Plant regeneration from protoplasts of Solanum tuberosum and Solanum brevidens.

Nelson, Robin Stephen

Award date:
1983

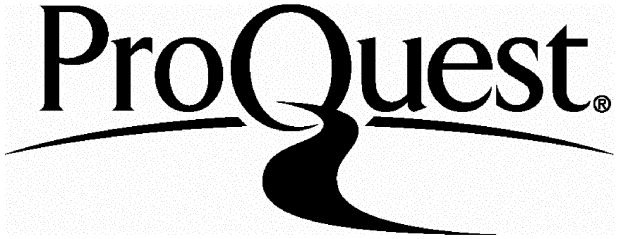
Awarding institution:
University of Bath

PLANT REGENERATION FROM PROTOPLASTS OF SOLANUM TUBEROSUM AND SOLANUM BREVIDENS

Submitted by Robin Stephen Nelson B.Sc. for the degree of

Doctor of Philosophy of the University of Bath

1983



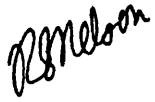
ACKNOWLEDGEMENTS

I would like to give my special thanks to Dr. Simon W.J. Bright, for his encouragement and guidance throughout the period of these studies, to Professor Graham G. Henshaw for his advice and constructive criticism, and to Dr. Angela Karp for her expertise as a cytologist.

It is a pleasure to acknowledge the friendship and co-operation of my colleagues in the Biochemistry Department at Rothamsted, in particular Mr. Gary P. Creissen, for problems shared (and the occasional hangover), Dr. Gert Ooms and Mrs. Susan Smith for their help with the amino acid analysis. Thanks also to Mr. Gordon T. Higgins (Photography Dept.) for his advice and skill as a photographer, to Dr. Peter Digby and Mr. Simon Harding (Statistics Dept.) for their statistical knowledge and to Mrs. Janet Why (Soil Microbiology Dept.) for typing this thesis. Finally I thank the Science and Engineering Research Council for financial support during the period 1980-1983 in which this work was performed.

PREFACE

This work is my own except where specific acknowledgement is made.



R.S. Nelson

This thesis is dedicated to the memory of Dr. Emrys Thomas.

PUBLICATIONS

1. KARP, A., NELSON, R.S., THOMAS, E. and BRIGHT, S.W.J. (1982). Chromosome variation in protoplast-derived potato plants. Theor. Appl. Genet **63**, 265-272.

2. JONES, M.G.K., BRIGHT, S.W.J., NELSON, R.S., FOULGER, D., CREISSEN, G.P. and KARP, A. (1983). Variation in plants regenerated from protoplasts and complex explants of potato. In: I. Potrykus, C.T. Harms, A. Hinnen, R. Hutter, P.J. King and R.D. Shillito (eds). Protoplasts 1983 Poster Proceedings, pp.150-151. Birkhauser Verlag, Basel, Switzerland.

3. NELSON, R.S., CREISSEN, G.P. and BRIGHT, S.W.J. (1983). Plant regeneration from protoplasts of Solanum brevidens. Plant Sci. Lett **30**, 355-362.

4. NELSON, R.S., KARP, A., CREISSEN, G.P. and BRIGHT, S.W.J. (1983). Plants regenerated from isolated protoplasts of Solanum brevidens. In: I. Potrykus, C.T. Harms, A. Hinnen, R. Hutter, P.J. King and R.D. Shillito (eds). Protoplasts 1983, Poster Proceedings, pp.68-69. Birkhauser Verlag, Basel, Switzerland.

5. BRIGHT, S.W.J., NELSON, R.S., KARP, A., JARRET, V.A., CREISSEN, G.P., OOMS, G., MIFLIN, B.J. and THOMAS, E. (1982). Variation in culture-derived potato plants. In: A. Fujiwara (ed). Plant Tissue Culture, 1982. pp.413-414. Japanese Assoc. for Plant Tissue Culture.

6. BRIGHT, S.W.J., JARRETT, V.A., NELSON, R.S., CREISSEN, G.P., KARP, A., FRANKLIN, J., NORBURY, P.B., KUEH, J.S.H., ROGNES, S.E. and MIFLIN, B.J. (1983). Modification of agronomic traits using *in vitro* technology. In: S.H. Mantell and H. Smith (eds). Plant Biotechnology. pp.251-265. SEB Seminar Series 18.

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

γAB	γ-Aminobutyric acid
ABA	Abscisic acid
Aec	S-2-Aminoethyl cysteine
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
Azc	Azetidine-2-carboxylic acid
BAP	6-Benzylaminopurine
ca	Circa
CoV	Coefficient of Variation
cv	cultivar
Cys	Cysteine
d	days
24D	2-4-Dichlorophenoxyacetic acid
FDA	Fluroscein diacetate
FTF	Protoplast-derived Fortyfold plant
FW	Fresh weight
GA_3	Gibberellic acid
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
His	Histidine
IAA	Indole-3-acetic acid
Ile	Isoleucine
L	Length
Leu	Leucine
Lys	Lysine
MES	2[N-Morpholino]ethane sulphonic acid
Met	Methionine
5-MT	5-methyltryptophan
NAA	σ-Naphthalene acetic acid
ND	Not Determined
NR	Nitrate reductase
ns	no significant difference
PE	Plating efficiency
PEG	Polyethylene glycol
pers. comm.	personal communication
Phen	Phenylalanine
Pro	Proline

Ser	Serine
spp	species
σ	standard deviation
Thr	Threonine
Tyr	Tyrosine
Val	Valine
VTSC	Virus tested stem cutting
W	Width
$\bar{x}$	mean

SUMMARY

1. The use of *in vitro* techniques as a supplement to conventional methods for potato breeding is discussed. The literature on protoplast, isolation, culture and somatic hybridisation is reviewed.
2. The materials and methods used are described.
3. Meristem-tip cultures of three *S. tuberosum* lines were used to initiate shoot cultures. Plants grown under different environmental regimes were assessed as protoplast sources. Isolations from shoot cultures were more reproducible but gave lower protoplast yields than leaves from growth room grown plants. Protoplasts from a tetraploid cultivar (Fortyfold) from both shoot cultures and leaves of whole plants were successfully cultured and regenerated to plants. Isolated protoplasts from two monohaploid lines failed to respond to the culture conditions used.
4. Shoot cultures of *S. brevidens* were suitable for the isolation of high yields of viable protoplasts, from which fertile plants were regenerated.
5. Isolated protoplasts of *S. brevidens* were used in attempts to improve the plating characteristics of cultured *Solanum* protoplasts. A protocol enabling the culture of protoplasts at low population densities and subsequently, more efficient regeneration of plants from protoplast-derived calluses was

developed. The final protocol was applied to the culture of protoplasts from a range of S. tuberosum genotypes.

6. Plants regenerated from protoplasts of S. brevidens and S. tuberosum showed phenotypic variation. Cytological analysis revealed some of this variation correlated with changes in ploidy, aneuploidy and chromosome structural changes. Variation was also present among plants with the normal chromosome number. Unstable changes in the phenotype of regenerated plants were also observed.
7. The final protocol described in this thesis is discussed in perspective with other methods for protoplast culture. The uses of potato protoplasts are discussed.

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Ever since plants were first taken into cultivation they have been subject to selection by growers, albeit often unintentionally. The gradual accumulation of knowledge in reproduction and heritability laid the foundations for modern plant breeding. Whilst there is still much scope for the improvement of crop plants by conventional methods, there is a limit, imposed by the presence of incompatibility barriers between and within groups of plants, to what can be attained by sexual crossing. The potentials of isolated protoplasts as a new breeding tool have often been heralded (e.g. Bajaj, 1974, 1977; Cocking 1977, 1981). Protoplast fusion, or somatic hybridisation may be used to bypass incompatibility mechanisms (Schieder and Vasil, 1980). Protoplasts can take up macromolecules or particles such as DNA, viruses, bacteria, and nuclei (Bajaj, 1977), although the use of organisms such as Agrobacterium tumefaciens, Agrobacterium rhizogenes and cauliflower mosaic virus as vectors may be necessary to facilitate the incorporation of new genetic information into the protoplast genome (see various articles in Kahl and Schell, 1982).

Many problems must still be overcome before protoplast technology can be used as a supplement to conventional plant breeding (Cocking, 1979; Thomas et al., 1979). Of the four major crop species (Wheat, Maize, Rice and Potatoes), so far conditions for the culture of protoplasts and regeneration of plants have only been elucidated for potato (Shepard and Totten, 1977). Techniques for the isolation of fusion products or transformed cells from mixed populations must be developed (Power and Cocking, 1977).

Biochemical mutants have been increasingly used to enhance our understanding of the genetics, biochemistry and physiology of a number of crop species (Nelson and Burr, 1973; Maliga, 1980; Miflin *et al.*, 1983). While mutants affecting the nutritional quality of crop plants may be agronomically desirable in their own right; the use of mutants as selectable markers offers one of the more efficient methods for the identification of desired genotypes in fusion or transformation studies.

1.2 POTATO BREEDING IN PERSPECTIVE

The limitations of conventional breeding are particularly apparent when applied to the potato (*Solanum tuberosum* L.). Historically the basic material from which most U.K. cultivars were bred was very small. The stock material was originally a small sample of tetraploid South American clones (*S. tuberosum* subspecies *andigena*). It is thought that clones which tuberised under long days (*S. tuberosum* subspecies *tuberosum*) were selected from these over a long duration (Hawkes, 1967). These were supplemented at intervals by new introductions (Hawkes, 1978a) but were decimated by the blight epidemics in the 1840's. Although resistant clones did emerge, there was a major loss of diversity in the process, and since then a number of pathological crises have occurred without any major accessions to increase the available gene pool (Simmonds, 1962). This resulted in the cultivated varieties of potato having a very narrow genetic base.

However, the global gene pool for the potato is large and at present virtually untapped. The genus *Solanum* contains over 2000 species including several species which are used for food, such as

aubergine (Solanum melongena), pepino (S. muricatum), chilli peppers (Capsicum spp) and tomato (Lycopersicon esculentum). There are seven cultivated and 160 wild species of potato recognised (Hawkes, 1978b). All the tuber-bearing species are grouped in subsection Potato, section Petota of the genus Solanum (Hawkes, 1978b and Figure 1), and are geographically confined to the South American continent. Hawkes (1979) divided the subsection Potato into 18 series, two of which, Juglandifolia and Etuberosum, contain species without stolons or tubers. A large number of the wild potato species have potentially useful traits, especially genes for resistance to pathogens of potato (Hawkes, 1978b).

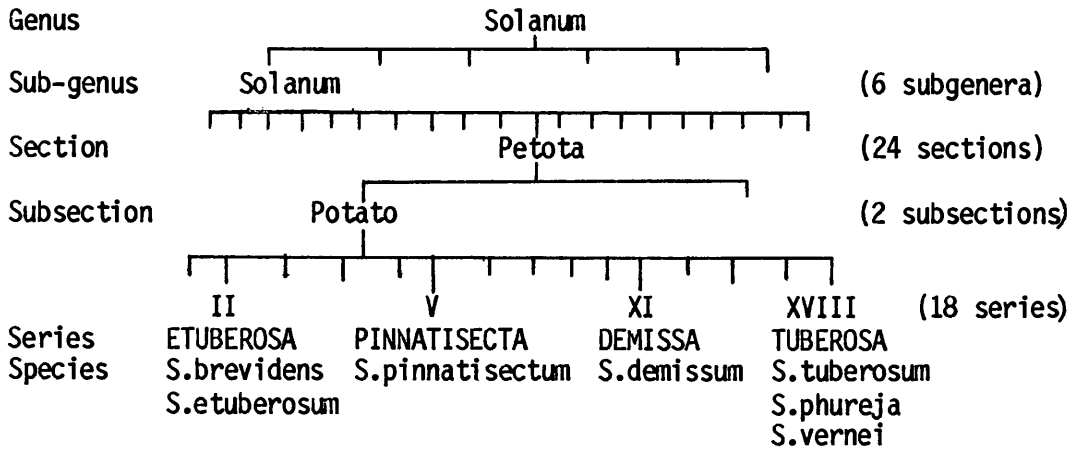


Fig. 1. Taxonomic relationships of the potato with some of its wild relatives. (references: Hawkes 1978b, Hawkes 1979, and Hawkes and Hjerting, 1969).

The use of wild species in breeding for disease resistance has been discussed (Howard, 1970, 1978). Many, but not all of the wild species can hybridize with S. tuberosum. Several cultivars with

traits introduced from wild species have been released. For example, in the U.K., 'Maris Peer'; in the U.S.A., 'Kennebec' and in the Netherlands 'Mentor' all resulted from S. tuberosum x S. demissum to introduce blight resistance. The new cultivar 'Klondyke' is a Dutch variety derived from a cross with S. vernei to incorporate nematode resistance. However, it must be emphasised that not all wild species can be successfully crossed with S. tuberosum. Furthermore the method is suitable for the introduction of resistance determined by major genes but is of less use if the resistance is polygenic (Howard, 1970).

The series Etuberosa contains two species, S. etuberosum and S. brevidens which, although non-tuberous, have attracted great interest from potato breeders because they carry genes for resistance to virus Y and leaf roll virus (Jones, 1979) and a high resistance to frost (Ross and Rowe, 1965). Pandey (1962) was unable to produce hybrid seeds in reciprocal crosses of S. brevidens with 13 tuber-bearing species. He concluded that failure to set seed was due to either i) inhibition of pollen germination or pollen tube growth in the style; ii) failure of berry set after normal fertilisation or iii) both. In spite of this Hermsen and Taylor (1979) attempted extensive crosses between S. brevidens and S. etuberosum on the one hand and 10 tuber-bearing species on the other. A highly vigorous but male sterile hybrid was obtained from the cross S. etuberosum and S. pinnatisectum. However the hybrid had an abnormal meiotic behaviour with a high percentage of univalents formed at metaphase 1 (Ramanna and Hermsen, 1979). To be of any practical use as a 'bridge' between the Etuberosa series and S. tuberosum, the hybrid had to be fully fertile, thus the valuable Etuberosa genes were still unavailable.

Potato breeding is at best an elaborate and difficult process often dependant upon chance. Many commercial potato cultivars are pollen sterile, preventing certain desirable crosses being made. Most cultivars ae extremely heterozygous tetraploids. For many characters studied, potatoes show simplex (Aaaa) inheritance (Howard, 1970). During the synthesis of a new cultivar, a large number of horticultural traits must be combined. Yield, size, shape, quality and storage characteristics of tubers are critical features, as are general growth characteristics of the foliage, environmental requirements for the onset of tuberisation and resistance to diseases. It can take at least 10 years to select a new variety, involving much valuable space and manpower. New cultivars, such as Maris Piper, with a good overall performance are quickly recognised, but frequently newly introduced varieties are commercially unsuccessful in that they do not displace established cultivars. Howard (1978), listed the ten most popular British potato varieties for 1976. The cultivar 'King Edward', although introduced in 1902 was then still more popular than many later cultivars. The same is true with the old varieties 'Russet Burbank' in the USA and 'Bintje' in the Netherlands. *In vitro* technology has already begun to supplement breeding programmes, and the use of protoplast systems will greatly increase the contribution in the future.

1.3 THE USE OF IN VITRO TECHNIQUES IN POTATO BREEDING

1.3.1. *In vitro* pollination and embryo culture

Work at the International Potato Centre (Peru) has sought to overcome pollen failure using *in vitro* methodology (Schmiediche,

P.E. pers. comm.). When the barriers hindering the process of pollen-tube growth lie in the stigma and style, they may be overcome if the style is excised and the pollen is placed on the surface of the ovary. Alternatively, the application of chemicals (plant hormones and vitamins) to stimulate pollen-tube growth, or the direct introduction of pollen into the ovary have proved successful in a few crosses (for a review see Zenkteller, 1980). Failure of berry set after fertilisation has been overcome in a number of interspecific crosses, including Solanum nigrum x S. luteum by using embryo culture (reviewed by Raghavan, 1977, 1980).

1.3.2. Meristem-tip culture and in vitro propagation

In nature potatoes are propagated vegetatively by means of tubers. Because the potato is susceptible to many viral, bacterial and fungal pathogens, routine production of disease free tubers is necessary for the propagation of high yielding cultivated forms. Thus plant breeders use virus-tested stem cuttings (VTSC) to multiply up desirable plants; this process takes up to three years to produce only 900 plants (Howard, 1978). The production of tubers for use as seed in the U.K. is restricted to areas where aphids do not present a serious threat as disease vectors. In vitro propagation, by means of axillary buds can produce many thousands of plants within a year, and maintain them in a disease-free condition. Meristem-tip cultures are the most suitable starting material for three reasons:

i) When combined with heat treatment, meristem-tip culture has been successfully used to obtain virus-free potatoes from infected stock (Mellor and Stace-Smith, 1977).

ii) Plants have been regenerated from a number of complex explants of

potato (Roest and Bokelmann, 1976), but for the production of genetically uniform propagules it is undesirable to go through a callus stage.

iii) The technique of meristem-tip culture and the media involved are relatively simple. As well as the production and rapid propagation of disease-free stocks, meristem-tip cultures may prove useful for the long term storage of potato germplasm (Westcott *et al.*, 1977).

An additional advantage of the use of shoot cultures for the propagation of disease-free potatoes is that the geographical limits imposed by disease vectors such as aphids no longer hold. The commercial implications of *in vitro* potato propagation have already been realised by at least one firm operating in the U.K.

1.3.3. The Manipulation of Ploidy

Krantz (1946) suggested that a logical way to breed potatoes was to obtain, by selfing, a series of inbred lines which would be more or less homozygous for desired characters. These lines could then be crossed to obtain vigorous offspring with the desirable characters of their inbred parents. This scheme suffered two basic shortcomings; first, few cultivars are pollen fertile, and second, much time and effort would be needed to test for combining ability between the lines. After Hougas and Peloquin (1957) discovered that diploid *S. phureja* (2n = 2x = 24) could be used as a pollinator in crosses with tetraploid *S. tuberosum* (2n = 4x = 48) to create dihaploid lines (2n = 2x = 24) parthenogenetically, Chase (1963) proposed a method of using dihaploids to produce inbred lines. Hermsen and Ramanna (1981) discussed the problems of working with dihaploids. Male sterility would be widespread, and they also suffer from a lowered female

fertility and poor flowering. In addition self-incompatibility is introduced at the dihaploid level, which hampers self-fertilisation and may restrict crossability. As dihaploids are restricted in any one individual to two alleles per locus it is doubtful that they could seriously compete with tetraploids.

The obvious advantages of dihaploids in breeding programmes overcome many of their shortcomings. Greatly simplified genetics and breeding occur at the diploid level, and the crossability with many valuable diploid wild species is improved. Since the early work of Hougas and Peloquin, the selection of parthenogenetic dihaploids from S. phureja x S. tuberosum hybrids has been facilitated by the use of selected 'superior pollinators' of S. phureja carrying a homozygous dominant marker either for purple hypocotyls, or for embryo spot (Hermsen and Verdenius, 1973). The extraction of dihaploids from tetraploids is now routine; using the same approach it is possible to obtain monohaploids (n = x = 12) (Jacobsen, 1978). Monohaploids are potentially very useful in both conventional breeding and in the selection of biochemical mutants for use as selectable markers in protoplast fusion studies. The presence of only one allele per locus allows the expression of recessive as well as dominant markers. However, using the parthenogenetic technique, the efficiency of monohaploid production is extremely low; only 2 seeds from 7×10^4 screened by Jacobsen (1978). An alternative method for producing monohaploids is by anther culture (for a recent review see Sunderland, 1980). Haploid plants were obtained from cultured anthers of the diploid species S. verrucosum, (Irikura and Sakaguchi, 1972) and later several other wild species (Irikura, 1975). Pollen embryoid, callus and root formation were observed on cultured anthers of three

tetraploid S. tuberosum cultivars, but only one dihaploid plantlet was obtained (Dunwell and Sunderland, 1973). Cultured anthers of dihaploid S. tuberosum, containing pollen at the uninucleate stage gave rise to a few monohaploid plants (Foroughi-Wehr et al., 1977). Further work demonstrated that by selecting the correct stage of microspore development and medium composition it was possible to obtain large numbers of monohaploid embryoids (Sopory et al., 1978). The technique is still not widely applicable however, and the importance of selecting the right genotype has been stressed (Jacobsen and Sopory, 1978).

Full use of haploidy in potato breeding is only feasible if tetraploids can be obtained from them. A number of methods for chromosome doubling have been reported, including colchicine treatment (Ross et al., 1967) and diplandroid producton (Jacobsen, 1980); both these methods are limited in application. Spontaneous chromosome doubling in plants regenerated from cultured leaf pieces of dihaploid potatoes was reported (Jacobsen, 1977), and further investigation revealed that the method could be exploited routinely (Jacobsen, 1982). Hermsen et al., (1981a) cultured leaf explants from the diploid S. etuberosum x S. pinnatisectum hybrid to obtain doubled regenerants. This resulted in fertile allotetraploid hybrids with nearly normal chromosome pairing at meiosis (Hermsen, et al., 1981b). Although the hybrid was successfully crossed with S. stoloniferum, S. polytrichon and S. verrucosum, crosses with S. tuberosum resulted in parthenocarpic berries only (Hermsen et al., 1981b)

Wenzel et al. (1979) proposed a scheme where parthenogenetic, androgenetic and protoplast techniques could be combined with

classical breeding approaches. S. phureja was used to produce a number of S. tuberosum dihaploid lines, followed by anther culture to obtain a total of 6 x 10^3 androgenetic clones (Wenzel et al., 1979; Wenzel and Uhrig, 1981). Wenzel and Uhrig (1981) reported that only 10% of these clones remained monohaploid, the other 90% had doubled up spontaneously producing completely homozygous, fertile clones with 24 chromosomes. They then subjected the dihaploid clones to tests for resistance to Globodera pallida (Pa3), potato virus Y (PVY), and potato leaf roll virus (PLRV). Clones derived from a S. tuberosum x S. vernei cross were shown to have retained a quantitatively inherited resistance to G. pallida (Wenzel and Uhrig, 1981). The final step in this breeding scheme would be to return the clones to the tetraploid level. This could be done by cultured leaf explants to produce homozygous tetraploids, or as suggested by Wenzel et al. (1979), to fuse protoplasts of two different dihaploid lines, thereby combining the useful characters from each line and introducing some heterozygosity. After evaluation of the tetraploids, any useful clones could be vegetatively propagated and would thus become a stable variety.

1.4 PROTOPLASTS: ISOLATION, CULTURE AND REGENERATION OF PLANTS

1.4.1. Historical Background

Plant cells possess a rigid cell wall enclosing the protoplast. Protoplasts were first isolated from plasmolysed cells of water soldier (Stratoites aloides). Intact protoplasts were released from cells in which incisions were restricted to the space between the cell wall and the protoplast (Klercker, 1892). However, such

mechanical isolation methods are limited to tissues with large cells in which a good separation of the protoplast from the cell wall takes place (Cocking, 1972). A method for producing large numbers of protoplasts was developed by Cocking (1960) who used an enzyme preparation from Myrothecium verrucaria to isolate tomato root protoplasts. The enzymatic method requires less osmotic shrinking of the cytoplasm than mechanical methods, thus living protoplasts can be obtained from a much wider range of tissues. Commercially produced cellulases and pectinases soon became available and were first used to isolate mesophyll cells (Takebe et al., 1968) and then protoplasts (Otsuki and Takebe, 1969) from tobacco leaves. Alternatively, a mixture of cellulases and pectinases may be used to isolate protoplasts in one step (Power and Cocking, 1970). When cultured, isolated protoplasts readily regenerated a cell wall and sometimes underwent a limited number of divisions (Cocking, 1972). Sustained divisions leading to colony formation and eventually plant regeneration were first reported from tobacco protoplasts (Takebe et al., 1971). The regeneration of plants from protoplasts has now been achieved in over 60 species, with the greatest success obtained with members of the Solanaceae (see Keller et al., 1982 and Table 1).

The removal of the cell wall leaves the plant cell membrane exposed as the only barrier between the external environment and the cell interior. This has meant that protoplasts have been extremely useful in physiological investigations ranging from studies of the physical properties of the plasmalemma to experiments on virus infection and the uptake of foreign particles (for a comprehensive review see Galun, 1981).

Table 1. Species in which plants have been regenerated from protoplasts since the review of Keller et al., 1982.

Species	Reference
Brassica oleracea	Xu et al., 1982
Cichorium intybus	Crepy et al., 1982
Citrus aurantium) Citrus paradisi) Citrus reticulata)	Vardi et al., 1982
Crotalaria juncea	Rao et al., 1982
Digitalis lanata	Li, 1981
Lactuca sativa	Berry et al., 1982
Lycopersicon esculentum	Morgan and Cocking, 1982
Manihot esculenta	Shahin and Shepard, 1980
Medicago coerulea) Medicago glutinosa)	Arcioni et al., 1982
Onobrychis viciifolia	Davey (unpublished)
Panicum maximum	Lu et al., 1981
Rehmannia glutinosa	Xu et al., 1983
Solanum melongena	Saxena et al., 1981 Jia and Potrykus, 1981
Solanum xanthocarpum	Saxena et al., 1982
Trifolium hybridum	Jones et al., (unpublished)
Trifolium pratense	Collins (unpublished)
Trigonella corniculata	Lu et al., 1982

1.4.2. Potato protoplasts

Potato protoplasts were first isolated from sterile tuber pieces (Lorenzini, 1973), but the first report of divisions leading to callus formation was from protoplasts isolated from leaves of the cultivar 'Sieglinde' (Upadhya, 1975). The calluses obtained differentiated roots, but the conditions necessary for shoot initiation were not ascertained. In 1977, three groups published protocols for the regeneration of plants from protoplasts of Solanum species. Butenko

et al., (1977a) isolated protoplasts from shoot cultures of the wild species S. chacoense and from shoot cultures and calluses of S. tuberosum. When cultured in a medium with a feeder layer of non-dividing γ-irradiated tobacco cells the protoplasts underwent divisions to form calluses which later differentiated shoots (Butenko et al., 1977b).

Binding and Nehls (1977) used shoot cultures to isolate protoplasts of bittersweet (S. dulcamara). Sustained divisions leading to callus formation were obtained when the protoplasts were cultured in a complex undefined medium, containing coconut milk and casein hydrolysate. On transfer to a second medium with reduced auxin concentration the calluses differentiated shoots which were then rooted by a further transfer to hormone-free medium. The system was later applied successfully to the regeneration of plants from protoplasts of four dihaploid S. tuberosum clones (Binding et al., 1978).

Shepard and Totten (1977) described a procedure for the regeneration of plants from mesophyll-derived protoplasts of the tetraploid S. tuberosum cultivar 'Russet Burbank'. To maintain a degree of physiological uniformity the protoplast source plants were grown from tubers in a controlled environment cabinet under a strict environmental and nutritional regime. Leaves taken from the plants were surface sterilised, then 'preconditioned' overnight at 4°C before protoplast isolation. Isolated protoplasts were cultured in a complex medium (containing casein hydrolysate), and a series of steps involving a total of five nutrient media was employed to obtain calluses, then shoot differentiation and eventually regeneration to plants. Subsequently a number of refinements to the technique and

media have been made (Shepard 1980a, 1980b, 1982) and the protocol has been found suitable for the regeneration of plants from protoplasts of five American cultivars (Shepard, 1982) and seven British cultivars (Gunn and Shepard, 1981). However, the same authors also found the procedure to be less suitable for several other cultivars.

Plant regeneration has also been achieved from mesophyll protoplasts of the hybrid S. phureja x S. chacoense (Grun and Chu, 1978) and from suspension culture-derived protoplasts of S. phureja (Schumann et al., 1980). At Rothamsted, protoplasts isolated from shoot cultures of S. tuberosum cv. Maris Bard, cultured in a completely defined nutrient medium underwent sustained divisions to form calluses from which plants were regenerated (Thomas, 1981).

1.4.3. Variation in regenerated plants.

There is much evidence that techniques employing meristem-tip culture successfully produce large numbers of genetically uniform plants whereas plants regenerated from callus cultures may show genetic variation (Bayliss, 1980). The extent of this variation covers changes in the ploidy, chromosome number and karyotype and is influenced by a number of factors including the ploidy level of the starting material, duration of the callus phase and the hormonal regimes used (D'Amato, 1977). Phenotypic variation has been observed in plants regenerated from calluses of sugar cane, tobacco, rice, oats, maize, barley and brassicas (reviewed by Larkin and Scowcroft, 1981). When such phenotypic variation occurs in agronomically valuable characters the variant may show an improvement over the parental line. Potentially useful variation amongst plants regenerated from calluses of sugar cane (Saccharum officinarum)

has been well documented (see Larkin and Scowcroft, 1981). In summary, regenerants displayed a variety of phenotypic alterations; some produced higher cane yields, a greater number of stalks or increased concentrations of sucrose when compared with the parents. Other regenerated plants were identified with higher levels of resistance to diseases including downy mildew (Sclerospora sacchari), eyespot (Dreschlera sacchari), smut (Ustilago scitaminea) and the Fiji disease virus. Tissue culture thus provided new impetus in the selection of improved sugar cane clones.

Plants regenerated from protoplasts of dihaploid S. tuberosum lines (Binding et al., 1978), were reported to be uniform in a number of morphological characters. Chromosome counts were made in 48 of the regenerants, two of the plants were aneuploid (with 47 and 49 chromosomes respectively) and the remaining 46 plants were shown to be tetraploid with 48 chromosomes (Wenzel et al., 1979). In contrast, plants regenerated from protoplasts of the tetraploid cultivar Russet Burbank showed considerable variation in a number of traits including growth habit, maturity date, tuber morphology, yield, photoperiodic requirements, and resistance to diseases. Clones with resistance to early blight (Alternaria solani) and late blight (Phytophthora infestans) were identified (Matern et al., 1978; Shepard et al., 1980; Secor and Shepard, 1981; Shepard, 1981). A cursory cytological examination of five regenerants which showed promise for high tuber yield revealed all five to have the normal tetraploid complement of 48 chromosomes (Shepard et al., 1980).

More recently, phenotypic variation has been reported in protoplast-derived plants of the tetraploid cultivar Maris Bard. None of the regenerants resembled each other in all of ten morphological

characters examined and only one resembled the parental Maris Bard type (Thomas et al., 1982). Plants regenerated from protoplasts of the USSR cv. Priekulsky rannii (Butenko et al., 1977b) were all reported to be aberrant in morphology and chromosome number (cited in Shepard, 1980a).

Many potato cultivars have a good overall performance but fall short because of one or two characters. It is not possible, using conventional technology to perform one-step improvement in potatoes whilst otherwise preserving all remaining characters. Thus Shepard et al., (1980) proposed that by using the variation observed in protoplast-derived plants it may be simpler to selectively enhance a popular variety rather than create a new one. With this in mind work in the U.S.A. and at the Plant Breeding Institute, Cambridge (R.E. Gunn, pers. comm.) is attempting to exploit such variation for potato improvement. However, for work involving the selection of biochemical mutants and for protoplast fusion studies it is desirable that any such spontaneous variation should be minimised or preferably eliminated.

1.5 PROTOPLASTS: FUSION AND SOMATIC HYBRIDISATION

1.5.1. Protoplast fusion

Attempts to fuse protoplasts were first made with protoplasts from algae (Vaucheria and Spirogyra) and higher plants (Elodea densa and Allium cepa) isolated using microdissection techniques (Kuster, 1909). Although handicapped by the small numbers of protoplasts isolated mechanically, fusions were observed, and calcium nitrate was found to be the most effective fusogen (fusion inducing

agent) among the chemicals tested (Kuster, 1910). The development of enzymatic methods for the isolation of large numbers of protoplasts enabled big advances to be made with protoplast fusion techniques. Sodium nitrate was reported to be a useful fusogen for root protoplasts from oats and maize (Power et al., 1970). Later calcium ions used in conjunction with high pH (10.5) and temperature (37°C) were shown to more effective (Keller and Melchers, 1973). The fusion technique used most often utilised calcium ions in conjunction with polyethylene glycol (PEG). Protoplasts treated with PEG solutions containing calcium ions adhered tightly and many (up to 10%) fused when the PEG solution was diluted with protoplast culture medium (Kao and Michayluk, 1974). The use of PEG treatment in combination with solutions containing high calcium ion concentrations at high pH and temperature resulted in enhanced fusion frequencies and increased prototoplast survival (Kao et al., 1974). Glimelius et al. (1978b) pretreated petri dishes with the lectin concanavalin A (con A) to aid adhesion of protoplasts to the dish surface. When used prior to PEG treatment, fusion frequencies of up to 100% were obtained. Several other chemicals have been tested for their ability to induce fusions (reviewed by Schieder and Vasil, 1980; and Keller et al., 1982), but at present PEG remains the most commonly used fusogen. Once fusion has taken place, the protoplasts are washed and suspended in a culture medium.

Electrical impulses applied to protoplasts induce rapid fusions; using this technique heterokaryons between Rauwolfia serpentina and Hordeum vulgare have been obtained (Senda et al., 1979). Zimmerman and Scheuriuch (1981) used dielectrophoresis to line up protoplasts of Vicia faba then applied a single electric pulse

that induced fusions with a high frequency. This new method may be a valuable alternative to PEG treatment although somatic hybrid plants have not yet been recovered from such a procedure.

In a suitable culture medium the protoplasts regenerate a cell wall and divide to give mixed populations of parental cells, homokaryons and heterokaryons. Somatic hybrids may be identified among mixed populations after shoots or plants have been regenerated (Gosch and Reinert, 1978; Melchers et al., 1978) but this is laborious and more efficient selection strategies are imposed earlier in the culture period. A medium was developed which favoured callus growth of the sexual hybrid Nicotiana glauca x Nicotiana langsdorfii over growth of callus from either parent (Carlson et al., 1972). When calluses from fusion experiments between the two species were placed on the selective medium, somatic hybrid callus behaved like the sexual hybrid and could thus be selected (Carlson et al., 1972; Smith et al., 1976; Chupeau et al., 1978). Similarly, sexual hybrids from the cross Petunia hybrida x Petunia parodii were used to develop a medium which gave enhanced growth of callus from the sexual or somatic hybrid between the two species (Power et al., 1977). However, the major advantage of somatic hybridisation is that it allows genetic interchange between sexually incompatible species. The use of sexual hybrids to develop media for the selection of somatic hybrids is obviously limited to sexually compatible species, thus it is important to develop other selection methods with wider applicability.

1.5.2. The identification of somatic hybrids

Heterokaryons may be isolated in the first few hours following

fusion. Products of fusions between suspension culture-derived protoplasts of Glycine max and mesophyll-derived protoplasts of Nicotiana glauca were identified on the basis that the heterokaryons consisted of chloroplast-free and chloroplast-containing zones (Kao, 1977). The protoplasts were isolated using a micropipette and cultured in microdrops. Each microdrop was then examined for the presence of heterokaryons. Similar methods have been used to identify and isolate heterokaryons of Arabidopsis thaliana with Brassica campestris (Gleba and Hoffmann, 1978), Nicotiana tabacum with Nicotiana knightiana (Menczel et al., 1981) and Atropa belladonna with Nicotiana chinensis (Gleba et al., 1982). Fluorescent cell membrane markers may be used to detect fusion products between different suspension cultures. Galbraith and Galbraith (1979) labelled two Glycine max populations with different fluorescent markers, then used fluorescence microscopy to detect the presence of both markers thereby distinguishing fusion products from unfused parental protoplasts. Fluorescent labels used in conjunction with a micromanipulation apparatus enable the isolation of individual heterokaryons from large mixed populations (Patnaik et al., 1982). Uhrig (1981) recommended the use of a herbicide (SAN 6706) to bleach potato shoot cultures. Mesophyll protoplasts obtained from such cultures are chlorophyll-free, allowing the detection of heterokaryons between bleached and unbleached mesophyll protoplasts. The method may provide a useful alternative to the use of suspension cultures which are often genetically unstable (Bayliss, 1980) and which take more time to establish.

The detection of heterokaryons at an early stage is invaluable for following the development of heterokaryons, but usually it is more

desirable to isolate hybrids at a stage when the manipulation of individual fusion products is less difficult and when large populations may be screened more efficiently. The optimum period for the manual isolation of somatic hybrids is after the callus becomes visible to the naked eye but before regeneration of whole plants. Smith et al. (1976) found that hybrid callus of Nicotiana glauca + Nicotiana langsdorfii grew more vigorously than callus from either parent and used this observation to preselect the best growing calluses before they employed the selective auxin-free medium. Interspecific hybrid callus of the genus Datura (Schieder, 1978a, b, 1980) and intergeneric hybrid callus of Datura innoxia with Atropa belladonna (Krumbiegel and Schieder, 1979) showed better overall growth when compared with their respective nonhybrid calluses ('heterosis') . When apparent, heterosis may serve as a useful selection marker, but it does not always occur.

1.5.3. The selection of somatic hybrids

The isolation of somatic hybrids relies on the appearance of a different phenotype or growth pattern when compared with the parental plant material. Several different groups of biochemical mutants have been used to provide a selectable marker for one or both of the fusion partners.

a) Chlorophyll deficient lines

Somatic hybrids obtained by fusion of protoplasts from two distinct recessive albino mutants of Nicotiana tabacum were easily distinguished as the non allelic mutations complemented each other to give the green wild type phenotype (Melchers and Labib, 1974). Non-

allelic albino mutants have since been used to select intraspecific hybrids of Datura innoxia (Schieder, 1977) and interspecific hybrids of Nicotiana tabacum + N. sylvestris (Melchers, 1977), Nicotiana tabacum + N. glauca (Evans et al., 1980), Nicotiana tabacum + N. rustica (Douglas et al., 1981) and Petunia parodii + P. hybrida (Cocking, 1978).

The use of an albino mutant as one fusion partner in combination with a different selectable marker for the other partner has also proved successful. Protoplasts of a chlorophyll-deficient mutant of Petunia hybrida, which can regenerate on a defined medium to form shoots were fused with wild type protoplasts of P. parodii which did not grow or form shoots on the defined medium. Green, shoot-forming calluses were selected and later confirmed as somatic hybrids using morphological traits and chromosome analysis (Cocking et al., 1977). The approach has become the most commonly used method of selection; somatic hybrids between a number of species have thus been isolated, including Petunia parodii + Petunia inflata (Power et al., 1979), P. parodii + Petunia parviflora (Power et al., 1980), Datura innoxia + Datura discolor and D. innoxia + Datura stramonium (Schieder, 1978a), D. innoxia + D. sanguinea and D. innoxia + D. candida (Schieder, 1980), Nicotiana tabacum + Nicotiana knightiana (Maliga et al., 1978). The use of albino mutants may be combined with morphological markers. Protoplasts of albino Datura innoxia were fused with wild type protoplasts of Atropa belladonna, then hybrid callus was selected by the presence of hairs from D. innoxia in combination with the green colour derived from A. belladonna (Krumbiegel and Schieder, 1979).

b) Auxotrophs

Somatic hybridisation between two different auxotrophic mutants may result in an autotrophic hybrid able to grow on unsupplemented medium. Protoplasts from two auxotrophic mutants of the moss Sphaerocarpos donnelli, one with a requirement for nicotinic acid, the other a pale green mutant with a requirement for sugar were fused. When transferred to a medium lacking both nicotinic acid and sugar both parental lines were eliminated leaving the autotrophic somatic hybrids (Schieder, 1974). In a series of experiments autotrophic somatic hybrids were selected on unsupplemented medium following protoplast fusion between several different auxotrophs of Physcomitrella patens (Grimsley et al., 1977). Similarly, Glimelius et al. (1978a) demonstrated that auxotrophic mutants could be used for complementation selection of higher plant somatic hybrids. Protoplasts were fused from two different nitrate reductase-deficient (NR^-) mutants of Nicotiana tabacum and hybrids were selected by their ability to grow in media containing nitrate as the sole nitrogen source. Following the isolation of nine NR^- mutants of Nicotiana plumbaginifolia, somatic hybridisation was used to help determine which lines were allelic and which were non-allelic (and therefore complementary). In hybrids between complementary lines, nitrate reductase activity was restored and plants were regenerated (Marton et al., 1982).

Auxotrophs have also been used in combination with other selection protocols. Protoplasts of a NR^- mutant and an albino mutant of Nicotiana tabacum were fused; when grown on a nitrate containing medium, green hybrid callus could be distinguished from the pale coloured, non-hybrid callus of the albino mutant (Glimelius and

Bonnett, 1981). Glimelius et al., (1981) fused protoplasts of a Nicotiana tabacum NR^- mutant with a cytoplasmically male-sterile (CMS) cultivar. Protoplasts of the non-hybrid CMS line did not survive the first few days in culture, thus small colonies obtained after 12-15 days were transferred to a nitrate containing medium on which only hybrid callus continued to grow. Recently auxotrophs requiring either isoleucine, uracil or leucine were isolated along with a number of albino mutants by mutagenising haploid protoplasts of Nicotiana plumbaginifolia (Sidorov and Maliga, 1982). Protoplasts from the different auxotrophs fused with the albinos proved the mutations to be recessive and showed that complementation between the auxotrophs and albinos could be used to isolate somatic hybrids.

c) Antibiotic resistant lines

Cell lines resistant to antibiotics have been selected from callus cultures of Nicotiana tabacum, Nicotiana sylvestris and Petunia hybrida (reviewed by Maliga, 1980), and of Nicotiana plumbaginifolia (Maliga et al., 1982). Antibiotics such as streptomycin, kanamycin and chloramphenicol are inhibitors of protein synthesis on prokaryotic type ribosomes, known to be present in chloroplasts and mitochondria. The majority of cell lines resistant to these drugs exhibit maternal inheritance (Maliga, 1980).

Protoplasts of a kanamycin resistant Nicotiana sylvestris line were fused with Nicotiana knightiana (Maliga et al., 1977). After culture for four weeks, protoplast-derived colonies were transferred to a selective medium on which the majority of N. knightiana colonies did not grow, the kanamycin resistant N. sylvestris colonies grew but remained unpigmented and the somatic

hybrids grew as green calluses. A further selection step was conducted by transferring the green calluses to a kanamycin-containing medium, where resistant somatic hybrids underwent morphogenesis. A similar selection procedure was also suitable for checking the hybrid nature of N. sylvestris + N. knightiana heterokaryons which had previously been isolated using microdrop procedures (Menczel et al., 1978).

Biochemical analyses of chloroplast DNA and proteins have shown that chloroplasts in fused protoplasts quickly segregate resulting in cells with only one type of parental chloroplast (Chen et al., 1977; Belliard et al., 1978; Melchers et al., 1978). Menczel et al. (1981) demonstrated that a maternally inherited resistance marker may be used to follow the fate of chloroplast populations. They fused protoplasts of streptomycin resistant Nicotiana tabacum (SR1) with streptomycin sensitive Nicotiana knightiana, isolated the heterokaryons after 72h and cultured them individually in microdrops, then tested the resulting calluses for streptomycin resistance. Resistant plants regenerated from somatic hybrid calluses were shown to be intermediate between N. tabacum and N. knightiana in morphology, but contained chloroplasts from N. tabacum SR1 only. Other somatic hybrid clones which proved to be streptomycin sensitive possessed chloroplasts from N. knightiana only.

At present all the reports using antibiotic resistance in fusion studies have used maternally inherited markers, but antibiotic resistance is not confined solely to chloroplast or mitochondrial genes, even with antibiotics that are known to act on these organelles. A streptomycin resistant diploid line was selected from callus of streptomycin sensitive, haploid Nicotiana sylvestris and

the resistance was shown to be a recessive Mendelian trait (Maliga, 1981).

d) Amino acid analogue resistant lines

Certain amino acids and amino acid analogues incorporated alone in the culture medium interfere with one or more of the cells metabolic processes resulting in growth inhibition. This growth inhibition may be relieved by the addition of other amino acids or the normal metabolite. In several species, resistant lines able to grow at normally inhibitory levels of amino acid analogues have been isolated (see reviews by Widholm, 1977; Maliga, 1980; Maliga et al., 1982). Although much information has been obtained from the study of these lines their usefulness has been limited as only a few plants have been regenerated. Even when plant regeneration has been successful, other problems have been encountered; the expression of resistance in culture is not always associated with expression in the plants and furthermore regenerated plants are frequently abnormal cytologically and morphologically, with reduced fertility (Miflin et al., 1983). Several fertile mutants showing changes in amino acid metabolism have been selected using resistance to amino acids or analogues from mutagenised mature embryos of barley (Bright et al., 1980) and seeds of Arabidopsis thaliana (Jacobs et al., 1982). These mutants have been characterised genetically and in some cases biochemically (reviewed by Miflin et al., 1983).

Two resistant lines were selected from cell suspensions of Nicotiana sylvestris; one resistant to S-2-aminoethyl-cysteine (Aec^R), the other to 5-methyl tryptophan ($5MT^R$). Protoplasts from the two lines were fused, then cultured for 30 - 60 days before the

protoplast-derived calluses were transferred to a selective medium containing both 5MT and Aec. Somatic hybrid calluses were resistant to both analogues, whereas nonhybrid calluses did not grow as they were resistant to only one of the two analogues. Resistant suspension cultures could be maintained, although plant regeneration was not obtained (White and Vasil, 1979). Resistances to 5MT and Aec in somatic hybrid lines of Daucus carota were used to demonstrate the dominant expression of resistance to these analogues, without regeneration of plants, (Harms et al., 1981). 5MT resistance was found to be semi-dominant and azetidine-2-carboxylic acid (Azc) resistance to be dominant in somatic hybrid plants of Daucus carota + Daucus capillifolius (Kameya et al., 1981).

e) Irradiation/Iodoacetate treatment

X-irradiated protoplasts from a cytoplasmically male-sterile (CMS) cultivar of Nicotiana tabacum were fused with protoplasts of Nicotiana sylvestris. Fusion products were selected on the basis that cell division was suppressed in protoplasts of N. tabacum due to the irradiation treatment whilst mannitol included in the medium was unfavourable to N. sylvestris protoplasts. Regenerated plants were characterised and somatic hybrids were shown to have the general morphology of N. sylvestris but the anthers resembled those of the male sterile N. tabacum (Zelcer et al., 1978).

Iodoacetate was used to inactivate protoplasts of the Nicotiana tabacum SR1 line which were then fused with untreated Nicotiana sylvestris protoplasts. After one month's culture, protoplast-derived calluses were placed on selective medium containing streptomycin, and the resistant clones were isolated. Regenerated

plants were examined and some were shown to have the N. sylvestris nucleus with SR1 chloroplasts (Medgyesy et al., 1980). X-ray irradiation and iodoacetate treatments may be combined. Protoplasts of X-irradiated Nicotiana tabacum were fused with iodoacetate-treated Nicotiana plumbaginifolia protoplasts (Sidorov et al., 1981). Inactivation prevented callus formation by both parental cell types, while interspecific fusion products formed calluses as a result of metabolic complementation. Nehls (1978) fused protoplasts of Solanum nigrum and Petunia hybrida which had been treated with the inhibitors diethylcarbamate and iodoacetate, respectively and subsequently selected three multicellular regenerants, one of which was a heterokaryon. Protoplasts of a cycloheximide resistant Daucus carota line were treated with iodoacetamide then fused with untreated protoplasts from a cycloheximide-sensitive albino carrot line. Somatic hybrid calluses were selected by the appearance of green calluses, and plants regenerated from these calluses exhibited hybrid morphology. Using the somatic hybrid callus cycloheximide resistance was found to be recessive (Lazar et al., 1981).

Following X-irradiation, protoplasts of Physalis minima and Datura innoxia were fused with protoplasts of an auxotrophic Nicotiana tabacum mutant lacking an active molybdenum cofactor for nitrate reductase. When cultured, inactivated unfused protoplasts of P. minima and D. innoxia did not divide. After two weeks culture calluses were transferred to a medium with nitrate as the sole nitrogen source, on which non-hybrid N. tabacum calluses did not survive. Calluses able to grow on nitrate were shown to have restored nitrate reductase activity. The high frequency of calluses with restored nitrate reductase activity (when compared with the frequency

of reversion to the wild type in the NR^- *N. tabacum* cell line) was used to suggest that the restoration of nitrate reductase activity was due to genetic transfer from the X-irradiated cells. Isoenzyme banding patterns of malate dehydrogenases, esterases and acid phosphatases remained the same as the recipient tobacco line indicating that there was only limited gene transfer (Gupta *et al.*, 1982).

1.5.4. Somatic hybrids of potato

Three different species, all within the *Solanaceae* have been hybridised with *S. tuberosum* using protoplast fusion techniques. Somatic hybrids of diploid *Lycopersicon esculentum* (2n = 2x = 24) with dihaploid and tetraploid *S. tuberosum* lines have been obtained (Melchers *et al.*, 1978; Shepard *et al.*, 1983). In the first report, mesophyll protoplasts of tomato were fused with protoplasts from suspension cultures of dihaploid potato. Protoplasts were cultured without selection resulting in a mixed population of calluses which were then plated onto a medium on which potato and somatic hybrid calluses produced shoots, but tomato calluses only produced roots. Somatic hybrid regenerants were selected from nonhybrid *S. tuberosum* regenerants on the basis of differing morphology. Fourteen shoots from four different calluses were identified as somatic hybrids. Root tip chromosome counts revealed all the hybrids to have more than 50 chromosomes: a perfect somatic hybrid between the two lines would have 48 chromosomes (Melchers *et al.*, 1978).

The potato-tomato somatic hybrids were further characterised by an analysis of the large (chloroplast encoded) and small (nuclear encoded) subunits of the Ribulose bisphosphate carboxylase protein; a

useful marker to trace the origin of both the nucleus and the chloroplasts. Isoelectric focusing patterns showed all the hybrids to have small subunit components from both potato and tomato, but large subunit components from only one parent. The use of endonuclease restriction enzyme patterns later confirmed that the chloroplasts were derived from only one of the two parents (Schiller et al., 1982). Of 12 hybrids examined, eight contained exclusively potato plastid DNA, and four contained exclusively tomato plastid DNA.

More recently, somatic hybrids were obtained following fusion of protoplasts from a variegated 'protoclone' of Russet Burbank (2n = 4x = 48) with protoplasts from two different tomato cultivars (Shepard et al., 1983). Protoplast-derived calluses were cultured on a medium which inhibited growth of potato nonhybrid callus, then a medium was used on which tomato calluses did not regenerate shoots. Four somatic hybrid plants were obtained, one of which was shown to have the correct number of chromosomes for a perfect somatic hybrid between the two lines (72 chromosomes). However vegetative cuttings taken from this somatic hybrid gave different chromosome counts (from 62-72) indicating a degree of mitotic instability (Shepard et al., 1983).

Mesophyll protoplasts of S. chacoense fused with callus derived protoplasts of tetraploid S. tuberosum were cultured in a medium in which S. chacoense protoplasts did not divide. Protoplast-derived calluses were transferred to a medium on which S. tuberosum calluses did not undergo morphogenesis. Three plants were regenerated, one of which was assumed to be a somatic hybrid with 'about' 60 chromosomes (Butenko and Kuchko, 1980).

Protoplasts of an atrazine-resistant biotype of Solanum nigrum (2n = 72) were fused with protoplasts of a dihaploid potato line. Shoots were regenerated before any form of selection was attempted. Somatic hybrids were characterised using morphological traits, sensitivity to atrazine and chromosome number (Binding et al., 1982).

These reports confirm that protoplast fusion techniques can be applied to the potato, but at the same time they demonstrate the need for efficient selection strategies allowing the screening of large populations. Several selection systems have been elucidated using 'model' species (Nicotiana, Datura and Petunia), and now need to be applied to Solanum species. The use of the herbicide 'SAN 6706' to bleach shoot cultures (Uhrig, 1982) may be of value as an aid to the visual selection of fusion products but it is unsuitable as a genetic marker as it is not permanent. At present there has been only one report of a biochemical variant of potato; a cell suspension resistant to 5MT, which unfortunately was nonmorphogenic (Carlson and Widholm, 1978). To my knowledge there have been no other reports of biochemical mutants of S. tuberosum suitable for use as selectable genetic markers.

Biochemical mutants may be selected at different levels of organisation, including single cells or protoplasts, calluses and whole plants (Miflin et al., 1983). Protoplasts offer an attractive system as they represent large populations of single cells in a relatively small volume (up to 10^7 ml^{-1}). For any single

gene, spontaneous mutation frequencies in the order of 10^{-6} to 10^{-7} may be expected, whilst various mutagenic treatments may be used to increase the frequency. Dominant mutations, including resistance to some amino acid analogues may be expressed in dihaploid or tetraploid potato lines, but recessive mutations will be hidden due to the presence of one or more different alleles. Thus for the selection of recessive biochemical mutations it is necessary to use monohaploid potato lines. This is probably the main reason for the lack of recessive markers such as chlorophyll-deficient lines, antibiotic resistant lines, and auxotrophs in S. tuberosum.

Preliminary results showed that the main obstacles to the in vitro selection of biochemical mutants and somatic hybridisation of Solanum species are the lack of efficient and reproducible protoplast techniques. Although published protocols were suitable for the regeneration of plants from protoplasts the percentage number of protoplasts which survived to undergo sustained divisions and eventually give rise to shoots were just not high enough to allow the direct application of these procedures to the selection of mutants. The work presented in thesis has therefore concentrated on the development of an efficient protocol for the regeneration of plants from protoplasts of several genotypes of S. tuberosum and the wild diploid species S. brevidens. Plants regenerated from protoplasts showed a range of morphological and chromosomal variation. This variation was evaluated in an attempt to understand its causes and controls.

CHAPTER 2

MATERIALS AND METHODS

2.1 PLANT MATERIAL

The protoplast source material was chosen to cover a range of ploidy levels, including wild and cultivated species.

S. tuberosum

At the tetraploid (2n = 4x = 48) level most work was done with the cultivars Fortyfold and Ukama, although cvs. Maris Bard, Majestic and Desiree were used occassionally. Fortyfold is an old cultivar, bred in the 1850's by W. Paterson [McIntosh, 1927; Salaman, 1949] and no longer grown commercially. Ukama is a modern cultivar which resulted from a cross between the Dutch cvs. Marijke and Sirtema. Ukama was donated in the form of axenic shoot cultures by R. Lyne (Shell Research Ltd., Sittingbourne). Tubers of Fortyfold, Maris Bard, Majestic and Desiree were supplied by J.H.W. Holden (Scottish Crops Research Institute). The dihaploid (2n = 2x = 24) lines PDH 119 and PDH 440 were derived parthenogenetically from S. tuberosum cv. Pentland Crown (2n = 2x = 48) using S. phureja as a pollinator. Tubers of the dihaploid lines were donated by M.J. De Maine (Scottish Crops Research Institute). The two monohaploid (n = x = 12) lines Mn 797322 and Mn 798261 were given to the late E. Thomas by G. Wenzel (Institute fur Resistenzgenetik, Grunbach, Germany). Both monohaploid lines were obtained via anther culture from a dihaploid line H78.01 which in turn was the result of a parthenogenetic cross between the tetraploid line HH 439 and S. phureja (G. Wenzel pers. comm.).

S. brevidens

Solanum brevidens Phil is a self-compatible, diploid (2n = 2x = 24), non-tuberous species which has been placed in the section Petota of the genus Solanum due to its morphological similarities with the tuber-bearing potatoes (Hawkes, 1978b). S. brevidens CPC 2451 originated from Argentina. Seeds were sent to Britain in 1952 (Hawkes and Hjerting, 1969) to form part of the Commonwealth Potato Collection. Solanum brevidens CPC 2451 x sibs. seeds were supplied by D.R. Glendinning of the Scottish Crop Research Institute. I gratefully acknowledge the generosity of everyone who supplied the material.

2.2 CHEMICALS AND EQUIPMENT

2.2.1 Chemicals

All chemicals used were of analytical grade, or similar, and obtained through the usual channels. Some of the more important or hard to obtain items are listed below. The formulations of all the media used in this thesis are provided in Appendix I.

Murashige and Skoog salts and vitamins		Flow Labs. Irvine, Scotland.
HORMONES		
ABA (Abscisic acid)	A-7383	Sigma London Chemical Co., Poole, Dorset
BAP (6-Benzylaminopurine)	B-6750	Sigma
24D (2,4-Dichlorophenoxyacetic acid)	D-2128	Sigma

GA_3 (Gibberellic acid)	G-3250	Sigma
IAA (Indole-3-acetic acid)	I-1250	Sigma
Kinetin	K-2875	Sigma
NAA (α-Naphthalene acetic acid)	N-0375	Sigma
Zeatin	Z-0500	Sigma

OTHER CHEMICALS

Agar-agar	Fisons Ltd., Loughborough, Leics.
Bacto-agar	Difco Labs, Michigan, USA
FDA (Fluoroscein diacetate) F-5502	Sigma
Mannitol	Merck mannitol, BDH, Poole, Dorset
MES (2[N-Morpholino]ethane sulphonic acid) M-8250	Sigma
Percoll	Pharmacia, Uppsala, Sweden

2.2.2 Equipment

OSMOMETER	'Osmette S'	Precision Systems Inc. Shuco Scientific Ltd., London

MEMBRANE FILTER UNITS

Autoclavable; 250 ml	Sartorius, W. Germany
Disposable; 125 ml	Nalgene, Techmate Ltd., Luton, Beds.

GLASSWARE

Beatson jars: 250 ml	French Flint and Ormco Ltd., London.
Duran bottles (100 ml, 250 ml, 500 ml and 1000 ml screw capped)	Schott Glass Ltd., Stafford

PLASTIC CONTAINERS

Petri-dishes; 9 cm	Sterilin Ltd., Teddington, Middlesex
Petri-dishes; (9 cm, 2 compartments)	Sterilin Ltd.
Multiwell dishes (100 x 18 mm, 25 compartment square petri-dishes)	Sterilin Ltd.
Specimen containers; 60 ml	Sterilin Ltd.

2.2.3 Enzymes

The enzymes used in this study for the isolation of protoplasts were all commercial preparations.

Cellulase R10 and Meicelase P are fungal cellulases derived from Trichoderma viride. The two preparations exhibit a range of enzyme activities including cellulase C, (attacking native and crystalline cellulose), cellulase Cx (attacking amorphous cellulose), cellobiase,

xylanase, glucanase, pectinase, lipase, phospholipase and nucleases (Cocking, 1972). Macerozyme R10 is a pectinase preparation obtained from a Rhizopus species and exhibits endo-polygalacturonase, hemicellulase and several other cell separation activities. Pectolyase Y23 is obtained from culture filtrates of Aspergillus japonicus and includes endo-polygalacturonase and endo-pectin lyase activities (Nagata and Ishii, 1979). Cellulase R10 and Macerozyme R10 are less crude preparations (obtained from Yakult Biochemical Co., Nishinomiya, Japan) which have been desalted by elution through a Biogel column (Evans and Cocking, 1977). Meicelase P was supplied by Meiji Seika Kasha, Tokyo, Japan, and Pectolyase Y23 by Seishin Pharmaceuticals, Tokyo, Japan. Freeze dried commercial enzymes were dissolved with mannitol in a solution containing the major salts of medium A (Appendix I). The insoluble material was removed by filtration through two layers of filter paper (Whatman no.1) and the osmoticum adjusted to the required level with the aid of an osmometer, (Section 2.6.1). The pH of the enzyme solution was adjusted to 5.6-5.7, and then the solution was filter sterilised (Section 2.3).

2.3 ASEPTIC TECHNIQUE

The composition of tissue culture media also makes them suitable for the growth of unwanted microorganisms, especially saprophytic bacteria and fungi. Thus all nutrient media, culture vessels and instruments used in handling the plant tissues must be sterile and the plant material must be free of contaminating organisms (aseptic). Plastic petri-dishes and culture vessels were obtained in pre-sterilised packs direct from the suppliers. Glassware and instruments

were sterilised by autoclaving at 121°C (103 KPa) for 30 min. Open glass containers and equipment such as metal sieves and pasteur-pipettes were sealed in a double layer of aluminium foil prior to autoclaving. Graduated glass-pipettes were sterilised in metal cylinders in a dry oven at 160°C for 1.5 h. Where possible, the nutrient media were sterilised by autoclaving at 110°C (68 KPa) for 20 min. Enzyme solutions and liquid media containing thermolabile compounds (such as glutamine and gibberellic acid) were sterilised by filtration through a cellulose nitrate membrane filter (0.22 μm pore size) mounted in an autoclavable filter unit (250 ml 'Sartorius' filter) or in a disposable 'Nalgene' (125 ml capacity) filter unit. When thermolabile compounds were included in agar medium they were filter sterilised separately, and then added to the bulk of the autoclaved medium just before it set (at about 40°C). Plant material was surface sterilised by immersing in a 10% v/v aqueous solution of household bleach ('Domestos') for 10-15 min, then rinsed thoroughly in sterile distilled water. All manipulations involving aseptic plant material and sterile nutrient media were conducted in a laminar airflow cabinet.

2.4 GROWTH AND MAINTENANCE OF PROTOPLAST SOURCE MATERIAL

2.4.1 Growth chamber grown plants

Tubers of Fortyfold and the dihaploid lines, all obtained from VTSC derived plants, were stored at 4°C in the dark until required. The tubers were then removed from the cold, covered in sand in aluminium-foil trays, and moistened with deionised water. When emerged, sprouts with roots were detached from the tuber and grown on

in vermiculite contained in 20 cm plastic-plant pots.

Plants of Mn 798261 and Mn 797322 were established from shoot culture after first transferring nodal stem segments to rooting medium (see Section 2.4.4). Seeds of S. brevidens were germinated on moist filter paper. Subsequently plants from these three lines were treated in the same way as those from tubers. Every two weeks the plants were fed with 50 ml Potato Fertiliser Solution (Appendix I), and between fertiliser applications the vermiculite was kept moist with deionised water. Protoplast-source plants were maintained in controlled-environment chambers (growth chambers) at a temperature of 18°C; irradiance at 250 μEm^{-2} s^{-1}; 12 h daylength and a relative humidity of 80%.

2.4.2 Shoot cultures

a) Solanum brevidens

Seeds of S. brevidens CPC 2451 were surface sterilised (Section 2.3). The seeds were germinated in plastic petri-dishes on basal medium (BM) consisting of the inorganic salts and vitamins of Murashige and Skoog (1962), with 20 g l^{-1} sucrose and 6 g l^{-1} agar (Appendix I).

b) Solanum tuberosum

The two monohaploid lines were grown and maintained as shoot cultures by Prof. Wenzel and then by Dr. Thomas. In addition, I initiated fresh stocks from the growth chamber grown plants via meristem-tip culture (Section 2.4.3.). Shoot cultures of Fortyfold were also established using meristem-tips isolated from plants grown in the growth chamber. Dihaploid potato shoot cultures were established from surface sterilised nodal stem segments taken from the

growth chamber grown plants.

Shoot cultures were grown in 'Beatson' jars (9 cm diameter, 6 cm high metal-capped glass jars) containing approximately 50 ml BM supplemented with BAP (0-0.25 mg l^{-1}) at pH 5.8. Every four to six weeks the shoot cultures were propagated by transferring several stem segments, each with an axillary bud to fresh medium. Shoot cultures were maintained in growth rooms under an irradiance of 70 μEm^{-2} s^{-1} (16 h day, 25°C).

2.4.3 Meristem-tip culture

The protocol used was adapted from that of Westcott et al., (1977). Stems from plants grown in the growth chamber were cut into 2 cm long nodal segments and surface sterilised (Section 2.3). Under a dissecting microscope the outer leaf primordia were cut away, using the tip of a hypodermic needle, to leave the youngest two or three leaf primordia and the meristem (together comprising the 'meristem-tip') intact. The meristem-tip was then excised from the stem segment and placed on the upper surface of a filter paper bridge with the ends immersed in 1 ml liquid medium in test-tubes (12 x 75 mm). The medium consisted of liquid BM supplemented with a range of BAP (0-0.5 mg l^{-1}) and GA_3 (0-0.2 mg l^{-1}) concentrations. The test-tubes were sealed with cotton wool plugs and aluminium caps, and the cultures maintained under an irradiance of 70 μEm^{-2} s^{-1} (16 h day, 25°C).

2.4.4 Establishment of plants from shoot cultures

Shoot pieces, each with an axillary bud were transferred to 60 ml plastic specimen bottles containing 20 ml BM supplemented with 0.05 mg l^{-1} NAA until they rooted. Plants were transferred to autoclaved vermiculite in plastic plant pots and maintained in propagators under light at 250 $\mu E\ m^{-2}\ s^{-1}$ (16 h day, 20°C/16°C night) until established. The plants were then transferred to potting compost and grown in the same environment as protoplast source plants (Section 2.4.1).

2.5 THE ISOLATION AND CULTURE OF PROTOPLASTS

A protocol for the isolation and culture of protoplasts released from either leaves of growth chamber grown plants or axenic shoot cultures was developed. Specific requirements for different genotypes of Solanum varied and are discussed in Chapters 3-5; the general features of the protocol are given below.

2.5.1 Protoplast isolation

The protoplast isolation procedure was modified from Thomas (1981). Leaves from growth chamber grown plants were surface sterilised (Section 2.3), separated into individual leaflets and placed in 50 ml medium A (Appendix I). Actively growing shoots from axenic cultures were cut into 2 cm pieces, then approximately 1 g material, weighed accurately, was placed in 50 ml medium A.

The protoplast source material was conditioned overnight at 4°C in medium A, then transferred to 50 ml 0.45 M mannitol solution (Appendix I) at 20°C to plasmolyse the cells. After 30 min. the

mannitol solution was replaced with 25-50 ml enzyme solution at 500-600 mOsm. In later isolations, enzyme solution EIV, (Appendix I) containing 1.5% Meicelase, 0.1% Pectolyase, the major salts of medium A and mannitol to give the required molality was used routinely. The material was incubated at 28°C for 3-6 h in a shaking water bath (40 strokes per min.) in the dark. The macerate was then filtered through 100 μm and 50 μm mesh stainless-steel sieves, dispensed in 10 ml aliquots in test-tubes and centrifuged at 100 xg for 5 min. The supernatant was discarded and each pellet resuspended in 1.5 ml mannitol at the same molality as used for the enzyme solution. To remove cell debris 1.5 ml protoplast suspension was layered over 2.5 ml 30% Percoll at the appropriate molality and centrifuged at 100 xg for 5 min. The protoplast band was removed from the Percoll/mannitol interface using a Pasteur-pipette and washed twice by centrifugation at 100 xg for 5 min. in mannitol before suspending in a small volume (~5 ml per test-tube) of culture medium. The yield of isolated protoplasts was determined with the aid of haemocytometer, then the protoplasts were diluted with culture medium to a known population density (1-4 x 10^4 ml^{-1}).

2.5.2 Protoplast culture

The protocol for protoplast culture and regeneration of shoots was developed from that of Shepard (Shepard and Totten, 1977; Shepard 1980b). Freshly isolated protoplasts, suspended in liquid culture medium (at 500-570 mOsm) were plated in 1.5 ml aliquots in 'Multiwell' dishes. The protoplasts were incubated for 2 days in the dark at 25°C, then transferred to a cabinet with irradiance at 20 μE m^{-2} s^{-1} (24 h day, 25°C). After 4-6 days the protoplast plating density and

osmotic pressure of the culture medium was lowered. This was done in one step by diluting with fresh culture medium (at the same molality as the protoplast suspension) and transferring 8 ml to one half of a divided 9 cm plastic petri-dish which contained in the other half, 15 ml of medium at 300 mOsm, solidified with 0.4% agar ('Bacto-agar'). Two slots (ca. 5 mm) in the dividing wall allowed exchange between the two media. Two weeks later 2 ml of protoplast-derived calluses in liquid medium were layered over 20 ml of solid medium C (Appendix I) in a 9 cm petri dish. After 6 days calluses were counted then picked off and plated on fresh medium C. Calluses were incubated on medium C under irradiance at 50 $\mu E\ m^{-2}\ s^{-1}$ (24 h day 20°C) for a total of 26-28 days, after which they were transferred to regeneration media.

2.6 OTHER TECHNIQUES

2.6.1 Determination of osmolality

A molal solution consists of one mole of solute dissolved in a kilogram of solvent, resulting in more than a litre of solution and is therefore slightly more dilute than a molar solution.

The osmotic pressure, vapour pressure, freezing point and boiling point of a solution are termed 'colligative' properties as they are all directly related to the concentration of the solution. As the concentration of the solution increases, the freezing point is lowered, and a measurement of the amount by which it is lowered is a simple measurement which can be used to determine the concentration of the solution or any of the other colligative properties. This is the principle on which a freezing point osmometer operates.

For every additional particle dissolved in a solvent the freezing

point will be lowered by a uniform amount. One mole of particles dissolved in a kilogram of water will lower the freezing point by 1.858°C. The measurement obtained using a freezing point osmometer is expressed in milliosmoles (mOsm) where one mOsm per kilogram change in concentration will cause a change of 1.858×10^{-3}°C in the freezing point.

In this thesis, mannitol was added to the protoplast culture media to give precise osmotic pressures, measured with an 'Osmette S' freezing point osmometer. Figure 2 shows the relationship between mannitol concentration and molality in different solutions.

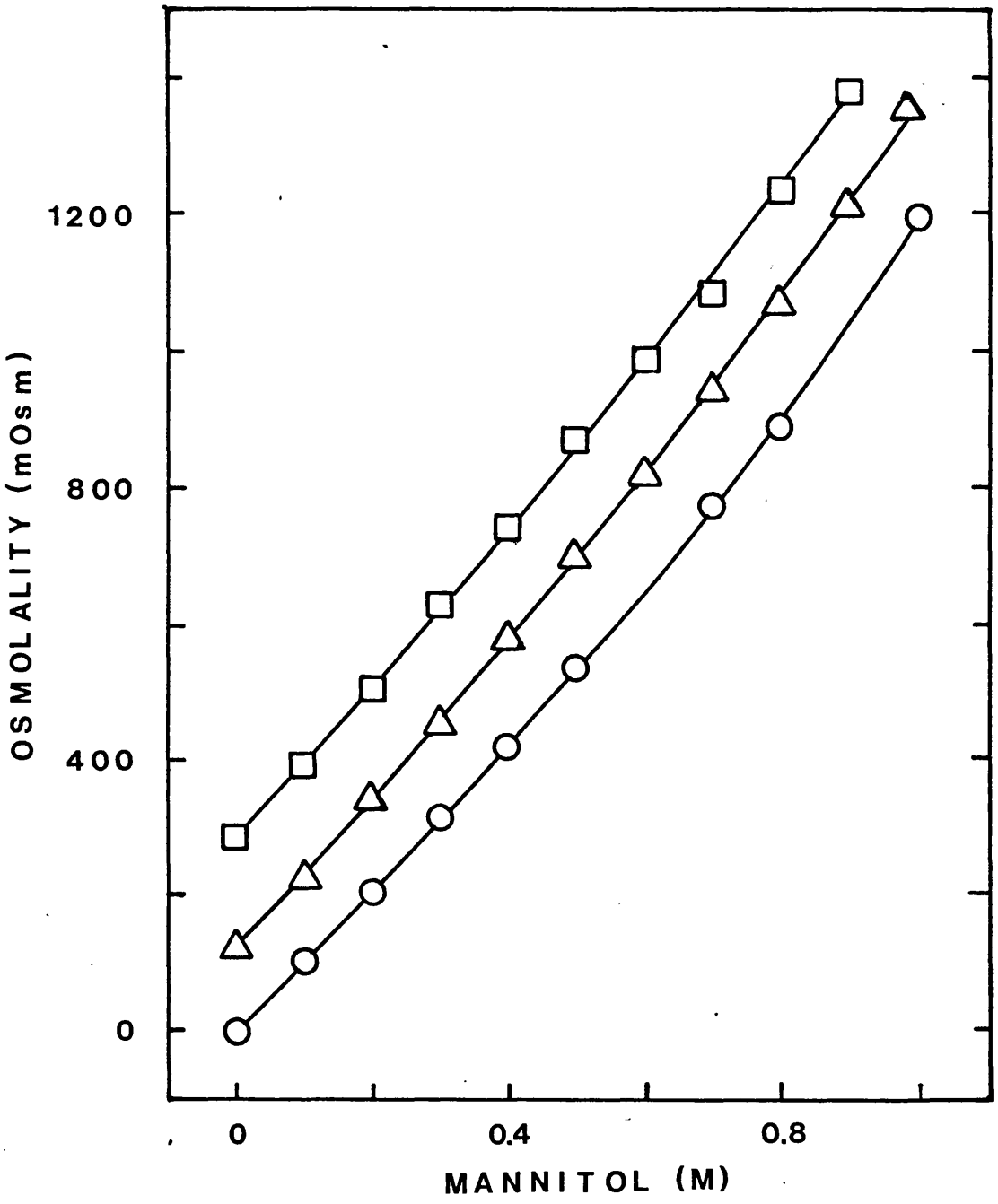


Fig. 2. The relationship between mannitol concentration (M) and osmolality in water (○); LO medium (△); and Rm medium (□).

2.6.2 Assessment of protoplast viability

Protoplast viability was assessed using fluoroscein diacetate (FDA) stain and UV light. FDA is absorbed by living cells and is then hydrolysed to fluoroscein by the action of esterases. Viable protoplasts thus accumulated fluoroscein, and when examined under blue light, emitted green fluorescence. A stock solution of FDA was made by dissolving the FDA in acetone to give a concentration of 5 mg ml^{-1}. When needed the FDA solution was added to the protoplast culture medium to give a concentration of 1 mg ml^{-1}. Protoplasts were incubated in medium with FDA for 5 min. and then were examined under a microscope fitted with a mercury vapour lamp and suitable excitation and suppression filters, (Section 2.6.6).

2.6.3 Measurement of plating efficiency.

Protoplast plating efficiency is defined as the percentage of the originally plated protoplasts which underwent sustained divisions to form calluses. The numbers of freshly isolated protoplasts were determined using a haemocytometer. Protoplast-derived calluses were counted 26 d after isolation (6 d after layering over solid medium C: Section 2.5.2).

2.6.4 Chromosome preparations

For cytological analysis, healthy root tips were collected from either freshly rooted plants in vitro (Section 2.4.4) or from plants established in potting compost. The roots were pretreated with 2 mM 8-hydroxyquinoline for 3-4 h at 18°C and then fixed in 3:1 absolute alcohol:glacial acetic acid for at least 24 h. The roots were then hydrolysed in 1 N HCl for 10 min. at 60°C and stained by the Feulgen

procedure (Feulgen and Rossenbeck, 1924). Squash preparations were made in aceto-carmine. Counts were obtained from a minimum of five well spread cells and from at least two separate roots.

2.6.5 Preparation of samples for free amino acid analysis

Approximately 400 mg leaf material was ground with a pestle and mortar in 2 ml MCW (12:5:3 methanol:chloroform:water) plus 10 mM mercaptoethanol. The homogenate was centrifuged at 2000 x g for 5 min. after which the supernatant was removed and retained. The pellet was twice reextracted in 2 ml MCW and all three supernatants were combined to give a total of 6 ml MCW. To the MCW extract was added 3 ml chloroform and then 4.5 ml water. This was mixed using a vortex mixer then centrifuged at 100 x g for 5 min. Two phases formed; the upper aqueous layer was retained and the lower chloroform layer discarded. To hydrolyse the asparagine and glutamine to aspartate and glutamate respectively, 3 ml 2 N HCl was added to the aqueous layer which was then incubated at 100°C for 1 h. The soluble amino acids were then vacuum dried at 45°C. This was done using an 'Evapomix', in which the extract was constantly vibrated in a 50 ml glass tube and the solvent removed under a reduced pressure. The dried samples were finally dissolved in 1 ml 0.01 N HCl for amino acid analysis. Norleucine (0.2 μmoles) was added at the start of the MCW extraction as an internal standard. Mercaptoethanol (10 mM) was added to the MCW and all the other solutions during extraction to reduce the oxidation of methionine to methionine sulphoxide. Samples were run by Mrs. Susan Smith using a LKB 4400 amino acid analyser.

2.6.6 Microscopy/Photography

Protoplasts, cells and developing colonies were examined under a Leitz Diavert inverted microscope. Larger calluses and tissues were viewed under a Wild M8 dissecting microcope. Cytological preparations were examined under a Zeiss Universal photomicroscope fitted with a x63 planapochromat objective. An Olympus BH2 transmission microscope with a mercury vapour lamp, an Olympus B(IF-490) excitation filter, a B(DM-500, O-515) dichroic mirror and a O-530 barrier filter was used for fluorescence microscopy. All these microscopes were fitted with a 35 mm camera attachment. A range of film types were used, but Kodak Ektachrome (ASA 160) was found most suitable for colour transparencies and Ilford Pan F (ASA 50) for monochrome photographs. Petri dishes and plants were photographed by Mr. Gordon Higgins (Photography Dept., Rothamsted).

CHAPTER 3

PROTOPLASTS TO PLANTS

I. SOLANUM TUBEROSUM

3.1 INTRODUCTION

Previous protocols for the regeneration of plants from isolated protoplasts of Solanum tuberosum differed in several respects, including the conditions in which the source material was grown, the isolation schedule, and the media used. Plants grown in two different environmental regimes (a glasshouse and a growth chamber) were compared with shoot cultures established from meristem-tips to find the most suitable protoplast source material. Procedures for the isolation of protoplasts were developed from the protocols of Thomas (1981) and Shepard (1982). The media formulations and sequence were modified from the protocol of Shepard (1982).

3.2 MERISTEM-TIP CULTURE

In two different experiments, isolated meristem-tips, 0.3-0.7 mm long, were cultured on basal medium (BM; Appendix I) with a range of BAP and GA_3 concentrations (Table 2). The addition of 0.25 mg l^{-1} BAP to the culture medium promoted shoot development but the resulting shoots were stunted with very little internodal expansion. Higher levels of BAP promoted callus development at the base of the meristem-tips. Shoot development was enhanced at all levels of GA_3 tested, the optimum being 0.1 mg l^{-1} GA_3 used together with 0.25 mg l^{-1} BAP (Table 2). In Experiment 3 meristem-tips of two monohaploid lines (Mn 797322 and Mn 798261) and of the tetraploid cv. Fortyfold were cultured in BM (Appendix I) supplemented with 0.25 mg l^{-1} BAP and 0.1 mg l^{-1} GA_3 (designated MT8), and the resulting

shoots were used to initiate shoot cultures of the three genotypes (Fig. 3a and b).

Table 2. Meristem-tip culture of S. tuberosum

Genotype	BM+ BAP ($mg\ l^{-1}$)	BM+ GA_3 ($mg\ l^{-1}$)	No. of meristems isolated	
Expt.1				
8261	0	0	4	Meristem swollen, no shoot growth apparent
	0.10	0	4	Meristem swollen, no shoot growth apparent
	0.25	0	4	Shoot developed (<5 mm); some callus at base
	0.50	0	4	Shoot developed, with much callus at base
7322	0	0	4	Meristem swollen, no shoot growth apparent
	0.10	0	4	Meristem swollen, no shoot growth apparent
	0.25	0	4	Shoot and roots developed
	0.50	0	4	Shoot developed, with much callus at base
Expt.2				
8261	0	0.05	4	Shoot developed (<10 mm); some roots
	0.25	0.05	4	Shoot developed with some roots
	0	0.10	4	Elongated shoot (>20 mm)
	0.25	0.10	4	Shoot developed (ca. 20 mm); very good structure
	0	0.15	4	Very elongated shoot (>50 mm)
	0.25	0.15	4	Good shoot development (ca. 20 mm)
	0	0.20	4	Very elongated (>50 mm) stolon like shoot
	0.25	0.20	4	Good shoot structure (ca. 20 mm)
Expt.3				
8261	0.25	0.10	12	8/12 developed healthy shoots
7322	0.25	0.10	12	11/12 developed healthy shoots
Fortyfold	0.25	0.10	12	7/12 developed healthy shoots

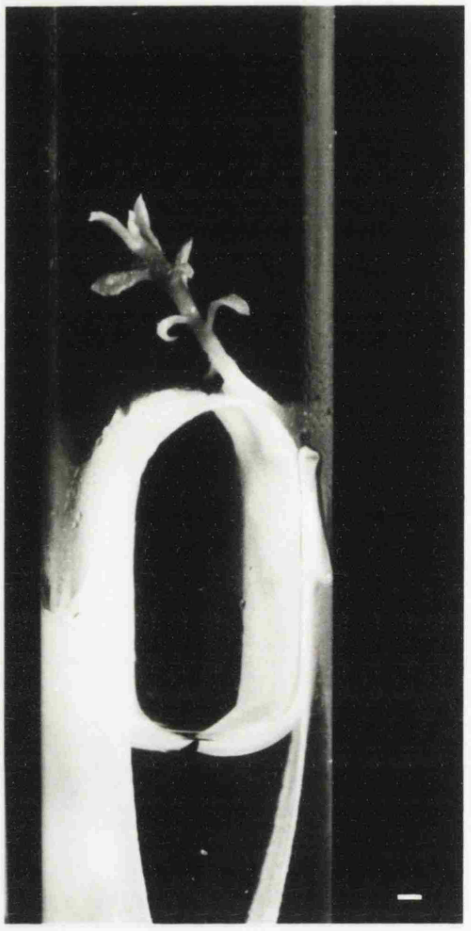


Fig. 3a

4 week old Meristem-tip culture of Fortyfold grown on BM with 0.25 mgl^{-1} BAP and 0.1 mgl^{-1} GA_3 (MT8). Scale bar = 1 mm

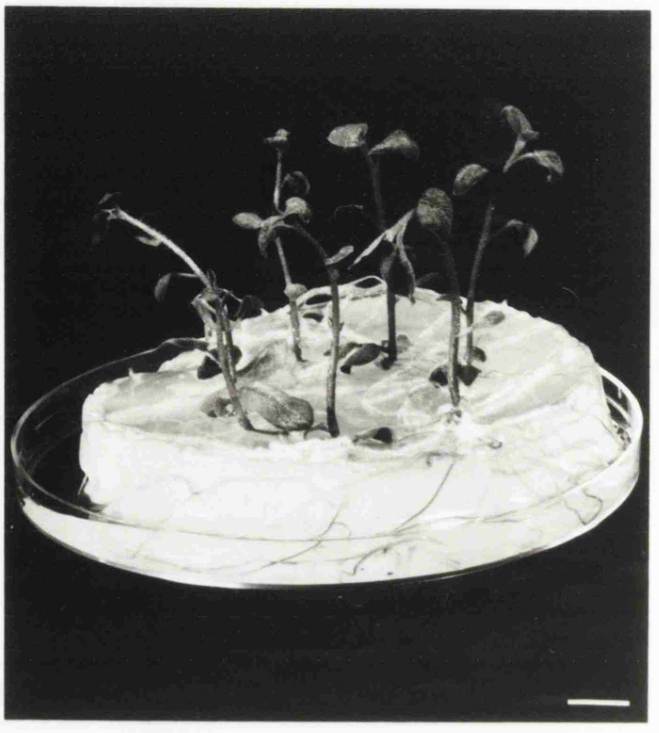


Fig. 3b

Meristem-tip derived shoot cultures of Fortyfold maintained on BM plus 0.05 mgl^{-1} BAP.
Scale bar = 1 cm

3.3 PROTOPLAST ISOLATION AND CULTURE

3.3.1 Protoplasts from glasshouse grown plants

The first fully expanded leaf (usually leaf 6 or 7) taken from plants grown from tubers in a glasshouse (at 20°C without supplementary lighting) was used for protoplast isolations.

Surface sterilised leaves were cut into strips, then incubated in enzyme solution at 25°C for up to 12 h. A number of combinations of cellulase R10 (0.15-2%) with either pectolyase Y23 (0.025-0.1%) or macerozyme (0.025-0.1%) were tested. Protoplasts of Desiree, Majestic and Maris Bard were obtained after incubation in 2% cellulase plus 0.1% pectolyase with 0.49 M sorbitol for 3-4 h. The protoplasts were cleaned of debris (Section 2.5.1), suspended in CL medium (Appendix I) at a known density of 1-5 x 10^4 ml^{-1} and placed in the dark at 25°C. Freshly isolated protoplasts stained with FDA and examined under blue light (Section 2.6.2) were 35-50% viable, but none of the protoplasts survived for longer than 2-3 days in culture.

3.3.2 Protoplasts from growth chamber plants

Preliminary results showed that the procedure used to isolate protoplasts from shoot cultures (Thomas, 1981) could be applied to leaves from plants maintained in a growth chamber with a controlled environment (Section 2.4.1). The youngest fully expanded leaf (leaf 6 or 7) was taken for protoplast isolation. Using leaves from the tetraploid cv. Fortyfold, viable protoplasts were obtained following a 4 h incubation in enzyme solution EIV (Appendix I) over a range of osmotic pressures (480-590 m Osm). To enable a better comparison of results, the osmotic pressure of the enzyme, wash solutions and

different protoplast culture media was standardised at 560 ± 5 mOsm using mannitol (Section 2.6.1).

Initially (isolation dates 29.1.81 and 3.2.81; Table 3) leaves of Fortyfold were prepared as recommended by Shepard (1980a). Leaves were positioned top side down on a solution containing 1 mM $CaCl_2$, 1 mM NH_4NO_3, 2 mg l^{-1} NAA and 1 mg l^{-1} BAP (designated 'Float solution'), and kept in the dark at 20°C for 48 h. The leaves were then surface sterilised (Section 2.3) and placed overnight at 4°C in medium A (Appendix I). After 4 h incubation in enzyme solution EIV at 560 mOsm the protoplast preparations were cleaned (Section 2.5.1), suspended in Rm medium (Appendix I) and examined under the microscope. In the first isolation (29.1.81) half of the material was plasmolysed for 30 min in 0.45 M mannitol solution prior to enzyme incubation, the other half was not. The yield of protoplasts obtained from the plasmolysed treatment was nearly four times that from the non-plasmolysed sample, thus a 30 min plasmolysis step was added to the isolation schedule.

The different pretreatments used by Shepard (1980a) were assessed by preparing leaf samples in four different ways (isolation date 10.2.83; Table 3):

i) The leaf was placed on Float solution for 2 days at 20°C, then surface sterilised and placed overnight at 4°C in medium A (i.e. as for isolations of 29.1.81 and 3.2.81).

ii) The leaf was surface sterilised then incubated overnight at 4°C in medium A.

iii) The leaf was sterilised then incubated overnight at 20°C in medium A.

iv) The leaf was surface sterilised.

Protoplast preparations following treatments iii) and iv) were visibly poorer with many more damaged protoplasts than those from treatments i) and ii). Freshly isolated protoplasts from i) and ii) appeared healthy with prominent chloroplasts (Fig. 4b) and even after prolonged culture there was little difference between the two preparations (isolation date 10.2.83 i) and ii); Table 3). In subsequent isolations a cold treatment, as in ii), was employed routinely. Several attempts to increase further the yield of viable protoplasts, including slicing the leaf material or peeling the epidermis from the leaf to facilitate enzyme digestion, were unsuccessful.

During the period January to May 1981, 17 isolations using leaves of Fortyfold were made (Table 3). Good preparations of viable protoplasts were obtained in 15 of the 17 isolations. However preparations from the last two isolations in May, and several subsequent isolations were poor, with a high proportion of damaged cells. Attempts to isolate protoplasts from leaves of Mn 797322 and Mn 798261 met with little success; only damaged cells and cell debris were found in the enzyme solution, even after extended incubation periods.

Table 3. Results of protoplast isolations from growth chamber leaves of Fortyfold.

Isolation Date	Protoplast Yield (per leaf)	Culture Medium (see Appendix 1)	Divisions	Plating Efficiency (%)
29.1.81	4×10^4	Rm	++	0.8
3.2.81	4×10^5	Rm	++	0.8
10.2.81 i)	2×10^5	Rm	++	0.5
ii)	2×10^5	Rm	++	0.4
11.2.81	8×10^4	Rm	++	0.4
18.2.81	1×10^5	Rm	++	1.0
20.2.81	9×10^5	Rm	+	Contaminated
27.2.81	1×10^5	Rm	+	Contaminated
6.3.81	6×10^5	Rm	++	Contaminated
	4×10^5	ET 11	++	
18.3.81	6×10^5	Rm	-	-
	2×10^5	ET 11	-	-
20.3.81	1×10^5	R	++	1.0
	1×10^5	Rm	++	0.8
	1×10^5	ET 10	++	0.6
	1×10^5	ET 11	++	1.0
25.3.81	2×10^5	Rm	++	Contaminated
	2×10^5	ET 11	++	
3.4.81	1×10^5	ET 11	-	-
14.4.81	3×10^5	Rm	+	Contaminated
	3×10^5	ET 11	+	
13.5.81	1×10^5	Rm	++	2.4
	1×10^5	CL/R	++	0.4
15.5.81	1×10^5	Rm	++	3.0
	1×10^5	CL/R	++	0.5

(-) No divisions (+) Initial divisions (++) Sustained divisions

Plating Efficiency (P.E.) = The percentage of protoplasts which underwent sustained divisions to form visible calluses.

3.3.3 Protoplasts from shoot cultures

a) Fortyfold

Shoot cultures initiated from meristem-tips (Section 3.2) were propagated and maintained on BM (Appendix I) supplemented with 0.25 mg l^{-1} BAP, in metal-capped glass jars (Beatson jars). Shoot cultures grown in these conditions were spindly with very small leaves. Nevertheless, using the methods established for leaves of growth chamber plants (Section 2.5.1 and 3.3.2) yields of 2-5 x 10^5 viable protoplasts per gram of shoot material were obtained (Table 4).

b) Monohaploids

A number of isolations from monohaploid shoot cultures grown on BM plus 0.25 mg l^{-1} BAP in 'Beatson' jars were attempted (Table 4). Protoplast quality varied greatly between isolations. Some were clean with a high proportion (80-90%) of viable protoplasts (Fig. 4b) but others were very messy with many damaged cells among the healthy protoplasts. The quality of the protoplasts was reflected in their subsequent survival; divisions were only observed in the cleaner preparations (Section 3.3.4 and Table 4). Monohaploid protoplasts tolerated a narrower range of osmotic pressures (520-575 m Osm) than those of Fortyfold; below 500 mOsm the isolate consisted mainly of cells, above 580 mOsm the protoplasts released were badly crumpled.

Table 4. Results of protoplast isolations from shoot cultures of 3 different lines grown on BM plus 0.25 mg l^{-1} BAP

Line	Isolation Date	Age of Shoot Culture (days)	Protoplast Yield (g^{-1} FW)	Culture Medium	Divisions	% P.E.
Fortyfold	30.11.81	28	2×10^5	Rm	++	0.2
	30.3.82	13	5×10^5	Rm	++	0.3
Mn 798261	7.1.81	51	2×10^5	R	+	0
	13.1.81	57	4×10^5	R	+	0
	16.1.81	60	3×10^5	R	-	0
	21.1.81	65	8×10^4	R	-	0
	30.1.81	21	1×10^4	Rm	++	5 calluses
	4.2.81	26	4×10^5	ET 9	-	0
	12.2.81	84	2×10^5	Rm	-	0
	26.2.81	21	2×10^5	Rm	++	7 calluses
	4.3.81	22	9×10^4	R	-	0
	11.3.81	26	4×10^4	ET 11	+	0
	16.3.81	25	4×10^5	Rm	-	0
	6.8.81	41	4×10^5	ET 11	++	Contaminated
	13.8.81	48	7×10^4	ET 11	-	0
	24.9.81	58	4×10^5	Rm	-	0
	28.10.81	21	5×10^5	Rm	-	0
Mn 797322	7.1.81	51	1×10^5	R	-	0
	30.1.81	21	1×10^4	Rm	-	0
	4.3.81	22	1×10^5	R	-	0

(-) No divisions (+) Initial divisions (++) Sustained divisions

P.E. = Plating Efficiency

3.3.4 Protoplast culture

a) Fortyfold

Freshly isolated protoplasts of Fortyfold (Fig. 4b) from leaves or shoot cultures were suspended in Rm medium (1.0 mg l^{-1} NAA, 0.4 mg l^{-1} BAP; Appendix I) at 560 mOsm. The initial population density was found to be optimum at 4 x 10^4 ml^{-1}; at higher densities (>6 x 10^4 ml^{-1}) the protoplasts clumped together, and at densities lower than 1 x 10^4 ml^{-1} the protoplasts did not survive for more than a few hours. After 2 days in the dark at 4 x 10^4 ml^{-1}, 25-50% of the protoplasts had survived and lost their spherical shape; an indication that they had synthesised a new cell wall. A few protoplast-derived cells underwent a limited number of divisions (Fig. 4d) but for sustained divisions, after 6 days it was necessary to dilute to 1 x 10^4 ml^{-1} with fresh Rm at 560 mOsm and gradually lower the osmoticum (Section 2.5.2). At 20 days protoplast-derived colonies (Fig. 4e) were layered over agar-solidified C medium (0.1 mg l^{-1} NAA, 0.5 mg l^{-1} BAP; Appendix I). Six days later (Fig. 4f) the protoplast plating efficiency was determined (Section 2.6.3), then calluses were transferred to fresh medium C.

Plating efficiencies of approximately 1% were obtained using Rm medium (Table 3 and 4). Several other media, based on the formulations of Thomas (1981) or Shepard (1980a) also supported sustained divisions, but none showed any improvement on the plating efficiencies obtained with Rm medium (Table 3).

Figure 4.

S. tuberosum protoplast culture.

a. Freshly isolated protoplasts of *S. tuberosum* Mn798261. (Scale bar = 50 μm).

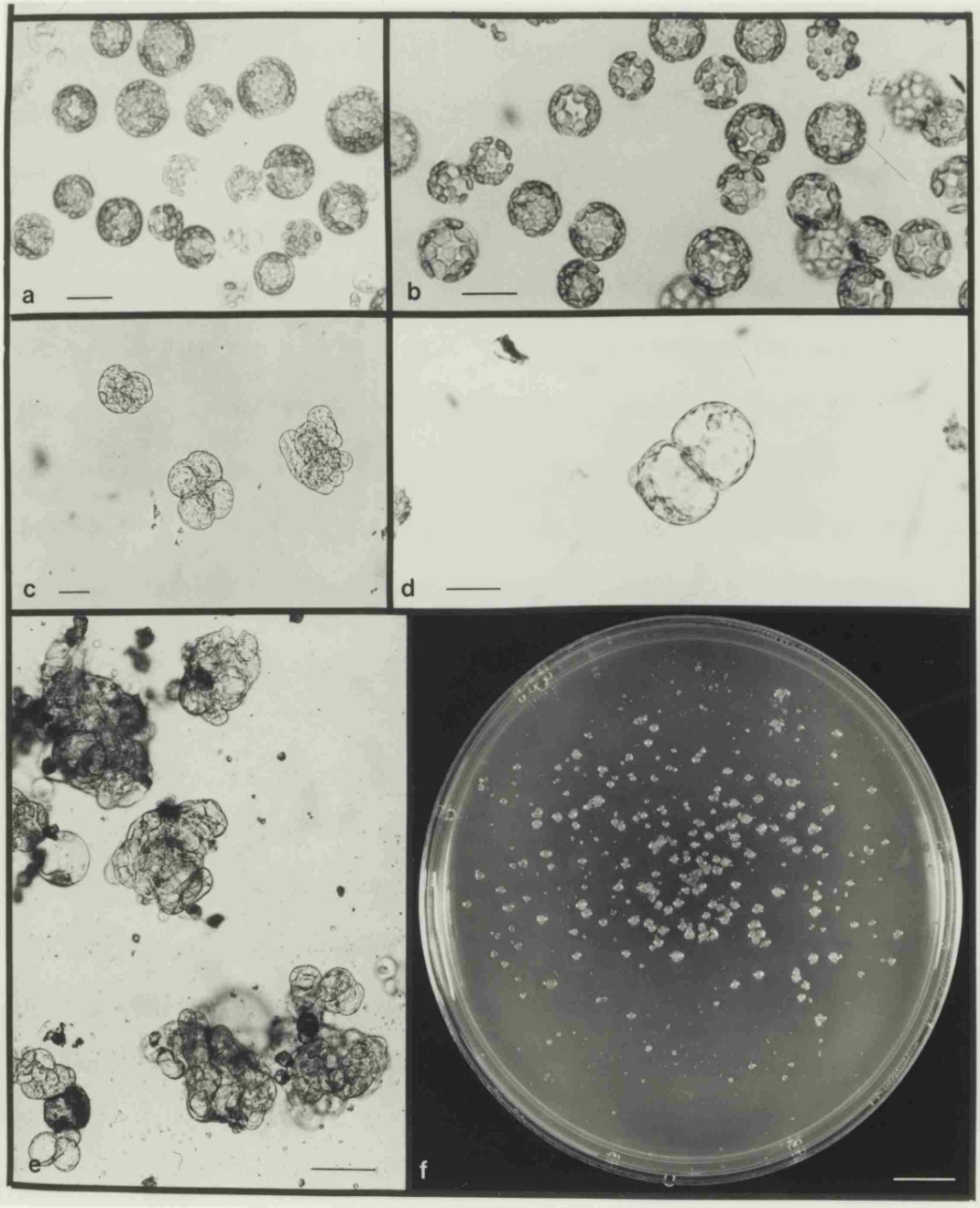
b. Freshly isolated protoplasts of *S. tuberosum* cv. Fortyfold. (Scale bar = 50 μm).

c. Protoplast-derived colonies of Mn 798261 after 10 d culture. (Scale bar = 50 μm).

d. Initial division in a protoplast-derived cell of Fortyfold. (Scale bar = 50 μm).

e. Multicellular colonies of Fortyfold after 20 d culture. (Scale bar = 50 μm).

f. Protoplast-derived calluses of Fortyfold 26 d from isolation. (Scale bar = 1 cm).


a
b
c
d
e
f

b) Monohaploids

Freshly isolated protoplasts of Mn 798261 (Fig. 4a) were plated in various media at a range of population densities (1-5 x 10^4 ml^{-1}). The majority of protoplasts did not survive the initial period of culture. When plated at 4 x 10^4 ml^{-1} a few protoplasts from cleaner preparations (Section 3.3.3) divided, but sustained divisions leading to callus formation were observed on only two occasions (Table 4 and Fig. 4c). On both occasions the plating efficiency was extremely low with only 12 calluses produced from 2.1 x 10^5 protoplasts (Table 4). The results obtained with protoplasts of Mn 797322 were also very poor; none of the protoplasts survived the first few days of culture.

3.4 PLANT REGENERATION

Protoplast-derived calluses of Fortyfold maintained on C medium for 28 d continued to grow and turned green (Fig. 5a). Following the report that ABA increased the efficiency of shoot morphogenesis in protoplast-derived calluses of Russet Burbank (Shepard, 1980b), calluses of Fortyfold were transferred to medium D (0.1 mg l^{-1} IAA, 0.5 mg l^{-1} zeatin) with different levels of ABA (0-0.2 mg l^{-1}). Shoot production commenced two to three weeks after transfer (Fig. 5b). Calluses were maintained on D medium for at least three months, and for one isolation (isolation date 3.2.81; Table 5) up to nine months, but few calluses produced shoots, and only a small improvement could be discerned due to the addition of 0.2 mg l^{-1} ABA (Table 5). When examined under a dissecting microscope more calluses were observed to have shoot primordia (Fig. 6a) which had not developed

into shoots. Calluses were therefore transferred to the medium found best for culture of meristem-tips (MT8 medium with 0.25 mg l^{-1} BAP, 0.1 mg l^{-1} GA_3; Section 3.2) solidified with 1% agar (Difco Bacto). After 4 weeks on MT8 the frequency of calluses with shoots increased to approximately 30%, although the figure varied with different isolations (Table 5 and Fig. 6b).

Shoots excised from calluses were used to initiate shoot cultures which were maintained on BM without hormones (Appendix I). The shoot cultures were then used to establish plants as described (Section 2.4.4).

Table 5. Regeneration of shoots from protoplast-derived calluses of Fortyfold

Isolation Date	Regeneration Media	No. of Calluses	% Calluses with Shoots After D Medium	% Calluses with Shoots After MT8 Medium
29.1.81	D	65	0	N.D.
	D + 0.1 mg l^{-1} ABA	190	7	N.D.
	D + 0.2 mg l^{-1} ABA	65	3	N.D.
3.2.81	D	286	14	43
	D + 0.2 mg l^{-1} ABA	286	24	44
10.2.81	D	98	10	19
	D + 0.1 mg l^{-1} ABA	135	12	34
	D + 0.2 mg l^{-1} ABA	25	8	64
11.2.81	D	40	4	40
	D + 0.1 mg l^{-1} ABA	31	10	25
	D + 0.2 mg l^{-1} ABA	12	12	39
18.2.81	D + 0.1 mg l^{-1} ABA	166	5	31
13.5.81	D	387	5	13
	D + 0.1 mg l^{-1} ABA	542	1	11
	D + 0.2 mg l^{-1} ABA	457	8	16
15.5.81	D	63	2	32
	D + 0.1 mg l^{-1} ABA	261	3	15
30.11.81	D + 0.1 mg l^{-1} ABA	138	5	24

The number of calluses with shoots were counted after at least 3 months on D medium, then the calluses were transferred to MT8 for one month, after which the numbers with shoots present were counted again. (N.D. = not determined.)

Figure 5

Shoot initiation on S. tuberosum protoplast-derived calluses

a. Green calluses after 20 d on C medium.
(Scale bar = 1 cm).

b. Shoot development after 12 weeks on D medium.
(Scale bar = 0.5 mm).

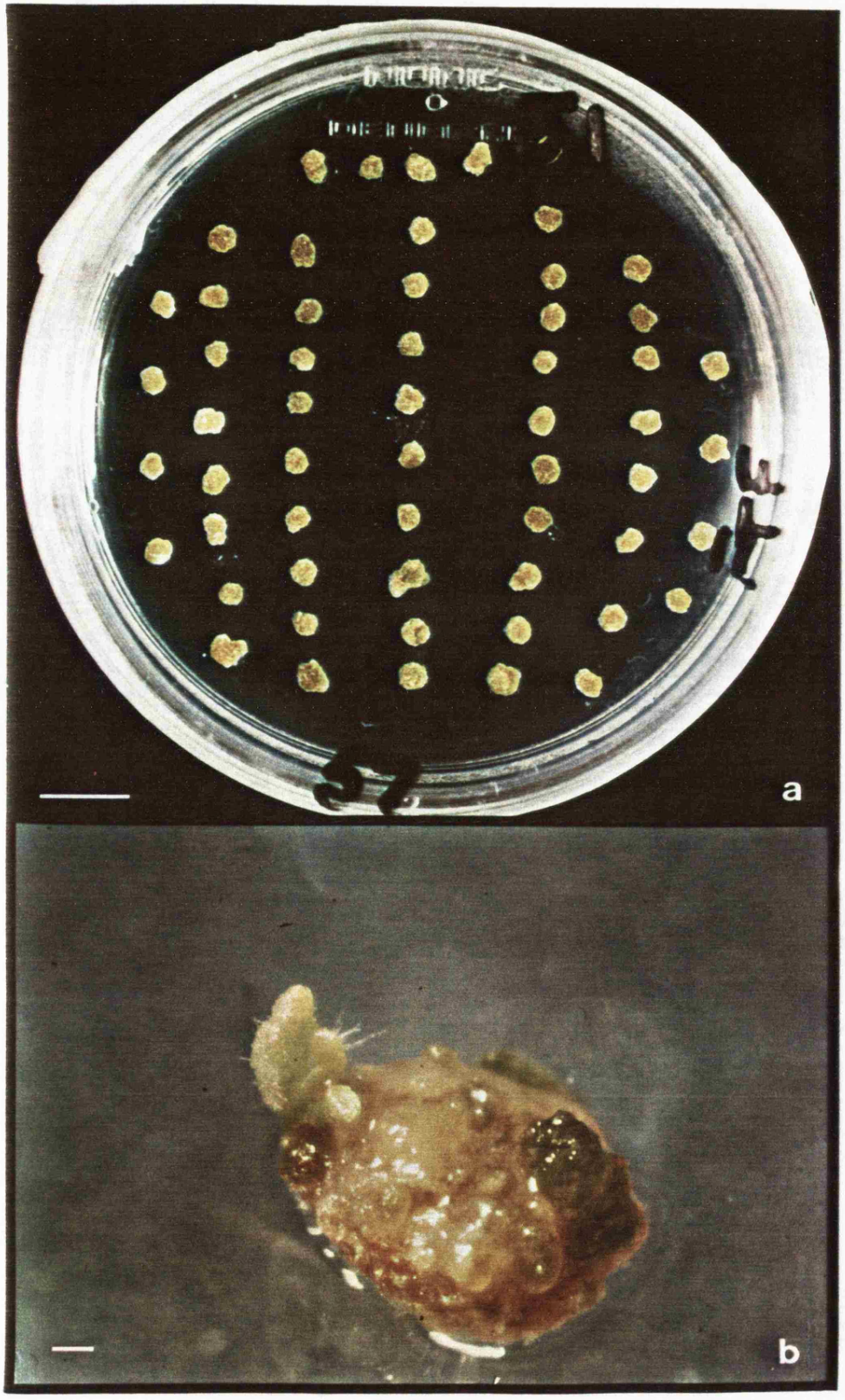
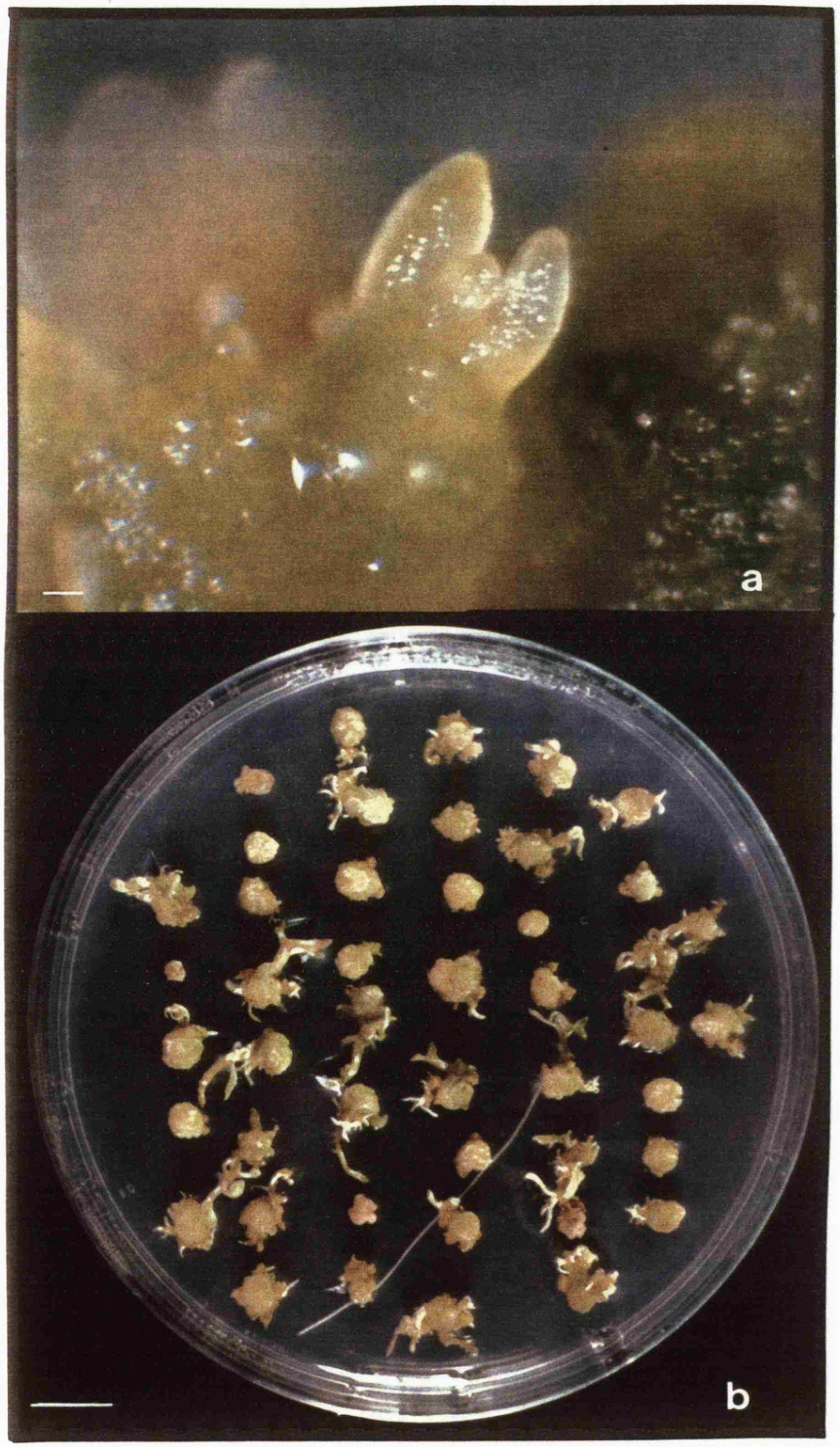

a
b

Figure 6

Shoot elongation on S. tuberosum protoplast-derived calluses

a. Shoot primordium after 6 weeks on D medium. (Scale bar = 0.1 mm).

b. Shoot elongation after 4 weeks on MT8 medium. (Scale bar = 1 cm).


a
b

3.5 DISCUSSION

It is well established that the conditions under which the donor material is maintained has a profound effect on protoplast quality (Keller et al., 1982; Shepard, 1980). In the present investigation potato plants grown in a glasshouse proved to be very unsatisfactory as a protoplast source material. Shepard and Totten (1977) used plants grown under strict environmental and nutritional regimes to obtain reproducible regeneration of plants from protoplasts of Russet Burbank. Plants of Fortyfold used for isolations all came from tubers lifted in the previous October. These tubers were stored at 2°C until needed, when they were treated as recommended by Shepard (1980a). Viable protoplasts, from which plants were eventually regenerated were obtained on 15 different occasions during the period January to May 1981. However, leaves of Fortyfold only gave viable protoplasts if they came from tubers less than 8 months old. After 7-8 months newly sprouted source plants of Fortyfold began to tuberise very quickly when maintained under Shepard's growth regime. The initiation of tuberisation in potatoes is most rapid under short days (less than 12 h), but most European cultivars will eventually tuberise under relatively long days (Hawkes, 1967). The onset of tuberisation in Fortyfold plants may be delayed by increasing the day length (to more than 16 h) under which they are grown, but tuberisation is also influenced by other factors including temperature, irradiance and the physiological age of the tubers from which the plants originated (Moorby, 1978). Even with tubers stored in the cold the physiological status changes gradually, until such tubers become too old to prevent rapid tuberisation (Moorby, 1978). To overcome this old stocks of

tubers would have to be replenished with newly lifted tubers every year.

A different approach to the problem of obtaining a uniform protoplast source is the use of shoot cultures (Binding et al., 1978; Thomas, 1981). Shoot cultures provide several advantages over intact plants as they are physiologically uniform, sterile and can be propagated and maintained with relative ease. Results with shoot culture protoplasts of Fortyfold showed that the protoplast plating and shoot production efficiencies were similar to those obtained with protoplasts from leaves of growth chamber plants.

Plating efficiencies of 0.4 to 3.0% obtained with Fortyfold are below those for Russet Burbank (where 'plating efficiencies' of 60% have been reported), but are comparable with results obtained with protoplasts from several other American (T. Barsby, pers. comm.), British (Gunn and Shepard, 1981; Thomas, 1981) and Dutch cultivars (Bokelmann and Roest, 1983). The majority of protoplasts did not survive the initial period of culture, although losses also occurred later.

At the dihaploid level, response to protoplast and anther culture was found to be dependant upon the genotype (Binding et al., 1978; Wenzel and Uhrig, 1981). This lead to the suggestion that 'tissue culture ability', or competence, may be genetically determined (Wenzel et al., 1982). With this in mind the poor response to protoplast culture obtained with the two monohaploid lines may be because neither has a suitable genotype. If this is so, a large number of monohaploids whould have to be screened to find lines amenable to protoplast culture. So far there have been no reports on the regeneration of plants from protoplasts of monohaploid potatoes.

The number of Fortyfold calluses which produced shoots on D medium, even after extended periods, was low, and was not greatly enhanced by the addition of ABA. This is in contrast to the findings of Shepard (1980b), who observed a three fold increase in shoot production with calluses of Russet Burbank on D medium plus 0.2 mg l^{-1} ABA, and with cv Atlantic on D medium plus 0.1 mg l^{-1} ABA, but confirms that different genotypes often respond differently to a given hormonal regime.

Fortyfold calluses transferred to medium with GA_3 (MT8 medium developed for meristem-tip culture) showed a significant increase in the number of calluses which produced shoots. Many Fortyfold calluses on D medium were observed to have shoot primordia on the surface, but others with no external evidence of shoot primordia also produced shoots on transfer to MT8 medium, indicating that shoot primordia also form within the callus. This is supported by histological studies on thin sections of potato leaf callus, which demonstrated the presence of meristematic regions within the callus (Osifo, 1983).

Plants regenerated from protoplasts of Fortyfold showed phenotypic variation, this is discussed in Chapter 6. For studies on mutant selection it is necessary to obtain, regularly, large numbers of viable protoplasts which will divide and form colonies. Until increased plating efficiencies are obtained, attempts to use potato protoplasts for the selection of biochemical mutants would be futile.

CHAPTER 4

PROTOPLASTS TO PLANTS

II. SOLANUM BREVIDENS

4.1 INTRODUCTION

A fundamental requirement for successful interspecific somatic hybridisation is the establishment of procedures for the isolation of protoplasts from both species involved. Furthermore a knowledge of the way protoplasts from the two genotypes respond in culture is important for the development of appropriate selection systems. Methods developed for S. tuberosum (Chapter 3) were applied to the isolation and culture of protoplasts from shoot cultures of S. brevidens. High yields of viable protoplasts were obtained from shoot cultures grown with a low concentration of cytokinin in the medium. Protoplasts cultured in Rm medium underwent divisions, leading to callus formation. Shoots initiated on protoplast-derived calluses gave rise to fertile plants.

4.2 PROTOPLAST ISOLATION

Protoplasts from shoot cultures of S. brevidens were isolated using the protocol developed from S. tuberosum (Section 2.5.1 and Chapter 3). Shoots of S. brevidens were cut into 2 cm segments, placed in A medium and kept overnight at 4°C in the dark, then placed in 0.45 M mannitol for 30 min. prior to enzyme treatment. Protoplasts were released after 3-4 h incubation in enzyme EIV (1.5% Meicelase, 0.1% Pectolyase; Appendix I) over a range of osmotic values (485-570 mOsm). Preliminary results revealed that protoplast plating efficiencies were highest when the molality was between 490 and 530 mOsm; subsequently all isolations were made using enzyme EIV at 500 ±5 mOsm.

Twelve isolations were made using shoot cultures maintained on BM (Appendix I) with 0.25 mg l^{-1} BAP, for 36 to 83 days from the time they were last subcultured. Protoplast yields of about 2 x 10^6 g^{-1} fresh weight (FW) were obtained (Fig. 7). When shoots were subcultured onto BM with 0.05 mg l^{-1} BAP for one or more passages before isolation, protoplast yields increased to about 6 x 10^6 g FW^{-1} (Fig. 7). Protoplast preparations from shoot cultures maintained on BM without BAP for more than one passage were of poor quality. Between 30 and 90 days, the protoplast yield was not affected by the age of the shoot cultures (Fig. 7); isolations were not attempted before 30 days as the shoots had not grown sufficiently, or after 90 days when the leaves began to turn brown and die.

Recently, the metal lids from the Beatson jars were replaced with the bottom of a 9 cm petri-dish, sealed with 'Parafilm'. This made no obvious difference to the growth of the shoot cultures or to protoplasts isolated from them. However when the 'Parafilm' was punctured with a scalpel blade the shoot culture leaves grew to more than twice the size, and higher protoplast yields (up to 1 x 10^7 g FW^{-1}) resulted.

4.3 PROTOPLAST CULTURE

Freshly isolated protoplasts were spherical and rich in chloroplasts (Fig. 9a). Protoplasts were suspended in Rm medium (1 mg l^{-1} NAA, 0.4 mg l^{-1} BAP; Appendix I) at 500 mOsm to give an initial population density of 4 x 10^4 ml^{-1}. After 48 h, between 50 and 75% of the protoplasts had commenced cell wall synthesis and lost their spherical shape. This was accompanied by chloroplast

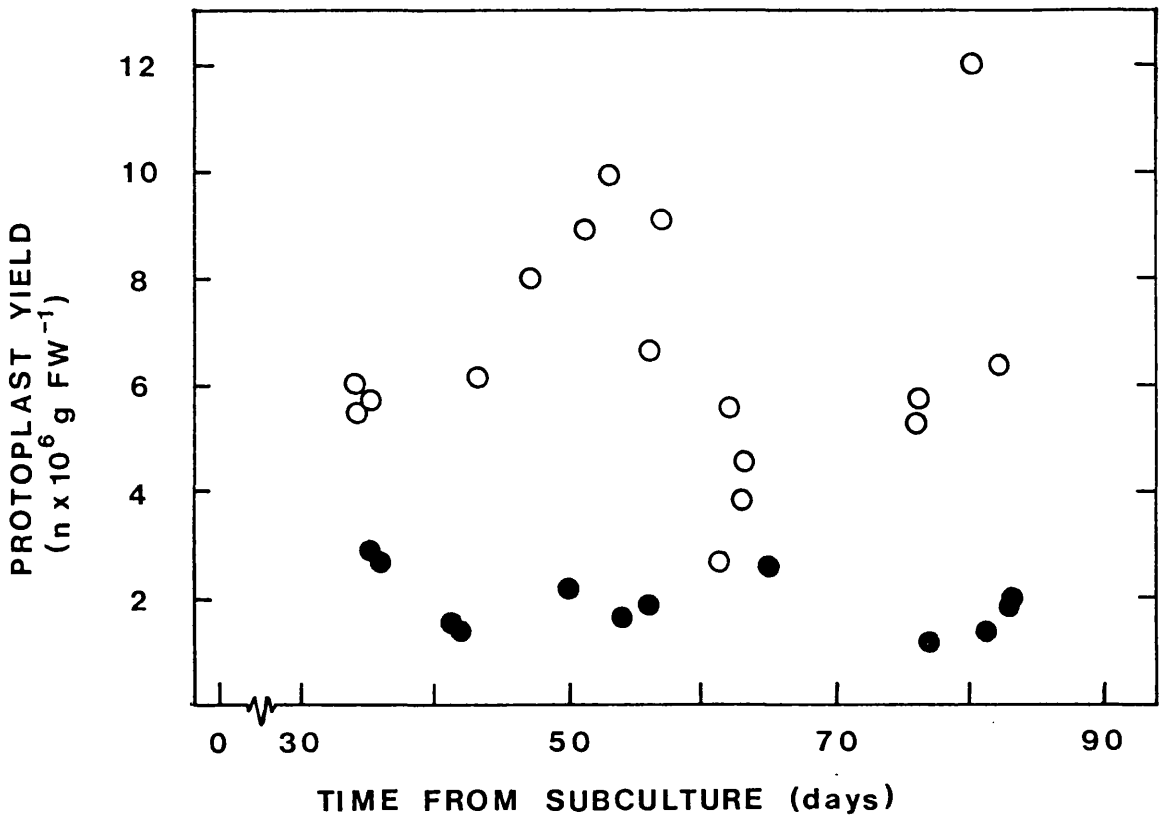
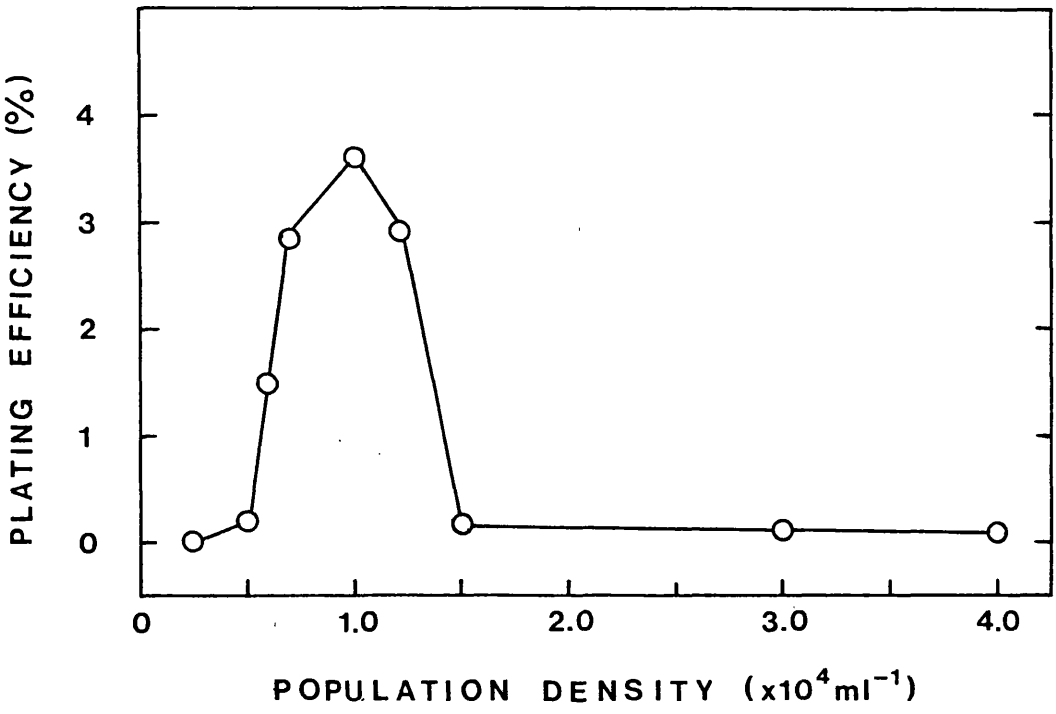


Fig.7 The effect of 0.25 mg l^{-1} BAP (●) and 0.05 mg l^{-1} BAP (○) in the shoot culture medium on the yield of S.brevidens protoplasts

degeneration. Protoplasts maintained at a density of 4 x 10^4 ml^{-1} for more than a week clumped together, and the culture medium turned brown. Very few divisions occurred, resulting in a low plating efficiency (Fig. 8). When the protoplast-derived cells were diluted after 4-6 days with fresh Rm at 500 mOsm, then plated next to Rm at 300 mOsm (Section 2.5.2), higher division frequencies were obtained. The optimum population density for cell division was 1 x 10^4 ml^{-1} at which about 50% of the protoplast-derived cells underwent divisions, although not all of these cells continued to divide. After 18-20 days culture small calluses had formed (Fig. 9b); 2 ml of the callus suspension was pipetted onto the surface of 20 ml C medium (0.1 mg l^{-1} NAA, 0.4 mg l^{-1} BAP; Appendix I) and six days later the plating efficiency (Section 2.6.3) was determined. In one isolation the highest plating efficiency was 3.6% at 1 x 10^4 protoplasts per ml; below 7 x 10^3 ml^{-1} or above 1.2 x 10^4 ml^{-1} the plating efficiency dropped rapidly (Fig. 8). Several subsequent isolations showed that plating efficiencies for S. brevidens protoplasts in Rm medium were fairly consistent and were not affected by the BAP concentration in the shoot culture medium or by the age of the shoots (Table 6).

As found with protoplasts of S. tuberosum cv. Fortyfold, S. brevidens protoplasts survived and formed calluses when suspended in several different media (Table 6) but none of the combinations tested were superior to Rm medium (see also Section 4.4.1).



Protoplasts plated at 4x10^{4}ml^{-1}, then after 5d diluted to above density with fresh Rm medium.

Fig.8 The effect of population density on the plating efficiency of S.brevidens protoplasts in Rm medium.

Figure 9.

The regeneration of fertile plants from protoplasts of S. brevidens.

a. Freshly isolated protoplasts in liquid Rm.
(Scale bar = 50 μm).

b. Multicellular colonies derived from protoplasts after 20 days.
(Scale bar = 50 μm).

c. Protoplast-derived calluses 6 days after layering over solid medium.
(Scale bar = 1 cm).

d. Shoots on protoplast-derived calluses.
(Scale bar = 1 cm).

e. Plants regenerated from protoplasts.
(Scale bar = 10 cm).

f. Flowers, berries and seed from a regenerated S. brevidens plant.
(Scale bar = 1 cm).

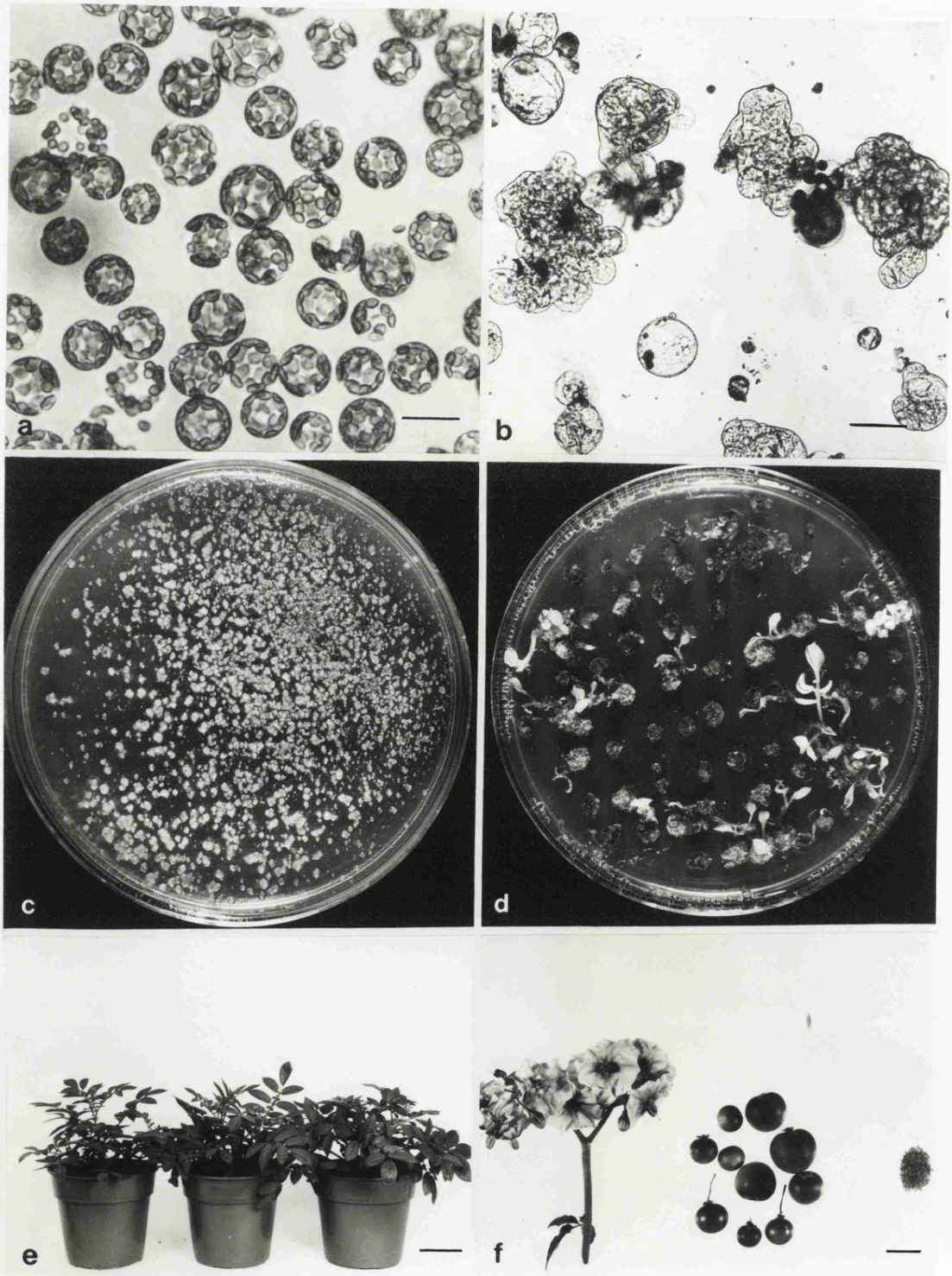

a
b
c
d
e
f

TABLE 6. Plating efficiencies from different S. brevidens protoplast isolations.

Isolation date	[BAP] in the shoot culture medium (mg l^{-1})	Age of shoot culture (days)	Protoplast culture medium	Plating efficiency (%)
5.6.81	0.25	35	Rm	5.5
			CL/R	3.0
3.7.81	0.25	36	Rm	3.6
			CL/R	1.7
17.9.81	0.25	83	Rm	3.6
			ET 11	7.0
4.11.81	0.25	41	Rm	4.6
			ET 9	3.2
			ET 11	4.0
18.11.81	0.05	34	Rm	1.0
21.1.82	0.25	65	Rm	4.6
	0.05	43	Rm	5.7
11.2.82	0.05	63	Rm	3.9
25.2.82	0.05	41	Rm	3.3

(Media formulations are given in Appendix I).

Protoplasts were plated at 4 x 10^4 ml^- for 4-6 days then the density was lowered to 1 x 10^4 ml^{-1} with fresh Rm medium.

4.4 PLANT REGENERATION

4.4.1 Shoot morphogenesis

Once the plating efficiency had been determined calluses between 0.1 and 1 mm in diameter were picked off from Rm layered over C medium and transferred to fresh C medium (to give about 50 calluses per 9 cm petri-dish). Calluses which developed in Rm medium were white. When maintained on C medium for 22 days they turned green and grew to 3-4 mm in diameter. Ten weeks after transfer to the D medium of Shepard (1982), with 0.1 mg l^{-1} IAA and 0.5 mg l^{-1} zeatin, only 3% of the calluses had developed shoots. Using the salts and organic constituents of D medium (Appendix I), a number of different hormone combinations were tested to find the optimum hormone concentrations for shoot morphogenesis (Fig. 10). After 6 weeks, many calluses developed shoot primordia, although very few of these had elongated (Fig. 10). When transferred from the regeneration medium to MT8 medium (0.25 mg l^{-1} BAP, 0.1 mg l^{-1} GA_3; Appendix I) for 4 weeks the number of calluses with shoots increased (Fig. 10). The results shown in Figure 10 are from one isolation, similar results were obtained in four subsequent isolations. The highest shoot production frequencies were routinely obtained using 1.0 mg l^{-1} zeatin and 0.1 mg l^{-1} IAA followed by MT8 medium (Fig. 9d and Fig. 10).

For reproducible shoot regeneration it was necessary to remove the calluses from the protoplast culture medium as soon as practicable. Calluses left on Rm layered over C medium for longer than 8 days began to turn brown, and failed to turn green when transferred to fresh C medium. Calluses grown in ET 11 (0.25 mg l^{-1} NAA, 0.5 mg l^{-1} 24D and 0.4 mg l^{-1} BAP; Appendix I) turned brown

Figure 10.

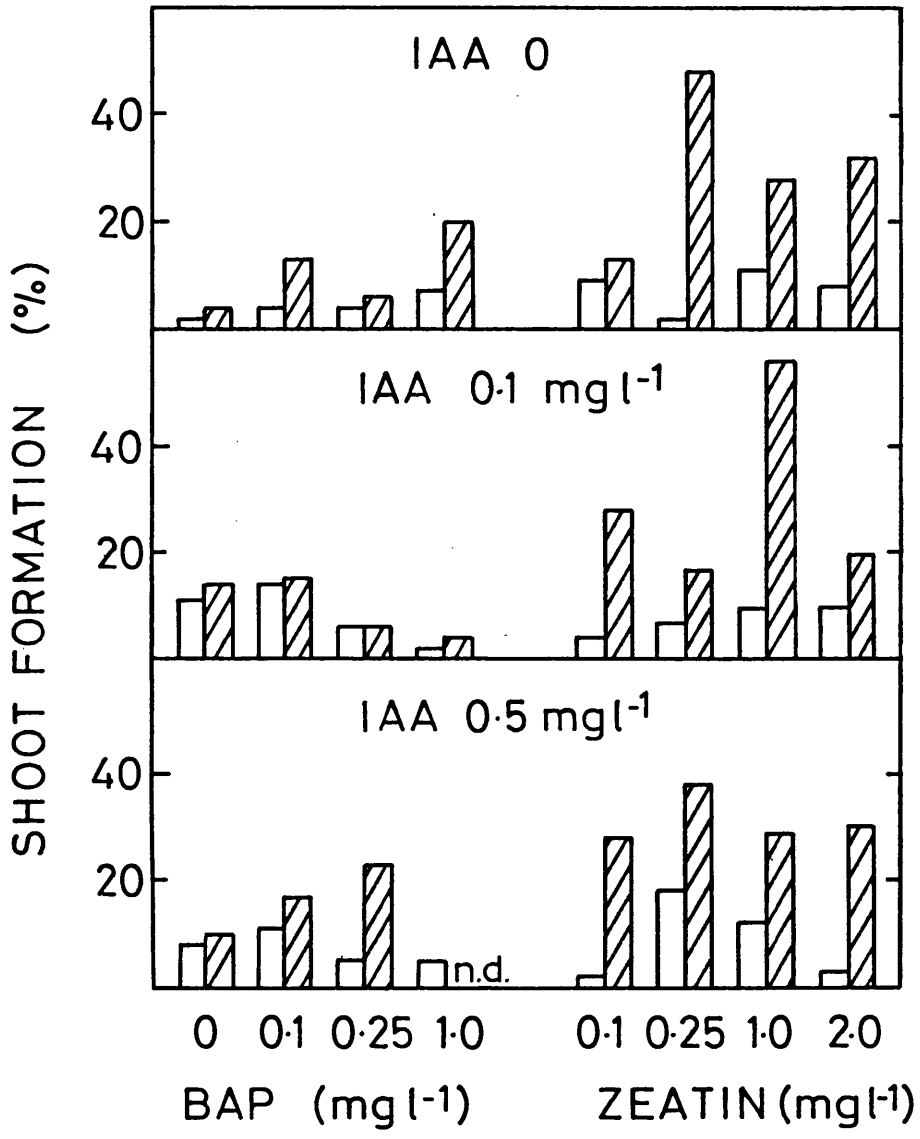
The effect of hormones on shoot morphogenesis.

Results are expressed as the percentage of plated calluses with at least one shoot,

A. after 6 weeks on medium D (□).

B. after 6 weeks on medium D plus 4 weeks on basal medium with 0.25 mg l^{-1} BAP, 0.1 mg l^{-1} GA_3 (▨).

At least 50 calluses were tested in each treatment.


IAA 0
IAA 0·1 mg l-1
IAA 0·5 mg l-1
SHOOT FORMATION (%)
40
20
n.d.
0 0·1 0·25 1·0
0·1 0·25 1·0 2·0
BAP (mg l-1)
ZEATIN (mg l-1)

even when the duration of culture in ET 11 was shortened by 4-5 days. No shoots were obtained on any of 1 x 10^3 calluses grown in ET 11.

4.4.2 Establishment of plants

Shoots were excised from the calluses and transferred to basal medium with 0.05 mg l^{-1} NAA for rooting (Section 2.4.4). Plants were then transferred to pots containing sterile vermiculite, watered with Potato Fertiliser Solution (Appendix I) and placed in a plant propagator under light at 250 $\mu E\ m^{-2}\ s^{-1}$ (16 h day 10°C/16°C night). Once established, plants were transferred to potting compost (Fig. 9e) and grown in a glasshouse at 18°C with natural lighting supplemented with 400 W SONT lamps at 180 $\mu E\ m^{-2}\ s^{-1}$ to maintain an 18 h day. The plants flowered and set berries, many of which contained seed (Fig. 9f).

4.5 DISCUSSION

The problems encountered with whole plants as a source of *S. tuberosum* protoplasts (Section 3.5) were avoided with *S. brevidens* by the use of shoot cultures. Shoot cultures of *S. brevidens* provide a uniform source material from which reproducibly high yields of protoplasts were obtained throughout the year. Previous reports where shoot cultures of different species were used as a protoplast source suggested that shoots should be subcultured frequently and grown for a specific period to obtain high yields of viable protoplasts (Schieder, 1975; Thomas, 1981; Saxena *et al.*, 1981). The yield and quality of protoplasts isolated from *S. brevidens* shoot cultures was unaffected by the time from subculture over a three

month duration. This allows a degree of flexibility in the procedure, increasing the value of shoot cultures as a source of S. brevidens protoplasts.

The presence of some cytokinin in the shoot culture medium was necessary to obtain good protoplast preparations of S. brevidens. Similarly, Wenzel (1979) included 0.5 mg l^{-1} BAP in the medium for growth of S. tuberosum and Saxena et al. (1981) used 2 mg l^{-1} kinetin for S. melongena cultures. Protoplast yields were consistently higher from S. brevidens cultures grown on basal medium with 0.05 mg l^{-1} BAP than with 0.25 mg l^{-1} BAP. The leaf sizes from shoots grown on the two BAP levels were not noticeably different, but after enzyme treatment leaves from shoots grown on 0.05 mg l^{-1} BAP were better digested than those grown on 0.25 mg l^{-1} BAP.

The yield of protoplasts was further increased by growing the shoot cultures in ventilated 'Beatson' jars. Ethylene content was significantly higher in the non-ventilated jars (G.P. Creissen, pers. comm.). Ethylene and cytokinin are known to affect the growth of leaves and the two types of hormone often have antagonistic roles (Wareing and Phillips, 1978). In well ventilated jars the addition of BAP to the shoot culture medium may be less important as the level of ethylene is lower, although this has not been tested.

The CL/R media system of Shepard (1982) was optimised for the culture of S. tuberosum cv. 'Russet Burbank' protoplasts. It has since been found suitable for the culture of protoplasts from eight different species as well as from several other potato cultivars (Shepard, 1981; Gunn and Shepard, 1981). Sustained divisions were obtained using the CL/R media, but higher survival and divisions were obtained using the modified medium (Rm). The principal differences

between the media are the concentrations of the major salts and the sugars used (Appendix I). The initial population density of 4×10^4 ml^{-1} was necessary for the survival of protoplasts, but the optimum density for divisions leading to callus formation was lower at 1×10^4 ml^{-1}. Relatively high population densities were also necessary for callus formation from protoplasts of tobacco (Nagata and Takebe, 1971), and Petunia (Hayward and Power, 1975). Plating efficiencies for protoplasts of S. brevidens in Rm medium were about 5%; comparable with the better plating efficiencies obtained for S. tuberosum cv. Fortyfold (Section 3.3.4). Plating efficiencies obtained with ET 11 medium were slightly higher (up to ~7%) but none of the resulting calluses underwent shoot morphogenesis. Even with calluses grown in Rm medium, the time spent in contact with the protoplast culture medium was critical for high shoot production frequencies. This may be because it is necessary to lower either the ratio of auxin to cytokinin or the absolute concentration of auxin early in the duration of culture to promote morphogenesis (see also Chapter 5). Shoot primordia developed on medium with a high cytokinin/auxin ratio, although not all the primordia elongated to form shoots until given a supply of exogenous GA_3. Zeatin gave a higher shoot production efficiency than BAP under this regime; 1.0 mg l^{-1} zeatin with 0.1 mg l^{-1} IAA gave the best results.

Up to 50% of the protoplasts isolated died in the early stages of culture without synthesising a new cell wall. One reason for this may be that although the physiological status of the shoot cultures was suitable for the isolation of high yields of protoplasts it was less than optimum for their survival. Another possibility is that the protoplasts may suffer physical damage during the isolation procedure.

Further work on the maintenance of the shoot cultures and improvements to the isolation procedure could reduce the high losses in the early stages. More protoplasts resynthesised a cell wall but failed to divide, whilst others only underwent limited divisions. Developments to the culture medium and refinements to the technique may increase the number of protoplast-derived cells which undergo sustained divisions. This is examined in Chapter 5.

CHAPTER 5

PROTOPLASTS TO PLANTS

III. IMPROVED PLATING CHARACTERISTICS

5.1 INTRODUCTION

In the previous two chapters procedures for the regeneration of plants from protoplasts of S. tuberosum and S. brevidens were described. While the demonstration of plant regeneration is significant in its own right, higher plating efficiencies giving increased numbers of cells regenerating to plants are desirable. The application of the procedures for the selection of somatic hybrids or biochemical mutants poses new problems. Selection systems are hampered by the high population densities necessary for protoplast survival. Any cells resistant to the selective agent would not survive simply because the culture medium did not support growth at low population densities. This problem has been overcome in various ways including the use of feeder layers (Raveh et al., 1973; Weber and Lark, 1979), complex media (Kao and Michayluk, 1975) and microdrop techniques (Gleba and Hoffmann, 1978). Attempts to develop a completely defined medium supporting the growth of protoplasts at low population densities have so far been unsuccessful (Caboche, 1980), but a procedure has been described which allows the growth of protoplast-derived cells at very low densities (1-4 ml^{-1}) in a defined medium, following an initial period of culture at high density (Caboche, 1980; Caboche and Muller, 1980). The essence of this procedure was to lower the NAA concentration to less than 0.3 mg l^{-1} NAA after an initial period at a higher auxin concentration.

As an experimental tool S. brevidens has two advantages over S. tuberosum; namely consistently higher protoplast yields and more reproducible plating efficiencies. Protoplasts of S. brevidens were therefore used to investigate the effect of the media composition on

the protoplast plating efficiency, and to begin to develop low density culture techniques for Solanum. The final protocol for protoplast isolation and culture was applied to different genotypes of S. tuberosum.

5.2 THE EFFECT OF DIFFERENT NITROGEN SOURCES ON PROTOPLAST PLATING EFFICIENCY

The nitrogen requirements of S. brevidens protoplasts were studied in medium Rm lacking casein hydrolysate and modified as shown in Table 7. Protoplasts of S. brevidens were isolated from shoot cultures as described (Section 2.5.1 and 4.2). Freshly isolated protoplasts were suspended at 4×10^4 ml^{-1} in Rm at 500 mOsm with ammonium chloride, potassium nitrate and glutamine added alone or in combination. Media omitting potassium nitrate were supplemented with potassium chloride (Table 7). After 6 days the population density was lowered to 1×10^4 ml^{-1} using the same medium. Protoplast plating efficiencies were determined after 26 d as before (Section 2.6.3). In Rm medium deprived of a major nitrogen source the protoplast plating efficiency was less than 1% (Treatment 1 and 2). The addition of potassium nitrate alone was superior to ammonium chloride or glutamine (Gln) supplied separately or both together (Treatments 7 and 8 compared with Treatments 3-6). Highest plating efficiencies were attained using potassium nitrate plus 10 mM glutamine (Treatments 9 and 10). The addition of 3 mM MES buffer to maintain the pH at 5.6 was also beneficial.

Table 7. The effect of different nitrogen sources on the growth of *S. brevidens* protoplasts

	KCl 46 mM	KNO_3 46 mM	NH_4Cl 15 mM	Gln 10 mM	MES 3 mM	No. of calluses	% P.E.
1	+	-	-	-	-	756	0.9
2	+	-	-	-	+	325	0.4
3	+	-	-	+	-	1963	2.4
4	+	-	-	+	+	2769	3.5
5	+	-	+	-	+	1197	1.5
6	+	-	+	+	+	1623	2.0
7	-	+	-	-	-	3225	4.0
8	-	+	-	-	+	3521	4.4
9	-	+	-	+	-	3685	4.6
10	-	+	-	+	+	4836	6.0

Results shown are from one isolation, 8×10^4 protoplasts per treatment were plated in Rm at 500 mOsm modified as above. Initial protoplast population density: 4×10^4 ml^{-1}; Final population density: 1×10^4 ml^{-1}. Plating efficiency (P.E.) was measured after 26 d.

To find the optimum concentration of glutamine, S. brevidens protoplasts were plated in Rm medium (with 46 mM KNO_3, but omitting casein hydrolysate) supplemented with different amounts of glutamine. The omission of casein hydrolysate led to a decrease in plating efficiency, but this was more than compensated for by the addition of glutamine. Protoplast growth was promoted over a range of glutamine concentrations (1 mM - 40 mM), with the highest plating efficiencies (up to 10%) obtained at a concentration of 5 mM glutamine (Figure 11). An increase in the growth rate of the colonies was also observed. In subsequent isolations medium with 46 mM KNO_3 and 5 mM glutamine (designated Rg; Appendix I) was used routinely for protoplast culture.

5.3 THE CULTURE OF PROTOPLAST-DERIVED CELLS AT LOW POPULATION DENSITIES

Freshly isolated protoplasts of S. brevidens were suspended in Rg medium (1.0 mg l^{-1} NAA, 0.4 mg l^{-1} BAP; Appendix I) at a population density of 1-4 x 10^4 ml^{-1}. After six days culture the protoplasts were either diluted with fresh Rg medium (1.0 mg l^{-1} NAA, 0.4 mg l^{-1} BAP) or transferred to LO medium (0.1 mg l^{-1} NAA, 0.4 mg l^{-1} BAP, Appendix I) by centrifugation at 100 x g for 5 min followed by resuspension in LO medium. At the same time the protoplasts were transferred to divided petri-dishes containing the respective Rg or LO medium at 300 mOsm, solidified with agar (Section 2.5.2). Figure 12a shows the relationship between population density and plating efficiency for protoplast-derived cells diluted with fresh Rg medium. In agreement with the results obtained using Rm medium

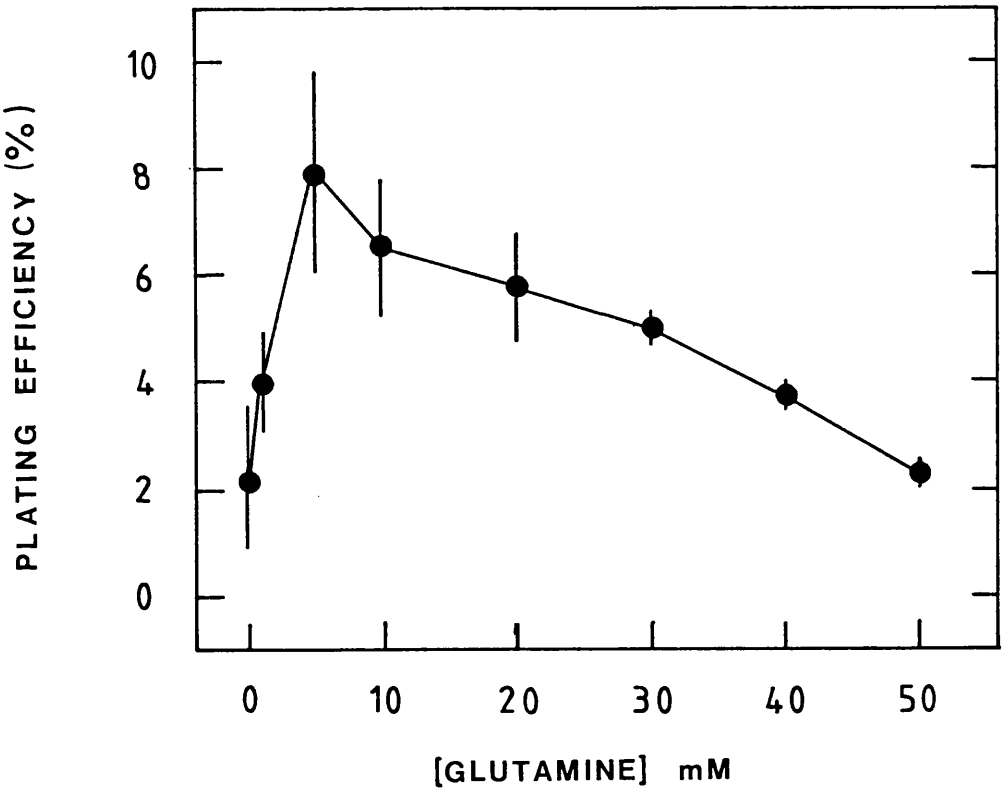


Fig.11 The effect of glutamine concentration on the plating efficiency of S.brevidens protoplasts

The bars represent standard deviation. Points for

0-10mM glutamine; mean of 6 determinations.

20-50mM glutamine; mean of 2 determinations.

Protoplasts plated in Rm at 4×10^4 ml^{-1} for six days then diluted to 1×10^4 ml^{-1} with fresh Rm.

(Figure 8 and Section 4.3) the plating efficiency was highest (approx. 10%) at 1 x 10^4 ml^{-1} and dropped rapidly with decrease in population density. Extrapolation of the graph suggests that no protoplasts would survive in Rg medium below 4 x 10^3 ml^{-1}. The results obtained with protoplasts initially cultured in Rg medium then transferred to LO medium are shown in Figure 12b. Plating efficiencies at high population densities (1 x 10^4 ml^{-1}) were approximately half those obtained with Rg medium. This was partially due to the fact that many of the protoplast-derived cells would not sediment upon centrifugation in Rg medium. However with protoplast-derived cells transferred to LO medium the plating efficiency was much less dependant upon the population density. At 1 x 10^3 ml^{-1} the plating efficiencies obtained using LO medium were about 1.5%; with protoplast-derived cells maintained in Rg medium the plating efficiency at 1 x 10^3 ml^{-1} was nil (Figure 12a and b). Freshly isolated protoplasts suspended in LO medium all died in the first 2-3 days, showing that an initial period of culture at high auxin concentration was necessary.

5.4 FREE AMINO ACID CONTENT IN RELATION TO PROTOPLAST CULTURE

Physiological studies using freshly isolated protoplasts have shown that there is a marked change in the polysomal profiles of protoplasts (compared with intact cells), associated with dedifferentiation, followed by an increase in RNA and protein synthesis leading to cell division (Galun, 1981). Any amino acids in short supply to the protoplasts would disrupt the synthesis of new

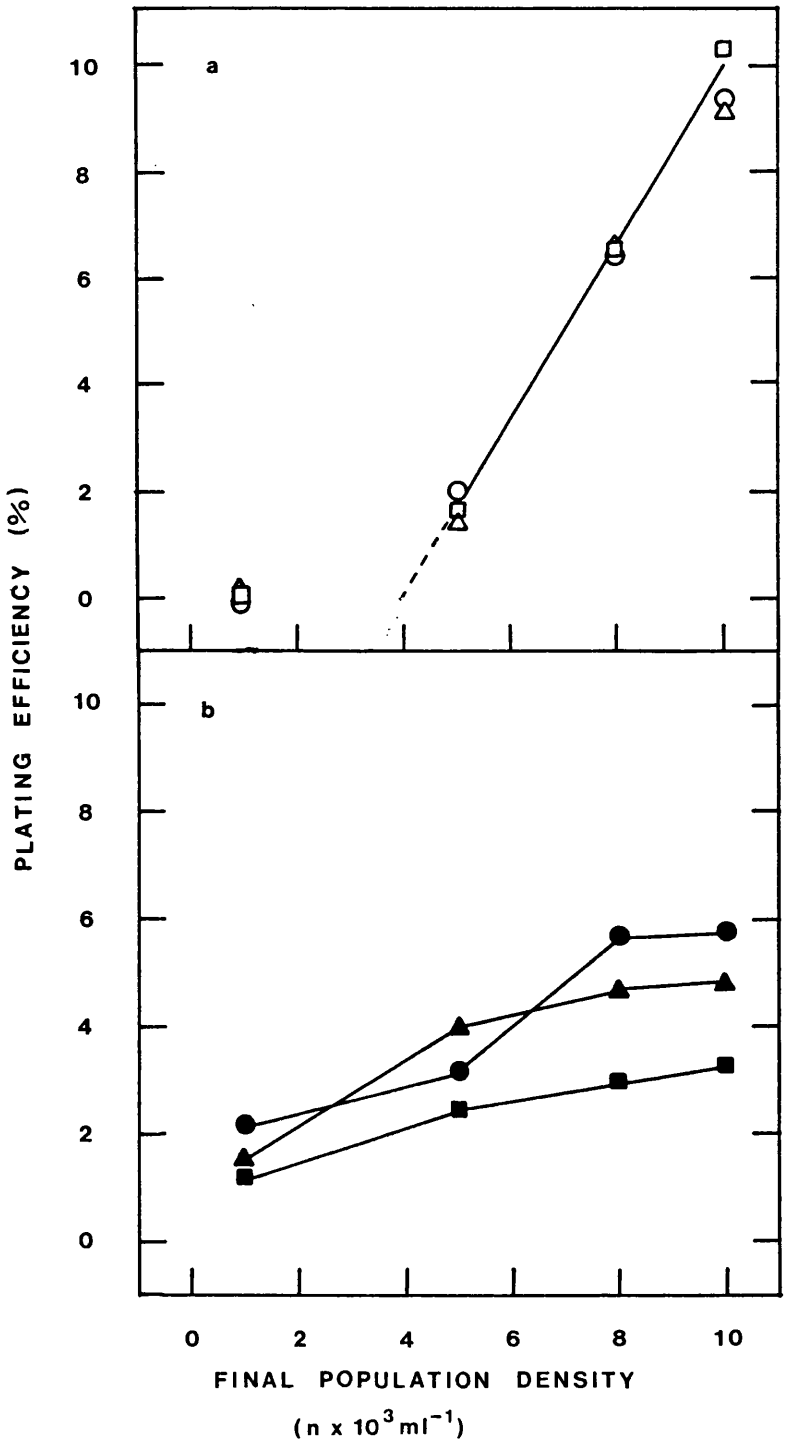
Figure 12.

The effect of population density on the plating efficiency of S. brevidens protoplasts in Rg medium and LO medium.

a) Protoplasts suspended in Rg medium then diluted with fresh Rg medium.

b) Protoplasts suspended in Rg medium then diluted with LO medium.

Initial population density: 4×10^4 ml^{-1} (□); 2×10^4 ml^{-1} (△); 1×10^4 ml^{-1} (○).


a
b
10
8
6
4
2
0
PLATING EFFICIENCY (%)
0
2
4
6
8
10
FINAL POPULATION DENSITY
(n x 10³ ml⁻¹)

proteins necessary for protoplast growth. A comparison of the free amino acid content of leaves and protoplasts was conducted (Section 5.4.2) for two reasons; firstly any amino acid which showed a decrease in concentration in protoplasts compared with leaves may have leaked out, and secondly amino acids present at high concentration may be particularly important. Addition of these amino acids to the protoplast culture medium may result in increased plating efficiencies.

5.4.1 Amino acids and the growth of protoplasts

An analysis of the free amino acid content of tobacco leaves revealed that levels of glutamine, glycine and γ-amino butyric acid (γAB) were relatively high (G. Ooms, pers. comm.). Previous results showed that 5 mM glutamine gave increased plating efficiencies for S.brevidens protoplasts (Section 5.2). In preliminary experiments several combinations of glutamine, glycine and γAB were added to the Rg/LO media to test their effects on protoplast growth (Table 8). The concentrations used were chosen to reflect the levels, relative to glutamine, observed in tobacco leaves. Protoplasts of S. brevidens were suspended at 1 x 10^4 ml^{-1} in Rg medium with modifications as shown, then after six days were diluted with the corresponding LO medium and transferred to divided petri-dishes. As found previously, 5 mM glutamine stimulated protoplast growth. The addition of glycine (2 mM) or γAB (3 mM) alone or in combination resulted in reduced plating efficiencies (Table 8). In one isolation, the addition of 5 mM glutamine, 2 mM glycine and 3 mM γAB together resulted in a plating efficiency of 8% (compared with only 4.5% obtained with glutamine alone), but in two subsequent isolations the plating efficiencies obtained were lower than with glutamine alone.

Table 8. Amino acids and protoplast growth.

Treatment	Glutamine 5 mM	Glycine 2 mM	γAB 3 mM	Plating efficiency %	± σ
1	-	-	-	1.6	± 0.9
2	+	-	-	3.5	± 0.8
3	-	+	-	0.8	± 1.3
4	-	-	+	0.8	± 0.4
5	+	+	-	2.5	± 1.2
6	+	-	+	1.6	± 0.1
7	-	+	+	0.6	± 0.3
8	+	+	+	4.2	± 3.0

S. brevidens protoplasts were suspended in Rg medium at 2×10^4 ml^{-1}, then diluted to 5×10^3 ml^{-1} with LO medium. Figures are means from three isolations, each with two replicates.

5.4.2 Amino acid analysis

Leaves of S. brevidens from plants grown in a controlled environment (as described for S. tuberosum, Section 2.4.1), shoot cultures, and protoplasts isolated from shoot cultures were taken for free amino acid analysis. The samples were extracted with methanol-chloroform-water solution as described (Section 2.6.5). The chloroform fractions obtained during the extraction were used to estimate the chlorophyll content and allow a comparison between shoot cultures and the protoplasts isolated from them (Table 9). The results of the amino acid analysis, expressed as μmol amino acid/g FW and as mol/100 moles of free amino acids are summarised in Table 10. The total free amino acid content of shoot cultures was nearly six times more than that of leaves from pot grown plants. Shoot culture leaves have less chlorophyll/g FW than leaves from whole plants. These two observations reflect the different physiological and developmental status of the two sources. Although the total amino acid content of shoot cultures was higher the percentage compositions were similar. In both shoot cultures and pot grown plants, glutamate/glutamine represented the highest proportion, followed by aspartate/asparagine, then γ-amino butyric acid (γAB), a derivative of glutamate (Table 10). The principal differences were in the levels of serine (higher in pot grown plants than in shoot cultures) and arginine (higher in shoot cultures).

Freshly isolated protoplasts from shoot cultures of S. brevidens (0.3 g shoot cultures yielded 3 x 10^6 protoplasts) were used for amino acid extraction, (Table 9). The total amino acid content of the protoplasts was approximately half that of shoot cultures. The amino acid composition of protoplasts reflected that

Table 9. Total chlorophyll content of leaves of S. brevidens

Source	Replicate	FW (g)	Absorbance A_{645}	Absorbance A_{663}	Chlorophyll content mg l^{-1}	Chlorophyll content mg Chl/g FW
Whole Plants	1	0.4	1.81	6.20	86.28	0.755
	2	0.4	1.87	6.36	88.75	0.777
	3	0.4	1.89	6.40	89.51	0.850
	$\bar{X}$					0.794
Shoot Cultures	1	0.4	1.75	4.90	74.65	0.709
	2	0.4	1.72	4.56	71.31	0.624
	3	0.4	1.65	4.20	67.01	0.620
	$\bar{X}$					0.651
Protoplasts	1	0.14*	0.86	1.12	26.35	-

$$\text{mg total chlorophyll/g tissue} = (20.2 \times A_{645}) + (8.02 \times A_{663}) \times \frac{V}{1000 \times FW}$$

(from Haas et al., 1979)

* Calculated using chlorophyll content of shoot cultures compared with protoplasts.

Table 10. Soluble amino acid content of leaves and protoplasts of *S. brevidens*

	nmol g FW^{-1}			mol%		
	Whole plant	Shoot cultures	Shoot culture Protoplasts	Whole plant	Shoot culture	Shoot cultu protoplast
Asp/Asn	101.9 ± 20.3	910.5 ± 19.1	390.1	16.2 ± 1.4	29.3 ± 2.9	15.0
Thr	15.1 ± 2.2	68.9 ± 5.7	76.8	2.4 ± 0.2	1.8 ± 0.0	2.9
Ser	61.4 ± 11.6	121.6 ± 18.1	89.6	9.8 ± 0.3	3.2 ± 0.4	3.5
Glu/Gln	167.4 ± 31.4	1313.0 ± 128.9	646.0	26.7 ± 0.8	34.2 ± 2.0	24.8
Pro	34.2 ± 12.0	110.3 ± 23.8	36.0	5.3 ± 1.4	2.9 ± 0.8	1.4
Gly	31.8 ± 3.9	62.5 ± 10.1	42.1	5.1 ± 1.3	1.6 ± 0.3	1.6
Ala	29.2 ± 6.5	96.9 ± 14.6	249.2	4.6 ± 0.4	2.5 ± 0.3	9.5
Cys	6.1 ± 1.2	53.2 ± 5.8	0	1.0 ± 0.2	1.4 ± 0.1	-
Val	12.9 ± 2.0	62.6 ± 5.4	54.2	2.1 ± 0.1	1.6 ± 0.2	2.1
Met	3.0 ± 0.8	28.4 ± 1.9	8.3	0.5 ± 0.2	0.7 ± 0.0	0.3
Ile	6.4 ± 1.7	39.1 ± 6.5	35.4	1.0 ± 0.2	1.0 ± 0.2	1.4
Leu	28.1 ± 3.2	211.3 ± 32.1	124.6	4.6 ± 0.7	5.5 ± 0.8	4.9
Tyr	9.1 ± 0.3	89.3 ± 21.2	30.9	1.5 ± 0.4	2.3 ± 0.7	1.2
Phe	18.4 ± 3.5	23.6 ± 8.6	18.8	3.0 ± 0.3	0.6 ± 0.3	0.7
γAB	78.9 ± 16.3	257.5 ± 87.7	591.8	12.7 ± 2.3	6.6 ± 2.3	22.7
His	11.8 ± 0.1	85.9 ± 23.2	76.2	1.9 ± 0.4	2.2 ± 0.5	2.9
Lys	6.7 ± 1.1	48.9 ± 7.5	56.1	1.1 ± 0.1	1.3 ± 0.1	2.2
Arg	2.64 ± 0.9	252.9 ± 72.2	76.2	0.4 ± 0.3	6.6 ± 2.1	2.9
Total	625	3836	2602			

Figures for whole plants and shoot cultures are the means of three replicates (± standard deviation).

Figures for nmol g FW^{-1} were calculated assuming 100% recovery of amino acids from the extraction.

from the source shoot cultures although the proportions of glutamate/glutamine and aspartate/asparagine were lower, partly due to an increase in the amount of γAB. The proportion of alanine was also higher in protoplasts than in shoot cultures, but cysteine was not detected in protoplasts.

5.5 SUMMARY OF TECHNIQUES AND RESULTS FOR PROTOPLAST ISOLATION AND CULTURE

The final protocol, incorporating all the modifications and improvements is outlined in Figures 13 and 14. Details of the protocol for protoplast isolation, culture and the regeneration of plants are given elsewhere (Sections 2.5 and 5.3) and formulations of the media used are provided in Appendix I.

Plating efficiencies for protoplasts of S. brevidens initially suspended in Rg medium and later diluted with LO medium were approximately 6%, although in one isolation a plating efficiency of 19% was obtained (Table 11); so far this has not been repeated. The tetraploid S. tuberosum genotypes Fortyfold and Ukama responded similarly, to give plating efficiencies of about 2% (Table 11). Using the Rg/LO media the optimum population density for initial culture was between 1 and 2 x 10^4 ml^{-1}, and when diluted after six days to half the initial population density plating efficiencies were fairly consistent. Results obtained with the dihaploid and monohaploid genotypes in this system were disappointing. Shoot cultures grown in ventilated glass jars on basal medium with 0.05 mg l^{-1} BAP gave high yields (~ 1.5 x 10^6 g^{-1}) of protoplasts, and the protoplasts obtained appeared healthy. A high proportion of the protoplasts

survived to synthesise a new cell wall, but very few divisions were observed and no calluses were recovered (Table 11).

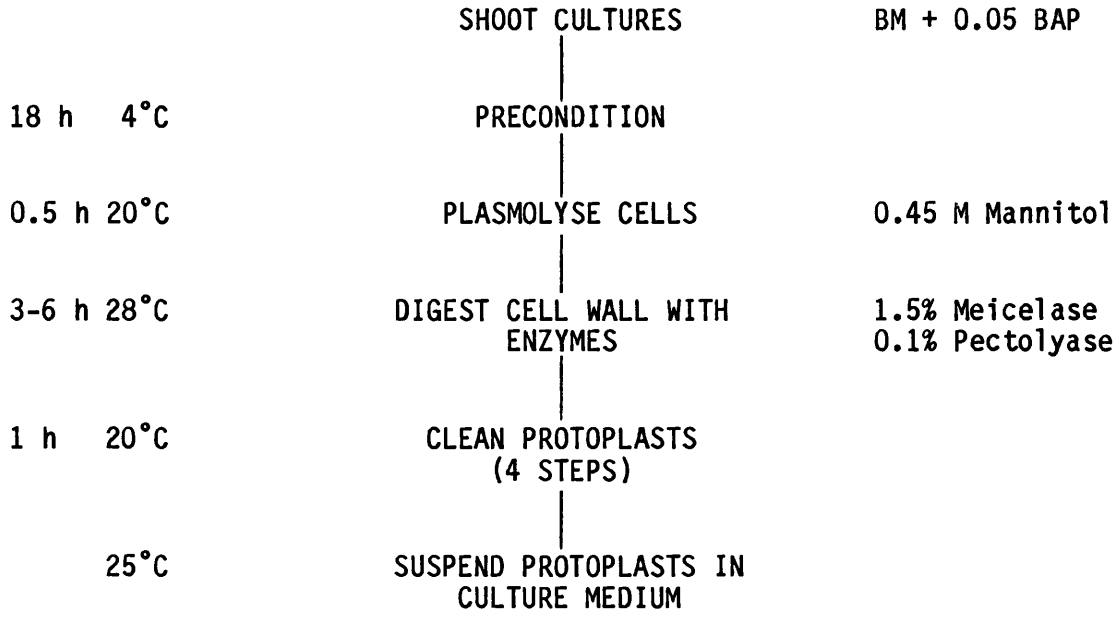


Figure 13. A summary of the procedure for protoplast isolation.

HORMONES	DAYS	
NAA 1, BAP 0.4	0	Protoplasts in culture medium
	2	Transfer to light
NAA 0.1, BAP 0.4	6	Dilute with fresh medium and lower osmoticum
NAA 0.1, BAP 0.5	20	Colonies on agar medium
	50	Green calluses
IAA 0.1, Z 1	80-100	Differentiated calluses
GA_3 0.1, BAP 0.25	20	Shoot elongation
	140	Plants

Concentration of hormones expressed in mg l^{-1}; Z = zeatin

Figure 14. A summary of the techniques for protoplast culture.

Table 11. Summary of results obtained with protoplasts cultured in Rg/LO media.

Genotype	Protoplast yield ($g\ FW^{-1}$)	Population density Initial (ml^{-1})	Final (ml^{-1})	No. of calluses	P.E. (%)
S. brevidens	$4x10^6$	$4x10^4$	$1x10^4$	4467	5.8
	$1x10^7$	$4x10^4$	$1x10^4$	9174	5.7
		$4x10^4$	$1x10^3$	200	1.3
	$5x10^6$	$4x10^4$	$1x10^4$	11350	7.2
		$1x10^4$	$5x10^3$	3682	4.8
	$3x10^6$	$1x10^4$	$5x10^3$	15131	19.0
S. tuberosum Fortyfold	$1.6x10^6$	$2x10^4$	$1x10^4$	4494	2.8
		$2x10^4$	$5x10^3$	2892	2.2
Ukama	$1.4x10^6$	$4x10^4$	$1x10^4$	1558	0.2
	$1.3x10^6$	$2x10^4$	$1x10^4$	4971	2.0
PDH119	$1.6x10^6$	$1x10^4$	$5x10^3$	0	-
	$1.1x10^6$	$2x10^4$	$1x10^4$	0	-
PDH440	$1.9x10^6$	$1x10^4$	$5x10^3$	0	-
Mn797322	$1.7x10^6$	$4x10^4$	$1x10^4$	0	-

Protoplasts were plated in Rg medium at 500 mOsm at an initial population density (shown above), then after 6 d were diluted with LO medium to give a final population density as above.

Protoplast-derived calluses of S. brevidens, Fortyfold and Ukama grown in LO medium turned green in a much shorter time than calluses maintained in Rg medium. Occasionally shoots were initiated on C medium, before transfer to 'regeneration' medium, this was never observed among calluses developed in Rg medium. The response to protoplast-derived calluses grown in Rg/LO medium to the shoot regeneration media tended to be much better. In one isolation, protoplasts of S. brevidens were diluted after six days with either fresh Rg medium or with LO medium, 300 calluses from each treatment were carried through to regenerate plants. The efficiency of shoot production among calluses developed in Rg/LO media was 93% compared with 55% with calluses from Rg/Rg. Similar high shoot production frequences were observed in two subsequent isolations using Rg/LO media. Shoot production efficiencies for protoplast-derived calluses of the tetraploid S. tuberosum cultivars in the Rg/LO system were 40% for Ukama and 70% for Fortyfold. With Fortyfold calluses from Rg/Rg medium, the average shoot production efficiency was only 30% (Table 5).

5.6 DISCUSSION

Protoplast plating efficiency may be influenced by many factors including the genotype involved, the physiological status and type of source material, osmotic pressure and type of osmoticum, pH, temperature, the type and concentration of enzymes used, protoplast population density and the composition of the culture media (for a review see Keller et al., 1982). Several of these factors were examined in Chapters 3 and 4; and in this Chapter some components of

the culture medium were examined. The nitrogen source is of particular interest with regard to Solanum protoplast culture as the reports in the literature are somewhat contradictory. The presence of ammonium ions was found to be toxic to protoplasts of S. tuberosum cvs. Sieglinde (Upadhya, 1975), Priekulsky ranii (Kuchko and Butenko, 1977), Russet Burbank (Shepard and Totten, 1977), and Bintje (Bokelmann and Roest, 1983). For culture of Russet Burbank protoplasts the concentration of potassium nitrate was raised to compensate for the omission of ammonium ions. In contrast plating efficiencies of the dihaploid line H^2140 increased following the omission of potassium nitrate (Binding et al., 1978), whilst protoplasts of S. tuberosum cv. Maris Bard (Thomas, 1981) and S. melongena (Saxena et al., 1981) were successfully cultured in the presence of both ammonium and nitrate ions. Protoplasts of S. brevidens divided in the presence of ammonium (supplied as ammonium chloride), but higher plating efficiencies were obtained using either potassium nitrate or glutamine as the major nitrogen source, (Table 7) supporting the case for omission of ammonium. In their work with soybean (Glycine max) suspension cultures, Gamborg and Shyluk (1970) showed that ammonium ions caused a rapid decrease in the pH of the culture medium unless the ammonium was added as a salt of citrate, malate, fumarate or succinate (the Krebs cycle acids). This was not tested for protoplasts of S. brevidens.

The addition of 1 mM glutamine was found to be beneficial for the culture of Nicotiana tabacum protoplasts (Caboche, 1980) and 7 mM glutamine was vital for sustained divisions in protoplasts of Asparagus officinalis (Bui-Dang-Ha and Mackenzie, 1973). Similarly 5 mM glutamine, in combination with potassium nitrate gave the highest

plating efficiencies for S. brevidens protoplasts. Glutamine is a key compound in the assimilation and reassimilation of nitrogen, formed from ammonia and glutamic acid by the enzyme glutamine synthetase. The reduction of nitrate via nitrite and ammonium to glutamine is expensive in energy terms whereas a direct supply of glutamine to the protoplasts bypasses such an energy requirement. Furthermore under anaerobic conditions nitrogen assimilation is drastically reduced (Streeter and Thomson, 1972) making a readily available supply of glutamine valuable. The relevance of this is discussed later in this section. Glutamine and asparagine are the major transport compounds found in plants (Lea and Miflin, 1980).

Amino acid analyses were carried out to compare leaves from different sources with protoplasts. The total amino acid content of leaves from shoot cultures was nearly six times that from pot grown plants, and this was to some extent reflected in the total amino acid content of shoot culture derived protoplasts. The amino acid composition expressed in mol% was similar in all three cases, with glutamate/glutamine and aspartate/asparagine representing the major components. The concentration of γAB and alanine was much higher in protoplasts than in leaves (Table 10). This is a strong indication that the protoplasts may be stressed as leaves of Raphanus sativus (Radish) accumulate both γAB and alanine under anaerobic conditions (Streeter and Thomson, 1972). Aspartate may be converted to alanine via oxaloacetate and pyruvate, and glutamate decarboxylation to γAB is accelerated under anaerobic conditions (Streeter and Thomson, 1972). In this context the reduction in the levels of aspartate/asparagine and glutamate/glutamine in protoplasts (compared with shoot cultures) may be important. Other amino acids also showed a decrease in

concentration following protoplast isolation. Cysteine was not detected in protoplasts, and arginine was much reduced. These differences may be accounted for by the fact that the protoplasts represent a particular population (ie. mesophyll cells) whilst shoot culture leaves contain a range of cell types (mesophyll, epidermis, phloem, etc.), or that these amino acids do show a genuine decrease and may be in short supply.

Based on analysis of tobacco leaves, preliminary experiments looked at the effects of glutamine, glycine and γAB on protoplast growth. Glycine and γAB inhibited growth when added alone but when added together in combination with 5 mM glutamine the inhibitory effects were negated (Table 8). Amino acid analysis of S. brevidens showed glycine levels in leaves and protoplasts to be fairly consistent (Table 10). Relative to the optimum concentration of glutamine (5 mM), the level of glycine used was nearly 10 times that observed in leaves suggesting that 2 mM glycine may have been supraoptimal. γAB was abundant in leaves and amino acid analysis indicated that the protoplasts may actually accumulate γAB. In retrospect neither glycine or γAB were the best amino acids to begin such an investigation. It would be interesting to examine the effect of adding aspartate (or asparagine), cysteine and arginine to the protoplast culture medium.

Protoplasts of S. brevidens cultured in Rg medium with 1.0 mg l^{-1} NAA and 0.4 mg l^{-1} BAP, gave plating efficiencies of about 10% at a population density of $1x10^4$ ml^{-1}. At the same density ($1x10^4$ ml^{-1}) protoplasts plated in LO medium (0.1 mg l^{-1} NAA, 0.4 mg l^{-1} BAP) gave a plating efficiency of only 5% (Figure 12). This was also found to be the case for protoplasts of Nicotiana tabacum

(Caboche, 1980). However the population density was far less critical for the growth of protoplasts cultured in the low auxin (LO) medium than in the high auxin (Rg) medium. The use of LO medium allowed growth of ~20 colonies ml^{-1} (2% of $1x10^3$ ml^{-1}; Figure 12b), compared with a minimum of ~100 colonies ml^{-1} (2% of $5x10^3$ ml^{-1}, Figure 12a) using Rg medium. Thus, in agreement with results obtained with Nicotiana and Petunia species (Caboche, 1980; Negrutiu and Muller, 1981; Muller et al., 1983), growth of Solanum protoplast-derived cells at low population densities was achieved by reducing the NAA concentration after an initial period of growth at high auxin. The initially high exogenous auxin concentration appears to be necessary for the first division of mesophyll protoplasts, and is not further required to support sustained division of the protoplast-derived cells. Experiments with ^{14}C labelled NAA demonstrated that high concentrations of NAA are toxic to tobacco protoplasts at low densities, but at high densities most of the NAA is detoxified by conjugation to amino acids (Caboche, 1980). Recent work has identified aspartic acid as the main amino acid involved in such conjugations (M. Caboche, pers. comm.). Caboche (1980) suggested that the need for an initial period of high NAA concentration may be related either to the dedifferentiation process in which mesophyll protoplasts may have to engage before entering new cycles of cell division, or to the unusual needs of stressed, wall-less plant cells.

The main difference between Rg and LO media is that the NAA concentration in Rg is 10 times that in LO medium. However there are also a number of smaller differences in the composition of salts and vitamins. LO medium was originally developed for N. tabacum and whilst the principle was successfully applied to other species,

modifications to the media improved the plating efficiencies at low densities, for example with Nicotiana sylvestris the additon of the Krebs cycle acids helped to increase the plating efficiency from approximately 20% to nearly 80% (Negrutiu and Muller, 1981). Such refinements may also improve the situation for Solanum species.

Protoplast-derived calluses grown in the Rg/LO media turned green sooner and gave higher shoot formation frequencies than calluses from the original Rg/Rg system (Section 5.5). This is supported by the earlier findings with S. brevidens (Section 4.5) that it was necessary to lower the auxin concentration as soon as possible for reproducible shoot regeneration.

The final protocol for isolation and culture of protoplasts was developed mainly using S. brevidens and was then applied to S. tuberosum. Monohaploid shoot cultures grown in ventilated glass jars gave higher yields of healthier looking protoplasts although they still proved recalcitrant to protoplast culture. Shoot cultures of the tetraploid cvs Fortyfold and Ukama grown this way also gave higher protoplast yields than with non-ventilated glass jars. Fortyfold protoplasts cultured in Rg/LO media showed improved plating characteristics and later on more efficient plant regeneration. Ukama also responded to this system. Freshly isolated S. brevidens showed signs of anaerobically induced stressed. Aeration of the protoplasts during isolation and washing may therefore be beneficial. Further improvements may result following experiments on the addition of amino acids (cysteine, aspartate and arginine) and the Krebs cycle acids (citrate, malate, fumarate and succinate). The addition of aspartate to the protoplast culture media is of particular interest as it is involved in the detoxification of NAA; this may be important for the

culture of the monohaploid lines which largely have failed to survive the first few days of culture.

CHAPTER 6

VARIATION IN PROTOPLAST-DERIVED PLANTS

6.1 INTRODUCTION

Phenotypic variation observed in protoplast-derived plants of S. tuberosum cvs Russet Burbank and Maris Bard (Section 1.4.3) was also evident amongst plants regenerated from protoplasts of cv. Fortyfold and S. brevidens. The extent of this variation was evaluated using protoplast-derived plants established in a glasshouse. Root-tip chromosome preparations were made to confirm the chromosome number of the different genotypes used as protoplast sources. A cytological analysis of the protoplast-derived plants, conducted by Dr. Angela Karp (Biochemistry Department, Rothamsted Experimental Station), revealed that much of the obvious morphological variation correlated with changes in chromosome number. The extent of the chromosome variation was partly influenced by the conditions used for protoplast culture.

Tubers from protoplast-derived Fortyfold plants grown in the glasshouse for two generations were put into a replicated field trial to assess the variation in field grown plants.

6.2 PROTOPLAST-DERIVED S. BREVIDENS PLANTS

Plants regenerated from protoplasts were maintained in a glasshouse, as described in Section 4.4.2. The chromosome number of S. brevidens was confirmed as 2n = 2x = 24 by screening a sample of plants established from shoot cultures (Section 2.6.4). Chromosome counts of 50 protoplast-derived plants (all taken from different calluses obtained from a single isolation) showed that 24% of the regenerants were normal diploids (2n = 2x = 24), 52% were tetraploids

(2n = 4x = 48) and 24% were aneuploids (Table 12). The proportion of normal plants regenerated from protoplasts transferred from Rg medium (1.0 mg l^{-1} NAA, 0.4 mg l^{-1} BAP) to LO medium (0.1 mg l^{-1} NAA, 0.4 mg l^{-1} BAP) five days after isolation was the same as for plants maintained in Rg for a full 20 days (see Section 5.3).

Leaf morphology correlated with chromosome number (Fig. 15). Leaves from the diploid regenerants were narrow (lanceolate) and resembled leaves from control S. brevidens plants. Leaves from the tetraploid regenerants were broader and easily distinguishable (Fig. 15). After two to three months in the glasshouse the regenerants flowered and eventually set berries. Berries collected from the euploid regenerants all contained seed, whereas berries from the aneuploids were parthenocarpic. Tetraploid regenerants were less fertile than the diploids producing an average of five seeds per berry, compared with more than 20 seeds per berry from the diploid regenerants. Twenty seeds from each of two diploid and two tetraploid regenerants were washed in tap water then surface sterilised and plated on BM as before (Section 2.4.2), where all the seeds germinated.

Table 12. Chromosome variation in protoplast-derived *S. brevidens* plants.

	Chromosome No.	No. of plants (% of total) High Auxin	Low Auxin
Euploids	24	6 (24%)	6 (24%)
	48	13 (52%)	13 (52%)
Aneuploids	≠48	4 (16%)	3 (12%)
	≠96	2 (8%)	3 (12%)

Plants were from one isolation. Protoplasts were suspended in Rg medium at 4×10^4 ml^{-1} then after 5 d were diluted to 1×10^4 ml^{-1} with either fresh Rg medium (High auxin) or with LO medium (Low auxin). Later, the first shoot arising on each of 25 calluses from both treatments was excised and rooted for chromosome analysis. (≠48 covers 44-47 and 49 chromosome plants; ≠96 covers 60-95 chromosome plants). Chromosomes counted by A. Karp.

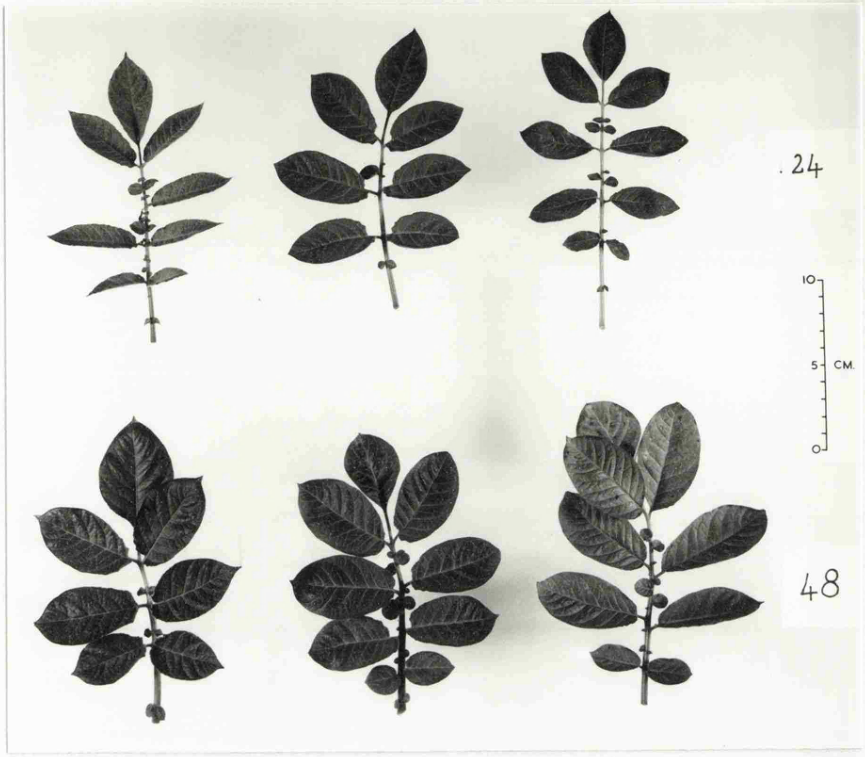


Figure 15. Leaves from Solanum brevidens protoplast derived plants. Leaf six taken from three diploid (2n = 2x = 24) and three tetraploid (2n = 4x = 48) protoplast-derived plants.

6.3 CHROMOSOME VARIATION IN PROTOPLAST-DERIVED S. TUBEROSUM PLANTS

6.3.1 Chromosome numbers in protoplast source material

The chromosome number of each genotype used as a source of protoplasts was checked using plants derived from shoot cultures or sprouted tubers. Chromosomes were prepared as described (Section 2.6.4). Cytological preparations of root-tips from Mn797322 consistently showed the monohaploid number of 12 chromosomes (Fig. 16a). The chromosome number of PDH 119 was confirmed as 2n = 2x = 24 (Fig. 16b), whilst Ukama (Fig. 16c) and Fortyfold both had the full tetraploid complement (2n = 4x = 48).

Preparations from root-tips of Mn798261 originally gave counts of 12 chromosomes, but after two years in culture the line was found to be unstable, some shoots gave rise to roots with 24 chromosomes whilst others gave counts of 48 chromosomes (A. Karp, pers. comm.).

6.3.2 Protoplast-derived plants

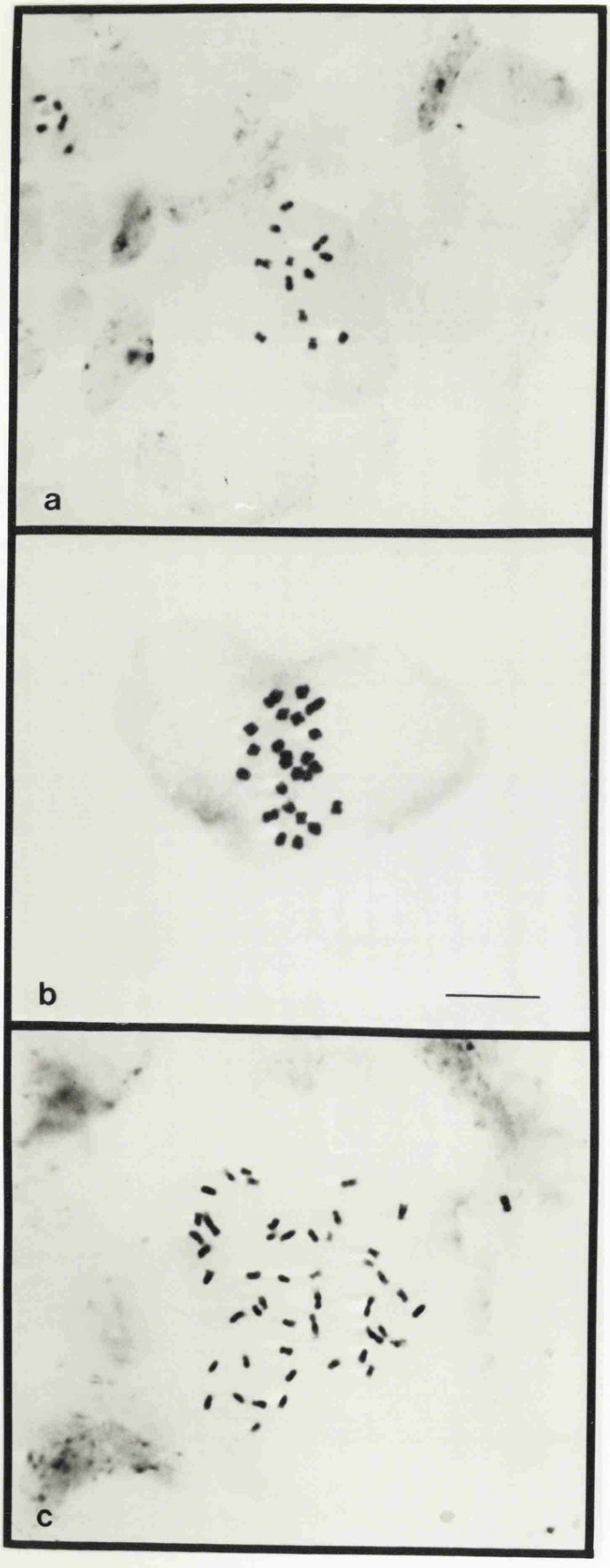
Protoplasts isolated from a single leaf of Fortyfold were cultured in Rm medium (1.0 mg l^{-1} NAA, 0.4 mg l^{-1} BAP) for 20 days before transfer to C medium (isolation date 3.2.81; Section 3.3.2). To investigate some of the origins of variation in protoplast-derived plants, calluses obtained from the protoplasts were numbered for identification, as were (in order) the different plants arising from each callus, which were then maintained as shoot cultures, and the different plants obtained from the shoot cultures. The numbering system enabled the genealogy of each Fortyfold regenerant to be traced back to the original shoot and callus from which it arose. The full

Figure 16. Chromosome numbers in protoplast source material (Scale bar = 10 μm)

a) Mn797322 (2n = x = 12)

b) PDH 119 (2n = 2x = 24)

c) Ukama (2n = 4x = 48)


a
b
c

description of each regenerant thus includes the callus number (given first), the shoot number (given second) and the number of the plant obtained from the shoot culture (given last). In addition some of the calluses fragmented prior to shoot formation and these were given a letter suffix e.g. 11a, 11b, 11c so that different subunits of the same callus could be identified. The letters FTF were used as a prefix to distinguish Fortyfold protoplast-derived plants from others.

Table 13a shows the chromosome numbers from 26 different Fortyfold calluses. In most cases the count quoted corresponds to the first plant from the first shoot obtained from each callus, but occasionally this died and the next available shoot was taken. Chromosome numbers varied between calluses; only 30% of the plants had the correct chromosome number (48), the remainder were aneuploids ranging between 46 and 49. The majority of the aneuploids had lost rather than gained chromosomes and monosomics were most frequent (2n = 47). An exception to this aneuploid range was callus 20 which gave rise to a plant (FTF 20) with 93 chromosomes.

Table 13. Chromosome numbers of plants regenerated from 26 separate protoplast-derived calluses of Fortyfold from a) protoplasts cultured in Rm medium, and b) protoplasts cultured in Rg/LO media.

Chromosome number	Number of plants	
	a	b
46	6	0
47	8	6
48	8	13
49	3	6
>50	1	0

(Chromosomes counted by A. Karp)

When different shoots from the same callus were analysed considerable variation was also revealed (Table 14), and this was true both for shoots from the same part of the callus (19a where six different shoots were counted) and for shoots from different subparts of the same callus (19c, e, f). In some cases different shoots from the same callus can have the same chromosome number. This is shown by callus 20 where both shoots obtained had 93 chromosomes. This could be due either to both shoots having originated from a very small region of the callus or to the change to 93 chromosomes having occurred very early on in the history of the callus.

Chromosome numbers were counted in 12 differnt plants derived from the same shoot culture (11a/1). Surprisingly, although nine of the plants scored had the same chromosome number (2n = 46), two of the plants had more than twice the number of chromosomes (93 and 94) and another (11a/1/4) consistently gave two counts (2n = 70 and 2n = 94).

In a separate isolation shoot culture-derived protoplasts of Fortyfold were suspended in Rg medium (1.0 mg l^{-1} NAA, 0.4 mg l^{-1} BAP) then after five days were diluted four times with LO medium (0.1 mg l^{-1} NAA, 0.4 mg l^{-1} BAP) as described in Section 5.5. The first shoots formed on 25 different protoplast-derived calluses were taken for chromosome counts (Table 13b). As found previously (Table 13a) chromosome numbers varied between calluses. However the range of aneuploids obtained was smaller and the proportion of calluses with the normal chromosome complement of 48 had increased to 52% (compared with only 30% in Table 13a). There are four possibilities which may account for this improvement.

i) In the first isolation (Table 13a) the protoplasts were isolated from a fully expanded leaf whereas in the second isolation

Table 14. Chromosome numbers for plants taken from shoot cultures in eleven cases where several arose from the same callus or sub-callus.

Callus number	Sub-callus	Shoot	Chromosome number
2	a	1	47
	a	2	47
	a	3	47
3	a	1	48
	a	2	44
6	a	1	47
	a	4	47
	b	5	47
	c	2	46
10	a	6	47
	b	1	49
11	a	1	46
	d	4	47
	e	3	44
	f	6	46
19	a	1	49
	a	2	48
	a	7	47
	a	8	48
	a	9	48
	a	10	47
	c	15	45
	e	4	49
	f	6	49
20	a	2	93
	a	4	93
23	a	1	48
	a	2	47
24	a	1	48
	a	3	47
29	a	1	46
	a	2	46
	a	3	46
74	a	1	46
	b	2	46

(Chromosomes counted by A. Karp)

(Table 13b) shoot culture leaves were used. If the results obtained were due to differences in the source material this would imply that there were more aneuploid cells present in the fully expanded leaf than in the immature shoot culture leaves. Although this seems unlikely, it cannot be ruled out.

ii) The culture regimes used in the two isolations were different. In the first isolation the protoplasts were cultured in Rm medium and in the second isolation protoplasts were grown in the low auxin Rg/LO system. Shoot morphogenesis was observed earlier on calluses from the Rg/LO system and after 10 weeks 70% of the calluses had produced shoots (Section 5.5), whereas with calluses from Rm only 30% of the calluses had produced shoots after 18 weeks (Secton 3.4). This agrees with the findings with dihaploid S. tuberosum, where the frequency of aneuploids increased when the duration of the callus phase was extended (Wenzel et al., 1979). It is unlikely that the differences in the Fortyfold regenerants were due directly to the different auxin levels as in a different experiment Majestic protoplasts were diluted with medium containing either 1.0 mg l^{-1} NAA or 0.1 mg l^{-1} NAA. Subsequently calluses from each treatment were treated identically. Chromosome numbers were obtained in more than 50 plants from each treatment but no different in the frequency of normal plants due to the different auxin levels could be discerned (A. Karp and G.P. Creissen, unpublished results).

iii) The differences could be a reflection of the variability which occurs between different isolations. Further isolations have been conducted to confirm or eliminate this possibility.

iv) The sample of 25 plants taken from each isolation was not representative of the whole population. Again results from repeat

experiments will help to confirm or eliminate this possibility.

I believe that the differences observed between the two Fortyfold isolations are a true reflection of improvements to the culture technique allowing morphogenesis to occur at an earlier stage than before.

6.4 PHENOTYPIC VARIATION IN FORTYFOLD REGENERANTS

6.4.1 First generation-pot grown plants

A total of 80 plants regenerated from 50 protoplast-derived calluses of Fortyfold were grown to maturity in a glasshouse. These included the plants for which chromosome numbers had been determined. Plants with obvious morphological abnormalities such as straggling growth habit, fused leaflets, lack of leaf formation and lack of tuber formation had been counted as aneuploids. Similar variation was also evident among protoplast-derived plants of Maris Bard (Thomas et al., 1982; Karp et al., 1982) and Bintje (Sree Ramulu et al., 1983). One regenerant, FTF36 which had the normal chromosome number (2n = 4x = 48) lacked any secondary or tertiary leaflets. Among the remaining Fortyfold regenerants morphological differences were not obvious. Whilst many of these plants were euploids (2n = 4x = 48) a proportion were found to be aneuploids. When mature, tubers from the regenerants were harvested. Control tubers from plants grown in pots under the same conditions as the regenerants were characteristically purple with white splashes. However all of 80 protoplast-derived plants (regenerants) produced tubers with white skins. This is discussed further in Section 6.4.4.

6.4.2 Second generation-pot grown plants

Two tubers from each regenerant with 48 or 47 chromosomes were sprouted as soon as possible after harvest and planted in compost in 25 cm plastic plant pots and kept in a 'cage' (consisting of a glass roof with open sides). The resulting plants were grown to maturity then measurements of 11 vegetative and seven tuber characteristics were taken for each plant (the primary data is given in Appendix II). A principle component analysis (Mardia et al., 1979) was used on the correlation matrix for the following 11 variables: Foliage-general impression; average internode length (cm); number of primary, secondary and tertiary leaflets; length to width ratio of the terminal leaflet; tubers-general impression; tuber shape; eye depth; tuber skin colour and average weight per tuber (g). Figure 17 shows the first two dimensions (together comprising 56% of the total variation) from the principal component analysis and gives the best two dimensional representation of the total variability in the data. The symbols represent the different populations; (48 chromosome regenerants, 47 chromosome regenerants and Fortyfold pot grown control plants maintained under the same conditions as the regenerants), whilst the numbers inside the symbols refer to the individual regenerants (two replicates). The contributions of the original variables to the principal axes are given in Table 15.

Several points are illustrated in Figure 17:

1) The scores for the control Fortyfold plants fall in a narrow band, giving an idea of the variability to be expected within a single euploid clone.

Table 15. Contributions of the variables to the principal axes shown in Figure 17.

Variable		Dimension 1	Dimension 2
Foliage-general impression	(V2)	-0.397*	-0.040
Average internode length	(V5)	-0.135	-0.423*
No. of 1° leaflets	(V6)	-0.375*	0.318
No. of 2° leaflets	(V7)	-0.406*	0.153
No. of 3° leaflets	(V8)	-0.279	0.342*
Terminal leaflet L/W	(V11)	-0.253	0.356*
Tubers-general impression	(V12)	-0.351	-0.264
Tuber shape	(V13)	0.147	0.278
Eye depth	(V14)	0.024	-0.542*
Skin colour	(V15)	-0.294	0.089
Average tuber weight	(V18)	-0.381*	-0.050
Percentage variance		39.4	17.4

The four variables contributing most to each dimension are denoted by *.

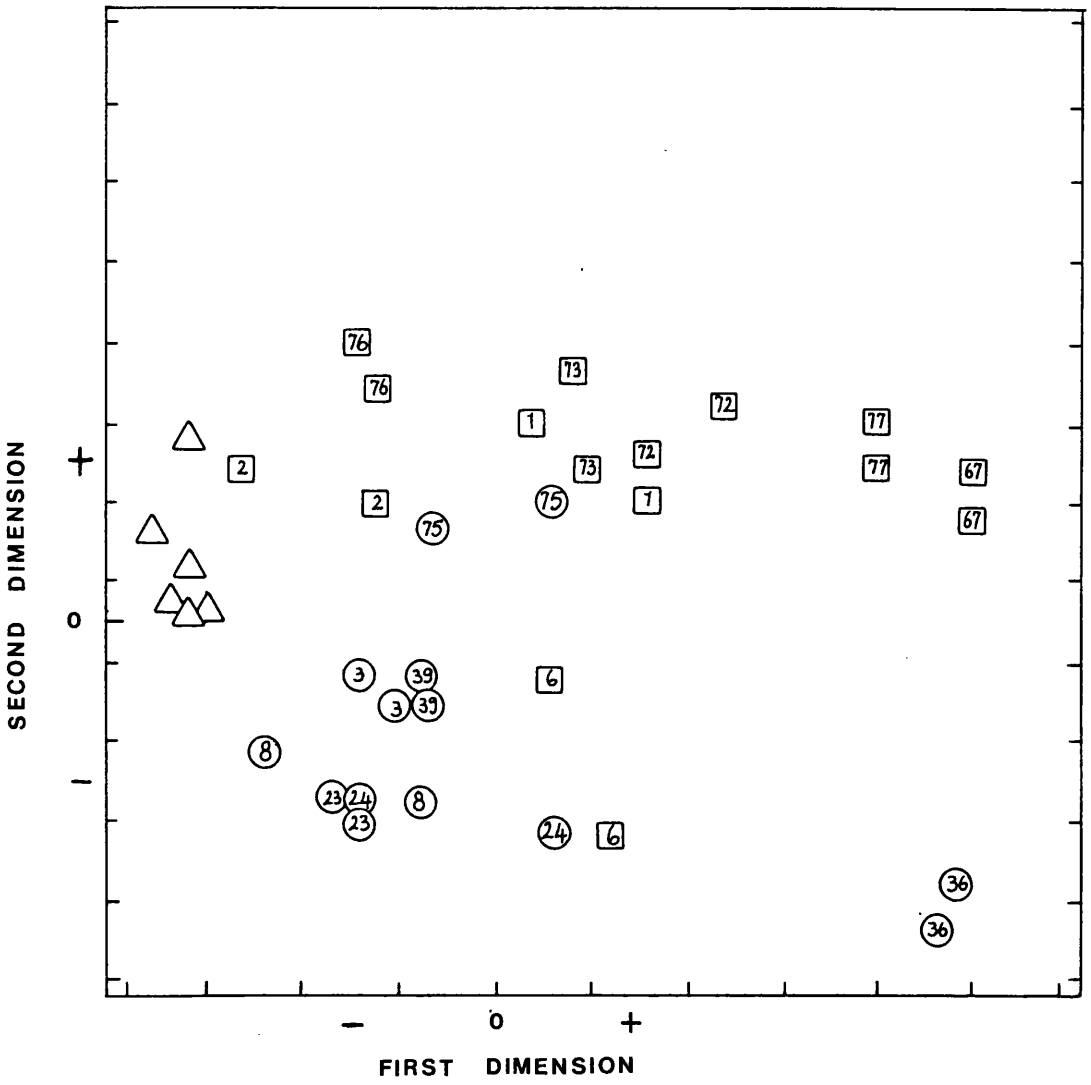


Fig.17 2-Dimensional Distribution Of Scores For Fortyfold Plants Following A Principal Component Analysis

Regenerants with 48 (○), and 47 (□) chromosomes ; Control plants (△)

2) The scores for the duplicate plants from each regenerant also lie very close together suggesting that the regenerant clones are quite uniform.
3) The distribution of scores along the first dimension separated the control population from the regenerants.
4) None of the scores for the regenerants coincided with those for the controls.
5) In the second dimension the distribution of scores allowed two populations to be discerned, one consisting mainly of 48 chromosome regenerants, the other mainly of 47 chromosome regenerants.

The variables which contributed most to the first dimension were average tuber weight, number of primary and secondary leaflets and general impression of the foliage (Table 15). The major components of the second dimension were average internode length, number of tertiary leaflets, length to width ratio of the terminal leaflet and eye depth of the tuber (Table 15).

Tuber Yield

The average tuber weight was derived from measurements of yield and number of tubers for each plant. The means for the total yield per plant and average tuber weight per plant were determined for the three populations (Controls, 48 and 47 chromosome regenerants), and compared using a students t-test (Table 16). The mean of the total yield per plant for the 48 chromosome population was not significantly different from that of the control population, but the average tuber weight was higher for the Controls. Both the total yield and average tuber weight were lower in the 47 chromosome population than in the controls. The means of total yield and average tuber weight for the

Table 16. Population means for tuber yield and internode length of Fortyfold plants.

Population	Total yield per plant (g)		Average tuber weight (g)		Average internode length (cm)	
	$\bar{x}$	CoV (%)	$\bar{x}$	CoV (%)	$\bar{x}$	CoV (%)
Controls	475	22.3	35.3	17.5	3.53	3.9
48	426	27.5	23.7	35.9	4.31	14.4
47	345	37.7	19.2	39.4	3.52	12.5
t-test						
Control v 48	$p>0.05$	(ns)	$p<0.01$	(**)	$p<0.01$	(**)
Control v 47	$p<0.05$	(*)	$p<0.001$	(***)	$p>0.05$	(ns)
48 v 47	$p>0.05$	(ns)	$p>0.05$	(ns)	$p<0.001$	(***)

CoV = Coefficient of Variation = $\frac{\sigma}{\bar{x}} \times 100$

ns = the means were not shown to differ

48 and 47 chromosome populations were not shown to differ. The coefficient of variation (CoV) of the means for average tuber weights for the 48 and 47 regenerant populations was twice that of the Controls. This confirms that the 48 regenerant population (and less importantly the 47 regenerant population) shows more variation than the control population. The ideal situation for a given character would be for the 48 regenerant population to have the same mean but a larger coefficient of variation than the control population, then one or more of the regenerants may show variation a plant breeder would consider to be useful.

Leaf characters

The arrangement and shape of the leaflets on a potato leaf are used by plant breeders to help identify a given variety (NIAB, 1975). A regenerant which gave a similar yield, but differed in a leaf character from the parental line would in theory be easier to classify as a new cultivar than a high yielding variant with no obvious changes in leaf morphology. When considered together the leaf characters (number of primary, secondary and tertiary leaflets, and the length to width ratio of the terminal leaflet) contribute a significant amount towards the overall variation (Table 15). One regenerant with 48 chromosomes (FTF36) was markedly different from the rest, with only three primary leaflets, and no secondary or tertiary leaflets (Appendix II). The yield of tubers from FTF36 was the lowest of the 48 chromosome regenerants. Two 47 chromosome regenerants (FTF67 and FTF77) had fewer primary leaves than the majority and leaves of FTF67 were a much darker green than any of the other regenerant clones. Both FTF67 and FTF77 gave low tuber yields. Variation in leaf

characters was also apparent among the other regenerants but was potentially more useful as it was less extreme.

Foliage - overall impression

The three regenerants (FTF36, FTF67 and FTF77) which differed most with regard to leaf character and yield were also shorter than the majority. The remaining plants were uniform and appeared healthy, though non were outstandingly better than the others. The 48 regenerants were taller overall than either the 47 or control populations, this is reflected in the measurements of average internode length (Table 16).

Tubers - depth of eye

With potatoes for domestic use it is desirable to have tubers with shallow eyes (Howard, 1978). One of the shortcomings of Fortyfold as a cultivar is that the eyes are fairly deep. Variation in eye depth towards shallower eyes would therefore be a useful form of variation in this old cultivar. A visual assessment of eye depth demonstrated that some variation was apparent but the extent of this variation would have to be evaluated properly in field grown material because of the limitations of looking at such a character in pot grown plants.

6.4.3 Third generation - Field grown plants

Second generation tubers were lifted in November 1982 then stored at 4°C in the dark until March 1983 when they were moved to a potato store to induce sprouting. Ten tubers from each regenerant, all 3-5 cm in diameter were sown in a replicated field trial in May 1983.

Thirty tubers, within the same size range from the Fortyfold plants (grown in pots with the regenerants for two generations) were sown at the same time. Figure 18a shows the field trial eight weeks after planting. Without exception the ten plants from each regenerant clone were uniform. Most of the regenerants appeared healthy with an upright bushy growth habit. The three regenerants (FTF36, FTF67 and FTF77) which performed badly in the second generation were markedly poorer in the field, none reached more than 25 cm in height. The poor growth of FTF77 is clearly shown in Figure 18a. Overall, the 47 chromosome plants were less vigorous than the 48 chromosome plants in the field, Figure 18b shows FTF24 (48 chromosomes) compared with FTF72 (47 chromosomes). Two regenerants with 47 chromosomes (FTF76 and FTF73) were as good as the 48 chromosome plants (regenerants or controls). A preliminary look at the leaf morphology of the field grown plants showed that the leaf characters observed in the second generation plants were carried over to the third generation field grown plants (Figure 19). A full analysis of the field grown plants will be conducted when the tubers have been harvested.

Figure 18. Field grown Fortyfold regenerants

a) Overall view of field plants showing FTF39, FTF8, FTF1 and FTF77 with two control (C) plots. Note the poor growth of FTF77.

b) FTF24 (48 chromosomes) and FTF72 (47 chromosomes). Note the uniformity among different plants from the same regenerant clone and the differences between the two regenerants.


8
C
77
39
1
C


47
48

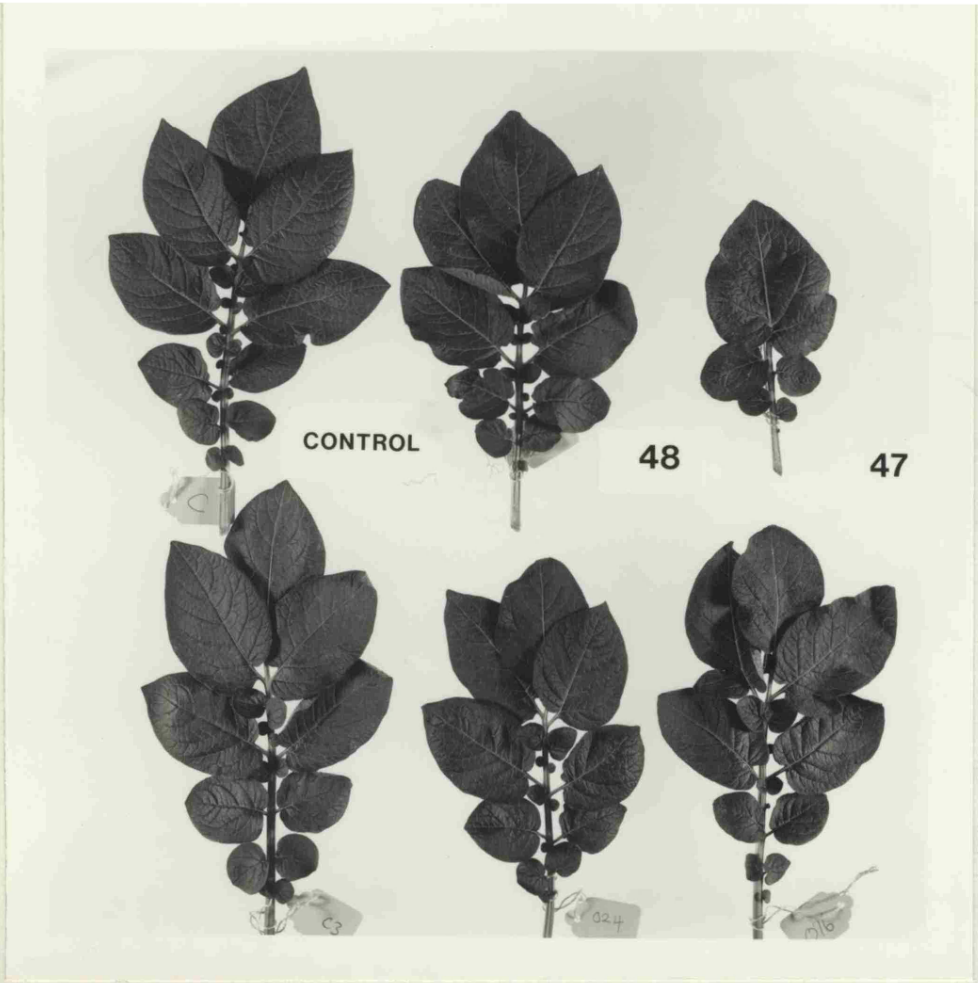


Figure 19. Leaves from field grown plants of Fortyfold Leaves are taken from two control plants, FTF8, FTF24 (48 chromosomes); FTF77 and FTF76 (47 chromosomes).

6.4.4 Tuber skin colour

When harvested tubers from the first and second generations of protoplast-derived Fortyfold plants were all white, whereas tubers from control Fortyfold plants grown under the same conditions (Section 6.4.1 and 6.4.2) were the characteristic purple splashed white (Figure 20a). First generation tubers were not stored at 4°C, but after storage of the second generation tubers at 4°C in the dark, purple patches were observed on the tubers from all but two of the regenerants (FTF36 and FTF67). Figure 20b shows the purple tubers from control Fortyfold plants and FTF6 alongside the white tubers from FTF36 after five months cold storage. A few tubers from FTF73, FTF8, FTF6, FTF24 and two control plants were dug up after four months in the field. Purple patches were present on tubers of FTF73, FTF8 and FTF6 but not FTF24. The control tubers were the normal purple splashed white.

There is evidence for the existence of at least six major genes controlling anthocyanin pigmentation in the tetraploid cultivated potatoes (Howard, 1970). Gene D is a basic gene which gives a brownish red colour in stems and inflorescences. Gene E gives a red colour in sprouts, stems and inflorescences; E-dddd plants have white tubers, but ED plants have tubers with a deep red colour in the periderm. Gene R causes a red colour in sprouts and stems. R-D plants have tubers with a deep red pigmentation in the phelloderm, no pigment in the periderm and colourless eyes; R-dddd plants have white tubers. Gene P converts the red of E and R to purple; without E, R or D, P will give purple sprouts and stems but not tubers. M restricts ED and PED tuber periderm pigmentation to areas around the eyes, causing the splashed effect. M has no effect on RD and PRD tuber

phelloderm pigmentation. F is a gene for flower colour (Howard, 1970).

The cultivar Fortyfold was bred by W. Paterson in the 1850's from a cross between a South American S. tuberosum line and the British cultivar 'Rock Potato' (McIntosh, 1927). I could not find any reference as to the genetics of tuber skin colour in Fortyfold, but the purple splashed white tubers are consistent with the genotype PEMD where D allows the expression of colour in the tubers, P in combination with E gives the purple coloration in the tuber periderm and M restricts the pigmentation to cause the splashed effect.

There are five possible explanations for the change from purple to white tubers in protoplast-derived Fortyfold plants.

1) An error was made and the leaf used as a protoplast source was not Fortyfold. This can be dismissed as many of the protoplast-derived plants have a leaf morphology very similar to that of Fortyfold and distinct from any other cultivar. Furthermore tubers from the regenerants were round with deep eyes and gave rise to sprouts and stems with purple pigmentation. All these traits are characteristic of the cultivar Fortyfold.

2) The possibility that the change to white tubers in Fortyfold regenerants was due to a mutation is unlikely because of the high frequency involved (all of 80 protoplast-derived plants gave white tubers). Frequencies at which induced mutations are recovered are usually about 1×10^5 for haploid genomes (Muller and Caboche, 1983; Steffen and Schieder, 1983).

3) A third possibility is that Fortyfold may be a periclinal chimera with a different genetic constitution in the L1 layer (which gives rise to the periderm and would thus have the genotype PEMD) to

that of the L2 and L3 layers (which make up the internal tissues including the tuber cortex and the leaf mesophyll). The sprouts on the white tubers from the Fortyfold regenerants were all purple. Sprout colour is due to anthocyanin pigmentation in the hypodermis which traces back to L2 (Asseyeva, 1931), thus gene P must still be present and the results could be explained if the L2 and L3 layers had the constitution P, PE, PEM or PMD. This would mean that all of the protoplast-derived Fortyfold plants originated from mesophyll protoplasts (L2) and none of the epidermal protoplasts (L1) survived to form shoots. Against the likelihood that Fortyfold is a periclinal chimera, eight plants regenerated from explants of tuber cortex (L2) all gave purple splashed white tubers suggesting that L2 does have the same genetic constitution as L1 (V.A. Wheeler, pers. comm.). Furthermore purple colour returned to the tubers following storage at 4°C, thus the genes for tuber colour must still be present, but for some reason are not expressed in the protoplast-derived plants.

4) Asseyeva (1931) found that after eye-excision of splashed tubers, so that adventitious bud formation took place, some of the resulting plants produced white tubers, and showed that the changes were not connected with any chimerical constitution of the starting material. Similar changes were also produced following X-ray treatment of splashed tubers (Howard, 1962, 1964 and 1967). When grown over several tuber generations the change was found to be stable (although a low rate of reversion to the original genotype was observed) but in sexual crosses the plants behaved in the same way as plants from untreated splashed tubers showing that the plants with white tubers still had the genes for splashed tubers (Howard, 1962). Howard (1967) showed that the phenomenon occured only when the gene M

was present, and proposed that the effect of M is due to some change connected with differentiation which occurs in the cytoplasm of cells and which somehow switches off the pigment producing enzyme system.

Can this phenomenon (termed the 'differentiation effect' by Howard, 1970) explain the results with Fortyfold? The splashed tuber phenotype of the cultivar Fortyfold is consistent with the presence of gene M. Secondly, Asseyeva (1931) found the effect to be common among a number of South American potato lines (S. tuberosum subspecies Andigena), and it is known that one of the parents of Fortyfold was a South American line (McIntosh, 1927). In that plants regenerated from protoplasts or from tuber explants originate from tissues which do not normally give rise to plants, the techniques may be compared to that of adventitious bud formation following eye excision. However, there are anomalies between the different systems: eight plants regenerated from tuber discs of Fortyfold all gave tubers with the normal purple splashed white phenotype. Of 12 plants from the eye excision experiments, eight gave white tubers and four gave the normal splashed phenotype (Howard, 1962) but all the tubers from 80 protoplast-derived plants were white. Also it does not explain the return of colour to tubers from the Fortyfold regenerants following cold storage. It may be that this is not linked as it is known that cold storage can stimulate colour expression in potatoes (Van Harten et al., 1981). In the present study an attempt to cross Fortyfold regenerants with a known white tubered variety to look at the inheritance pattern failed as the Fortyfold plants did not flower. The protoplast-derived plants used in this study all came from one isolation; plants have now been established from a second isolation and will be used to confirm the present results. Furthermore, plants regenerated from leaf pieces of

Fortyfold using the protocol of Webb et al. (1983) will be grown to maturity and tuber colour will be examined.

5) Other explanations include the possibility that the white tubers are due to a tissue culture carry over effect, and that in future field grown generations the purple splashed white phenotype will fully return. This would not explain why plants from tuber explants gave the normal tuber phenotype for Fortyfold.

Figure 20. Second generation Fortyfold tubers, showing differences in tuber skin colour.

a) Freshly harvested tubers from two 48 chromosome and two 47 chromosome regenerants. (Controls in the middle.)

b) Tubers after 5 months cold storage.

Top row: Five control tubers.

Bottom row: Five tubers from FTF6 (left) and FTF36 (right)


47
48
47
48
0 5 10 15
CM.

6.5 DISCUSSION

Following the regeneration of plants from protoplasts of the diploid species S. brevidens a cytological analysis showed that 24% of the regenerants were normal diploids (2n = 2x = 24), 52% had doubled up to become tetraploid (2n = 4x = 48) and the remaining plants were aneuploids with chromosome numbers between 46 and 96 (Table 12). The chromosome variation observed among S. brevidens regenerants was similar to that among plants regenerated from protoplasts of dihaploid S. tuberosum where the majority (99%) of the plants had doubled up in chromosome number (Wenzel et al., 1979). It had been suggested that spontaneous doubling of chromosomes may be caused by the presence of auxin in the protoplast culture medium (Wenzel et al., 1979). A ten fold decrease in the exogenous auxin concentration after six days culture (Section 5.3) increased the number of S. brevidens calluses which later underwent shoot morphogenesis but did not alter the pattern of chromosomal variation among the regenerated plants (Table 12). This implies either that the chromosome doubling occurs during the first six days of cultue or that the level of auxin is not important in this context.

Chromosome numbers of plants regenerated from protoplasts of the tetraploid S. tuberosum cultivar Fortyfold revealed that 30% of the plants were normal tetraploids (2n = 4x = 48) and the remaining plants were aneuploids within the range 2n = 48 ± 2 (Table 13). As found with S. brevidens a reduction in the auxin concentration after six days culture improved the efficiency of shoot production from Fortyfold calluses, but in contrast to S. brevidens it also led to an increase in the proportion of normal plants recovered (Table 13b and Section

6.3.2).

The nature of the chromosomal variation in Fortyfold regenerants differed from that observed among regenerants from the dihaploid *S. tuberosum* lines (Wenzel *et al.*, 1979) and *S. brevidens* where chromosome doubling was common. The differences are probably due to the difference in ploidy levels of the starting material and are consistent with results for plants regenerated from leaf explants of potato, where all of plants regenerated from leaf pieces of the monohaploid Mn797322 had doubled up whereas the majority of plants regenerated from leaf explants of the tetraploid cultivar Desiree were still tetraploid (A. Karp and S.W.J. Bright, unpublished results). Similarly, with *Crepis capillaris* a higher frequency of change in ploidy was found in callus derived from a haploid plant compared with callus from a diploid plant (Sacristan, 1971).

The pattern of chromosomal variation in Fortyfold regenerants was very different from that observed in plants regenerated from protoplasts of the tetraploid cv Maris Bard which were characterised by high chromosome numbers and a wide range of aneuploidy (46-93); only one Maris Bard regenerant from 26 examined had the normal chromosome complement (Karp *et al.*, 1982). It was concluded that such a large difference was more likely to be the consequence of using two different methods of regeneration than to differences between cultivars (Karp *et al.*, 1982). In support of this, plants regenerated from protoplasts of cv Majestic (2n = 4x = 48), using the same technique as used for Fortyfold, showed a similar pattern of chromosome variation to that obtained for Fortyfold regenerants (G.P. Creissen and A. Karp, unpublished results).

In their discussion Karp *et al.* (1982) explored the possible

origins of chromosome variation. One possibility which could not be eliminated was that the changes in chromosome number occurred at part of the differentiation of the leaf and by forcing the cells into regeneration these changes were revealed. Since then cytophotometric measurements of DNA content in protoplasts isolated from S. tuberosum cv. Astarte demonstrated that all the cells contained the correct amount of DNA for the tetraploid level (Jacobsen et al., 1983). This data eliminates the possibility that cells with different ploidy levels are present in the leaf, although the method is not accurate enough to detect aneuploids within the range 48 ± 2. The possibility that the differences between regenerants of Maris Bard and Fortyfold were because Maris Bard protoplasts were isolated from shoot cultures whereas Fortyfold protoplasts were isolated from leaves of whole plants can now be discounted as in a different isolation shoot cultures of Fortyfold were used as a protoplast source, but the pattern of chromosome variation in the regenerants was similar to that from the first isolation (Table 13).

By recording the pedigree of each shoot taken from a protoplast-derived callus (Section 6.3.2) it was possible to look at the variation between plants from different calluses and between different plants from the same callus. Using this approach, a cytological analysis revealed there to be chromosome variation both between (Table 13) and within (Table 14) calluses of Fortyfold. Variation in different plants from the same callus provides strong evidence that the variation originated during culture, (although the possibility that the callus arose from more than one protoplast cannot be eliminated). There is considerable evidence that chromosome changes do occur in the callus phase (Orton, 1980; Constantin, 1981).

The levels of exogenous auxin and cytokinin are known to influence chromosomal variation (Bayliss, 1980). Protoplast-derived calluses of Fortyfold grown in the low auxin Rg/LO system showed a higher morphogenetic capability and gave rise to a higher proportion of normal tetraploid plants than calluses grown in the orginal high auxin system (Table 13). Ths demonstrates that suitable manipulation of the hormones used during protoplast culture and plant regeneration can increase the stability of the system. In turn this may provide the more exacting conditions necessary for the growth of lines which have so far failed to respond.

Phenotypic variation has been reported in protoplast-derived plants of Russet Burbank (Shepard, 1980a; Shepard et al., 1980; Secor and Shepard, 1981), Maris Bard (Thomas et al., 1982), Bintje (Sree Ramulu et al., 1983) and now Fortyfold. Morphological variation in regenerants of Maris Bard correlated with changes in chromosome number, only one of the regenerants had the correct chromosome number (Karp et al., 1982). Similarly, in the report on phenotypic variation in Bintje regenerants, observations were made mainly on plants showing changes in chromosome number and gross variation (Sree Ramulu et al., 1983). Although a number of plants with the normal chromosome complement were regenerated, these remain to be characterised. It is doubtful whether plants showing gross variation will be of any use in potato improvement; to assess the true value of somaclonal variation it is necessary to examine the variation among plants with the normal chromosome complement. In their work with Russet Burbank, Shepard and his coworkers discarded the wild aberrant plants (which probably included many aneuploids) and looked at the variation among the remaining plants. Chromosome counts

revealed that five lines (from a population of 1700 regenerants) which showed promise for higher yield all had the tetraploid number of chromosomes (Shepard, 1980a). Much of the obvious phenotypic variation among Fortyfold protoplast-derived plants correlated with aneuploidy. However not all of the aneuploids could be readily distingusihed from the tetraploid regenerants. In the second generation phenotypic variation among the 47 and 48 chromosome regenerants was examined in more detail with the aid of a principal component analysis. This enabled a comparison of the variation between the 47 regenerant and 48 regenerant populations. A significant difference was observed in the average internode length, (which reflected differences in height), the 47 chromosome regenerants being shorter than the 48 chromosome regenerants. This was confirmed in the following field grown generation. Variation was also observed in a number of characters, including leaf morphology and tuber yield, among the 48 chromosome regenerants. Analysis of the field grown material will be necessary to assess the full extent of this variation.

The presence of variation in plants regenerated from tissue culture raises the question as to its basis. Several factors including a) karyotype changes, b) chromosome or gene rearrangements, c) gene amplification or depletion d) single gene mutation and e) extranuclear changes may be involved (Larkin and Scowcroft, 1981). Changes in chromosome number were evident in protoplast-derived potato plants of cv Fortyfold and S. brevidens but they cannot be the sole explanation as variation was also present among regenerants with the normal chromosome number. Despite the small size of potato chromosomes, translocations have been detected in at least three of the Fortyfold regenerants (A. Karp, pers. comm.). There have been

several reports of single gene mutations in plants regenerated from complex explants, including potato (Van Harten et al., 1981) and tomato (Evans and Sharp, 1983) and from protoplasts of Nicotiana sylvestris (Prat, 1983). It would seem that a whole spectrum of genetic changes may occur as a result of tissue culture. However, our understanding of what is happening is far from complete. For instance the basis for the change in tuber skin colour in Fortyfold regenerants is not known even though a similar phenomenon was observed following eye excision experiments on splashed tubers over fifty years ago (Section 6.4.4). Whether or not variation in protoplast-derived plants is useful for plant breeding it is clear that an understanding of the causes and basis of variation will increase the usefulness of protoplast techniques.

CHAPTER 7

GENERAL DISCUSSION

7.1 INTRODUCTION

In the continuing quest for improved agronomic performance, plant breeders have been quick to realise the value of new techniques which may increase their ability to manipulate crop plants. Increasingly, *in vitro* techniques have been employed alongside more traditional methods. The latest of the *in vitro* methods to attract the interest of the plant breeder is that of protoplast culture. Studies with 'model' species including *Nicotiana*, *Petunia* and *Hyoscyamus* have demonstrated the potential of protoplasts for the production of somatic hybrids (Evans, 1983), biochemical mutants (Bourgin *et al.*, 1982) and as a basic tool for plant genetic engineering (Cocking *et al.*, 1981; De Block and Herrera-Estrella, 1983; Hain *et al.*, 1983). While there is still a need for developmental work with these model species, the time is now right for the application of such techniques to the improvement of crop plants. A prerequisite for such work is the ability to regenerate plants from protoplasts of a range of cultivars of the crop species in question. In this thesis I have focussed on one crop, the potato, and a related wild species, *S. brevidens*, which has valuable genes for resistance to Leaf Roll Virus (Jones, 1979). During the period of my work several groups have also been interested in the application of techniques for the culture of protoplasts to a range of potato cultivars (Shepard, 1980a; Gunn and Shepard, 1981; Bokelmann and Roest, 1983; Carlberg *et al.*, 1983). The methodology will now be discussed.

7.2 PROTOPLAST ISOLATION

In a series of reports Shepard and co-workers (Shepard and Totten, 1977; Shepard, 1980a; Shepard, 1980b; Shepard *et al.*, 1980) described a technique for the regeneration of plants from mesophyll protoplasts of *S. tuberosum* cv Russet Burbank. The success of this protocol relied on the use of a uniform starting material. This was achieved by growing the protoplast source plants from tubers in a carefully controlled environmental and nutritional regime followed by a series of conditioning steps prior to protoplast isolation. The technique was then applied, without modification, to the regeneration of plants from protoplasts of several other American (Shepard, 1980a) and British cultivars (Gunn and Shepard, 1981).

An alternative approach to the same problem was developed by Binding (1974) who used shoot cultures of *Petunia hybrida* as a source of protoplasts. The use of shoot cultures was then shown to be suitable for the isolation and culture of several dihaploid (Binding *et al.*, 1978) and tetraploid lines of potato including Maris Bard (Thomas, 1981) and Bintje (Bokelmann and Roest, 1983; Carlberg *et al.*, 1983).

My experiments with Fortyfold confirmed that the conditions stipulated by Shepard (Section 2.4.1) for the growth of protoplast source material were applicable to U.K. cultivars, but problems wre encountered due to tuberisation of the plants which meant that the method was not suitable all year round (Section 3.5). The use of shoot cultures of Fortyfold as a source of protoplasts overcame the problems found with whole plants to make protoplast isolations more reproducible, and when grown in ventilated glass jars the protoplast

yields were as high as those from leaves of whole plants. Shoot cultures grown on BM with 0.05 mg l^{-1} BAP in ventilated jars gave protoplast yields in the order of 1.5 x 10^6 g FW^{-1} for several *S. tuberosum* lines and ca. 6 x 10^6 g FW^{-1} for *S. brevidens* (Section 5.5; Table 11).

7.3 PROTOPLAST CULTURE

The basal medium of Murashige and Skoog (1962) was used by Shepard to develop the media employed for the culture of Russet Burbank protoplasts (Shepard, 1980a). Freshly isolated protoplasts of Russet Burbank were suspended in CL medium (with incresed concentrations of major salts, some additional sugars, 1.0 mg l^{-1} NAA and 0.4 mg l^{-1} BAP) then plated in divided petri dishes next to a reservoir of R medium. In my hands this system was not any better for the culture of Fortyfold or *S. brevidens* protoplasts than the use of Rm medium (effectively a mixture of CL and R media: Appendix I), suggesting that the elevated concentrations of major salts may be less important for cultivars other than Russet Burbank (Section 3.3.4). Protoplasts cultured in Rm at 4 x 10^4 ml^{-1} then diluted to 1 x 10^4 ml^{-1} with fresh Rm medium gave plating efficiencies of ca. 1% for Fortyfold (Section 3.3.4) and 5% for *S. brevidens* (Section 4.3). These results are comparable with results obtained for other cultivars e.g. plating efficiencies of 0.3% for Maris Piper and Feltwell (Gunn and Shepard, 1981), 0.1% for Maris Bard (Thomas *et al.*, 1982) and 5% for Bintje (Bokelmann and Roest, 1983). Plating efficiencies of up to 50% were reported for Russet Burbank (Shepard, 1980a) and the dihaploid line H^2140 (Binding *et al.*, 1978) but it

is difficult to compare these directly with the others as neither report defines how the plating efficiencies were calculated.

Experiments on the effects of different nitrogen sources on the growth of S. brevidens protoplasts revealed a combination of potassium nitrate and glutamine to be the most suitable. The concentration of glutamine was optimal at 5 mM, resulting in plating efficiencies of ca. 10% at 1 x 10^4 ml^{-1} (Section 5.2). Thus Rg medium (Rm plus 5 mM glutamine) was used for the culture of Solanum protoplasts.

A major limitation of the standard protocols for potato protoplasts is that population densities of 1-4 x 10^4 ml^{-1} are necessary for protoplast growth (Binding et al., 1978; Shepard, 1980a; Thomas, 1981; Bokelmann and Roest, 1983). At population densities below 1 x 10^4 ml^{-1} the plating efficiency for S. brevidens protoplasts in Rg medium dropped rapidly with decreasing population density to give a plating efficiency of 2% at 5 x 10^3 ml^{-1} and zero at densities less than 4 x 10^3 ml^{-1} (Section 5.3). When the protoplasts were diluted after six days culture with a low auxin medium (LO medium modified from the Ag medium of Caboche, 1980) the plating characteristics improved considerably to give a plating efficiency of 2% at 1 x 10^3 ml^{-1}; thus supporting growth at only 20 colonies ml^{-1} (Section 5.3). This with the results obtained for four other Solanaceous species (Muller et al., 1983) suggests that the use of low auxin media for growth of protoplast-derived cells at low densities may be generally applicable (at least within the Solanaceae). The improved plating characteristics obtained will facilitate the use of potato protoplasts in selection, fusion and transformation studies. In three separate isolations protoplasts of

Fortyfold or Ukama were cultured using the Rg/LO system. In each case callus formation followed by shoot morphogenesis was observed (Section 5.5). Protoplasts isolated from shoot cultures of PDH 119, PDH 440 and Mn797322 were also cultured in the Rg/LO system but did not undergo sustained divisions (Section 5.5).

A comparison of the free amino acid content in leaves and freshly isolated protoplasts showed that the levels of four amino acids; glutamine, asparagine, cysteine and arginine, were noticeably lower in protoplasts than in the source leaves. The addition of 5 mM glutamine was shown to increase the plating efficiency of S. brevidens protoplasts (Section 5.2). Similar experiments with the other three amino acids may prove to be beneficial. Further increases may result from studies on the effects of Krebs cycle acids on protoplast growth (Section 5.6). Recently the use of agarose as a gelling agent in the protoplast culture medium was shown to improve the plating efficiency of Hyoscyamus muticus protoplasts and to stimulate divisions in protoplasts of a mutator gene line of Petunia hybrida which had previously proved unresponsive (Shillito et al., 1983). Increased plating efficiencies were also obtained for protoplasts of tomato and potato cultured in medium with agarose (Adams and Townsend, 1983). Work in these areas may provide the conditions necessary for the successful culture of the monohaploid and dihaploid lines, as well as leading to higher plating efficiencies for Fortyfold, Ukama and S. brevidens.

7.4 PLANT REGENERATION

Following the original procedure of Shepard (1980a) protoplast-

derived colonies were transferred to C medium with 0.1 mg l^{-1} NAA and 0.4 mg l^{-1} BAP after 20 days thereby reducing the auxin concentration. In my experiments calluses on C medium turned green over a period of one month, and when transferred to D medium underwent morphogenesis. With the Rg/LO system described in this thesis (Section 5.5) the auxin concentration was lowered after only six days in culture. As well as improving the plating characteristics, this meant that the calluses started to turn green before transfer to C medium. As a result calluses after one month on C medium were a deeper green and when transferred to regeneration medium, a higher percentage formed shoots.

Calluses of S. brevidens transferred to regeneration medium developed shoot primordia over a range of cytokinin/auxin combinations, but the highest percentage shoot production was obtained on medium with 1.0 mg l^{-1} zeatin and 0.1 mg l^{-1} BAP, followed by transfer to medium containing GA_3 (MT8 medium). Shoot production efficiencies of 90% for S. brevidens, 70% for Fortyfold and 40% for Ukama were obtained using this regime (Section 5.5). Shepard (1982) increased the level of zeatin in D medium to 2.0 mg l^{-1} (from 0.5 mg l^{-1} in earlier reports) on which 30% of 'Superior' calluses and 40% of Russet Burbank calluses formed shoots. The use of GA_3-containing medium after a regeneration medium was also found to enhance shoot production in protoplast derived calluses of Bintje (Bokelmann and Roest, 1983) and several British potato lines (R.E. Gunn, pers. comm.).

The protocol developed in this thesis has now been used for the successful regeneration of plants from protoplasts of ten U.K. cultivars, Fortyfold and Ukama (Section 5.5), King Edward, Majestic,

Maris Piper and Champion (Bright et al., 1983), Desiree, Pentland Crown, Record and Myatts Ashleaf (G.P. Creissen, D. Foulger and V.A. Wheeler, unpublished results). The plating efficiencies I have reported here for S. tuberosum and S. brevidens are still low when compared with the plating efficiencies obtained for some model species e.g. Nicotiana tabacum 80-95%, N. sylvestris 7-28%, N. plumbaginifolia 60-70%, Petunia "Mitchell" 9-36% (Muller et al., 1983) and Hyoscyamus muticus 40% (Wernicke and Thomas, 1980). Thus further improvements would be desirable for work involving the use of potato protoplasts. However the system I have developed is now more reproducible, with plants obtained from every isolation using the tetraploid cultivars Fortyfold and Ukama and the wild relative S. brevidens. The uses of potato protoplasts will now be considered.

7.5 USES OF POTATO PROTOPLASTS

7.5.1 Somaclonal variation

Plants regenerated from protoplasts of Fortyfold showed considerable phenotypic variation (Section 6.4). Much of the gross variation correlated with aneuploidy, as found with protoplast-derived plants of Maris Bard (Karp et al., 1982) and Bintje (Sree Ramulu et al., 1983). A more detailed examination of the Fortyfold regenerants in the second vegetative generation revealed that plants with the normal chromosome number showed variation in one or more of a number of morphological characters including plant height, leaf morphology, tuber yield and eye depth. This confirms the earlier findings with Russet Burbank that plants regenerated from protoplasts show potentially useful variation in a number of agronomically important

characters (Shepard, 1980a; Shepard *et al.*, 1980; Secor and Shepard, 1981). Bright *et al.* (1983) mentioned two criteria which must be satisfied if *in vitro* methods are to make an impact on any breeding scheme. Firstly, the methods should produce a plant which is fit to enter a breeding or screening programme and compete effectively with plants produced by conventional improvement programmes. Secondly, the 'improved' plants should be obtained in an economical and efficient way so that the methods may be applied, in conjunction with conventional breeding and assessment programmes with the minimum need for highly skilled manpower or expensive equipment.

Howard (1978) described a potato breeding scheme as used by the Plant Breeding Institute, Cambridge. Starting from an initial population of 25,000 seedlings it is possible after nine years to end up with one clone worthy of entry into statutory performance trials. In comparison, in 1977 an initial population of 1,700 protoplast-derived Russet Burbank clones were tested. After three years 65 of the clones had survived for further evaluation (Secor and Shepard, 1980). After only six years, one of the clones was selected for performance trials (D. Bidney, pers. comm.). The results at this stage are very promising, although it is still too early to know what the full outcome will be.

There is mounting evidence that *in vitro* techniques involving a callus stage are mutagenic (Van Harten *et al.*, 1981; Prat, 1983; Evans and Sharp, 1983), thus it would seem pertinent to compare variation induced by tissue culture with that obtained by mutation breeding programmes. In their work with tomato, Evans and Sharp (1983) demonstrated that the mutations observed in plants regenerated from complex explants were similar to those that occur spontaneously

or after mutagenic treatment. They found the level of chimerism to be very low in tissue culture derived tomatoes whereas it presents a real problem in mutation breeding programmes. On this basis, tissue culture techniques may be preferable to mutation breeding. However, results reported in this thesis for protoplast-derived plants of Fortyfold have shown that a whole spectrum of changes may be found in tissue culture regenerants ranging from changes in ploidy, aneuploidy, chromosome structural changes and changes which may be attributed to classical single gene mutations. Some of the observed variation, such as the changes in tuber skin colour does not seem to comply with normal genetic behaviour (Section 6.4.4).

The results of several groups (Evans and Sharp, 1983; Van Harten et al., 1981; Bright et al., 1983) emphasise that somaclonal variation is not unique to plants regenerated from protoplasts. The techniques used for the regeneration of plants from complex explants are both simpler and more rapid than that of the protoplast culture system. Furthermore, the frequency of aneuploidy from complex explants tends to be much lower than with protoplast-derived plants (Jones et al., 1983). At present, it would appear that the use of complex explants may be preferable for breeding purposes, although it may be that protoplasts give a wider spectrum of changes. There is now a need to investigate and compare the variation present in plants derived from complex explants or protoplasts from the same source.

Whilst such changes as single gene mutations may be desirable as far as somaclonal variation is concerned, it is evident that other changes are less desirable. A reduction in the proportion of aneuploid plants regenerated from protoplasts would be certain to enhance the usefulness of protoplast systems, as would a greater

understanding of events such as changes in tuber colour.

7.5.2 Somatic hybridisation

A number of somatic hybrid plants have been recovered from fusion experiments with S. tuberosum as one of the fusion partners (Melchers et al., 1978; Butenko and Kuchko, 1980; Shepard et al., 1983; Binding et al., 1983). These experiments were discussed in Section 1.5.4. In each case chromosome instability was evident in the somatic hybrid plants; indeed aneuploidy is common and has been observed in most somatic hybrid combinations (Evans, 1982). In a few cases loss of chromosomes proved to be useful as with somatic hybrids between Arabidopsis thaliana and Brassica campestris where chromosome elimination was necessary for shoot morphogenesis (Hoffman and Adachi, 1981). Although such plants have been of immense value in the study of the behaviour of fusion products and subsequently somatic hybrids, it is dubious whether any of these will make an impact as far as potato improvement is concerned.

One factor known to be important in studies on somatic hybridisation is the taxonomic relationship of the parents (Evans, 1982; Power and Chapman, 1983). A second factor would seem to be the ploidy level of the starting material. Cytological analysis of protoplast-derived potato plants revealed a range of chromosome variation (Karp et al., 1982; Sree Ramulu et al., 1983). In both reports the frequency of aneuploids about the hexaploid (2n = 72) and octaploid levels was much higher than the frequency of euploid plants suggesting these high levels to be inherently unstable in potato. There are seven hexaploid species in the section Petota, (none of which are placed in the series Tuberosa) but there is no record of an

octaploid potato species (Hawkes, 1978b). In three of the reports on somatic hybridisation of potato the material was polyploid to begin with (Butenko and Kuchko, 1980; Shepard *et al.*, 1983; Binding *et al.*, 1983); it is not surprising then that chromosome instability was observed in the somatic hybrid plants. In only one report, (Melchers *et al.*, 1978) was a dihaploid *S. tuberosum* line fused with a diploid species (tomato) which could theoretically result in the production of a stable tetraploid, unfortunately though, a suspension culture of potato was used which was probably aneuploid to start with.

To produce a somatic hybrid between *S. tuberosum* and *S. brevidens* it should be possible to fuse diploid *S. brevidens* with either tetraploid, dihaploid or monohaploid *S. tuberosum*. The protocol for the isolation and culture of protoplasts described in this thesis now works for a range of tetraploid cultivars, although it has not yet been applied successfully to the haploids. The use of dihaploid *S. tuberosum* as a fusion partner would appear to be the most desirable as it increases the chances of obtaining a stable, tetraploid somatic hybrid which may even prove to be fertile.

If protoplast fusion techniques are to be of practical use in plant breeding, the development of suitable selection systems is vital. Without selectable markers it could prove difficult to distinguish somatic hybrids from protoplast regenerants exhibiting somaclonal variation. This will be an important consideration in the evaluation of breeding schemes such as that proposed by Wenzel *et al.* (1979) where protoplasts of different dihaploid cultivars are to be fused to create new tetraploid cultivars. As the first step towards a selection system for potato I have developed a protocol for

growth of protoplast-derived cells at low population densities enabling the selection of colonies after six days culture.

7.5.3 Mutant selection

At the start of the work described in this thesis, potato protoplast techniques were too inconsistent for work involving the use of protoplasts. Over a period of three years the techniques for tetraploid S. tuberosum and diploid S. brevidens were made more reproducible, with consistently higher protoplast yields, higher plating efficiencies, improved plating characteristics (low density plating) and more efficient plant regeneration. Will it now be possible to use the protocol for the selection of mutants? Spontaneous mutation frequencies for resistance to 5-methyltryptophan of 3×10^{-7} in carrot and 1.5×10^{-6} in tobacco were reported (Widholm, 1977). Similarly, the spontaneous mutation frequency for valine resistance in tobacco was less than 3×10^{-6} (Caboche and Muller, 1980). For 1 ml of S. brevidens protoplasts at 1×10^{4} ml^{-1}, a 6% plating efficiency would give 600 colonies, of which ca. 90% should undergo morphogenesis to give 540 plants. Assuming a spontaneous mutation frequency of 5×10^{-6}, nearly 10 l of S. brevidens protoplast suspension would be needed to recover one mutant plant' Different mutagenic treatments may be used to give increased mutation frequences of up to 4×10^{-4} (Weber and Lark, 1980; Caboche and Muller, 1980; Muller and Caboche, 1983; Negrutiu, 1981). With mutagen dosages causing 50% lethality, 150 ml of S. brevidens protoplasts would give a putative mutant. However, there is evidence that a tissue culture cycle may be mutagenic (Van Harten et al., 1981; Evans and Sharp, 1983; Prat, 1983) and variants have been

recovered at very high frequencies. In potato, from a population of 4 x 10^4 calluses, 36 showed resistance to culture filtrate of Phytophthora infestans (Benkhe, 1979). With Nicotiana tabacum tissue culture variability affecting a single locus (the sulphur locus) was found in the frequency of 1-6 x 10^{-2} (Lorz and Scowcroft, 1983). At the highest frequency of 6 x 10^{-2}, 1 ml of S. brevidens protoplasts at 1 x 10^4 ml^{-1} should result in the recovery of 32 putative mutants' Whilst the lack of success with plant regeneration from protoplasts of monohaploid potatoes means that selection for recessive mutations is still not practicable, it may now be possible to use the protocol described in this thesis to select for dominant mutations in S. brevidens and tetraploid S. tuberosum.

7.5.4 Transformation

Various methods have been described for the transfer of a limited amount of specified genetic information into plant cells (Cocking et al., 1981). A number of methods involve the manipulation of protoplasts (e.g. De Block and Herrera-Estrella, 1983; Hain et al., 1983; Paszkowski et al., 1983; Krens et al., 1983). As yet none of these techniques have been successfully applied to S. tuberosum, although transformed potato plants have been obtained following infection of S. tuberosum shoot cultures with Agrobacterium tumefaciens (Ooms et al., 1983). In the near future it is highly probable that a vector system applicable to the potato will be developed. A major limitation of such work must first be overcome before the technique will be really useful; that is the lack of suitable genetic material with which to transform the potato.

Over the next 20 years the world needs to double its output of food simply to keep pace with the increase in population (Tudge, 1983). Thus the work of the plant breeder has never been more important. Protoplast techniques will in one way or another prove an invaluable extension to conventional breeding methods.

APPENDIX I

MEDIA

LIST OF MEDIA

Media were either autoclaved or filter sterilised, as shown with formulations.
To add agar to filter sterilised medium, agar made up in 100 ml medium was autoclaved and then added to the bulk of the filter sterilised medium.

	BASAL MEDIUM (BM)		POTATO FERTILISER SOLUTION		A MEDIUM	
	mgl^{-1}	mM	mgl^{-1}	mM	mgl^{-1}	mM
NH_4NO_3	1650	48.5	1650	48.5	-	-
KNO_3	1900	18.8	1900	18.8	190	1.88
$CaCl_2.2H_2O$	440	3.0	880	6.0	44	0.30
$MgSO_4.7H_2O$	370	1.5	370	1.5	37	0.15
KH_2PO_4	170	1.25	350	2.5	17	0.125
Na_2EDTA	37.3	0.2	37.3		3.7	0.02
$FeSO_4.7H_2O$	27.8	0.1	27.8		2.8	0.01
H_3BO_3	6.2	0.1	6.2		0.6	0.01
$MnSO_4.4H_2O$	22.3	0.1	22.3		2.2	0.01
$ZnSO_4.6H_2O$	8.6	0.03	8.6		0.9	$3x10^{-3}$
KI	0.83	$5x10^{-3}$	0.83		0.08	$0.5x10^{-3}$
$Na_2MoO_4.2H_2O$	0.25	$1x10^{-3}$	0.25		0.03	$0.1x10^{-3}$
$CuSO_4.5H_2O$	0.025	$0.1x10^{-3}$	0.025		0.003	$0.01x10^{-3}$
$CoSO_4.6H_2O$	0.025	$0.1x10^{-3}$	0.025		0.003	$0.01x10^{-3}$
Glycine	2.0	0.03_{-3}	-	-	0.2	$3x10^{-3}$
Nicotinic Acid	0.5	$4x10^{-3}$	-	-	0.5	$4x10^{-3}$
Pyridoxin HCl	0.5	$2.4x10^{-3}$	-	-	0.05	$0.3x10^{-3}$
Thiamin HCl	0.1	$0.3x10^{-3}$	-	-	0.05	$0.15x10^{-3}$
Folic Acid	-	-	-	-	0.05	$0.1x10^{-3}$
Biotin	-	-	-	-	0.005	$0.002x10^{-3}$
Sucrose	20 gl^{-1}	58.0	-	-	-	-
Myo-Inositol	100	0.55	-	-	10.0	0.055
NAA	-	-	-	-	2.0	
BAP	-	-	-	-	0.5	
Agar	6 gl^{-1}		-	-	-	-
pH	5.6		5.6		5.6	
Autoclave (A)/ Filter Sterilise (FS)	A		A		A	
Reference	Murashige & Skoog, 1962		Shepard, 1980a		Shepard & Totten, 1977	

DERIVATIVES OF BASAL MEDIUM

BASAL MEDIUM PLUS	SHOOT CULTURE MEDIUM	ROOTING MEDIUM	MT8 MEDIUM
NAA (mgl^{-1})	-	0.05	-
BAP (mgl^{-1})	0.05	-	0.25
GA_3 (mgl^{-1})	-	-	0.1
Agar (gl^{-1})	6.0	6.0	10.0 (Bacto-agar)
pH	5.8	5.6	5.6
Autoclave (A) or Filter sterilise (FS)	A	A	A FS GA_3

0.45 M MANNITOL SOLUTION

KNO_3	190 mgl^{-1}
$CaCl_2.2H_2O$	44
$MgSO_4.7H_2O$	37
KH_2PO_4	17
Mannitol	82 gl^{-1} (0.45 M)
pH	5.6

Autoclave

ENZYME SOLUTION EIV

KNO_3	190 mgl^{-1}
$CaCl_2.2H_2O$	44
$MgSO_4.7H_2O$	37
KH_2PO_4	17
MEICELASE P	1.5%
PECTOLYASE Y23	0.1%
Mannitol	Vary to required osmolality
pH	5.6

Filter sterilise

PROTOPLAST CULTURE MEDIA

	CL		R		Rm		Rg		LO	
	mgl^{-1}	mM	mgl^{-1}	mM	mgl^{-1}	mM	mgl^{-1}	mM	mgl^{-1}	mM
NH_4NO_3	-	-	-	-	-	-	-	-	800	10
KNO_3	7600	75.2	1900	18.8	4700	46.5	4700	46.5	1010	10
$CaCl_2.2H_2O$	1760	12.0	440	3.0	1100	7.5	1100	7.5	440	3.0
$MgSO_4.7H_2O$	1480	6.0	370	1.5	950	3.9	950	3.9	790	3.0
KH_2PO_4	680	5.0	170	1.25	430	3.2	430	3.2	140	1.0
	mgl^{-1}	mM							mgl^{-1}	mM
Na_2EDTA	18.5	0.1	18.5		18.5		18.5		37	0.2
$Fe SO_4.7H_2O$	13.9	0.05	13.9		13.9		13.9		27	0.1
	mgl^{-1}	µm							mgl^{-1}	µm
H_3BO_3	3.0	50	3.0		3.0		3.0		3.0	50
$MnCl_2.4H_2O$	9.9	50	9.9		9.9		9.9		0.4	2.0
$ZnSO_4.7H_2O$	4.6	20	4.6		4.6		4.6		3.0	10
KI	0.42	2.5	0.42		0.42		0.42		0.03	0.18
	mgl^{-1}	µm							mgl^{-1}	µm
$Na_2MoO_4.2H_2O$	0.13	0.5	0.13		0.13		0.13		0.10	0.4
$CuSO_4.5H_2O$	0.013	0.05	0.013		0.013		0.013		0.09	0.3
$CoSO_4.7H_2O$	0.015	0.05	0.015		0.015		0.015		0.01	0.03
	mgl^{-1}	µm							mgl^{-1}	µm
Glycine	2.0	26	2.0		2.0		2.0		-	-
Nicotinic Acid	5.0	40	5.0		5.0		5.0		1.0	8.0
Pyridoxin HCl	0.5	2.4	0.5		0.5		0.5		1.0	5.0
Thiamin HCl	0.5	1.5	0.5		0.5		0.5		1.0	3.0
Folic Acid	0.5	1.0	0.5		0.5		0.5		-	-
Biotin	0.05	0.2	0.05		0.05		0.05		0.01	0.04
Ca Pantothenate	-	-	-		-		-		1.0	2.0
	gl^{-1}	mM	gl^{-1}	mM	gl^{-1}	mM	gl^{-1}	mM	gl^{-1}	mM
Casein hydrolysate	0.05	-	0.1	-	0.1	-	-	-	-	-
Glutamine	-	-	-	-	-	-	0.73	5.0	0.73	5.0
Sucrose	68.4	200	17.1	50	50	150	50	150	20	60
Myo-Inositol	4.5	25	-	-	1.2	6.7	1.2	6.7	0.1	6.0
Mannitol	4.5	25	18.2	0.1			Vary to given molality			
Sorbitol	4.5	25	-	-	-	-	-	-	-	-
Xylitol	4.5	25	-	-	-	-	-	-	-	-
MES	-	-	-	-	-	-	3mM Vary to given molality			3mM
	mgl^{-1}	µm	mgl^{-1}		mgl^{-1}		mgl^{-1}		mgl^{-1}	µm
NAA	1.0	5.3	1.0		1.0		1.0		0.1	0.53
BAP	0.4	1.8	0.4		0.4		0.4		0.4	1.8
	gl^{-1}		gl^{-1}						gl^{-1}	
Agar (Bacto-agar)	2.0		4.0						4.0	
pH	5.6		5.6		5.6		5.6		5.6	
				FILTER STERILISE						
Reference	Shepard, 1980a		Shepard, 1980a						Caboche, 1980	

PROTOPLAST CULTURE MEDIA

ET MEDIA

	mgl^{-1}	mM
KNO_3	600	6.0
$CaCl_2.2H_2O$	894	6.0
$MgSO_4.7H_2O$	300	1.2
KCl	300	
KH_2PO_4	170	1.25

	mgl^{-1}	mM
Na_2EDTA	74	0.4
$FeSO_4.7H_2O$	28	0.1

	mgl^{-1}	µM
H_3BO_3	3	50
$MnSO_4.4H_2O$	10	50
$ZnSO_4.7H_2O$	2	8.6
KI	0.75	4.5

	mgl^{-1}	µM
$Na_2MoO_4.2H_2O$	0.25	1.0
$CuSO_4.5H_2O$	0.025	0.1
$CoSO_4.7H_2O$	0.025	0.1

	mgl^{-1}	µM
Glycine	2.0	26
Nicotinic Acid	5.0	40
Pyridoxin HCl	0.5	2.4
Thiamin HCl	0.5	1.5
Folic Acid	0.5	1.0
Biotin	0.05	0.2

	gl^{-1}	mM
Glutamine	0.1	0.7
Sucrose	50	150
Glucose	10	55
Myo-Inositol	0.1	0.6
Mannitol	25-40	0.14-0.22

HORMONES	NAA		24D		BAP	
	mgl^{-1}	µM	mgl^{-1}	µM	mgl^{-1}	µM
ET9	1.0	5.3			0.4	1.8
ET10			0.75	3.4	0.4	1.8
ET11	0.25	1.3	0.50	2.3	0.4	1.8

pH 5.6 FILTER STERILISE

Reference Thomas, 1981

MEDIA FOR CALLUS GROWTH AND SHOOT REGENERATION

	C		D	
	mgl^{-1}	mM	mgl^{-1}	mM
NH_4Cl	100	1.9	270	5.0
KNO_3	1900	18.8	1900	
$CaCl_2$	440	3.0	440	
$MgSO_4.7H_2O$	370	1.5	370	
KH_2PO_4	170	1.25	170	
	mgl^{-1}	mM	mgl^{-1}	
Na_2EDTA	18.5	0.1	18.5	
$FeSO_4.7H_2O$	13.9	0.05	13.9	
	mgl^{-1}	µM	mgl^{-1}	
H_3BO_3	3.1	50	3.1	
$MnCl_2.4H_2O$	9.9	50	9.9	
$ZnSO_4.7H_2O$	4.6	20	4.6	
KI	0.42	2.5	0.42	
	mgl^{-1}	µM	mgl^{-1}	
$Na_2MoO_4.2H_2O$	0.13	0.5	0.13	
$CuSO_4.5H_2O$	0.013	0.05	0.013	
$CoSO_4.7H_2O$	0.013	0.05	0.013	
	mgl^{-1}	µM	mgl^{-1}	
Glycine	2.0	26	2.0	
Nicotinic Acid	5.0	40	5.0	
Pyridoxin HCl	0.5	2.4	0.5	
Thiamin HCl	0.5	1.5	0.5	
Folic Acid	0.5	1.0	0.5	
Biotin	0.05	0.2	0.05	
	gl^{-1}	mM	gl^{-1}	mM
Glutamine	0.73	5.0	0.15	1.0
Adenine Sulphate	0.04	0.2	0.08	0.4
Sucrose	2.5	7.3	2.5	7.3
Myo-Inositol	0.1	0.6	0.1	0.6
Mannitol	55.0	0.3	36.0	0.2
MES	0.976	5.0	0.976	5.0
	mgl^{-1}	µM	mgl^{-1}	µM
IAA	-	-	0.1	0.6
NAA	0.1	0.6	-	-
BAP	0.5	2.2	-	-
ZEATIN	-	-	1.0	4.8
Agar (Bacto-agar)	10 gl^{-1}		10 gl^{-1}	

pH 5.6 Autoclave; Filter sterilise Glutamine

Reference Shepard, 1982.

APPENDIX II

VARIATION

Measurements on second generation of Fortyfold protoplast-derived plants.

V2 Foliage: general impression 1. V. poor - 5. V. good

V3 Stem length (cm)

V5 Average internode length (cm)

V6 No. of 1° leaflets

V7 No. of 2° leaflets

V8 No. of 3° leaflets

V11 Length/Width ratio terminal leaflet

V12 Tubers: general impression 1. V. poor - 5. V. good

V13 Tuber shape

1. Round; 2. Oval; 3. Flat; 4. Long; 5. Square

V14 Eye depth 1. Shallow - 5. V. deep

V15 Tuber skin colour

1. White/no purple
2. <5% purple
3. 5<10% purple
4. 10<50% purple
5. 50<100% purple

V16 Tuber weight (total):g

V17 Tuber weight (average):g

		VARIABLES												
		V2	V3	V5	V6	V7	V8	V11	V12	V13	V14	V15	V16	V18
PLANTS														
CONTROL	1	4	63	3.50	6	9	3	1.58	4	1	4	5	560	35.0
	2	4	71	3.55	5	10	3	1.58	3	1	4	5	325	46.4
	3	4	75	3.75	6	7	3	1.52	4	1	4	5	595	35.0
	4	4	70	3.33	6	9	4	1.53	4	1	3	5	408	29.1
	5	4	61	3.59	6	9	5	1.44	4	1	4	5	544	36.3
	6	4	66	3.47	6	9	4	1.52	4	1	4	5	421	30.1
48 FTF	3	4	72	3.60	5	6	3	1.49	4	1	4	1	523	40.2
	3	4	70	4.12	5	7	4	1.58	4	1	4	1	469	19.5
	8	4	73	4.06	5	7	1	1.69	4	1	4	1	416	23.1
	8	4	85	5.00	5	9	5	1.55	4	1	4	2	477	28.1
	23	4	79	4.39	6	8	1	1.54	3	1	5	2	440	31.4
	23	4	81	4.76	5	6	2	1.53	4	1	4	2	424	30.3
	24	4	78	4.59	4	3	1	1.43	4	1	4	2	515	23.4
	24	4	91	5.69	5	5	4	1.43	4	1	4	2	493	27.4
	36	2	74	4.35	3	0	0	1.41	2	1	5	1	418	19.0
	36	2	29	3.22	3	0	0	1.37	4	1	5	1	35	5.8
	75	4	74	3.89	6	8	5	1.42	3	1	4	1	442	17.0
	75	4	72	3.79	6	5	3	1.12	3	1	4	1	438	18.2
	39	4	91	4.55	5	6	3	1.51	4	2	4	1	438	31.3
	39	4	79	4.39	5	8	3	1.46	4	1	4	1	439	17.6
47 FTF	1	3	77	3.50	5	4	3	1.46	3	1	3	1	274	12.4
	1	3	72	3.27	5	5	6	1.37	3	2	4	2	398	30.6
	2	4	64	4.00	6	10	4	1.11	4	1	4	1	514	22.3
	2	4	62	3.44	6	10	6	1.34	4	1	4	2	411	31.6
	6	4	66	3.30	5	4	1	1.58	3	1	4	2	187	18.7
	6	4	65	4.33	4	3	0	1.43	4	1	4	2	377	16.4
	67	1	61	3.05	4	5	2	1.16	1	1	4	1	152	13.8
	67	1	60	3.16	4	4	2	1.29	1	1	4	1	132	12.0
	72	3	89	4.45	6	3	2	1.35	3	2	3	1	397	18.0
	72	3	81	3.52	5	5	2	1.06	3	1	3	1	504	14.8
	73	4	74	3.08	5	3	0	1.45	3	2	3	2	376	31.3
	73	4	77	3.35	5	6	2	1.28	3	2	3	2	477	23.8
	76	4	76	3.80	6	6	6	1.37	3	1	3	2	497	23.7
	76	4	66	3.67	6	7	7	1.39	3	1	3	2	386	20.3
	77	3	50	2.94	4	2	2	1.31	2	2	4	2	195	9.8
	77	3	52	3.47	4	3	1	1.31	2	2	3	2	240	8.0

REFERENCES

ADAMS, T.L. and TOWNSEND, J.A. (1983) A new procedure for increasing efficiency of protoplast plating and clone selection. Plant Cell Reports **2** 165-168.

ARCIONI, S., DAVEY, M.R., DOS SANTOS, A.V.P. and COCKING, E.C. (1982) Somatic embryogenesis in tissues from mesophyll and cell suspension protoplasts of Medicago coerulea and M. glutinosa. Z. Pflanzenphysiol **106** 105-110.

ASSEYEVA, T. (1931) Bud mutations in the potato. Bull. appl. Bot., Leningrad. **27** 135-217.

BAJAJ, Y.P.S. (1974) Potentials of protoplast culture work in agriculture. Euphytica **23** 633-649.

BAJAJ, Y.P.S. (1977) Protoplast isolation, culture and somatic hybridisation. In J. Reinert & Y.P.S. Bajaj (eds). Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture pp.467-496. Springer-Verlag, Berlin.

BAYLISS, M.W. (1980) Chromosomal variation in plant tissues in culture. Intern. Rev. Cytol. Suppl. **11A** 113-144.

BEHNKE, M. (1979) Selection of potato callus for resistance to culture filtrates of Phytophthora infestans and regeneration of resistant plants. Theor. Appl. Genet. **55** 69-71.

BELLIARD, G., PELLETIER, G., VEDEL, F. and QUETIER, F. (1978) Morphological characteristics and chloroplast DNA distribution in different cytoplasmic parasexual hybrids of Nicotiana tabacum. Molec. Gen. Genet. **165** 231-237.

BERRY, S.F., LU, D.Y., PENTAL, D. and COCKING, E.C. (1982) Regeneration of plants from protoplasts of Lactuca sativa L. Z. Pflanzenphysiol **108** 31-38.

BINDING, H., JAIN, S.M., FINGER, J., MORDHORST, G., NEHLS, R. and GRESSEL, J. (1982) Somatic hybridisation of an atrazine resistant biotype of Solanum nigrum with Solanum tuberosum. Part 1: Clonal variation in morphology and in atrazine sensitivity. Theor. Appl. Genet. **63** 273-277.

BINDING, H. and NEHLS, R. (1977) Regeneration of isolated protoplasts to plants in Solanum dulcamara L. Z. Pflanzenphysiol. **85** (3) 279-280.

BINDING, H., NEHLS, R., SCHIEDER, O., SOPORY, S.K. and WENZEL, G. (1978) Regeneration of mesophyll protoplasts isolated from dihaploid clones of Solanum tuberosum. Physiol. Plant **43** 52-54.

BOKELMANN, G.S. and ROEST, S. (1983) Plant regeneration from protoplasts of potato (Solanum tuberosum cv. Bintje). Z. Pflanzenphysiol. **109** 259-265.

BOURGIN, J.P., CHUPEAU, M.C. and MISSONIER, C. (1982) Amino acid resistant plants from tobacco cells selected in vitro. In E.D. Earle and Y. Demarly (eds). Variability in plants regenerated from tissue culture. pp.163-174. Praeger Publ.

BRIGHT, S.W.J., JARETT, V.A., NELSON, R.S., CREISSEN, G.P., KARP, A., FRANKLIN, J., NORBURY, P.B., KUEH, J.S.H., ROGNES, S.E. and MIFLIN, B.J. (1983) Modification of agronomic traits using in vitro technology. In H. Smith and S. Mantell (eds). Plant Biotechnology SEB Seminar Series Symp. pp.251-265. Cambridge Univ. Press.

BRIGHT, S.W.J., NORBURY, P.B. and MIFLIN, B.J. (1980) Isolation and characterisation of barley mutants resistant to aminoethylcysteine and lysine plus threonine. In F. Sala, B. Parisi, R. Cella and O. Ciferri (eds). Plant Cell Cultures: Results and Perspectives. pp 179-182. Elsevier/North Holland Biomedical Press.

BUI-DANG-HA, D. and MACKENZIE, I.A. (1973) The division of protoplasts from Asparagus officinalis L. and their growth and differentiation. Protoplasma **78** 215-221.

BUTENKO, R.G. and KUCHKO, A.A. (1980) Somatic hybridisation of Solanum tuberosum L. and Solanum chacoense Bitt. by protoplast fusion. In L. Ferenczy and G.L. Farkas (eds). Advances in Protoplast Research. pp.293-300 Pergamon Press, Oxford.

BUTENKO, R.G., KUCHKO, A.A., and VITENKO, V.A. (1977a) Isolation, culture and plant regeneration studies on protoplasts from two potato species. Abstr. Intern. Conf. on Regulation of development processes in plants. p.111. Halle, German Democratic Republic.

BUTENKO, R,G., KUCHKO, A,A., VITENKO, V,A. AND AVETISOV, V,A. (1977b) Production and cultivation of isolated protoplasts from the mesophyll of leaves of Solanum tuberosum L and Solanum chacoense Bitt. Soviet Plant Phys. **24** 541-6.

CABOCHE, M. (1980) Nutritional requirements of protoplast-derived, haploid tobacco cells grown at low cell densities in liquid medium. Planta **149** 7-18.

CABOCHE, M. and MULLER, J.F. (1980) Use of a medium allowing low cell density growth for in vitro selection experiments: Isolation of

valine-resistant clones from nitrosoguanidine mutagenized cells and gamma-irradiated tobacco plants. In F. Sala, B. Parisi, R. Cella and O. Ciferri (eds). Plant Cell Cultures: Results and perspectives. pp.133-138. Elsevier/N. Holland Biomedical Press.

CARLBERG, I., GLIMELIUS, K. and ERIKSSON, T. (1983) Improved culture ability of shoot culture derived protoplasts of Solanum tuberosum L. by use of activated charcoal. In I. Potrykus, C.T. Harms, A. Hinnen, R. Hutter, P.J. King and R.D. Shillito (eds). Protoplasts 1983, Poster Proceedings. pp.258-259. Birkhauser Verlag, Basel.

CARLSON, P.S., SMITH, H.H. and DEARING, R.D. (1972) Parasexual interpsecific plant hybridisation. Proc. Natl. Acad. Sci. **69** 2292-2294.

CARLSON, J.E. and WIDHOLM, J.M. (1978) Separation of two forms of Anthranilate Synthetase from 5-Methyltryptophan susceptible and resistant cultured Solanum tuberosum cells. Physiol. Plant **44** 251-255.

CHASE, S.S. (1963) Analytical breeding in Solanum tuberosum L. Can. J. Genet. Cytol. **5** 359-363.

CHEN, K. WILDMAN, S.G. and SMITH, H.H. (1977) Chloroplast DNA distribution in parasexual hybrids as shown by polypeptide composition of fraction 1 protein. Proc. Nat. Acad. Sci. **74** 5109-5112.

CHUPEAU, Y., MISSONIER, C., HOMMEL, M-C. and GOUJAUD, J. (1978) Somatic hybrids of plants by fusion of protoplasts. Observations on the model system "Nicotiana glauca-Nicotiana langsdorfii". Molec. Gen. Genet. **165** 239-245.

COCKING, E.C. (1960) A method for the isolation of plant protoplasts and their vacuoles. Nature **187** 962-963.

COCKING, E.C. (1972) Plant cell protoplasts - Isolation and development. Ann. Rev. Plant Physiol. **23** 29-50.

COCKING, E.C. (1977) Plant protoplast fusion: Progress and prospects for agriculture. In R.F. Beers and E.G. Bassett (eds). Recombinant molecules: Impact on Science and Society pp.195-208. Raven Press, New York..

COCKING, E.C. (1978) Selection and somatic hybridisation. In T.A. Thorpe (ed) Frontiers of Plant Tissue Culture pp.151-158. Univ. of Calgary Press, Alberta.

COCKING, E.C. (1979) Advanced and new breeding methods. Proc. Conf. Broadening Genet. Base Crops pp.333-337. Pudoc, Wageningen..

COCKING, E.C. (1981) Opportunities from the use of protoplasts. Phil. Trans. R. Soc. Lond. B **292** 557-568.

COCKING, E.C., DAVEY, M.R., PENTAL, D. and POWER, J.B. (1981) Aspects of plant genetic manipulation. Nature **293** 265-269.

COCKING, E.C., GEORGE, D., PRICE-JONES, M.J. and POWER, J.B. (1977) Selection procedures for the production of interpsecies somatic hybrids of Petunia hybrida and Petunia parodii II. Albino complementation selection. Plant Sci. Lett. **10** 7-12.

CONSTANTIN, M.J. (1981) Chromosome instability in cell and tissue cultures and regenerated plants. Environ. Exp. Bot. **21** 359-368.

CREPY, L., CHUPEAU, M-C. and CHUPEAU, Y. (1982) The isolation and culture of leaf protoplasts of Cichorium intybus and their regeneration into plants. Z. Pflanzenphysiol. **107** 123-131.

D'AMATO, F. (1977) Cytogenetics of differentiation in tissue and cell cultures. In J. Reinert and Y.P.S. Bajaj (eds). Applied and Fundamental Aspects of Plant Cell Tissue and Organ Culture. pp.343-357. Springer-Verlag, Berlin.

DE BLOCK, M. and HERRERA-ESTRELLA, L. (1983) Chimeric genes as dominant selectable markers in plant cells. In I. Potrykus, C.T. Harms, A. Hinnen, R. Hutter, P.J. King and R.D. Shillito (eds). Protoplast 1983 Poster Proceedings. pp.130-131. Birkhauser Verlag, Basel.

DOUGLAS, G.C., KELLER, W.A. and SETTERFIELD, G. (1981) Somatic hybridisation between Nicotiana rustica and N. tabacum II: Protoplast fusion and selection and regeneration of hybrid plants. Can.J. Bot. **59**(2) 220-227.

DUNWELL, J.M. and SUNDERLAND, N. (1973) Anther culture of Solanum tuberosum L. Euphytica **22** 317-333.

EVANS, D.A. (1982) Plant regeneration and genetic analysis of somatic hybrid plants. In Earle E.D. and Demarly, Y. (eds) pp.303-323. Variability in plants regenerated from tissue culture. Praeger Publ. USA.

EVANS, D.A. (1983) Agricultural applications of plant protoplast fusion. Biotechnology **1** 253-261.

EVANS, D.A. and SHARP, W.R. (1983) Single gene mutations in tomato plants regenerated from tissue culture. Science **221** 949-951.

EVANS, D.A., WETTER, L.R. and GAMBORG, O.L. (1980) Somatic hybrid plants of Nicotiana glauca and Nicotiana tabacum obtained by protoplast fusion. Physiol. Plant. **48** 225-230.

EVANS, P.K. and COCKING, E.C. (1977) Isolated plant protoplasts. In H.E. Street (ed). Plant tissue and cell culture. pp.103-135. Blackwell Sci. Pub., Oxford.

FEULGEN, R. and ROSSENBECK, H. (1924) Mikroscopisch-chemischer Nachweis einer Nucleinsaure vom Typus der Thymonucleinsaure. Hoppe-Seyl **135** 203-248.

FOROURGHI-WEHR, B., WILSON, H.M., MIX, G. and GAUL, H. (1977) Monohaploid plants from anthers of a dihaploid genotype of Solanum tuberosum L. Euphytica **26** 361-367.

GALBRAITH, D.W. and GALBRAITH, J.E.C. (1979) A method for identification of fusion of plant protoplasts derived from tissue cultures. Z. Pflanzenphysiol. **93** 149-158.

GALUN, E. (1981) Plant protoplasts as physiological tools. Ann. Rev. Plant Physiol. **32** 237-266.

GAMBORG, O.L. and SHYLUK, J.P. (1970) The culture of plant cells with ammonium salts as the sole nitrogen source. Plant Physiol. **45** 598-600.

GLEBA, Y.Y. and HOFFMANN, F. (1978) Hybrid cell lines Arabidopsis thaliana + Brassica compestris: no evidence for specific chromosome elimination. Mol. Gen. Genet. **165** 257-264.

GLEBA, Y.Y., MOMOT, V.P., CHEREP, N.N. and SKARZYNSKAYA, M.V. (1982) Intertribal hybrid cell lines of Atropa belladonna (x) Nicotiana chinensis obtained by cloning individual protoplast fusion products. Theor. Appl. Genet. **62** 75-79.

GLIMELIUS, K. and BONNETT, H.T. (1981) Somatic hybridisation in Nicotiana: restoration of photoautotrophy to an albino mutant with defective plastids. Planta **153** 497-503.

GLIMELIUS, K., CHEN, K. and BONNETT, H.T. (1981) Somatic hybridisation in Nicotiana: Segregation of organellar traits among hybrid and cybrid plants. Planta **153** 504-510.

GLIMELIUS, K., ERIKSSON, T., GRAFE, R. and MULLER, A.J. (1978a) Somatic hybridisation of nitrate reductase-deficient mutants of Nicotiana tabacum by protoplast fusion. Physiol. Plant. **44** 273-277.

GLIMELIUS, K., WALLILN, A. and ERIKSSON, T. (1978b) Concanavalin A improves the polyethylene glycol method for fusing plant protoplasts. Physiol. Plant. **44** 92-96.

GOSCH, G. and REINERT, J. (1978) Cytological identification of colony formation of intergeneric somatic hybrid cells. Protoplasma **96** 23-38.

GRIMSLEY, M.J., ASHTON, N.W. and COVE, D.J. (1977) The production of somatic hybrids by protoplast fusion in the moss Physcomitrella patens. Molec. Gen. Genet. **154** 97-100.

GRUN, P. and CHU, L.J. (1978) Development of plants from protoplasts of Solanum (Solanaceae). Am. J. Bot. **65**(5) 538-543.

GUNN, R.E. and SHEPARD, J.F. (1981) Regeneration of plants from mesophyll-derived protoplasts of British potato (Solanum tuberosum L.) cultivars. Plant Sci. Lett. **22** 97-101.

GUPTA, P.P., GUPTA, M. and SCHIEDER, O. (1982) Correction of nitrate reductase defect in auxotrophic plant cells through protoplast mediated intergeneric gene transfers. Mol. gen. Genet. **188** 378-383.

HAAS, R., HEINZ, E., POPOVICI, G. and WEISSENBOECK, G. (1979) Protoplasts from oat Avena sativa primary leaves as tools for

experiments on the compartmentation in lipid and flavenoid metabolism. Z. Naturforsch. **34** 854-864.

HAIN, R., STABEL, P. and STEINBISS, H.H. (1983) Expression of foreign genes in plant cells following fusion of Agrobacterium spheroplasts with isolated mesophyll protoplasts of Nicotiana tabacum. In I. Potrykus, C.T. Harms, A. Hinnen, R. Hutter, P.J. King and R.D. Shillito (eds). Protoplasts 1983 Poster Proceedings. pp.136-137. Birkhauser Verlag, Basel.

HARMS, C.T., POTRYKUS, I. and WIDHOLM, J.M. (1981) Complementation and dominant expression of amino acid analogue resistance markers in somatic hybrid clones from Daucus carota after protoplast fusion. Z. Pflanzenphysiol. **101** 377-390.

HAWKES, J.G. (1967) The history of the potato. Masters Memorial Lecture 1966. J. Roy. Hort. Soc. **92** 207-224, 249-262, 288-302, 364-365..

HAWKES, J.G. (1978a) History of the potato. In P.M. Harris (ed). The Potato Crop: The Scientific Basis for Improvement pp. 1-14. Chapman Hall, London.

HAWKES, J.G. (1978b) Biosystematics of the potato. In P.M. Harris (ed). The Potato Crop: The Scientific Basis for Improvement. pp.15-69. Chapman Hall, London.

HAWKES, J.G. (1979) Evolution and polyploidy in potato species. In J.G. Hawkes, R.N. Lester, A.D. Skelding (eds). The Biology and Taxonomy of the Solanaceae. Linnean Soc. Symp. Series **7** 637-646.

HAWKES, J.G. and HJERTING, J.P. (1969) The Potatoes of Argentina, Brazil, Paraguay and Uruguay. A Biosystematic study. Annals of Botany Memoir No. 3. Clarendon Press, Oxford.

HAYWARD, C. and POWER, J.B. (1975) Plant production from leaf protoplasts of Petunia parodii. Plant Sci. Lett. **4** 407-410.

HERMSEN, J.G.Th. and RAMANNA, M.S. (1981) Haploidy and plant breeding. Phil. Trans. R. Soc. Lond. B **292** 499-507.

HERMSEN, J.G.Th., RAMANNA, M.S., ROEST, S. and BOKELMANN, G.S. (1981a) Chromosome doubling through adventitious shoot formation on in vitro cultivated leaf explants from diploid interspecific potato hybrids. Euphytica **30** 239-246.

HERMSEN, J.G.Th., RAMANNA, M.S. and SAWOR. Z. (1981b) The effect of chromosome doubling on fertility, meiotic behaviour and crossability of S. etuberosum x S. pinnatisectum. Euphytica **30** 33-39.

HERMSEN, J.G.Th. and TAYLOR, L.M. (1979) Successful hybridisation of non-tuberous Solanum etuberosum Lind. and tuber-bearing S. pinnatisectum Dun. Euphytica **28** 1-7.

HERMSEN, J.G.Th. and VERDENIUS, J. (1973) Selection from Solanum tuberosum group phureja of genotypes combining high frequency haploid induction with homozygosity for embryo spot. Euphytica **22**, 244-259.

HOFFMANN, F. and ADACHI, T. (1981) "Arabidobrassica": Chromosomal recombination and morphogenesis in asymmetric intergeneric hybrid cells. Planta **153** 586-593.

HOUGAS, R.W. and PELOQUIN, S.J. (1957) A haploid plant of the variety Katahdin. Nature **180** 1209-1210.

HOWARD, H.W. (1962) Experiments with potatoes on the effect of the pigment-restricting gene - M. Heredity **17** 145-156.

HOWARD, H.W. (1964) Further effects of the pigment-restricting gene M in potatoes. Heredity **19** 349-356.

HOWARD, H.W. (1967) Differentiation in potatoes: Hidden-spotted and spectacled. Heredity **22** 57-64.

HOWARD, H.W. (1970) Genetics of the Potato Solanum tuberosum. Logos Press Ltd. London, 126 pages.

HOWARD, H.W. (1978) The production of new varieties In P.M Harris (ed.) The Potato Crop: The Scientific Basis for Improvement pp.607-646, Chapman Hall, London.

IRIKURA, Y. (1975) Induction of haploid plants by anther culture in tuber bearing species and interspecific hybrids of Solanum. Potato Res. **18** 133-140.

IRIKURA, Y. and SAKAGUCHI, S. (1972) Induction of 12-chromosome plants from anther culture in a tuberous Solanum. Potato Res. **15** 170-173.

JACOBS, M., CATTOIR-REYNAERTS, A., NEGRUTIU, I., VERBRUGGEN, I. and DEGRYSE, E. (1982) Comparison of selection schemes for the isolation of resistant mutants to aspartate-derived amino acids and S(2-aminoethyl)cysteine in various cell culture and "whole plant" systems. In A. Fujiwara (ed.) Plant Tissue Culture, 1982. pp.453-454. Japanese Association for Plant Tissue Culture.

JACOBSEN, E. (1977) Doubling dihaploid potato clones via leaf tissue culture. Z. Pflanzensuchtg. **80** 80-82.

JACOBSEN, E. (1978) Haploid, diploid and tetraploid parthenogenesis in interspecific crosses between Solanum tuberosum interdihaploids and S. phureja. Potato Res. **21** 15-17.

JACOBSEN, E. (1980) Diplandroid formation and its importance for the seed set in 4x X 2x crosses in potato. Z. Pflanzensuchtg **84** 240-249.

JACOBSEN, E. (1982) Polyploidization in leaf callus tissue and in regenerated plants of dihaploid potato. Plant Cell Tissue and Organ Culture **1** 76-84.

JACOBSEN, E. and SOPORY, S.K. (1978) The influence and possible recombination of genotypes on the production of microspore embryoids in anther cultures of Solanum tuberosum and dihaploid hybrids. Theor. Appl. Genet. **52** 119-123.

JACOBSEN, E., TEMPELAAR, M.J. and BIJMOLT, E.W. (1983) Ploidy levels in leaf callus and regenerated plants of Solanum tuberosum determined by cytophotometric measurements of protoplasts. Theor. Appl. Genet. **65** 113-118.

JIA, J.-F. and POTRYKUS, I. (1981) Mesophyll protoplasts from Solanum melongena var depressum. Bailey regenerate to fertile plants. Plant Cell Reports **1** 71-72.

JONES, M.G.K., BRIGHT, S.W.J., NELSON, R.S., FOULGER, D., CREISSEN, G.P., KARP, A. and OOMS, G. (1983) Variation in plants regenerated from protoplasts and complex explants of potato. In I. Potrykus, C.T. Harms, A. Hinnen, R. Hutter, P.J. King and R.D. Shillito (eds). Protoplasts 1983 Poster Proceedings. pp.150-151. Birkhauser Verlag, Basel.

JONES, R.A.C. (1979) Resistance to poato leaf roll virus in Solanum brevidens. Potato Res. **22** 149-152.

KAHL, G. and SCHELL, J.S. (1982) Molecular Biology of Plant Tumours. (eds. Kahl, G. and Schell, J.S.) Academic Press, London. 615 pages.

KAMEYA, T., HORN, M.E. and WIDHOLM, J.M. (1981) Hybrid shoot formation from fused Daucus carota and D. capillifolius protoplasts. Z. Pflanzenpohysiol. **104** 459-466.

KAO, K.N. (1977) Chromosomal behaviour in somatic hybrids of Soybean-Nicotiana glauca. Molec. gen. Genet. **150** 225-230.

KAO, K.N., CONSTABEL, F., MICHAYLUK, M.R and GAMBORG, O.L. (1974) Plant protoplast fusion and growth of intergeneric hybrid cells. Planta **120** 215-227.

KAO, K.N. and MICHAYLUK, M.R. (1974) A method for high-frequency intergeneric fusion of plant protoplasts. Planta **115** 355-367.

KAO, K.N. and MICHAYLUK, M.R. (1975) Nutritional requirements for growth of Vicia hajastana cells and protoplasts at very low population density in liquid media. Planta **126** 105-110.

KARP, A., NELSON, R.S., THOMAS, E. and BRIGHT, S.W.J. (1982) Chromosome variation in protoplast-derived potato plants. Theor. Appl. Genet. **63** 265-272.

KELLER, W.A. and MELCHERS, G. (1973) The effect of high pH and calcium on tobacco leaf protoplast fusion. Z. Naturforsch. **28**(C) 737-741.

KELLER, W.A., SETTERFIELD, G., DOUGLAS, G., GLEDDIE, S. and NAKAMURA, C. (1982) Production, characterisation, and utilisation of somatic hybrids of higher plants. In D.T. Tomes, B.E. Ellis, P.M. Hanney, K.J. Kasha and R.L. Peterson (eds). Application of Plant Cell and Tissue Culture to Agriculture and Industry. pp.81-114. University of Guelph, Guelph..

KLERCKER, J. (1892) Eine Methode zur isolierung lebender protoplasten. Ofvers. Vetensk. Akad. Forh. Stockholm. **49** 463-475.

KRANTZ, F.A. (1946) Potato breeding methods: III - A suggested procedure for potato breeding. Techn. Bull. Minn. Agric. Exp. Sta. No. **173** 24.

KRENS, F.A., WULLENS, G.J. and SCHILPEROORT, R.A. (1983) A reproducible method for the introduction of nucleic acids in tobacco protoplasts. In I. Potrykus, C.T. Harms, A. Hinnen, R. Hutter, P.J. King and R.D. Shillito (eds). Protoplasts 1983 Poster Proceedings. pp.140-141. Birkhauser Verlag, Basel.

KRUMBIEGEL, G. and SCHIEDER, O. (1979) Selection of somatic hybrids after fusion of protoplasts from Datura innoxia Mill. and Atropa belladonna L. Planta **145** 371-375.

KUCHKO, A.A. and BUTENKO, R.G. (1977) The culture of protoplasts isolated from Solanum tuberosum L. and Solanum chacoense. Bitt. In F.J. Novak (ed). Uses of tissue culture in plant breeding. pp.441-461. Check. Acad. Sci. Inst. Exp. Bot., Prague.

KUSTER, E. (1909) Uber die Verschmelzung nackter protoplasten. Ber. Deutsch. Bot. Ges. **27** 589-598.

KUSTER, E. (1910) Eine methode fur gewinnung abnorm grosser protoplasten. Arch. Entwicklungsmech. Org. **30** 351-355.

LARKIN, P.J. and SCOWCROFT, W.R. (1981) Somaclonal Variation - a novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet. **60** 197-214.

LAZAR, G.B., DUDITS, D. and SUNG, Z.R. (1981) Expression of cycloheximide resistance in carrot somatic hybrids and their segregants. Genetics **98** 347-356.

LEA, P.J. and MIFLIN, B.J. (1980) Transport and metabolism of asparagine and other nitrogen compounds within the plant. In B.J. Miflin (ed). The biochemistry of plants a comprehensive treatise Vol. 5. Amino acids and derivatives. pp.569-607. Academic Press, London.

LI, X.H. (1981) Plantlet regeneration from mesophyll protoplasts of Digitalis lanata Ehrh. Theor. Appl. Genet. **60** 345-347.

LORENZINI, M. (1973) Physiologie Vegetale. "Obtention de protoplastes de tubercule de Pomme de terre". C.R. Acad. Sc. Paris t. **276** 1839-1842.

LORZ, H. and SCOWCROFT, W.R. (1983) Variability among plants and their progeny regenerated from protoplasts of Su/Su heterozygotes of Nicotiana tabacum. Theor. Appl. Genet. **66** 67-75.

LU, C., VASIL, V. and VASIL, I.K. (1981) Isolation and culture of protoplasts of Pannicum maximum Jacq. (Guinea Grass): somatic embryogenesis and plantlet formation. Z. Pflanzenphysiol. **104** 311-318.

LU, D.Y., DAVEY, M.R. and COCKING, E.C. (1982) Somatic embryogenesis from mesophyll protoplasts of Trigonella corniculata (Leguminosae). Plant Cell Reports **1** 278-280.

LU, D.Y., PENTAL, D. and COCKING, E.C. (1982) Plant regeneration from seedling cotyledon protoplasts. Z. Pflanzenphysiol **107** 59-63.

MALIGA, P. (1980) Isolation, characterization and utilization of mutant cell lines in higher plants. Intern. Rev. Cytol Suppl. 11A, 225-249.

MALIGA, P. (1981) Streptomycin resistance is inherited as a recessive mendelian train in a Nicotiana sylvestris line. Theor. Appl. Genet. **60** 1-3.

MALIGA, P., KISS, Z.R., NAGY, A.H. and LAZAR, G. (1978) Genetic instability in somatic hybrids of Nicotiana tabacum and Nicotiana knightiana. Mol. gen. Genet. **163** 145-151.

MALIGA, P., LAZAR, G., JOO, F., NAGY, A.H. and MENCZEL, L. (1977) Restoration of morphogenic potential in Nicotiana by somatic

hybridisation. Mol. gen. Genet. **157** 291-296.

MALIGA, P., MENCZEL, L., SIDOROV, V., MARTON, L., CSEPLO, A., MEDGYESY, P., MANH DUNG, T., LAZAR, G. and NAGY, F. (1982) Cell Culture mutants and their uses. In I.K. Vasil, K.J. Frey and W.R. Scowcroft (eds). Plant Improvement and somatic cell genetics. Academic Press, London (In press).

MARDIA, K.V., KENT, J.T. and BIBBY, J.M. (1979) Multivariate analysis. Academic Press, London. 521 pages.

MARTON, L., SIDOROV, V., BIASINI, G. and MALIGA, P. (1982) Complementation in somatic hybrids indicates four types of Nitrate Reductase deficient lines in Nicotiana plumbaginifolia. Mol. gen. Genet. **187** 1-3.

MATERN, U., STROBEL, G. and SHEPARD, J. (1978) Reaction to phytotoxins in a potato population derived from mesophyll protoplasts. Proc. Natl. Acad.Sci. U.S.A. **75** 4935-4939.

MCINTOSH, T.P. (1927) The potato: its history, varieties, culture and diseases. Oliver and Boyd, London. 264 pages.

MEDGYESY, P., MENCZEL, L. and MALIGA, P. (1980) The use of cytoplasmic streptomycin resistance: chloroplast transfer from Nicotiana tabacum into Nicotiana sylvestris and isolation of their somatic hybrids. Mol. gen. Genet. **179** 693-698.

MELCHERS, G. (1977) Microbial techniques in somatic hybridisation by fusion of protoplasts. In Brinkley, B.R. and Porter, K.R. (eds). International Cell Biology. 1976-1977. pp.207-215. Rockefeller Univ. Press, N.Y. USA.

MELCHERS, G. and LABIB, G. (1974) Somatic hybridisation of plants by fusion of protoplasts 1. Selection of light resistant hybrids of

"haploid" light sensitive varieties of tobacco. Mol. gen. Genet. **135** 277-294.

MELCHERS, G., SACRISTAN, M.D., HOLDER, A.A (1978) Somatic hybrid plants of potato and tomato regenerated from fused protoplasts. Carlsberg Res. Commun **43**(4) 203-218.

MELLOR, F.C. and STACE-SMITH, R. (1977) Virus-free potatoes by tissue culture In J. Reinert and Y.P.S. Bajaj (eds). Applied and Fundamental Aspects of Plant Cell, Tissue and Organ culture. pp.616-636. Springer-verlag, Berlin.

MENCZEL, L., LAZAR, G. and MALIGA, P. (1978) Isolation of somatic hybrids by cloning Nicotiana heterokaryons in nurse cultures. Planta **143** 29-32.

MENCZEL, L., NAGY, F., KISS, Z.R. and MALIGA, P. (1981) Streptomycin resistant and sensitive somatic hybrids of Nicotiana tabacum + Nicotiana knightiana: correlation of resistance to N. tabacum plastids. Theor. Appl. Genet. **59** 191-195.

MIFLIN, B.J., BRIGHT, S.W.J., ROGNES, S.E. and KUEH, J.S.H. (1983) Amino acids, nutrition and stress; the role of biochemical mutants in solving problems of crop quality. In A. Hollaender, T. Kosuge, and C. Meredith (eds). Genetic Engineering of Plants. pp.391-414. Plenum Publ. Corp., N. York.

MOORBY, J. (1978) The physiology of growth and tuber yield. In P.M. Harris (ed). The potato crop: the scientific basis for improvement. pp.153-194. Chapman and Hall, London.

MORGAN, A. and COCKING, E.C. (1982) Plant regeneration from protoplasts of Lycopersicon esculentum Mill. Z. Pflanzenphysiol **106** 97-104.

MULLER, J.F. and CABOCHE, M. (1983) Isolation of tobacco clones resistant to Naphthalene acetic acid and affected in root morphogenesis. In I. Potrykus, C.T. Harms, A. Hinnen, R. Hutter, P.J. King and R.D. Shillito (eds). Protoplasts 1983 Poster Proceedings. pp.156-157. Birkhauser Verlag, Basel.

MULLER, J.F., MISSIONIER, C. and CABOCHE, M. (1983) Low density growth of cells derived from Nicotiana and Petunia protoplasts: influence of the source of protoplasts and comparison of the growth-promoting activity of various auxins. Physiol. Plant **57** 35-41.

MURASHIGE, T. and SKOOG, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15** 473-497.

NAGATA, T. and ISHII, S. (1979) A rapid method for the isolation of mesophyll protoplasts. Can. J. Bot. **57** 1820-1823.

NATIONAL INSTITUTE OF AGRICULTURAL BOTANY (1975) Guide to the identification of potato varieties, Cambridge, U.K.

NEGRUTIU, I. (1981) Improved conditions for large scale culture, mutagenesis, and selection of haploid protoplasts of Nicotiana plumbaginifolia. Z. pflanzenphysiol. **104** 431-442.

NEGRUTIU, I. and MULLER, J.F. (1981) Culture conditions of protoplast-derived cells of Nicotiana sylvestris for mutant selection. Plant Cell Reports **1** 14-17.

NEHLS, R. (1978) The use of metabolic inhibitors for the selection of fusion products of higher plant protoplasts. Molec. gen. Genet. **166** 117-118.

NELSON, O.E. and BURR, B. (1973) Biochemical genetics of higher plants Ann. Rev. Plant Physiol. **24** 493-518.

OOMS, G., KARP, A. and ROBERTS, J. (1983) From tumour to tuber; tumour cell characteristics and chromosome numbers of crown gall-derived tetraploid potato plants (Solanum tuberosum cv 'Maris Bard'). Theor. Appl. Genet. **66** 169-172.

ORTON, T.J. (1980) Chromosomal variability in tissue cultures and regenerated plants of Hordeum. Theor. Appl. Genet. **56** 101-112.

OSIFO, E.O. (1983) In vitro morphogenetic studies with the potato cv Disiree, S. brevidens and Dioscorea rotundata. Ph.D. Thesis, University of Birmingham.

OTSUKI, Y. and TAKEBE, I. (1969) Isolation of intact mesophyll cells and their protoplasts from higher plants. Plant Cell Physiol. **10** 917-921.

PANDEY, K.K. (1962) Interspecific incompatibility in Solanum species. Am. J. Bot. **49** 874-882.

PASZKOWSKI, J., SHINSHI, H., KOENIG, I., LAZAR, G.B., HOHN, T., MANDAK, V. and POTRYKUS, I. (1983) Proliferation of cauliflower mosaic virus in protoplast-derived clones of turnip (Brassica rapa). In I. Potrykus, C.T. Harms, A. Hinnen, R. Hutter, P.J. King and R.D. Shillito (eds). Protoplasts 1983 Poster Proceedings. pp.138-139. Birkhauser Verlag, Basel.

PATNAIK, G., COCKING, E.C., HAMILL, J. and PENTAL, D. (1982) A simple procedure for the manual isolation and identification of plant heterokaryons. Plant Sci. Lett. **24** 105-110.

POWER, J.B., BERRY, S.F., CHAPMAN, J.V. and COCKING, E.C. (1980) Somatic hybridisation of sexually incompatible Petunias: Petunia parodii, Petunia parviflora. Theor. Appl. Genet. **57** 1-5.

POWER, J.B., BERRY, S.F. CHAPMAN, J.V., COCKING, E.C. and SINK, K,C. (1979) Somatic hybrids between unilateral cross-incompatible Petunia species. Theor. Appl. Genet. **55** 97-99.

POWER, J.B. BERRY, S.F., FREARSON, E.M. and COCKING, E.C. (1977) Selection procedures for the production of inter-species somatic hybrids of Petunia hybrida and Petunia parodii. 1. Nutrient media and drug sensitivity complementation selection. Plant Sci. Lett. **10** 1-6.

POWER, J.B. and CHAPMAN, J.V. (1983) Intra and Intersubfamilial somatic hybridisation within the Solanaceae. In I. Potrykus, C.T. Harms, A. Hinnen, R. Hutter, P.J. King and R.D. Shillito (eds). Protoplasts 1983 Poster Proceedings. pp.276-277. Birkhauser Verlag, Basel.

POWER, J.B. and COCKING, E.C. (1970) Isolation of leaf protoplasts: macro-molecule uptake and growth substance response. J. Exp. Bot. **21** 64-70.

POWER, J.B. and COCKING, E.C. (1977) Selection systems for somatic hybrids. In J. Reinert, Y.P.S. Bajaj (eds). Applied and Fundamental Aspects of Plant Cell Tissue and Organ Culture pp.497-505. Springer-Verlag, Berlin.

POWER, J.B., CUMMINS, S.E. and COCKING, E.C. (1970) Fusion of isolated plant protoplasts. Nature **225** 1016-1018.

PRAT, D. (1983) Genetic variability induced in Nicotiana sylvestris by protoplast culture. Theor. Appl. Genet. **64** 223-230.

RAGHAVAN, V. (1977) Applied aspects of embryo culture. In J. Reinert and Y.P.S. Bajaj (eds). Applied and Fundamental aspects of Plant Cell Tissue and Organ Culture. pp.375-397. Springer-Verlag, Berlin.

RAGHAVAN, V. (1980) Embryo culture. Intern. Rev. Cytol. Suppl. **11B** 209-240.

RAMANNA, M.S. and HERMSEN, J.G.Th. (1979) Unique meiotic behaviour in F_1 plants from a cross between a non-tuberous and a tuberous Solanum species in section Petota. Euphytica **28** 9-15.

RAO, I.V.R., MEHTA, U. and RAM, H.Y.M. (1982) Whole plant regeneration from cotyledonary protoplasts of Crotalaria juncea. In A. Fujiwara (ed). Plant tissue culture 1982. pp.595-596. Japanese Assoc. for plant tissue culture.

RAVEH, D., HUBERMAN, E. and GALUN, E. (1973) In vitro culture of tobacco protoplasts: use of feeder techniques to support division of cells plated at low frequencies. In vitro **9** 216-220.

ROEST, S., BOKELMANN, G.S. (1976) Vegetative propagation of Solanum tuberosum L. in vitro. Potato Res. **19** 173-178.

ROSS, R.W., DIONNE, L.A. and HOUGAS, R.W. (1967) Doubling the chromosome number of selected Solanum genotypes. Eur. Potato J. **10** 37-52.

ROSS, R.W. and ROWE, P.R. (1965) Frost resistance among the Solanum species in the IR-1 potato collection. Amer. Potato J. **42** 177-185.

SACRISTAN, M.D. (1971) Karyotypic changes in callus cultures from haploid and diploid plants of Crepis capillaris (L.). Wallr. Chromosoma **33** 273-283.

SALAMAN, R.N. (1949) The history and social influence of the potato. Cambridge Univ. Press. 685 pages.

SAXENA, P.K., GILL, R., RASHID, A. and MAHESHWARI, S.C. (1981) Plantlet formation from isolated protoplasts of Solanum melongena L. Protoplasma **106** 355-359.

SAXENA, P.K., GILL, R., RASHID, A. and MAHESHWARI, S.C. (1982) Plantlets from mesophyll protoplasts of Solanum xanthocarpum. Plant Cell Reports **1** 219-220.

SCHIEDER, O. (1974) Selektion einer somatischen Hybride nach Fusion von Protoplasten auxotropher Mutanten von Sphaerocarpos donnellii Z. Pflanzenphysiol. **74** 357-365.

SCHIEDER, O. (1975) Regeneration von haploiden und diploiden Datura innoxia Mill. Mesophyll-Protoplasten su Pflanzen. Z. Pflanzenphysiol. **76** 462-466.

SCHIEDER, O. (1977) Hybridisation experiments with protoplasts from chlorophyll-deficient mutants of some Solanaceous species. Planta **137** 253-257.

SCHIEDER, O. (1978a) Somatic hybrids of Datura innoxia Mill. + Datura discolor. Bernh. and of Datura innoxia Mill + Datura stramonium L. var tatula L. 1. Selection and Characterisation. Molec. gen. Genet. **162** 113-119.

SCHIEDER, O. (1978b) Production and uses of metabolic and chlorophyll deficient mutants. In T.A. Thorpe (ed). Frontiers of Plant tissue culture, 1978 pp.393-401. Univ. of Calgary Press, Calgary, Alberta.

SCHIEDER, O. (1980) Somatic hybrids between a herbaceous and two tree Datura species. Z. Pflanzenphysiol. **98** 119-127.

SCHIEDER, O. and VASIL, I.K. (1980) Protoplast fusion and somatic hybridisation. Intern. Rev. Cytol. Suppl. 11B 21-46.

SCHILLER, B., HERRMANN, R.G., and MELCHERS, G. (1982) Restriction endonuclease analysis of plastid DNA from Tomato, Potato and some of their somatic hybrids. Mol. Gen. Genet. **186**, 453-459.

SCHUMANN, U., KOBLITZ, H., and OPATRNY, Z. (1980) Plant recovery from long term callus cultures and from suspension culture derived protoplasts of Solanum phureja. Biochem. Physiol. Pflanzen **175** 670-675.

SECOR, G.A. and SHEPARD, J.F. (1981) Variability of protoplast-derived potato clones. Crop Science **21** (1) 102-105.

SENDA, M., TAKEDA, J., SHUNNOSUKE, A. and NAKAMURA, T. (1979) Induction of cell fusion of plant protoplasts by electrical stimulation. Plant and Cell Physiol. **20**(7) 1441-1443.

SHAHIN, E.A. and SHEPARD, J.F. (1980) Cassava mesophyll protoplasts: isolation, proliferation and shoot formation. Plant Sci. Lett. **17** 459-465.

SHEPARD, J.F. (1980a) Mutant selection and plant regeneration from potato mesophyll protoplasts. In I. Rubenstein, B. Gengenbach, R.L. Phillips and C.E. Green (eds). Genetic Improvement of crops. Emergent Techniques pp.185-219. Univ. of Minnesota Press, Minneapolis.

SHEPARD, J.F. (1980b) Abscisic acid-enhanced shoot initiation in protoplast-derived calli of potato. Plant Sci. Lett. **18** 327-333.

SHEPARD, J.F. (1981) Protoplasts as sources of disease resistance in plants. Ann. Rev. Phytopathol. **19** 145-166.

SHEPARD, J.F. (1982) Cultivar dependant cultural refinements in potato protoplast regeneration. Plant Sci. Lett. **26** 127-132.

SHEPARD, J.F., BIDNEY, D., BARSBY, T. and KEMBLE, R. (1983) Genetic transfer in plants through interspecific protoplast fusion. Science **219** 683-688.

SHEPARD, J.F., BIDNEY, D. and SHAHIN, E.A. (1980) Potato protoplasts in crop improvement. Science **208**(4) 17-24.

SHEPARD, J.F. and TOTTEN, R.E. (1977) Mesophyll cell protoplasts of potato: Isolation, proliferation and plant regeneration. Plant Physiol. **60** 313-316.

SHILLITO, R.D., PASZKOWSKI, J. and POTRYKUS, I. (1983) Culture in agarose improves protoplast plating and proliferation and permits division in otherwise unresponsive systems. In I. Potrykus, C.T. Harms, A. Hinnen, R. Hutter, P.J. King and R.D. Shillito (eds). Protoplasts 1983 Poster Proceedings. pp.266-267. Birkhauser Verlag, Basel.

SIDOROV, V.A. and MALIGA, P. (1982) Fusion-complementation analysis of auxotrophic and chlorophyll-deficient lines isolated in haploid Nicotiana plumbaginifolia protoplast cultures. Mol. gen. Genet. **186** 328-332.

SIDOROV, V.A., MENCZEL, L., NAGY, F. and MALIGA, P. (1981) Chloroplast transfer in Nicotiana based on metabolic complementation between irradiated and iodoacetate treated protoplasts. Planta **152** 341-345.

SIMMONDS, N.W. (1962) Variability in crop plants, its use and conservation. Biol. Rev. **37**, 422-465.

SMITH, H.H., KAO, K.N., and COMBATTI, N.C. (1976) Interspecific hybridization by protoplast fusion in Nicotiana. J. Heredity **67** 123-128.

SOPORY, S.K., JACOBSEN, E. and WENZEL, G. (1978) Production of monohaploid embryoids and plantlets in cultured anthers of Solanum tuberosum. Pl. Sci. Lett. **12** 47-54.

SREE RAMULU, K., DIJKHUIS, P. and ROEST, S. (1983) Phenotypic variation and ploidy level of plants regenerated from protoplasts of tetraploid potato (Solanum tuberosum L. cv Bintje). Theor. Appl. Genet. **65** 329-338.

STEFFEN, A. and SCHIEDER, O. (1983) Selection and characterisation of nitrate reductase deficient mutants of Petunia. In I. Potrykus, C.T. Harms, A. Hinnen, R. Hutter, P.J. King and R.D. Shillito (eds). Protoplasts 1983 Poster Proceedings. pp.162-163. Birkhauser Verlag, Basel.

STREETER, J.G. and THOMPSON, J.F. (1972) Anaerobic accumulation of γ-aminobutyric acid and alanine in radish leaves (Raphanus sativus L.). Plant Physiol. **49** 572-578.

SUNDERLAND, N. (1980) Anther and pollen culture 1974-1979. In: D.R. Davies and D.A. Hopwood (eds). The Plant Genome. pp.171-183. John Innes Charity.

TAKEBE, I., LABIB, G. and MELCHERS, G. (1971) Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. Naturwiss. **58** 318-320.

TAKEBE, I., OTSUKI, Y., and AOKI, S. (1968) Isolation of tobacco mesophyll cells in intact and active state. Plant Cell Physiol **9**, 115-124.

THOMAS, E. (1981) Plant regeneration from shoot culture-derived protoplasts of tetraploid potato (Solanum tuberosum cv. Maris Bard). Plant Sci Lett. **23** 81-88.

THOMAS, E., BRIGHT, S.W.J., FRANKLIN, J., GIBSON, R., LANCASTER, V. and MIFLIN, B.J. (1982) Variation amongst protoplast-derived potato plants (Solanum tuberosum cv. 'Maris Bard'). Theor. Appl. Genet. **62** 65-68.

THOMAS, E., KING, P.J. and POTRYKUS, I. (1979) Improvement of crop plants via single cells in vitro - an assessment. Z. Pflanzenzuchtg. **82** 1-30.

TUDGE, C. (1983) The future of crops. New Scientist **98** 547-553.

UHRIG, H. (1981) Regeneration of protoplasts of dihaploid potato plants bleached by a herbicide (SAN 6706). Mol. Gen. Genet. **181** 403-405.

UPADHYA, M.D. (1975) Isolation and culture of mesophyll protoplasts of potato (Solanum tuberosum L.). Potato Res. **18** 438-445.

VAN HARTEN, A.M., BOUTER, H. and BROERTJES, C. (1981) In vitro adventitious bud techniques for vegetative propagation and mutation breeding of potato (Solanum tuberosum L.) II. Significance for mutation breeding. Euphytica **30** 1-8.

VARDI, A., SPIEGEL-ROY, P. and GALUN, E. (1982) Plant regeneration from Citrus protoplasts: variability in methodological requirements among cultivars and species. Theor. Appl. Genet. **62** 171-176.

WAREING, P.F. and PHILLIPS, I.D.J. (1978) The control of growth and differentiation in plants. 2nd Edition. Pergamon Press, Oxford. 347 pages.

WEBB, K.J., OSIFO, E.O. and HENSHAW, G.G. (1983) Shoot regeneration from leaflet discs of six cultivars of potato (Solanum tuberosum subsp. tuberosum). Plant Sci. Lett. **30** 1-8.

WEBER, G. and LARK, K.G. (1979) An efficient plating system for rapid isolation of mutants from plant cell suspensions. Theor. Appl. Genet. **55** 81-86.

WEBER, G. and LARK, K.G. (1980) Quantitative measurement of the ability of different mutagens to induce an inherited change in

phenotype to allow maltose utilisation in suspension cultures of the soybean Glycine max (L.) Merr. Genetics **96** 213-222.

WENZEL, G. (1980) Protoplast techniques incorporated into applied breeding programmes. In L. Ferenczy and G.L. Farkas (eds). Advances in protoplast research. pp.327-340. Pergammon Press, Oxford.

WENZEL, G., MEYER, C., PRZEWOZNY, T., UHRIG, H. and SCHIEDER, O. (1982) Incorporation of microspore and protoplast techniques into potato breeding programmes. In E.D. Earle and Y. Demarly (eds). Variability in plants regenerated from tissue culture. pp.290-302. Praeger Publ. USA.

WENZEL, G., SCHIEDER, O., PRZEWOZNY, T., SOPORY, S.K. and MELCHERS, G. (1979) Comparison of single cell culture derived Solanum tuberosum L. plants and a model for their application in breeding programs. Theor. Appl. Genet **55**, 49-55.

WENZEL, G. and UHRIG, H. (1981) Breeding for nematode and virus resistance in potato via anther culture. Theor. Appl. Genet. **59** 333-340.

WERNICKE, W. and THOMAS, E. (1980) Studies on morphogenesis from isolated plant protoplasts: shoot formation from mesophyll protoplasts of Hyoscyamus muticus and Nicotiana tabacum. Plant Sci. Lett. **17** 401-407.

WESTCOTT, R.J., HENSHAW, G.G. and ROCA, W.M. (1977) Tissue culture storage of potato germplasm: Culture initation and plant regeneration. Plant Sci. Lett. **9** 309-315.

WHITE, D.W.R. and VASIL, I.K. (1979) Use of amino acid analogue resistasnt cell lines for selection of Nicotiana sylvestris somatic cell hybrids. Theor. Appl. Genet. **55**, 107-112.

WIDHOLM, J.M. (1977) Selection and characterisation of biochemical mutants. In W. Barz., E. Reinhard and M.H. Zenk (eds). Plant tissue culture and its biotechnological application. pp 112-122. Springer-Verlag, Berlin.

XU, X.H. and DAVEY, M.R. (1983) Shoot regeneration from mesophyll protoplasts and leaf explants of Rehmannia glutinosa. Plant Cell Reports **2**, 55-57.

XU, Z.H., DAVEY, M.R. and COCKING, E.C. (1982) Plant regeneration from root protoplasts of Brassica. Plant Sci. Lett. **24** 117-121.

ZELCER, A., AVIV, D. and GALUN, E. (1978) Interspecific transfer of cytoplasmic male sterility by fusion between protoplasts of normal Nicotiana sylvestris and X-ray irradiated protoplasts of male-sterile N. tabacum. Z. Pflanzenphysiol. **90** 397-407.

ZENTKELLER, M. (1980) Intraovarian and in vitro pollination. Intern. Rev.Cytol. Suppl. **11B** 137-156.

ZIMMERMANN, U. and SCHEURICH, P. (1981) High frequency fusion of plant protoplasts by electric fields. Planta **151** 26-32.

ERRATA