0.10	9±2	0.39±0.07	17±2
21	0.09±0.001	3±1	0.18±0.03	9±3
22	0.28±0.11	8±1	0.37±0.07	15±4

* See footnote Table 5.3.

Table 5.5 Effect of pre-plasmolysis treatment on yield and viability of protoplasts from newly-expanded leaves of cv. The Countryman.

Enzyme Solution	Normal		Pre-plasmolysis	
	Yield x 10^{6*}	% Viability	Yield x 10^{6*}	% Viability
1	0.12±0.07	18±2	0.15±0.07	22±2
2	0.16±0.08	14±3	0.19±0.05	14±1
3	0.11±0.06	11±1	0.12±0.05	14±2
4	0.07±0.02	5±2	0.10±0.06	7±1
5	**0.66±0.11**	**41±3**	**0.80±0.08**	**54±2**
6	0.49±0.12	32±3	0.50±0.10	39±3
7	0.38±0.09	29±3	0.34±0.09	39±2
8	0.20±0.05	17±1	0.27±0.09	20±2
9	0.16±0.07	14±1	0.18±0.07	19±3
10	0.19±0.05	16±1	0.22±0.10	15±3
11	0.09±0.08	8±2	0.13±0.11	12±3
12	0.11±0.06	10±2	0.25±0.08	16±3
13	0.10±0.03	15±3	0.20±0.05	19±4
14	0.15±0.05	12±3	0.17±0.05	16±3
15	0.18±0.07	17±3	0.25±0.06	13±3
16	0.13±0.05	22±3	0.11±0.04	29±3
17	0.06±0.01	8±1	0.10±0.05	14±3
18	0.08±0.02	12±2	0.12±0.05	20±2
19	0.13±0.04	18±2	0.26±0.08	22±2
20	0.11±0.03	14±2	0.20±0.07	19±1
21	0.02±0.007	4±1	0.18±0.09	9±2
22	0.09±0.005	11±3	0.15±0.08	24±3

* See footnote Table 5.3.

5.3.2 Protoplasts from rose cell suspension cultures and callus.

5.3.2.1 Rose cell suspension protoplasts.

Pre-plasmolysis of cells in CPW 13M solution, coupled with slow shaking (40 rpm), was essential for the production of large numbers of viable protoplasts. Optimum results for both cultivars (Mary Rose and Phyllis Byde), were with cells incubated in the dark (17 h) with Enzyme Solution 22 [2.0% (w/v) Cellulase R-10, 0.1% (w/v) Pectolyase Y-23 and 1.0% (w/v) Hemicellulase (see Table 5.6 and Plate 5.1b)]. Serial sieving did not give debris-free preparations, but protoplasts were successfully purified on CPW 21S solution. Protoplast diameters ranged from 27 - 34 μm.

Table 5.6 Isolation of cell suspension protoplasts from the cvs. Mary Rose and Phyllis Byde (all with pre-plasmolysis).

Enzyme Solution	Mary Rose		Phyllis Byde	
	Yield x 10^7*	% Viability	Yield x 10^7*	% Viability
1	1.8±0.18	73±2	1.6±0.15	70±3
2	1.3±0.11	78±2	1.1±0.19	72±3
3	1.7±0.22	74±3	1.9±0.17	71±4
4	1.1±0.18	69±2	1.3±0.18	73±3
5	1.5±0.16	76±4	1.9±0.15	74±5
6	1.5±0.13	77±4	1.7±0.15	79±3
7	1.2±0.11	72±3	1.5±0.13	60±3
8	1.4±0.22	80±4	1.9±0.25	74±3
9	1.3±0.19	81±2	1.1±0.19	79±3
10	1.2±0.18	76±5	0.9±0.15	80±3
11	1.2±0.27	79±3	1.1±0.20	85±5
12	2.2±0.24	89±4	1.6±0.17	84±3
13	1.7±0.19	90±2	1.4±0.15	79±3
14	2.5±0.43	84±4	2.2±0.31	80±3
15	2.7±0.35	89±3	2.5±0.21	84±3
16	3.1±0.49	91±3	2.4±0.19	86±3
17	2.9±0.38	84±3	2.4±0.18	80±5
18	2.9±0.29	86±4	2.6±0.24	80±2
19	3.1±0.41	87±2	3.0±0.27	82±4
20	2.8±0.39	81±3	2.1±0.31	72±5
21	3.3±0.69	94±5	3.5±0.37	83±5
22	**3.8±0.76**	**97±4**	**3.6±0.33**	**92±5**

* Number of protoplasts per g f. wt. Data are means ±standard error. All enzyme incubations were carried out in the dark with shaking (40 rpm).

5.3.2.2 Rose callus protoplasts.

Following pre-plasmolysis of tissues in CPW 13M solution, protoplasts could only be obtained using Enzyme Solutions 12 or 22 (Table 5.7), with optimum release with a 16 h incubation in the dark with shaking (40 rpm). Protoplasts tended to be vacuolated and had realtively low viabilities in comparison to those isolated from other tissue sources. Purification was with CPW 21S solution.

Table 5.7 Protoplasts release from callus tissues of cvs. Abraham Darby, Mary Rose and Phyllis Byde.

Enzyme	Abraham Darby		Mary Rose		Phyllis Byde	
Solution	Yield x 10^4	% Viability	Yield x 10^4	% Viability	Yield x 10^4	% Viability
12	2.0±0.5	16±3	2.1±0.1	13±3	1.3±0.5	21±3
22	1.5±0.3	8±2	1.8±0.5	11±2	2.0±0.5	17±3

Yields are number of protoplasts per g f. wt. Data are means ±standard error. All incubations were carried out in the dark with shaking (40 rpm).

5.3.3 Protoplast size.

Figure 5.1 shows the size distribution of protoplasts isolated from all sources.

Figure 5.1 Size distributions of protoplast populations.

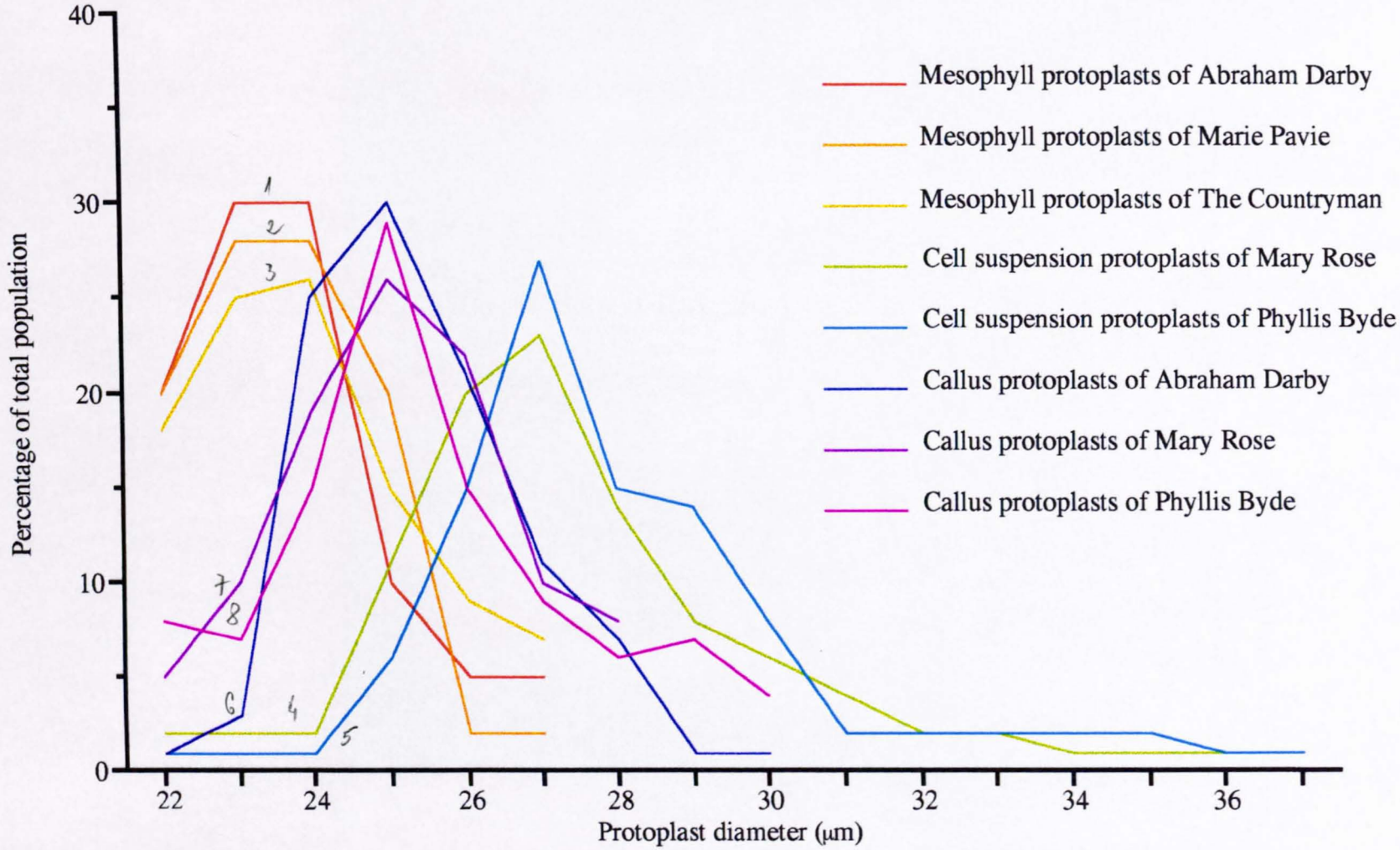


Plate 5.1.

a. Mesophyll protoplasts of English rose cv. Abraham Darby isolated using Enzyme Solution 5 and suspended in CPW 13M solution (x 200).

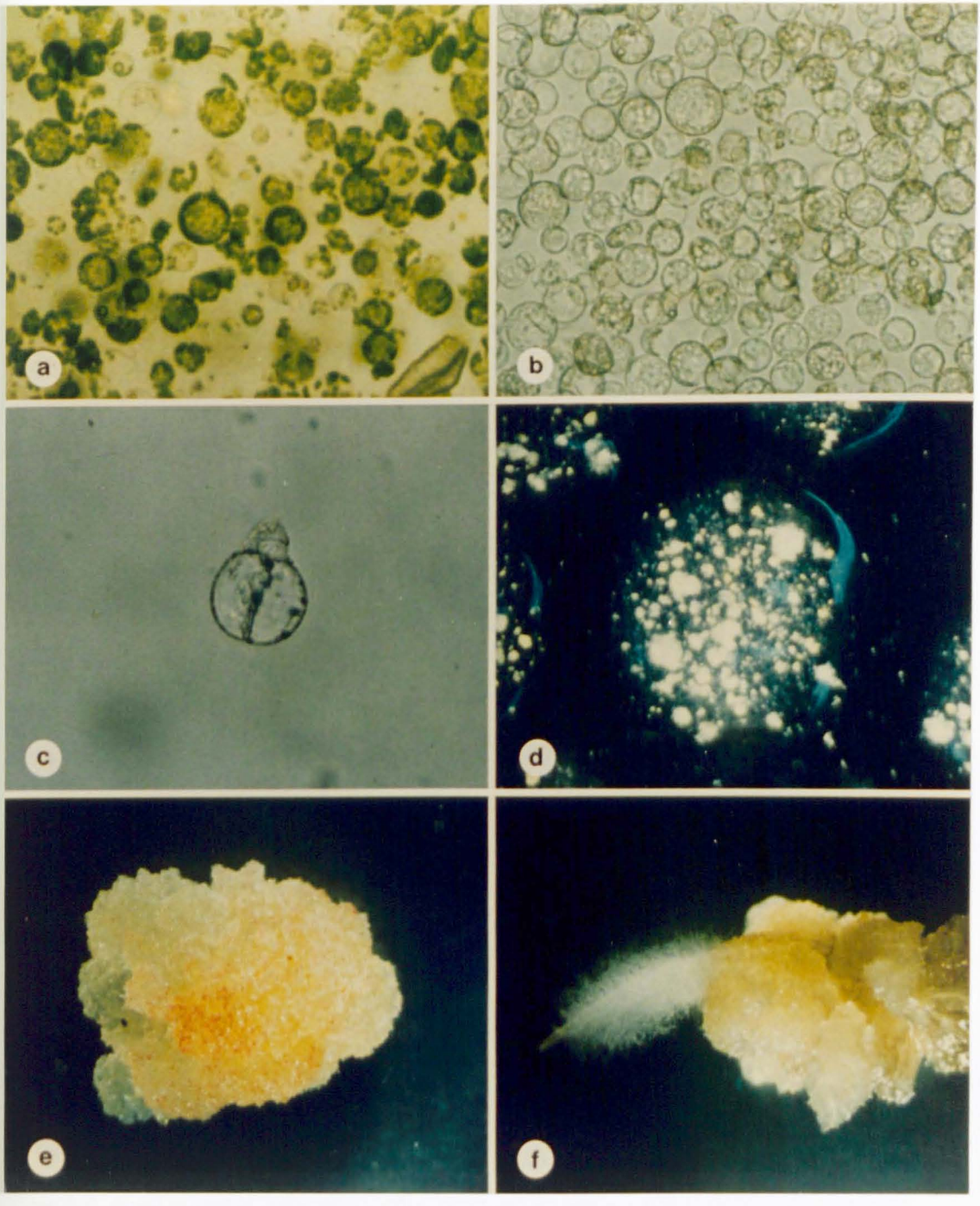
b. Cell suspension protoplasts of English rose cv. Mary Rose isolated using Enzyme Solution 22 suspended in CPW 13M solution (x 200).

c. First division of mesophyll-derived protoplasts of cv. Abraham Darby after 10 d culture in the dark at a density of 0.5 x 10^5 protoplasts ml^{-1} in KM-based medium (x 375).

d. Mesophyll protoplast-derived microcalli of cv. Abraham Darby, in bead culture (KM-based medium) after 1 month (x 6).

e. Mesophyll protoplast-derived calli of cv. Abraham Darby, after 2 weeks in the dark on callus proliferation medium (x 3.3).

f. Rhizogenesis in mesophyll protoplast-derived calli of cv. Abraham Darby after 18 d culture on SH medium containing 3.0 mg l^{-1} 2,4-D (x 4).


a
b
c
d
e
f

5.3.4 Protoplast culture and attempts at plant regeneration.

5.3.4.1 Leaf mesophyll protoplasts.

Extensive browning during culture was a persistent problem with mesophyll protoplasts in all media. In the majority of cases, cell wall regeneration was observed in 10 -20% of the cultured protoplasts by 7 d with small cell colonies (4-8 cells) by 21 d. Such colonies browned and subsequently died. Protoplast/cell viability assessments revealed that by day 10, viability was reduced by up to 80%. Protoplasts were subsequently cultured with antioxidants (Section 5.2.2.1), whereupon protoplast/cell division was observed, with most media, within 7 d (Plate 5.1c). Sustained cell division beyond the 16-cell stage was only observed in the cvs. Abraham Darby and Marie Pavié in agarose bead culture or in semi-solid layers of KM-based media. Division beyond the 10-cell stage was not observed with MS- or SH-based media. Protoplasts in liquid medium or in alginate layers failed to divide beyond the 16-cell stage irrespective of media composition.

With cvs. Abraham Darby and Marie Pavié, the optimum plating density was 0.5 x 10^5 protoplasts ml^{-1} in agarose bead culture with KM-based media lacking coconut milk, with 30 g l^{-1} sucrose and 90 g l^{-1} mannitol, supplemented with 1.0% PVP-10 and 250 μg ml^{-1} cefotaxime, pH 5.8. Development of protoplast-derived cells to the true microcolony stage (Plate 5.1d) was only observed with BAP and NAA, with FPE values being maximal (Abraham Darby: 6.0 x $10^{-2}\pm1.2$ x 10^{-5}, Marie Pavié: 5.0 x $10^{-2}\pm1.2$ x 10^{-5}) with 2.0 mg l^{-1} NAA and 1.0 mg l^{-1} BAP (Fig 5.2). Dark culture (25°C) for 1 month followed by transfer to light (1.0 $\mu Em^{-2}s^{-1}$) was optimal. Both IPE and FPE values were reduced when protoplasts were plated at densities either greater or lower than 0.5 x 10^5 ml^{-1} (Fig 5.3).

Irrespective of concentration, glycine failed to control phenolic browning, whilst 8-HQS negated visible browning, but did not result in as large an increase in plating efficiency as with 1.0% PVP-10 (Table 5.9). Cefotaxime proved not to be essential,

for growth although bacterial contamination was suppressed. For this latter reason, it was used routinely.

Table 5.9 Effects of PVP-10 and 8-HQS as media supplements mesophyll protoplast FPE.

	PVP-10 (%, w/v)			8-HQS (%, w/v)		
Cultivar	0.5	1.0	2.0	0.05	0.1	0.2
Abraham Darby	2.6±1.1	5.8±0.5	3.1±0.8	1.1±0.8	1.6±0.4	0.2±0.1
Marie Pavié	2.0±0.2	4.3±0.7	3.5±0.4	1.0±0.5	0.9±0.1	0.5±0.1

FPE for control protoplasts (i.e. no antioxidants) Abraham Darby: 0.8 x 10^{-2} ±0.2 x 10^{-2}; Marie Pavié: 0.3 x 10^{-2} ±0.1 x 10^{-2}. Medium composition was as in Section 5.3.4.1. Values are % FPE x 10^{-2}. Data are means of five replicates ±standard error.

Continued growth of protoplast-derived microcalli (both cultivars) on callus proliferation medium was only achievable in the dark. Despite previous exposure to light (1.8 $\mu Em^{-2}s^{-1}$), upon transfer to callus proliferation media, microcalli browned, whereas in the dark such microcalli continued to proliferate (Plate 5.1e).

With calli of both cultivars and after 14 -21 d on regeneration media containing only 3.0 mg l^{-1} 2,4-D, root proliferation occurred (for Abraham Darby 75% of calli, for Marie Pavié 62% of calli) irrespective of the basal medium (Plate 5.1f). Maximal root formation (75% of calli exhibiting rhizogenesis) was observed with cv. Abraham Darby on SH medium with this level of 2,4-D. Production of such roots declined with time and, three months after initiation, rhizogenesis ceased. Shoot regeneration was never induced from such roots. The addition of antioxidants, such as PVP-10, did not resolve this problem and the addition of silver thiosulphate and TIBA to the range of regeneration medium failed to promote shoot organogenesis.

Protoplasts of both cultivars showed no significant increase in their FPE following the application of electrical currents of varying field strengths. There were no significant differences ($p < 0.001$) when the results obtained after the application of a pulse of 20.0 μs duration are compared to those obtained with a 40.0 μs duration (Tables 5.10, 5.11). The application of field strengths of 250 and 500 V cm^{-1} resulted in a significant decrease ($p < 0.01$) in FPE with both cultivars, irrespective of pulse duration (Tables 5.10, 5.11).

Tables 5.10 and 5.11 Effects of electrical field strength and pulse duration on FPE of Abraham Darby (5.10) and Marie Pavié (5.11) mesophyll protoplasts.

Table 5.10

Pulse Duration (μs)	Field Strength (V cm^{-1})			
	250	500	750	1000
20.0	2.2±0.8	3.2±0.3	4.9±0.8	5.7±1.1
40.0	2.1±0.1	2.9±1.1	5.0±1.3	5.9±0.9

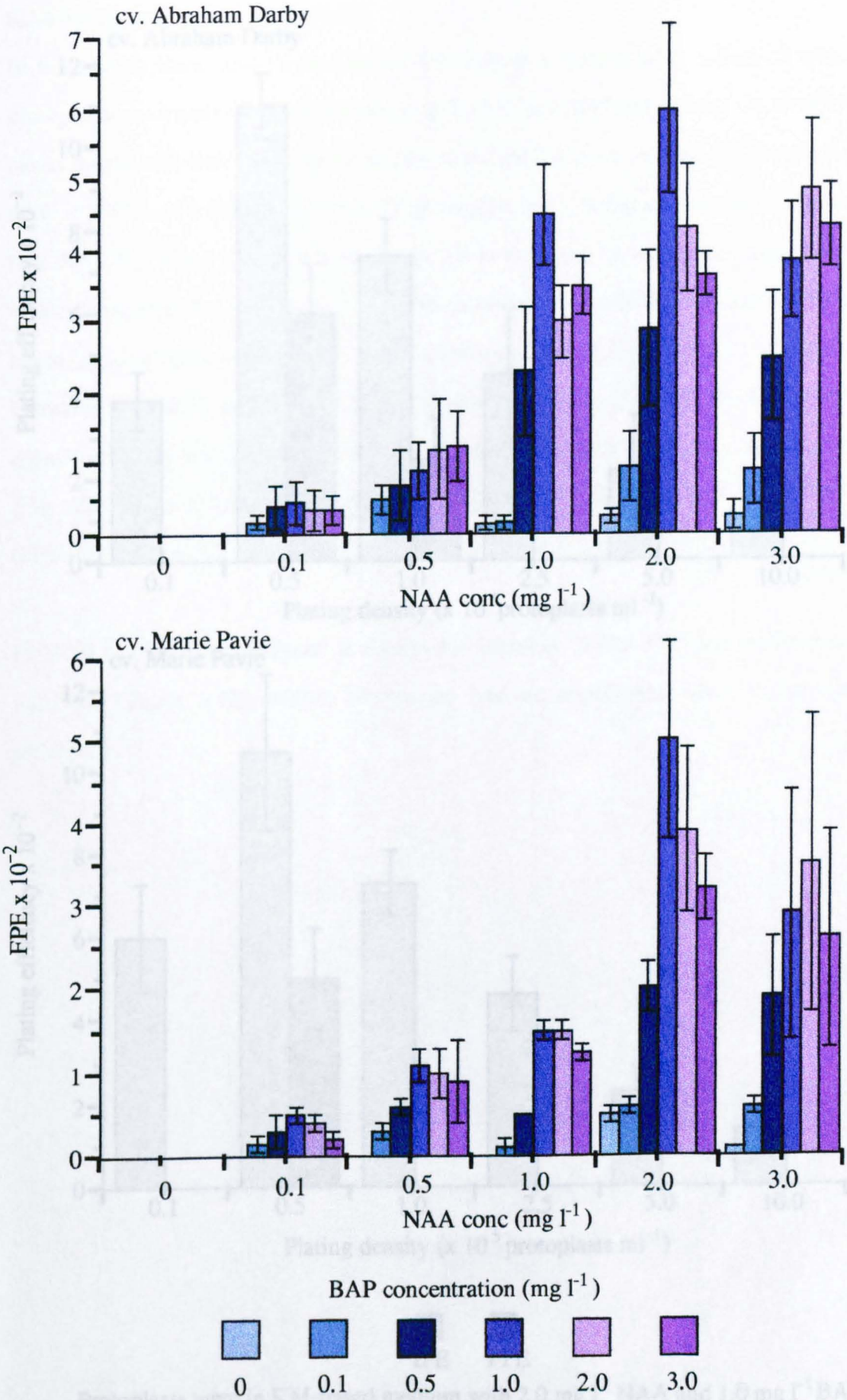
FPE control protoplasts: $5.9 \times 10^{-2} \pm 1.1 \times 10^{-2}$.

Table 5.11

Pulse Duration (μs)	Field Strength (V cm^{-1})			
	250	500	750	1000
20.0	3.0 ±1.1	3.6±0.5	5.8±0.7	5.0±1.2
40.0	3.1±1.4	3.2±0.8	6.1±0.9	4.1±0.7

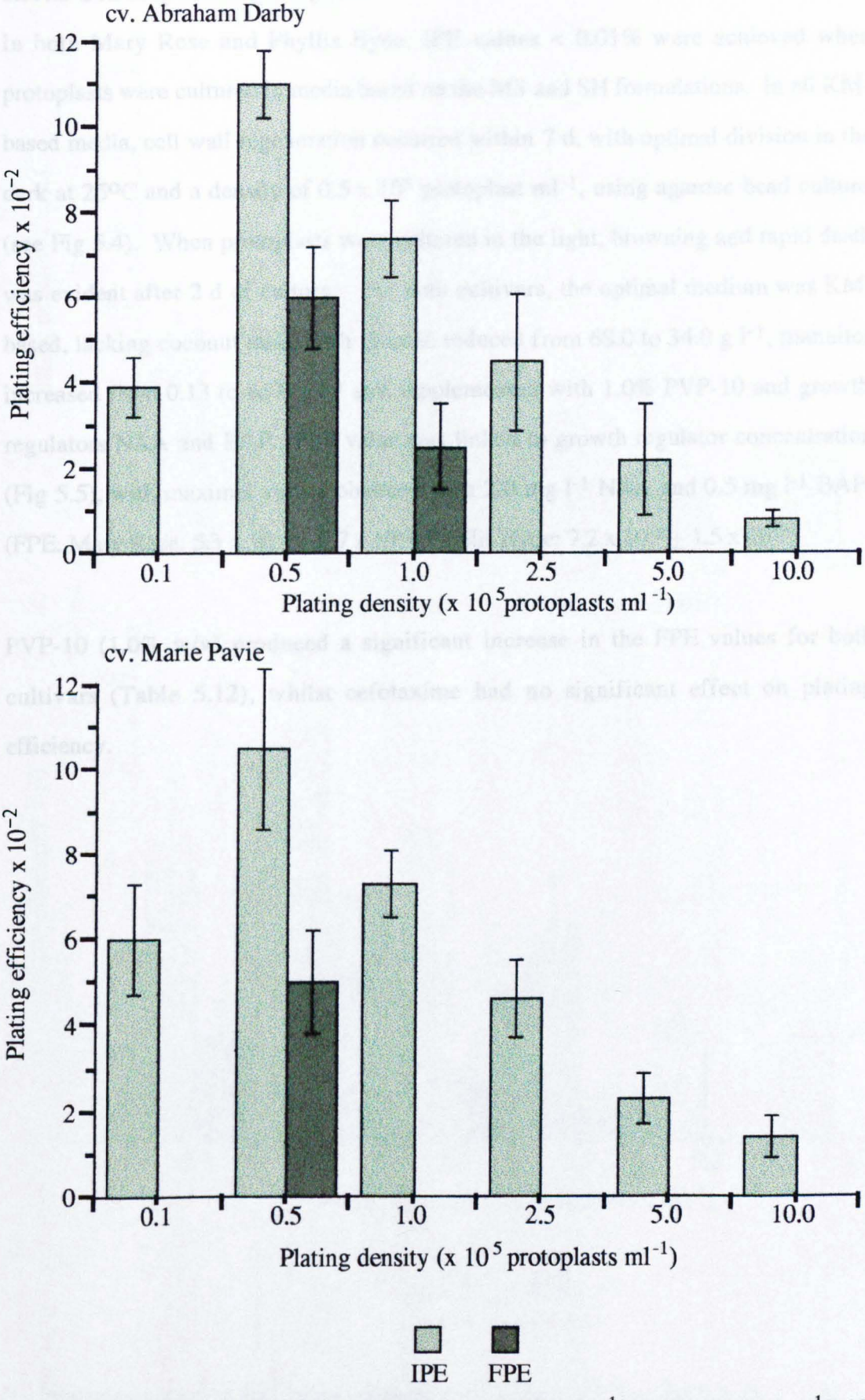
FPE control protoplasts: $4.9 \times 10^{-2} \pm 1.0 \times 10^{-2}$. Values are % FPE x 10^{-2}. Data are means of three replicates±standard deviation.

Figure 5.2 Effect of NAA and BAP concentrations on FPE of mesophyll protoplasts of two English rose cultivars.



Protoplasts were plated at a density of 0.5 x 10^5 protoplasts ml^{-1} in KM-based media. Data are the mean of three replicates. Bars represent standard error.

Figure 5.3 Effect of plating density on IPE and FPE of mesophyll protoplasts of the rose cvs. Abraham Darby and Marie Pavie.



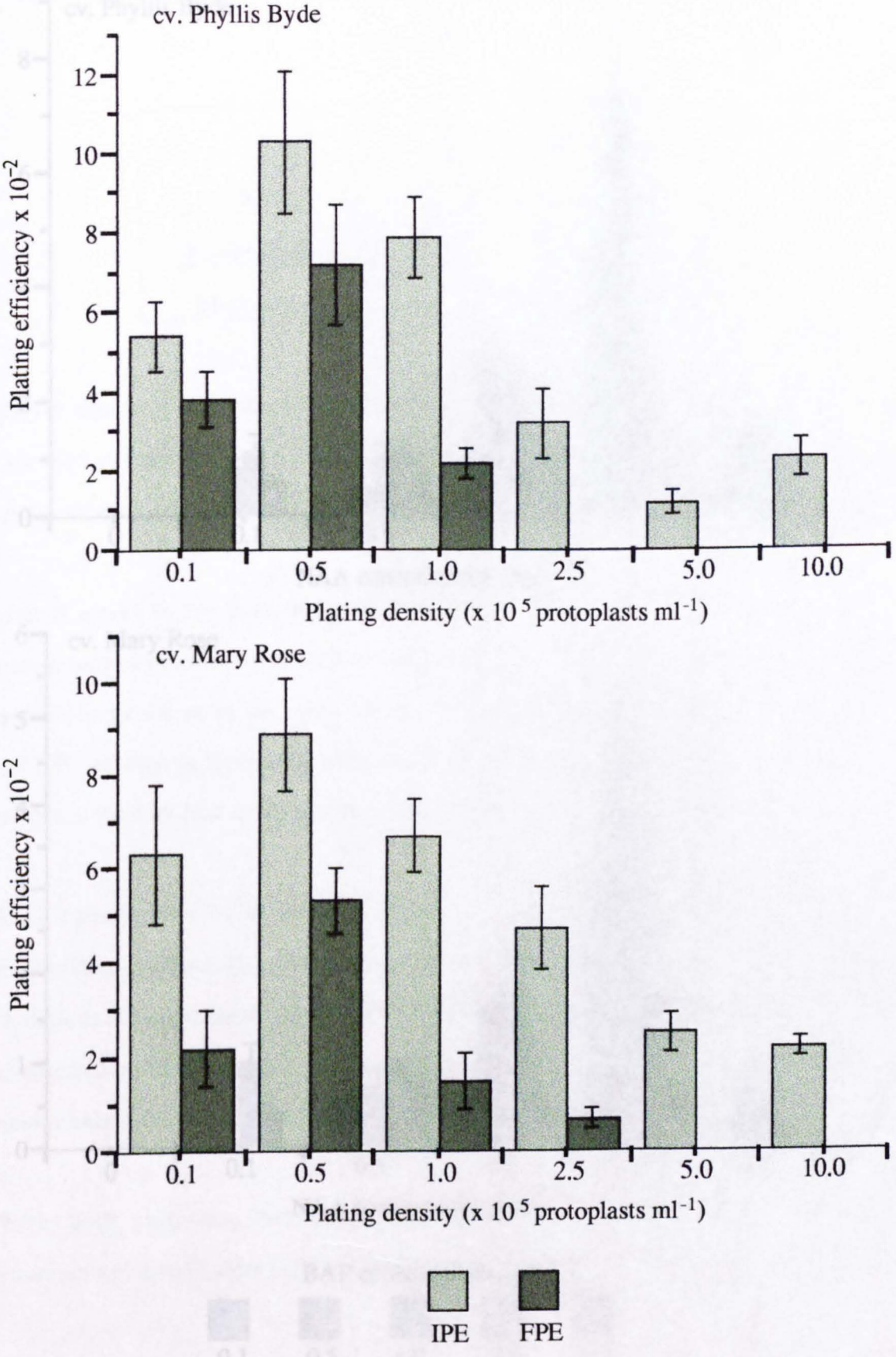
Protoplasts were in KM-based medium with 2.0 mg l^{-1} NAA and 1.0 mg l^{-1} BAP. Data are the mean of six replicates. Bars represent standard error.

5.3.4.2 Cell suspension protoplasts.

In both Mary Rose and Phyllis Byde, IPE values < 0.01% were achieved when protoplasts were cultured in media based on the MS and SH formulations. In all KM-based media, cell wall regeneration occurred within 7 d, with optimal division in the dark at 25°C and a density of 0.5×10^5 protoplast ml^{-1}, using agarose bead culture (see Fig 5.4). When protoplasts were cultured in the light, browning and rapid death was evident after 2 d of culture. For both cultivars, the optimal medium was KM-based, lacking coconut milk, with glucose reduced from 68.0 to 34.0 g l^{-1}, mannitol increased from 0.13 to 65.0 g l^{-1} and supplemented with 1.0% PVP-10 and growth regulators NAA and BAP. FPE value was linked to growth regulator concentration (Fig 5.5), with maximal values obtained with 2.0 mg l^{-1} NAA and 0.5 mg l^{-1} BAP (FPE: Mary Rose: $5.3 \times 10^{-2} \pm 0.7 \times 10^{-2}$; Phyllis Byde: $7.2 \times 10^{-2} \pm 1.5 \times 10^{-2}$).

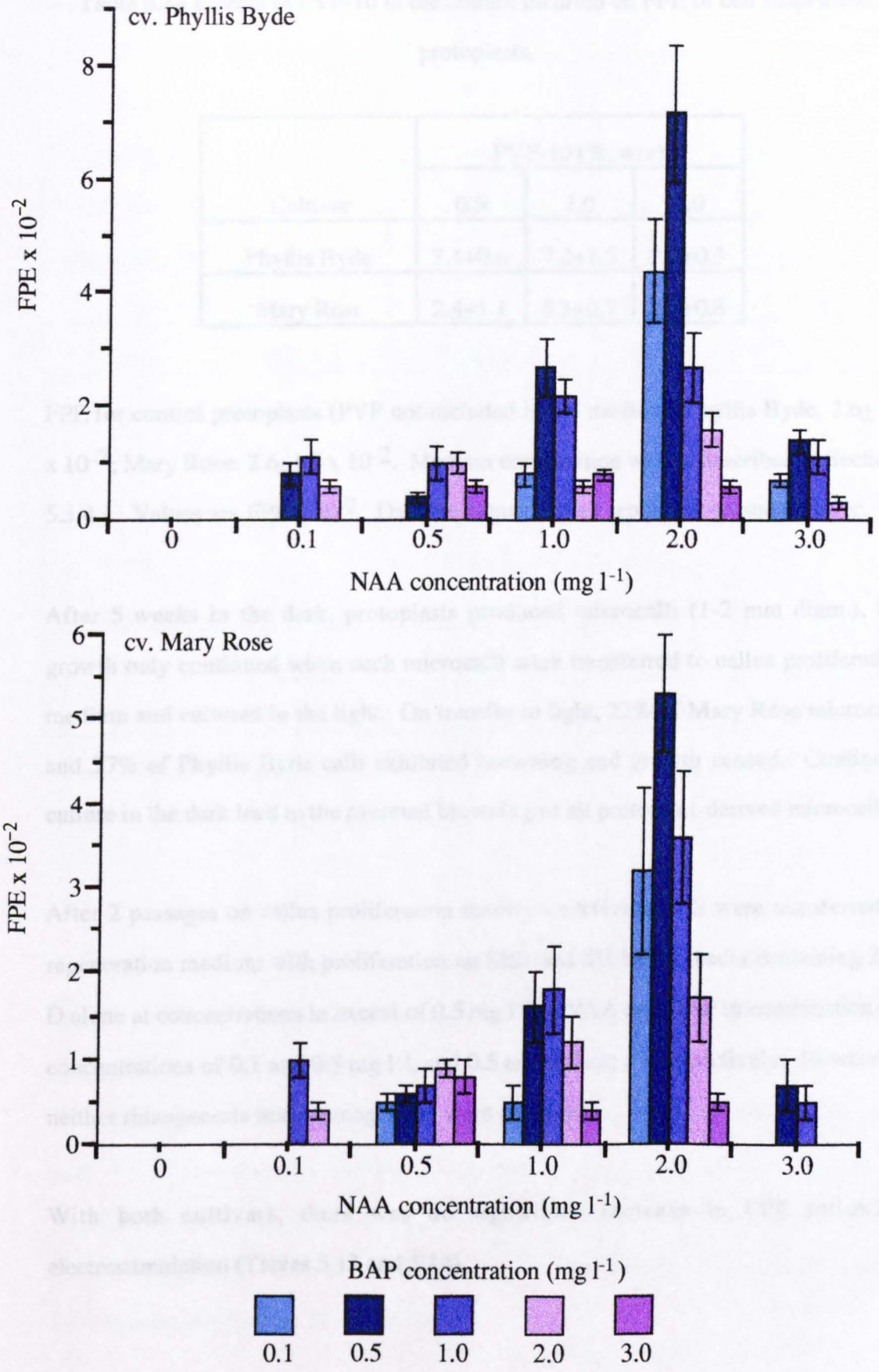
PVP-10 (1.0% w/v) produced a significant increase in the FPE values for both cultivars (Table 5.12), whilst cefotaxime had no significant effect on plating efficiency.

Figure 5.4 Effect of plating density on IPE and FPE of cell suspension-derived protoplasts of the rose cvs. Phyllis Byde and Mary Rose.



Protoplasts were in KM-based medium with 2.0 mg l^{-1} NAA and 0.5 mg l^{-1} BAP. Data are the mean of six replicates. Bars represent standard error.

Figure 5.5 Effect of NAA and BAP concentrations on FPE of cell suspension-derived protoplasts of two English rose cultivars.



Protoplasts were plated at a density of 0.5 x 10^5 protoplasts ml^{-1} in KM-based media. Data are mean of three replicates. Bars represent standard error.

Table 5.12 Effects of PVP-10 in the culture medium on FPE of cell suspension protoplasts.

Cultivar	PVP-10 (%, w/v)		
	0.5	1.0	2.0
Phyllis Byde	3.1+0.6	7.2+1.5	2.2+0.3
Mary Rose	2.4+1.1	5.3+0.7	2.9+0.8

FPE for control protoplasts (PVP not included in the medium) Phyllis Byde: $2.6 \pm 1.3 \times 10^{-2}$; Mary Rose: $2.6 \pm 1.5 \times 10^{-2}$. Medium composition was as described in Section 5.3.4.1. Values are FPE $\times 10^{-2}$. Data are means of five replicates $\pm$standard error.

After 5 weeks in the dark, protoplasts produced microcalli (1-2 mm diam.), but growth only continued when such microcalli were transferred to callus proliferation medium and cultured in the light. On transfer to light, 22% of Mary Rose microcalli and 37% of Phyllis Byde calli exhibited browning and growth ceased. Continued culture in the dark lead to the eventual browning of all protoplast-derived microcalli.

After 2 passages on callus proliferation medium, surviving calli were transferred to regeneration medium with proliferation on MS- and SH-based media containing 2,4-D alone at concentrations in excess of 0.5 mg l^{-1} or NAA and BAP in combination (at concentrations of 0.1 and 0.5 mg l^{-1}, and 0.5 and 2.0 mg l^{-1}, respectively. However, neither rhizogenesis nor organogenesis were observed.

With both cultivars, there was no significant increase in FPE following electrostimulation (Tables 5.13 and 5.14).

Tables 5.13 and 5.14 Influence of electrical field strength and pulse duration on FPE for Phyllis Byde (5.13) and Mary Rose (5.14) cell suspension protoplasts.

Table 5.13

Pulse Duration (μs)	Field Strength (V cm^{-1})			
	250	500	750	1000
20.0	6.9±1.4	6.3±1.1	6.0±1.9	7.1±0.9
40.0	6.7±1.2	6.3±1.5	7.2±1.5	6.7±1.1

FPE control protoplasts: 6.9 x 10^{-2} ±1.7 x 10^{-2}.

Table 5.14

Pulse Duration (μs)	Field Strength (V cm^{-1})			
	250	500	750	1000
20.0	5.5±1.9	5.9±1.3	5.7±0.5	6.1±1.6
40.0	5.0±1.3	5.0±2.0	5.5±1.2	5.3±1.0

FPE control protoplasts: 5.1 x 10^{-2} ±1.7 x 10^{-2}. Values are % FPE x 10^{-2}. Data are means of three replicates±standard deviation.

5.3.4.3 Callus protoplasts.

After 10 d, callus protoplasts (both cultivars) regenerated a new cell wall only when in KM-based media, with all of the culture methods (Section 5.2.2.2) giving an IPE of 15-20% after 12 d. Protoplast-derived cells in agarose beads of KM-based media with 2,4-D in excess of 1.0 mg l^{-1} divided to the 32 cell stage. The use of antioxidants (Section 5.2.2.1), and the use of a variety of cultural conditions, failed to prevent browning. Electrical treatment did not extend cessation of colony development beyond the 32 celled stage.

5.4 Discussion.

5.4.1 Isolation of English rose protoplasts.

5.4.1.1 Mesophyll protoplasts.

This is the first report of the successful isolation of mesophyll protoplasts for any English rose cultivars. The enzyme solutions developed here have also been used for a number of other woody plants (Park *et al.*, 1990) and at concentrations comparable to those for other Rosaceous genera, such as *Prunus* (Ochatt, 1992) and *Malus* (Patat-Ochatt *et al.*, 1993). The majority of enzyme mixtures used successfully for the isolation of protoplasts from woody plants incorporate Cellulase R-10 rather than Cellulase RS (Park *et al.*, 1990). In the present study, Cellulase RS was effective for English rose, probably reflecting the higher enzyme (cellulase) activity (Appendix III).

The physiological status of source material has been shown to be a decisive factor in the isolation of protoplasts of many woody species (James *et al.*, 1984; Ochatt and Caso, 1986; McCown and Russell, 1987; Ochatt, 1991). The need to use newly-expanded leaf material, rather than fully-expanded leaves, for the isolation of rose protoplasts is clear from the results presented here. This requirement for fast-growing tissues could be explained by the fact that during such growth, the cell wall is thinner, since wall elongation outstrips biosynthesis (Roberts and Butt, 1968). However, variations in the balance between endogenous growth regulators and also in the phenolic compounds (the concentration of which increases with the age of the material [Porter, 1991]) within such tissues, should not be ruled out as another possible factor.

The benefits associated with preplasmolysis of leaf tissues prior to enzyme incubation, in terms of increased protoplast yield and viability, were clear from the results. Where assessed, similar treatments were beneficial for apple (Doughty and Power, 1988) and wild pear (Ochatt and Caso, 1986) and such treatments are now

routine (for review, see Ochatt and Power, 1992). Enzymatic digestion of rose leaf tissues with constant shaking gave optimal results for all three cultivars investigated. Such results concur with those observed in many other woody species such as *Lonicera nitida* (Ochatt, 1991a) and *Malus* x *domestica* Borkh. (Patat-Ochatt, 1993).

Likewise, the inclusion of PVP-10 in the enzyme solutions, also increased the viability and the number of rose protoplasts which could be routinely isolated. PVP probably acts by reducing (or eliminating) the oxidation of phenolic compounds that are produced as a result of inevitable tissue/cell wounding. The reduction in phenol oxidation may also be a consequence of the use of dark conditions, as such reactions are light-induced. This agrees with studies on other woody species (Ochatt and Power, 1992).

The general method established for the purification of rose mesophyll protoplasts was similar to that of other woody plants ie. sieving followed by sucrose floatation (Ochatt and Power, 1992), but with 25% (w/v) sucrose, instead of the usual 21% (w/v). Such a requirement probably reflected the highly cytoplasmic nature of rose protoplasts. For cv. Marie Pavié, a reduction in viability was observed when Percoll was used. Percoll (and Ficoll) have generally been regarded as being preferable to sucrose floatation, since as they are osmotically inert, the same osmotic strength can be maintained throughout the isolation procedure thereby enhancing viability (Ochatt and Power, 1992). However, Ochatt *et al.* (1989b) reported a reduction in the viability of cherry mesophyll protoplasts following Percoll gradient treatment. Such a reduction in viability was attributed to physical damage resulting from repeated centrifuging/resuspension, which is necessary to remove all residues of Percoll. This may also account for the situation with rose.

5.4.1.2 Cell suspension protoplasts.

Results of this study illustrate that the optimal enzyme solution for the isolation of English rose cell suspension protoplasts was a mixture which consisted of Cellulase R-10, Pectolyase Y-23 and Hemicellulase. Cellulase R-10 and Hemicellulase [1.0% (w/v)], together with Pectinase [0.5% (w/v)], have previously been shown to be effective for the isolation of cell suspension protoplasts of *Rosa hybrida* cv. 'Paul's Scarlet' (Strauss and Potrykus, 1980) and for embryogenic cell suspension cultures of *R. persica* x *xanthia* (Matthews *et al.*, 1991). The pectolytic enzyme Pectolyase Y-23 has been extensively used for protoplast isolation from various Rosaceous species, such as *Pyrus communis* (Ochatt and Power, 1988a) and *Malus* x *domestica* (Patat-Ochatt *et al.*, 1988), and for several *Prunus* species (James *et al.*, 1984; Matsuta *et al.*, 1986; Ochatt and Power, 1988b). Exceptions were for *Pyrus communis* (Ochatt and Power, 1988c) and *Prunus avium* x *pseudocerasus* (Ochatt *et al.*, 1987). Although not essential for the isolation of protoplasts from cell suspensions of the English rose cultivars studied, namely Mary Rose and Phyllis Byde, the use of Pectolyase Y-23, when combined with Cellulase R-10 and Hemicellulase, did result in substantially higher yields of viable protoplasts.

Purification of English rose cell suspension protoplasts (floatation on CPW 21S solution) was consistant with a number of other woody species (see review, Ochatt and Power, 1992), including *R. persica* x *xanthia* protoplasts from embryogenic cell suspensions (Matthews *et al.*, 1991).

5.4.1.3 Callus protoplasts.

The fact that protoplasts could be obtained only from callus of the English rose cvs. Abraham Darby, Mary Rose and Phyllis Byde, using Enzyme Solutions 12 and 22, indicated a requirement, not only for effective mixtures of Hemicellulase, Cellulase and Pectolyase, but that these enzymes were required at relatively high concentrations. Krishnamurthy *et al.* (1979) isolated callus protoplasts from *Rosa*

hybrida cv. Soraya using an enzyme solution consisting of Macerozyme and Cellulase. Attempts to isolate English rose callus protoplasts using similar mixtures of enzymes (Enzyme Solutions 1, 3 or 4) failed, even when supplemeted with additional enzyme types (as Enzyme Solutions 5 - 7, 9 or 11). This suggests that the biochemical make-up and/or the structure of cell walls of English rose callus tissue is, in some way, different from that of rose cv. Soraya. Contrasting results for cell suspension protoplasts compared to callus protoplasts would seem to suggest that, within one cultivar, there are significant differences in wall structure and/or composition. Callus cells converted to cell suspensions are likely to have walls with reduced cellulose levels which may explain why a larger range of enzyme mixtures were effective in producing protoplasts from cell suspensions as compared to callus.

5.4.2 Protoplast culture.

The beneficial effects of including antioxidants in protoplast culture media were clearly illustrated in the results. The exclusion of antioxidants for mesophyll protoplasts/protoplast-derived cells led to browning and no further development. Only when 1.0% (w/v) PVP-10 was added could sustained division be achieved. For rose tissue culture, PVP-10 was most effective in preventing phenolic oxidation, in line with the observations of Ochatt (1991) who showed that, with mesophyll protoplasts of *Prunus avium*, the inclusion of 1.0% (w/v) PVP-10 resulted in a greater plating efficiency.

The failure of English rose protoplasts to respond to cefotaxime contrasts sharply with the observations of d'Utra Vaz *et al.* (1993), who reported increased IPE values, and sustainable division of protoplasts/cells of the woody species *Passiflora edulis* fv flavicarpa Degener. No reports satisfactorily explain such effects, although the contrasting results with *Passiflora* reflects species-specific responses at this level.

The requirement for a low plating density for maximal plating efficiency in both mesophyll and cell suspension protoplasts of English rose, may, in part, be associated with phenolic production, since at higher plating densities, the diffusion of phenolic compounds from non-viable cells will adversely affect the growth of neighbouring cells (Kirby *et al.*, 1989). The optimal plating densities, determined in this study, were comparable to those employed for the culture of cell suspension-derived protoplasts of *R. persica* x *xanthia* (Matthews *et al.*, 1991), but fall at the lower end of the range usually used with woody Rosaceous species such as *Prunus* ($0.5 - 5.0 \times 10^5$; Ochatt, 1992) and *Malus* ($0.5 - 2.0 \times 10^5$; Patat-Ochatt *et al.*, 1993). It may be that plating at higher densities ellicits a nutritional-limitation to growth since this was suggested for protoplasts of the rose cv. Paul's Scarlet when they were maintained at high densities (Strauss and Potrykus, 1980). In some woody plants, a protoplast-to-protoplast relationship appears to play a key role in their cultural responses and therefore a high plating density is required (Ochatt, 1989). The proliferation of protoplast-derived rose cells used in this study, would seem to indicate that such an effect either fails to occur or that it is not the limiting factor.

Sustained division of protoplast-derived cells of English rose was maximised when cultured in agarose beads surrounded by liquid medium. This technique was also beneficial for protoplast culture of other woody species, including *Passiflora* (d'Utra Vaz, 1992) *Pyrus* (Ochatt and Caso, 1986) and *Prunus* (Ochatt and Power, 1988b). The exact reason for this is uncertain, but an improved oxygen supply (d'Utra Vaz, 1992) or the better diffusion of toxic metabolites, may be important (Dons and Colijn-Hooymans, 1989). In all previous reports (Krishnamurthy *et al.*, 1979; Strauss and Potrykus, 1980; Matthews *et al.*, 1991), rose protoplasts were cultured in layered, semi-solid media. However, it is unclear, from these reports, as to why this culture option was retained since no authors reported the use of other culture strategies.

NAA and BAP, in different forms of modified KM8p were most effective for the culture of English rose protoplasts. This generally agrees with reports of other woody species (d'Utra Vaz *et al.*, 1993; Ochatt, 1993) and, in particular, Rosaceous species (Patat-Ochatt *et al.*, 1988; Ochatt *et al.*, 1989a). The claimed promotary effect of zeatin on protoplast division of several Rosaceous woody species (Ochatt and Power, 1988b; Ochatt, 1992) contrasts markedly with the inhibitory effect of zeatin on rose protoplasts. This was also consistent for studies on protoplasts of *R. persica* x *xanthia* (Matthews *et al.*, 1991). A requirement for KM-based media in line with other other woody species [*Passiflora* (d'Utra Vaz, 1992), *Malus* (Patat-Ochatt *et al.*, 1993) and *R. persica* x *xanthia* (Matthews *et al.*, 1991)], may suggest a familial requirement for an organic-rich medium. However since, in this study, organic suplementation of MS medium was ineffective other factors may be important. It is well documented that a reduction in the ammonium ion concentration in protoplast culture media is beneficial (or even essential) for the successful culture of woody plant protoplasts (see review by Ochatt and Power, 1992) but for English rose this was not the case. However, KM-based medium still contains approximately one-third the concentration of ammonium ions as compared to MS medium.

The successful culture of rose mesophyll protoplasts in the KM-based medium, with reduced glucose and increased mannitol, was also reported for the *R. persica* x *xanthia* protoplast system (Matthews *et al.*, 1991). In the present study, for both mesophyll and cell suspension protoplasts, media with relatively high concentrations of mannitol and a low concentration of sugar were optimal. A higher concentration of mannitol suggests a species-specific osmotic potential requirement in the medium. This coupled with the inclusion of a sugar which can be readily metabolised, is typical of protoplasts of many woody species (Ochatt and Power, 1992).

Electrical treatment of protoplasts has been reported for many species, including Colt cherry, Conference pear (Rech *et al.*, 1987) and passionfruit (Manders, 1992), with an

early onset of division and increased FPE values being observed. English rose failed to respond. Indeed, electrical treatment of mesophyll protoplasts (field strengths below 500 V cm^{-1}), independent of pulse duration, resulted in a significant decrease in FPE. A similar effect has been observed with mesophyll protoplasts of *Passiflora edulis* (Manders, 1992). An analysis of other studies on the electrical treatment of protoplasts would suggest that a positive growth response is linked to enhanced DNA synthesis (Rech *et al.*, 1988), coupled with semi-permanent membrane modifications leading to a sustained capacity for a larger/more efficient uptake of media components (Ochatt *et al.*, 1988). It is not unreasonable, therefore, to suggest that electrotreatment of protoplasts may well be species, variety or tissue-type dependant. Different electro-responses could also simply be size-linked (Rech *et al.*, 1987).

The finite growth response of callus protoplasts of three of the English rose cultivars to the 32-celled stage is difficult to explain, although it is far from unique. Antioxidants did not prevent such death associated with browning, as at the protoplast stage. The cessation of division, even at low cell densities and/or in media rich in macro- and micro-elements, suggests that this was not attributable to media component limitation, or, since the same response occurred at high plating densities, to lack of a 'nurse effect'. It is also unlikely that genetic aberrations would result in a total growth cessation specifically in callus cells.

5.4.3 Culture and regeneration of protoplast-derived tissues.

Protoplast-derived calli of all English rose cultivars/sources were capable of sustained proliferation on MS-based media and this concurs with previously published work on other species (Ochatt, 1989; Ochatt, 1991; Manders, 1992; Patat-Ochatt *et al.*, 1993). These authors have also shown that, small, protoplast-derived microcalli of woody species were unable to undergo organogenesis if transferred directly to plant regeneration medium (Ochatt, 1989). The need for a critical callus mass, prior to the initiation of plant (or root) regeneration, may be linked to the fact that, in woody

species, plant regeneration from true dedifferentiated callus is rare (Bajaj, 1986) and that reports for many species were, in fact, based on calli where portions of the initial explant were still present (James, 1987). In the absence of an explant portion, therefore, a longer time in culture seems to be required for protoplast-derived calli before heterogeneity and subsequent polarity can be established thus leading to organogenesis (Bunning, 1957). However, any proliferation period must be restricted to a minimum, as there is always the risk that long-term culture would reduce expression of organogenic totipotency. Such a loss of organogenic competence may have occurred in this study for rose cell suspension protoplast-derived calli in which regeneration could not be established. By contrast, English rose mesophyll protoplasts were not cultured for periods in excess of those for other (woody or herbaceous) species, and thus, a loss of shoot organogenic competence due to prolonged culture/somaclonal variation seems extremely unlikely.

Rhizogenesis was observed in calli of rose mesophyll protoplasts of the English rose cvs. Abraham Darby and Marie Pavié, in line with other Rosaceous species such as *Prunus cerasus* L. (Ochatt and Power, 1988). However, in this study the rhizogenic response occurred after culture on MS-based medium containing NAA, BAP and zeatin. In the present study on English rose, a comparable rhizogenic response was observed only on media containing 3.0 mg l^{-1} 2,4-D. Despite the screening of several media types containing a wide range of growth regulator types and/or concentrations/combinations, many of which had been previously used to attain regeneration, the production of shoots from English rose protoplasts was not achieved. This, in part, could be due to the rhizogenic nature of calli or to the roots themselves, in that root tips are sites of cytokinin synthesis (Davies, 1987; Lethan *et al.*, 1978) and, as such, cytokinins may be present at supraoptimal levels, thus inhibiting direct or indirect shoot formation.

Attempts to obtain shoot regeneration based on supplementation of media with PVP-10, sodium thiosulphate and TIBA in part designed to minimise the possible oxidation of phenolic compounds suppressing shoot regeneration, were unsuccessful. Ethylene production has also been shown to reduce morphogenic responses in callus of species such as *Zea mays* (Vain *et al.*, 1989) and for various *in vitro* growth responses in rose, such as shoot proliferation (Mekers *et al.*, 1984; Kevers *et al.*, 1992). It was assumed that rose callus tissues in this study contained high levels of endogenous auxins which, in turn, are known to stimulate ethylene production in many species (Yu and Yang, 1979), including *R. hybrida* (Wulster and Secalis, 1980). Sodium thiosulphate, an inhibitor of ethylene action (Leshem *et al.*, 1986), was thus included. TDZ, a potent cytokinin, reported to stimulate shoot formation in callus of many species (Lu *et al.*, 1993), failed to induce shoot regeneration in protoplast-derived calli of English rose and, as a consequence of this result, TIBA was evaluated as a media supplement. TIBA can drive bud formation in both herbaceous or woody explants (McCown, 1989; Belaizi *et al.*, 1991) and inhibit polar transport of auxin (Thompson *et al.*, 1973) in a non-competitive way (Depta *et al.*, 1983), by binding with the anion efflux carrier situated at the basal end of the cell (Davies, 1987). Such a fixation modifies the ligand conformation and prevents its translocation through the plasmalemma (Depta and Rubery, 1984). TIBA added to the regeneration medium, might have inhibited the transport of auxin to the potential regeneration sites, thus leading to a more favourable balance between cytokinins and auxins for shoot regeneration. Combined effects of such additives were not apparent for English rose.

Once callus becomes rhizogenic the chance of caulogenesis occurring is severely limited (Fruit Tree Biotechnology Meeting, 1986, France). Researchers in the past have therefore usually discarded such rhizogenic tissues. However, shoots have been regenerated from explant-root calli (where a portion of the initial root explant was required to be retained) in *Prunus* (Druart, 1980; Jones *et al.*, 1984). Ochatt and Power (1988) encountered rhizogenic responses in protoplast-derived calli of *Prunus*

cerasus, and were able to convert the roots produced, via a further culture phase, to adventive shoots. This protocol (Ochatt and Power, 1988) was therefore extended to rose, together with the use of the aforementioned media supplements. Irrespective of root portion, this option was not successful for English rose.

Although this study has clearly illustrated the possibility of isolating and culturing protoplasts of several English rose cultivars, the negative regeneration responses that were encountered reflect a persistent recalcitrance, common to many woody species.

5.5 Summary.

i) Strategies were developed for the isolation of large numbers of viable protoplasts from *in vitro* derived leaf tissues, cell suspensions and calli of several cultivars of English rose.

ii) Enzyme solutions, containing Hemicellulase, Macerozyme, Cellulase RS and Pectolyase Y-23 in CPW 13M solution, were optimal for the isolation of mesophyll protoplasts (cvs. Abraham Darby and Marie Pavié).

iii) Highly viable protoplasts were sucessfully isolated from callus and cell suspensions using enzyme solutions based on Cellulase R-10, Pectolyase Y-23 and Hemicellulase.

iv) Mesophyll protoplasts were sucessfully cultured to the microcalli stage in agarose bead cultures with KM-based medium containing 30 g l^{-1} sucrose, 90 g l^{-1} mannitol, 2.0 mg l^{-1} NAA and 1.0 mg l^{-1} BAP.

v) Cell suspension protoplasts were also sucessfully cultured to the microcalli stage in agarose bead cultures with KM-based medium containing 34 g l^{-1} glucose, 65 g l^{-1} mannitol, 2.0 mg l^{-1} NAA and 0.5 mg l^{-1} BAP.

vi) Plating density, growth regulator concentration and the use of antioxidants were all demonstrated to significantly affect protoplast plating efficiency.

vii) Transfer of mesophyll protoplast-derived calli to SH medium containing 3.0 mg l^{-1} 2,4-D stimulated rhizogenesis. Shoot regeneration could not be established.

CHAPTER 6.
Towards somatic hybridisation in rose by chemical and electrical fusion, and the subsequent selection of fusion products.

6.1 Introduction.

Woody species are ideal candidates for somatic hybridisation, as improvement by conventional sexual crossing methods is constrained by their complex and often prolonged life-cycles and by sexual incompatibility (Chapter 1, Section 1.2.1). For several years, somatic hybridisation has been suggested as a possible method for the improvement of rose (Strauss and Potrykus, 1980; Matthews *et al.*, 1991), as sexual incompatibility has frequently presented a barrier to the breeding of many new cultivars. Sexual incompatibility in crosses between English rose cultivars had been demonstrated to be the result of a pollen-style incompatibility mechanism operating to arrest pollen tube growth within the stylar tissues (Chapter 2, Sections 2.3.4 and 2.4.2). In the light of these observations, and concomitant with investigations into the culture and regeneration of English rose protoplasts, somatic hybridisation was investigated in order to provide a possible alternative for the production of intervarietal hybrids between cultivars previously regarded as sexually incompatible. In particular, the cvs. Abraham Darby, Mary Rose and Marie Pavié were employed in this study, as they had been shown to be amenable to *in vitro* culture techniques (Section 4.3.1), such as micropropagation (source material), and because they were confirmed as being sexually incompatible (Chapter 2, Sections 2.3.4 and 2.4.2). In addition, the production of hybrids between cvs. Abraham Darby and Marie Pavié, and between Mary Rose and Marie Pavié, is regarded as highly desirable by breeders as this may facilitate the production of plants, which would be suitable for incorporation into subsequent breeding programmes, by combination of several desirable attributes (David Austin; pers. comm.).

The numerous strategies which are available for the fusion of plant protoplasts and for the subsequent identification/selection of fusion products, along with the advantages and disadvantages of the different techniques have been described (Marchant *et al.*, 1993; see enclosures-this thesis). In the present study, chemical fusion using purified polyethylene glycol (PEG) and electrically-mediated fusion were investigated and the manual selection of heterokaryons was performed using micromanipulation. This technique relied on the visual discrimination of morphologically distinct, or fluorescently-labelled, protoplasts and their resultant fusion products. Following the fusion of green mesophyll protoplasts with protoplasts from cell suspensions, which lack chlorophyll, heterokaryons can either be identified as possessing both normal chloroplasts and colourless plastids. More precisely when both fusion partners are cell suspension-derived (and thus colourless), heterokaryons can be identified by a dual yellow-green fluorescence (FDA) labelling of one parent combined with the use of the orange-red fluorescent label rhodamine isothiocyanate (RITC) for the other partner (Barsby *et al.*, 1984). Micromanipulation is advantageous as a selection method in that it eliminates the need to employ more complex techniques such as introduced antibiotic resistance mediated by transformation. It also avoids the potential drawbacks associated with flow cytometric methods (Marchant *et al.*, 1993) in which adhering, non-fused protoplasts (which arise with the use of chemical fusogens) cannot be discriminated from heterokaryons. Despite the fact that micromanipulation has not been widely reported, it has been effective for heterokaryon/hybrid selection in the genera *Nicotiana* (Galbraith and Mauch, 1980) and *Medicago* (Gilmour *et al.*, 1987; Mendis *et al.*, 1991).

6.2 Materials and Methods.

6.2.1 Origin of protoplasts.

Protoplasts were isolated from *in vitro* shoot cultures of cvs. Abraham Darby and Marie Pavié and from cell suspension cultures of cvs. Mary Rose and Phyllis Byde, using previous procedures (Chapter 5, Section 5.2.1).

6.2.2 Fluorescence-labelling of protoplasts prior to fusion.

Mesophyll-derived protoplasts of cv. Abraham Darby and cell suspension-derived protoplasts of cv. Mary Rose were labelled with FDA. FDA (2 μl of a 10 μg ml^{-1} stock made up in acetone) was added to 10 ml of CPW 13M solution containing protoplasts at a density of 1 x 10^6 protoplasts ml^{-1}, and incubated at room temperature (15 min). In order to remove excess FDA, protoplasts were washed 3 times by centrifugation and resuspension in either CPW 9M solution (for subsequent chemical fusion procedure) or electrofusion solution (for subsequent electrofusion procedure). RITC (Sigma, Poole, Dorset, UK) (12 μl of a 20 μg ml^{-1} stock made up in acetone) was added to 10 ml of CPW 13M solution containing either mesophyll-derived protoplasts of cv. Marie Pavié or cell suspension-derived protoplasts of cv. Phyllis Byde, both at a density of 1.0 x 10^6 protoplasts ml^{-1} (30 min). Excess RITC was removed by washing 5 times using the same procedure as for FDA. Labelled protoplasts were visualised under UV illumination as described in Section 6.2.7 (Plate 6.1a and b). Unfused, labelled protoplasts of all types were used as controls.

6.2.3 Protoplast fusion combinations.

The following combinations of protoplasts were subjected to both chemical and electrical fusion procedures:

i) Abraham Darby mesophyll-derived protoplasts [+] Phyllis Byde cell suspension-derived protoplasts (no fluorescent labels).

ii) Abraham Darby mesophyll-derived protoplasts (FDA label) [+] Marie Pavié mesophyll-derived protoplasts (RITC label).

iii) Mary Rose cell suspension derived protoplasts (FDA label) [+] Phyllis Byde cell suspension-derived protoplasts (RITC label)

iv) Mary Rose cell suspension-derived protoplasts [+] Marie Pavié mesophyll-derived protoplasts (no fluorescent labels).

6.2.4 Chemical fusion of protoplasts.

Protoplasts of each fusion partner (combinations as described in the previous section) were resuspended separately in 16 ml centrifuge tubes in CPW 9M solution (Appendix II) at a density of 4.0 x 10^5 protoplasts ml^{-1}. Prior to fusion, they were dispensed as either 4 ml aliquots (for viability controls of each genotype), 8 ml aliquots (for homofusions of each genotype) and as two 4 ml aliquots of each partner mixed together (1:1 for heterofusion) into screw-capped glass centrifuge tubes. Following centrifugation (100 xg, 10 min), 2 ml of polyethylene glycol (PEG) fusion solution (see Table 6.1) was added to each of the tubes (with the exception of the viability controls). After 10 min the PEG fusion solution was gradually diluted by addition to each tube of 0.5, 1.0, 2.0, 3.0, 4.0 ml aliquots of CPW 13M solution (Appendix II). Samples of protoplasts (200 µl approx.) were removed and examined microscopically in order to determine fusion frequencies. Protoplasts were finally resuspended in 10 ml of the appropriate double-strength protoplast culture medium (Chapter 5, Section 5.3.4) in preparation for micromanipulation.

Table 6.1 Composition of PEG fusion solution[1].

30 % (w/v) polyethylene glycol (m. w. 6000; Koch Light Ltd., Haverhill, UK.)
4.0% (w/v) sucrose
0.01 M $CaCl_2.2H_20$

[1]. Sterilised by autoclaving (121°C, 20 min); stored in dark at 4°C until required.

6.2.5 Electrofusion of protoplasts.

Protoplast combinations (described in Section 6.2.3) were subjected to electrofusion, employing the apparatus of Jones *et al.* (1994). The AC field was produced by a commercial function generator with a 15 V peak output. This was increased to a potential field strength of 170 V cm^{-1} at 1 MHz by inclusion of a parallel series of

high frequency pulse transformers and a radio frequency amplifier. The DC unit provided 1 - 4 sequential pulses which were individually pre-selected, and the duration of each pulse and the inter-pulse duration was independently controlled by a logic circuit. The unit was fitted with a Gould DSO-420 oscilloscope (Gould, Ilford, Essex, UK) for the direct monitoring of the released AC and DC voltages and discharges were performed using a remote fire switch. The voltages were applied to the protoplasts using 7 parallel nickel-silver plate electrodes, each with a 0.27 mm Perspex separator (as described by Watts and King, 1984). The electrode was sterilised by immersion in absolute ethanol (at least 30 min), followed by drying in a sterile air flow prior to protoplast electrofusion.

Protoplasts of each parental type were resuspended in electrofusion solution (0.5 mM $CaCl_2$, 13 % w/v mannitol, pH 5.6) at a density of 2.5 x 10^5 ml^{-1}. The parental protoplast suspensions were mixed in the ratio 1:1 (v/v) and 1 ml aliquots dispensed into the 8 central compartments of a 5 x 5 well plastic dish (Sterilin, Hounslow, UK), which was located on the stage of a Nikon inverted microscope contained within a laminar air flow cabinet. The electrode assembly was placed, in turn, into the compartments containing the protoplast samples. In each compartment, the protoplasts were aligned with the application of an AC field. The DC pulse(s) were applied and the AC field reduced. Following fusion, the protoplasts were left undisturbed (30 min) and 1 ml of double-strength protoplast culture medium (Chapter 5, Section 5.3.4) was added to each well. After 30 min, the electrofusion solution/culture medium mixture was pipetted away and replaced with 2 ml of fresh, double strength protoplast culture medium in preparation for micromanipulation. Non-fused (but, where appropriate, fluorescently labelled) protoplasts of both parent types, suspended in double-strength culture medium, were used as controls.

6.2.6 Evaluation of the optimum electrofusion parameters.

6.2.6.1 Determination of protoplast alignment and fusion.

Both during fusion, and immediately after the application of a DC pulse, it was possible to observe the response of protoplasts (in terms of alignment and fusion) by the use of the inverted microscope. Protoplasts were aligned in an 80 Vcm^{-1} sine wave AC field. After application of pulse(s) (400 - 1200 V cm^{-1}; 500 - 1500 μs duration) the AC field was reduced to 40 V cm^{-1} to avoid post-pulse displacement of protoplasts. Where multiple fusion pulses were applied, the interpulse duration was 0.25 s. With each treatment combination, a low magnification (x40) photomicrographic was produced. From this photographic record, it was possible to determine the percentage of the total population of protoplasts that underwent i) dimer formation (ie. protoplasts forming pearl chains consisting of just one protoplast of each type), and ii) the proportion of the total number of protoplasts that underwent fusion. Evaluation of protoplast response for all electrofusion parameters was taken from three experimental replicates.

6.2.6.2 Determination of the FPE of fused, non-micromanipulated protoplasts.

Fusions were performed on all the combinations (Section 6.2.3) using both electrofusion parameter combinations (Section 6.2.6.1) and chemical fusion procedure (Section 6.2.4). Fusion products were subsequently cultured (without micromanipulation) at a density of 5.0 x 10^4 protoplasts ml^{-1} in agarose-solidified droplets surrounded by liquid culture medium (Section 5.2.2.2). The media and culture conditions employed were those that had previously been determined to be optimal (Chapter 5, Section 5.3.4). Fusion products arising from mesophyll- and cell suspension-derived protoplasts were always cultured in media and conditions determined optimal for the mesophyll protoplast fusion partner (Chapetr 5, Section 5.3.4). The FPE of protoplasts was evaluated when the majority of protoplast derived colonies had reached a size of 1 - 2 mm diam. (Section 5.2.2.4). Twenty replicate dishes of protoplasts were cultured for each combination of fusion parameters, and

the experiment was repeated three times. Calli were subsequently subjected to the regeneration protocol previously described (Chapter 5, Section 5.2.3).

6.2.7 Micromanipulation for the sorting of heterokaryons and their subsequent culture.

Apparatus, used for the micromanipulation of fused rose protoplasts, was a modified version of that previously described for single cell engineering (Koop and Schweiger, 1985; Schweiger *et al.*, 1987; Koop and Spangenberg, 1989). The apparatus was obtained via Dr. H. U. Koop, University of Munich, Germany. Briefly, the equipment consisted of a Zeiss Axiovert 35M microscope (fitted with a UV light source, excitation filter B [IF-490], dichroic mirror, barrier filter 0530 and a Sony CMA-D1CE close-circuit video camera in turn connected to a television monitor) with a stepmotor driven stage (with 1μm accuracy), a stepmotor driven microtool holder (Bachofer 7410) for the vertical positioning of a microneedle in the optical axis of the microscope and a electric micropump (Hamilton Pumps, Bonaduz, Switzerland) capable of delivering nanolitre volumes (Plate 6.1c). The nanolitre pump, microscope stage and microstage actions were all automatically controlled by a microcomputer running software designed by Peter Kraus (University of Munich, Germany). Disposable 100 μl pipettes ('Drummond Microcaps', Drummond Scientific Co, Broomall, PA, USA) were each drawn into a fine point (80 - 100 μm diam.) using a Kopf vertical microelectrode puller (Kopf Instruments, Tujunga, California, USA.). A 90° bend was created by heating the microcapillary in a gas flame. The microcapillary was then, in turn, connected using 1.5 mm diam. silicon tubing to the nanolitre pump and was primed with fine hydraulic oil.

A sample of protoplasts/heterokaryons resulting from the fusion experiments and suspended in double-strength culture medium, was placed in a 3 cm plastic Petri dish mounted on the microscope stage. Using either the eyepieces of the microscope or the TV monitor, it was possible to visualise heterokaryons (depending on the fusion

combination that was being examined) either as having dual orange-green fluorescence (Plate 6.1d), or by possessing mixed chloroplasts and plastids. When a heterokaryon was identified, the microneedle was brought into position alongside, using the joystick control driving the stepmotor. Once the needle was in place (this was determined either by viewing through the microscope or by watching the TV monitor), a footswitch was pressed. This activated the nanopump to collect the heterokaryon along with 100 nl of the surrounding double-strength liquid culture medium. This procedure was repeated until 100 heterokaryons had been collected in the tip of the needle. When this was accomplished the computer controller automatically removed the needle and deposited the heterokaryons and the accompanying medium as a droplet in a separate, 3 cm diam. Petri dish. The needle was then automatically returned to a different location in the original dish. This procedure was repeated, each time placing the droplet of heterokaryons in the same place to form a droplet of ever increasing size. Once sufficient heterokaryons were present to culture (ie. to give the relevant optimum plating density), an equal volume of molten (approx. 40°C) double-strength SeaPlaque agarose [1.2% (w/v) in water] was added and mixed using a sterile yellow Gilson tip to ensure even gelling and heterokaryon distribution. Once solidified, the droplet was surrounded with liquid culture medium as described in Chapter 5, Section 5.2.2.2 and cultured as previously described (Chapter 5, Section 5.3.4). After 5 - 7 weeks, visible microcalli formed and FPE values were determined. Subsequent growth of heterokaryon-derived microcalli (approx. 2 mm diam.) and attempts to regenerate plants from this material followed procedures previously described (Chapter 5, Section 5.2.3). In order to assess possible deleterious (handling) effects of micromanipulation on rose protoplasts, the same procedures were adopted for non-fused (but, where appropriate, fluorescently labelled) protoplasts of all the parental types to act as controls. In all experiments, a minimum of three replicates were tested and each experiment was repeated at least twice.

6.2.8 Analysis of putative somatic hybrid material.

6.2.8.1 Protein banding analysis of tissues derived from electrofused protoplasts.

6.2.8.1.1 Extraction of proteins.

In order to assess the hybrid nature of the putative somatic hybrid callus tissues recovered by micromanipulation, total protein profiles derived from extracted proteins were obtained following their isoelectric focusing (see 6.2.8.1.2).

Randomly selected, putative somatic hybrid calli, derived from micromanipulated heterokaryons and calli derived from non-fused micromanipulated parent-type protoplasts, were used for protein extraction. Samples of callus (0.25 - 0.5 g f. wt.) were individually homogenised with 200 µl of ice-cold protein extraction buffer (Appendix V) in sterile 1.5 ml Eppendorf tubes using sterile disposable grinders (Bel-art Products, Pequannock, NJ, USA). Once the callus material had been thoroughly disrupted, the Eppendorf tubes were briefly centrifuged (6000 rpm; 3 min) and the supernatants transferred to fresh tubes which were stored on ice until required.

6.2.8.1.2 Isoelectric focusing of proteins.

'Isogel' agarose isoelectric focusing (IEF) plates (pH range 3 - 7) (FMC BioProducts, Rockwell, USA), cast onto 0.2 mm thickness 'Gelbond' film, were used for the rapid focusing of high molecular weight proteins (≥ 200 kD). An IEF plate (stored at 4°C) was placed onto the pre-wetted (2 ml distilled water) platter of an LK3 Broma 2117 IEF chamber (Multiphor, Sweden). Two electrode wicks (supplied by FMC BioProducts) were soaked in either catholyte solution (0.1 M L-histidine free-base) or anolyte solution (0.5 M acetic acid, pH 2.6) and the excess solution blotted off. The wicks were placed parallel to each other at the ends of the gel corresponding to the cathode and anode electrodes respectively. A glass plate, slightly larger than the gel, was laid (for 1 min approx.) on top of the gel and wicks, to ensure uniformity of contact between the wicks and the gel surface. Sample wicks were placed directly onto the gel, parallel and approx. 1.5 cm adjacent to the cathode wick. Aliquots (20 -

40 µl) of protein extract were pipetted onto the sample wicks, and the refrigerated circulator bath (10°C approx.) of the IEF chamber was started. The electrodes were placed in contact with the appropriate electrode-wicks and a DC voltage was applied (500 V; 15 mA) for 10 min. The sample wicks were carefully removed using fine forceps and the voltage reapplied (1 h).

6.2.8.1.3 Staining of IEF plates for total protein.

The total protein banding profile was visualised by staining the gels with PAGE blue dye (0.2% w/v PAGE blue, 70% v/v absolute ethanol and 8% v/v glacial acetic acid) for 16 h. This was followed by repeated rinses (10 min each) in the same solution lacking the PAGE blue dye, until the clarity of the blue bands, corresponding to the proteins, was optimised and the blue background colour of the gel was reduced. The gel was photographed on Ilford FP4 film, through a 3x yellow filter.

6.2.8.2 Ploidy level assessments of putative somatic hybrid tissues.

The ploidy levels of putative somatic hybrid calli (in the absence of plants) derived from all fusion combinations (Section 6.2.3) were evaluated. Pieces of calli (approx. 3 mm^3) were immersed in a solution comprising 0.1% (w/v) colchicine (Sigma, Poole, UK) in basal MS medium. After a period, not exceeding 3 h, calli were transferred to fixative (1 part glacial acetic acid: 3 parts absolute ethanol) and incubated for 16 h (25±3°C). Following fixation, calli were hydrolysed for 30 min in 5 M HCl (60°C) and then transferred to Feulgen solution (BDH, Poole, UK) for 1 h. The stained callus was placed in a drop of Feulgen solution on a microscope slide and dispersed using a mounted needle before being overlaid with a coverslip. The coverslip was tapped gently using the flattened end of a glass rod in order to further disperse the cells, and pressed firmly between several sheets of paper towel. The coverslip was gently prised off using a mounted needle. The exposed cellular material was fixed to the slide by warming in the flame of a spirit burner. The cytoplasm was counter-stained by immersing the slide in 1% (w/v) toluidine blue (20

min), followed by washing by immersion in two changes of distilled water (15 min each). The slide was air dried in a laminar air flow cabinet for 30 min. A drop of distilled water was applied and a coverslip placed over the preparation prior to sealing with nail varnish. Using a Vickers M41 Photoplan microscope, typically 45 callus cells of calli resulting from each fusion combination were examined (15 cells from each of three randomly selected calli).

6.2.8.3 Random amplified polymorphic DNA (RAPD) analysis of putative rose somatic hybrid tissues.

6.2.8.3.1 DNA extraction from putative hybrid tissues.

The DNA was extracted from samples of parent tissues and putative somatic hybrid tissues using a modified version of the procedure described by Edwards *et al.*, 1991. Fresh tissues (0.25 g f. wt.) were macerated (15 min) at room temperature in sterile 1.5 ml Eppendorf tubes using sterile disposable grinders. Following maceration, 400 µl of extraction buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, 0.1% mercaptoethanol) were added to each sample and the latter vortexed for 5 s. The samples were left at room temperature for at least 30 min (usually until all the sample extractions had been completed). The extracts were centrifuged (13000 rpm; 1 min) and 300 µl of the resulting supernatants were transferred to clean, sterile Eppendorf tubes. An equal volume (300 µl) of isopropanol was added and the samples left at room temperature for a further 2 min. The sample were centrifuged (13 000 rpm, 5 min) and the supernatants removed by evaporation in the sterile air flow of a laminar air flow cabinet. The resulting pellets were each dissolved in 100 µl of TE buffer (10 mM Tris HCl; 1 mM EDTA; pH 8.0). Ten µl of 3M Na acetate were added and the samples well mixed. Two hundred and fifty µl of ice-cold 100% absolute ethanol were added and the samples were again thoroughly mixed. The samples were stored at -20°C (10 min) prior to centrifuging (13 000 rpm, 5 min). Following centrifugation, the supernatants were removed (by pouring away) and the resultant pellets washed in ice-cold 70% (v/v) absolute ethanol. The samples were

again centrifuged (13 000 rpm, 2 min) and the pellets dried by placing the open Eppendorf tubes inside a laminar air flow cabinet. The DNA pellets remaining were each rehydrated in 50 ml of TE buffer and stored at -70°C until required for polymerase chain reaction (PCR) amplification.

6.2.8.3.2 Amplification of extracted DNA.

An OP-AA-10 primer was used (obtained from Operon Technologies Inc, Alameda, California, USA). The primer sequence was: 5'- TGG TCG GGT G -3'. The concentration of dNTPs used in the PCR was 28 μl of each, and primers were at 6 μl each. Dynazyme thermostable DNA polymerase (2 μl; Flowgen, Sittingbourne, Kent, UK.) was added immediately before the first cycle. PCR cycling was performed for 1.0 min at 93°C (30°C min^{-1}), 1.0 min at 37°C (30°C min^{-1}) and 1 min 20 s at 72°C (20°C min^{-1}). In the final cycle, polymerisation time was extended to 5.0 min at 72°C in order to complete synthesis of PCR fragments, followed by 10.0 min at 4°C The total number of cycles was 46. PCR was performed using a Techne PHC 3 thermal cycler (Techne Ltd., Cambridge, UK.).

6.2.8.3.3 Analysis of PCR products.

One-tenth of the PCR products were separated by electrophoresis through 1% agarose (SeaKem, FMC BioProducts, USA) gels run on a BRL Horizon mini-gel system with a Consort Microcomputer Electrophoresis Power Supply. Gels were stained with ethidium bromide (0.5 μg ml^{-1} from a 10 mg ml^{-1} stock solution) and visualised under UV illumination.

6.2.9 Statistical Analysis.

Where appropriate, the statistical significance was determined using analysis of variance.

6.3 Results.

6.3.1 Effects of fluorescence-labelling of protoplasts.

The labelling of protoplasts with the fluorescent stains FDA and RITC (Section 6.2.2), did not significantly affect the FPE of the protoplast types used in this study.

6.3.2 Chemical fusion of protoplasts.

Viable fusion products were obtained from all the fusion combinations described in Section 6.2.3. Fusion frequencies were lowest when both fusion partners were of mesophyll origin. The fusion of mesophyll-derived protoplasts with cell suspension-derived protoplasts resulted in increased fusion frequencies, which were further increased when both fusion partners were of cell suspension origin (Table 6.2). Protoplast agglutination was common and the majority of the protoplasts underwent multiple fusion (ie. more than two protoplasts fused). The products of all fusion combinations showed significantly reduced FPEs compared to unfused protoplasts (Table 6.2). On culture, fusion products of all combinations underwent division to the 16-cell stage (approx.), after which division ceased.

Table 6.2 Chemical fusion of English rose protoplasts.

Protoplast Fusion Combination	Fusion Frequency (%)	FPE (%)[1]
i) Abraham Darby mesophyll [+] Phyllis Byde cell suspension	0.9 ± 0.10	$2.2 \times 10^{-2} \pm 1.5 \times 10^{-7}$
ii) Abraham Darby mesophyll [+] Marie Pavié mesophyll	0.5 ± 0.08	$1.9 \times 10^{-2} \pm 0.8 \times 10^{-7}$
iii) Mary Rose cell suspension [+] Phyllis Byde cell suspension	1.3 ± 0.17	$2.1 \times 10^{-2} \pm 1.3 \times 10^{-7}$
iv) Mary Rose cell suspension [+] Marie Pavié mesophyll	1.0 ± 0.12	$1.3 \times 10^{-2} \pm 2.1 \times 10^{-7}$

[1] Determined as described in Section 5.2.2.4. FPE values (%) of control, non-fused protoplasts: Abraham Darby; $6.0 \times 10^{-2} \pm 1.5 \times 10^{-5}$, Phyllis Byde; $7.2 \times 10^{-2} \pm 1.3 \times 10^{-2}$, Marie Pavié; $4.9 \times 10^{-2} \pm 1.2 \times 10^{-5}$, Mary Rose; $5.5 \times 10^{-2} \pm 1.0 \times 10^{-2}$.

6.3.3 Electrofusion of protoplasts.

The frequency of protoplast fusion was highest (1.62%) when cell suspension-derived protoplasts of the cvs. Mary Rose and Phyllis Byde were electrofused using a single pulse (1500 μs) at a field strength of 1200 V cm^{-1} (Fig 6.1). Minimum fusion frequencies (0.08%) were encountered when protoplasts were fused with a field strength of 400 V cm^{-1} (Fig 6.1). In general, with all the fusion combinations attempted, the fusion frequency increased as the applied field strength increased. At lower field strengths (400 and in some cases 800 V cm^{-1}) fusion frequencies generally increased as the pulse duration increased. However, at a greater field strength (1200 V cm^{-1}), maximal fusion frequencies were generally obtained with a 1000 μs pulse duration (Fig 6.1). Higher fusion frequencies were obtained when both fusion partners were derived from cell suspensions; fusions in which one partner was

cell suspension-derived generally resulted in a higher fusion frequency than those in which both partners were of a mesophyll origin (Fig 6.1). At field strengths of 800 V cm^{-1} or less, the largest proportion of potential heterokaryons resulted (Fig 6.2). However, at field strengths of 1200 V cm^{-1}, although the overall fusion frequency was increased, the greatest proportion of fusion products resulted from multiple fusions (Fig 6.2). Field strength or number of pulses applied during the electrofusion procedure did not have any significant effect on the FPE of the fusion products (Table 6.3). The FPE values of electrofused protoplasts were not significantly different ($p<0.05$) from their non-fused parent controls (Table 6.3). The time required for the development of microcalli (as defined in Section 5.2.2.4) from the fusion products was 30 - 40 d. This was comparable to that required for the formation of microcalli from non-fused protoplasts. Organogenic responses have not been observed at the time of writing.

Figure 6.1 The effects of the number of DC pulses, field strength and pulse duration on the electrofusion frequency of four English rose cultivars.

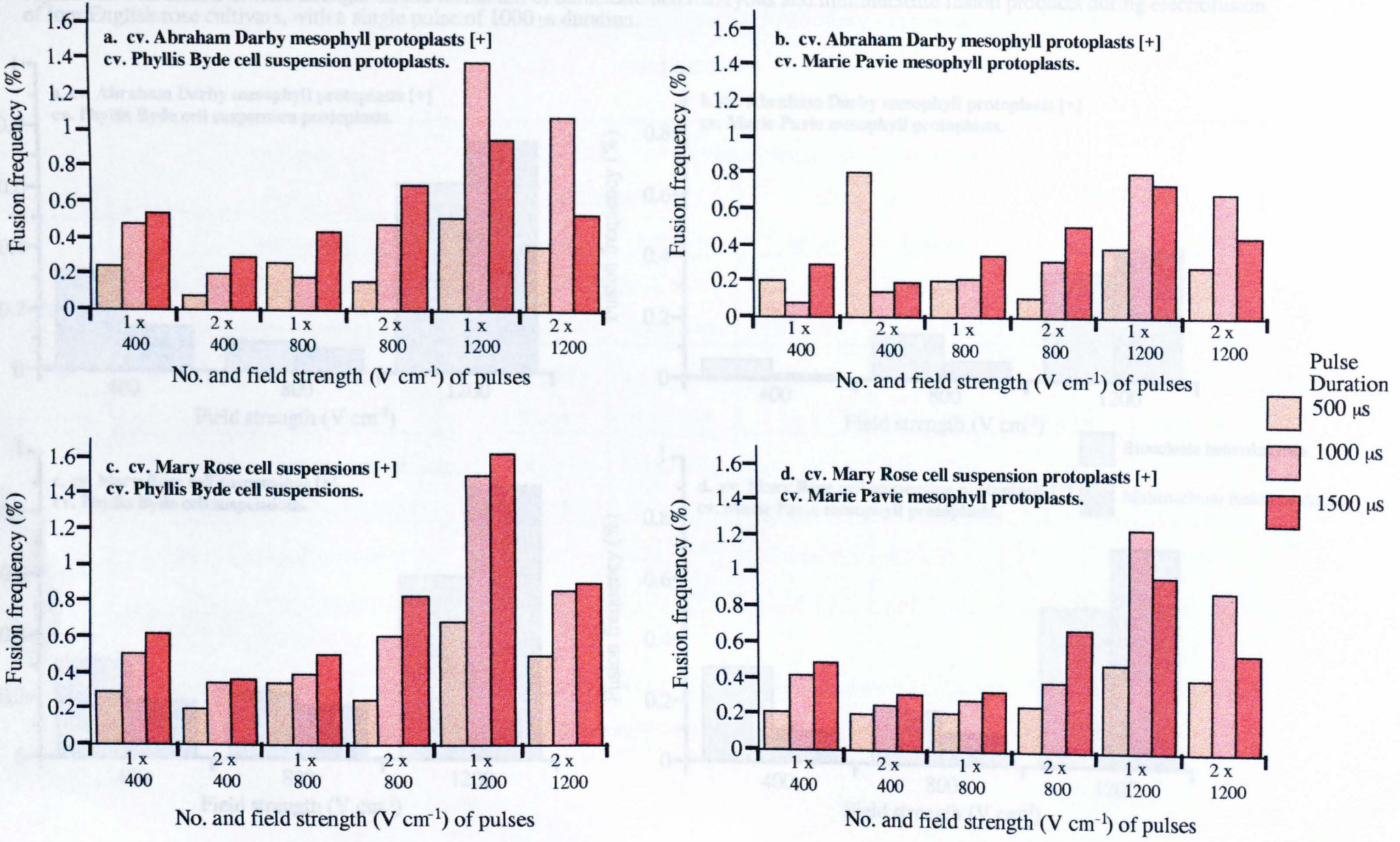


Figure 6.2 The effects of field strength on the formation of binucleate heterokaryons and multinucleate fusion products during electrofusion of four English rose cultivars, with a single pulse of 1000 μs duration.

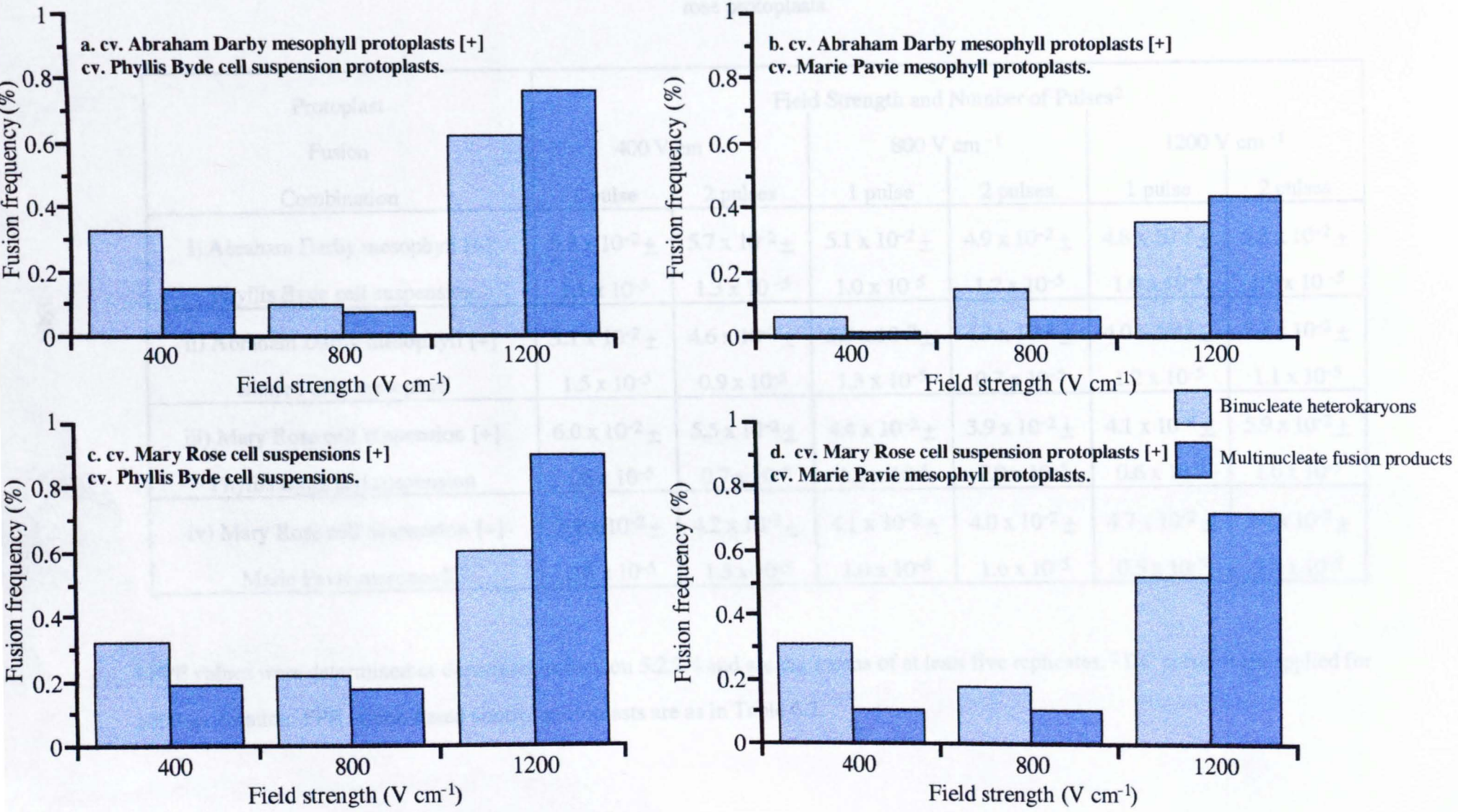


Table 6.3 Effects of the DC field strength and number of pulses on the FPE[1] (%) of non-micromanipulated fusion products of English rose protoplasts.

Protoplast Fusion Combination	Field Strength and Number of Pulses[2]					
	400 V cm $^{-1}$		800 V cm $^{-1}$		1200 V cm $^{-1}$	
	1 pulse	2 pulses	1 pulse	2 pulses	1 pulse	2 pulses
i) Abraham Darby mesophyll [+] Phyllis Byde cell suspension	$5.5 \times 10^{-2} \pm 1.1 \times 10^{-5}$	$5.7 \times 10^{-2} \pm 1.3 \times 10^{-5}$	$5.1 \times 10^{-2} \pm 1.0 \times 10^{-5}$	$4.9 \times 10^{-2} \pm 1.2 \times 10^{-5}$	$4.8 \times 10^{-2} \pm 1.0 \times 10^{-5}$	$5.3 \times 10^{-2} \pm 1.9 \times 10^{-5}$
ii) Abraham Darby mesophyll [+] Marie Pavié mesophyll	$3.1 \times 10^{-2} \pm 1.5 \times 10^{-5}$	$4.6 \times 10^{-2} \pm 0.9 \times 10^{-5}$	$5.0 \times 10^{-2} \pm 1.3 \times 10^{-5}$	$4.3 \times 10^{-2} \pm 0.7 \times 10^{-5}$	$4.0 \times 10^{-2} \pm 1.2 \times 10^{-5}$	$5.1 \times 10^{-2} \pm 1.1 \times 10^{-5}$
iii) Mary Rose cell suspension [+] Phyllis Byde cell suspension	$6.0 \times 10^{-2} \pm 1.6 \times 10^{-5}$	$5.5 \times 10^{-2} \pm 0.7 \times 10^{-5}$	$4.4 \times 10^{-2} \pm 1.2 \times 10^{-5}$	$3.9 \times 10^{-2} \pm 0.9 \times 10^{-5}$	$4.1 \times 10^{-2} \pm 0.6 \times 10^{-5}$	$5.9 \times 10^{-2} \pm 1.6 \times 10^{-5}$
iv) Mary Rose cell suspension [+] Marie Pavié mesophyll	$3.9 \times 10^{-2} \pm 0.8 \times 10^{-5}$	$4.2 \times 10^{-2} \pm 1.5 \times 10^{-5}$	$4.1 \times 10^{-2} \pm 1.0 \times 10^{-5}$	$4.0 \times 10^{-2} \pm 1.6 \times 10^{-5}$	$4.7 \times 10^{-2} \pm 0.5 \times 10^{-5}$	$5.4 \times 10^{-2} \pm 1.3 \times 10^{-5}$

[1] FPE values were determined as described in Section 5.2.2.4 and are the means of at least five replicates. [2] DC pulses were applied for 1000 μs duration. FPE of non-fused control protoplasts are as in Table 6.2.

Plate 6.1.

a. FDA labelled mesophyll protoplasts of cv. Abraham Darby exhibiting yellow-green fluorescence under UV illumination (x 50).

b. RITC labelled mesophyll protoplasts of cv. Marie Pavié exhibiting orange-red fluorescence under UV illumination (x 50).

c. Micromanipulation apparatus (as described in Section 6.2.7) employed for the selection of heterokaryons (x 0.05).

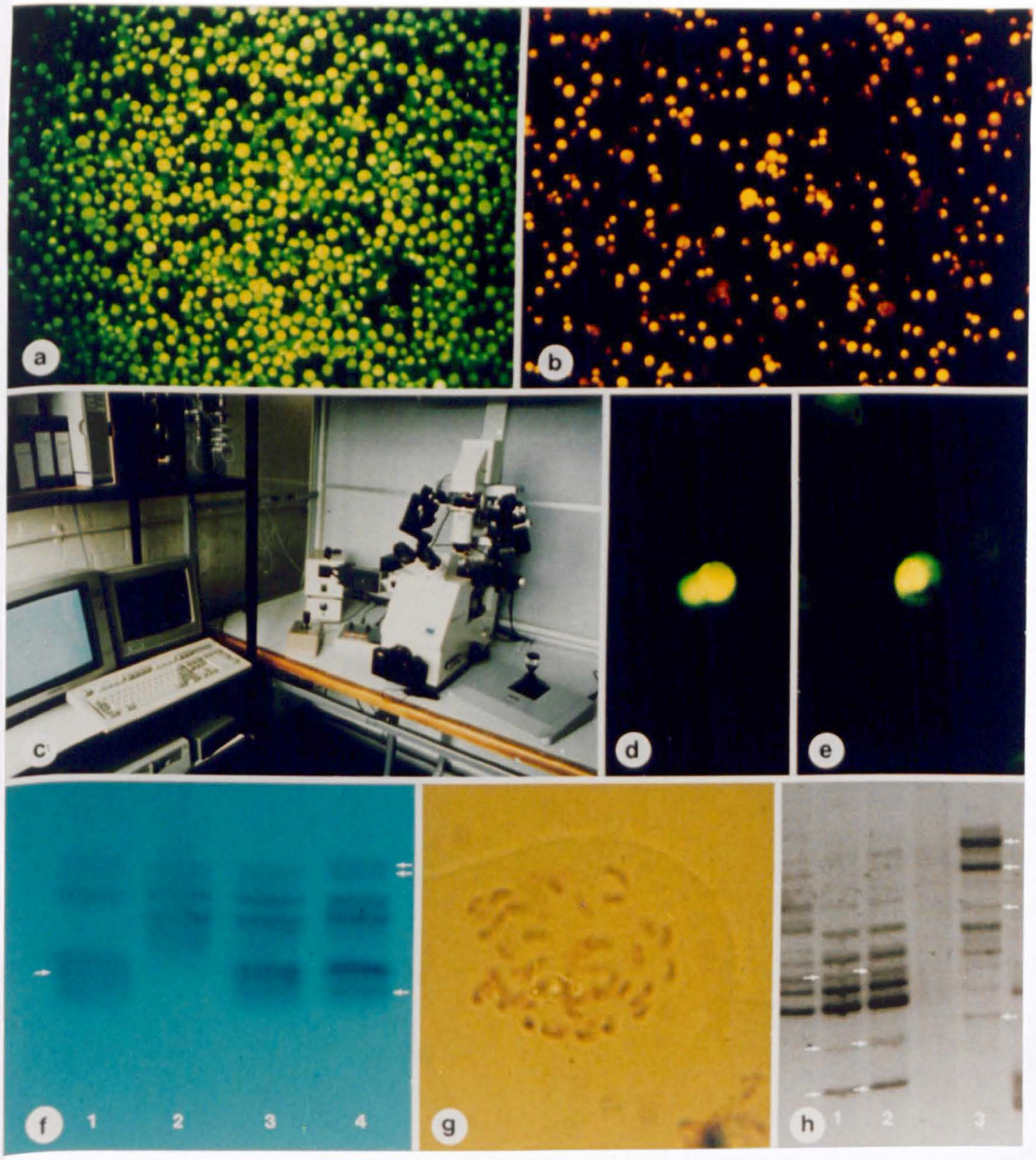
d. FDA labelled mesophyll protoplast of cv. Abraham Darby and RITC labelled mesophyll protoplast of cv. Marie Pavié aligning just prior to electrical fusion (x 170).

e. Heterokaryon resulting from the electrofusion of mesophyll protoplasts of cvs. Abraham Darby and Marie Pavié exhibiting dual orange-green fluorescence under UV illumination (x 200).

f. Isoelectric focusing banding patterns of parental and putative hybrid callus material of cvs. Abraham darby and Marie Pavié; Lane 1: cv. Abraham Darby, Lane 2: cv. Marie Pavié, Lane 3: physical mixture, Lane 4: putative somatic hybrid. The arrows (—>) indicate the bands from each parental cultivar that are missing in the somatic hybrid, and the arrows (<—) indicate the new (hybrid) bands present in the putative somatic hybrid callus.

g. Micrograph of cell from putative somatic hybrid callus resulting from the fusion of mesophyll protoplasts of cvs. Abraham Darby and Marie Pavié stained using the procedure described in Section 6.2.8.2 (x 1000).

h. Banding pattern obtained after separation of PCR RAPD amplification products through agarose gel; Lane 1: cv Abraham Darby, Lane 2: cv. Marie Pavié, Lane 3: putative somatic hybrid. The arrows (—>) indicate the bands from each parental cv. that are missing in the somatic hybrid, and the arrows (<—) indicate the new (hybrid) bands present in the putative somatic hybrid.


a
b
c
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6.3.4 Micromanipulation of heterokaryons and their subsequent culture.

6.3.4.1 Micromanipulation of the products of chemical fusion.

The isolation of heterokaryons resulting from chemical fusion by micromanipulation proved extremely difficult. A large proportion of protoplasts exhibited agglutination which resulted in the formation of large clusters of unfused protoplasts. Frequently, heterokaryons would also adhere to these clusters and it was impossible to separate these without excessive damage. Clusters of protoplasts (containing both heterokaryons and unfused parental types) often accidentally entered the micromanipulation needle and prevented either selected heterokaryons from being discharged or heterokaryons from being collected. The addition of volumes of culture medium in order to reduce the density of protoplasts did not rectify these problems. Because of these problems chemical fusion was not pursued.

6.3.4.2 Micromanipulation of the products of electrical fusion.

By contrast, micromanipulation of the products of electrical fusion was relatively straightforward due to lack of agglutination. Between 1.5 - 3 h was required to collect sufficient heterokaryons to produce one agarose bead (as described in Section 5.2.2.2). The FPE of selected fusion products was not significantly different ($p<0.05$) from those obtained with non-micromanipulated controls (Section 6.3.3). Thus, one bead gave 4 - 12 calli. Using micromanipulation, it was possible to recover putative somatic hybrid callus material from electrofusion for all the cultivar combinations. To date, plants have yet to be recovered.

6.3.5 Analysis of putative somatic hybrid material.

6.3.5.1 Protein banding analysis by isoelectric focusing.

In all cases, the protein banding patterns, derived from putative somatic hybrid material, contained bands which were common to protoplast-derived calli of one of the unfused parent types. Zymograms of the parents contained a number of bands which were absent in the putative somatic hybrids. Additionally, a number of unique

bands, which were not present in profiles of either of the parents, were present for the somatic hybrid material (Plate 6.1e). These observations initially suggest that calli may truly be of heterokaryon origin.

6.3.5.2 Ploidy level assessments.

Accession records and cytological studies by the breeder (D. Austin) had previously indicated that the rose cultivars used in this study were either diploid (2n=2x=14) or tetraploid (2n=4x=28) (Table 6.4). Additional analyses, as part of this study, confirmed these observations to be correct. On examination, cells of calli derived from isolated protoplast fusion products had mixaploid chromosome numbers ranging from (2n=6x≈42-52) (Table 6.5) within one callus mass, although the majority (in excess of 91%) of cells possessed a (2n=6x=42) chromosome complement (Table 6.5; Plate 6.1f). As it was expected that the chromosome number of somatic hybrid material (calli cells or plants) would be a summation of the two parent cultivars (ie. 2n=6x=42 chromosomes), the observation that chromosome numbers in putative hybrid material were ca. 42 was taken as a strong indicator of the somatic hybrid nature of the calli.

Table 6.4 Cell chromosome number of protoplast-derived calli of unfused parent English rose cultivars.

Cultivar	No. of Chromosomes	Ploidy designation[1]
Abraham Darby	28	Tetraploid
Phyllis Byde	14	Diploid
Marie Pavié	14	Diploid
Mary Rose	28	Tetraploid

[1] Consistent with the known cultivar data supplied (D. Austin; pers. comm.).

Table 6.5 Distribution of somatic chromosome numbers of putative somatic hybrid callus materials.

Callus Origin [1]	Proportion of Cells with 2n=6x=42 Chromosomes [2] (%)	Distribution of Chromosome Numbers other than 2n=6x=42
Abraham Darby [+] Phyllis Byde	93.3	43, 43, 45
Abraham Darby [+] Marie Pavié	88.8	43, 44, 47, 48, 52
Mary Rose [+] Phyllis Byde	95.5	45, 45
Mary Rose [+] Marie Pavié	91.1	45, 46, 49, 50

[1] Protoplast fusion combination. [2] Percentage per 45 cells (15 cells/callus) examined.

6.3.5.3 RAPD analysis.

Using the OP-AA-10 primer, DNA amplification products were obtained only for the parent cultivars Abraham Darby, Phyllis Byde and Marie Pavié, and from products arising from fusion combinations (i) and (ii), as described in Section 6.2.3. After fractionation of products by agarose gel electrophoresis, only a low level of polymorphism was observed between the parent cultivars. However, the fusion products showed a relatively higher degree of polymorphism, with unique amplification products that were not present in either of the parent cultivars, along with fragments that were present in one or both of the parents (Plate 6.1g). Such a result would seem to suggest that the putative hybrid lines resulting from the cultivar cross combinations (i) and (ii) (Section 6.2.3) were indeed, somatic hybrids. Although it can be concluded that the putative somatic hybrid material has a different

genetic background to the parent tissues it was not possible to confirm, beyond all reasonable doubt, the presence of both parental genomes in the somatic hybrid material because of the lack of unambiguous polymorphic amplification products from both parents in a given sample.

6.4 Discussion.

6.4.1 Methods of protoplast fusion.

The merits (and limitations) of both electrical and chemical methods of protoplast fusion must be considered when deciding which approach can ultimately be the most appropriate for the fusion of any two particular protoplast types. Chemical fusion has been commonly used for protoplasts of woody species such as *Citrus* (Ohgawara *et al.*, 1989; Deng *et al.*, 1992) and for intergeneric somatic hybridisation between *Pyrus* and *Prunus* genera (Ochatt *et al.*, 1989a). However, in this study on English roses, electrical fusion was found to be far superior. Firstly, chemical fusion methods resulted in a large amount of protoplast agglutination, which made heterokaryon selection by micromanipulation virtually impossible. Secondly, electrical fusion generally produced a higher fusion frequency than chemical fusion and, thirdly, electrical fusion did not affect FPE. The reduction in FPE values that was observed with chemical fusion was probably caused by the phytotoxic effects of contaminants such as oxidative decomposition products (Chand *et al.*, 1988). However, the use of purified forms of PEG would still result in protoplast agglutination which, for rose, would have prevented the use of micromanipulation.

The optimal fusion frequencies and parameters were comparable here for rose to those with other woody species such as *Passiflora* (d'Utra Vaz, 1992). Increasing field strength resulted in an increase in the number of multiple fusion products and since one-to-one fusion was a priority it was evident that optimisation of electrofusion parameters had to be a compromise. Although leaf-derived protoplasts generally fuse more readily than cell suspension-derived protoplasts (Marchant *et al.*, 1993), the

fusion frequencies encountered here for cell suspension-derived protoplasts (both cultivars) probably reflected differences in size of the protoplasts (see Chapter 5, Fig 5.1). This confirmed the observations of Tempelaar and Jones (1985), who noted that, in general, larger protoplasts can be electrofused more easily than can smaller protoplasts. Enhancement of FPE values following the electrofusion of English rose protoplasts was not observed, which was consistent with earlier findings (Section 5.3.4). This contrasts with the findings of Manders (1992) and d'Utra Vaz (1992), who reported an enhancement of FPE values after electroporation and/or electrofusion of *Passiflora* protoplasts.

6.4.2 Selection of fusion products.

A key requisite of somatic hybridisation is to selectively encourage heterokaryon division at the expense of homokaryons or unfused protoplasts. As resistance markers (transformed cells) were not available for rose, the development of manual methods (ie. micromanipulation) was the only option. Although time consuming, it can eliminate the presence of multiple fusion products. A low optimum plating density, combined with the fact that the agarose-bead method of culture required relatively small numbers of protoplasts, made micromanipulation an ideal option.

The fact that the use of fluorescent labels and the physical handling effects of micromanipulation did not significantly reduce the FPEs of protoplasts in this study, also increased the validity of this method for rose hybridisation. Flow cytometry, for example, can damage protoplasts resulting in FPE reductions (Marchant *et al.*, 1993).

6.4.3 Confirmation of hybridity.

Until recently, isozyme analysis alone was widely employed as a reliable method of confirming hybridity of tissues arising from protoplast fusion products. In this study, isozyme analysis had suggested that material initially designated as being putatively hybrid had indeed arisen from the protoplast fusion. However, the use of isozymes

alone cannot be conclusive proof of hybridity as it has been demonstrated that banding patterns are subject to shifts by environmental factors and also by the physiological status of tissues (Brown *et al.*, 1993). In addition, the use of isozymes in distinguishing between closely-related cultivars, such as those used in this study, has also been questioned (Brown *et al.*, 1993). It was important therefore, that the results of the isozyme analyses carried out as part of this study were only indicators of hybridity, rather than as 'stand-alone' evidence of somatic hybridity.

Recently, the use of RAPD markers has been employed to identify somatic hybrids (Baird *et al.*, 1992; Waugh and Powell, 1992) and is seen as a more equivocal method of confirming somatic hybridity. In the present study, it was not possible to obtain amplification products from all the samples. This may have been due either to phenolic degradation of extracted DNA, or to the use of inappropriate primers. It was also not possible to obtain a clear difference in the banding patterns of DNA amplified from the parental callus materials. Once again, this may be due to the poor quality of extracted DNA or due to the use of inappropriate primers. However, such a result indirectly confirms the assumed close relationship between the parent English rose cultivars. As the banding patterns obtained from the putative somatic hybrid tissues were so different from that obtained for the two parent cultivars, it is clear that the genetic background of this material is quite different and, in all probability, resulted from an isolated heterokaryon. It is highly improbable that this could be attributed to somaclonal variation in all cases.

Karyological examination of the putative somatic hybrid materials provided evidence of true hybridity. Variation from the original parental chromosome number can of course occur by pathways other than fusion, for example, by somaclonal variation (Karp, 1991) which has been demonstrated to occur in many species, including rose (Moyne *et al.*, 1993). Additionally, karyological examination may not absolutely discriminate between heterokaryon-derived ploidy changes and those arising from

homokaryons that may have come through the selection procedure, especially when superimposed onto a background of possible somaclonal variation/chromosome elimination (Marchant *et al.*, 1993). If the chromosome numbers encountered in this study had arisen from the type of chromosome doubling reported for *R. hybrida* callus by Moyne *et al.* (1993), then it would be expected that the chromosome numbers of the majority of cells within the callus material would have been ca. 28 (2n=4x=28) or 56 (2n=4x=56). This clearly was not the case. In this study, the minimum number of chromosomes was 42, and the fact that the majority of cells (of independent events) possessed this chromosome complement would seem to confirm somatic hybridity.

It can be seen that, when taken alone, none of the three pieces of evidence, used for confirmation of somatic hybridity, may unequivocally confirm true (nuclear) somatic hybrids, but, collectively, the case is compelling.

6.5 Summary.

i) Electrofusion was demonstrated to be effective for the production of rose heterokaryons from protoplasts of four English rose cultivars, with a maximum fusion frequency of 1.62%.

ii) Using micromanipulation, it was possible to manually select heterokaryons resulting from electrofusion. Selected, heterokaryons underwent division to produce microcalli and subsequently callus. The final plating efficiency of microcalli resulting from protoplast electrofusion was not significantly different from untreated control samples. Calli resulting from electrofusion did not exhibit any form of regeneration.

iii) Chemical fusion was less effective than electrical as it resulted in agglutinated protoplasts that were not suitable for micromanipulation and resulted in reduced final plating efficiencies.

iv) Examination of putative somatic hybrid material by isozyme analysis, determination of ploidy level and RAPD analyses confirmed the material to be somatic hybrid in nature.

CHAPTER 7.
Transformation of rose.

7.1 Introduction.

The benefits of genetically transforming rose have already been discussed (Chapter 1, Sections 1.2.2 and 1.5.6). This chapter describes attempts to transform English rose using two methodologies; namely *Agrobacterium*-mediated transformation and high velocity DNA delivery by microprojectile bombardment. Such techniques were selected as the lack of a reliable protoplast-to-plant system in English rose excluded the transformation of rose based on protoplast systems, such as microinjection and/or direct DNA uptake.

7.1.1 *Agrobacterium*-mediated transformation.

The natural ability of the soil-borne Gram negative phytopathogenic bacterium *Agrobacterium* to infect plants, and to mediate transfer and integration of DNA (genes) into host plant genomes, has been exploited in plant genetic engineering for some time now. Wild-type strains of *Agrobacterium* may confer abnormal growth on the host plant either in the form of tumours as caused by *A. tumefaciens* (De Cleene and De Ley, 1976; Hooykaas and Schilperoort, 1992) or as a proliferation of adventitious roots as induced by *A. rhizogenes* (De Cleene and De Ley, 1981). The genes responsible for virulence are carried on large bacterial plasmids, classified either as tumour-inducing plasmids (pTi) or root-inducing plasmids (pRi) and are conveyed as part of a region of the plasmid DNA referred to as the transfer DNA (T-DNA), which can undergo stable integration into the host plant genome (Chilton *et al.*, 1977; Chilton *et al.*, 1982). Transfer of DNA is facilitated by a complex of virulence proteins, encoded elsewhere on the Ti plasmid (*vir* genes, Klee *et al.*, 1983) and the bacterial chromosome (*cvh* genes). Expression of these genes is induced by the wound response of plant cells and, in particular, by the production of phenolic compounds such as acetosyringone (Stachel *et al.*, 1986).

Genetic engineering techniques have made it possible to insert foreign genes into the T-DNA, enabling their cointegration into the host genome, thus providing a system of transformation limited only by the host specificity of the agrobacteria (Porter, 1991), the length of the foreign DNA fragment, and the ability to recover plants from transformed tissues.

7.1.2. Microprojectile-mediated transformation.

The recently developed microprojectile method for gene transfer into intact plant cells has been successfully used to transform plant species including some which previously resisted attempts using *Agrobacterium*- and protoplast-mediated techniques (Birch and Franks, 1991). As described by Sanford *et al.* (1987), this technique relies upon the acceleration of small, DNA coated particles (frequently referred to as microprojectiles) into target cells. The microprojectiles usually consist of fragments of inert metal, such as tungsten or gold, with diameters of 0.2 - 4 μm. In order to achieve microprojectile acceleration, a number of different devices have been employed, including apparatus employing gunpowder (Sanford, 1987), an airgun (Oard, 1990), a stream of flowing helium gas (Finer, 1992) and electrical discharge (McCabe and Christou, 1993; Aragao *et al.*, 1993). However, the most commonly used instruments are those powered by a burst of helium gas generated by a rupture-membrane mechanism (Kikkert, 1993) or by a shock wave generated by a high voltage discharge through a water droplet (McCabe and Christou, 1993). In both cases, a macrocarrier, upon which DNA coated microprojectiles have been dried, is accelerated towards a perforated stopping screen. The microprojectiles continue at high velocity into the target tissue. Usually, this procedure is carried out under vacuum in order to reduce air resistance on the macro and microprojectiles.

A number of factors have been identified which may affect the efficiency of transformation using microprojectile-mediated DNA transfer. These include type and

size of microprojectile (Sanford *et al.*, 1993), the density of microprojectiles (Aragao *et al.*, 1993) and adherence of DNA to the microprojectiles (Birch and Franks, 1991), microprojectile velocity (Reggiardo *et al.*, 1991), distance between the discharge chamber and the sample (Aragao *et al.*, 1993), and DNA concentration (Klein *et al.*, 1988). Other factors include the choice of explant type (Genga *et al.*, 1991) and DNA plasmids/promoters (Charest *et al.*, 1993).

The present study aimed to examine the possibility of transforming readily available rose explants using *Agrobacterium* and microprojectile-mediated DNA delivery techniques and to investigate factors affecting such methods of gene delivery in rose.

7.2 Materials and Methods.

7.2.1 *Agrobacterium*-mediated transformation.

7.2.1.1 Plant materials and their propagation by tissue culture.

Unrooted axenic shoot cultures of the English rose cvs. Abraham Darby and Marie Pavié (propagated as described in Chapter 4, Section 4.3.1.2), were used for infection with *A. rhizogenes*. Freshly excised axillary buds, excised from *in vitro* propagated shoots of cvs. Abraham Darby and Marie Pavie, were used for infection with *A. tumefaciens*.

7.2.2.2 Bacterial cell cultures.

7.2.2.2.1 *Agrobacterium rhizogenes.*

A. rhizogenes strain R1601, which carries pRiA4b containing a chimaeric *nptII* gene cointegrated into HindIII fragment 21 of the TL-DNA and pTVK291 *in trans* conferring a supervirulent phenotype (Pythoud *et al.*, 1987), was used. Bacteria were grown in APM medium (5.0 g l^{-1} yeast extract, 0.5 g l^{-1} casein (enzymatic) hydrolysate, 2.0 g l^{-1} $(NH_4)_2SO_4$, 3.0 g l^{-1} NaCl and 8.0 g l^{-1} mannitol, pH 6.6) supplemented with 100 mg l^{-1} kanamycin sulphate and 100 mg l^{-1} ampicillin.

7.2.2.2.2 *Agrobacterium tumefaciens.*

The *A. tumefaciens* strain HAT1065 was used, which carries pVDH65 containing a chimaeric *gus* gene under the control of the CaMV35S promoter, the *nptII* gene under the control of a *nos* promoter as well as pToK47 which confers supervirulence, thus enabling this strain to infect a wide range of plants (Jin *et al.*, 1987). Bacteria were cultured in APM medium (as in Section 7.2.2.2.1) supplemented with 40.0 mg l^{-1} rifampicin, 50.0 mg l^{-1} kanamycin and 2.0 mg l^{-1} tetracycline.

7.2.2.2.3 Culture conditions and maintenance of bacteria.

A loopful of bacteria from a stock culture was inoculated into 7.5 ml of the respective culture medium in a 30 ml screw-capped tube and grown overnight in the dark on a horizontal shaker (120 rpm) at 26±2°C. Serial dilutions were made so as to give single cell colonies (10^{-8} dilution) on the appropriate semi-solidified [1.5% (w/v) Difco-Bacto agar] medium (15 ml in a 9 cm Petri dish). Dishes were maintained at 26±2°C until single colonies developed (24-72 h) and then transferred to dark, cold (4°C) conditions, where they were held for 4 weeks, until the next subculture.

A single colony from each plate was inoculated into 7.5 ml of fresh liquid medium, where it was grown in the dark (120 rpm, 25°C) for 24 h, and subsequently used to produce stock cultures [bacterial suspension: glycerol, 1:1 (v/v)]. Stocks were kept at -20°C, in the dark, for long-term storage.

For assessments of viability and growth of stock cultures, 500 µl of an overnight culture (produced as previously described) was inoculated into 100 ml of liquid medium and incubated in the dark on a shaker (120 rpm) at 26°C. Samples (1 ml) were removed immediately after inoculation and every 90 min thereafter. A 100 µl aliquot of such samples was serially diluted, with 100 ml of the 10^{-8} dilution, plated in duplicate, onto 10 ml (in 9 cm Petri dishes) of the respective semi-solid medium for each strain. The number of colonies per dish was counted after 24 - 72 h

incubation (26±2°C). The remaining 900 µl aliquot was transferred to a 10 mm quartz cuvette for spectrophotometric measurements of optical density (at 455 nm) against a blank of sterile liquid culture medium. Bacterial growth was determined using the mean results obtained for the colony number per ml and the absorbance data, over 12 h.

An overnight bacterial culture was initiated as previously described and the bacterial concentration monitored by optical densitometry using a Pye Unicam SP6-500 spectrophotometer at 455 nm against a blank of sterile culture medium, and with reference to a calibration curve. When bacteria had grown to a density of either 10^5 or 10^{10} ml^{-1}, the bacterial suspension was centrifuged (2500 rpm) in a 16 ml screw-capped glass centifuge tube and the culture medium replaced with either identical culture medium lacking antibiotics or with *Agrobacterium*-induction broth (AIB; Appendix VI). Cultures were subsequently incubated for a further 5 h (120 rpm, 26±2°C), prior to explant inoculation.

7.2.2.3 Bacterial inoculation of explants.

7.2.2.3.1 Microinjection of shoots with *A. rhizogenes*.

Axenic shoots of both rose cultivars, were taken at the end of a subculture passage and were decapitated and all leaves excised, thus leaving only the basal portion of petioles attached to the stem. Such shoots were inoculated, at the apical and/or subapical internodal surfaces, by 3 - 5 micro-injections of bacterial suspension (each approx. 10 µl in volume), using a 23 gauge hypodermic needle. Groups of 5 inoculated shoots were transferred to 175 ml glass jars with 50 ml of growth regulator-free 0.8% (w/v) agar-solidified MS medium and maintained, for up to 12 weeks, at 25°C with constant cool white fluorescent illumination (27 $\mu Em^{-2}s^{-1}$). Five hundred explants of each cultivar were subjected to each treatment.

A series of control, un-inoculated shoots were produced in order to ensure that any induced responses were not a result of normal rooting or healing responses. Thus, decapitated shoots were left either uninoculated or were inoculated with sterile bacterial culture media, whereupon they were transferred to growth regulator-free medium as previously described. All cultures were maintained under the same conditions as described for shoots inoculated with bacteria. Results were assessed in terms of the percentage of surviving shoots that exhibited hairy root growth.

7.2.2.3.2 Cocultivation of axillary buds with *A. tumefaciens*.

Axillary buds of both cultivars, were excised from axenic shoots at the end of a subculture passage. The scale buds were removed using fine forceps, thus leaving only the exposed meristematic area and associated leaf primordia attached to a small section of lateral shoot. Freshly excised axillary buds were immediately placed into a 1:10 (v:v) dilution (cultured bacteria:fresh medium) of an overnight culture of *A. tumefaciens* in a 5.5 cm Petri dish (50 buds per 10 ml of bacterial culture). After 30 min cocultivation, groups of 20 buds were transferred to 9 cm Petri dishes containing 20 ml of the appropriate RSC shoot culture medium (Chapter 4, Section 4.3.1.2) supplemented with 200μM acetosyringone. Dishes were maintained for 16 h in darkness at 25°C. Subsequently, the axillary buds were transferred onto identical medium supplemented with 250 mg l^{-1} cefotaxime and 500 mg l^{-1} carbenicillin and were maintained at the same temperature with a 16 h photoperiod (27 $\mu Em^{-2}s^{-1}$). A total of 100 axillary buds of each cultivar were subjected to each treatment. In addition, 50 axillary buds of each cultivar were subjected to identical cocultivation treatments, but with sterile medium to act as controls. After four weeks in culture, β-glucuronidase (GUS) activity was determined in shoots proliferating from the axillary buds, using a histochemical assay modified from Jefferson *et al.* (1987). Explants were vacuum infiltrated for 15 min with a solution which consisted of 0.01M $NaPO_4$ (pH 7.5), 10mM EDTA, 0.1% (v/v) Triton X-100, 0.5mM ferrocyanide, 0.5mM ferricyanide and 0.5 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-

Gluc; Gold Biotechnology, St. Louis, USA), 500 mg l^{-1} cefotaxime and incubated overnight at 37°C. After staining, explants were rinsed in 50% and 75% (v/v) ethanol for 30 min each, and kept in 96% (v/v) ethanol for microscopic examination.

7.2.2 Microprojectile-mediated transformation.

7.2.2.1 Plant materials and their propagation by tissue culture.

Explants subjected to microprojectile bombardment were axillary buds of cv. Abraham Darby with the scale buds removed as described in Section 7.2.2.3.2, and rescued embryos resulting from sexual crosses between English rose cvs. Heritage and Lucetta, as described in Chapter 2, Section 2.2.1.3.

7.2.2.2. Plasmids used in microprojectile-mediated transformation.

Investigations to determine the optimal parameters for transformation employed pMJD67. This plasmid was based on the vector pTZ18 (2.9 kb) with the CaMV35S promoter (526 bp), upstream of the *gus* coding sequence (1885 kb) as described by Rech *et al.* (1991) and was supplied by Dr. M. Dowson-Day (AFRC-IPSR, Laboratory of Nitrogen Fixation, University of Sussex, Brighton, UK). Experiments examining the effects of promoter sequences on gene expression used pBI 221.1479. The construction of this plasmid was similar to that of pMJD67, except that it carried three CaMV35S promoters upstream of the *gus* gene and also carried the ADH intron. pBI 221.1479 was supplied by Dr. R. Westcott (Unilever Research, Colworth House, Sharnbrook, Bedford, UK) and was used with the permission of Pioneer Hybred International Inc., Des Moines, Iowa, USA., from where the ADH intron originated.

7.2.2.3 Isolation of plasmid DNA from *Escherichia coli.*

The competent bacterial strain of *E. coli*, HB101, harbouring the specific plasmid constructs used in this study was maintained as a glycerol stock. Stocks were prepared by inoculating 10 ml aliquots of LB medium (Appendix VI) with a loopful of bacteria taken from the surface of semi-solid (1.5 % w/v Difco Bacto agar) LB

medium plates. The appropriate antibiotic(s) was/were added for selected growth of the desired bacteria (Appendix VI). The cultures were incubated (125 rpm) for 16 h at 37°C and subsequently mixed (1:1, v/v) with sterile glycerol prior to storage at -20°C.

A few drops of the glycerol stock were added to 10 ml of fresh LB medium. After incubation (16 h, 37°C), 2.5 ml aliquots were transferred to 150 ml of pre-warmed (37°C) LB medium (supplemented with the same antibiotic mixture) in 250 ml Erlenmeyer flasks. Flasks were incubated for 16 h at 125 rpm (37°C) until the OD_{600} of the suspension was 0.7. Chloramphenicol was added (170 $\mu g\ ml^{-1}$ from a 34 mg ml^{-1} ethanolic stock) to each flask, and cultures incubated overnight as previously described. The bacterial suspension was transferred to 4, sterile, screw-capped bottles (400 ml capacity, Nalgene Co., Rochester, New York, USA) and pelleted by centrifugation in a JA-10 rotor using a Beckman J2-21 centrifuge (10 000 rpm) for 20 min at 4°C. Following discarding of the supernatant, the plasmid was isolated from the pelleted bacteria using the Promega Wizard Megaprep DNA purification system (Promega, Southampton, UK.), according to the manufacturer's instructions.

7.2.2.4 Transformation methodology.

7.2.2.4.1 Particle acceleration device.

The apparatus used was a laboratory-built electrical discharge device, the design of which was based on that of McCabe *et al.* (1988), and which has been described by Rech *et al.* (1991). In this device, the electrical power is provided by a pulse generator containing two banks of capacitors (Plate 7.1b). The electrical discharge causes evaporation of a water droplet which bridges two electrodes and, in so doing, propels a small section of Mylar film (the macrocarrier), carrying DNA coated particles (microcarriers), upwards towards a mesh screen which retains the macrocarrier, but which allows passage of the microcarriers. The microcarriers subsequently reach the surface of the plant sample which is held inverted in a 5.5 cm

Petri dish on a plate holder (Plate 7.1c). The distances separating the electrical discharge chamber, the retaining screen and the plate holder can all be varied. The electrode/plate holder assembly are all held within a vacuum desiccator and are sterilised with 70% (v/v) ethanol for 30 min prior to use.

7.2.2.4.2 Preparation of microprojectiles.

A calcium-spermidine method was used for the coating of microprojectiles with DNA. Particles (12.5, 25.0, 37.5 or 50.0 µl from a 10 mg ml^{-1} sterile water stock) of either tungsten [1 µm diam.] or gold powder [1-3 µm diam.]; (Aldrich Chemical Co., Gillingham, Dorset, UK) were mixed with 0, 2.5, 5.0, 7.5 or 10.0 µl of plasmid DNA (from a 0.8 mg ml^{-1} stock in TE buffer) in a 1.5 ml Eppendorf tube. While being vortexed, 25 µl of $CaCl_2$ (2.5 M) was added followed by 10 µl of spermidine (0.1 M). Tubes were allowed to stand at room temperature for 10 min, after which they were centrifuged (6000 rpm) for 2 s. The supernatant was removed and the pellet was resuspended in 70 µl of absolute ethanol. Centrifugation was repeated after which the supernatant was removed and replaced with 34 µl of absolute ethanol. The pellet was then resuspended by gentle pipetting (for 5 min), using a Gilson micropipettor fitted with a wide bore yellow tip. Once the DNA coated particles were fully resuspended, 7.5 ml of the suspension was pipetted onto the surface of each macrocarrier (a 12 mm x 12 mm sheet of Mylar film; Dupont , Wilmington, Delaware, USA) and allowed to dry.

7.2.2.4.3 Particle delivery into target tissues.

In all experiments, prior to firing, the electrode gap was set at 1.7 mm and the electrodes were bridged with a 9.0 µl droplet of sterile water. The macrocarrier was placed on the top of the electrical discharge chamber. Tissues to be bombarded were placed in the centre of a 5.5 cm Petri dish containing 12 ml of water-agar (10 embryos/axillary buds per dish). The Petri dish was, in turn, inverted onto the Petri plate holder and secured with the retaining screw. The distance between the

discharge chamber and the stopping screen was adjusted to 2.5 (the minimum), 5.0, 6.5, 7.5, 37.5 or 72.5 mm, after which the lid was placed on the dessicator chamber (in which the apparatus was held) and the vacuum switched on. Once the chamber had been evacuated to 500 mm of Hg, a voltage of 2.25 kV was discharged from the pulse generator through the electrodes, resulting in particle acceleration. Following bombardment, samples were placed directly into culture. Embryos were placed in the dark on embryo culture medium (Chapter 2, Section 2.3.2.1) at 25°C for 2 d, while axillary buds were cultured for 2 or 14 d at 25°C, 16 h photoperiod (27 $\mu Em^{-2}s^{-1}$) on RSC culture medium. The latter was identical to that used to maintain the original *in vitro* cultures (Chapter 4, Section 4.3.1.2). In addition, embryos were cultured for 3 weeks following the procedure described in Chapter 2 Section 2.3.2 prior to assaying for GUS activity. Each treatment was replicated 5 times (ie. 5 Petri dishes = 50 embryos or axillary buds).

7.2.2.5 Histochemical localisation of β-glucuronidase.

Following culture, *in situ* localised β-glucuronidase (GUS) activity was analysed in bombarded embryos and shoots proliferating from bombarded axillary buds and germinated embryos using a histochemical assay modified from Jefferson *et al.* (1987) as described in Section 7.2.2.3.2. Five explants were placed in 0.5 ml of stain solution contained within each of the wells of a Sterilin 25 well dish and incubated for 36 h at 37°C. Following incubation, green tissues were rinsed in 50% and 75% (v/v) ethanol for 30 min to remove chlorophyll and facilitate visualisation of blue histochemical staining. Transformation efficiency was expressed as the number of blue spots per bombarded axillary bud or embryo. Each blue spot (a single, isolated cell or an aggregate of cells) was scored as one 'expression unit', as defined by Klein *et al.* (1988).

7.3 Results.

7.3.1 *Agrobacterium rhizogenes*-mediated transformation.

Out of 2000 shoots that were inoculated with *A. rhizogenes* R1601 hairy root production was observed in 10 shoots (all cv. Marie Pavié; Plate 7.1a). Of those shoots that did produce hairy roots, 6 had been inoculated with bacteria cultured on APM medium. The remaining 4 had been inoculated with bacteria cultured on AIB medium. *A. rhizogenes* inoculated shoots that failed to produce hairy roots displayed an apparently normal phenotype and continued normal growth *in vitro*. Shoots, from which hairy roots were produced, showed signs of necrosis and grew at an extremely slow rate. When excised from the shoots, and placed on MS-based medium lacking growth regulators, all roots displayed necrosis and died. Control shoots, inoculated with sterile culture medium, did not produce hairy roots.

7.3.2 *Agrobacterium tumefaciens*-mediated transformation.

After 10 d of culture, all axillary buds showed some degree of shoot proliferation. However, with each bacterial treatment, between 1 and 12% of explants exhibited visual bacterial contamination (presumed to be residual *Agrobacterium*), despite being cultured on medium containing the antibiotics cefotaxime and carbenicillin (Section 7.2.2.3.2). These contaminated shoots displayed severely retarded growth compared to their non-contaminated counterparts. Bacterial contamination was not observed in control explants 'cocultivated' in sterile culture medium.

The blue colour associated with GUS activity after staining with X-Gluc substrate (as described in Section 7.2.2.3.2) was not observed in shoots subjected to any of the treatments described. Control shoots did not exhibit any blue histological staining.

7.3.3 Microprojectile-mediated transformation.

Following bombardment, small clusters (approx. 20-40 μm diam.) of microprojectiles were observed on the surface of the explants, when examined microscopically (Fig

7.1d). These were particularly evident in explants subjected to bombardment with high concentrations of DNA (ie. 1.5 and 2.0 μg per shot). When incubated in X-Gluc solution, bombarded axillary buds and shoots that had arisen following the culture of bombarded axillary buds, did not show any blue colour. However, following incubation in X-Gluc solution, embryos subjected to microprojectile bombardment with DNA coated particles expressed blue colour, which is indicative of GUS activity. Embryos which displayed this blue colour appeared to have a blue hue when viewed with the naked eye (Fig 7.1e). Individual expression units (blue spots) were only visible when embryos were viewed under magnification. Distinct blue spots, caused either by aggregates of cells harbouring the foreign gene or by diffusion of the GUS reaction products as described by Klein *et al.* (1988), were rarely observed (Fig 7.1g). Embryos bombarded with particles which were not coated with DNA, embryos that were bombarded with DNA solution (without particles) and embryos which were not subjected to any form of bombardment, did not exhibit any blue colour after incubation in X-Gluc solution (Fig 7.1f). A number of parameters were shown to affect the degree of transient expression (number of expression units) in rose embryos, as detailed in Sections 7.3.3.1 - 7.3.3.3. Shoots resulting from embryos that had been subjected to microprojectile-mediated DNA delivery did not express any blue colour.

7.3.3.1 Effect of microprojectile concentration on GUS activity in microprojectile bombarded English rose embryos.

Fig 7.1 shows the effect of particle concentration on the number of expression units following bombardment at a constant firing distance and DNA concentration. The use of gold particles gave significantly greater numbers of expression units at all particle concentrations, except at 125 μg per shot. The number of expression units was maximal using gold particles at a concentration of 62.5 μg per shot and significantly lower at reduced particle concentrations. At concentrations above 62.5 μg of gold per shot, the number of expression units was also reduced. The greatest

number of expression units obtained when tungsten microcarriers were employed was obtained at the maximum concentration of particles (125 μg per shot).

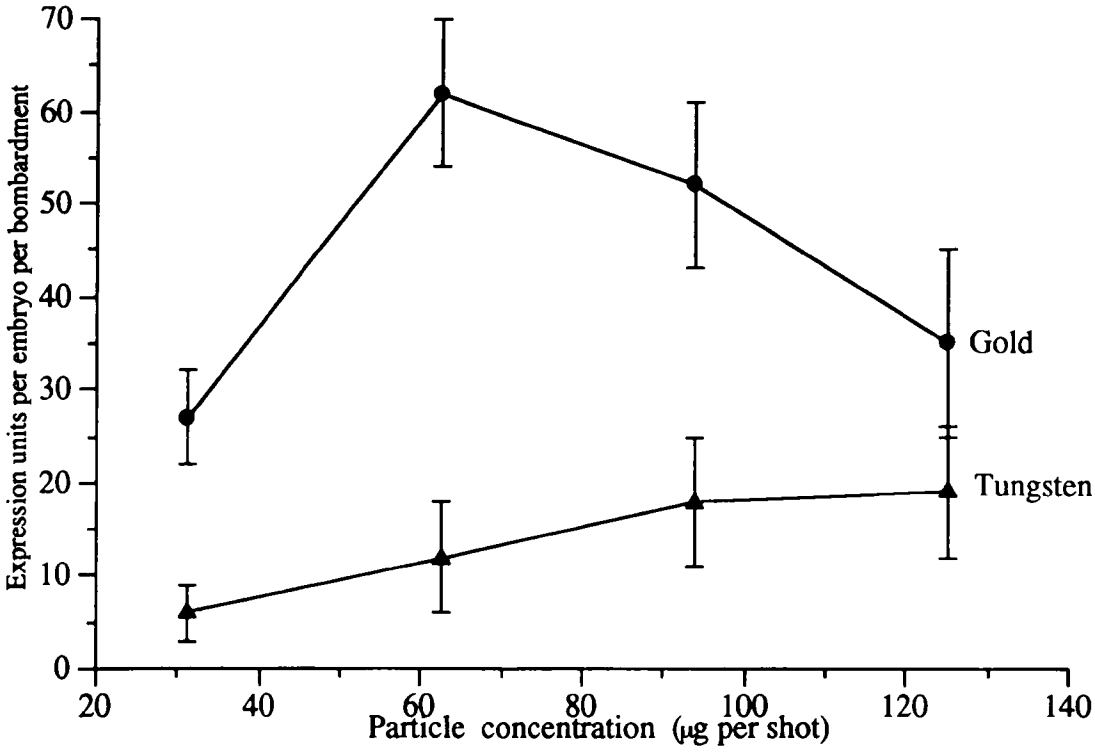
7.3.3.2 Effect of DNA concentration and plasmid type on GUS activity in microprojectile bombarded English rose embryos.

Fig 7.2 shows the effect of DNA concentration and plasmid type on the number of expression units following bombardment at a constant firing distance and microprojectile concentration. At all DNA concentrations, higher numbers of expression units were obtained using pMJD67 as compared to pBI221.1479. The maximum number of expression units was obtained using pMJD67 at 1.5 μg per shot. Concentrations of plasmid either greater or less than 1.5 μg per shot resulted in a decline in the number of expression units obtained. The maximum number of expression units obtained when plasmid pBI221.1479 was used were obtained at of 1.0 μg of DNA per shot. Expression units were not observed on embryos subjected to bombardment with 2.0 μg per shot of pBI221.1479.

7.3.3.3 Effect of firing distance on GUS activity in microprojectile bombarded English rose embryos.

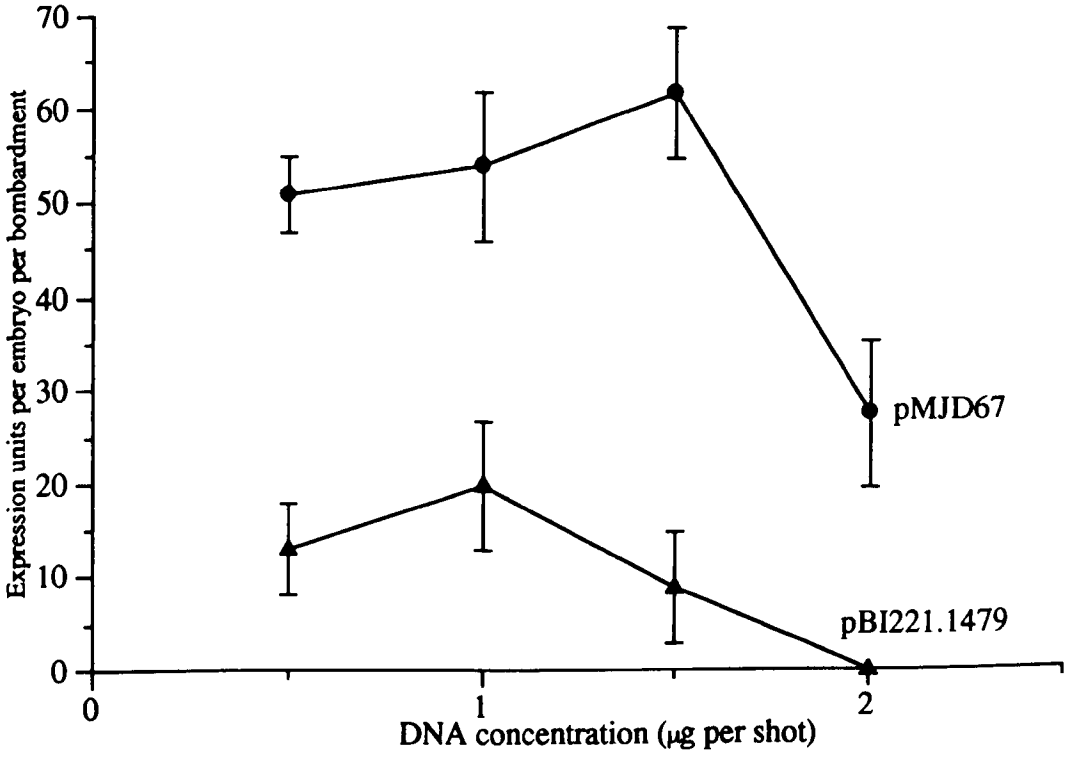
Fig 7.3 shows the effect of distance between the discharge chamber to the retaining screen on GUS expression in English rose embryos following bombardment with a constant concentration of gold particles and DNA. The maximum number of expression units was obtained when the distance between the discharge chamber and the retaining screen was at its minimum (2.5 mm). Increasing the distance between the discharge chamber and retaining screen resulted in a decrease in the subsequent number of expression units.

Figure 7.1 Effect of particle concentration on GUS expression in English rose embryos.



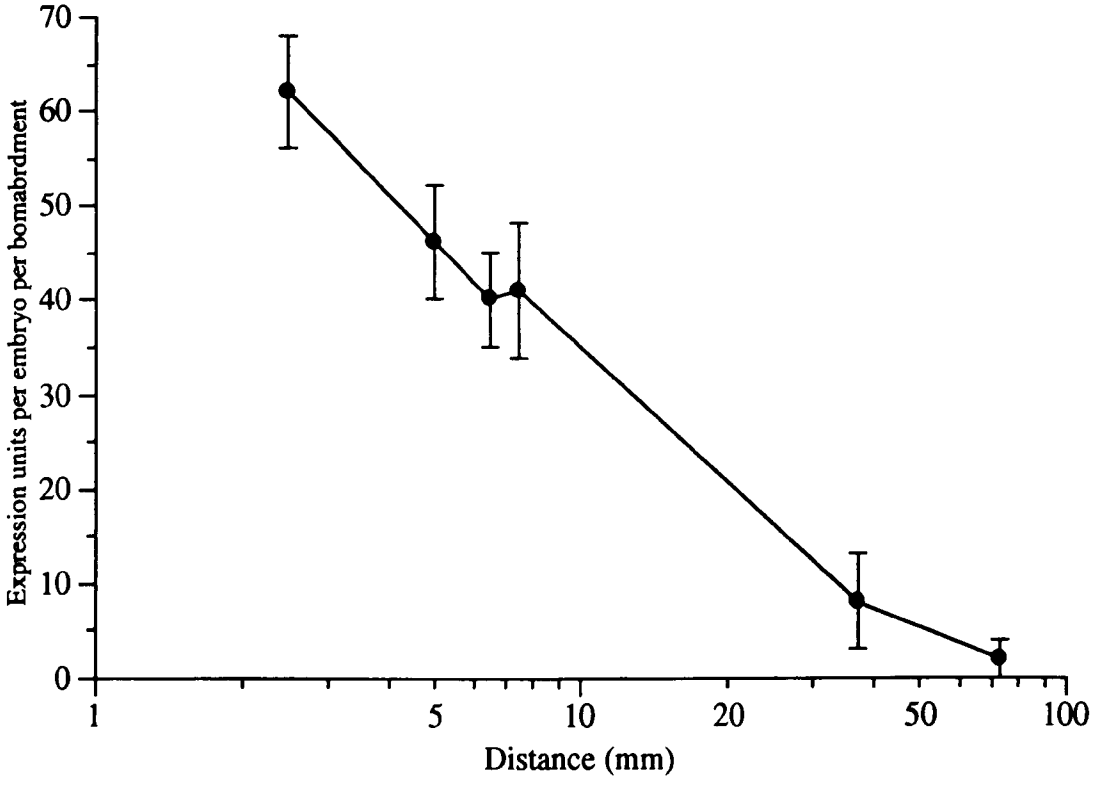
Each bombarded plate had 10 embryos. Firing distance was constant at 2.5 mm and DNA concentration at 1.5 μg per shot. Data are means of 5 replicates. Bars represent standard error.

Figure 7.2 Effect of DNA concentration and plasmid type on GUS expression in English rose embryos.



Each bombarded plate had 10 embryos. Firing distance was constant at 2.5 mm and concentration of gold particles at 62.5 μg per shot. Data are means of 5 replicates. Bars represent standard error.

Figure 7.3 Effect of distance between the discharge chamber to the retaining screen on GUS expression in English rose embryos.



Each bombarded plate had 10 embryos. Concentration of gold particles and DNA was constant at 62.5 and 1.5 μg per shot respectively. Data are means of 5 replicates. Bars represent standard error.

Plate 7.1.

a. Hairy root production from *in vitro* shoots of the English rose cv. Marie Pavié 8 weeks after inoculation with *Agrobacterium rhizogenes* strain R1601 (x 3).

b. Electrical discharge device (as described in Section 7.2.2.4.1) used for microprojectile-mediated gene delivery into rose embryos (x 0.1).

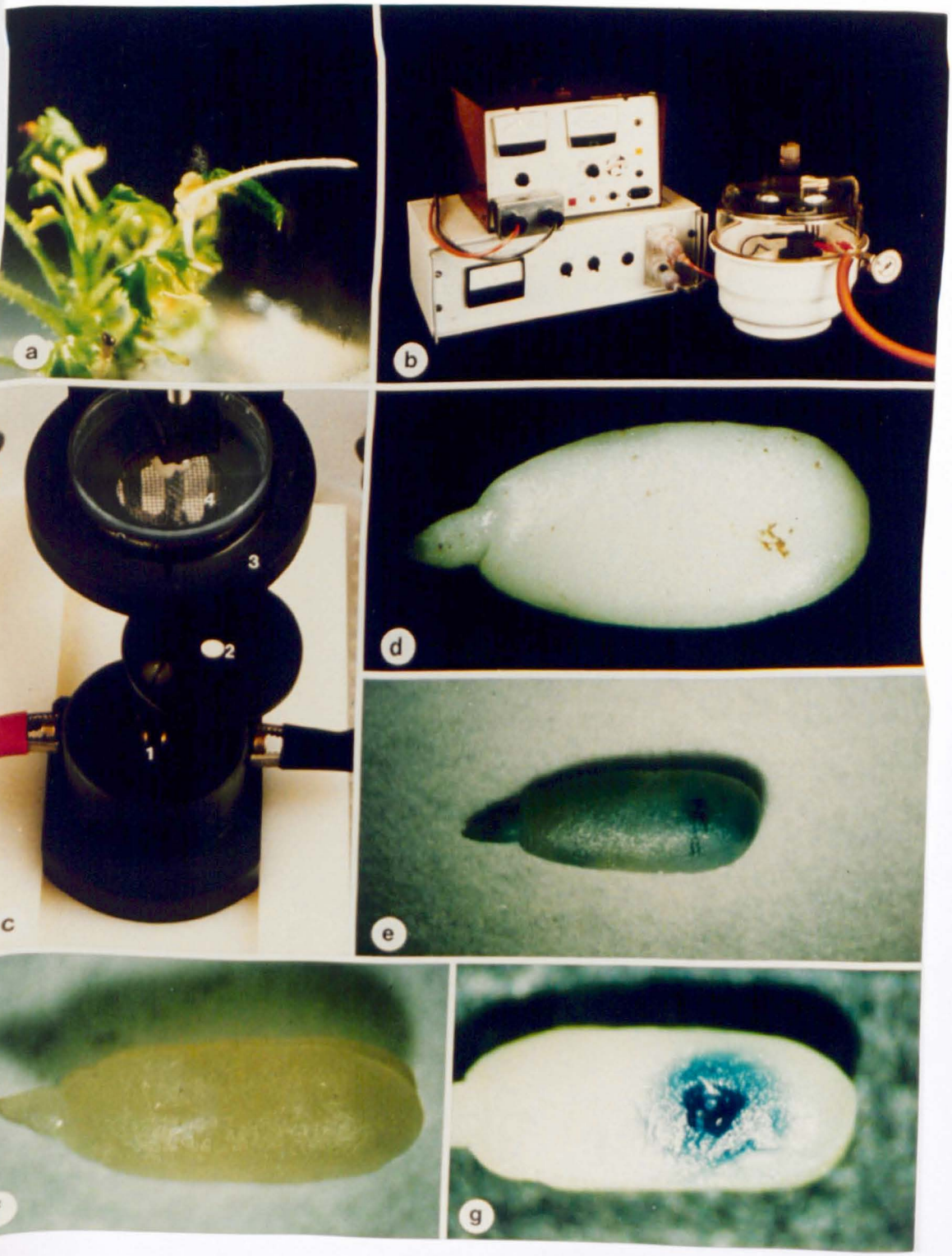
c. Detail of electrical discharge device showing 1: electrodes, 2: aperture over which is placed the macrocarier, 3: Petri plate holder with inverted Petri dish containing the plant sample to be bombarded, 4: macrocarrier retaining screen (x 0.6).

d. Aggregates of gold microprojectiles on the surface of rose embryos following particle bombardment (firing distance=2.5 mm, gold concentration=62.5 μg per shot, DNA concentration=2 μg per shot) (x 15).

e. Embryo after 36 h incubation in X-Gluc solution (Section 7.2.2.3.2) and after bombardment with 1.5 μg per shot DNA, 62.5 μg per shot gold particles and at a firing distance of 2.5 mm (x 9).

f. Control embryo after 36 h incubation in X-Gluc solution (Section 7.2.2.3.2) and after bombardment with 0 μg per shot DNA, 62.5 μg per shot gold particles and at a firing distance of 2.5 mm (x 13).

g. Blue expression unit on an embryo after bombardment and following incubation in X-gluc solution. The large size of expression unit is possibly caused by microprojectile agglutination and/or diffusion of the GUS reaction product (x 15).


a
b
c
4
3
2
1
d
e
f
g

7.4 Discussion.

7.4.1 *Agrobacterium*-mediated transformation.

The frequency with which explants, in this study, expressed infection with agrobacteria was negligible when compared to similar studies on other Rosaceous species, such as Cherry (Ochatt, 1989), in which up to 81% of shoots produced hairy roots after inoculation with *A. rhizogenes* strain LBA 9402. However, Ochatt (1989) concluded that the strain of *A. rhizogenes* used in the present study (R1601), was too virulent as its use resulted in death of the majority of cherry shoots within 2 weeks of inoculation. Conversely, other workers such as Manders (1992), reported that R1601 failed to infect apple and inoculated shoots continued to grow normally. In the present study, the majority of rose explants survived post-inoculation with *A. rhizogenes* or *A. tumefaciens* and exhibited continued growth *in vitro* suggesting that 'over-virulence' was not a problem and that the failure to produce hairy roots/express GUS activity was probably due to lack of infection. There are a number of possible factors which may have affected infection with agrobacteria. Firstly, different strains of agrobacteria can have vastly different levels of virulence (Godwin *et al.*, 1992). Although certain strains of *A. rhizogenes* are known to infect rose *ex vitro* (Ohta, 1986, van der Mark *et al.*, 1990) there are no previous reports of strain R1601 resulting in infection. Therefore, it is possible that despite harbouring pTVK291, which should result in strain R1601 having a wide host range (Pythoud *et al.*, 1987), the failure to infect rose reflects host range limitations. The use of another strain of *A. rhizogenes*, such as LBA 9402, may have resulted in successful infection. Likewise, there are no reports of the *in vitro* infection of shoot explants of rose with *A. tumefaciens*, except for those using strains such as LBA4404 to infect callus (Firoozabady *et al.*, 1991). It should also always be borne in mind that the rose cultivars employed in this study result from a long and complex breeding programme which selects for disease resistance. The failure to infect plants in this study with agrobacteria may simply reflect attempts by breeders to reduce the susceptibility of cultivars to infection.

The use of *in vitro* cultured rose explants rather than *ex vitro* material may also explain the failure of *Agrobacterium* to infect in this study. A number of compounds, primarily phenolics, are known to play a key role in chemotaxis and virulence induction in agrobacteria (see Porter, 1991 and Godwin *et al.*, 1992). Transcription of the *vir* region is induced by various phenolic compounds, such as acetosyringone and alpha-hydroxy-acetosyringone (Stachel *et al.*, 1985). It is possible that the amounts of phenolic substances produced by some plants reflect the degree of lignification (Porter, 1991). As the plant material used in the present study was cultured *in vitro* (and as a result is probably not heavily lignified), it is possible that it produced/contained reduced amounts of phenolic compounds, compared to identical material produced *ex vitro*. Indeed, one reason why *in vitro* cultured material was used for protoplast isolation was that it apparently contained lower levels of phenolic compounds (see Chapter 4 Section 4.1). The results, in the present study, may therefore be caused by a lack of *vir* gene transcription upon infection with agrobacteria due to the absence of sufficient levels of phenolic compounds from the plant tissues. The phenolic compound acetosyringone, which has been demonstrated to enhance *Agrobacterium*-mediated transformation of other Rosaceous species such as apple (James *et al.*, 1993), was included in the AIB medium (Appendix VI) used in the present study. However, Godwin *et al.* (1991) have demonstrated that the response of *Agrobacterium* to compounds such as acetosyringone varies between different bacterial strains and also between plant species, and it may be that in the present study, the incorporation of one of many other compounds that have been suggested to induce *vir* transcription, such as syringaldehyde, ferulic acid or syringic acid (Holford *et al.*, 1992), into the culture medium, may have resulted in an increase in the transformation frequency.

7.4.2 Microprojectile-mediated transformation.

As the mechanisms by which foreign DNA leaves the microcarrier and enters the host plant genome are unknown, microprojectile-mediated transformation relies on maximising the number of DNA coated particles entering the recipient plant material and on maximising the expression of DNA, once integrated into the genome, by the selection of effective gene promoters. Blue expression units were not observed in axillary buds or in shoots derived from these axillary buds. This is probably due to a failure of the microprojectiles to effectively penetrate the surface of the plant material and enter the plant cells. This is confirmed by the observation of microprojectiles on the surface of the explants, and would also explain why shoots arising from bombarded axillary buds failed to express any GUS activity.

Blue expression units, indicating GUS activity, were observed on bombarded excised embryos incubated in X-Gluc solution. The number of expression units observed was clearly influenced by the various parameters under investigation. However, before discussing these factors, some consideration should be made of the method being used to assess transformation efficiency, namely the GUS gene reporter system.

The *gus* gene, isolated from *Escherichia coli* (Jefferson *et al.*, 1986), is one of the most widely exploited reporter genes in use today. It apparently fulfils the basic requirements for the effective use of reporter genes (Jefferson, 1989) including, it has been assumed, the lack of background activity in higher plants. However, it is not common knowledge that GUS activity was detected in plants by Neuberg and Niemann as early as 1905 (cited by Hoffmann-Ostenhof, 1954) and that, more recently, background GUS activity has been detected in histochemical assays of various plant species and tissues (Plegt and Bino, 1989; Hu *et al.*, 1990). Hu *et al.* (1990) report the occurrence of intrinsic GUS activity in the seed coat and mature embryos of an unidentified species of rose. However, in the present study it can be assumed, with confidence, that the observed GUS activity was the result of the

expression of an introduced foreign gene and not of intrinsic GUS gene activity, as the recommendations suggested by Alwen *et al.* (1992) were closely followed. Specifically, the untreated control embryos used in the study did not show any blue colouration when incubated in X-Gluc solution, indicating a lack of intrinsic GUS activity. Secondly, all histochemical assays were performed in the presence of a buffer which maintains the pH of the X-Gluc solution at 7.0-7.5, exploiting the difference in pH optima; pH 5.0 for plant activity and close to neutral (pH 7.0) for the bacterial enzyme (Alwen *et al.*, 1992). In addition, the X-Gluc solution also contained the antibiotic cefotaxime in order to eliminate any contaminating bacteria which may have given rise to blue coloration on incubation.

The blue expression units observed on the embryos in this study are considerably smaller than those seen after the bombardment of other plant species (Klein *et al.*, 1988, Wilmink *et al.*, 1992; Aragao *et al.*, 1993). This may reflect the small, compact nature of the cells and the waxy coating of the embryo, which may act to prevent diffusion of the blue reaction product. Similar patterns of histochemical staining were observed by Bommineni *et al.* (1994) in black spruce somatic embryos.

This study highlighted a number of factors that affect the number of expression units obtained after particle bombardment of rose embryos. Both the quantity and type of microprojectile used had an effect. The response to particle concentration obtained in this study is similar to that noted by other workers (Klein *et al.*, 1988; McCown *et al.*, 1991; Aragao *et al.*, 1993). In particular, there is an optimal particle concentration for each system, above and below which the number of expression units is reduced. When using a similar particle acceleration device to introduce pMJD67 into bean, Aragao *et al.* (1993) noted an almost identical response although the number of expression units obtained was considerably higher in their study. In addition, the optimal microprojectile concentration was 125 μg of gold per shot rather than 62.5 μg per shot as in the present study. It appears that, below the optimal concentration, the

number of expression units is directly related to the number of particles being introduced into the sample. The decline in expression units above the optimal particle concentration is probably caused by two factors. Firstly, the higher number of particles cause a greater amount of tissue damage, which prevents gene expression. Secondly, the aggregates of particles that form may be either larger or occur more frequently. Such aggregates are too large to reach sufficient velocity to penetrate the cells of the target tissue, which will result in a reduction in the level of transformation. Additionally, such large aggregates coming into contact with the plant sample will increase the level of tissue damage.

The difference that was observed in terms of the numbers of expression units that were obtained when gold microprojectiles were employed as opposed to those of tungsten was probably due to several factors. Because of availability, tungsten particles could only be obtained as a powder consisting of particles, all of which were 1 μm in diameter. In contrast, the gold particles used had a range of diameters (1-3 μm). As previous workers have demonstrated that different sizes of cells require different sizes of microprojectile for efficient gene delivery (Klein *et al.*, 1988; Sanford *et al.*, 1993), it is possible that the particle size of tungsten was sub-optimal while the gold microprojectiles included a range of sizes some of which were optimal. In addition, tungsten has been demonstrated to have toxic effects, which may have also resulted in the reduced number of expression units observed in this study. Tungsten will catalytically degrade the DNA bound to it (Sanford *et al.*, 1993), and it can also be toxic to the recipient plant cells. Indeed, in their study comparing the use of tungsten and gold microprojectiles, Russell *et al.* (1992) noted a similar effect in the microprojectile transformation of tobacco and demonstrated that this was, in part, due to the toxicity of tungsten to the plant cells. The exact nature of this toxicity is unknown. Russell *et al.* (1992) demonstrated that tungsten caused acidification of the medium surrounding bombarded cells and as a result incorporated the buffer MES into the medium to overcome this. However, cell death was not eliminated,

indicating that the toxic effects of tungsten cannot be fully attributed to acidification of the medium.

The effect of DNA concentration on the number of expression units has been examined both by Klein *et al.* (1988) and by Aragao *et al.* (1993). Both groups of workers noted that the number of expression units increased as the DNA concentration increased to an optimal point. Increases in the DNA concentration above the optimum resulted in a rapid decline in the number of expression units. A similar effect was observed in this study on rose using both pMJD67 and pBI221.1479. It would appear that at concentrations below the optima, the number of expression units is related to the amount of DNA present and that increasing the DNA concentration simply increases the proportion of microcarriers that are coated with DNA. This, in turn, will lead to a greater number of expression units following particle delivery. If this principle is correct, then it can be assumed that at any given particle concentration there will be a concentration of DNA which will enable all the available particles to be coated with DNA. Such a concentration could be referred to as the DNA saturation point. Beyond such a point, increasing the DNA concentration will fail to result in any increase in the number of expression units. However, in the present study on English rose, and in those by Klein *et al.* (1988) on maize and Aragao *et al.* (1993) on bean, there was a decrease in expression units, if the DNA concentration exceeded a particular level. Such a decrease probably results from the agglutination of microprojectiles, which has been reported to occur at high DNA concentrations (Klein *et al.*, 1988, Sanford *et al.*, 1993; Aragao *et al.*, 1993). Such agglutination will result in large aggregates of microprojectiles which, as previously explained, will fail to penetrate the target tissues.

Results in the present study would seem to indicate that the use of pMJD67 with a single CaMV35S promoter is preferable to pBI221.1479 containing a triple CaMV35S promoter, since the former plasmid gave substantially higher numbers of

expression units than the latter. Such results contrast with the recommendations of Charest *et al.*, (1993) and Sanford *et al.*, (1993), both of whom suggest the use of plasmids containing multiple CaMV35S promoters is preferable to the use of plasmids containing single CaMV35S promoters. Charest *et al.*, (1993) demonstrated that, in various *Picea* species, the use of a double CaMV35S promoter gave a three to six fold increase in the number of GUS expression units, while Sanford *et al.*, (1993) suggested that the use of a GUS-NPT II plasmid driven by a double 35S promoter (plus a leader sequence from alfalfa mosaic virus) yielded a ten to one hundred fold increase in the number of transiently transformed tobacco cells compared with pBI121 containing a single CaMV35S promoter. In the present study, the smaller number of expression units observed when a plasmid containing a triple CaMV35S promoter was used, may be relate to plasmid size. Mendel *et al.*, (1989) demonstrated that smaller plasmids were preferable in the microprojectile-mediated transformation of barley. This preference reflected the higher transformation frequencies that were observed using smaller plasmids, which were presumed to be more stable than larger ones. In the current study on rose, pMJD67 had a size of 5.6 kb while pBI221.1479 was larger, at 6.4 kb. The assumption that the relatively smaller number of expression units when pBI221.1479 was used was due to the larger size of the plasmid would therefore appear legitimate.

Several workers have noted that the firing distance (ie. distance between the discharge chamber and the retaining screen) influences the number of expression units obtained (Klein *et al.*, 1988; Iida *et al.*, 1990; Aragao *et al.*, 1993). Others have suggested that microparticle velocity (which is probably at least in part related to firing distance) also has an effect (Franks and Birch, 1991). Klein *et al.* (1988) and Iida *et al.* (1990), using an explosive driven device and a pneumatic air device respectively, both obtained maximum numbers of expression units at their minimal chosen firing distance. Aragao *et al.* (1993), using a device similar to that used in this experiment on rose, obtained maximum expression units at the second smallest firing distance (ie.

4.0 mm) and demonstrated a large reduction in the number of expression units at a smallest firing distance (2.5 mm). All of these workers reported that, as in this study with rose, there is a decline in the number of expression units when the firing distance is increased beyond an certain point. Such a decline is probably due to the reduction in particle velocity that occurs due to air resistance. This will be greater over longer firing distances. This decline in velocity will result in a reduction in the number of particles reaching the target at a sufficient velocity to penetrate the tissues. One possible method of overcoming the reduction in particle velocity due to air resistance, and thus theoretically increasing the number of expression units, may be to increase the vacuum under which the discharge takes place. However, this option was not possible on the apparatus used in the present study and that used by Aragao *et al.* (1993) as in both cases the apparatus were being operated at their maximum permissible vacuum. The smaller number of expression units observed by Aragao *et al.* (1993) at the lowest firing distance was suggested to have been caused by excessive tissue damage that occurred due to the extreme velocity of the particles at this distance. This phenomena was not observed in the present study on rose and may be due to the different composition of the bombarded material. The rose embryos possibly being more able to withstand the high velocity of the microprojectiles than the bean embryos used by Aragao *et al.* (1993).

Despite this study demonstrating that particle delivery is an effective method of introducing DNA into rose cells there remains room for refinement and improvement. Indeed, assuming that the microprojectiles are perfectly spherical and do not adhere together, the number of gold particles accelerated from the surface of each macroprojectile used in this study has been calculated to be between 9.2×10^5 and 7.4×10^6 (P. Drake, University of Nottingham; pers. comm.). This means that, even with the maximum number of expression units obtained, only between 8.35×10^{-6} and 6.5×10^{-5} % of the particles actually effected a transformation event. A number of possible ways of improving the technique, and consequently increasing the number of

expression units, have been suggested. The use of helium driven devices, in particular the Biolistic®/PDS-1000 He device (marketed by Bio-Rad Laboratories, St Albans, UK), has been shown to both increase the yield of transformants 4 to 300 fold in a number of species (Sanford *et al.*, 1991), and to reduce the variations which occur between different delivery events that are associated with many devices (Kikkert, 1993).

The use of gold microparticles with a considerably larger range of particle sizes may also improve the frequency of transformation. However, at the present time, such mixtures are difficult to obtain. Several workers, including Perl *et al.* (1992) and Aragao *et al.* (1993), have emphasised the effect of different microprojectile DNA coating procedures on the number of expression units obtained, and this area warrants further investigation.

The osmotic conditioning of embryogenic cell suspension cells of maize prior to particle bombardment has been demonstrated to increase GUS expression (Vain *et al.*, 1993). It has been suggested that, following osmotic treatment, plasmolysed cells may be less likely to extrude their cytoplasm following penetration of the cells by the microprojectiles (Vain *et al.*, 1993). In addition, Yamashita *et al.* (1991) have demonstrated that gold particles were preferentially delivered into the vacuole of bombarded tobacco material. Therefore, an osmoticum treatment, which would reduce the size of the vacuole, would increase the likelihood of particles entering the nucleus and thus increase the amount of foreign gene expression. Such treatment should be applied to other explants and different species as it is possible that a similar beneficial effect may be observed.

One concern with the use of microprojectile-mediated plant transformation has been the production of chimaeric plants consisting of non-transformed and transformed sectors of tissue (Birch and Franks, 1991). Such plants were obtained in studies by

Fitch *et al.* (1990) and Christou *et al.* (1990) on papaya and soybean respectively. In this respect, rose may prove to be an ideal subject for particle-mediated DNA delivery. The asexual method of propagation (ie. grafting buds onto a rootstock of a different cultivar) could virtually eliminate problems relating to the production of chimaeras. Following the recovery of a chimaerally transformed plants after particle-mediated DNA delivery, the transformed sectors could be identified using a non-destructive assay. For instance, the non-destructive assay for GUS activity, based on the substrate 4-methylumbelliferyl β-D-glucoronic acid (4-MUG), could be employed (Martin *et al.*, 1992). Providing transformed sectors included some bud tissue, they could then be excised, grafted onto a rootstock in the normal manner and cultivated following standard procedures. Thus, a transformed, non-chimaeral plant would be produced in which all but the rootstock tissue contained a foreign gene. A similar approach was followed by McCabe and Martinell (1993) who used selective pruning, following GUS analysis, to produce transformed plants of cotton using microprojectile-mediated gene delivery. These methods of eliminating chimaeras rely on the observations of Christou and McCabe (1992) who demonstrated that whenever any part of the vascular system of a soybean leaf, produced from meristem bombardment, expressed a reporter gene it was able to pass that gene to subsequently regenerated shoots.

An additional advantage in the use of microprojectile-mediated DNA delivery in rose, with explants such as embryos or axillary buds, is that it minimises or eliminates a tissue culture stage, thus reducing the chances of somaclonal variation occurring and hence ensuring that varietal fidelity is maintained. The microprojectile bombardment approach of gene delivery, therefore appears to have considerable potential for generating transgenic rose plants with desirable horticultural traits.

7.5 Summary.

i) *Agrobacterium rhizogenes* strain R1601, injected into *in vitro* shoots of the English rose cv. Marie Pavié, induced a low frequency of formation of hairy roots.

ii) *Agrobacterium tumefaciens* strain HAT1065 was demonstrated to be ineffective in the transformation of *in vitro* shoots of the English rose cvs. Abraham Darby and Marie Pavié.

iii) Microprojectile-mediated DNA delivery, using an electrical discharge device, was an effective method for introducing foreign genes into zygotic embryos of English rose.

iv) Microprojectile-mediated gene delivery into rose embryos was shown to be affected by microprojectile concentration, DNA concentration, firing distance and the plasmid constructs employed.

CHAPTER 8.

Future developments: Rose as an ornamental market leader.

Rose breeding, perhaps more than any other horticultural industry, must respond to frequently changing consumer demands. Changes in preferences, fashion and aesthetic appeal result in an ever-increasing demand for plants and flowers which have unusual or novel characteristics. The rapid introduction of a new cultivar is, thus, a major objective within the industry (as discussed in Chapter 1). Conventional breeding and selection, with mutation breeding and the selection of natural mutations (sports), have been the main sources of the abundant array of rose cultivars currently available. The embryo rescue technique developed as part of this study, provides breeders with an easily applied method of increasing the number of plants being recovered from a crossing programme. Inevitably, this will lead to a greater probability of a new cultivar being obtained. This technique may serve not only to increase the scope of plants available to the rose breeder, but could be applied in a similar manner to any breeding programme in which low percentage seed germination is a barrier to obtaining the maximal number of hybrid plants. Additionally, the pollen cryopreservation techniques, successfully devised as part of this study, when introduced into a breeding programme, will increase the parental cultivars that the breeder can utilise in hybridisation programmes, as differences in cultivar flowering times or spatial separation will no longer impede pollen availability.

Micropropagation strategies have, for some time, been applied to many rose cultivars (see Chapter 1, Section 1.5.1) and, as part of this study, have been extended to several cultivars of English rose. Micropropagation, resulting in a rapid turnover of plants, is independent of season and produces uniform specimens notionally devoid of disease. At present, the market for micropropagated roses is restricted and the future of rose micropropagation will be entirely determined by market demands. Micropropagated

plantlets can be sold only at a premium if they bring tangible benefits to the grower. Clearly, the ability to respond rapidly to market demands, particularly in reducing the time taken to bring new cultivars to market, will appeal to all growers. Others may reap the benefits of reduced production costs, particularly in terms of reducing the personnel required for labour intensive stages of propagation, such as bud grafting. However, this study has demonstrated that the *in vitro* culture of rose is not easily applied to all cultivars. This reflects the recalcitrance encountered with many woody plants and suggests that further work will be required before such techniques become universally applicable to the majority of rose cultivars. The difficulties encountered with the *in vitro* propagation of some rose cultivars will, almost certainly, deter producers, especially those handling a wide range of germplasm, from adopting these innovative techniques. As a result, it is likely that the significance of *in vitro* culture in rose production and/or breeding will increase gradually but over many years. Micropropagation may also become an important tool with other woody floricultural species. Dirr (1989) has identified over 70 woody species with potential as flower crops and micropropagation will be essential if such species are to be rapidly exploited and brought to the consumer.

The recalcitrance, encountered with the basic tissue culture of English rose in this study, was reflected in difficulties in regenerating plants from protoplasts. Nonetheless, this study has clearly indicated the possibilities that may be attained if protoplast technologies are applied to rose breeding. Techniques relying on the use of rose protoplasts will undoubtedly be restricted by the cultivar/species dependent recalcitrance that is common to many woody species. In addition, the future use of protoplast-based technologies on species such as rose may be restricted, as the use of protoplasts, either in somatic fusions or as targets for direct gene delivery (by methods such as microinjection), will almost certainly result in a disruption of varietal fidelity. Therefore, the development of technologies, in which a single gene is

introduced into the rose genome with minimal somaclonal variation, is highly desirable.

This study has demonstrated the effective use of microprojectile-mediated DNA delivery using an electrical discharge device. Since the completion of experimental work for this thesis, further investigations with rose using a helium powered device (BioRad PDS1000/He) have given promising results; this will undoubtedly be further exploited in rose transformation in the future. Unlike many methods of transformation, microprojectile-mediated gene delivery can exploit a large range of explants making it applicable to systems, such as those in English rose, in which a reliable protoplast-to-plant system has yet to be realised. Additionally, the transformation of English roses, and other previously resistant cultivars, may become possible as an increasing number of *Agrobacterium* strains are tested. One possible route may be to employ strains of agrobacteria that have been isolated from naturally infected rose plants. The use of such strains would then eliminate or reduce the problems relating to host specificity. Likewise, the exploitation of an ever-increasing number of *Agrobacterium* helper plasmids will almost certainly increase the host range of established strains (Hood *et al.*, 1993).

Because of public concern over the consumption of genetically engineered plants, it is likely that the majority of genetically engineered plants that are cultivated in the near future will be ornamental types (Bains, 1993), and that the regulatory hurdles will probably be less stringent in floral as compared to food or fodder crops. Already, a large number of genes have been identified and isolated, which, if introduced into rose, could result in cultivars with novel or improved characteristics. The introduction into rose of genes, such as those coding for bean chitinase or β-1,3-glucanase (Melchers *et al.*, 1993), may result in plants with increased resistance to common rose fungal pathogens, such as blackspot and mildew. Genes, such as the snowdrop lectin gene (which has conferred resistance to predation by insects such as

the aphid *Myzus persicae* in tobacco [Gatehouse *et al.*, 1993]) should give protection against a number of insect pests, particularly aphids, when introduced into rose. Although the introduction of these genes into rose would obviously have major benefits for all involved in rose cultivation, it is probably the modification of floral traits by genetic engineering which will continue to attract most interest.

Flower colour is one of the most important floral attributes for customers and, not surprisingly, has long been a major target for conventional breeding. As the pathways controlling flower colour are progressively understood (Stevenson, 1991; van Tunen and Mol, 1991, Holton and Tanaka, 1994), so the possibility of modifying flower colour has become a more realistic proposition. The first successful application of genetic engineering to modify flower colour was in *Petunia hybrida* (Meyer *et al.*, 1987). Antisense gene technology has been used for the modification of flower colour in other species. For example, Van der Krol *et al.* (1988) and Napoli *et al.* (1990) transferred antisense and sense chalcone synthase genes respectively into *Petunia hybrida* to produce transgenic plants with several different types of flower colouration. More recently, this technology has been extended to alter the flower colour of *Gerbera hybrida* (Elomaa *et al.*, 1993) and *Dendranthema grandiflora* (florist's chrysanthemum) (Courtney-Gutterson *et al.*, 1994) and undoubtedly, in the future will be applied to rose. Calgene Pacific Inc. (Collingwood, Victoria, Australia) has recently claimed to be near to producing the long sought-after blue rose by the use of genetic engineering (Holton and Tanaka, 1994). However, definitive evidence of this remains to be seen, and there are many in the ornamentals industry who are sceptical about the actual demand for such a product (Kidd and Davis, 1993).

One of the most obvious differences between many groups of roses is their flower morphology, such as the difference in petal number between the wild-types (5 petals) and multi-petalled English rose cultivars. An understanding of the molecular basis for flower morphology and organ development, which is emerging from studies on

mutant plants may, in the future, offer an alternative and more precise method of changing flower morphology. The isolation of the *deficiens* gene from *Antirrhinum majus* (Sommer *et al.*, 1990) and the *agamous* gene from *Arabidopsis thaliana* (Yanofsky *et al.*, 1990) might prove to be a useful advance. Transfer of the *deficiens* gene could result in rose flowers where petals are replaced by a ring of sepals and the stamens are converted to carpels. The *agamous* gene affects the developmental sequence of stamen and carpel whorls (Bowman *et al.*, 1991), and its transfer into rose could produce flowers in which the stamen whorl develops into an additional whorl of petals and the carpel whorl is altered into repeat mutant flowers.

It is known that the control of flower fragrance relies on a pathway similar to that which governs flower colour (Bauer and Garbe, 1985). Rose breeders have generally regarded fragrance as being of secondary importance when compared with attributes such as flower colour, the number of heads or repeat flowering. Thus, many cultivars, particularly the Hybrid Teas, lack fragrance. Inevitably, as research into the chemical pathways controlling fragrance takes place, so the opportunity for genetic modification will arise. It is possible that the highly desirable fragrance characteristics of some rose cultivars, such as the English roses, could be introduced into the less fragrant cultivars, or that a cultivar possessing a particular fragrance will be modified to produce a different one. However, to date, no example exists for the manipulation of a fragrance chemical in any species.

In the cut-flower industry, vase life is important. The mechanism of flower ageing differs widely amongst plant species. In some cases, the onset of senescence is known to be associated with a burst in ethylene production. This can be delayed by inhibitors of ethylene biosynthesis (aminoethyoxyvinyl glycine) or action (Ag^+ ions). Ag^+, in the form of silver thiosulphate, is widely used in the cut-flower industry to extend vase life. Calgene Pacific Inc. is currently involved in attempts to extend the vase life of several cut-flower species, including rose, by the introduction of a gene

inhibiting ethylene biosynthesis (Kidd and Davis, 1993). This work is presently restricted to cut-flower varieties, but, in the future, such genes may be introduced into rose plants to extend the life of flowers whilst retained on the bush.

In the cultivation of cut-flower roses, the number of flowers produced per bush is of prime commercial importance. Genes influencing the number of flowers which develop have been identified and are being introduced into roses cultivated for cut flower production. Plants expressing these genes apparently produce between 2 and 4 times the number of flowers produced in non-transformed control specimens (Dr. M. Boulay, L.V.M.H Recherche, Colombes, France; pers. comm.). In the future, this gene may also be introduced into other groups of rose. This may result in the production of plants with floribunda-type characteristics (in terms of flower number), whilst retaining the other characteristics associated with specific groups of roses.

By exploiting genetic engineering to manipulate endogenous genes involved in pigmentation and senescence, longer-lasting, novel cultivars of rose will, in the future, become available. The large-scale production of rose (whether cut-flowers or plants) will also undoubtedly benefit from the introduction of pest and disease resistance genes. Developments presented in this thesis, such as embryo rescue and pollen cryopreservation, can be applied immediately to give benefit to rose breeding programmes. However, the challenge for the future will be to obtain further knowledge on the effects of growth regulators, other compounds and regulatory genes on aspects of *in vitro* culture, such as plant regeneration, as well as on phenotypic features such as floral shape, plant form and habit and time of flowering. Once achieved, exciting opportunities will arise for cell biologists, molecular biologists and plant breeders to combine their knowledge to produce roses with improved horticultural and consumer traits.

APPENDIX I.

AI.1 Media preparation.

All media based on MS (Murashige and Skoog, 1962) salts were made using commercially available preparations (Flow Laboratories, Irvine, Scotland), to which sucrose (3.0% w/v), agar (Type IV; Sigma Chemical Co Ltd., Poole, Dorset, UK.; except when otherwise stated), any organic or inorganic supplements and growth regulators were added, as appropriate, and the pH of the medium adjusted prior to sterilisation. Any thermolabile componds, incorporated into semi-solid media, were filter-sterilised and added to autoclaved medium whilst still molten (45-50°C).

AI.2 Aseptic technique.

All *in vitro* manipulations including filter-sterilisation, surface sterilisation, inoculation and subculturing of axenic plant material were performed under aseptic conditions in a laminar air-flow cabinet (Slee, London, UK) providing an airflow of 0.5 m sec^{-1} using sterilised media and equipment.

AI.3 Sterilisation of media and equipment.

Media were dispensed into the appropriate containers and sterilised by autoclaving, for 20 min at 121°C (118 KPa nominal steam pressure). Alternatively, media containing thermolabile compounds, such as some vitamins, amino acids, or antibiotics, were filter-sterilised using a Millipore (Millipore UK Ltd., Harlow, Middlesex, UK) or Sartorius (Sartorius Instruments Ltd., Belmont-Sutton, Surrey, UK) vacuum pressure polycarbonate filter assembly with a Whatman cellulose nitrate membrane filter (0.22 mm pore diameter). For filter sterilised media, agar/agarose solidification was achieved by combining equal quantities of the appropriate double-strength medium with autoclaved agar/agarose (at a concentration double the final one required for culture) in distilled water.

For culture handling, all equipment and glassware was sterilised either by autoclaving or by exposure to hot air (160°C, 2 h).

Instruments routinely used for inoculation and transfer of axenic plant materials were sterilised by dipping in ethanol and flaming prior to use. Those used for the handling of bacteria in transformation experiments were autoclaved before and after use.

APPENDIX II.

AII.1 Composition of Murashige and Skoog (1962) salts (without growth regulators).

Component	mg l^{-1}	Component	mg l^{-1}
$CaCl_2.2H_2O$	440.4	Sucrose	3000.0
NH_4NO_3	1650.0	Glycine	2.0
KNO_3	1900.0	myo-Inositol	100.0
KI	0.83	Nicotinic acid	0.5
$CoCl_2.6H_20$	0.025	Pyridoxine HCl	0.5
KH_2PO_4	170.0	Thiamine HCl	0.1
H_3BO_3	6.2		
Na_2MoO_4	0.25		
$MgSO_4.7H_2O$	370.0		
$MnSO_4.4H_2O$ 2	2.3		
$CuSO_4.5H_2O$	0.025		
$ZnSO_4.7H_2O$	8.6		
FeNaEDTA	0.1M		

AII.2 Composition of CPW salts solution (Power *et al.*, 1989).

Component	mg l^{-1}
KH_2PO_4	27.2
KNO_3	101.0
$CaCl_2.2H_2O$	1480.0
$MgSO_4.7H_2O$	246.0
KI	0.16
$CuSO_4.5H_2O$	0.025

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

CPW 13M add 13% (w/v) mannitol

CPW 21S add 21% (w/v) sucrose

CPW 25S add 25% (w/v) sucrose

For all CPW solutions; pH 5.8, sterilize by autoclaving.

AII.3 Salt composition of KM-Based tissue culture media (Kao and Michayluk, 1975).

Component	mg l^{-1}
$CaCl_2.2H_2O$	600.0
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_20$	0.025
H_3BO_3	3.0
KCl	300.0
KH_2PO_4	170.0
KI	0.75
KNO_3	1900.0
$MgSO_4.7H_2O$	300.0
$MnSO_4.H_2O$	10.0
$NaMoO_4.2H_2O$	0.25
NH_4NO_3	600.0
$ZnSO_4.7H_2O$	2.0
Sequestrine-330 Fe	28.0
Ascorbic acid	1.0
Biotin	0.005
Choline chloride	0.5
Folic acid	0.2
Inositol	100.0
Nicotinamide	1.0
p-ABA	0.01
D-Ca Pantothenate	0.5
Pyridoxine HCl	1.0
Riboflavin	0.1
Thiamine HCl	1.0
Vitamin A	0.005
Vitamin B12	0.01
Vitamin D3	0.005
Citric acid	10.0
Fumaric acid	10.0
Malic acid	10.0
Na-pyruvate	5.0
Cellobiose	125.0
Fructose	125.0
Mannose	125.0
Rhamnose	125.0
Ribose	125.0
Xylose	125.0
Mannitol	125.0
Sorbitol	125.0
Casamino acids	125.0
Coconut milk	10.0 ml l^{-1}

APPENDIX III.

AIII.1 Enzymes used for protoplast isolation.

Enzyme (Commercial Name)	Enzyme Activity	Source	Manufacturer
Driselase	Contains laminarase, xylanase and cellulase.	*Basidiomyc-etes*	Sigma, Poole, Dorset, UK.
Hemicellulase	Crude with some cellulase activity; active at pH 5.5 (37°C).	*Apergillus niger*	Sigma, Poole, Dorset, UK.
Macerozyme R-10	Contains high pectinase activity with some hemicellulase activity; active between pH 5.0-6.0 at 40-50°C.	*Rhizopus* spp.	Yakult Honsha Co, Ltd., Higashi-Shinbashi, Minato-Ku, Tokyo, Japan.
Meicellase	A crude cellulase which contains small amounts of cellobiase, phospholipase, xylanase, protease, amylase, glucanase and lipase as imurities; active pH between 3.0 and 6.0 at 20-55°C.	*Trichoderma viridae*	Meiji Seika Kaisha Ltd., Tokyo, Japan.

Continued...

Cellulase Onozuka R-10	A cellulase containing 10,000 units g^{-1} filter paper decomposing activity and appreciable hemicellulase activity. Optimum activity is at a pH between 4.0 and 5.0, at 40 -50°C.	*T. viridae*	Yakult Honsha Co, Ltd., Higashi-Shinbashi, Minato-Ku, Tokyo, Japan.
Cellulase RS	A more purified form of cellulase, having higher enzyme activity than Cellulase R-10	*T. viridae*	Honshu Co Ltd., Yakult, Japan.
Pectolyase Y-23	Highly purified, with high activity of endopolygalac-turonase and endo-pectin lyase, active at pH 4.5-6.5 with optima at pH 5.5.	*Aspergillus japonicus*	Seishim Pharmaceutical Co Ltd., Tokyo, Japan.

APPENDIX IV.

AIV.1 Embedding of protoplasts in Sodium Alginate.

Following isolation and purification, protoplasts were resupended at twice the required final plating density in 13% (w/v) mannitol solution. A solution consisting of 13% (w/v) mannitol and 2.1% (w/v) sodium alginate was then added in an equal proportion to the protoplast supension and thoroughly mixed. The resulting mixture was then dispensed as 0.25 ml aliqots onto the surface of agar-solidified (1.0 % w/v) CPW 13M solution supplemented to give a final concentration of 20 mM $CaCl_2$ (pH 5.8). After the sodium alginate had solidified by the action of the divalent calcium ions (approx. 1 h), the alginate discs were transferred to 5 cm Petri dishes containing 5 ml of the appropriate liquid culture medium.

APPENDIX V.

AV.1 Composition of protein extraction buffer.

20% (v/v) glycerol

50 mM Tris HCl (pH 5.8)

2.0 mM $MgCl_2$

10.0 mM dithiothrietol (DTT)

10 mM leupeptin

10 mM antipain

1.0% (w/v) PVP-40

0.1% (v/v) mercaptoethanol

Appendix VI.

AVI.1 Composition of *Agrobacterium*-Induction Broth.

Component	mg l^{-1}
NH_4Cl	1000.0
$MgSO_4.7H_2O$	1000.0
KCl	150.0
$CaCl_2.2H_2O$	10.0
$FeSO_4.7H_2O$	2.5
$NaH_2PO_4.H_2O$	300.0
MES	3904.0
Glucose	5000.0
Sucrose	20000.0
Acetosyringone	200 mM
pH 5.5	

AVI.2 Composition of LB medium.

Component	g l^{-1}
Tryptone	10.0
Yeast Extract	5.0
NaCl	5.0
pH 7.0	

AVI.3 Antibiotics used for the selection of bacteria.

***A. rhizogenes* strain R1601.**

	ampicillin	50 μg ml^{-1}

***A. tumefaciens* strain HAT 1065.**

(Solid medium)	rifampicin	100 μg ml^{-1}
	tetracycline	5 μg ml^{-1}
	kanamycin	50 μg ml^{-1}
(liquid medium)	rifampicin	40 μg ml^{-1}
	tetracycline	2 μg ml^{-1}
	kanamycin	50 μg ml^{-1}

E. coli **strain HB101.**

(Non-transformed)	ampicillin	50 $\mu g\ ml^{-1}$
(with pMJD67)	carbenicillin	100 $\mu g\ ml^{-1}$
(with pBI221.1479)	ampicillin	100 $\mu g\ ml^{-1}$

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ADDENDUM.

The following changes should be made:

Page 3, lines 13 and 14 should read; Juvenility is a major problem with most woody plant species as they have prolonged juvenile periods prior to the production of flowers, fruits and/or marketable

Page 12, line 21 arbitary should read; arbitrary.

Page 14, line 17 persuit should read; pursuit.

Page 20, line 17 should read; reduce leaf chlorosis, abscission, shoot tip necrosis and have a beneficial effect on ....

Page 22, line 11 should read; upon transfer to half-strength MS basal medium containing 1 mg l^{-1} kinetin ....

Page 27, line 4 petolyase should read; pectolyase.

Page 30, line 27 should read; frequencies. Rose transformation systems may well have been developed by ....

Page 33, line 25 senario should read; scenario.

Page 38, Table 2.1 The primary carbohydrate is measured in g l^{-1}.

Page 47, line 10 initialy should read; initially.

Page 47, line 19 subsequenty should read; subsequently

Page 50, final line should read; actually entered the nucellus via the micropyle to effect fertilisation.

Page 53, lines 17 & 18 should read; to germinate. In normal seeds, there are amounts of soluble substrates that are normally present in the form of raffinose. However it is possible that the seeds used ....

Page 55, line 11 should read; The quantity and choice of carbohydrate can also affect the osmolarity of the medium. In this ....

Page 58, line 20 apperture should read; aperture.

Page 58, line 26 should read; scarification (Tinker, 1935), which thin or partially remove part of the pericarp, are ....

Page 61, line 11 lead should read; led.

Page 63, line 2 should read; greater. As this was not the case, these results suggested that an incompatibility ....

Page 70, line 9 dessicator should read; desiccator.

Page 81, final line fascets should read; facets.

Page 85, line 14 should read; influenced by cryopreservation. There was a close similarity in the number of ....

Page 102, Table 4.4 Motley and Keen (1987) should read; Douglas *et al.* (1989).

Page 121, line 21 additionally should read; additionally.

Page 127, line 16 should read; effectively induce adventitious shoot regeneration in previously recalcitrant woody ....

Page 141, line 11 Z should read; z.

Page 167, line 10 ellicits should read; elicits.

Page 170, line 24 Lethan *et al.*, should read; Letham *et al.*,.

Page 184, line 3 50 ml should read; 50 µl.

Page 192 zymograms should read; total protein profiles.

Page 197, line 9 should read; (Baird *et al.*, 1992; Waugh and Powell, 1992) and is seen as a more unequivocal method ...

Pages 197 and 199 isozyme should read; total protein.

Page 211 references to Fig 7.1 should be to Plate 7.1.

Page 235, AII.1 MS medium contains 30000.0 mg l^{-1} sucrose.

Page 242 The following reference should be inserted; Ball, E. (1950) Experimental dividing of a shoot apex. Am. J. Bot. 37: 660.

R. Marchant, September 1994.