TISSUE CULTURE AND FROST TOLERANCE STUDIES IN *SOLANUM*

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ABBREVIATIONS

ABA	Abscisic acid
BA	6-benzyladenine
BAP	6-benzylaminopurine
BDMA	Benzyldimethylamine
CCC	Chlorocholine chloride, Cycocel
C.L. Med.	Cell layering medium
Cond. Med.	Conditioning medium
cv(s)	Cultivar(s)
2,4-D	2,4-dichlrophenoxyacetic acid
DDSA	Dodecenyl succinic anhydride
Dig. Med.	Digestion medium
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethyl methanesulfonate
ENU	N-ethyl-N-nitrosourea
FDA	Fluorescein diacetate
FKT	Frost-killing temperature
g	1) Gravity; employed in describing centrifugal force, e.g. 200 g refers to a sedimentation force 200 times than of gravity 2) gram
GA, GA_3	Gibberellic acid
Gy	A unit for measuring the dose of gamma radiation, 1 Gy = 0.1 krad.

Hyp	Hydroxyproline
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
2-ip	N-isopentylaminopurine or 6-γ-dimethylallylamninopurine
KM	Kao and Michayluk (1975) medium
lux	Unit of illuminance in metric system equal to a luminous flux of 1 lumen per square metre. (1 lux = 1/10.764 foot candle)
M	Molar solution (moles/litre)
mM	Millimolar solution
MES	2(N-morpholino) ethanesulphonic acid
MS	Murashige and Skoog (1962) medium
N	Normal solution
NAA	γ-Naphthaleneacetic acid
nm	Nanometer
No.	Number
OD	Optical density
PCV	Packed cell volume
PEG	Polyethylene glycol
pH	A measure of hydrogen ion concentration $[H]^+$, and hence of the acidity or alkalinity of an aqueous solution.
Prim. Med.	Primary medium
p.s.i.	Pounds per square inch
PVP	Polyvinylpyrrolidone
Res. Med.	Reservoir medium

Rin. Med.	Rinse medium
S.D.	Standard deviation
RNA	Ribonucleic acid
r.p.m.	Revolutions per minute
TEM	Transmission electron microscopy
TTC	2,3,5-triphenyltetrazolium chloride
UV	Ultra-violet wavelength
v/v	Volume per volume
w/v	Weight per volume

ABSTRACT

In vitro shoot cultures of two commercial cultivars of *Solanum tuberosum*, Desiree and Maris Piper, and of two wild species *S. commersonii* and *S. acaule*, were established from single nodal explants and seedling tissues respectively. Callus cultures were initiated from potato stem, leaf and tuber explants. Cell suspension cultures were obtained from tuber and stem-derived calluses of *S. tuberosum*. Protoplasts were isolated from leaves of greenhouse-grown plants and from suspension-cultured cells of *S. tuberosum*, and *in vitro* shoot cultures of *S. tuberosum* and *S. commersonii*. Plantlets were regenerated from tuber discs, internodal explants, growing calluses, suspension-cultured cells and protoplast-derived calluses. Microtubers were induced from nodal explants of these *Solanum* species.

Attempts were made to select frost-tolerant cell lines through resistance to hydroxyproline, by direct transfer of axillary buds, callus cultures and suspension-cultured cells to media containing different concentrations of hydroxyproline. Selection was also made after exposure of suspension-cultured cells to a freezing temperature (-6°C), and to gamma-irradiation (20 Gy). Several hyp-resistant cell lines were established from callus cultures and suspension-cultured cells but not from axillary buds. Most of these selected cell lines were found to show increased tolerance to frost. Plants were regenerated from one of the hyp-tolerant, frost-tolerant cell lines. The cellular damage to *S. tuberosum* cv. Desiree callus cells due to freezing temperatures was examined, and the cellular structure of the callus of three *Solanum* species and one frost-tolerant cell line was compared by electron microscopy.

1. INTRODUCTION

1.1 The potato crop

1.1.1 Economic importance of the potato

The potato is a crop of worldwide importance and is an integral part of the diet of a large proportion of the world population. In terms of production, potato is the world's fourth largest food crop after rice, wheat and maize. It is known for high yield, valuable starch content, for use both as a food and for a range of industrial purposes, and for remunerative income to the growers. Potatoes supply at least 12 essential vitamins and minerals including an extremely high content of vitamin C. Potatoes also provide significant amounts of protein and iron (Gray and Hughes, 1978). It is estimated that about 65% of the potato harvest is consumed by humans, 15% is processed and used as fodder, 12.5% is retained for "seed", and 7.5% is wasted due to spoilage (Horton and Sawyer, 1985).

1.1.2 Potato production in Pakistan

Potatoes are grown in Pakistan on an area of 76 thousand hectares with the total production of 861 thousand metric tonnes (Anonymous, 1992). Nature has gifted Pakistan with excellent edaphic and favourable environmental conditions. Due to this, fresh tubers remain available throughout the year. This is achieved by growing two crops each year in the plains and one in the hill regions. Of the two crops in the plains, the spring crop is planted in January and harvested in April while the autumn crop is planted in September and harvested in January. The crop known as the hill crop relates to the months of May to September. In this way potato cropping continues

the whole year round.

1.1.3 Problems of potato production in Pakistan

The potato crop is vulnerable to attack by a very large number of pests and diseases and is frequently threatened by frost, hail, drought and or heat as well as nutrient deficiencies and toxicities. These constraints reduce yield and increase production cost per unit of output. Pakistani growers, particularly in the plains, are confronted by a serious problem of severely low temperatures during December to February. During this period, due to the occurrence of frost and temperatures falling below 0°C, most of the foliage dies and tubers in the soil may remain under-developed, hence, yield is decreased. Therefore, production of varieties resistant to lower temperatures becomes desirable.

1.1.4 Potato genetics and breeding problems

Generally the term potato refers to all the tuber-bearing *Solanum* species in addition to the commonly known *Solanum tuberosum L.* Genetically, the potato is a complex and diverse group of tuber-bearing species and subspecies belonging to the genus *Solanum.* Collectively, over 160 species are known (Hawkes, 1978) but members of *Solanum tuberosum L.* (mainly the subspecies tuberosum) occupy most cultivated acreage worldwide. Virtually all commercial cultivars are extremely heterozygous tetraploids (2n=4X=48)(Ugent, 1970), vegetatively propagated and as such pose several problems for plant breeders. These include the high level of heterozygosity, the common occurrence of pollen sterility, incompatibility, selection difficulties in the seedling and first clonal years, build-up of viruses, and difficulties in germplasm

storage (Miller and Lipschutz, 1984).

1.1.5 Possible new breeding strategies

The transfer of desirable traits from wild into cultivated species is one method of improving crops. Regarding potato breeding for frost tolerance, there is no dearth of material to meet this challenge because several non-commercial potato species, e.g., *Solanum acaule, S. commersonii, S. multidissectum,* and *S. chomatophilum* are available as frost-resistant and low temperature-tolerant plants. Several efforts have been made to evolve frost-tolerant varieties through conventional methods but the progress for potatoes in this direction seems to be rather impeded and no single potato cultivar has emerged with, for example, tolerance to environmental stresses and quick adaptability to new geographic setting coupled with desirable horticultural traits, including yield potential. Fortunately, tissue culture *in vitro* appears to have come to our rescue and a great deal of work is under way in this highly sophisticated technology.

Plant protoplasts are now considered as a valuable tool for plant improvement. Their totipotent nature and absence of cell wall offer unique possibilities for the improvement of crop plants. The fusion of protoplasts from different plants to form somatic hybrid cells and subsequent regeneration of plants from callus tissues produced from proliferation of the fusion product, offer great promise. Many somatic hybrids have been produced, most of which are not of direct agricultural importance but they may be valuable as sources of desirable traits for further breeding programmes.

Spontaneous variability occurs in cell cultures and can be exploited in mutant selection for crop improvement. The use of well-recognized variability of cultured plant cells (somaclonal variation), especially in the light of the tendency of potatoes to undergo potentially valuable somatic changes (Shepard, 1980) have suggested answers to the problem. The frequency of mutations can also be increased several times by exposing the cells to suitable mutagens and any promising frost-tolerant mutants can be selected. During the present studies efforts have been made to select frost-tolerant cell lines from potato callus and cell suspension cultures and regenerate plants from those cell lines.

1.2 Plant tissue culture

The maintenance or growth of tissues *in vitro* in a way that allows differentiation and preservation of the structure and/or function is termed tissue culture (Torres, 1989). This is generally used for the cultivation of plant parts (cells, tissues, organs) under aseptic conditions in synthetic medium *in vitro*. Current widespread interest in the utilization of plant tissue culture techniques for a wide range of research problems and objectives is due to advances and developments in its technology. These techniques are now successfully applied in the fields of agriculture and horticulture for the rapid multiplication of plants, production of pathogen-free plants, storage of germplasm, in a few cases in industry for the synthesis of pharmaceutically-useful metabolites on a commercial scale, and in basic science for the understanding and solution of basic problems in plant physiology, biochemistry, anatomy, genetics and cytogenetics. Methods have been developed for the cellular cloning, generation and screening of desirable traits, extending the range of genetic variability by means of induced

mutations and somatic clones and the formation of isolated callus and cell cultures for studies on the effects of nutrients, vitamins and growth regulators on cell growth and differentiation. Furthermore, it has become an integral part of "plant biotechnology" in which research and techniques are successfully applied for the development of improved plants for agriculture, horticulture and forestry.

1.3 Potato tissue culture

The potato is highly responsive to many tissue culture techniques, which have been applied extensively in all aspects of production and improvement. Some of these are reviewed in following sections.

1.3.1 Potato micropropagation

The most economically-successful tissue culture technique is the alternative means of plant vegetative propagation known as micropropagation. The most significant advantage offered by micropropagation over conventional methods is that in a relatively short time and space a large number of plants can be produced from a single individual independently of the seasons (Smith and Drew, 1990). Micropropagation is very important for highly heterozygous species such as potato for producing uniform plants (Warren, 1991). Potatoes can be micropropagated rapidly on a large scale by meristem and shoot-tip culture (Roca *et al.*, 1978; Goodwin *et al.*, 1980), proliferation by axillary shoots developed from *in vitro* cultured nodal cuttings (Roca *et al.*, 1978; Hussey and Stacey, 1981; Espinoza *et al.*, 1986; Al-Wareh *et al.*, 1989), production of adventitious shoots directly on explants or indirectly via a callus phase (Lam, 1977a; Roest and Bokelmann, 1976; Al-Wareh *et al.*, 1989) and *in vitro* mass

tuberization (Wang and Hu, 1982; Hussey and Stacey, 1984; Rosell *et al.*, 1987).

As endogenous contaminants cannot easily invade or rapidly multiply in meristems, meristem culture has been used successfully to eliminate viruses from plants. When combined with micropropagation techniques, a large number of disease-free plants can be produced from meristematic explants. Several techniques have been developed for the rapid propagation of potatoes from shoot tips. At least 50 plantlets may be regenerated from a single shoot tip utilizing the "multimeristem" technique (Roca *et al.*, 1978). Although this method involves induction of callus from shoot tips before regenerating shoots, varietal characteristics appeared to be stable. Dodds (1985) described two standard methods of potato micropropagation; by single node cuttings and shaken shoot cultures. When nodal sections were inoculated onto agar-solidified MS culture medium (Murashige and Skoog, 1962) supplemented with GA_3, Ca-pantothenic acid and sucrose, the number of nodes increased 6- to 7-fold within 3 - 4 weeks. When nodal sections were cultured on a liquid MS medium supplemented with GA_3, BAP, NAA, Ca-pantothenic acid and sucrose and gently agitated, after 2 - 3 weeks the number of nodes increased 15- to 20-fold (Espinoza *et al.*, 1986).

As described above, Lam (1977a) reported the formation of high numbers of adventitious shoots from tuber discs by adjusting the auxin-cytokinin ratio in modified MS medium, and Roest and Bokelmann (1976) obtained adventitious shoots (47 - 60 shoots/explant) on potato explants from the rachis of a compound leaf cultivated *in vitro*. Shepard (1982) also reported the clonal propagation of potatoes on a large scale by regeneration from isolated leaf protoplasts. Later, potato micropropagation was

achieved by culturing different explants on a suitable culture medium, but the genetic stability of regenerants is still in question. Potter and Jones (1991) attempted to assess the genetic stability of potato *in vitro* by molecular and phenotypic analysis. Their results confirmed the existence of variants when regenerated via a callus phase. No variation was found in any of the population of plants derived directly from meristems.

In vitro-produced tubers are used in seed-multiplication programmes and for germplasm distribution (Tovar *et al.*, 1985). Wang and Hu (1982) harvested about 36,000 dormant, miniature tubers from about 1,200 culture flasks incubated on a 10 m^2 bench area in a four-month period, and Rosell and his colleagues (1987) also obtained mass tuberization on plantlets growing from nodal cuttings cultured *in vitro*.

1.3.2 Potato callus culture

The first successful establishment of potato callus culture *in vitro* was achieved by Steward and Caplin (1951). Tuber explants were cultured on a basal medium containing 2,4-D and coconut milk. After 5 weeks, actively-growing calluses, 50-fold larger than the explant, were produced. Four years later similar results were reported by Chapman (1955), following which many workers initiated callus from various explants of one or more potato cultivars on agar-solidified medium containing auxin (Okazawa *et al.*, 1967; Anstis and Northcote, 1973; Lam, 1977b; Patrascu, 1981; Austin and Cassells, 1983; Chung and Sim, 1986; Al-Wareh *et al.*, 1989; Hagen *et al.*, 1990), or an auxin in combination with cytokinin (Bragdo-Aas, 1977; Gavinlertvatana and Li, 1980; Chandra *et al.*, 1981; Ahloowalia, 1982; Ochatt and Caso, 1986; Lindeque *et al.*, 1991).

Usually, callus growth starts with small, round bodies over the surface of the potato tuber discs. After transferring to fresh medium, the proliferation continues more irregularly (Bragdo-Aas, 1977). Callus initiation from an explant also depends upon the origin of the explant. Chandra *et al.* (1981) observed callus initiation within 3 - 4 days on leaf tissue and 6 - 8 days on tuber tissue on certain growth media. Remarkable differences among potato clones, such as rate of callus growth, have been reported (Bragdo-Aas, 1977). Chandra *et al.* (1981) observed differences in callus growth between dihaploid and tetraploid genotypes, but in general, actively-growing calluses in both genotypes were light yellow in colour and friable in texture.

In potato it has been possible to initiate and maintain callus from tissues of leaf, petiole, stipule, root, tuber, apical meristem, stem, rachis, peduncle, flower bud, sepal, ovary, anther, embryo and hypocotyl of most of the genotypes (Chandra *et al.*, 1981; Flick *et al.*, 1983).

Potato callus is normally maintained through subculturing on the same medium used for callus initiation. Some workers initiated callus on a medium containing only auxin but for healthy proliferation of induced callus through periodic subcultures, they found that a low concentration of cytokinin was also necessary (Okazawa *et al.*, 1967; Patrascu, 1981).

1.3.3 Potato cell suspension culture

Most suspension cultures are initiated by transferring preferably friable callus to an agitated liquid medium of the same composition used for callus growth and usually

require regular subcultures at more frequent intervals than the callus cultures from which they are derived. Anstis and Northcote (1973) initiated suspension cultures of cv. King Edward by transferring small pieces of solid potato tuber callus to liquid medium, incubating at 26°C and shaking continuously at a speed of 90 rpm. Due to the agitation, callus separated easily to give a white suspension culture. The growth curve appeared to be relatively independent of the size of initial volume, beyond an inoculum size (fresh weight) of 16 mg callus/ml medium. Later, research workers initiated potato cell suspension cultures from actively-growing calluses and maintained these for different experimental aims (e.g. Lam, 1977b; Bragdo-Aas, 1977; Austin and Cassells, 1983; De Vries and Bokelmann, 1986; Chung and Sim, 1986; Tavazza *et al.*, 1988; Gerard *et al.*, 1991; Lindeque *et al.*, 1991).

In the suspension culture of potato cv. King Edward, Anstis and Northcote (1973) observed a lag phase of 2 days, followed by a phase of rapid growth. After 19 days the cells were entering a stationary phase. Chung and Sim (1986) also obtained similar results with the cell suspension cultures of potato cv. Dejima. In order to increase the growth rate of cells in suspension cultures, Gerard *et al.* (1991) suggested a high ratio for nitrate/ammonium in MS medium and the addition of the auxins 2,4-D and NAA.

1.3.4 Potato plantlet regeneration

Potato plants have been successfully regenerated from different explants including tuber discs, stem segments, leaf pieces, and callus tissue, suspension-cultured cells, and protoplasts. It is very difficult to generalize the method of inducing adventitious shoots and regeneration *in vitro* because the process of organogenesis may vary among

species, cultivars (clones) and especially the donor tissue. Both one-step and sequential methods have been used in plantlet regeneration from organized tissue. In a sequential method explants are transferred through two or more different culture media or incubating conditions.

Okazawa and coworkers (1967) reported on the formation of adventitious shoots from large explants of tuber pith tissue cultured in the absence of auxin. Multiple shoot regeneration from tuber discs was first achieved by Lam (1975). Embryoid bodies and shoots were produced from the calluses on tuber explants cultured on modified MS medium containing kinetin and BAP. However, the shoots produced were abnormal and subsequently the medium was refined to allow the production of normal plants (Skirvin *et al.*, 1975). Fully-developed shoots were observed only when tuber discs were cultured on a complex medium containing several auxins and cytokinins (Lam, 1977a). Jarret *et al.* (1980b) initiated adventitious shoots from tuber discs of 8 out of 10 cultivars tested on modified MS medium supplemented with NAA, BAP and GA_3. Adventitious shoots were formed only from the discs of cortical and perimedullary tissue. Discs of pith tissue either did not survive or failed to undergo morphogenesis (Jarret *et al.*, 1980a). Later, Kikuta and Okazawa (1982) succeeded in regenerating adventitious shoots from the tuber pith tissue by preparing explants from the central core of potato tuber (presumably containing mostly pith tissue) and culturing on modified White's medium supplemented with IAA and zeatin. Bragdo-Aas (1977) used a sequential regeneration method with modified MS medium plus NAA, and kinetin in the callus formation stage, IAA, kinetin, BAP and GA_3 in the shoot-forming stage, and hormone-free medium in the rooting stage. Wheeler *et al.* (1985) also recovered

a few plants from tuber discs of seven out of 12 cultivars tested by a two-step procedure method using Shepard (1980) C/D media.

Although plants can be regenerated from various explants of potato, explants of the compound leaf (petiole, rachis and leaflet discs) have the highest shoot regeneration potential. Roest and Bokelmann (1976) reported a high incidence of shoot development from explants of rachis of the clone EM-52 cultured on MS medium containing NAA and BAP. In some cases even 100 shoots were produced on one explant when GA_3 was added to the basal medium. Maroti *et al.* (1982) reported plantlet regeneration from shoot segments of four potato cultivars; Bintje, Desiree, Gracia and Ostara. The highest number of plantlets was developed from the explants cultured on MS medium supplemented with NAA and kinetin. Webb *et al.* (1983) cultured leaflet discs on the medium developed by Roest and Bokelmann (1976) both with and without GA_3. Only a low level of shoot regeneration was achieved (1 or 2 per explant). They then used a sequential method and cultured explants on MS medium with different combinations of NAA and BAP both with and without GA_3. After 14 days explants were transferred to another medium with a different combination of growth regulators. Optimal shoot regeneration was achieved when both BAP and GA_3 were present in the second medium. All the tested cultivars except Pentland Crown produced shoots but the frequency of shoot formation was highest in Desiree. Later, many workers (e.g., Karp *et al.*, 1984; Wheeler *et al.*, 1985; Fish and Jones, 1988; Cardi *et al.*, 1992) regenerated plantlets from various explants (leaf, rachis, stem pieces) of dihaploid and tetraploid clones using sequential methods. Plants have also been regenerated from stem and leaf explants of wild *Solanum* species. Kaburu M'Ribu and Veilleux (1990)

regenerated plants from various *in vitro* anther-derived monoploids (2n = x = 12) of *Solanum phureja* from stem and leaf explants under various environmental conditions using a two-step regeneration method. Leaf explants regenerated at high frequencies and the number of shoots per explant was greater when explants were incubated at 20°C compared to 25°C. Iapichino *et al.* (1991) examined the influence of growth regulators on plant regeneration from leaf and stem explants of *Solanum commersonii*. Various auxins and cytokinins in different combinations were used and nearly all explants produced shoots with an average of 12 shoots per explant when IAA and zeatin were used in the medium.

Regeneration of plantlets from callus has proved to be quite difficult and was not achieved for many years. Wang and Huang (1975) regenerated plantlets from stem and shoot tip-derived callus of cv. Norina No. 1 using kinetin and IAA in MS medium. Patrascu (1981) used zeatin alone in the modified MS medium to induce shoot formation, while Ahloowalia (1982) used zeatin with 2,4-D in a half-strength MS medium and obtained multiple shoot primordia in the proliferating calluses that stayed regenerative even after routine subculturing during more than 3 years. These shoot primordia did not develop into shoots until they were transferred to hormone-free medium. Al-Wareh *et al.* (1989) transferred calluses of potato cvs; Red LaSoda, Norgold"M" and Viking on different shoot media, when regeneration occurred only from Red LaSoda leaf callus cultured on MS medium supplemented with IAA, BA and GA_3. Only 30% of the calluses transferred produced one to three shoots per callus. The media developed by Roest and Bokelmann (1976), Webb *et al.* (1983), and Wheeler *et al.* (1985) failed to regenerate shoots from any of the cultivars tested.

Plants have also been regenerated from single, isolated, suspension-cultured cells and from protoplasts. Lam (1977b) regenerated plantlets from isolated single cells of tuber tissue origin. The frequency of shoot initiation varied from 10% in *S. tuberosum* to 70% in *S. demissum*. Austin and Cassells (1983) regenerated shoots from individual calluses produced from separated cells of potato stem callus. The procedure developed by Lam (1977b) to regenerate plantlets from suspension-cultured cells was used by several workers. Lindeque *et al.* (1991) established suspension cultures by inoculating friable callus, initiated from stem internode sections of potato cv. BP1, into a MS liquid containing 2,4-D, NAA and kinetin. They also regenerated plantlets from these suspension-cultured cells and reported phenotypic variation among regenerated plants.

1.3.5 Potato protoplast isolation, culture and plantlet regeneration

Potato protoplasts have been isolated, cultured and regenerated into whole plants from cell suspension cultures, callus cultures, and tubers, but mainly from leaf mesophyll tissue. Leaves have been taken either from greenhouse-grown plants or *in vitro* shoot cultures.

Potato protoplast isolation was first reported by Lorenzini (1973) from tuber tissue, but isolated protoplasts failed to divide in the culture medium. Two years later, Upadhya (1975) isolated protoplasts from leaves of greenhouse-grown plants, which after culturing in the liquid medium, synthesised new cell walls, divided and formed calluses. In some of these cultures, root formation was observed but no shoot regeneration occurred. Successful regeneration of plantlets from potato protoplasts was first achieved by Shepard and Totten (1977). They isolated protoplasts from leaves of

potato cv. Russet Burbank taken from greenhouse-grown plants. Binding *et al.* (1978) isolated mesophyll protoplasts from *in vitro* shoot cultures of six dihaploid potatoes, and plant regeneration was also reported in four clones. Later, isolation, culture and plant regeneration from mesophyll protoplasts of potato (*Solanum tuberosum L.*) was reported for several cultivars by a number of authors (e.g. Bokelmann and Roest, 1983; Haberlach *et al.*, 1985; Foulger and Jones, 1986; Tavazza and Ancora, 1986; Taylor and Secor, 1988; Briza and Machova, 1991).

Cultured cells have also frequently been used for protoplast isolation and culture, and plant regeneration has been achieved from protoplasts isolated from both callus tissue and suspension-cultured cells. Melchers (1978) isolated protoplasts from suspension-cultured cells of dihaploid potato and successfully regenerated plants from the protoplast-derived calluses. Later, protoplasts were successfully isolated and regenerated into whole plants from leaf or stem-derived callus tissue (De Vries and Bokelmann, 1986; Avetisov *et al.*, 1987) of different potato cultivars and suspension cultures (e.g. De Vries and Bokelmann, 1986; Tavazza *et al.*, 1988) including some wild species (Handley and Sink, 1985; Tan *et al* ., 1987)

Although at first protoplasts were isolated from tuber tissue, plant regeneration from tuber tissue-derived protoplasts was not possible until 1989. Opatrny *et al.* (1980) isolated protoplasts from tuber-derived cell suspension cultures, and obtained calluses but no shoot regeneration. Jones *et al.* (1989) isolated protoplasts from storage parenchyma cells of potato tubers grown *in vitro*, and obtained viable calluses, regenerated shoots, and intact plants.

Protoplasts have also been isolated from the apices of *in vitro* cultured roots of *S. tuberosum* and plants have also been regenerated from such protoplasts (Laine and Ducreux, 1987).

Protoplast isolation, culture and subsequent plant regeneration have also been achieved from a number of wild *Solanum* species including *S. brevidens* (Barsby and Shepard, 1983; Nelson *et al.*, 1983; Helgeson *et al.*, 1984; Haberlach *et al.*, 1985), *S. demissum* (Haberlach *et al.*, 1985), *S. etuberosum* (Barsby and Shepard, 1983; Pellow and Towill, 1984, 1986; Haberlach *et al.*, 1985), *S. phureja* (Schumann *et al.*, 1980; Schumann and Koblitz, 1983) and *S. commersonii* (Cardi *et al.*, 1990).

1.3.6 *In vitro* tuberization

The first report on *in vitro* tuberization in potato was published by Barker in 1953 (Chandra *et al.*, 1988). Since then different aspects of *in vitro* tuberization have been studied but there is considerable disagreement in the conclusions of these studies. Different factors have been reported to induce the formation of *in vitro* tubers. These include;

a) growth regulators (cytokinins, auxins, gibberellins, ethylene)

b) growth retardants (abscisic acid, chlorocholine chloride, paclobutrazol)

c) culture conditions (temperature, photoperiod, light intensity), and

d) the carbon source (sucrose, glucose)

For inducing potato tuberization *in vitro*, much attention has so far been focused on the use of growth regulators, in particular the role of cytokinins such as BAP (Wang

and Hu, 1982; Hussey and Stacey, 1984; Tovar *et al.*, 1985; Abbot and Belcher, 1986; Estrada *et al.*, 1986; Rosell *et al.*, 1987; Lentini and Earle, 1991; Harvey *et al.*, 1991), 2ip (Levy *et al.*, 1993), kinetin (Kwiatkowski *et al.*, 1988; Pelacho and Mingo-Castel, 1991), and zeatin (Koda and Okazawa, 1983).

Compounds that inhibit vegetative growth frequently stimulate tuberization. Abscisic acid (ABA) in the medium increases the tuberization rate progressively with the increase in concentration (Koda and Okazawa, 1983). Chlorocholine chloride (CCC) in combination with BAP in the medium can easily induce tuberization in a broad range of genotypes in a period of 4 weeks (Tovar *et al.*, 1985; Estrada *et al.*, 1986; Lentini and Earle, 1991; Rosell *et al.*, 1987). Harvey *et al.* (1991) tested the effects of CCC, daminozide, ancymidol and paclobutrazol, all stimulated tuberization *in vitro* at lower concentrations but CCC and daminozide reduced microtuber fresh weight in some cultivars tested. Simko (1991) also found a speeding-up effect of paclobutrazol on tuber initiation, and the percentage of tuber-forming plantlets increased from 53% to 100%.

In general, 18 - 25°C is considered a suitable temperature range for *in vitro* tuberization (Bottini *et al.*, 1981; Hussey and Stacey, 1981; Tovar *et al.*, 1985; Estrada *et al.*, 1986; Lentini and Earle, 1991), and a low light intensity (100 - 500 lux) with a short photoperiod (8 hours) is most effective (Wang and Hu, 1982; Garner and Blake, 1989). However, many workers have induced tubers *in vitro* in the dark (e.g. Mangat *et al.*, 1984; Tovar *et al.*, 1985, Estrada *et al.*, 1986; Rosell, *et al.*, 1987).

The carbon source directly influences the carbohydrate synthesis needed for tuberization. Different concentrations of sugars in the medium have been used, e.g. sucrose at 4 - 12% has been used widely for tuberization *in vitro* (Garner and Blake, 1989). Hussey and Stacey (1981 & 1984) used 6% sucrose in the medium in combination with BAP. Other workers (Wang and Hu, 1982; Tovar *et al.*, 1985; Estrada *et al.*, 1986; Rosell *et al.*, 1987; Lentini and Earle, 1991; Harvey *et al.*, 1991; Levy *et al.*, 1993) have used sucrose with additives like CCC, ABA, paclobutrazol or a cytokinin in the medium to induce tuberization *in vitro* while Mangat *et al.* (1984) used sucrose with different concentrations of 2,4-D, and Garner and Blake (1989) used sucrose in combination with a photoperiod without any growth regulator.

Most of the *in vitro* tuberization studies have been conducted on different clones of *S. tuberosum*, but other tuber-bearing *Solanum* species such as *S. microdontum, S. pinnatisectum* (Kwiatkowski *et al.*, 1988) and *S. berthaultii* (Lentini and Earle, 1991) have also been studied.

1.4 Low-temperature tolerance in plants

There are two kinds of low temperature stress; chilling stress and freezing stress. A low-temperature damage to the plant in the absence of freezing is called a chilling injury and a chilling temperature can be defined as any temperature that is cool enough to produce injury but not enough to freeze the tissue. However, a chilling injury may occur when a plant is exposed to any temperature between about 10-15°C and 0°C, and the ability of a plant to survive when exposed to chilling temperature is known as

chilling tolerance. Only plants originating from tropical and subtropical regions are vulnerable to chilling injury. Two major symptoms of chilling injury are sudden loss of semipermeability of cell membranes and alteration of respiratory activity (Levitt, 1980). Plants cannot avoid chilling injury, only tolerance mechanisms enable plants to survive the effect of chilling temperature. Low temperature can cause injury through changes in the fluidity of membranes, which may disrupt ion transport, denature proteins, and induce imbalance in normal metabolism (Lindsey and Jones, 1989).

Frost tolerance on the other hand, is defined as the ability of leaf tissue to survive ice formation when subjected to subzero temperatures. Frost tolerance in plants depends upon their ability to tolerate freeze-induced dehydration and the resultant mechanical stresses, and to avoid intracellular freezing. In potatoes, the term frost tolerance is generally used to describe their ability to survive -4°C or lower without injury (Li and Fennell, 1985).

Most of the tuber-bearing *Solanum* species can survive a few degrees below zero, but some species cannot tolerate even a slight frost and are considered to be frost sensitive. Among the frost-hardy *Solanum* species, some are not only frost-hardy but also able to cold acclimate, while others are unable to cold acclimate (Chen and Li, 1980a). On the basis of leaf frost hardiness and ability to cold acclimate, tuber-bearing *Solanum* species have been classified into five groups (Table 1);

a) frost resistant, able to cold acclimate

b) frost resistant, unable to cold acclimate

c) frost sensitive, able to cold acclimate

Table 1. Classification of tuber-bearing *Solanum* species in terms of cold tolerance and cold acclimation (adapted from Chen and Li, 1980a).

Groups & Species	Killing temperature (°C)	
	Before acclimation	After acclimation
Group 1: Frost resistant, able to cold acclimate		
S. acaule	-6.0	-9.0
S. commersonii	-4.5	-11.5
S. multidissectum	-4.0	-8.5
S. chomatophilum	-5.0	-8.5
Group 2: Frost resistant, unable to cold acclimate		
S. bolviense	-4.5	-4.5
S. megistacrolobum	-5.0	-5.0
S. sanchae-rosae	-5.5	-5.5
Group 3: Frost sensitive, able to cold acclimate		
S. oplocense	-3.0	-8.0
S. polytrichon	-3.0	-6.0
Group 4: Frost sensitive, unable to cold acclimate		
S. brachistotrichum	-3.0	-3.0
S. cardiophyllum	-3.0	-3.0
S. fendleri	-3.0	-3.0
S. jamesii	-3.0	-3.0
S. kurtzianum	-3.0	-3.0
S. microdontum	-3.0	-3.0
S. pinnatisectum	-3.0	-3.0
S. stenotomum	-3.0	-3.0
S. stoloniferum	-3.0	-3.0
S. sucrense	-3.0	-3.0
S. tuberosum	-3.0	-3.0
S. venturii	-3.0	-3.0
S. vernei	-3.0	-3.0
S. verrucosum	-3.0	-3.0
Group 5: Chilling sensitive		
S. trifidum	-3.0	dead

Note: Plants were grown at 20/15°C day/night under 14 h light. Cold acclimation was done at 2°C day/night under 14 h light for 20 days.

d) frost sensitive, unable to cold acclimate, and

e) chilling sensitive.

There are two types of mechanism of freezing resistance when exposed to freezing stress; avoidance and tolerance. Plants may avoid freezing stress by the ability to supercool or to accumulate enough antifreeze to maintain their freezing points below that of the environment. Plants capable of supercooling can actually become hardened to freezing temperatures by increasing their supercooling ability. Supercooling is favoured by a low water content, and during hardening all cells are known to decrease in water content. Cell wall thickening may also occur during the hardening period, and so native plants are adapted to episodic frosts by freeze-avoidance mechanisms of insulation, ability to supercool, and lowered freezing point of the tissues (Levitt, 1980).

In potatoes, exposure to subzero temperatures without the presence of ice does not cause frost injury. Leaflets of *S. tuberosum* show freezing injury at -3°C but can be supercooled to -4°C for several days with no apparent injury. Several other *Solanum* species can also survive supercooling for shorter periods below their freeze-killing temperatures (Rajashekar *et al.*, 1983).

Tolerance to intracellular freezing is not possible in plants presumably because of rupture of the cell membranes by internal ice crystals, with the destruction of the semipermeability essential for life. The only tolerance developed by the plant is, therefore, tolerance to extracellular freezing. Any increase in freezing tolerance is due

to increase in the avoidance or tolerance of dehydration stress or both, and an increase in freezing tolerance during hardening is mainly due to an increase in the tolerance of freeze-induced dehydration.

1.5 Frost injury / freezing injury

Freezing injury depends on the rate of freezing and thawing and the length of the freezing period. Rapid cooling increases the freezing injury, and rapid warming may also affect freezing injury, although this has been observed less frequently than injury due to rapid cooling. A direct relationship also exists between length of time frozen and the amount of injury (Levitt, 1980).

Freezing injury itself consists of two main types; primary freezing injury and secondary freezing injury. Primary injury is a result of intracellular (or intra-protoplasmal) freezing, which is always followed by death of cells if ice crystals are large enough to be detected microscopically. Intracellular injury has rarely been observed in nature because the rate of freezing is not too rapid (Levitt, 1980).

Because in extracellular freezing there is no direct contact between ice and cytoplasm, primary freezing injury does not occur but secondary freezing injury may result. Secondary freezing injury is a result of (a) ice pressure in the wall and the intercellular spaces which exerts mechanical stress, causing cell crushing and collapse, (b) freeze-dehydration by withdrawal of water from the cells which results in the increased concentration of cell sap, particularly the concentration of salts or acids, or (c) freeze smothering, when plants are covered or imbedded in a sheet of ice which disrupts

normal respiration due to oxygen deficiency. Damage occurring to the cell membranes during extracellular freezing is presumably a result of mechanical stress and/or freeze-dehydration (Li and Fennell, 1985).

In potatoes, after a damaging freeze-thaw cycle, the first sign of freezing injury is a water-soaked appearance which is due to infiltration of intercellular spaces with liquid and loss of turgor. It is assumed that this infiltration of the intercellular spaces is the result of rupture or loss of semipermeability of the cell membrane (Li and Fennell, 1985). When *S. tuberosum* leaflets were frozen to -3°C and examined in the electron microscope, the cell membranes, chloroplasts, mitochondria and nuclei appeared to be normal when compared with those of unfrozen cells despite 75% leakage of cellular ions. When the temperature was further decreased, to a point below that initiating damage, swelling of cytoplasm, including chloroplasts, and mitochondria, and finally physical damage was observed in both *S. tuberosum* and *S. acaule* (Palta *et al.*, 1983).

1.6 Factors related to frost tolerance in potato

Unfortunately, the factors involved in freezing tolerance are not fully known, but many have been investigated, including morphological, anatomical, physiological and genetic factors.

1.6.1 Morphology

The morphology of several frost-hardy and non-hardy potato species has been studied and it has frequently been noticed that hardiness varies inversely with the plant height, internode length and leaf length. However, although frost-hardy potato species tend

to have shorter and highly branched stems with smaller and thicker leaves, no consistent relationship has been found between morphological characters and frost tolerance (Palta and Li, 1979). Such a relationship could be expected if the characters measured are the result of development during the hardening period but an opposite relationship may be true for species of native herbaceous plants that complete their growth before the hardening period. Thus the taller plant may possess greater freezing tolerance presumably due to the selective effect of low temperatures to which they are exposed (Levitt, 1980).

1.6.2 Anatomy

Small cell size, thick cell walls and low stomatal density have been reported to be associated with frost tolerance (Chen *et al.*, 1977; Estrada, 1982; Li and Fennell, 1985). This relationship does not hold true for all potato species because contradictory results have also been reported and it is concluded that cell size is probably a minor factor in relation to frost tolerance. Difference in the number of palisade layers in potato leaves has also been reported (Palta and Li, 1979). Frost-hardy potato leaves had two layers of palisade parenchyma while less cold hardy only one layer and this character was also found to be genetically transmittable (Estrada, 1982).

1.6.3 Physiological factors

The most commonly investigated factor in relation to frost tolerance is the amounts of specific substances and their accumulation during hardening. A number of studies have revealed that in general, cell sap concentration increases with freezing tolerance, and this trend has also been observed during hardening (Levitt, 1980). Increase in cell sap

concentration may sometimes be a factor, but it is unable by itself to account for all the accompanying increase in frost tolerance. Decrease in water content has been reported during cold treatment in both cold acclimating and non-acclimating tuber-bearing *Solanum* species (Li and Palta, 1978). As the decrease was also observed in non-acclimating species with no increase in frost tolerance, no real relationship between water content and frost tolerance can be concluded. The reduction in water content may be the result of decreased water uptake or due to the accumulation of cellular solutes (Chen and Li, 1980b).

It has long been thought that sugars must play some role in the mechanism of frost tolerance, and at least in some plants, increase in sugar level during cold acclimation is associated with increased frost tolerance. In the leaves of tuber-bearing *Solanum* species, an increase in sugar and starch content has been recorded during cold acclimation. The increase in sugars was greater in cold acclimating potato species (*S. commersonii*) than in non-acclimating species (*S. tuberosum*) (Chen and Li, 1980b; Chen and Li, 1982). In cold acclimating species the increase in sugars was not proportional to the increased frost hardiness.

Some early investigators also attempted to correlate the amino acid contents of plants with frost tolerance, but the results presented a confusing picture (Levitt,1980). In some plants the amino acid content increased with increase in frost hardiness, in others no relationship was found. Of the amino acids, proline has been reported by several investigators to accumulate during hardening or to increase more in hardy plants (Stefl *et al.*, 1978; van Swaaij *et al.*, 1985). Some workers also reported significant

correlations between proline concentration and frost tolerance in different species, e.g., potato (van Swaaij *et al.*, 1985), winter barley (Dobslaw and Bielka, 1988), and winter wheat (Dorffling *et al.*, 1990). Exogenous application of proline also increased both the proline content and frost tolerance of *S. andigena* leaves (van Swaaij *et al.*, 1985) and cell cultures of *S. tuberosum* (Hellergren and Li, 1981).

It appears that specific protein changes occur during cold acclimation. According to Chen and Li (1980b), increase in frost hardiness during cold acclimation in cold acclimating *Solanum* species (*S. acaule* and *S. commersonii*) was linearly correlated with increase in total soluble proteins. Such a correlation did not exist in the non-acclimating species (*S. tuberosum*) during the same treatment, suggesting that soluble proteins are synthesized during the cold acclimation process. However, no specific proteins directly involved in the development of frost hardiness in potato are known.

In early studies a positive correlation between lipid content and frost tolerance was reported in many plant species (Levitt, 1980). In potato, during cold acclimation an increase in total lipid content was recorded in cold acclimating *S. acaule* but no change occurred in non-acclimating *S. tuberosum* (Chen and Li, 1980b). Among the lipids, phospholipid content has been correlated with frost tolerance. Chen and Li (1980b) also found higher phospholipid content in acclimated *S. acaule* followed by non-acclimated *S. acaule* plants when compared with cold-treated and untreated *S. tuberosum* plants.

Several investigators have reported that with a number of different species hardening

is accompanied by an increase in inhibitor content, e.g. ABA, and decrease in auxin and gibberellin levels. Chen *et al.* (1983) reported that in potato, ABA level increased when plants were subjected to acclimating temperatures. Exogenously applied ABA also increased frost hardiness in stem-cultured plants of *S. tuberosum* and *S. commersonii* (Chen *et al.*, 1979; Chen and Li, 1982) and cell cultures (Lee *et al.*, 1992) of *S. commersonii*. ABA is probably not directly involved in frost tolerance but may be involved in the synthesis of specific protein responsible for frost tolerance (Li and Fennell, 1985).

1.6.4 Genetic factors

It is still unclear which gene or genes control frost tolerance in potatoes. However, genetic investigations have so far succeeded only in pointing to a multifactor relationship. As so many generations of crosses and the use of recurrent selection are required to achieve frost tolerance, combined with other horticultural traits in determined individuals or clones, Estrada (1982) supposed that frost tolerance is a very complex trait with polygenic inheritance. Stone *et al.* (1992 & 1993) investigated the mode of inheritance of frost tolerance and cold acclimation and found that these two traits are genetically distinct, suggesting independent genetic control.

1.7 Somaclonal variation

Somaclonal variation is a general phenomenon of all plant regeneration systems that involve a callus phase, whether regeneration occurs through somatic embryogenesis or by adventitious shoot formation (Lindsey and Jones, 1989). It was noticed several years ago that plants regenerated from somatic cells via tissue culture are not

genetically identical but express variability. Therefore, plant tissue culture techniques can be used to produce new variation in existing crop varieties. This is particularly true in a species that is traditionally propagated asexually or for which few cultivars are available (Smith and Drew, 1990). The main advantage of utilizing somaclonal variation is that change occurs only in one or a few characters of an otherwise outstanding cultivar without necessarily altering the remaining, and often unique part of the genotype. Hence the technique can be used to uncover new variants that retain the favourable qualities of an existing variety while adding one additional trait such as disease resistance or frost tolerance. By using this approach it is possible to produce new breeding lines with desirable traits in a short period of time. There are instances where somaclonal variation has produced agriculturally useful variants for several traits in crop plants, for example improvement of tuber shape, colour and uniformity, and late blight resistance in potatoes (Shepard et al., 1980), increased solids, male sterility and resistance to *Fusarium* race 2 in tomatoes, yield and male sterility in rice, earliness in maize, disease resistance in wheat, sugarcane and celery, and freezing tolerance in wheat (Evans, 1989).

If the selection is for simply-inherited and easily-selected traits such as herbicide resistance or disease resistance, selection from somaclonal variation can be coupled with a variation-inducing process. Under ideal circumstances, preselected plants transferred to the field would express desirable traits. Stability of somaclones is, however, not always the rule. Desirable characteristics in newly-regenerated plants may be gradually lost over successive generations, and in such cases the mechanisms determining the newly arisen phenotype are likely to be different from those

responsible for stable changes (Stafford, 1991). It is probable that somaclonal variation will be used widely for crop improvement, because the techniques are simple and plants can be transferred directly to the field and evaluated as part of an ongoing breeding programme.

1.8 In vitro mutagenesis

Induced mutations have been extensively used in plant breeding either directly or via cross-breeding schemes. Seeds have most frequently been used for mutagen treatment and many commercial crop varieties have been produced. In vegetatively-propagated crops, plant parts with buds or whole plants are used and mutagen treatment often results in a chimera instead of a solid mutant organ, which is undesirable to plant breeders. Mutation is a one-cell event, and as *in vitro* regenerated plantlets frequently have a single-cell origin, tissue culture techniques can be used to obtain solid mutants. In higher plants, mutant selection from cell cultures also has the advantage over whole plant selection that a very large population can be screened out at one time for a desirable trait (Flick, 1983).

The genetic instability of cultured plant cells frequently produces sufficient variants within a population without the need for artificial mutagenic treatment. For plant species with a low frequency of somaclonal variation, it may be necessary to induce mutants in cells before regeneration. Mutagenic agents such as ultraviolet light, X-rays, gamma rays, ethylmethane sulphonate and nitrosoguanidine are sometimes applied to increase the frequency of variants, but evidence for their efficacy is uncertain (Dix, 1986; Cresswell, 1991).

1.9 *In vitro* selection

In vitro selection is a useful tool in identifying plants showing resistance or tolerance to specific environmental stresses such as low and high temperatures, salinity, drought, herbicides, toxic metals and pathogens (Tomes and Swanson, 1982; Chaleff, 1983). Traditional breeding and selection at the whole plant level has been successful in incorporating many of these traits into cultivated varieties. *In vitro* selection will complement these traditional methods and should be of most use where no natural resistance is found within a species or where conventional breeding practices are difficult.

In vitro selection usually involves subjecting a population of cells to a suitable selection pressure, recovering any variant lines which have developed resistance or tolerance to the stress and then regenerating plants from the selected cells. This approach presumes that tolerance operating at unorganized cellular level can act, at least to some degree of effectiveness, in the whole plant. If the tolerance has a genetic basis then the trait could be transferred to other plants. Many stress-tolerant cell lines of crop plants have so far been selected from cell cultures. Most of these were selected for resistance or tolerance to stress produced by cold or high temperatures, drought, aluminium, manganese, and salt toxicity (Table 2).

Plant cell cultures can be useful systems for isolating and studying the cellular response to various environmental stresses. As the whole plant response is a combination of many factors, so great care must be taken in the analysis of the system because cellular activities may not be similar to the whole plant response (Smith, 1992).

Table 2. Stress-tolerant cell lines (adapted from Dix, 1986 and updated).

Stress	Species	Reference
Freezing	*Hedera helix* (ivy)	Steponkus, 1972
	Picea excelsa (spruce)	Tumanov *et al.*, 1977
	Daucus carota (carrot)	Templeton-Somers *et al.*, 1981
	Triticum aestivum (wheat)	Kendall *et al.*, 1990
Chilling	*Nicotiana sylvestris*	Dix & Street, 1976; Dix, 1977
	Capsicum annuum (sweet pepper)	Dix & Street, 1976
Temperature extremes	*Antirrhinum majus*	Melchers & Bergmann, 1959
Salt	*N. sylvestris*	Zenk, 1974; Dix & Street, 1975
	N. tabacum (tobacco)	Nabors *et al.*, 1975, 1980; Hasegawa *et al.*, 1980; Watad *et al.*, 1983
	C. annuum	Dix & Street, 1975
	Medicago sativa (alfalfa)	Croughan *et al.*, 1978
	Datura innoxia	Tyagi *et al.*, 1981
	Glycine max (soybean)	Jia-Ping *et al.*, 1981
	Citrus sinensis (orange)	Ben-Hayyim & Kochba, 1982
	Oryza sativa (rice)	Yano *et al.*, 1982
	Avena sativa (oat)	Nabors *et al.*, 1982
	Colocasia esculenta (taro)	Nyman *et al.*, 1983
	Pennisetum americanum (pearl millet)	Rangan & Vasil, 1983
Aluminium	*Lycopersicon esculentum* (tomato)	Meredith, 1978
	D. carota	Ojima & Ohira, 1983
Manganese	*D. carota*	Ojima & Ohira, 1983
Mercury	*Petunia hybrida*	Colijn *et al.*, 1979
Drought (PEG-induced water stress)	*N. tabacum*	Heyser & Nabors, 1979
	L. esculentum	Handa *et al.*, 1983

1.9.1 *In vitro* selection methods

A. Resistance selection

This is the most widely practised method of *in vitro* selection and is applied when lines have a selective advantage over the wild type under certain conditions. Simply, mass cultures are grown under the defined conditions (e.g. toxic level of drugs) or after being subjected to adverse culture conditions (e.g. chilling). The end result may be that cell lines capable of withstanding the selective conditions will grow, and can be recovered. This method has frequently been used to select lines resistant to disease, herbicides and environmental stresses. This approach is applicable not only to the selection of resistant cell lines but also to the selection of other classes such as nuclear and cytoplasmic hybrids, transformed cells and to obtain genetic markers (Colin and Dix, 1990).

B. Visual selection

This method is used when the required phenotype is readily identified by appearance, permitting rapid visual screening of a large number of cells, cell aggregates or colonies (Colin and Dix, 1990). This method has been most commonly used to select cell lines accumulating or losing pigments (chlorophyll, anthocynin, carotenoids etc.). This technique has also been used for the identification of heterokaryons after protoplast fusion (Menczel *et al.*, 1978), cell sorting on the basis of optical characteristics, obtaining antibiotic (Cseplo and Maliga, 1982) or herbicide resistant (Arntzen *et al.*, 1982) cell lines with combined features of resistance and visual selection.

C. Counterselection

This method is used when there is no condition in which the mutant/variant cells have a selective advantage over the wild type. For example for many lethal mutants (auxotrophs, temperature-sensitive mutants etc.) when there is no direct approach for selection (Dix, 1986). To overcome this, an indirect selection procedure (counterselection) has been developed, depending on the use of agents which kill only dividing cells so that others, division of which is inhibited, will survive (Colin and Dix, 1990).

D. Total selection

Total selection is applied to those lines which cannot be subjected to selection pressures such as growth tests on non-permissive medium (for identifying auxotrophs) or immunological tests (for identification of lines producing secondary metabolites) (Dix, 1986). This method involves the systematic screening of a large number of individual cell colonies using different screening procedures appropriate to that phenotype.

E. Other selection methods

A comprehensive knowledge of the physiological or biochemical basis of a particular phenotype, together with its biosystematic pathways may lead to a selection approach. For example, an understanding of metabolic pathways and their control may suggest means of selecting lines which accumulate certain compounds. The production of amino acid-overproducing cell lines by selection for resistance to amino acid analogues

is possible. Widholm (1972a) first reported the production of tryptophan-overproducing lines of tobacco by selection with 5-methyltryptophan and later this approach has been used to obtain cell lines accumulating various amino acids, secondary metabolites and auxin autotrophic lines.

1.10 Amino acid and amino acid analogue resistance

Many cell lines resistant to amino acids or amino acid analogues have so far been selected. Tobacco and carrot have frequently been used for selecting amino acid analogue-resistant cell lines (Table 3). Variant cell lines resistant to amino acid analogues have been used to elucidate biochemical pathways of amino acid biosynthesis. The general aim of selection has been to obtain feed-back-insensitive mutants which overproduce specific amino acids. Most amino acid analogue-resistant cell lines have not been genetically characterized, but are often quite stable, even when cultures are re-initiated from regenerated plants (van Swaaij *et al.*, 1987a & b). The potential use of such mutants could be selection for environmental stresses, secondary metabolite production, or increased amino acid content of seeds.

In many instances increases of amino acid pools have been reported in amino acid analogue-resistant cell lines. Carlson (1973) isolated methionine sulphoximine-resistant mutants in *Nicotiana tabacum*, overproducing methionine and with increased resistance to *Pseudomonas tabaci* (the causative agent of wildfire disease). Increased levels of proline in proline analogue-resistant lines, e.g. hydroxyproline-resistant potato (van Swaaij *et al.*, 1986) and wheat lines (Tantau and Dorffling, 1991) may lead to plants resistant to freezing stress. Therefore, through the use of amino acid analogues,

Table 3. Amino acid analogue-resistant cell lines selected for in crop plants.*

Amino acid	Analogue	Species	Reference
Glycine	Glycine hydroxamate	*Nicotiana tabacum* (tobacco)	Lawyer *et al.*, 1980
Lysine	S-(aminoethyl)-L-cysteine	*Oryza sativa* (rice)	Chaleff & Carlson, 1975 Schaeffed & Sharpe, 1981
		N. tabacum	Widhholm, 1976
		Daucus carota (carrot)	Widholm, 1978 Mathews *et al.*, 1980
		N. sylvestris	White & Vasil, 1979 Negrutiu *et al.*, 1981
		N. plumbaginifolia	Negrutiu, 1981
	Δ-hydroxylysine	*N. tabacum*	Widholm, 1976
Methionine	Ethionine	*N. tabacum*	Gonzale *et al.*, 1984
		D. carota	Widholm,1977, 1978
		Medicago sativa (alfalfa)	Reisch *et al.*, 1981
	Methionine sulphoximine	*N. tabacum*	Carlson, 1973
Phenylalanine	p-fluorophenylalanine	*N. tabacum*	Palmer & Widholm, 1975 Berlin & Widholm, 1978 Berlin, 1980 Flick *et al.*, 1981
		D. carota	Palmer & Widholm, 1975
		Datura innoxia	Evans & Gamborg, 1979

Table 3. Amino acid analogue-resistant cell lines selected for in crop plants (continued).

Amino acid	Analogue	Species	Reference
Phenylalanine	Thienylalanine	*N. sylvestris*	Vunsh *et al.*, 1980
Proline	Hydroxyproline	*D. carota*	Widholm, 1976
		N. sylvestris	Dix *et al.*, 1984
		Hordeum vulgare	Kueh and Bright, 1981
		Solanum tuberosum	van Swaaij *et al.*, 1986 & 1987a
		Triticum aestivum	Tantau & Dorffling, 1991
	Azetidine-2-carboxylic acid	*D. carota*	Cella, *et al.*, 1982
		N. sylvestris	Breiman *et al.*, 1982
		N. tabacum	Dridze *et al.*, 1991
Tryptophan	5-methytryptophan	*D. carota*	Widholm, 1972a, 1974
			Sung, 1975, 1979
		N. tabacum	Widholm, 1972b
		S. tuberosum	Carlson & Widholm, 1978
		N. sylvestris	White & Vasil, 1979
		D. innoxia	Ranch *et al.*, 1983
	6-fluorotryptophan	*Petunia hybrida*	Colijn *et al.*, 1979

* After Flick *et al.*, 1983; Dix, 1986 and updated

normally toxic to plant cells, it may be possible to select for economically important characters in crop plants which result from resistance to certain amino acid analogues.

1.11 Proline as a selective marker for frost tolerance

Accumulation of proline occurs in plant tissues as a consequence of environmental stresses (e.g. salt, drought). Increase in proline level has also been observed in many plant species subjected to cold hardening (Stefl *et al.*, 1978; van Swaaij *et al.*, 1985) but the cause and effect in terms of proline production in response to stress is unclear. In plant cells proline accumulates in both cytoplasm and vacuole (Fricke and Pahlich, 1990), and cytoplasmic proline is considered as being more related to stress tolerance than vacuolar, or overall cellular proline concentration.

Correlation between the amount of proline in the cell and frost tolerance has been studied. Significant correlation between proline level and frost tolerance has been reported in many genotypes from potato (van Swaaij *et al.*, 1985), winter barley (Dobslaw and Bielka, 1988) and winter wheat (Dorffling *et al.*, 1990). Exogenous application of proline results in an increase in frost tolerance in leaves (van Swaaij *et al.*, 1985) and cell cultures (Hellergren and Li, 1981). The positive correlation between proline content and frost tolerance suggests the involvement of this amino acid in the protection mechanism against frost injury, therefore, high proline level may serve as a selection criterion in screening for frost tolerance in potato.

A protective effect of proline against freezing stress was demonstrated by Hellergren and Li (1981), and further support of the involvement of proline in adaptation

mechanisms against frost came from *in vitro* studies. From cell cultures of potato (van Swaaij *et al.*, 1986, 1987a & b) and of wheat (Tantau and Dorffling, 1991) a number of hydroxyproline resistant cell lines were selected, and all were found to be proline-overproducing and showed increased frost tolerance. Therefore, hydroxyproline resistance could be used as an indirect route to the production of frost-tolerant lines.

1.12 *In vitro* selection for frost tolerance

Research in this area has so far focused more on the mechanism of the induction of short-term effects rather than obtaining stable lines possibly useful in the field. Some efforts have been made but the success has been limited.

Steponkus (1972) made attempts to select low temperature-tolerant lines in cultures using callus of *Hedera helix*. By acclimating and freezing callus cultures at various temperatures, he isolated callus masses with varying degrees of freezing tolerance. None of the selected lines displayed stable enhanced resistance to freezing temperatures (Dix, 1980). Tumanov and his colleagues (1977) selected cells resistant to freezing temperatures from the callus cultures derived from roots and needles of spruce (*Picea excelsa L.*) seedlings. Root callus tissue remained alive after -20 and -25°C, while needle callus tissue remained alive after -20°C. Hardening after selection further increased freezing tolerance to -32.5°C. As no plants were regenerated from resistant callus, it is not possible to demonstrate the inheritance of this resistance. Templeton-Somers *et. al.* (1981) isolated cold-resistant lines from carrot cell suspensions following treatment with the mutagen, ethyl methanesulfonate (EMS). Mutagen-treated cells were exposed to -2°C for 3 weeks and grown at 23°C. Freezing

tolerance was tested on the basis of callus survival test and embryo survival test after germination. Results of the embryo survival test indicated that cold tolerance expressed by cells in suspension and by callus was not expressed in the embryos. Kendell *et al.* (1990) selected freezing-tolerant cell lines from spring wheat (*Triticum aestivum*) callus by immersion in liquid nitrogen without cryoprotectants. Seed lines from five of 11 regenerated lines exhibited significantly enhanced tolerance to freezing at -12°C.

One reason for the difficulties in relation to selection for frost tolerance is that selection is based on survival after selection pressure rather than growth during selection pressure, as in e.g. salinity resistance. Survival of the individual cells in the population may relate to their physiological state at the commencement of freezing treatment and be maintained until the stress is lifted (Dix, 1986). This could be one reason why a trait selected in unorganized callus culture may fail to be exhibited in the organized differentiated plants. Therefore, a correlation between stress tolerance and proline in an indirect approach may be an improvement (Hellergren and Li, 1981). van Swaaij *et al.* (1986, 1987a & b) selected a number of hydroxyproline-resistant cell lines from cell cultures of potato (*Solanum tuberosum*) after treating cells with 10 or 50 μM N-ethyl-N-nitrosourea (ENU). All the selected lines were proline over-producing and retained this character even after transfer onto non-selective media. These lines also showed an increased frost tolerance. One line was regenerated into plants, and showed an increased frost tolerance in the leaves accompanied by a higher leaf proline content. Callus initiated from leaves of the regenerated clone still showed more resistance to hydroxyproline and frost than the control. Tantau and Dorffling (1991) selected a number of hydroxyproline-resistant cell lines of wheat (*Triticum*

aestivum), which showed increased levels of free proline, and also showed increased frost tolerance.

1.13 The experimental aims

The aims of the present study were:

1. To induce and establish callus cultures of *Solanum tuberosum* cultivars; Desiree and Maris Piper and the wild species *S. acaule* and *S. commersonii* from different plant organs, viz., tuber, stem, and leaf, and to regenerate plantlets from differentiated and undifferentiated tissues.

2. To initiate and maintain cell suspension cultures from the calluses and to regenerate plantlets from suspension-cultured cells.

3. To select suitable material for protoplast isolation, and to culture isolated protoplasts and regenerate to the plantlet stage.

4. To obtain a large number of clonal plants in aseptic culture through axillary bud culture and to produce tubers *in vitro* from stem segments of potato and its wild relatives.

5. To attempt the selection of frost-tolerant lines from axillary bud, callus and cell suspension cultures through resistance to hydroxyproline.

6. To find out whether any cytological differences between frost-sensitive and frost-tolerant *Solanum* species, and between normal and selected cell lines, can be observed, and to examine the effects of freezing on cell structure by electron microscopy.

2. MATERIALS AND METHODS

2.1 Biological materials

Virus-free tubers of potato (*Solanum tuberosum L.*) cultivars, Desiree and Maris Piper were kindly supplied by Scottish Crop Research Institute (SCRI), Pentland Field, Edinburgh, Scotland. Tubers were either used freshly or kept in a coldroom at 4 ± 1°C for later use.

Seeds of wild potato species *S. commersonii Dun.* (CMM 5858, from Uraguay) and *S. acaule Bitt.* (ACL 129, from Bolivia) were obtained from SCRI, Invergowrie, Dundee, Scotland. Seed stock was also stored at 4 ± 1°C.

2.2 Chemical compounds

Murashige and Skoog basal salt mixture, Murashige and Skoog modified basal salt mixture (NH_4-free), sucrose, glucose and agar were purchased from Sigma Chemical Company, Poole, Dorset, England. Macro- and micro-nutrients were obtained either from Sigma Chemical Company or BDH Chemicals Ltd. Poole, England. Vitamins, vitamin solutions, growth regulators, coconut water, proline, hydroxyproline, and fluorescein diacetate and other staining dyes, were also purchased from Sigma Chemical Company. Enzymes; cellulase (Onozuka R-10) and Macerozyme (R-10) were obtained from Yakult Pharmaceutical Co. Ltd. Tokyo, Japan and pectolyase (Y-23) was purchased from Seishin Pharmaceutical Co. Ltd., Tokyo, Japan. Other chemicals used in the present studies were purchased mainly from Sigma Chemical Company.

2.3 Culture vessels

Gamma-irradiated disposable plastic petri dishes (Ø 90 mm), autoclavable membrane rafts (53 x 53 mm, Sigma Plant Tissue Culture), culture tubes (25 x 92 mm) and culture jars (60 mm in height) were used for explant and callus cultures, and plant regeneration experiments. For culturing cell suspensions 250 ml conical flasks were used. Disposable plastic culture tubes (25 x 92 mm), culture jars (107 mm in height), and Magenta B-cap glass culture jars (72 mm in height, Sigma Plant Tissue Culture) were used for axillary bud cultures. For protoplast isolation and culture, gamma-irradiated plastic centrifuge tubes (15 ml) and plastic petri dishes (Ø 55 mm and 90 mm) were used respectively.

Glassware, petri dishes, culture vessels, pipettes, syringes and "Millipore" filter assemblies and "Durapore" membrane filters were purchased mainly from Sterilin and Sigma Chemical Company.

2.4 Preparation of culture media

MS basal salt mixture, MS modified basal salt mixture (containing macro- and micro-nutrients except NH_4NO_3), and MS vitamin solution (Table 4) and Nitsch and Nitsch vitamin solution (Table 5) were used as commercially available powder mixes and solutions respectively.

In addition to those commercially manufactured, some media {e.g. Lam (1977b) cell suspension and cell plating media, MS-KM medium} were prepared in the laboratory using stock solutions which were made in advance.

Table 4. Composition of Murashige and Skoog (1962) basal medium.

Constituents	Concentration (mg l^{-1})
1. Macronutrients	
NH_4NO_3	1650.0
KNO_3	1900.0
$CaCl_2.2H_2O$	440.0
KH_2PO_4	170.0
$MgSO_4.7H_2O$	370.0
2. Micronutrients	
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
$Na_2.EDTA.2H_2O$	37.3
$FeSO_4.7H_2O$	27.8
3. Vitamins	
Myo-inositol	100.0
Thiamine-HCl	0.1
Pyridoxine-HCl	0.5
Glycine	2.0
Nicotinic acid	0.5

Table 5. Composition of Nitsch and Nitsch (1969) vitamin solution.

Constituents	Concentration (mg l^{-1})
Myo-inositol	100.0
Thiamine-HCl	0.5
Pyridoxine-HCl	0.5
Folic acid	0.5
Biotin	0.05
Nicotinic acid	5.0
Glycine	2.0

Stock solutions of macronutrients and micronutrients were prepared at 10 and 100 times their final concentrations in the medium respectively. Vitamin stock solutions were made as 100x or 1000x concentrations. For growth regulators, stock solutions 1 mg ml^{-1} were prepared in distilled water. Some growth regulators are not soluble in water and were therefore dissolved in small amounts of 50% ethanol, 1N NaOH or 1N HCl and made to the final volume with distilled water. Enzyme solutions were prepared by weighing appropriate amounts of individual enzyme powder and dissolving in distilled water. All the stock solutions were stored at 4 $\pm$ 1°C except vitamin stock solutions and enzyme solutions which were divided into small vials and deep frozen.

Some chemicals were weighed and directly added to the main flask in media preparation. These include:

a) sucrose

b) glucose

c) casein hydrolysate

d) myo-inositol

e) agar

One litre of medium was prepared each time, either used at once or stored in aliquots at 4 $\pm$ 1°C for later use. The medium was prepared as below.

a) Salt mixture was dissolved in about 800 ml of distilled water or required amounts of different stock solutions were mixed in a 2 litre conical flask.

b) While stirring on a magnetic stirrer, appropriate amounts of sucrose and other directly-added chemicals were added.

c) The required amount(s) of relevant growth regulator(s) was added, if heat-stable.

d) Additional distilled water was added to bring the medium to a final volume of 1 litre.

e) While stirring, the pH of the medium was adjusted (5.5 to 5.9, as required) using 1N NaOH or 1N HCl.

f) If a solidified medium was needed, agar was added and heated over an electric hot plate until clarity of solution was obtained.

g) The medium was distributed among six 250 ml conical flasks and sterilized in an autoclave at 121°C at a pressure of 1.06 Kg/cm^2 (15 p.s.i.). After sterilization the medium was allowed to cool to around 60°C.

h) Thermo-labile compounds (pre-sterilized through membrane filters) were added to the autoclaved media and finally poured into culture vessels under aseptic conditions.

2.5 Sterilization of material

2.5.1 Distilled water and culture media

Distilled water and culture media (excluding heat-labile substances) were sterilized in bottles with caps, or conical flasks capped with aluminium foil, using an autoclave at 121°C at a pressure of 1.06 Kg/cm^2 (15 p.s.i). The times taken for the sterilization of small (up to 250 ml), medium (250-500 ml) and larger (500-1000 ml) volumes were 20, 25 and 30 minutes respectively.

A few chemical compounds were recognized as thermo-labile in all experiments and were sterilized using ultrafiltration membrane filters. These include vitamins (thiamine-HCl, Ca-pantothenate and nicotinic acid), enzymes, growth regulators (IAA, zeatin,

zeatin riboside and GA_3) and sucrose (when used as an osmoticum). Disposable "Millipore" filter assemblies (Sterifid D-GS) with 0.22 μm filter unit were used for large volumes of liquid. For smaller volumes, "Durapore" membrane filters of hydrophobic materials with pore diameters of 0.45 and 0.22 μm were used in sequence.

2.5.2 Dry goods

Heat-resistant labware (e.g. polypropylene, polymethyl-pentene) such as membrane rafts, centrifuge tubes etc., Magenta B-cap glass culture jars, nylon meshes, filter papers, aluminium foil, and cotton wools were sterilised by wet heat using an autoclave at 121°C at a pressure of 1.06 Kg/cm^2 for about 20 minutes.

Metallic instruments such as forceps, scalpels etc. were sterilised by dry heat in an oven at 160-180°C for at least 3 hours. Glassware was sterilised either by wet heat or dry heat as found convenient. Before sterilization all the above dry goods were either wrapped in two layers of aluminium foil or put inside sealed glass containers.

2.5.3 Plant tissue and organs

Potato stems were cut into segments of about 1-1.5 cm and their leaves were removed. These segments were washed with distilled water and sterilized for 15-20 minutes in 20% Domestos solution. Segments were rinsed at least four times with sterile distilled water and allowed to become surface dry.

Fully-expanded leaves excised from greenhouse-grown plants were kept in distilled

water until being surface sterilized. Leaflets were submerged in 10% Domestos solution plus 3-4 drops of Tween 20 for 15 minutes, rinsed 3-4 times with sterile distilled water and allowed to dry.

Potato tubers were washed under running tap water, peeled and trimmed to 4 x 1.5 x 1.5 cm pieces. These pieces were submerged in 20% Domestos solution for 20-30 minutes, washed 3 times with sterile distilled water and surface-dried with sterile filter papers to remove excessive water.

Seeds of *S. commersonii* and *S. acaule* were surface-sterilized in 20% Domestos solution for 10 minutes (it was made sure that seeds sank), washed 5 times with sterile distilled water and dried on sterile filter papers.

2.5.4 Sterility control during manipulation

To avoid contamination, a laminar air-flow cabinet was used for all sterile operations. The cabinet was switched on at least one hour before starting the work. The work surface was swabbed down every time with 95% ethanol or methylated spirit prior to use. Hands were carefully washed and sleeves were rolled back.

During working, breathing into the transfer chamber was avoided as far as possible. While transferring material from one container to another, care was taken not to pass the hands or unsterilized parts of the instruments over, or to touch the inside surface of any culture container. Throughout the sterile operations forceps, scalpels and other small instruments were kept in 95% ethanol and flamed before use.

2.6 Gamma irradiation

2.6.1 Dose estimation

Cobalt 60 was used as a source of gamma irradiation and Fricke dosimetry was used for calibrating the dose. Ferric dosimeter solution was prepared by dissolving 0.39 g ferrous ammonium sulphate (1 mM), 0.06 g sodium chloride (1 mM) and 22 ml concentrated sulphuric acid (0.4 M) per litre of solution (Swallow, 1973).

Twelve tubes were used, each containing 10 ml of ferric dosimeter solution. These were placed at different distances from the irradiation source and exposed to ^{60}Co source. Samples were withdrawn at 2 minute intervals giving the exposure times 2, 4, 6 and 8 minutes. Absorbance of irradiated solutions was determined in a spectrophotometer at 304 nm against the reference blank (un-irradiated ferric dosimeter solution) for each sample. The absorbed dose for each sample was calculated by using the following formula.

$$D_{(Gy)} = 4.42 \times 10^5 [1-0.007 (t-20)] OD \times 10^{-2} / G$$

Where OD is the absorbance (optical density) of the irradiated solution at 304 nm, measured in 1 cm cell with the un-irradiated solution as blank, G is the yield of the reaction for the irradiation used and is constant (for ^{60}Co γ-rays = 15.5) and t is the temperature in °C at which absorbance was measured.

A dose rate of 10 Gy min^{-1} at two points from the irradiation source was determined and used for irradiating the experimental material.

2.6.2 Irradiation of plant material

Cell suspensions, cultured in Lam (1977b) medium were transferred to sterilized tubes. Each tube was placed at the same distance from the source where the dose was calculated to be 10 Gy min^{-1}. The samples were exposed to the required dose, e.g. for a dose of 20 Gy, tubes were exposed for 2 minutes. After the irradiation, cell suspensions were plated on Lam (1977b) cell plating medium containing 5 or 10 mM hydroxyproline.

2.7 Potato micropropagation

2.7.1 *In vitro* shoot culture

Virus-free tubers of potato cvs. Desiree and Maris piper of about uniform size were planted in plastic pots (Ø 15 cm) containing John Innes No. 2 compost and kept in a greenhouse between 25-30°C with a 12/12 h light and dark cycle. The pots were kept well moistened by watering every 2-3 days. After about 3 weeks when shoots had grown, these were removed from the plants. Each shoot was cut into segments about 1.5 cm in length, each segment containing an axillary bud. Leaves were removed and segments were sterilized as described in Section 2.5.3. These sterile single nodes were transferred to culture jars or culture tubes containing shoot culture medium. The media used for culturing these nodal cuttings were as follows.

1. Medium of Espinoza *et al.* (1986):

MS mineral salts + MS vitamins + 30 g l^{-1} sucrose + 8 g l^{-1} agar + 2 mg l^{-1} Ca-pantothenic acid + 0.25 mg l^{-1} GA_3 (pH 5.8).

2. Medium PM of Shahin (1984):

MS mineral salts + Nitsch and Nitsch vitamins + 30 g l^{-1} sucrose + 7 g l^{-1} agar

(pH 5.8).

These media were designated in the experiment as Med. NA and NB respectively.

Cultures were incubated at 25 ± 1°C in 16 h photoperiod with a light intensity of approximately 1000 lux. After about 3-4 weeks when the buds had developed into shoots with leaves, each shoot was cut into pieces, each containing at least one leaf with an axillary bud and subcultured on fresh medium. These cultures were multiplied by sub-dividing and subculturing every 4-6 weeks.

Seeds of *S. commersonii* and *S. acaule* were surface-sterilized as described in Section 2.5.3 and placed on Malt Extract agar medium (33.6 g l^{-1}) in Ø 90 mm petri dishes. The petri dishes were incubated at 20 ± 1°C in a 16 h photoperiod for seed germination. When the seedlings shoots were about 4 cm, these were cut into segments containing one or more axillary buds and transferred to culture jars or culture tubes containing shoot culture medium NA or NB. These cultures were maintained and multiplied as described above.

2.7.2 Growth assessment of *in vitro* shoot cultures

For growth estimation, only one explant was cultured in each culture jar containing medium NA or NB and growth of cultures was assessed on the basis of; a) stem height (cm), b) number of nodes, c) number of roots d) root length (cm) and e) fresh weight (g) of culture. In case of stem height, the height of the dominant shoot, and for root length, the length of the longest root was recorded.

2.8 Initiation and maintenance of callus cultures

2.8.1 Callus initiation

A. From stem explants

Callus was induced from stem sections of *S. tuberosum* cvs. Desiree and Maris Piper, and wild species *S. acaule*. Explants consisting of short pieces of stem about 1 cm in length without an axillary bud (internodal cutting) were prepared from greenhouse-grown plants and *in vitro* shoot cultures. Internodal cuttings taken from greenhouse-grown plants were sterilized as described in Section 2.5.3. These segments were aseptically cultured on the surface of solidified culture medium in disposable plastic petri dishes (Ø 90 mm).

B. From leaf explants

S. tuberosum cvs. Desiree and Maris Piper leaves were collected from greenhouse-grown plants. Leaves of *S. commersonii* were taken from *in vitro* shoot cultures. Leaves collected from greenhouse-grown plants were sterilized as described in Section 2.5.3. Leaflets were cut into segments (about 1 x 1 cm), each including the midrib, and cultured on the surface of solidified culture medium.

C. From tuber explants

Non-growing, virus-free potato tubers were used for preparing the explants. Tubers were cut into 4 x 1.5 x 1.5 cm pieces and sterilized as described in Section 2.5.3. Cut surfaces where cells had been killed, were discarded and 5 x 5 x 2 mm explants were removed from the remaining tissue. These explants were cultured in disposable plastic petri dishes (Ø 90 mm) on the surface of solidified MS medium and on autoclavable

polypropylene membrane rafts over liquid MS medium. Petri dishes were sealed with Parafilm while membrane raft vessels were covered with a lid.

For all the callus initiation and maintenance experiments MS medium (Murashige and Skoog, 1962) (Table 4) containing 30 g l^{-1} sucrose, 8 g l^{-1} agar and 3 mg l^{-1} 2,4-D was used except in callus induction medium from leaf explants in which 0.3 mg l^{-1} kinetin was also added, and in liquid medium under porous polypropylene membranes where agar was omitted from the medium. At all times, cultures were incubated at 25 $\pm$ 1°C. Tuber callus cultures were kept in darkness and leaf and stem callus cultures were kept in 16 h photoperiod under a light intensity of 1000 lux.

2.8.2 Callus establishment and maintenance

After 5-6 weeks of culture, callus was removed from the explants, divided into about 3 x 3 x 3 mm pieces and subcultured onto fresh medium of the same composition in the same type of culture vessel as used for callus induction. To maintain actively-growing and friable calluses periodic subcultures were made at 4 week intervals under aseptic conditions and maintained under same conditions.

2.8.3 Growth assessment of callus cultures

To assess the growth of callus cultures, the criterion of fresh weight was used. Measurements of the fresh weight of cultured explants plus callus (if any) were made at 7 day intervals using a non-sacrificing method in which weighing was carried out under aseptic conditions and explant with callus was returned to the culture medium (Yeoman, 1977). Same technique was used to determine the growth of established

calluses.

2.9 Cell suspension cultures

2.9.1 Initiation and establishment of suspension cultures

For initiation of cell suspension cultures from tuber callus, a medium of the same composition was used as for the production of callus but without agar, while from stem callus, Lam (1977b) cell suspension culture medium (Table 6) was used in which the concentration of micronutrients was increased from 1/5 to 1/2 of MS. The cultures were initiated from actively-growing calluses maintained on solidified MS medium with 3 mg l^{-1} 2,4-D. Pieces of friable callus of approximately 2 g weight were removed from the petri dishes and transferred into 250 ml conical flasks containing 50 ml of culture medium. The culture flasks were sealed with two layers of aluminium foil and Parafilm. Cultures initiated from tuber callus and stem callus were incubated in the dark and 16 h photoperiod respectively on a rotary shaker at 110 r.p.m. at 25 $\pm$ 1°C.

2.9.2 Subculture and maintenance of cell suspensions

The first subculture was performed 10 days after initiation of the culture. The suspended cells were filtered through a double layer of 250 μm nylon mesh into a 100 ml measuring cylinder and allowed to settle down for 5-10 minutes. The supernatant was poured off and the cells were subcultured into a 250 ml conical flask containing 50 ml of fresh medium. After incubation for 10 days on a rotary shaker at 110 r.p.m. at 25 $\pm$ 1°C, the cell suspension was again filtered through a double layer of 250 μm nylon mesh. At this time, only 15 ml of filtrate was used as inoculum for every 50 ml

Table 6. Composition of Lam (1977b) cell suspension, cell plating and shoot regeneration media (mg l^{-1}).

Components	Suspension culture	Cell plating	Shoot regeneration
1. Inorganic salts			
Major Elements	MS (without NH_4NO_3)	MS (without NH_4NO_3)	MS
Minor salts	1/5 of MS	1/2 of MS	MS
2. Organic constituents			
Organic Addenda	Nitsch and Nitsch vits.	Nitsch and Nitsch vits.	Nitsch and Nitsch vits.
Casein Hydrolysate	1000	1000	1000
Sucrose	10000	10000	10000
Glucose	10000	10000	10000
D-Mannitol	40000	40000	40000
Agar	-	6000	10000
3. Growth regulators			
NAA	-	2.0	0.1
IAA	-	0.1	0.05
BAP	0.5	0.5	0.5
Zeatin	-	-	0.5
Kinetin	0.01	0.1	0.2
GA_3	0.2	0.2	0.2
2,4-D	2.0	-	-

pH adjusted to 5.7 $\pm$ 0.1

of fresh medium. Subsequent subcultures were made at 14 day intervals and were incubated in the same way.

2.9.3 Growth assessments of cultures

In order to determine the growth of cells in suspension cultures, the following techniques were used.

A. Packed cell volume (PCV)

This method is used to measure the total volume of cells present at any time during the growth cycle (Allan, 1991). To determine the packed cell volume, small samples (up to 10 ml) of evenly-dispersed suspension were transferred aseptically to 15 ml graduated centrifuge tubes, tapering at the bottom. Centrifuge tubes were centrifuged at 200x g for 5 minutes. PCV is the volume of the pellet produced and is expressed as a percentage of the total volume in the tube.

B. Cell number

Cell counts were made by using a modified Fuchs-Rosenthal haemocytometer with a chamber depth of 0.2 mm. Cell aggregates were disrupted by treating the cell suspension with 8% (w/v) aqueous chromic trioxide solution. 1 volume of culture was mixed with 2 volumes of 8% chromic trioxide, heated to 70°C for up to 15 minutes, cooled to room temperature and shaken vigorously for c. 10 minutes. Finally the mixture was macerated by pumping it repeatedly through the orifice of a pasteur pipette. The haemocytometer was loaded and cell counts were made in the chambers at 100 x magnification.

Each square of the haemocytometer with cover-slip in place represents a total volume of 0.2 mm^3 or 1/5,000 of 1 ml. Since 1 cm^3 is equivalent to approximately 1 ml, the subsequent cell concentration was determined using the following calculation.

No. of cells ml^{-1} = Average count/square x 5,000 x dilution factor

2.9.4 Viability estimation

The viability of cultured cells was estimated using fluorescein diacetate, phenosafranine (Widholm, 1972b) and Evans' blue (Gaff and Okong'O-ogola, 1971).

A. Viability estimation using fluorescein diacetate

A stock solution (5 mg ml^{-1} of acetone) of fluorescein diacetate was prepared and stored in the freezer. 0.01% fluorescein diacetate solution was prepared freshly by 50-fold dilution of an aliquot of stock solution. An equal volume of cell suspension was added. After 5 minutes, the haemocytometer was loaded and the viable cells were estimated by fluorescence microscopy. Each sample was counted twice; once for the total number of cells (using white light) and once for the number of living cells (using UV light). The viability was expressed as the percentage of the total number of cells fluorescing.

B. Viability estimation using phenosafranine

Phenosafranine stains the cellular contents of dead cells while viable cells remain unstained, and was used to estimate the proportions of dead and live cells. 0.1% phenosafranine was prepared in a sample of culture medium and an equal volume of cell suspension was added. After loading into a haemocytometer, cells were viewed

through a compound microscope, and the viability was expressed as the percentage of unstained cells.

C. Viability estimation using Evans' blue

0.4% Evans' blue, which is also excluded from living cells, was prepared in culture medium, and viability was estimated in the same way as with phenosafranine.

2.10 Protoplast isolation

2.10.1 Protoplast isolation from mesophyll cells

In order to isolate viable mesophyll protoplasts, leaves were either collected from greenhouse-grown plants or obtained from *in vitro* shoot cultures. If leaves were taken from greenhouse-grown plants, their midribs were discarded and laminas were surface-sterilized as described in Section 2.5.3.

Two grams of sterilized leaf material or shoot cultures were collected, transferred to a 250 ml conical flask containing 150 ml of conditioning medium (Table 7) and incubated in the dark at 4 ± 1°C for 24 hours (Haberlach *et al.*, 1985; Feher *et al.*, 1989). The leaves were taken out from the conditioning medium, cut into small pieces (approximately 2 mm^2), and transferred into a 500 ml filter flask containing 20 ml of digest medium (Table 7). Enzyme solution was vacuum-infused into the leaf tissue for c. 2-4 minutes and incubated for 14 - 16 hours at 28°C on a rotary shaker set at 40 r.p.m. for cell wall digestion.

The digested leaves were filtered through two nylon meshes with pore sizes of 250 and

Table 7. Composition of protoplast isolation media (mg l^{-1}) developed by Haberlach *et al.* (1985).

Constituents	Prim. Med.	Cond. Med.	Dig. Med.	Rin. Med.
Micronutrients				
NH_4NO_3	1650.0	-	-	-
KNO_3	1900.0	190.0	190.0	3800.0
$CaCl_2.2H_2O$	440.0	44.0	44.0	880.0
KH_2PO_4	170.0	17.0	17.0	340.0
$MgSO_4.7H_2O$	370.0	37.0	37.0	740.0
Micronutrients				
$MnSO_4.4H_2O$	22.3	2.23	2.23	5.58
$ZnSO_4.7H_2O$	8.6	0.86	0.86	2.12
H_3BO_3	6.2	0.62	0.62	1.55
KI	0.83	0.083	0.083	0.21
$Na_2MoO_4.2H_2O$	0.25	0.025	0.025	0.063
$CuSO_4.5H_2O$	0.025	0.0025	0.0025	0.0063
$CoCl_2.6H_2O$	0.025	0.0025	0.0025	0.0063
Na_2.EDTA	37.26	3.726	3.726	9.32
$FeSO_4.7H_2O$	27.86	2.786	2.786	6.97
Vitamins				
Myo-inositol	100.0	10.0	-	-
Thiamine-HCl	0.5	0.05	-	-
Pyridox.-HCl	0.5	0.05	-	-
Glycine	2.0	0.2	-	-
Nicot. acid	5.0	0.5	-	-
Folic acid	0.5	0.05	-	-
Biotin	0.05	0.005	-	-
Phytohormones				
NAA	-	2.0	2.0	2.0
BAP	-	0.5	0.5	0.5
Others				
Casein hyd.	-	100.0	-	-
Sucrose	30 g l^{-1}	-	103 g l^{-1}	103 g l^{-1}
Agar	10 g l^{-1}	-	-	-
PVP	-	-	20 g l^{-1}	-
Cellulase	-	-	5 g l^{-1}	-
Macerozyme	-	-	1 g l^{-1}	-

pH adjusted to 5.8 $\pm$ 0.1

75 μm consecutively. The filtrate was transferred into sterile 15 ml plastic centrifuge tubes, capped, and centrifuged at 350 g for 8 minutes. During centrifugation, viable protoplasts floated to the meniscus while most debris settled to the bottom or remained suspended. Protoplasts were collected from the surface with a wide-bore pasteur pipette, rinsed twice by re-suspending in the rinse medium (Table 7) and centrifuging as before at 350 g but for 5 minutes (Haberlach *et al.*, 1985).

2.10.2 Protoplast isolation from suspension-cultured cells

Cell suspension cultures were established as described in Section 2.9. For isolating the protoplasts, 5-7 days old cell cultures were used and cell samples were obtained by centrifuging at 100 g for 5 minutes (Chung and Sim, 1986). Two millilitres packed cell volume (PCV) was mixed with 10 ml of enzyme solution for digestion. Three different enzyme compositions were examined (Table 29). The mixtures were incubated at 27°C in the dark on a rotary shaker at 40 r.p.m. The incubation period (Table 29) varied with the enzyme composition used. Protoplasts were separated from cell debris by filtration through a nylon mesh of pore size 70 μm. The filtrate was transferred to sterile plastic centrifuge tubes (15 ml) and centrifuged at 70 g for 5 minutes. Protoplasts were washed twice by suspending the pellet containing protoplasts in washing medium (0.5 M mannitol) and centrifuging at 70 g for 3 minutes (De Vries and Bokelmann, 1986).

2.10.3 Protoplast yield

Protoplast yield was determined by haemocytometer counts. Three counts were made each time and the number of protoplasts ml^{-1} was estimated in same way as described

for cultured cells (Section 2.9.3). Number of protoplasts g^{-1} wet weight of leaf tissue was calculated by using the following formula.

$$\text{Protoplasts ml}^{-1} \times \frac{\text{Volume of suspension produced}}{\text{Fresh weight of leaf tissue}}$$

2.10.4 Protoplast viability

For estimation of protoplast viability, fluorescein diacetate (Kikuta *et al.*, 1986; Jones *et al.*, 1989) and Evans' blue (Chung and Sim, 1986; Kikuta *et al.*, 1986) were used and viability was determined as described in Section 2.9.4.

2.11 Protoplast culture

2.11.1 Mesophyll cell protoplasts

Protoplasts were adjusted to a density of 2.5 x 10^5 protoplasts ml^{-1} with cell layering medium (Table 8) and allowed to equilibrate for 2 hours. Finally the protoplast suspension was mixed with two parts of warm (40 - 45°C) cell layering medium containing 1.05% agar. Two ml of protoplast suspension were distributed evenly over solidified reservoir medium (Table 8) contained in Ø 55 mm plastic petri dishes and incubated at 25 ± 1°C under continuous illumination of 1000 lux. After two weeks 1 ml of cell layering medium was added to the petri dishes and incubated for a further two weeks. When developing calluses were about 2 mm in diameter, they were transferred to the protoplast culture medium (Table 8) contained in Ø 90 mm petri dishes and incubated at 25 ± 1°C under continuous light of 3000-4000 lux for a further two weeks.

Table 8. Composition of protoplast culture media (mg l^{-1}) developed by Haberlach *et al.* (1985).

Constituents	Prim. Med.	C.L. Med.	Res. Med.	Cult. Med.
Macronutrients				
NH_4NO_3	1650.0	-	-	-
KNO_3	1900.0	7600.0	1900.0	1900.0
$CaCl_2.2H_2O$	440.0	1760.0	440.0	440.0
KH_2PO_4	170.0	680.0	170.0	170.0
$MgSO_4.7H_2O$	370.0	1480.0	370.0	370.0
NH_4Cl	-	-	-	107.0
Micronutrients				
$MnSO_4.4H_2O$	22.3	11.15	11.15	11.15
$ZnSO_4.7H_2O$	8.6	4.30	4.30	4.30
H_3BO_3	6.2	3.10	3.10	3.10
KI	0.83	0.42	0.42	0.42
$Na_2MoO_4.2H_2O$	0.25	0.13	0.13	0.13
$CuSO_4.5H_2O$	0.025	0.013	0.013	0.013
$CoCl_2.6H_2O$	0.025	0.013	0.013	0.013
Na_2.EDTA	37.26	18.63	18.63	18.63
$FeSO_4.7H_2O$	27.86	13.93	13.93	13.93
Vitamins				
Myo-inositol	100.0	4.505 g l^{-1}	100.0	100.0
Thiamine-HCl	0.5	0.5	0.5	0.5
Pyridox.-HCl	0.5	0.5	0.5	0.5
Glycine	2.0	2.0	2.0	2.0
Nicot. acid	5.0	5.0	5.0	5.0
Folic acid	0.5	0.5	0.5	0.5
Biotin	0.05	0.05	0.05	0.05
Adenine sulfate	-	-	-	40.0
Phytohormones				
NAA	-	1.0	1.0	0.1
BAP	-	0.4	0.4	0.5
Others				
Casein hyd.	-	-	100.0	100.0
Sucrose	30 g l^{-1}	68.66 g l^{-1}	17.17 g l^{-1}	2.50 g l^{-1}
D-mannitol	-	4.55 g l^{-1}	45.55 g l^{-1}	54.70 g l^{-1}
Sorbitol	-	4.55 g l^{-1}	-	-
Xylitol	-	3.80 g l^{-1}	-	-
Agar	10 g l^{-1}	7.00 g l^{-1}	10.00 g l^{-1}	10.00 g l^{-1}

pH adjusted to 5.8 $\pm$ 0.1

2.11.2 Cultured-cell protoplasts

After washing, protoplasts were suspended in MS-KM liquid culture medium (Table 9) at a final density of 1 x 10^5 protoplasts ml^{-1}, and 2 ml of protoplast suspension was plated into plastic petri dishes (Ø 55 mm). The petri dishes were sealed with Parafilm and incubated at a temperature of 25 ± 1°C at continuous light of 1000 lux. After one week, the protoplast cultures were diluted to twice their volume with fresh liquid culture medium. The following week, an equal volume of MS-KM culture medium plus 0.4% agar was added, and the cultures incubated for further 2 weeks. Subsequently, the small calluses about 0.5 mm in diameter were plated in Ø 90 mm plastic petri dishes on solidified MS medium containing 8 g l^{-1} agar, 30 g l^{-1} sucrose, 1.0 mg l^{-1} BAP, and 0.2 mg l^{-1} NAA, and incubated at 25 ± 1°C under continuous light at 3000-4000 lux (Bokelmann and Roest, 1983).

2.12 Plantlet regeneration

2.12.1 From tuber discs

Virus-free tubers of potato cultivars Desiree and Maris Piper were used to obtain the explants, prepared and sterilized as described previously. Explants were cultured in jars (60 mm in height) containing regeneration medium. Three different regeneration media, as developed by Jarret *et al.* (1980a) (Table 10), Iapichino *et al.* (1991) (Table 11) and Ahloowalia (1982) (Table 12), were used and designated as Med. RA, RB and RC. The culture jars were capped and incubated at 25 ± 1°C under approximately 1000 lux light intensity in a 16 h photoperiod.

Table 9. Composition of MS-KM protoplast culture medium.*

Constituents	Concentration (mg l^{-1})
1. Macronutrients	
KNO_3	1900.0
$CaCl_2.2H_2O$	440.0
KH_2PO_4	170.0
$MgSO_4.7H_2O$	370.0
2. Micronutrients	
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
$Na_2.EDTA.2H_2O$	37.26
$FeSO_4.7H_2O$	27.86
3. Vitamins	
D-Calcium pantothenate	1.0
Choline chloride	1.0
Ascorbic acid	2.0
p-Aminobenzoic acid	0.02
Nicotinamide	1.0
Thiamine-HCl	10.0
Pyridoxine-HCl	1.0
Folic acid	0.4
Biotin	0.01
Vitamin A	0.01
Vitamin D3	0.01
Vitamin B12	0.02
4. Organic acids	
Sodium pyruvate	20
Fumaric acid	40
Citric acid	40
Malic acid	40

Continued .....

Table 9. Composition of MS-KM protoplast culture medium (continued).

Constituents	Concentration (mg l^{-1})
5. Sugars and sugar alcohols	
Mannitol	250
Sorbitol	250
Sucrose	250
Fructose	250
Ribose	250
Xylose	250
Mannose	250
Rhamnose	250
Cellobiose	250
Myo-inositol	100
Glucose	88300
6. Growth regulators	
2,4-D	0.2
NAA	1.0
Zeatin riboside	0.5
7. Supplements	
Casein hydrolysate	250
Coconut water	20 ml l^{-1}

pH adjusted to 5.7 ± 0.1

* This medium (MS-KM) is composed of macro- and micronutrients of MS medium (Murashige and Skoog, 1962) without NH_4NO_3 and other nutrients of the KM-8P medium (Kao and Michayluk, 1975) without riboflavin.

2.12.2 From stem segments

Stem segments (without axillary buds) about 1 cm in length were prepared from *in vitro* shoot cultures as described previously and cultured in Ø 90 mm petri dishes containing different regeneration media. Two different media designated as Med. RA (Table 10) and RB (Table 11) were used. Petri dishes were sealed with Parafilm and incubated at 25 ± 1°C in a 16 h photoperiod under approx. 1000 lux light intensity.

2.12.3 From callus cultures

Potato tuber and leaf-derived calluses of Desiree and Maris Piper cultivars were cultured in media contained in culture jars (60 mm in height) or petri dishes (Ø 90 mm). For plantlet regeneration from tuber-derived callus, three media designated as Med. RA (Table 10), RB (Table 11) and RC (Table 12) were used while for leaf-derived callus Med. RB (Table 11), RC (Table 12) and RD (Table 6) were used. All the cultures were kept at 25 ± 1°C and a light intensity of 1000 lux with a 16 h photoperiod.

2.12.4 From suspension-cultured cells

Plantlets were also regenerated from cell suspension cultures of Desiree and Maris Piper derived from stem calluses. Cell suspensions were prepared as described previously and plated onto the surface of Lam (1977b) cell plating medium (Table 6) at a plating density of 0.5×10^5 cells ml^{-1}. Petri dishes were sealed with Parafilm and incubated at 25 ± 1°C in 16 h photoperiod with low light intensity of about 250 lux. After 6 weeks calluses were transferred either to the Lam (1977b) shoot regeneration medium (designated as Med. RD) (Table 6) or to the shoot regeneration medium

Table 10. Composition of plant regeneration medium developed by Jarret *et al.* (1980a).

Constituents	Concentration (mg l^{-1})
1. Macronutrients	
NH_4NO_3	1650.0
KNO_3	1900.0
$CaCl_2.2H_2O$	440.0
KH_2PO_4	170.0
$MgSO_4.7H_2O$	370.0
2. Micronutrients	
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
$Na_2.EDTA.2H_2O$	37.3
$FeSO_4.7H_2O$	27.8
3. Vitamins	
Myo-inositol	100.0
Thiamine-HCl	0.1
Pyridoxine-HCl	0.5
Glycine	2.0
Nicotinic acid	0.5
Folic acid	0.5
D-biotin	0.05
4. Growth regulators	
NAA	0.03
BAP	3.0
GA_3	0.5
5. Supplements	
Casein hydrolysate	1000
Sucrose	25000
Agar	9000

pH adjusted to 5.6 $\pm$ 0.1

Table 11. Composition of plant regeneration medium developed by Iapichino *et al.* (1991).

Constituents	Concentration (mg l^{-1})
1. Macronutrients	
NH_4NO_3	1650.0
KNO_3	1900.0
$CaCl_2.2H_2O$	440.0
KH_2PO_4	170.0
$MgSO_4.7H_2O$	370.0
2. Micronutrients	
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
$Na_2.EDTA.2H_2O$	37.3
$FeSO_4.7H_2O$	27.8
3. Vitamins	
Myo-inositol	100.0
Thiamine-HCl	0.1
Pyridoxine-HCl	0.5
Glycine	4.0
Nicotinic acid	0.5
4. Growth regulators	
IAA	1.0
Zeatin	2.0
5. Supplements	
Adenine sulphate	40
Casein hydrolysate	1000
Sucrose	20000
Agar	10000

pH adjusted to 5.7 $\pm$ 0.1

Table 12. Composition of plant regeneration medium developed by Ahloowalia (1982).

Components	Concentration (mg l^{-1})
1. Inorganic salts	
Major Elements	1/2 of MS
Minor salts	1/2 of MS
2. Organic constituents	
Organic Addenda	1/2 of MS vitamins
Sucrose	10000
Agar	5000
3. Growth regulators	
2,4-D	0.5
Zeatin	1.0

pH adjusted to 5.9 $\pm$ 0.1

developed by Bokelmann and Roest (1983) designated as Med. RE (Table 13) and incubated in 16 h photoperiod with approx. 3000-4000 lux illumination provided by Cool White fluorescent lights at 20 ± 1°C.

2.12.5 From protoplast-derived callus

For the initiation of shoots from the callus of protoplast origin, two different media, as used for shoot regeneration from suspension-cultured cells, were used. Calluses were subcultured onto the surface of solidified regeneration medium in Ø 90 mm petri dishes and incubated at 3000-4000 lux light intensity in a 16 h photoperiod at 20 ± 1°C.

Regenerated adventitious shoots about 1-2 cm long were excised and transferred to shoot culture medium (Med. NA) for root formation. The rooted plantlets were then transferred to plastic pots containing sterilized compost under high humidity. After 1-2 weeks plants were transferred to pots containing soil mix (John Innes No. 2) and moved to a greenhouse under non-sterile conditions.

2.13 *In vitro* tuberization

2.13.1 Induction of tubers

Stem segments about 1 cm in length each containing an axillary bud were prepared from *in vitro* shoot cultures and transferred onto the surface of solidified medium in Magenta GA-7 culture jars. Media used for the induction of tuberization were as follows.

1. MS basal medium (Table 4) + 80 g l^{-1} sucrose + 7 g l^{-1} agar

Table 13. Composition of shoot regeneration medium developed by Bokelmann and Roest (1983).

Constituents	Concentration (mg l^{-1})
1. Macronutrients	
NH_4NO_3	1650.0
KNO_3	1900.0
$CaCl_2.2H_2O$	440.0
KH_2PO_4	170.0
$MgSO_4.7H_2O$	370.0
2. Micronutrients	
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
$Na_2.EDTA.2H_2O$	37.3
$FeSO_4.7H_2O$	27.8
3. Vitamins	
Myo-inositol	100.0
Thiamine-HCl	0.1
Pyridoxine-HCl	0.5
Glycine	2.0
Nicotinic acid	0.5
4. Growth regulators	
NAA	0.01
Zeatin riboside	1.0
GA_3	0.01
5. Plus the followings:	
Sucrose	30000
Agar	8000

pH adjusted to 5.7 $\pm$ 0.1

2. MS basal medium + 80 g l^{-1} sucrose + 7 g l^{-1} agar + 5 mg l^{-1} BAP.

3. MS basal medium + 80 g l^{-1} sucrose + 7 g l^{-1} agar + 5 mg l^{-1} BAP + 0.5 mg l^{-1} 2,4-D.

The culture jars were incubated at 25 ± 1°C in 16 h photoperiod of the same light intensity used for maintenance of *in vitro* shoot cultures (Lentini and Earle, 1991). After 2 weeks when cultures had become established, these were transferred to 20 ± 1°C with 8 h photoperiod at 250 lux (Chandra *et al.*, 1988).

2.13.2 Assessment of cultures

Tuber initiation and tuberization efficiency were estimated on the basis of; a) time taken for tuber initiation, b) shoot height (cm) of plantlet, c) no. of tubers/jar, and d) average weight/microtuber.

2.14 Frost tolerance experiments

For the selection of frost tolerant cell lines, an indirect approach was made. Cell lines were first selected for different hydroxyproline concentrations and then their frost tolerance was evaluated.

2.14.1 *In vitro* selection for hyp-resistant cell lines

A. Axillary bud cultures

Axillary buds together with a small portion of stem were grown either directly in culture tubes on shoot culture medium (Med. NA) containing 10, 20 or 30 mM hydroxyproline (hyp) or were first grown on shoot culture medium containing 0.05 M

proline and after 4 weeks subcultured onto shoot culture medium containing 10, 20 or 30 mM hyp by subdividing into segments each containing an axillary bud. Hyp-tolerant axillary buds which developed into shoots were subdivided and subcultured on fresh medium containing the same concentration of hydroxyproline.

B. Callus cultures

For *in vitro* selection studies, in one experiment, small pieces of leaf callus of potato cvs. Desiree and Maris Piper were grown on the surface of solidified MS medium containing 10, 20 or 30 mM hyp. In another experiment small pieces of leaf callus were first grown on solidified MS medium containing 0.05 M proline or mannitol. After 2 weeks calluses growing on medium containing 0.05 M proline were subcultured onto solidified MS medium containing 10, 20 or 30 mM hyp. Hyp-resistant cell clusters were detected by either vigorous growth or by their white colour. The selection procedure lasted 5 to 6 months with transfer of callus pieces to fresh hyp-containing medium at 4 week intervals. Hyp-resistant callus pieces were then transferred, either to hyp-free medium or used for a frost tolerance assay.

C. Cell suspension cultures

Cell suspension cultures were initiated and maintained in Lam (1977b) suspension culture medium (Table 6), as described in Section 2.9, from leaf calluses of potato cvs. Desiree and Maris Piper. For *in vitro* selection studies 7 day-old cultures were used. In one experiment cell suspensions were plated at a plating density of 0.5×10^6 cells ml^{-1} on Lam (1977b) cell plating medium (Table 6) containing 5 or 10 mM hyp. In a second experiment, cell suspensions were frozen in a freezer down to -6°C at the

rate of 1°C h^{-1} and kept at this temperature for at least 1 hour, thawed at 4°C for 1 hour and plated on Lam (1977b) cell plating medium at a plating density of 0.5 x 10^6 cells ml^{-1}. After 4-6 weeks growing colonies were transferred to solidified MS medium containing 5 or 10 mM hyp.

In a third experiment, cell suspensions were irradiated at a dose of 20 Gy (at the rate of 10 Gy $min.^{-1}$) and plated on Lam (1977b) cell plating medium containing 5 or 10 mM hyp at a density of 0.5 x 10^6 cells ml^{-1}. Resistant colonies were transferred to solidified MS medium containing the same concentration of hyp and maintained by subculturing onto fresh medium at 4-6 week intervals.

2.14.2 Determination of frost tolerance

A. From leaves

Leaflets of recently-matured leaves were excised from 3-4 plants and frozen to predetermined temperatures (from 0°C to -10°C) with a cooling rate of 2°C h^{-1}. To prevent supercooling, samples were seeded with a small piece of crushed ice when -1°C was reached. At each test temperature, samples (4 leaflets per treatment) were removed, thawed for 1 h at 4°C (Tantau and Dorffling, 1991) and transferred into a flask containing 50 ml of deionized water. Flasks were shaken for 1 h in a rotary shaker at 30 r.p.m. and 25°C and the conductivity of decanted leachate was measured using a digital conductivity meter (JENWAY, Model, 4070). The leachate was poured into other flasks separately and flasks containing the leaflets were then dipped in liquid nitrogen for 10 minutes to kill samples. The original leachate was poured back into the flasks, again shaken for 1 h and the conductivity of final leachate measured

(Sukumaran and Weiser, 1972). Per cent electrolyte leakage was calculated according to the formula.

$$\text{Electrolyte leakage \%} = \frac{\text{Conductivity after test freeze}}{\text{Final conductivity after killing}} \times 100$$

The temperature value which resulted in electrolyte leakage equivalent to 50% of the control (frozen in liquid nitrogen) was obtained from plots of freezing temperature vs relative electrolyte leakage % by graphical analysis (Tantau and Dorffling, 1991) and designated as the frost killing-temperature (FKT).

B. From callus

For estimation of freezing tolerance, callus was frozen as 200 mg pieces in closed tubes and care was taken to prevent damage to callus structure (van Swaaij *et al.*, 1987a & b). To avoid supercooling, samples were cooled at -1°C for 1 h and inoculated with a small piece of crushed ice to initiate freezing. Then the temperature was decreased at the rate of 2°C h^{-1} to various subzero temperatures and samples were exposed to each test temperature for 1 h. Samples were removed at each test temperature and thawed overnight at 4°C (Lee *et al.*, 1992). Freezing damage was determined using the TTC-viability assay of Towill and Mazur (1975), as follows.

Two ml of 0.8% 2,3,5-triphenyltetrazolium chloride (TTC), prepared in 0.05 M K-phosphate buffer, pH 7.5, was added to the tubes containing callus withdrawn at different freezing temperatures and incubated for 18 h in the dark at 25°C without shaking. After incubation, cells were pelleted and washed once with deionized water. The red formazan was extracted by adding 3 ml of 95% ethanol to the pelleted cells

and incubating at 60°C for 1 h. Brief heating (60°C) for about 15-30 minutes was necessary to remove formazan from large cell clumps. Absorbance of the extract was recorded at 485 nm in a digital spectrophotometer (CECIL, CE 1020). Cell survival at each test temperature was calculated according to the formula:

$$\text{Per cent survival} = \frac{\text{TTC reduction at freeze temperature}}{\text{TTC reduction at 25°C (control)}} \times 100$$

Freezing temperature that resulted in 50% cell death was designated as the frost-killing temperature (FKT).

2.15 Preparation of material for electron microscopy

For cytological studies, normal, frozen and hyp-resistant calluses of *S. tuberosum* cv. Desiree, and *S. acaule* and *S. commersonii* calluses were used and specimens were prepared for observation by electron microscopy in the following way.

A. Fixation

The double fixative glutaraldehyde-osmium (Juniper *et al.*, 1970) was used as follows.

1. 2.5% glutaraldehyde was prepared in 0.1M Na cacodylate buffer pH 7.2.
2. Callus was trimmed into cubes, each c. 2 mm^3.
3. Callus tissues were transferred to a specimen tube containing glutaraldehyde.
4. After 1.5 h the fixative was removed by three washes of 0.1M Na cacodylate buffer, 5 minutes each change.
5. Tissues were then post-fixed in 1% osmium tetroxide in 0.1M Na cacodylate buffer for about 2 hours.
6. Three washings of buffer were given each for 5 minutes to remove all the traces

of fixative from the tissues.

B. Dehydration

Specimens were dehydrated through a series of 30, 50, 70 and 90% acetone at room temperature, each change for 5 minutes, followed by 5 changes of absolute acetone.

C. Embedding

Epoxy resin as formulated by Mollenhauer (1964) was used for embedding the specimens and was prepared by mixing 40 g agar 100, 22 g Araldite CY212, 72 g DDSA and 2 g BDMA.

1. Absolute acetone was replaced by 1:1 acetone/resin mixture and specimens were placed on a slowly-rotating machine to assist penetration of resin mixture into the tissue.
2. After one hour the acetone/resin mixture was replaced by fresh resin mixture and the specimens rotated overnight.
3. Each specimen was placed at the bottom of a BEEM capsule, which was filled with fresh resin and placed in an oven at 60°C overnight.

D. Microtomy

1. For cutting the sections an LKB Ultratome III was used.
2. Glass knives were prepared by LKB Knifemaker, and made just before cutting the sections.
3. Sections were floated onto water held behind the cutting edge with a strip of PVC tape.

4. Blocks were trimmed by razor to expose the specimen in an orientation to present a suitable face to the knife.

5. Sections were cut and mounted on 3.05 mm copper mesh grids for viewing in the electron microscope. Only silver and gold-coloured sections were selected.

E. Staining

Grids with sections were washed with distilled water and stained for 30 minutes in uranyl acetate (Gibbons and Grimestone, 1960), after which the grids were again washed with distilled water and stained with lead citrate for 5 minutes (Reynolds, 1963). Specimens were again washed with distilled water and dried.

Finally, the stained sections were observed in a Cornith 500 transmission electron microscope and selected areas were photographed.

3. EXPERIMENTAL RESULTS

3.1 *In vitro* shoot cultures and plant multiplication

Potato shoot cultures were established from nodal segments of *Solanum tuberosum* cvs. Desiree and Maris Piper, *S. commersonii* and *S. acaule*. Virus-free tubers of *S. tuberosum* cvs. Desiree and Maris Piper were planted in the greenhouse, in plastic pots containing John Innes No. 2 compost. After about 10 - 12 days buds started to grow, and after 3 weeks, shoots reached about 10 - 15 cm in height, with 8 - 10 leaves. At this stage, the shoots were removed and nodal explants were prepared, sterilized and cultured on the media developed by Espinoza *et al.* (1986) (Med. NA) and by Shahin (1984) (Med. NB).

Seeds of *S. commersonii* and *S. acaule* were surface-sterilized and sown on Malt Extract agar medium. After 3 weeks, the seeds started to germinate. Seed germination was 60% in *S. commersonii* and 30% in *S. acaule*. When the seedling stems were about 4 - 5 cm, these were cut aseptically into segments, each containing at least one axillary bud, and cultured either on medium NA or NB.

Axillary buds started to grow after 3 - 5 days of culture on nutrient medium in *S. commersonii* and 5 - 7 days in Desiree, Maris Piper and *S. acaule*. In most cases root development from the nodes was also observed after 5 - 6 days. In two to three weeks time, roots were well branched and often extended several times around the base of the container. Roots also developed at nodes well above the medium surface and this was observed frequently in *S. commersonii*.

Table 14. Shoot height (cm) of potato plantlets after culturing nodal cuttings on media developed by Espinoza *et al.* (1986) (Med. NA) and Shahin (1984) (Med. NB).

Weeks of culture	Desiree		Maris Piper		*S. acaule*		*S. commersonii*	
	Med. NA	Med. NB	Med. NA	Med. NB	Med. NA	Med. NB	Med. NA	Med. NB
2	2.52 ± 0.50	2.30 ± 0.67	2.45 ± 0.32	1.68 ± 0.34	2.70 ± 0.65	2.38 ± 0.42	4.32 ± 0.35	3.12 ± 0.35
4	4.05 ± 0.89	3.95 ± 0.74	3.98 ± 0.39	2.88 ± 0.61	4.97 ± 0.84	3.82 ± 0.61	7.85 ± 0.41	5.82 ± 0.55
6	8.35 ± 1.85	7.88 ± 0.92	7.08 ± 0.88	4.88 ± 0.97	6.67 ± 1.34	4.98 ± 0.67	11.62 ± 0.80	8.38 ± 1.46
8	10.15 ± 2.18	9.80 ± 1.19	8.45 ± 0.97	6.15 ± 1.16	8.38 ± 1.15	5.85 ± 0.71	13.88 ± 1.33	12.50 ± 1.76

Data represent means ± SD of 4 replicates

Table 15. Number of nodes on potato plantlet shoots after culturing nodal cuttings on media developed by Espinoza *et al.* (1986) (Med. NA) and Shahin (1984) (Med. NB).

Weeks of culture	Desiree		Maris Piper		*S. acaule*		*S. commersonii*	
	Med. NA	Med. NB	Med. NA	Med. NB	Med. NA	Med. NB	Med. NA	Med. NB
2	5.50 ± 0.50	5.00 ± 0.71	4.00 ± 0.71	3.25 ± 0.43	3.75 ± 0.83	2.50 ± 0.50	4.50 ± 0.50	5.25 ± 0.83
4	9.75 ± 0.43	9.25 ± 1.48	7.25 ± 1.64	5.00 ± 1.00	10.50 ± 1.50	7.75 ± 1.09	11.75 ± 1.92	11.75 ± 1.09
6	13.75 ± 1.48	13.00 ± 0.71	9.50 ± 1.50	7.50 ± 1.12	14.00 ± 2.35	10.25 ± 0.83	15.25 ± 1.30	16.50 ± 1.80
8	18.75 ± 2.68	16.50 ± 2.29	11.75 ± 1.48	10.25 ± 1.78	17.00 ± 2.55	12.75 ± 0.83	17.50 ± 1.12	18.50 ± 1.12

Data represent means ± SD of 4 replicates

Table 16. Number of roots on potato plantlets after culturing nodal cuttings on media developed by Espinoza *et al.* (1986) (Med. NA) and Shahin (1984) (Med. NB).

Weeks of culture	Desiree		Maris Piper		*S. acaule*		*S. commersonii*	
	Med. NA	Med. NB	Med. NA	Med. NB	Med. NA	Med. NB	Med. NA	Med. NB
2	2.25 ± 0.43	1.50 ± 0.50	2.50 ± 0.50	1.75 ± 0.43	4.00 ± 0.71	3.25 ± 0.83	3.75 ± 0.43	4.00 ± 0.71
4	2.75 ± 0.83	2.25 ± 0.43	3.25 ± 0.43	2.25 ± 1.09	4.25 ± 0.83	4.00 ± 1.00	4.50 ± 0.50	4.75 ± 0.83
6	4.75 ± 1.09	3.75 ± 0.50	4.75 ± 0.43	2.75 ± 0.83	4.75 ± 1.09	4.50 ± 1.12	5.75 ± 0.43	6.00 ± 0.71
8	6.25 ± 0.83	4.50 ± 0.50	6.00 ± 0.71	3.25 ± 0.83	5.00 ± 0.71	4.75 ± 0.83	6.25 ± 0.83	6.50 ± 0.50

Data represent means ± SD of 4 replicates

The axillary bud present in a nodal cutting developed into a complete plantlet within 3 - 4 weeks of culture. Depending on the number of nodes per shoot, 5 - 10 plantlets could be regenerated from a single shoot. Cultures were multiplied by subculture of nodal cuttings at 4 to 6-week intervals depending upon the rates of growth.

To assess the growth of potato types on the different culture media, single nodal explants were prepared from the middle portions of 5-week-old shoots and transferred either onto medium NA or medium NB. Only one nodal explant was cultured per culture jar on the surface of solidified medium. Cultures were evaluated over a period of 8 weeks for length of the dominant shoot and number of its nodes, roots, mean root length and mean total fresh weight of cultures. Results are presented in Tables 14, 15, 16, 17 and 18.

Medium NA proved better for the growth of axillary buds and their subsequent development into plantlets than medium NB (Fig. 1), and therefore for subsequent multiplication of cultures only medium NA was used. The potato types differed for all the growth parameters measured, and was most vigorous in *S. commersonii* axillary bud cultures, followed by Desiree, while Maris Piper and *S. acaule* axillary buds resulted in poor shoot growth on both media tested. There were some exceptions, where growth of the dominant shoot became limited by the height of the culture jar and this appeared to break apical dominance, possibly due, at least in part, to the shading effect of the opaque jar lids.

These cultures were multiplied by subdividing shoots as before into nodes, each with

Fig. 1. Potato shoot cultures on media developed by Espinoza *et al.* (1986) (medium NA), and by Shahin (1984) (medium NB). Six nodal cuttings each containing an axillary bud were cultured in each jar. Photographed after 5 weeks.

Table 17. Mean root length (cm) on potato plantlets after 2 weeks of culturing nodal cuttings on media developed by Espinoza *et al.* (1986) (Med. NA) and Shahin (1984) (Med. NB).

Potato type	Med. NA	Med. NB
Desiree	3.82 ± 0.96	2.87 ± 0.78
Maris Piper	3.95 ± 0.51	2.17 ± 1.02
S. acaule	1.68 ± 0.19	1.55 ± 0.13
S. commersonii	4.00 ± 0.66	3.95 ± 0.68

Data represent means ± SD of 4 replicates

Table 18. Fresh weight (mg) of potato plantlets after 8 weeks of culturing nodal cuttings on media developed by Espinoza *et al.* (1986) (Med. NA) and Shahin (1984) (Med. NB).

Potato type	Med. NA	Med. NB
Desiree	322.5 ± 98.3	197.0 ± 37.3
Maris Piper	123.5 ± 16.4	88.0 ± 8.6
S. acaule	91.5 ± 21.9	86.5 ± 33.5
S. commersonii	357.2 ± 46.1	337.5 ± 91.6

Data represent means ± SD of 4 replicates

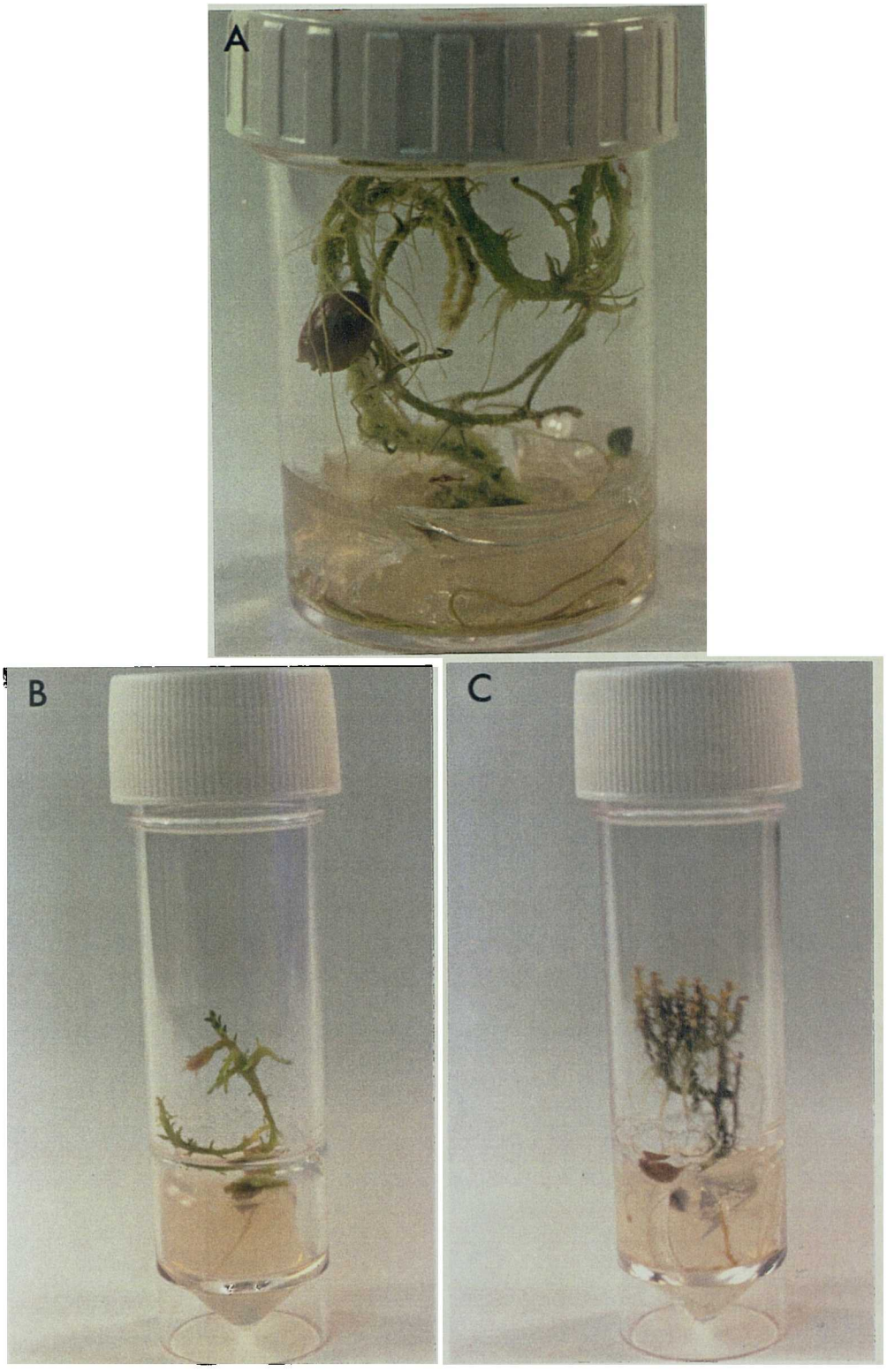


Fig. 2. Shoot morphology of potato plantlets on medium developed by Espinoza *et al.* (1986) (medium NA) in tightly-closed culture jars. Photographed after 10 weeks. A. Desiree B. Maris Piper C. *S. commersonii* Tuberization can be seen in Desiree and Maris Piper.

an axillary bud, and subculturing on fresh medium. If the culture jars were tightly closed, shoot morphology became altered and growth was also reduced (Fig. 2). In some cultures of cvs. Desiree and Maris Piper, 1 - 2 microtubers were also produced after 8 - 10 weeks of culture (Fig. 2, A & B).

3.2 Callus cultures

3.2.1 Callus initiation

A. From stem explants

Explants of stems of *S. tuberosum* cvs. Desiree and Maris Piper, and *S. acaule* prepared according to the methods described in Section 2.8.1 A: Materials and Methods, were cultured on solidified MS medium containing 8 g l^{-1} agar, 30 g l^{-1} sucrose and 3 mg l^{-1} 2,4-D. Callus initiation was observed from both ends of the explants after 4 - 5 days of culture on nutrient medium, finally covering the whole explant within 4 weeks (Fig. 3). At this stage most of the explant tissue had become de-differentiated and turned into a mass of unorganized cells, except the central stele. Callus induction from *S. acaule* explants was slow compared to Desiree and Maris Piper, and the callus was compact and hard in texture. After six weeks of culture, callus was removed from the explants, divided into pieces approximately 3 x 3 x 3 mm and these subcultured onto fresh nutrient medium in order to multiply the cultures. It was found that this increased the friability of the callus.

B. From leaf explants

When leaf explants prepared from leaves of greenhouse-grown plants of *S. tuberosum* cvs. Desiree and Maris Piper, and of *in vitro* shoot cultures of *S. commersonii* (see

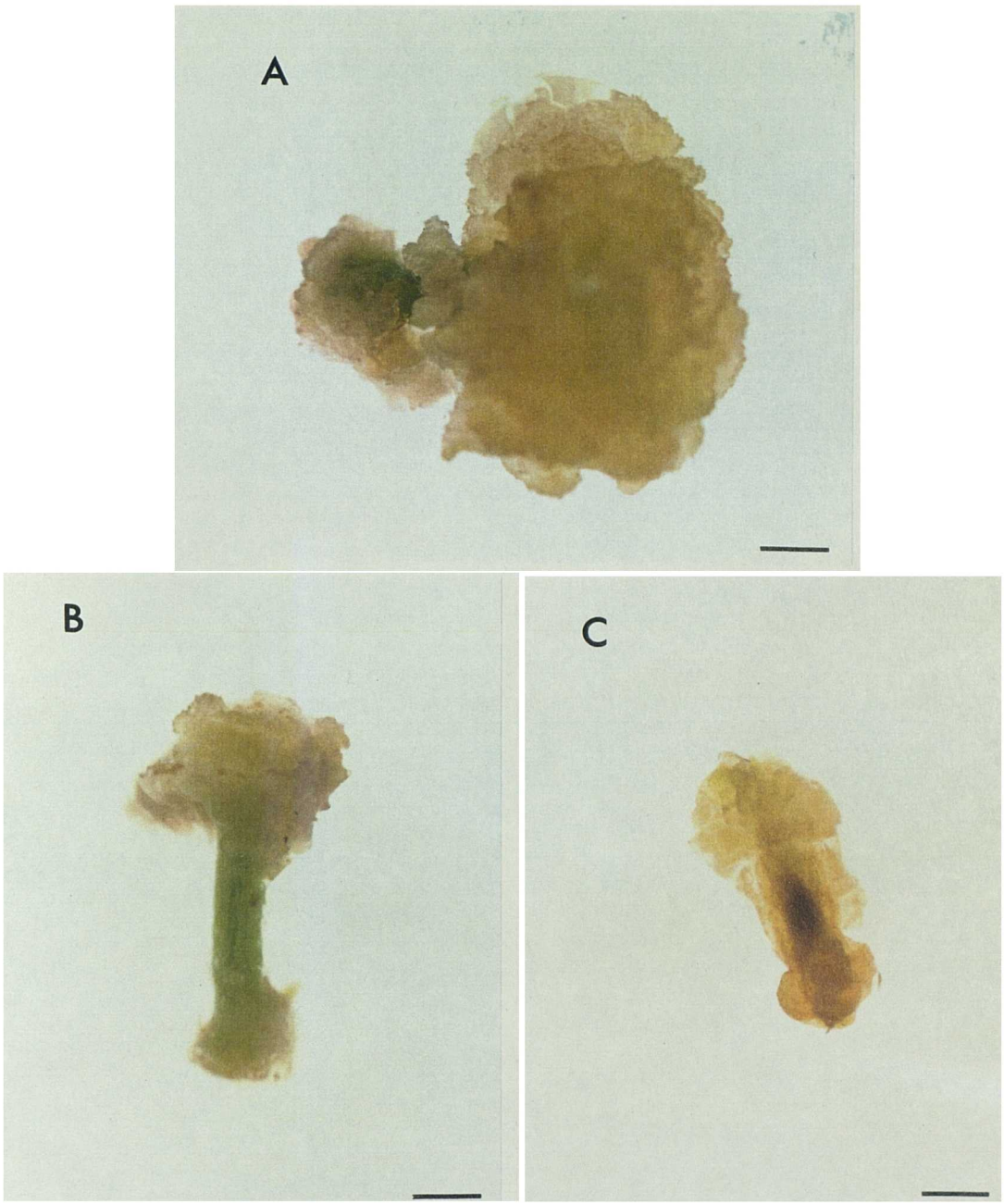


Fig. 3. Callus production from potato stem explants cultured on MS medium supplemented with 3 mg l^{-1} 2,4-D, 30 g l^{-1} sucrose and 8 g l^{-1} agar. Photographed after 4 weeks. (Scale bar = 2 mm)

A. Desiree B. Maris Piper C. *S. acaule*

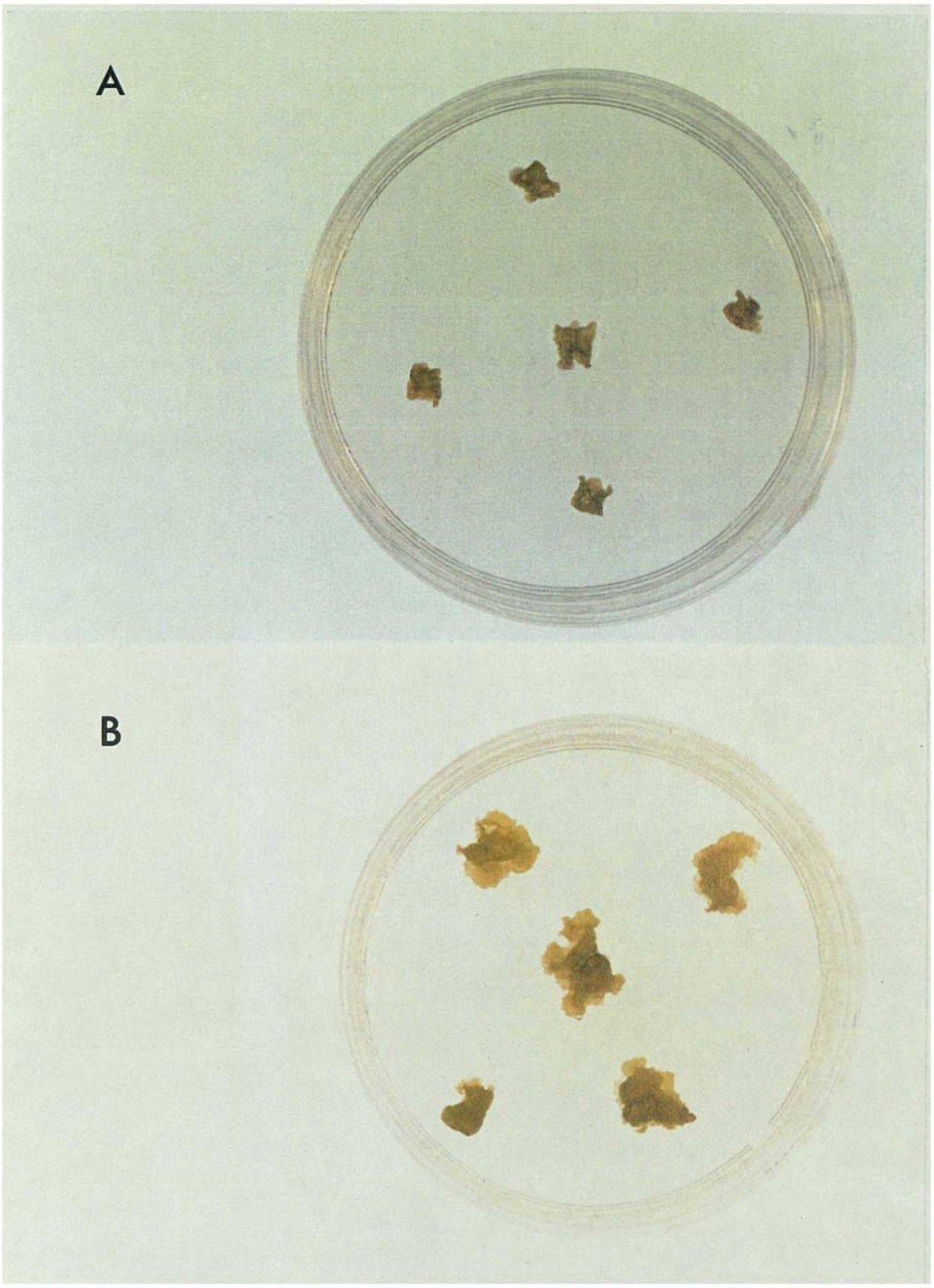


Fig. 4. Callus production from Desiree leaf explants cultured on solidified MS medium containing

A. 3 mg l^{-1} 2,4-D but without kinetin.

B. 3 mg l^{-1} 2,4-D and 0.3 mg l^{-1} kinetin.

Photographed after 6 weeks. (Petri dish Ø 90 mm)

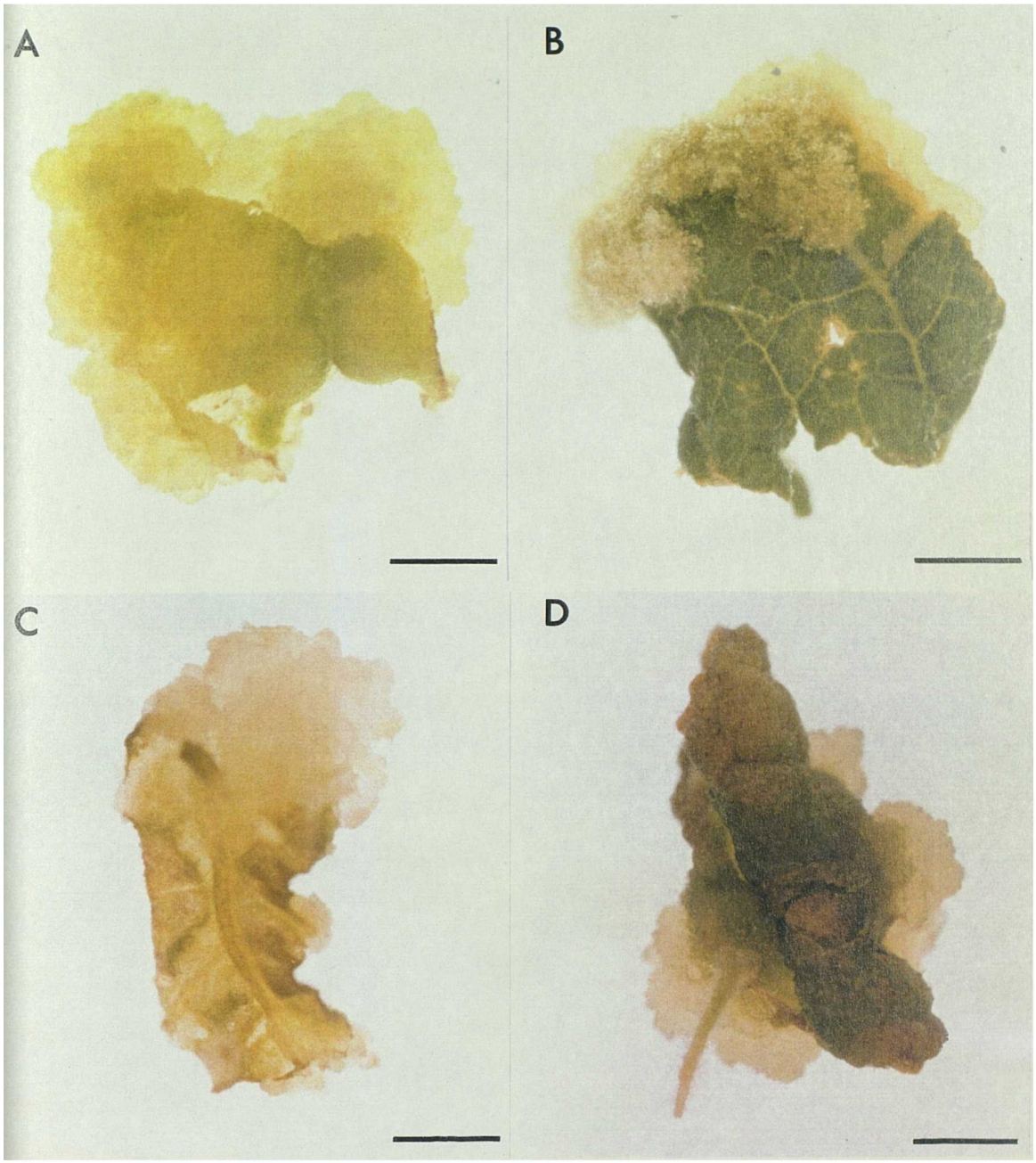


Fig. 5. Proliferated leaf explants cultured on MS medium supplemented with 3 mg l^{-1} 2,4-D, 0.3 mg l^{-1} kinetin, 30 g l^{-1} sucrose and 8 g l^{-1} agar. Photographed after 5 weeks. (Scale bar = 2 mm)

A. Desiree C. *S. commersonii*

B. Maris Piper D. Root initiation from a leaf culture of cv. Desiree.

Section 2.8.1 B) were cultured on MS medium containing 3 mg l^{-1} 2,4-D as the only growth regulator, callus initiation started from the edges and midrib of each explant 8 - 10 days after transferring to nutrient medium. Callus initiation was very slow, and even after six weeks of culture, only a small amount of callus was produced on each explant (Fig. 4). When 0.3 mg l^{-1} kinetin was added to the nutrient medium, callus initiation started from the edges of each explant after about 8 - 10 days of culture, eventually covering the lower surface. No callus was formed on the upper surfaces of the explants, except on the midribs (Fig. 5). Roots were also produced from some explants with the callus growths (Fig. 5, D). After six weeks, sufficient callus was produced from most of the explants to enable subculture onto fresh MS medium supplemented with 3 mg l^{-1} 2,4-D, but without kinetin.

C. From tuber explants

Callus formation in both Desiree and Maris Piper cultivars was initiated from tuber explants (see Section 2.8.1 C) within 7 days of placing the explants on MS medium with 3 mg l^{-1} 2,4-D. The growth started as small, globular bodies which appeared near the edges of the explants, finally spreading all around the edges. At this early stage it looked shiny under the microscope. Calluses grew actively after induction without any regular form, and were creamy white in colour in both cultivars. Roots were also produced in some cultures (Fig. 7). After 5 weeks of culture, there was sufficient callus on each explant (Fig. 6) to allow subculture onto fresh nutrient medium for culture multiplication.

The rate of callus production from explants of both Desiree and Maris Piper cultured

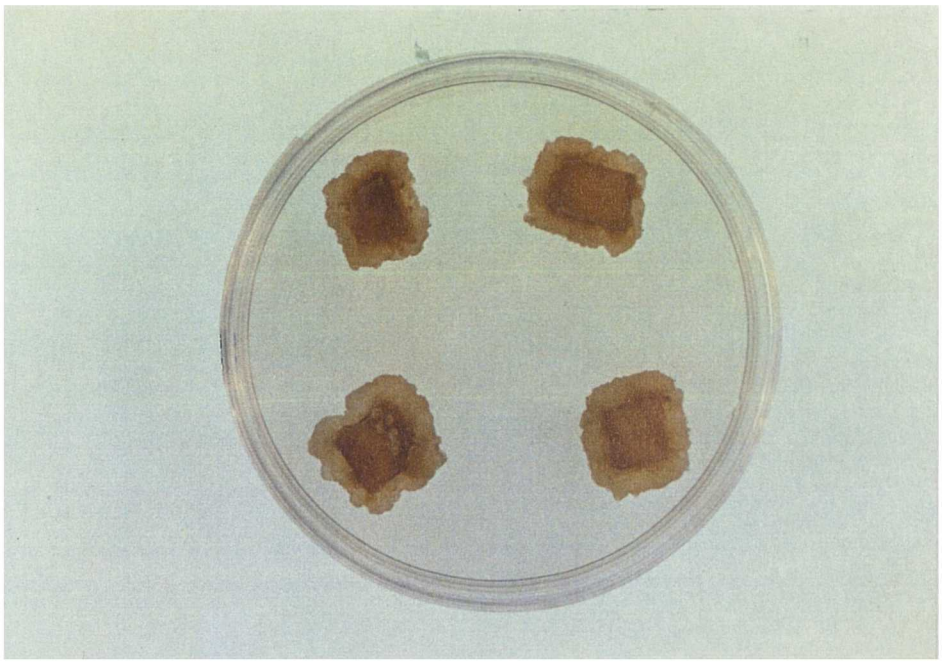


Fig. 6. Proliferated Desiree tuber explants cultured on solidified MS medium supplemented with 3 mg l^{-1} 2,4-D, 30 g l^{-1} sucrose and 8 g l^{-1} agar. Photographed after 5 weeks. (Petri dish Ø 90 mm)

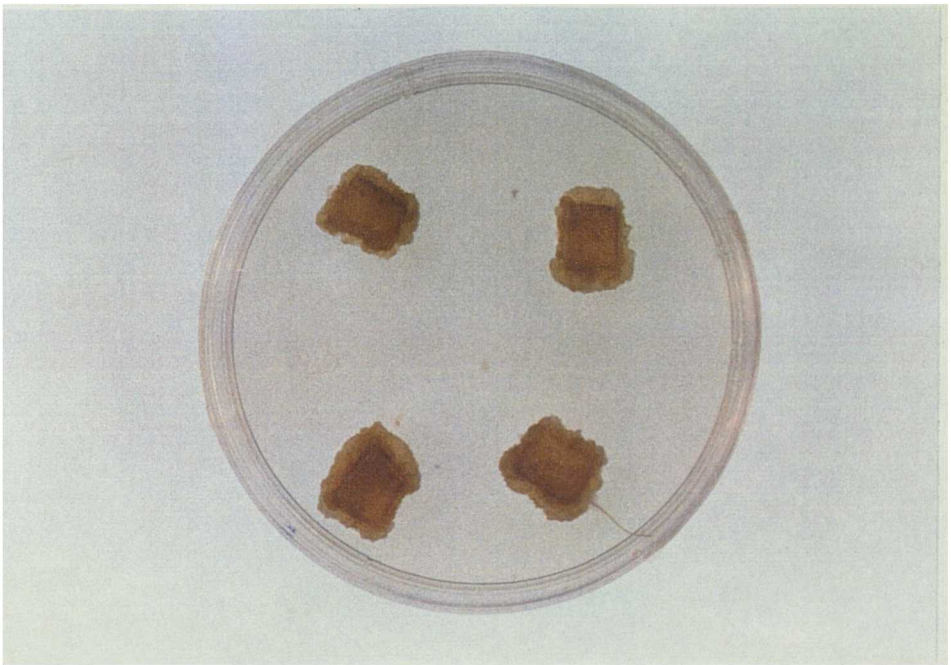


Fig. 7. Proliferated Maris Piper tuber explants cultured on solidified MS medium supplemented with 3 mg l^{-1} 2,4-D, 30 g l^{-1} sucrose and 8 g l^{-1} agar. Photographed after 5 weeks. Root produced from one explant can also be seen. (Petri dish Ø 90 mm)

in disposable Ø 90 mm petri dishes on agar-solidified medium, and on autoclavable polypropylene membrane rafts (Sigma Chemical Co.) over liquid medium, otherwise of the same composition, was assessed on the basis of increases in their fresh weights. In each of these assessments, the average fresh weight of eight replicate cultures was obtained every 7 days and the percent increases in fresh weights were calculated. Results are summarized in Table 19, and Figure 8.

Callus formation from Desiree tuber explants was more rapid than that from Maris Piper explants, and the rate of callus formation was higher on rafts over liquid medium than on agar-solidified medium. Explants from Desiree cultivar cultured on membrane rafts over liquid medium produced the maximum amount of callus followed by the explants of Desiree cultured on agar-solidified medium, with 266% and 234.4% increases in fresh weights respectively. Explants taken from Maris Piper tubers and cultured on membrane rafts over liquid medium gave 146.5% increase in fresh weight. The minimum amount of callus was produced from Maris Piper explants cultured on agar-solidified medium, with only 125.5% increase in fresh weight.

3.2.2 Callus culture multiplication

After 5-6 weeks of culture, callus tissue was removed from the original explants and subcultured onto fresh nutrient medium. Subsequent subcultures were made regularly after 28 day intervals. Calluses induced from Desiree explants grew more actively compared to others. After 3 - 4 subcultures, the calluses became quite friable and at this stage growth of established calluses was assessed on a fresh weight basis. The fresh weights of 8 cultures of callus were determined after every 7 days under aseptic

Table 19. Increases (%) in fresh weights of explants + callus produced from tuber explants of commercial potato cultivars cultured on agar-solidified (8 g l^{-1}), and on liquid MS medium, both supplemented with 3 mg l^{-1} 2,4-D and 30 g l^{-1} sucrose.

Physical state of culture medium	Day 7		Day 14		Day 21		Day 28		Day 35	
	Desiree	M. Piper	Desiree	M. Piper	Desiree	M. Piper	Desiree	M. Piper	Desiree	M. Piper
Solidified	9.27	9.58	61.12	36.47	111.89	63.61	168.13	97.92	234.36	125.46
Liquid	18.09	14.06	104.27	43.91	165.58	79.64	219.35	116.03	265.95	146.52

Data represent means of 8 replicates

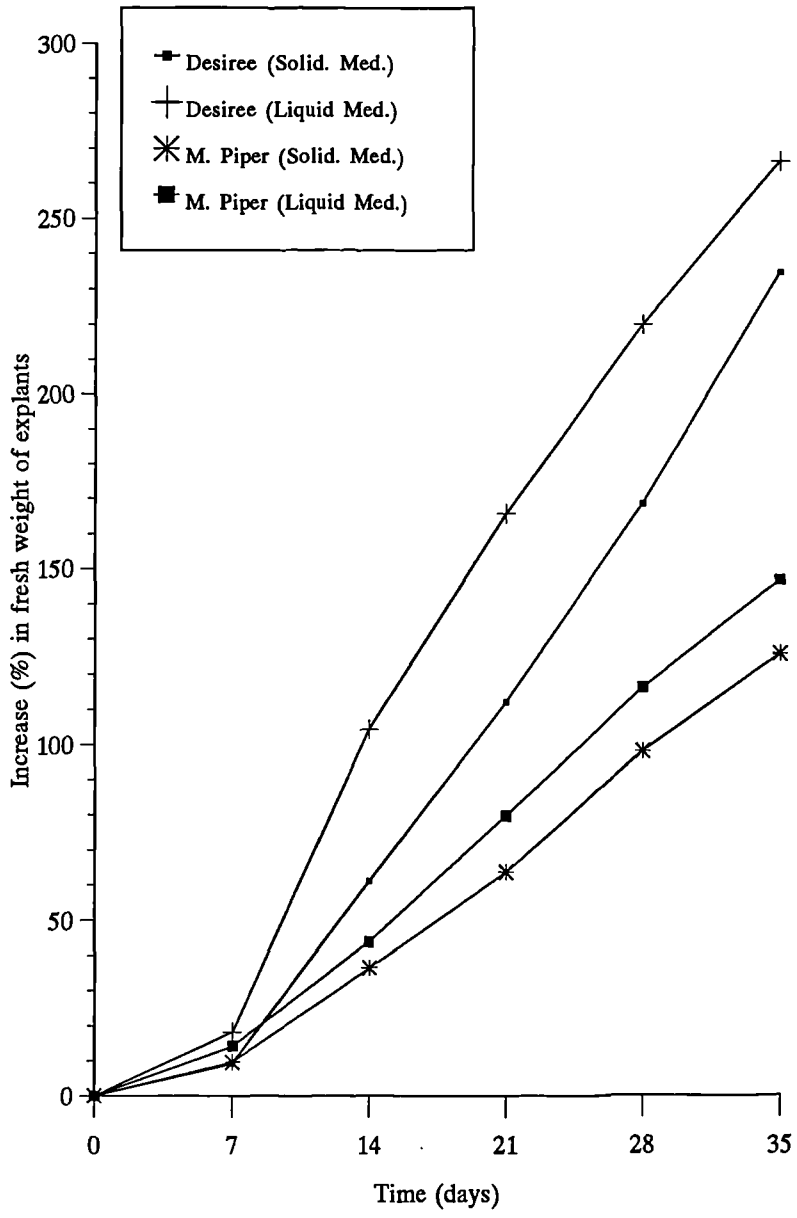


Fig. 8. Rate of callus formation on *S. tuberosum* cvs. Desiree and Maris Piper tuber explants on agar-solidified (8 g/l), and liquid MS medium supplemented with 3 mg/l 2,4-D and 30 g/l sucrose.

Table 20. Fresh weights (mg) of *Solanum tuberosum* cvs. Desiree and Maris Piper, and *S. acaule* stem callus cultures on MS medium supplemented with 3 mg 2,4-D, 30 g sucrose and 8 g l^{-1} agar.

Days of culture	Desiree	Maris Piper	*S. acaule*
0	200 ± 4.64	200 ± 6.52	200 ± 6.40
7	490 ± 29.58	449 ± 20.32	300 ± 17.11
14	1008 ± 105.62	836 ± 55.17	489 ± 36.16
21	1909 ± 184.24	1655 ± 139.21	843 ± 92.98
28	2532 ± 182.46	2311 ± 102.06	1376 ± 97.70
35	2953 ± 211.91	2685 ± 94.59	1688 ± 135.27

Data represent means ± SD of 4 replicates

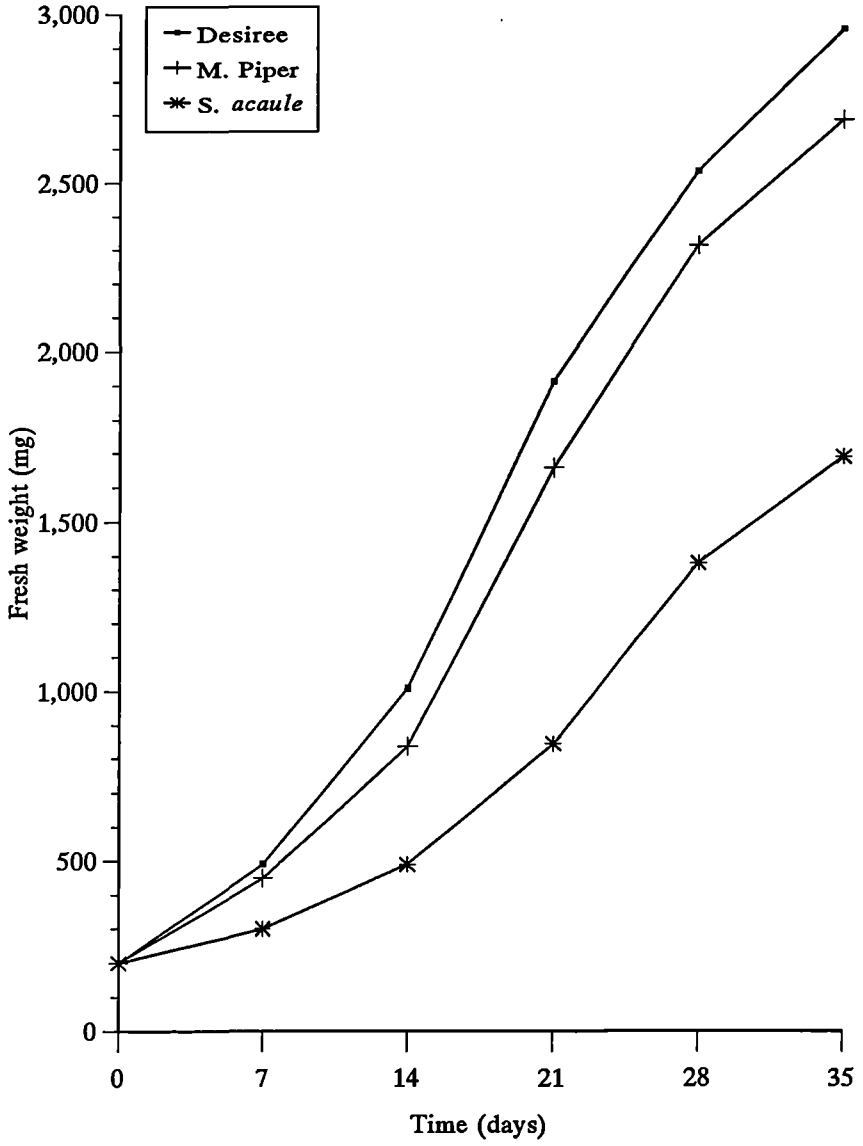


Fig. 9. Growth of *S. tuberosum* cvs. Desiree and Maris Piper, and *S. acaule* stem callus cultures on MS medium supplemented with 3 mg/l 2,4-D , 30 g/l sucrose and 8 g/l agar.

Table 21. Fresh weights (mg) of *S. tuberosum* cvs. Desiree and Maris Piper, and *S. commersonii* leaf callus cultures on MS medium supplemented with 3 mg 2,4-D, 30 g sucrose and 8 g l^{-1} agar.

Days of culture	Desiree	Maris Piper	*S. commersonii*
0	200 ± 3.24	200 ± 4.74	200 ± 3.32
7	476 ± 31.95	400 ± 28.35	316 ± 25.17
14	878 ± 78.84	799 ± 60.51	486 ± 32.58
21	1718 ± 134.23	1520 ± 109.33	929 ± 54.87
28	2281 ± 239.99	2193 ± 190.00	1415 ± 96.90
35	2616 ± 274.91	2563 ± 227.17	1729 ± 107.38

Data represent means ± SD of 4 replicates

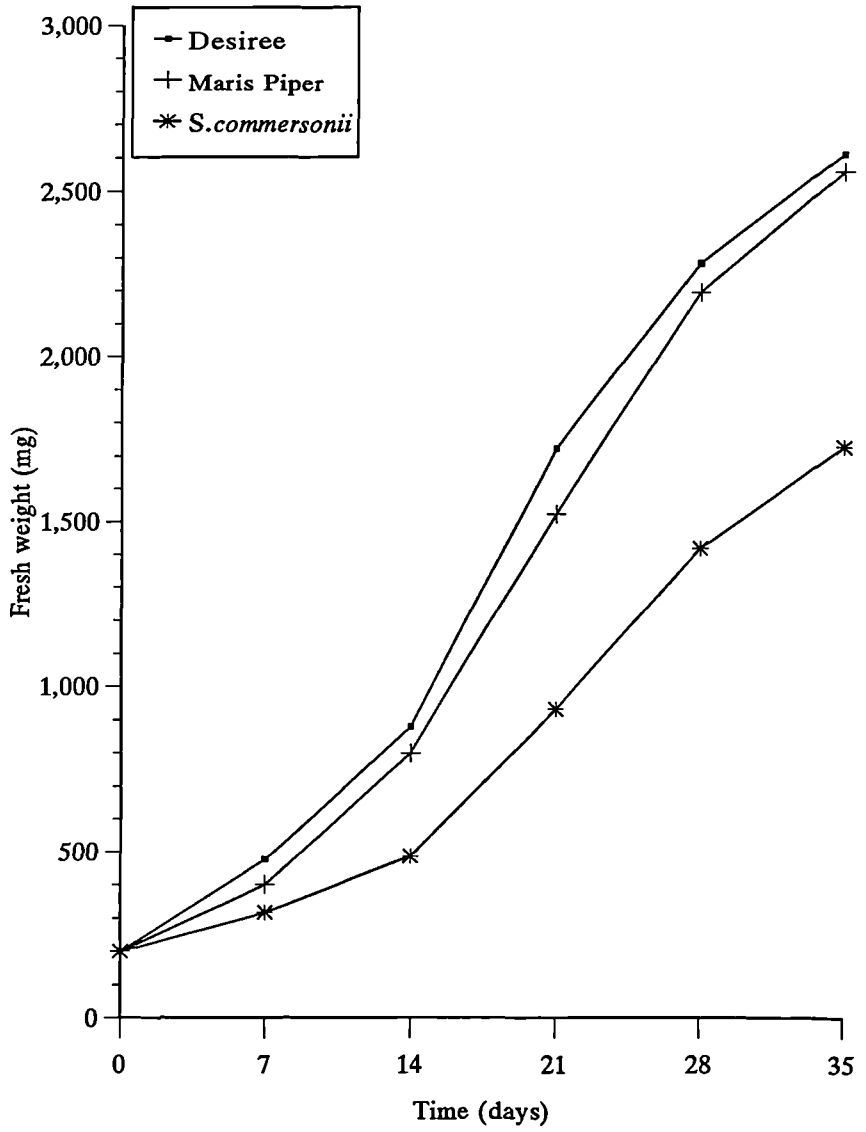


Fig. 10. Growth of *S. tuberosum* cvs. Desiree and Maris Piper, and *S. commersonii* leaf callus cultures on MS medium supplemented with 3 mg/l 2,4-D, 30 g/l sucrose, and 8 g/l agar.

conditions using a non-sacrificing method.

The growth of established stem callus cultures of *S. tuberosum* cvs. Desiree and Maris Piper, and *S. acaule*, is presented in Table 20. The growth of Desiree, Maris Piper and *S. commersonii* leaf callus cultures is given in Table 21, and growth of Desiree and Maris Piper tuber callus cultures on MS nutrient media is presented in Table 22. Respective curves, on the basis of fresh weight measurements, are plotted as Figures 9, 10 and 11.

It is clear from the data that the growth rate of Desiree callus was greater than those of other genotypes regardless of the origin of an explant. This was followed by Maris Piper callus, while growth rates of *S. acaule* and *S. commersonii* calluses were lower still.

The growth of established tuber calluses of *S. tuberosum* cvs. Desiree and Maris Piper was also compared on agar-solidified vs. liquid nutrient media on a fresh weight basis, again recorded at weekly intervals (Table 22).

The results of the present study demonstrate that the growth rate of callus was much higher over liquid medium than on agar-solidified medium. An initial inoculum of 150 mg of callus grew and increased its fresh weight up to 2520 mg in Desiree and 1963 mg in Maris Piper after 35 days of growth on liquid medium over membrane rafts, while on agar-solidified medium in petri dishes they grew up to fresh weights of 1358 mg and 1230 mg in Desiree and Maris Piper respectively at the end of the period of

Table 22. Fresh weight production (mg) of *S. tuberosum* cvs Desiree and Maris Piper tuber callus cultures on solidified (8 g l^{-1} agar), and on liquid MS medium, both supplemented with 3 mg l^{-1} 2,4-D and 30 g l^{-1} sucrose.

Physical state of culture medium	Day 0		Day 7		Day 14		Day 21		Day 28		Day 35	
	Desiree	M. Piper	Desiree	M. Piper	Desiree	M. Piper	Desiree	M. Piper	Desiree	M. Piper	Desiree	M. Piper
Solidified	150 ±3.64	150 ±3.39	238 ±21.56	228 ±32.15	417 ±36.36	369 ±47.80	827 ±92.32	777 ±79.04	1184 ±115.10	1074 ±132.12	1358 ±142.15	1230 ±141.46
Liquid	150 ±2.18	150 ±3.39	359 ±29.28	336 ±16.08	628 ±83.98	519 ±67.28	1185 ±119.57	989 ±119.76	1962 ±157.55	1636 ±218.11	2520 ±239.24	1963 ±217.85

Data represent means ± SD of 8 replicates

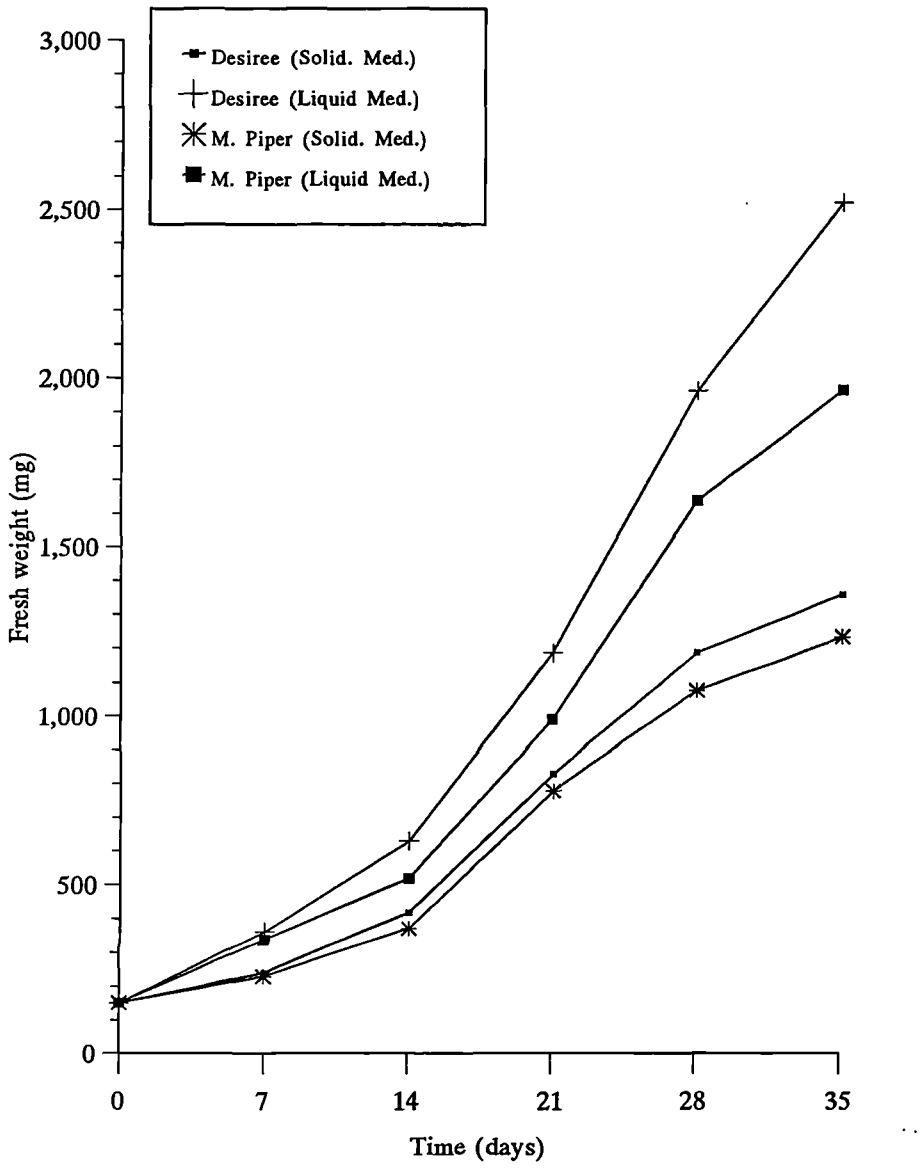


Fig. 11. Growth of *S. tuberosum* cvs. Desiree and Maris Piper tuber calluses on solidified (8 g/l agar) and liquid MS medium supplemented with 3 mg/l 2,4-D and 30 g/l sucrose.

observation.

3.3 Cell suspension cultures

3.3.1 Tuber callus-derived cell suspensions

Tuber-derived calluses of *S. tuberosum* cultivars Desiree and Maris Piper were used to initiate the cell suspension cultures. Cell suspensions were obtained by transferring one-week-old friable callus, to fresh liquid nutrient medium of the same composition, which is described in Section 2.8.1 for tuber callus induction and maintenance, but without agar. The callus pieces readily separated into cells and cell aggregates in the liquid nutrient medium when agitated, giving an off-white cell suspension. The growth of the suspension cultures was assessed after 2 or 3 subcultures. The changes in packed cell volume and cell number were recorded over a period of 20 days and are presented in Tables 23 and 24, and the relevant growth curves are plotted as Figures 12 and 13.

Data regarding packed cell volume revealed that there was a lag phase of about 2 days followed by a sudden increase in growth rate of both potato cultivars which resulted in rapid increases in the packed cell volumes. Increase in packed cell volume was greater in Desiree cell suspensions than in Maris Piper at all stages of growth. 16 days after subculture, the rate of increase in packed cell volume diminished, which indicated that the cultures were entering a stationary phase.

Data concerning cell number also showed a lag phase of 2 - 3 days in both cultivars followed by a rapid growth phase. During this period (first 2 - 3 days after subculture)

Table 23. Changes in packed cell volumes (%) of tuber callus-derived cell suspension cultures of *S. tuberosum* maintained in MS medium, plus 3 mg l^{1} 2,4-D and 30 g l^{-1} sucrose.

Days of culture	Desiree	Maris Piper
0	2.0	1.9
2	2.0	1.9
4	2.5	2.4
8	4.1	3.7
12	7.7	6.8
16	11.9	10.3
20	13.8	12.4

Data represent means of 4 replicates

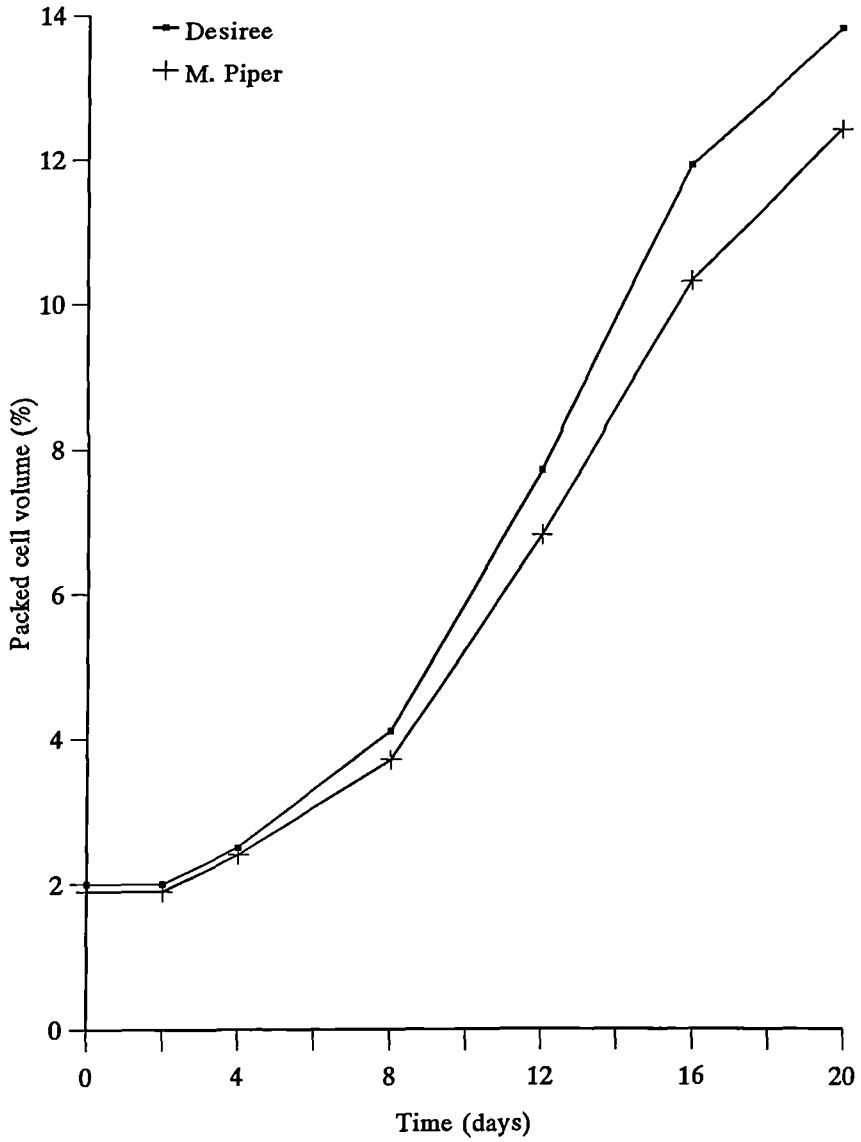


Fig. 12. Increases in packed cell volumes of tuber callus-derived cell suspension cultures of *S. tuberosum* cvs. Desiree and Maris Piper in MS medium plus 3 mg/l 2,4-D.

Table 24. Increases in cell number (x 10^5) ml^{-1} in tuber callus-derived cell suspension cultures of *S. tuberosum* maintained in MS medium, plus 3 mg l^{-1} 2,4-D and 30 g l^{-1} sucrose.

Days of culture	Desiree	Maris Piper
0	1.0	0.95
2	1.0	0.95
4	1.4	1.3
8	2.8	2.5
12	5.3	4.2
16	7.4	6.5
20	7.9	7.2

Data represent means of 4 replicates

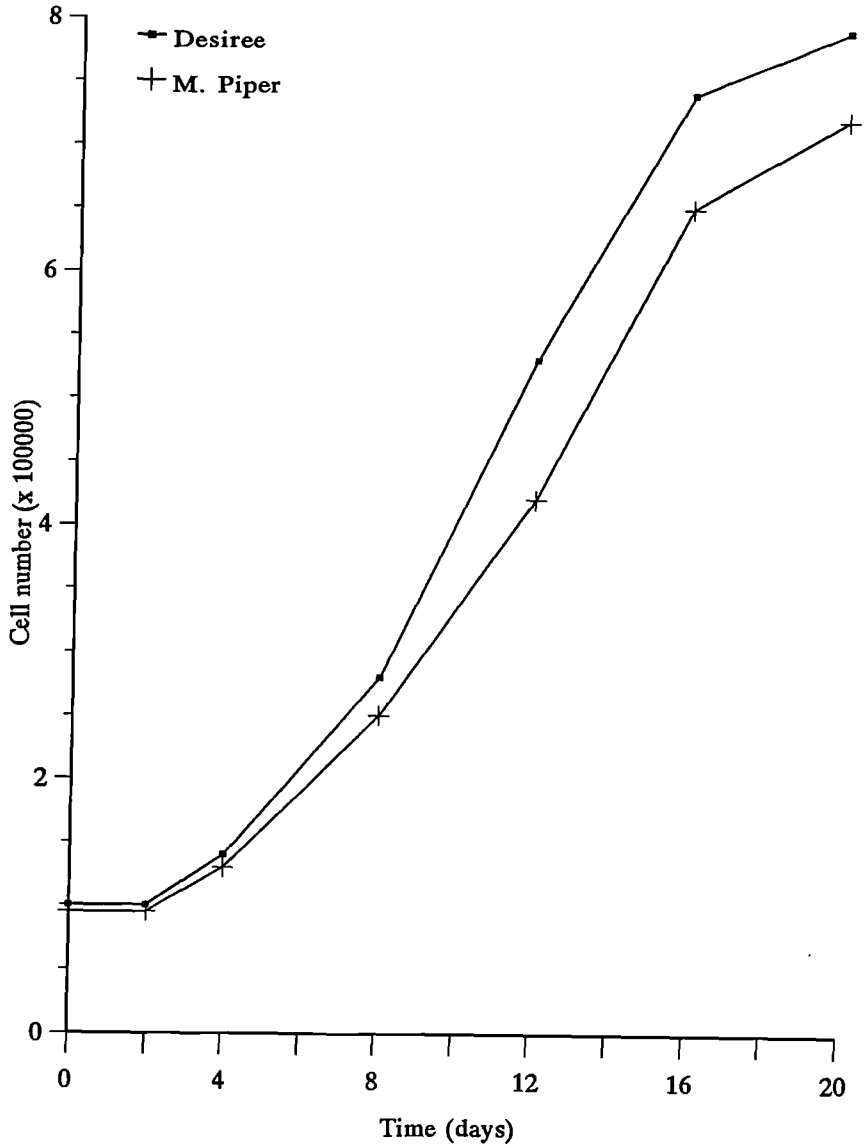


Fig. 13. Increases in cell number in tuber callus-derived cell suspension cultures of *S. tuberosum* cvs. Desiree and Maris Piper in MS medium plus 3 mg/l 2,4-D.

cells might be preparing for cell division. Despite continuous shaking (orbital), on 10th day of subculture, cell aggregates appeared in the cultures, while after two weeks of subculture some cell clumps or callus masses were also seen.

3.3.2 Stem callus-derived cell suspensions

After one week of subculture of calluses on fresh medium, 2 - 3 g pieces of actively-growing, friable callus were removed and transferred to Lam (1977b) medium. Due to the agitation, callus pieces dispersed into single cells and small cell aggregates in the liquid nutrient medium. By filtering through a 250 μm nylon mesh, the larger cell clumps were removed, and after a series of two to three subcultures, fine cell suspensions were obtained. These cultures were maintained by regularly subculturing every 14 days. The growth of each culture was estimated after 2 - 3 subcultures, using packed cell volume (PCV) to determine the increases in growth. The cell populations were determined as cell number using a haemocytometer. Results are presented in Tables 25 and 26 and the relevant growth curves are plotted as Fig. 14 and 15.

A lag phase of 2 - 3 days was observed in suspension culture growth of both cultivars followed by a rapid increase in culture growth. The growth of Desiree cell suspension cultures was greater compared with Maris Piper.

Although some cell aggregates were observed in the cultures after two weeks of subculture, no large cell clumps were seen until the end of the study period. Lam (1977b) medium appeared to be more suitable and was used later in plant regeneration and frost tolerance experiments.

Table 25. Changes in packed cell volumes (%) of *S. tuberosum* stem callus-derived cell suspension cultures maintained in Lam (1977b) medium.

Days of culture	Desiree	Maris Piper
0	1.80	1.75
2	1.80	1.70
4	2.10	2.00
8	4.10	3.70
12	7.05	6.30
16	8.95	8.10
20	9.80	9.15

Data represent means of 4 replicates

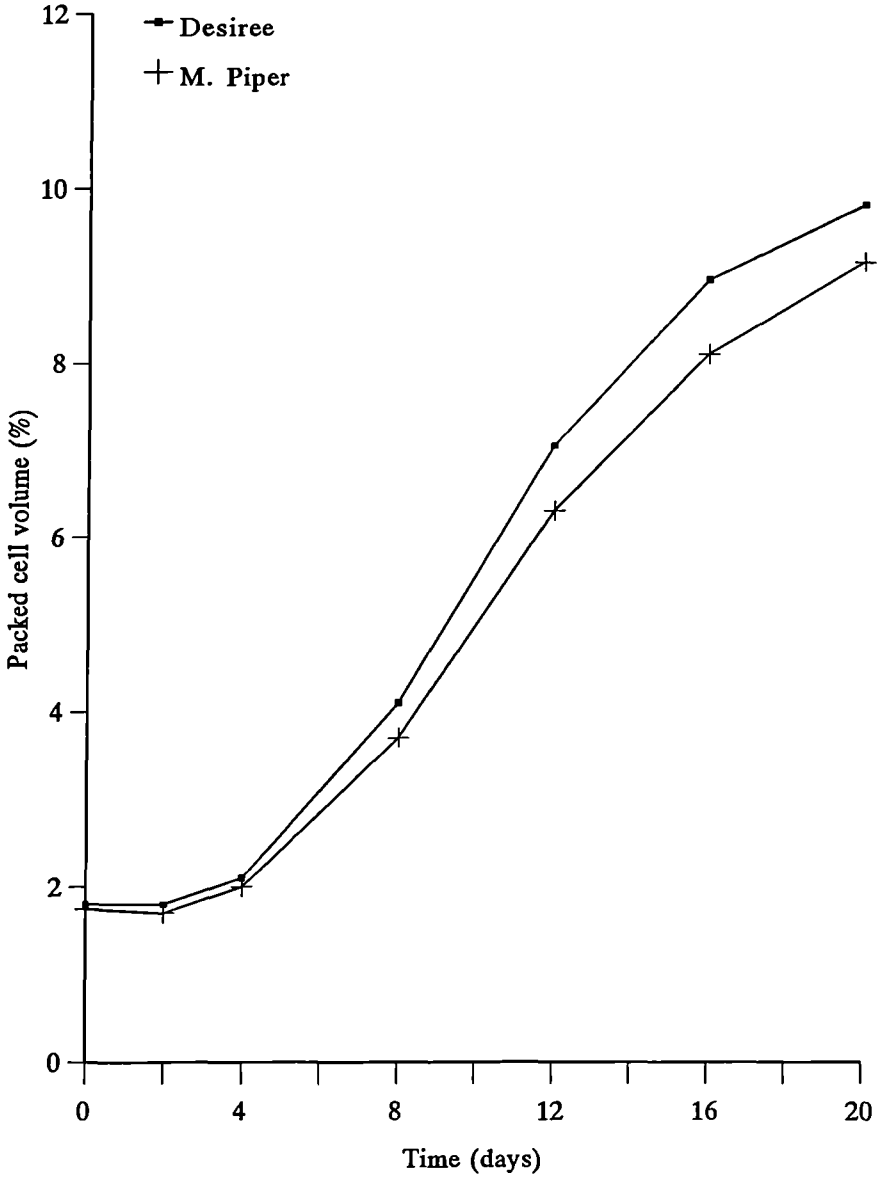


Fig. 14. Increases in packed cell volumes of stem callus-derived cell suspension cultures of *S. tuberosum* cvs. Desiree and Maris Piper in Lam (1977b) medium.

Table 26. Increases in cell number (x 10^5) ml^{-1} in *S. tuberosum* stem callus-derived cell suspension cultures maintained in Lam (1977b) medium.

Days of culture	Desiree	Maris Piper
0	1.10	1.05
2	1.10	1.10
4	1.25	1.20
8	2.45	2.35
12	4.70	4.40
16	6.35	6.00
20	6.90	6.55

Data represent means of 4 replicates

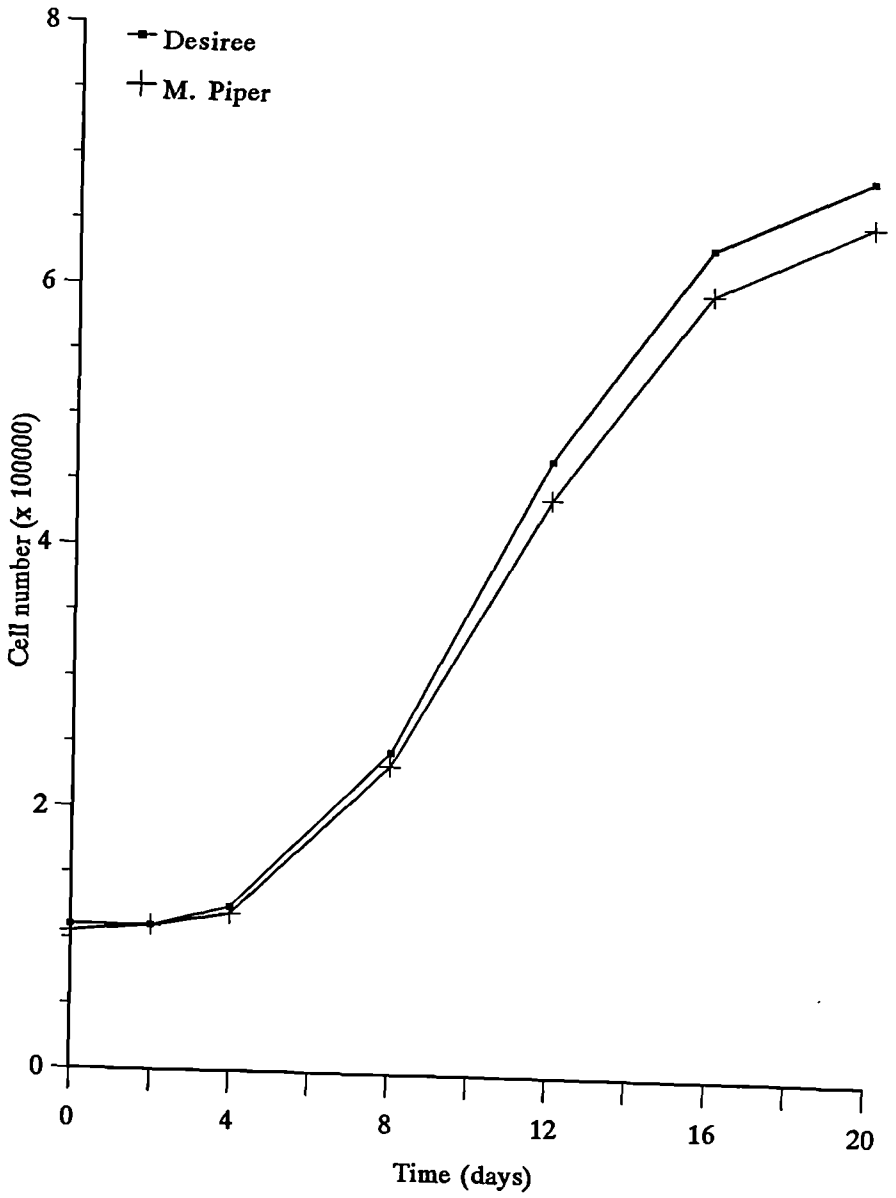


Fig. 15. Increases in cell number in stem callus-derived cell suspension cultures of *S. tuberosum* cvs. Desiree and Maris Piper in Lam (1977b) medium.

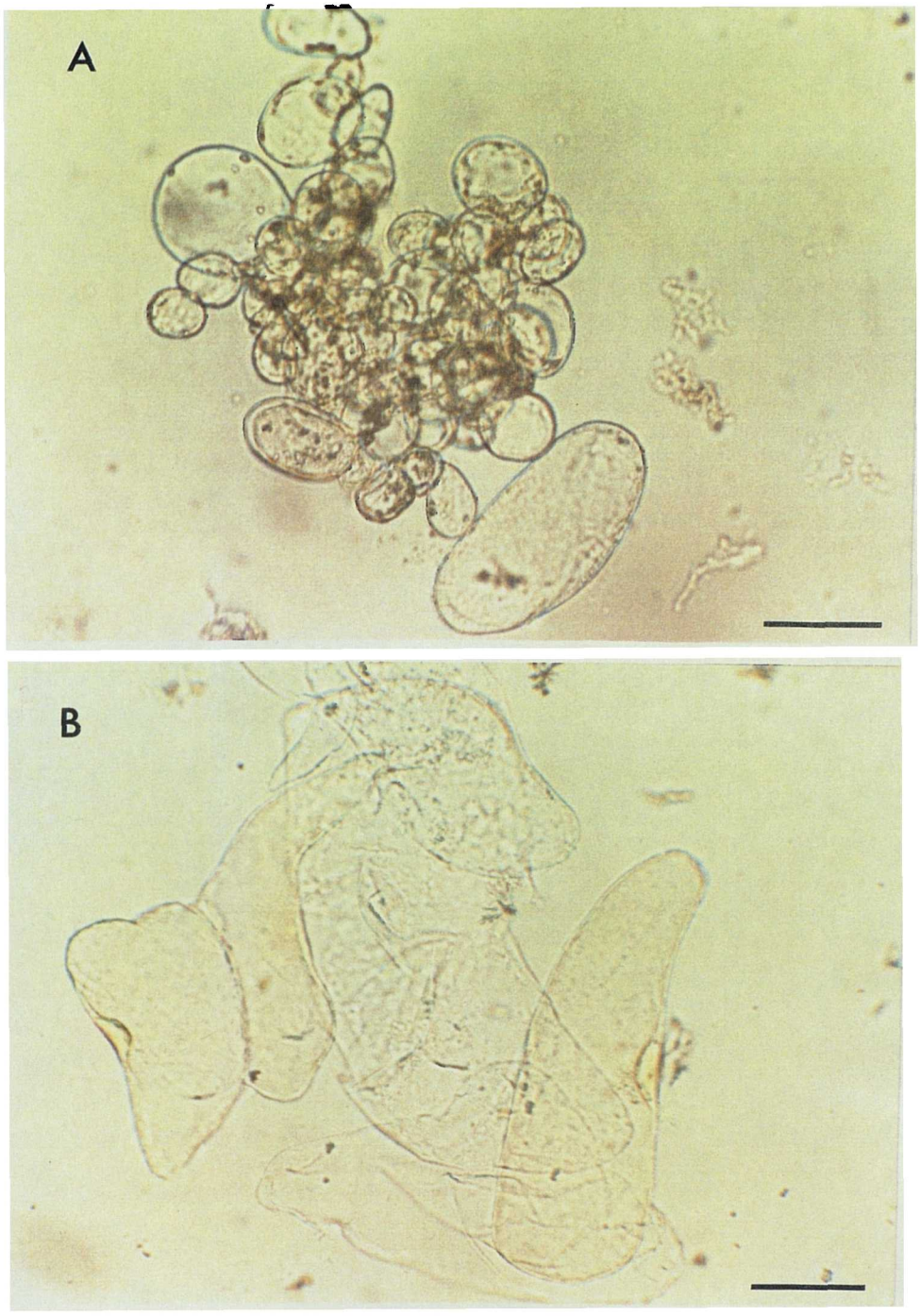


Fig. 16. Tuber cell suspension-cultured cells, 10 days after last subculture in MS medium supplemented with 3 mg l^{-1} 2,4-D and 30 g l^{-1} sucrose. Cells in (A) are dividing showing a large proportionate increase in their cytoplasmic contents. (Scale bar = 50 μm)

A. Desiree B. Maris Piper

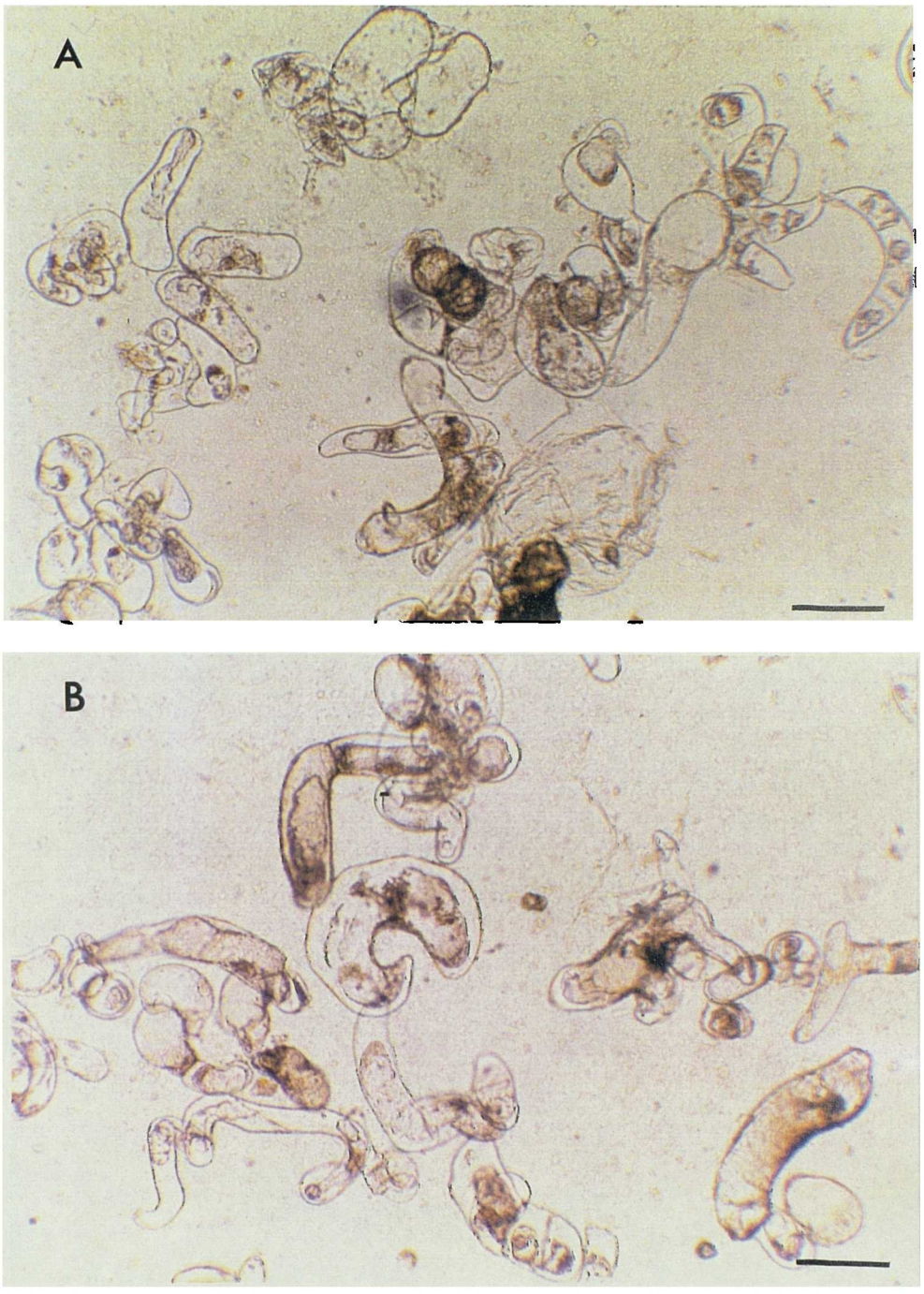


Fig. 17. Stem cell suspension-cultured cells, 10 days after last subculture in Lam (1977b) medium. (Scale bar = 100 μm)

A. Desiree B. Maris Piper

3.3.3 Morphology of cultured cells

When cells were observed under the microscope, the shapes and sizes of cells were seen to be highly variable. Rounded, elongated, pear-shaped or filamentous cells (Fig. 16 and 17) were frequent while some even more irregularly-shaped cells were also observed. Furthermore, in tuber-derived cell suspensions in MS medium, cell aggregates (Fig. 16) were more frequently seen under the microscope compared with stem-derived cell suspensions in Lam (1977b) medium, which consisted mostly of single cells (Fig. 17). Streaming of the cytoplasm could also be seen in several instances.

3.3.4 Viability of cultured cells

The viability of suspension-cultured cells was determined using fluorescein diacetate (Fig. 18 and 19), phenosafranine (Fig. 20) and Evans' blue (Fig. 21). In tuber callus-derived cell suspensions, the viability estimated was close to 90% in cv. Desiree and 84% in cv. Maris Piper, while in stem callus-derived cell suspensions, viabilities of 85 and 80% were recorded in Desiree and Maris Piper respectively.

3.4 Protoplast isolation

3.4.1 Protoplast yield

A. Mesophyll cell protoplasts

Protoplasts were isolated from the leaves of greenhouse-grown plants and *in vitro* shoot cultures using the method of Haberlach *et al.* (1985). Leaves were taken from greenhouse-grown *S. tuberosum* cv. Desiree and Maris Piper plants of a range of ages and were only collected from positions 4 - 7 from the shoot apex. The results are

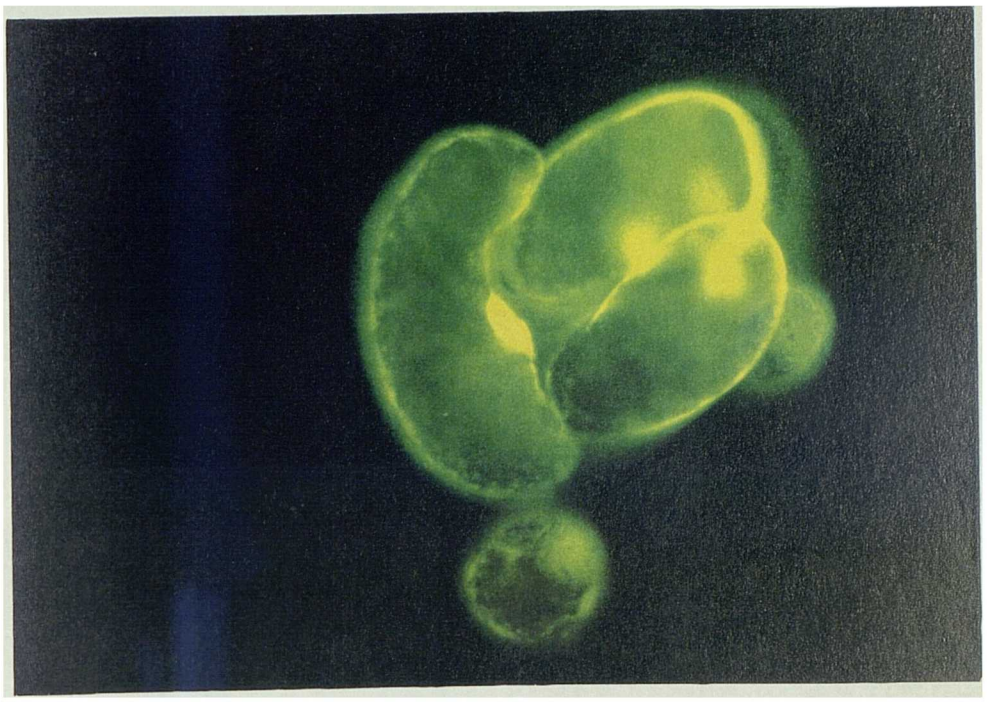


Fig. 18. Fluorescence microscope photograph of 10-day-old tuber suspension-cultured cells of cv. Desiree treated with FDA. (Scale bar = 50 μm)

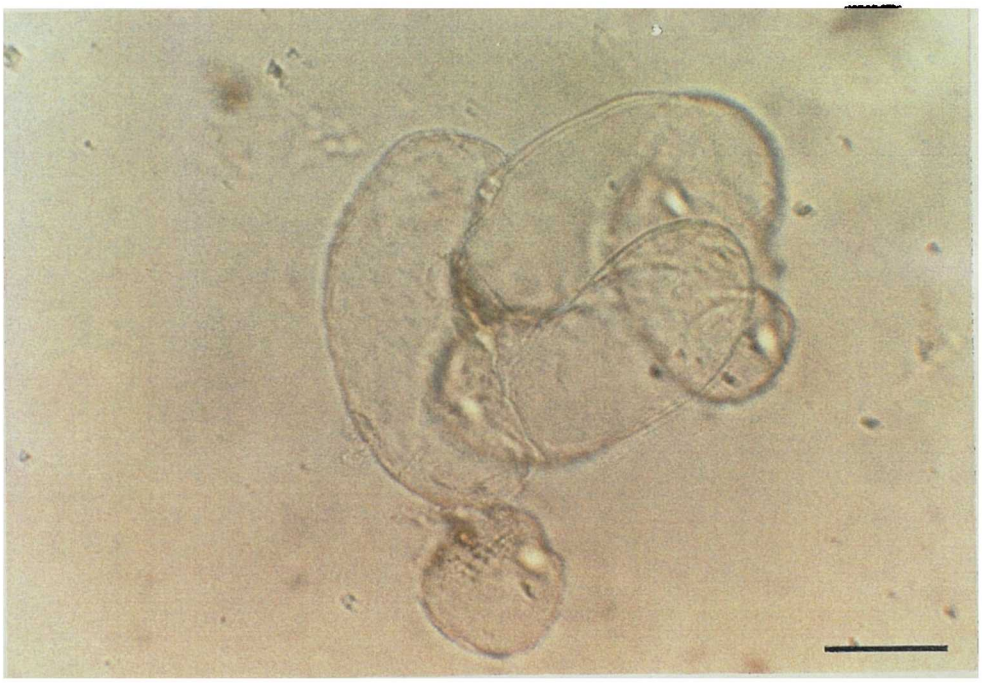


Fig. 19. The same group of cells under the microscope, illuminated with tungsten light. (Scale bar = 50 μm)

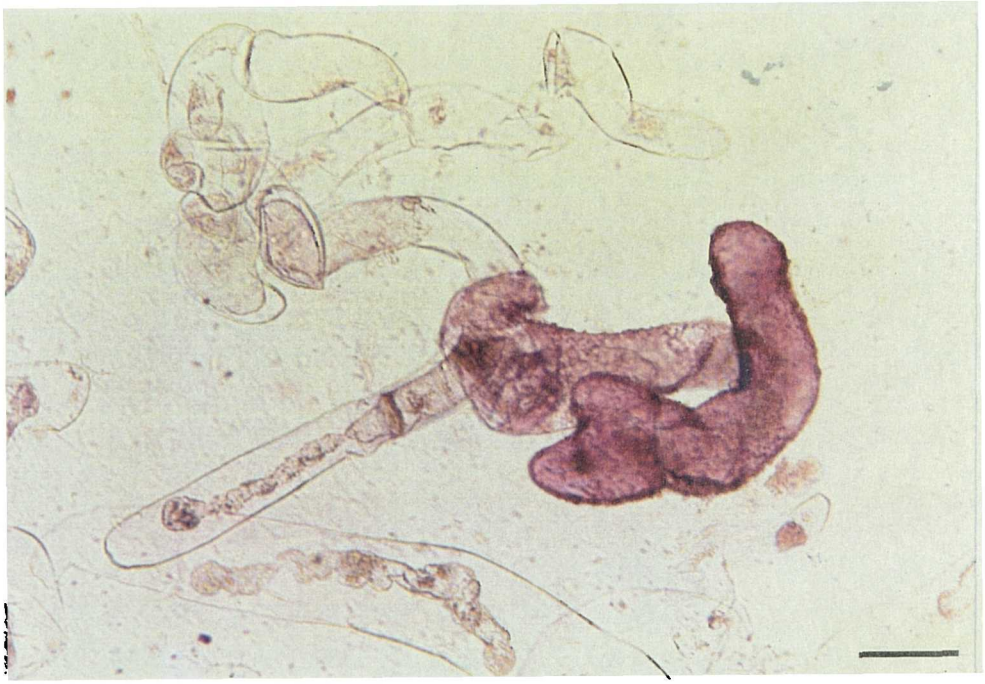


Fig. 20. 10-day-old, stem suspension-cultured cells of cv. Desiree treated with phenosafranine. The red-stained cells are dead, while those unstained are alive. (Scale bar = 100 μm)

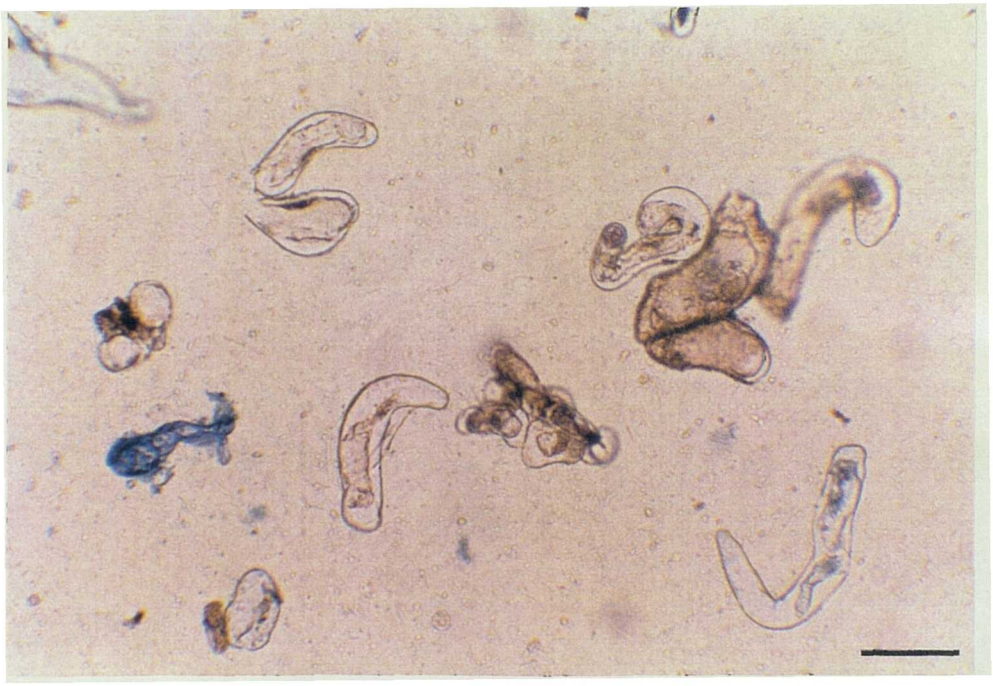


Fig. 21. 10-day-old, stem suspension-cultured cells of cv. Maris Piper treated with Evans' blue. The blue-stained cells are dead, while those unstained are alive. (Scale bar = 100 μm)

summarised in Table 27.

It is apparent from the data that leaves taken from greenhouse-grown plants of cv. Maris Piper resulted in higher protoplast yields. Protoplast yield varied in both cultivars with plant age. In cv. Maris Piper, protoplast yield increased with the age of plant (45 - 59 days) but in cv. Desiree, maximum yield was obtained when leaves were taken from plants aged 52 days. Fig. 22 and 23 show protoplasts isolated from cv. Desiree while, Fig. 24 and 25 show protoplasts from cv. Maris Piper.

When protoplasts were isolated from the leaves of 35-day-old *in vitro* shoot cultures of *S. tuberosum* cvs. Desiree and Maris Piper, and of *S. commersonii*, the highest protoplast yield (2.25×10^6) was obtained from leaves of Desiree followed by *S. commersonii* (1.85×10^6), while leaves from Maris Piper resulted in the lowest protoplast yield (1.20×10^6)(Table 28). Leaves produced in *S. acaule* shoot cultures were few and narrow (Fig. 26), and therefore were not used for protoplast isolation.

B. Cultured cell protoplasts

Three enzyme mixtures, with different incubation periods, (Table 29) were tested for protoplast isolation from stem callus-derived cell suspensions of *S. tuberosum* cvs. Desiree and Maris Piper. The number of protoplasts isolated from ml^{-1} PCV of suspension-cultured cells varied depending upon the enzyme mixture used. The highest protoplast yield was obtained when enzyme mixture I (cellulase and pectolyase) was used, this was followed by enzyme mixture III, while enzyme mixture II resulted in the lowest protoplast yield (Table 30). The yield was higher from cv. Desiree

Table 27. Yields/gram fresh weight of leaf, and viabilities, of mesophyll protoplasts isolated from leaves taken from greenhouse-grown *S. tuberosum* plants of increasing ages.

Potato cultivar	Plant age (days)	Yield (x 10^5)	Viability (%)
Desiree	45	2.10	85
	52	3.73	92
	59	3.26	88
Maris Piper	45	3.00	86
	52	3.75	90
	59	4.99	85

Data represent means of 3 replicates

Table 28. Yields/gram fresh weight of plant material, and viabilities of potato mesophyll protoplasts isolated from *in vitro* shoot cultures.

Potato type	Yield (x 10^6)	Viability (%)
Desiree	2.25	90
Maris Piper	1.20	90
S. commersonii	1.85	86

Data represent means of 3 replicates

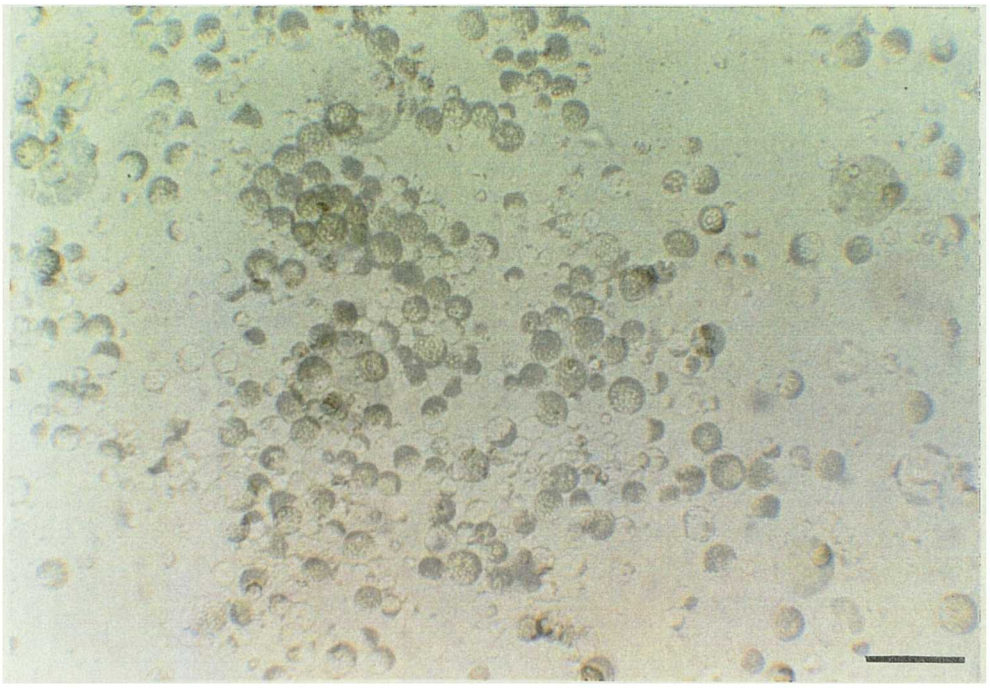


Fig. 22. *S. tuberosum*, cv. Desiree, protoplasts isolated from leaves of greenhouse-grown plants. Seen at low magnification. (Scale bar = 100 μm)

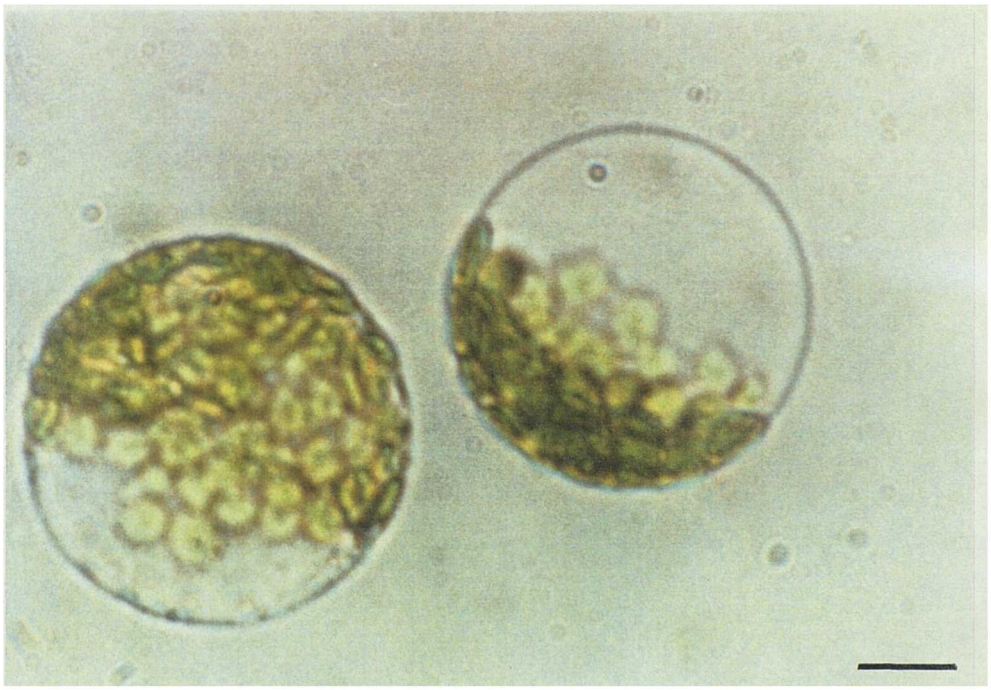


Fig. 23. Two protoplasts of cv. Desiree isolated from leaves of greenhouse-grown plants. Seen at higher magnification. (Scale bar = 10 μm)

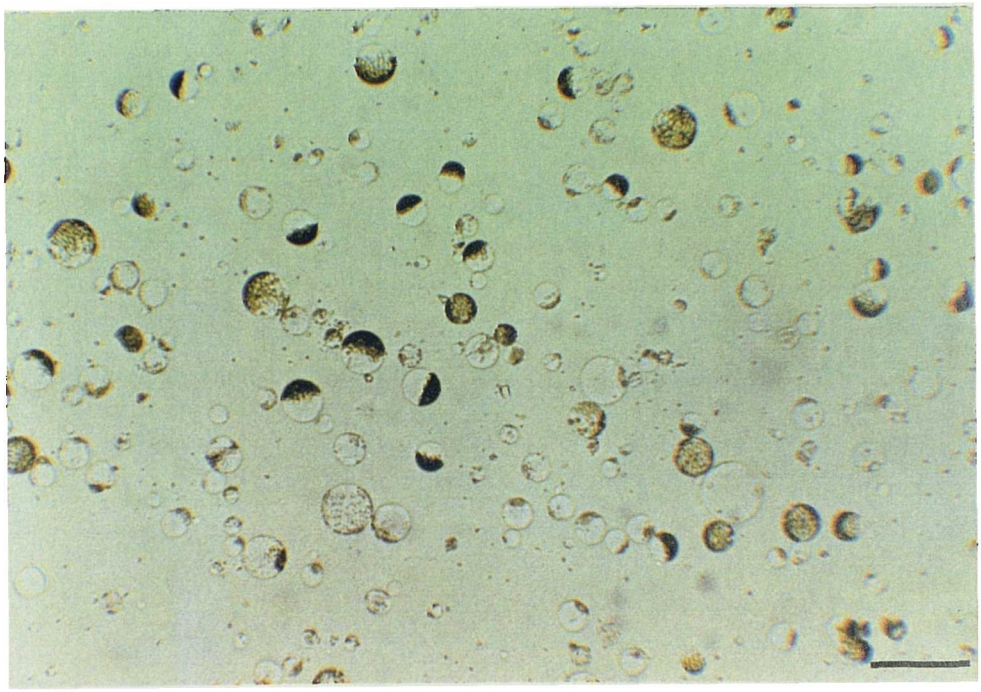


Fig. 24. *S. tuberosum*, cv. Maris Piper, protoplasts isolated from leaves of greenhouse-grown plants. Seen at low magnification. (Scale bar = 100 μm)

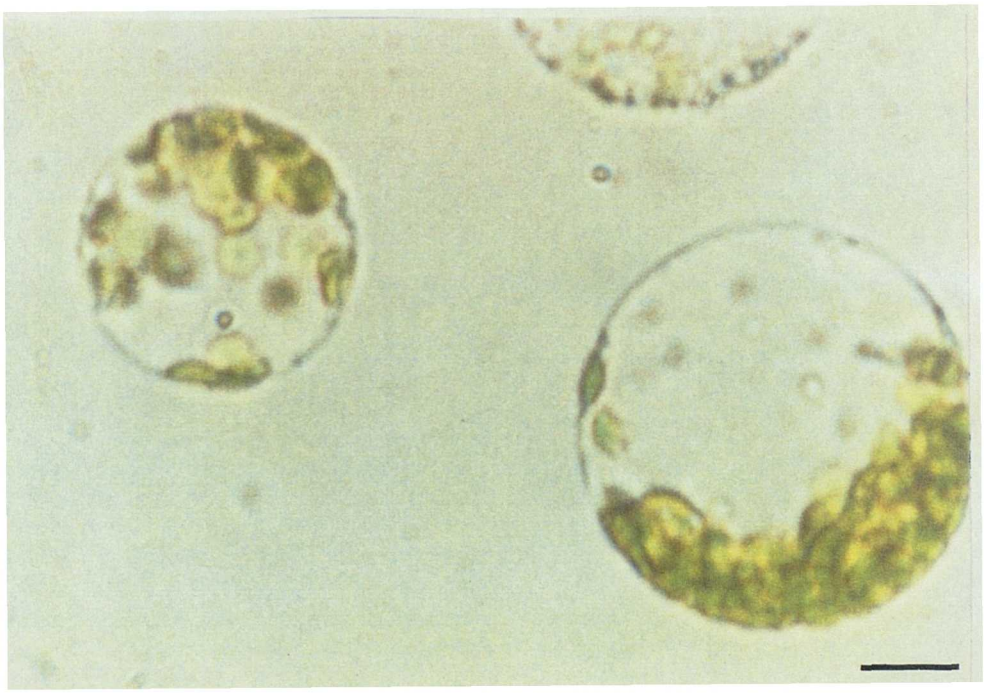


Fig. 25. Protoplasts of cv. Maris Piper isolated from leaves of greenhouse-grown plants. Seen at higher magnification. (Scale bar = 10 μm)

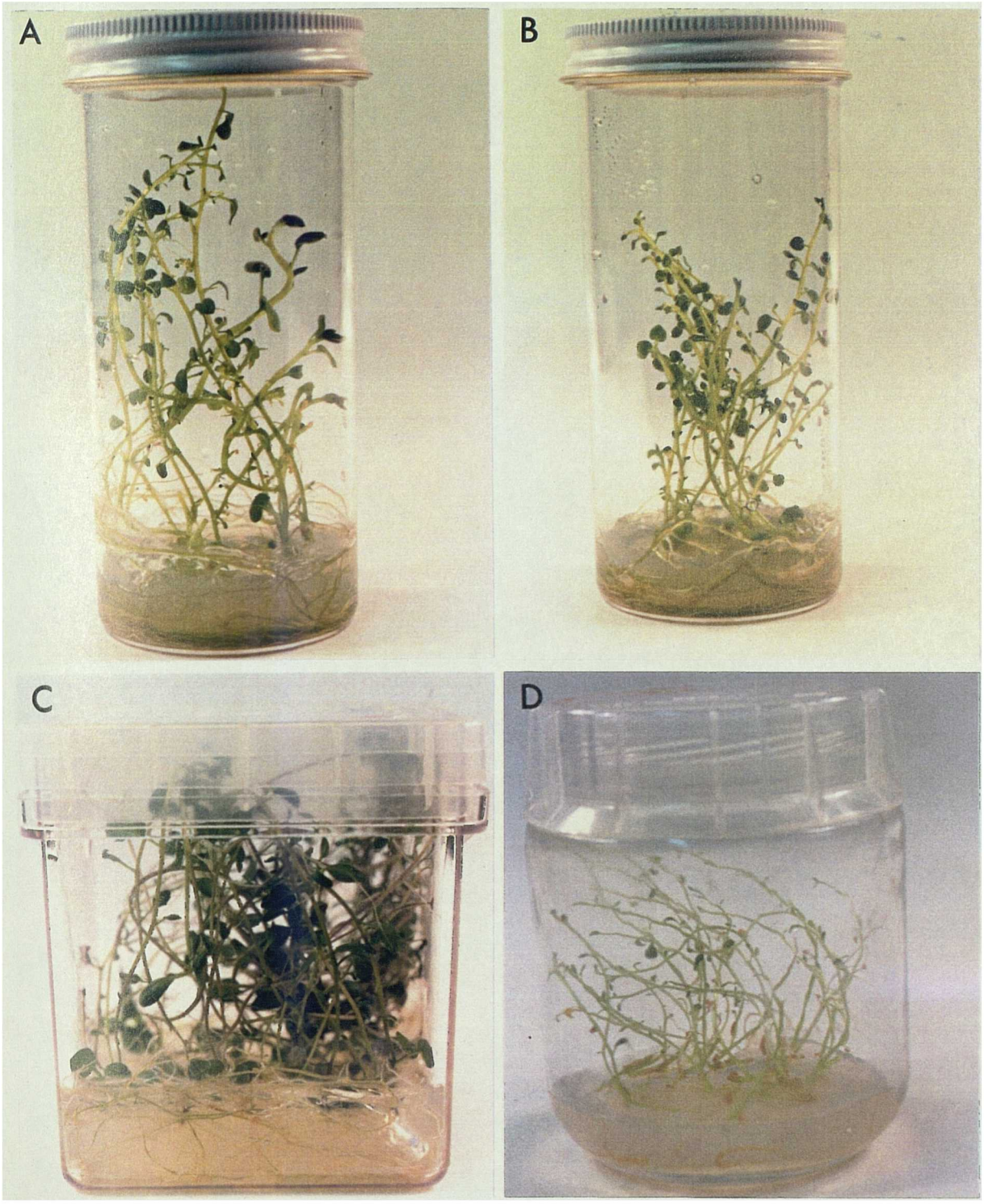


Fig. 26. Potato shoot cultures, 5 weeks old, used for protoplast isolation.

A. Desiree B. Maris Piper C. *S. commersonii*

D. *S. acaule* cultures, showing that the leaves were small and unexpanded, and therefore not used.

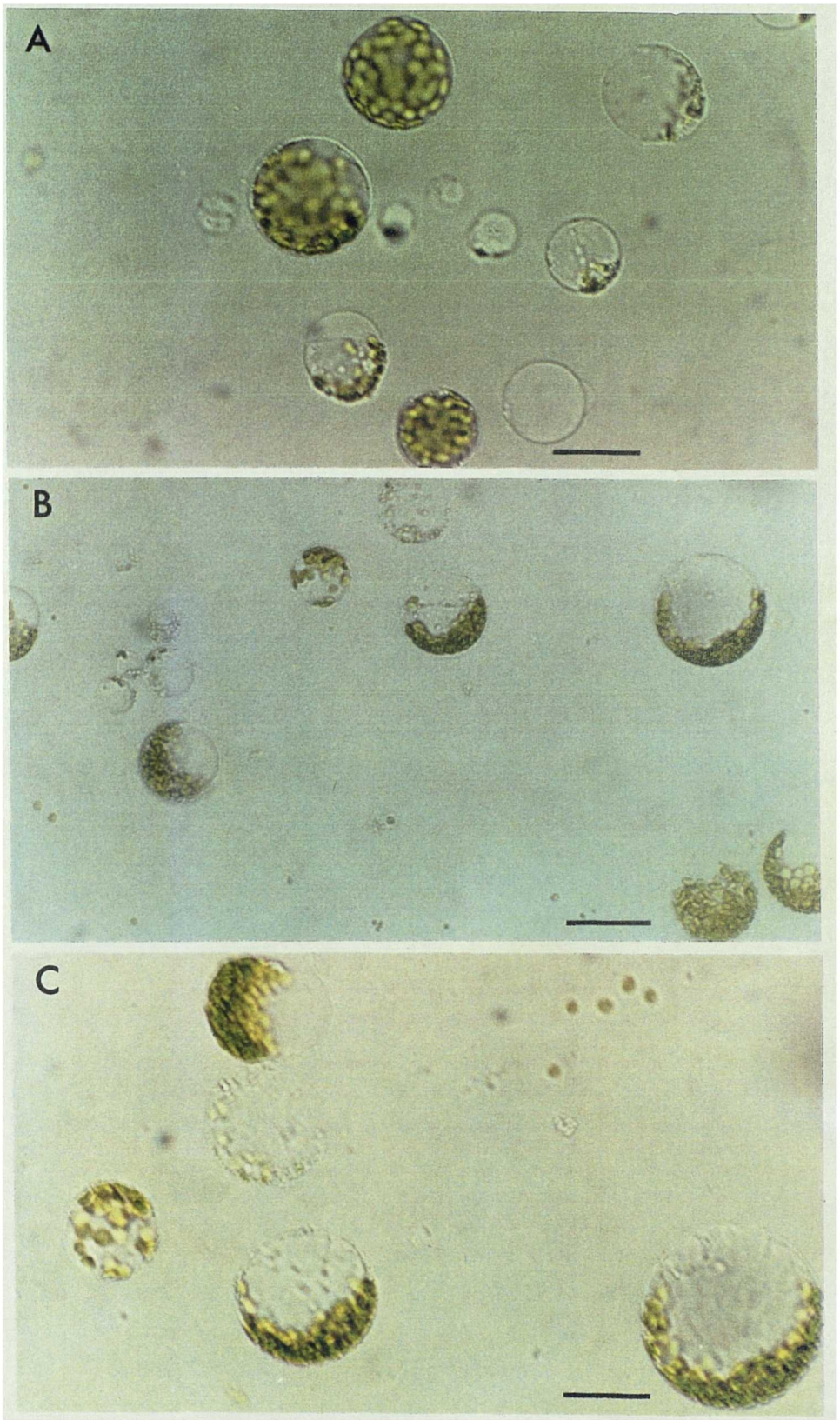


Fig. 27. Mesophyll cell protoplasts isolated from leaves of *in vitro* shoot cultures. (Scale bar = 25 μm)

A. Desiree B. Maris Piper C. *S. commersonii*

compared with Maris Piper when enzyme mixtures I and III were used, but when enzyme mixture II was used, Maris Piper gave a higher protoplast yield.

3.4.2 Protoplast viability

A. Mesophyll cell protoplasts

The viabilities of freshly isolated protoplasts were assessed by the ability to exclude the vital stain Evans' blue (0.4%) or to accumulate the dye from a 0.01% w/v solution of fluorescein diacetate. The viabilities of protoplasts isolated from leaves taken from greenhouse-grown plants ranged from 85 - 92% (Table 27) and was slightly better in cv. Desiree protoplasts. The viabilities of protoplasts isolated from *in vitro* shoot cultures were 90% in *S. tuberosum* and 86% in *S. commersonii* (Table 28).

B. Cultured cell protoplasts

The viabilities of protoplasts isolated from suspension-cultured cells ranged from 72 to 85% in both Desiree and Maris Piper. In enzyme mixtures I and II, with incubation periods 3 and 5 hours respectively, protoplast viabilities were 80 - 85% but for protoplasts isolated using enzyme mixture III, with an incubation period of 12 hours, the viabilities of protoplasts decreased to 72 - 75% (Table 30).

3.5 Protoplast culture

A. Mesophyll cell protoplasts

For protoplast culture, the media developed by Haberlach *et al.* (1985) were used with a slight modification in procedure. Protoplasts at a density of 0.82×10^5 protoplasts ml^{-1}, in warm (40 - 45°C) cell layering medium (Table 8) containing 0.7% agar, were

Table 29. Composition of enzyme mixtures; I (De Vries and Bokelmann, 1986), II (Chung and Sim, 1986), and III (Tavazza *et al.*, 1988) and incubation period used for protoplast isolation from cell suspension cultures.

	Enzyme mixture		
	I	II	III
Enzymes (w/v):			
Cellulase ("Onozuka" R-10)	1%	2%	3%
Macerozyme (R-10)	--	1%	2%
Pectolyase Y-23	0.1%	--	--
Mineral salts:			
Salt mixture	--	MS (exc. NH_4NO_3)	--
$CaCl_2.H_2O$	--	--	408 mM
Osmoticum:			
Mannitol	0.5 M	0.5 M	0.3 M
Buffer:			
MES	--	--	5 mM
pH	5.5	5.7	5.5
Incubation time	3 hours	5 hours	12 hours

Table 30. Yields/ml PCV and viabilities of *S. tuberosum* cvs. Desiree and Maris Piper protoplasts isolated from stem-derived cell suspensions by using different enzyme mixtures.

	Enzyme mixture		
	I	II	III
Yield (x 10^5):			
Desiree	3.2	0.85	2.7
Maris Piper	2.8	1.0	2.5
Viability (%):			
Desiree	85	82	75
Maris Piper	80	84	72

Data represent means of 3 replicates

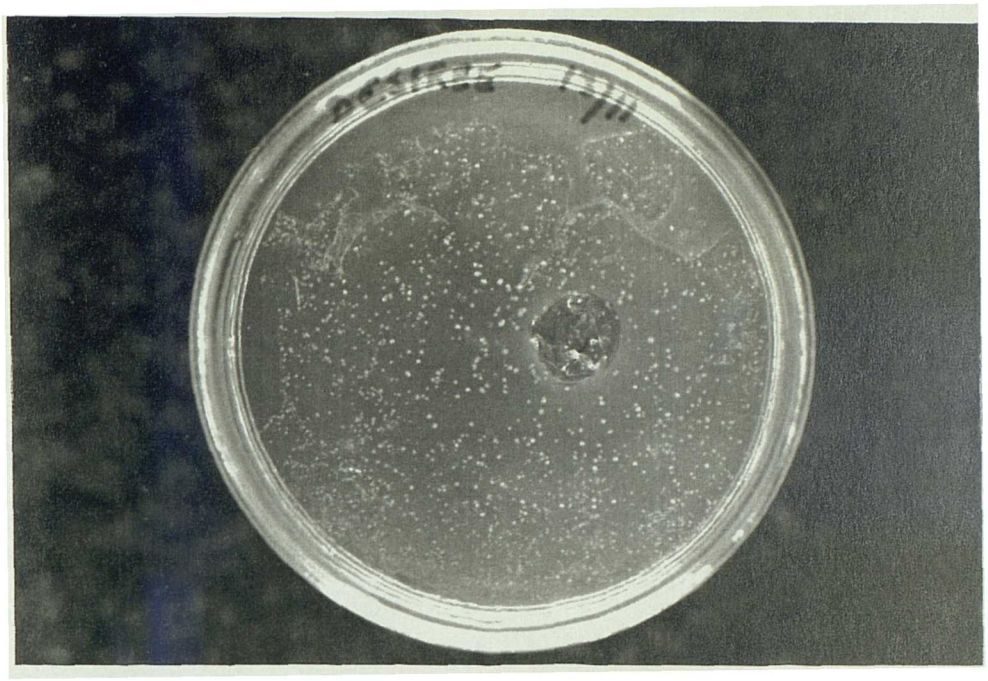


Fig. 28. Mesophyll protoplast-derived colonies of cv. Desiree, 2 weeks after culture on reservoir medium of Haberlach *et al.* (1985). (Petri dish Ø 90 mm)

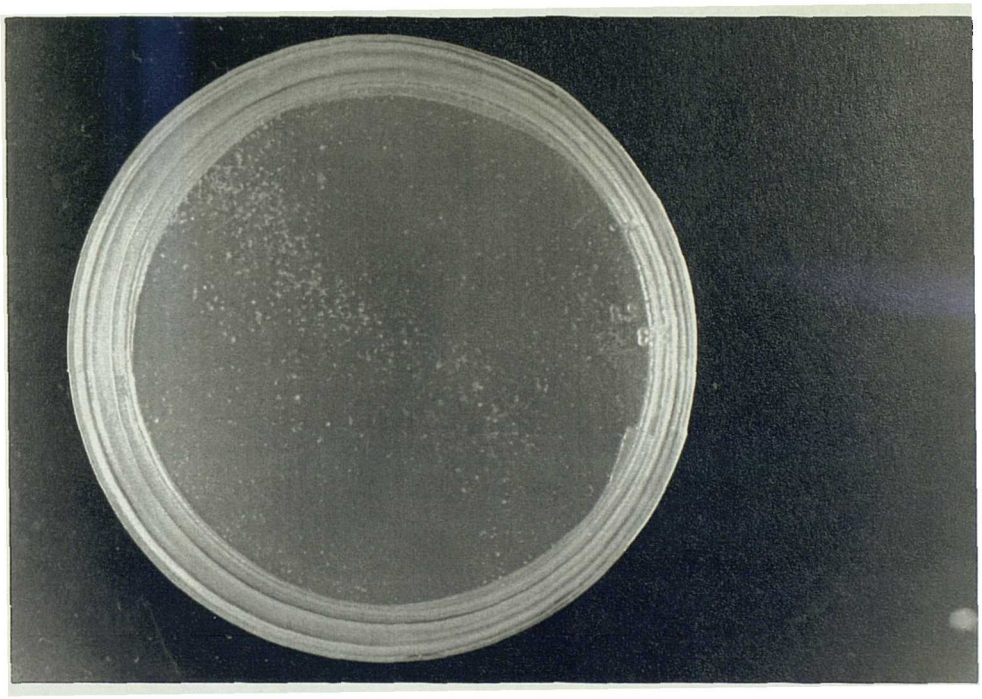


Fig. 29. Mesophyll protoplast-derived colonies of cv. Maris Piper, 2 weeks after culture on reservoir medium of Haberlach *et al.* (1985). (Petri dish Ø 90 mm)

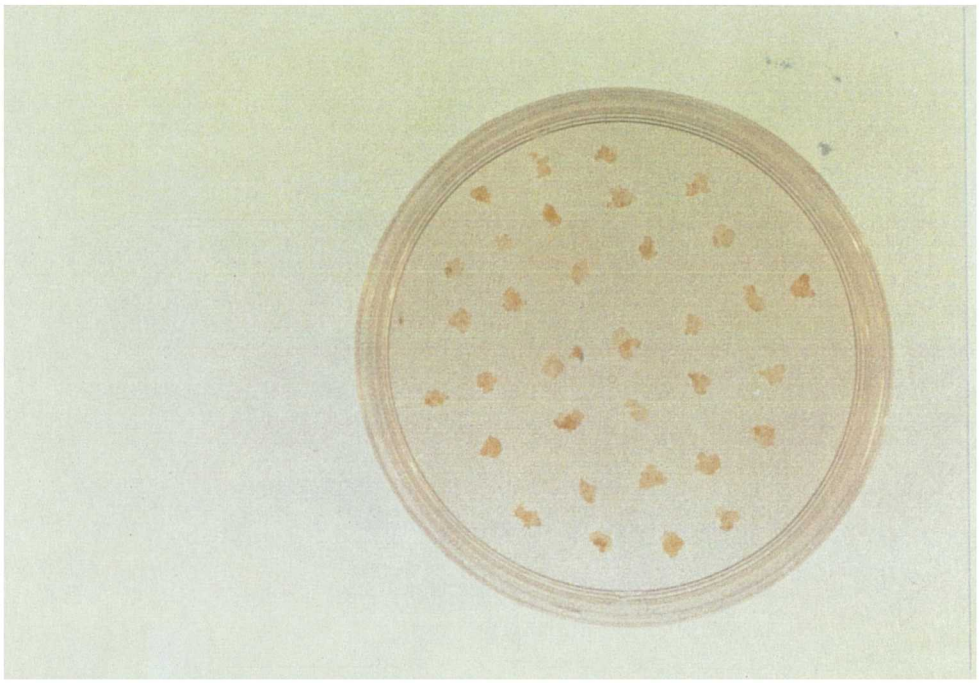


Fig. 30. Mesophyll protoplast-derived calluses of cv. Desiree, transferred to protoplast culture medium of Haberlach *et al.* (1985). Photographed after 1 week. (Petri dish Ø 90 mm)

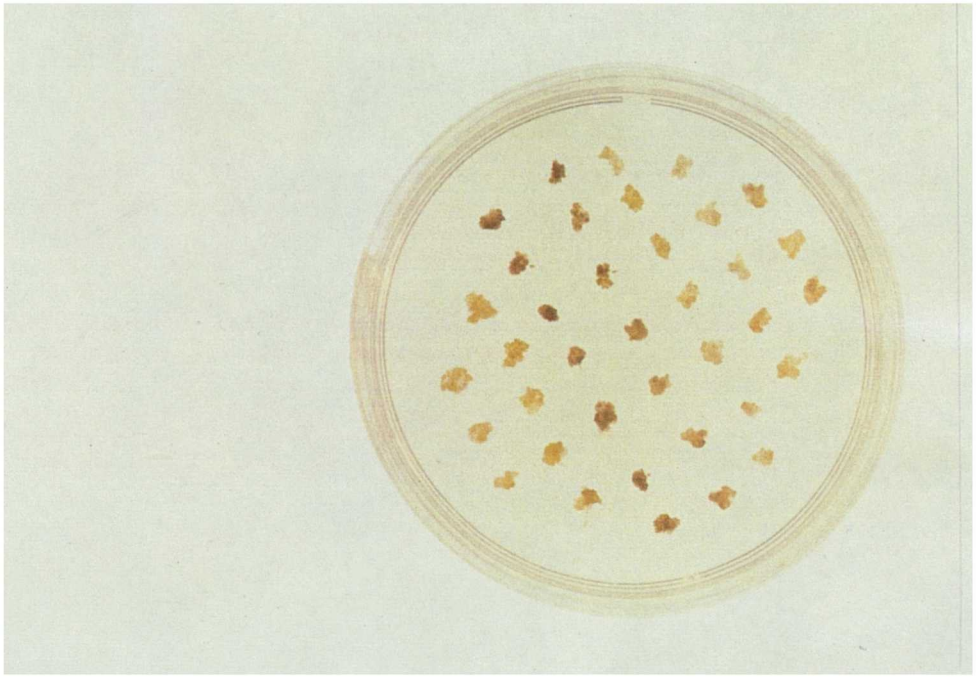


Fig. 31. Mesophyll protoplast-derived calluses of cv. Maris Piper, transferred to protoplast culture medium of Haberlach *et al.* (1985). Photographed after 1 week. (Petri dish Ø 90 mm)

plated over solidified reservoir medium (Table 8) in Ø 55 mm petri dishes. Two millilitres of protoplast suspension were evenly distributed in each petri dish and at least 10 petri dishes were prepared for each type and incubated at 25 ± 1°C under 1000 lux continuous illumination. Cultures of both Desiree and Maris Piper protoplasts, isolated from leaves taken from greenhouse-grown plants became contaminated after 10 days. The experiment was repeated three times and in each case progress was hampered by contamination.

Protoplast plating efficiency (the percentage of plated protoplasts which underwent cell division), in cultures of protoplasts isolated from *in vitro* shoot cultures, was determined after 2 weeks and ranged between 30 and 35% in Desiree and Maris Piper and 10 - 15% in *S. commersonii*. At this stage, 1 ml of cell layering medium was added to each petri dish and incubated for a further 2 weeks. None of the dividing protoplasts of *S. commersonii* developed into callus. The experiment was repeated three times, and after 2 - 3 divisions, cell division stopped and cell colonies failed to form callus. Growing calluses of Desiree and Maris Piper were transferred onto the culture medium (Table 8) and incubated at 25 ± 1°C under continuous light of 3000 - 4000 lux. After 2 weeks, calluses grew and became light green in colour and were transferred to shoot regeneration medium for differentiation.

B. Cultured cell protoplasts

MS-KM medium, composed of inorganic salts of MS medium (Murashige and Skoog, 1962) without NH_4NO_3 and other nutrients of KM-8P medium (Kao and Michayluk, 1975), was used for protoplast culture.

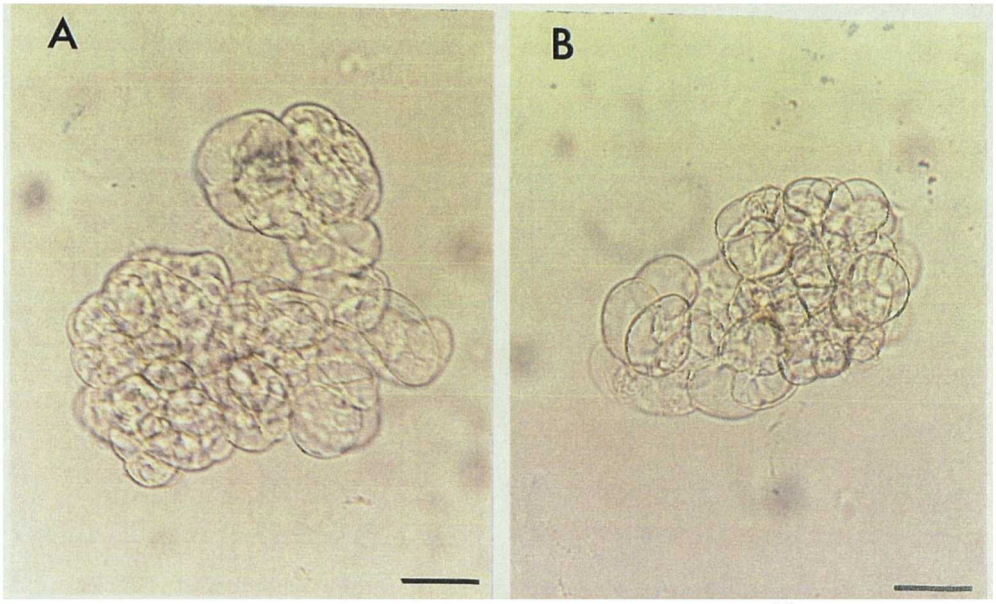


Fig. 32. Cell colonies from *S. tuberosum* cultured-cell protoplasts, after 10 days of culture in MS-KM protoplast culture medium. (Scale bar = 50 μm)

A. Desiree B. Maris Piper

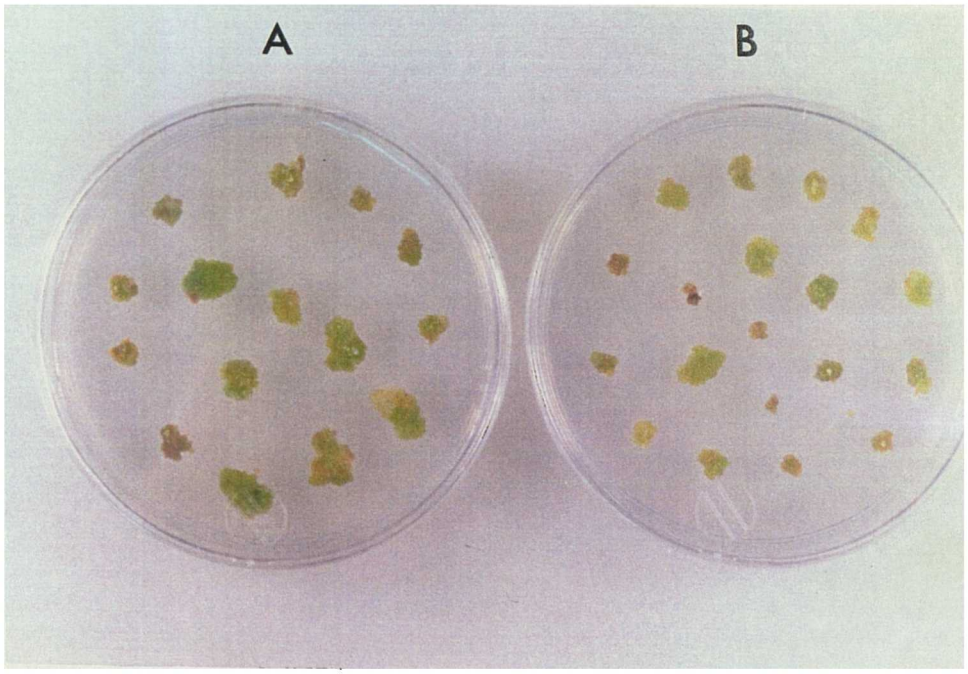


Fig. 33. Cultured-cell protoplast-derived calluses transferred to protoplast culture medium (MS medium supplemented with 1 mg l^{-1} BAP, 0.2 mg l^{-1} NAA, 30 g l^{-1} sucrose and 8 g l^{-1} agar). Photographed after 2 weeks. (Petri dish Ø 90 mm)

A. Desiree B. Maris Piper

The protoplasts were adjusted to a density of 1 x 10^5 ml^{-1} in the culture medium (Table 9). 2 ml of protoplast suspension was cultured in Ø 55 mm petri dishes and incubated at 25 ± 1°C under continuous illumination of 1000 lux. The protoplasts began dividing after 3 days of culture. In the second week, the cultures were diluted by adding an equal volume of fresh culture medium. Protoplast plating efficiency was determined after 14 days of culture and was 21% in Desiree and 15% in Maris Piper.

Cell colonies (0.5 - 1 mm in diameter) were obtained two weeks after adding soft medium (MS-KM plus 0.4% agar), and were subsequently plated over solidified MS medium (see Materials and Methods) for further growth. Due to the higher illumination (3000 - 4000 lux), small calluses became light green and grew about 2 - 3 mm in diameter in 2 weeks. These calluses were then transferred to regeneration medium for shoot development (see Section 3.6E, below).

3.6 Plantlet regeneration

3.6.1 From tuber discs

Explants prepared from Desiree and Maris Piper virus-free tubers were cultured on three regeneration media viz., those of Jarret *et al.* (1980a)(Med. RA, Table 10), Iapichino *et al.* (1991) (Med. RB, Table 11), and Ahloowalia (1982) (Med. RC, Table 12), and were kept at 25 ± 1°C in a 16 hours photoperiod. After 2 weeks, explants developed from creamy white to light green in colour, indicating chlorophyll content. After 4 weeks of culture, small compact green protuberances, possibly meristemoid bodies appeared along the outer edges of explants, and after 6 weeks, shoot primordia were visible under the dissecting microscope on the explants cultured on medium RC.

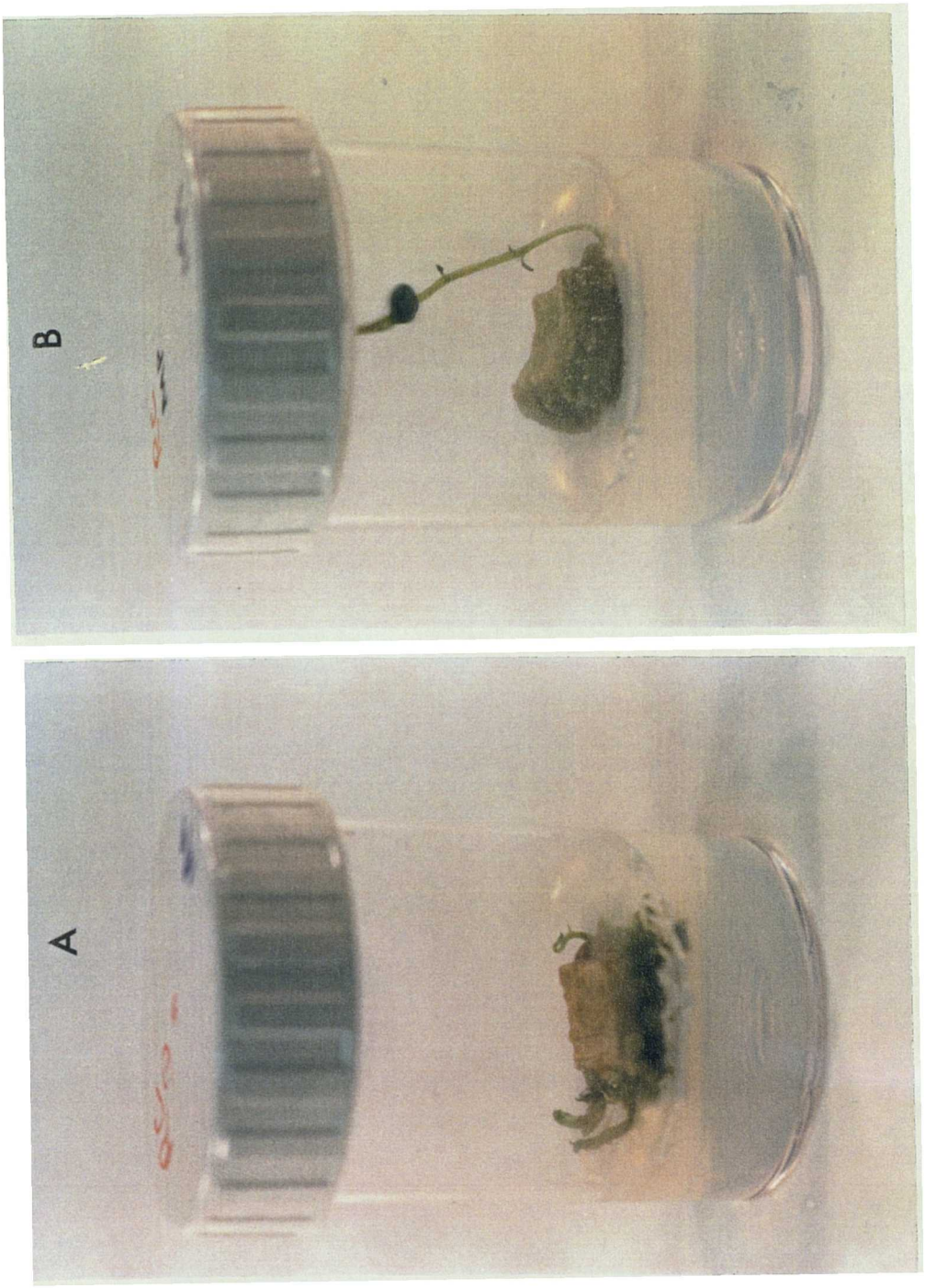


Fig. 34. Shoot regeneration from potato tuber explants cultured on Ahloowalia (1982) medium. Photographed after 10 weeks.

A. Desiree B. Maris Piper

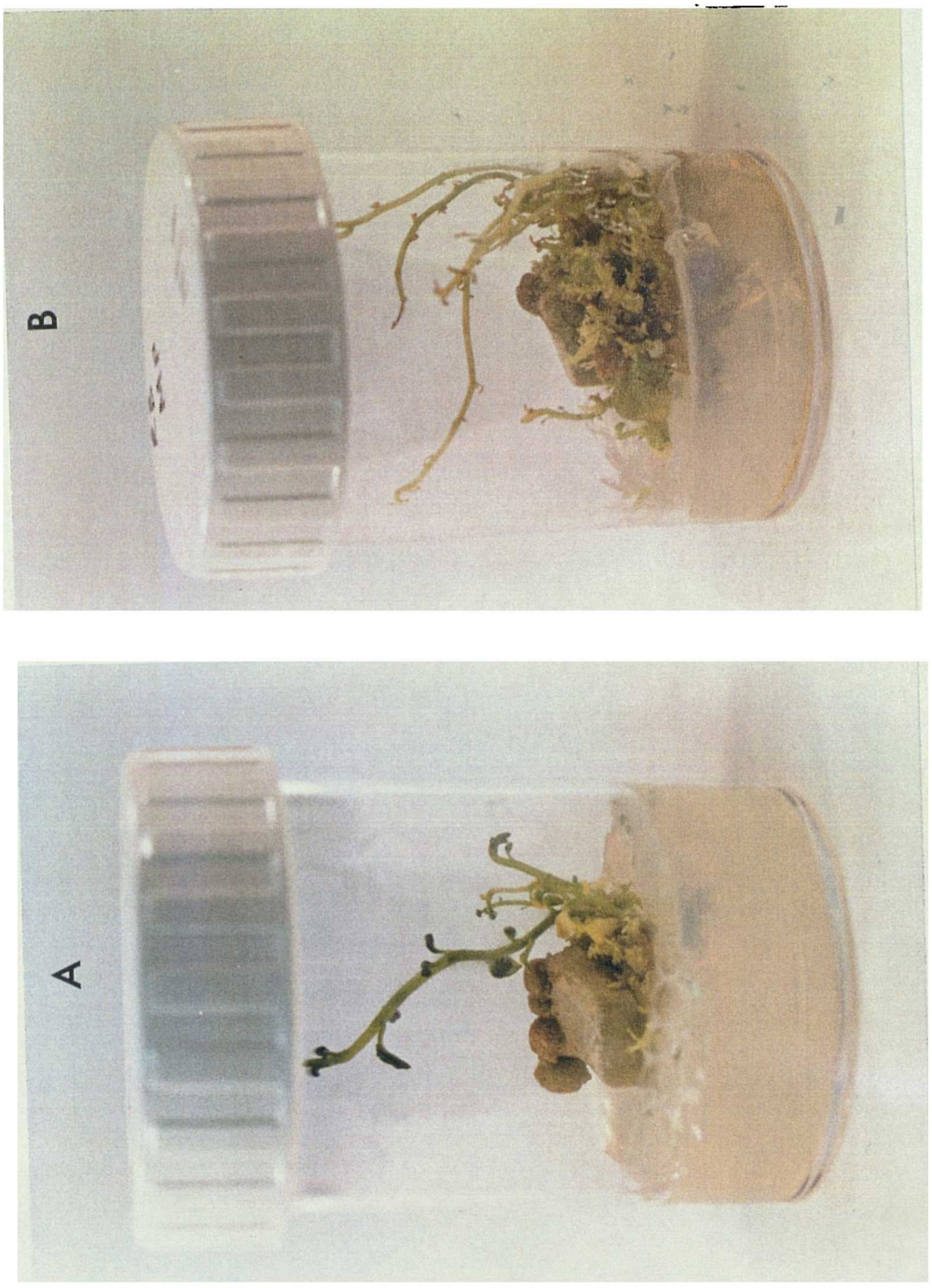


Fig. 35. Plantlet regeneration from potato tuber explants cultured on shoot regeneration medium of Iapichino *et al.* (1991). Photographed after 12 weeks.

A. Desiree B. Maris Piper

After 7 weeks shoots started appearing from the explants (Fig. 34). Shoots appeared after 10 weeks of culture from explants cultured on medium RB (Fig. 35), but no shoot regeneration occurred from explants cultured on medium RA. After 12 weeks of culture shoot regeneration efficiency was assessed on the basis of the number of shoots per explant. Shoot regeneration was higher on medium RB. On medium RA, no shoots were produced from explants of either Desiree or Maris Piper. On medium RB, averages of 7.5 shoots/disc were produced in Desiree and 14 shoots/disc in Maris Piper, while on medium RC, 4 shoots/disc in Desiree and 3 shoots/disc in Maris Piper were produced. Regenerated shoots were removed and transferred to shoot culture medium NA for rooting.

3.6.2 From stem explants

Two different media, viz., those of Jarret *et al.* (1980a) (Med. RA), and Iapichino *et al.* (1991) (Med. RB), were used to induce shoot regeneration from *S. tuberosum* cvs. Desiree and Maris Piper , and *S. commersonii* and *S. acaule* internodal explants.

On medium RA, after one week incubation, callus initiation was observed which continued to grow. Shoot initiation was observed after 7 weeks of culture in Maris Piper and 8 weeks in Desiree (Fig. 36 and 37). In *S. commersonii*, explants produced callus but no shoot regeneration occurred. However, some roots were produced (Fig. 38). In *S. acaule*, after 6 weeks of culture, explants became necrotic, and after 12 weeks almost all the explants were dead (Fig. 39).

On medium RB, swelling at the wound sites of explants appeared after one week of

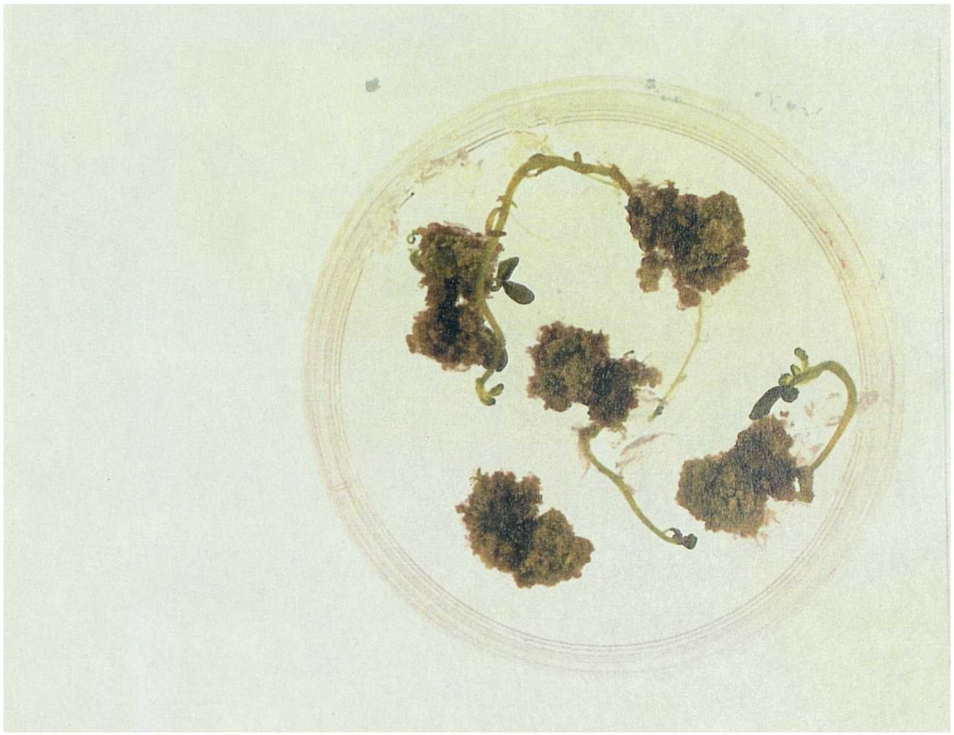


Fig. 36. Shoot regeneration from Maris Piper internodal explants cultured on Jarret *et al.* (1980a) medium. Photographed after 10 weeks. (Petri dish Ø 90 mm)

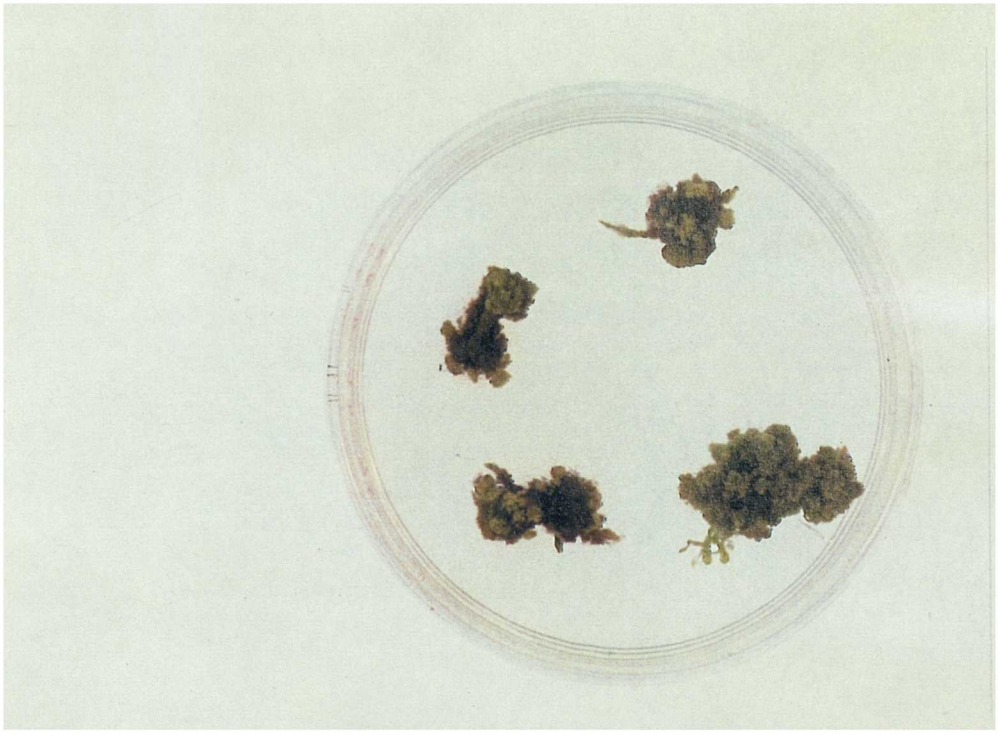


Fig. 37. Shoot regeneration from Desiree internodal explants cultured on Jarret *et al.* (1980a) medium. Photographed after 10 weeks. (Petri dish Ø 90 mm)

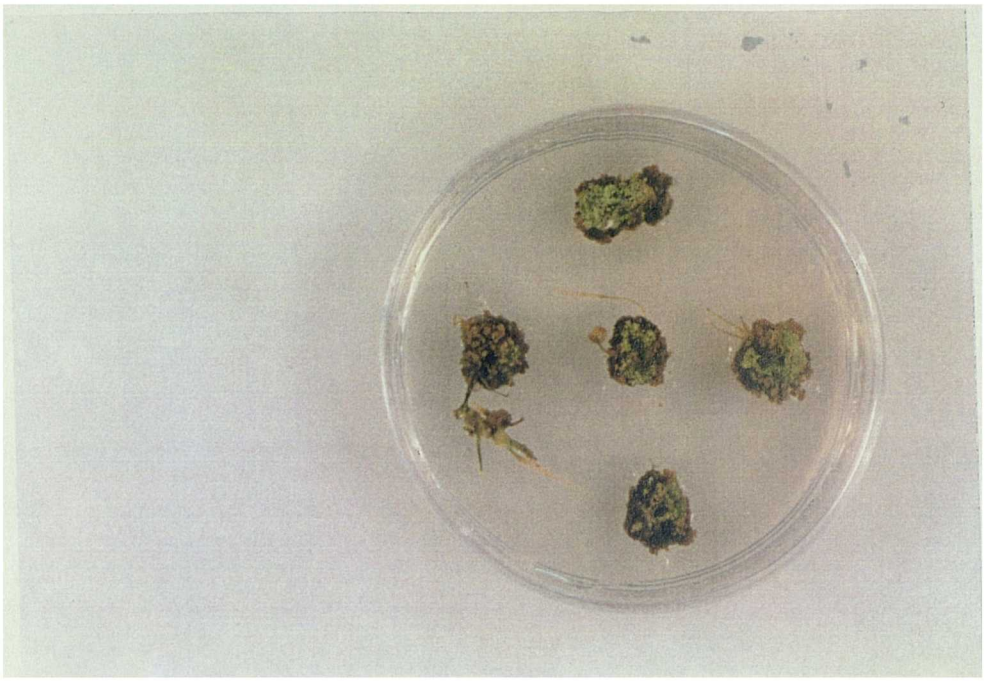


Fig. 38. *S. commersonii* internodal explants cultured on Jarret et al. (1980a) medium. Roots can be seen but no shoots were generated. Photographed after 12 weeks. (Petri dish Ø 90 mm)

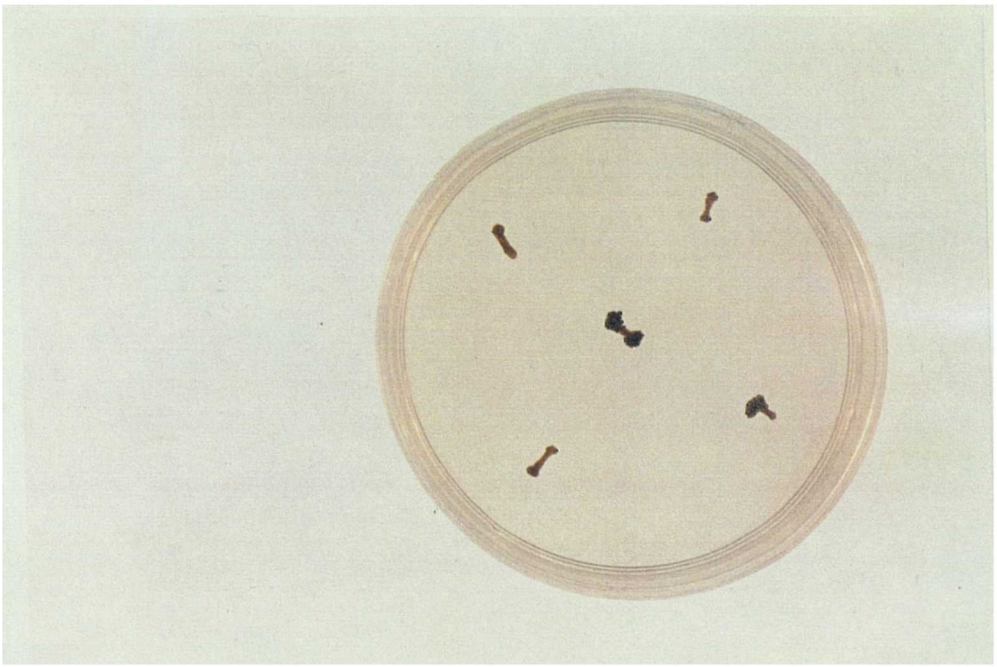


Fig. 39. *S. acaule* internodal explants cultured on Jarret *et al.* (1980a) medium. Explants died on this medium. Photographed after 12 weeks. (Petri dish Ø 90 mm)

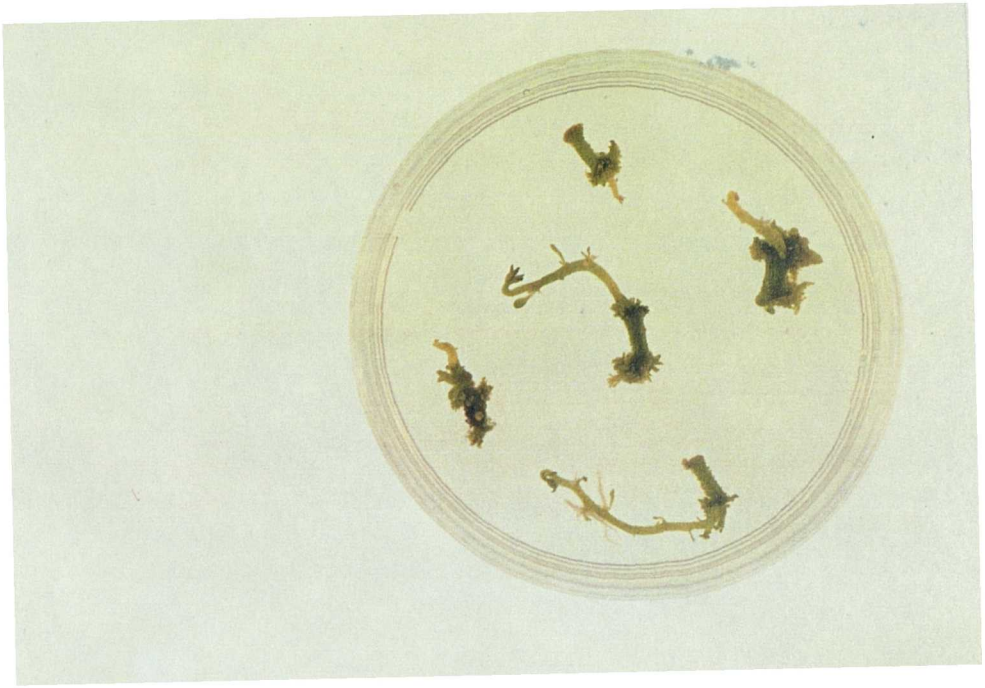


Fig. 40. Shoot development from Maris Piper internodal explants cultured on shoot regeneration medium of Iapichino *et al.* (1991). Photographed after 5 weeks. (Petri dish Ø 90 mm)

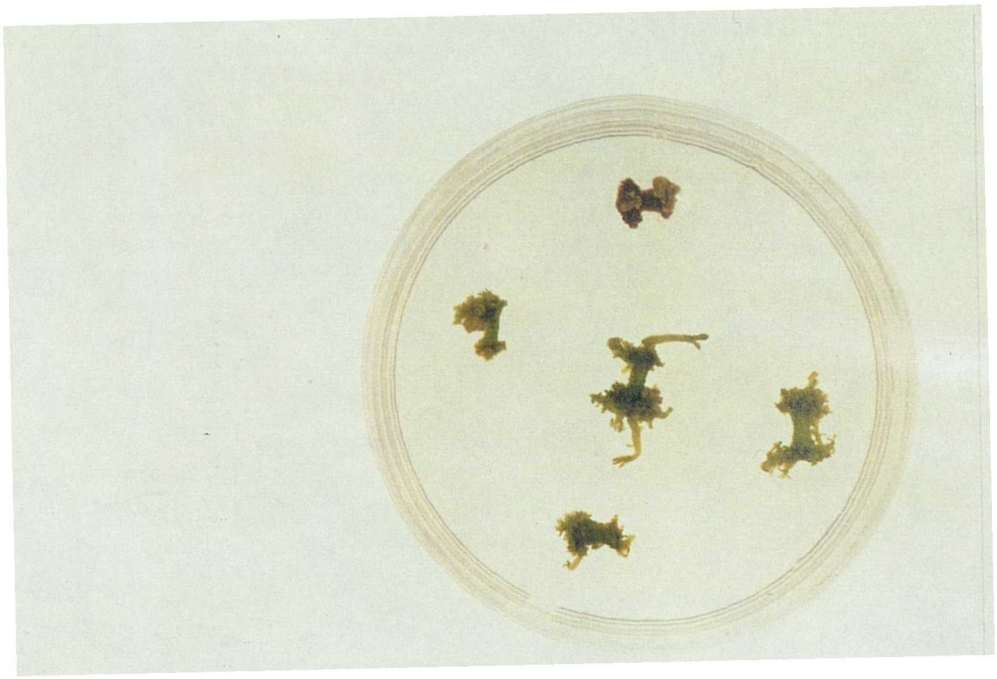


Fig. 41. Shoot development from Desiree internodal explants cultured on shoot regeneration medium of Iapichino *et al.* (1991). Photographed after 7 weeks. (Petri dish Ø 90 mm)

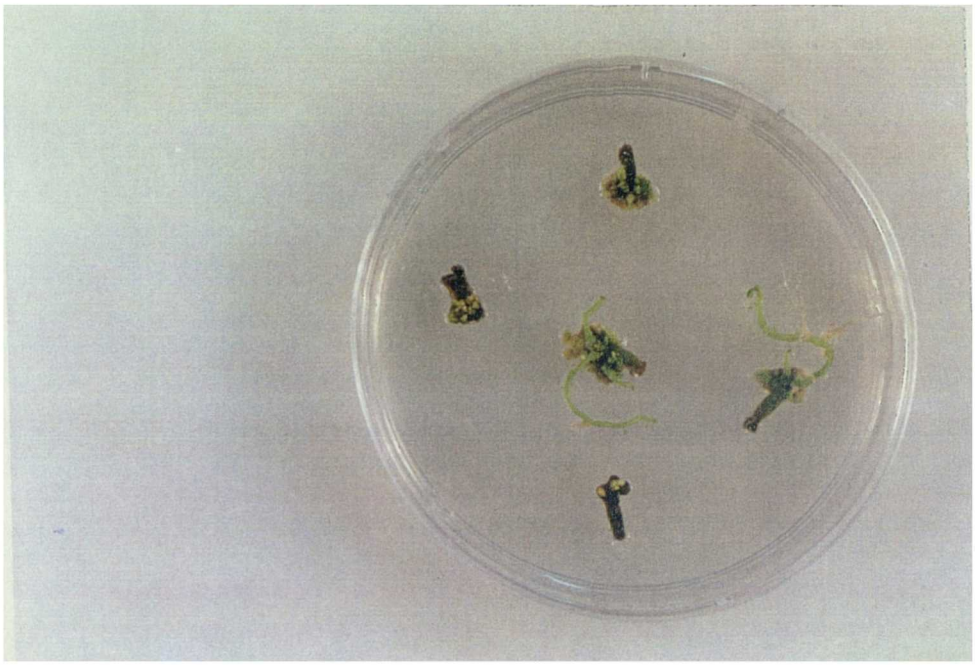


Fig. 42. Shoot development from *S. commersonii* internodal explants cultured on shoot regeneration medium of Iapichino *et al.* (1991). Photographed after 8 weeks. (Petri dish Ø 90 mm)

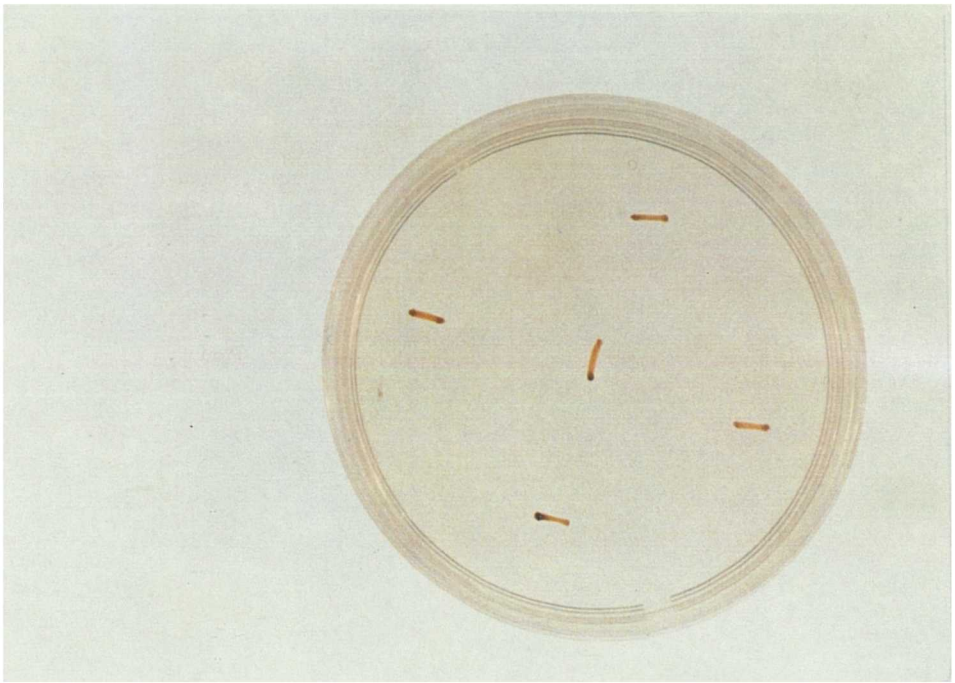


Fig. 43. *S. acaule* internodal explants cultured on shoot regeneration medium of Iapichino *et al.* (1991). All the explants died after 4 weeks of culture. (Petri dish Ø 90 mm)

culture and multiple shoots with minimal callus appeared after 3 weeks in Maris Piper. In Desiree shoots appeared after 5 weeks and in *S. commersonii* after 6 weeks. In *S. acaule*, no shoot regeneration occurred and almost all explant died after 4 weeks of culture (Fig. 43).

The main difference in shoot regeneration on these two media was that on medium RA, explants first produced callus and then differentiated into shoots, while on medium RB, very little callus was produced and shoots arose from swellings or protuberances on the cut sites of explants (Fig. 36 to 43). Medium RB also took less time to initiate shoots from explants compared with medium RA with all types of potato used. Among the genotypes, shoot regeneration was in general quicker in Maris Piper on both media tested.

3.6.3 From growing calluses

Attempts to regenerate plantlets from growing tuber calluses proved unsuccessful. Tuber calluses were subcultured onto three regeneration media, viz., RA, RB and RC. These media failed to regenerate shoots from calluses of either Desiree or Maris Piper, even after six months, and calluses remained creamy white or turned brown instead of green, without any indication of shoot regeneration.

For shoot regeneration from leaf calluses, three media as developed by Iapichino *et al.* (1991) (Med. RB), Ahloowalia (1982) (Med. RC) and Lam (1977b) (Med. RD, Table 6) were used. After every 4 weeks calluses were transferred onto fresh medium.

Fig. 44. Multiple shoots developed from leaf-derived callus of cv. Maris Piper cultured on shoot regeneration medium of Iapichino *et al.* (1991). Photographed after 13 weeks.

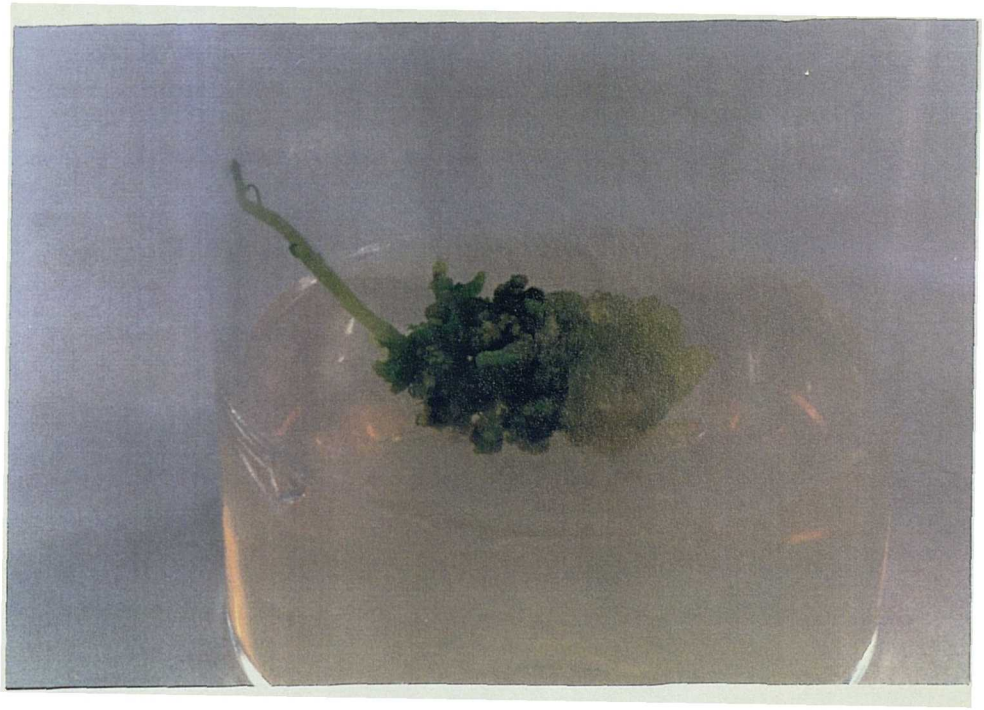


Fig. 45. Multiple shoots developing from leaf-derived callus of cv. Desiree cultured on shoot regeneration medium of Iapichino *et al.* (1991). Photographed after 13 weeks.

Fig. 46. Shoot regeneration from leaf-derived callus of cv. *Maris Piper* cultured on shoot regeneration medium of Lam (1977b). Photographed after 18 weeks. (Scale bar = 2 mm)

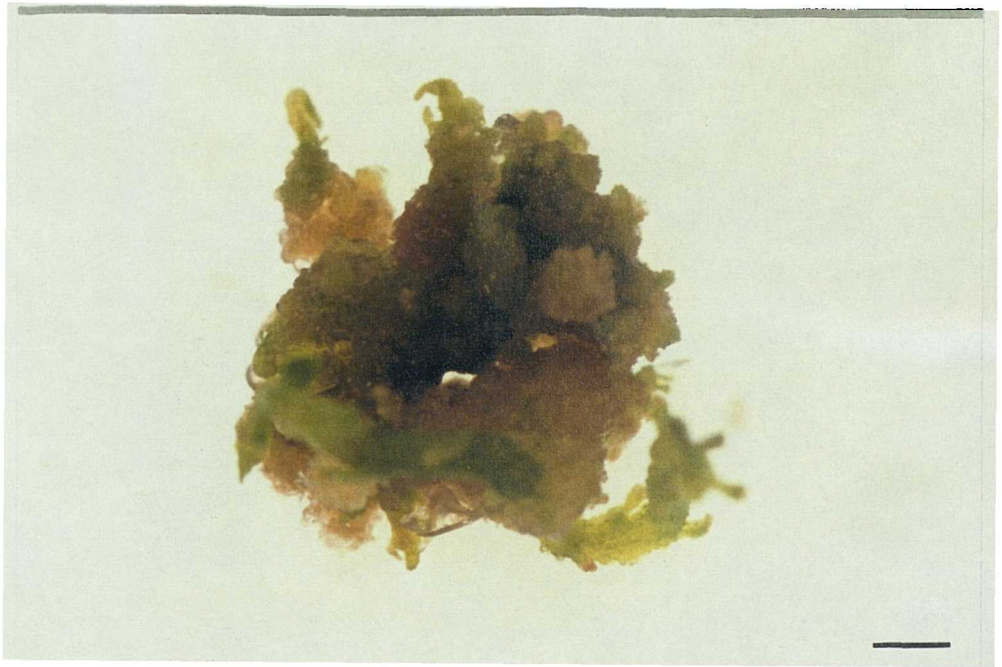


Fig. 47. Shoot regeneration from leaf-derived callus of cv. Desiree cultured on shoot regeneration medium of Lam (1977b). Photographed after 19 weeks. (Scale bar = 2 mm)

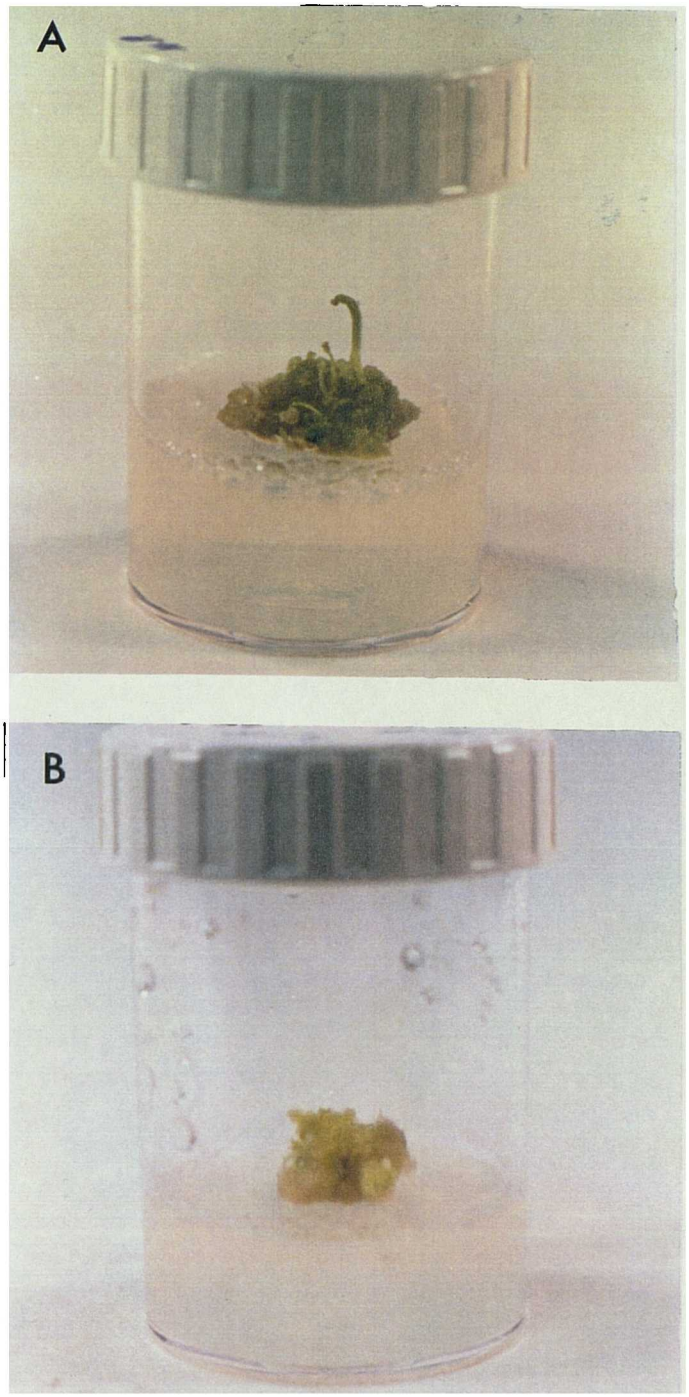


Fig. 48. Shoot regeneration from leaf-derived calluses cultured on shoot regeneration medium of Ahloowalia (1982). Photographed after 23 weeks.

A. Maris Piper B. Desiree

Calluses changed to a light green colour within 4 weeks of transferring to regeneration media. Shoot initiation started from the edges of calluses after 10 weeks in Maris Piper and 12 weeks in Desiree on medium RB. No shoot differentiation was observed on medium RD until the 15th week when shoot primordia appeared. Shoots were visible after 16 weeks in Maris Piper and 17 weeks in Desiree. Shoot development on medium RC was achieved after 20 weeks in Maris Piper and after 22 weeks in Desiree. When regenerated shoots were 1 - 2 cm in length, they were excised and transferred to shoot culture medium NA for root development.

3.6.4 From suspension-cultured cells

Stem callus-derived cell suspensions of Desiree and Maris Piper were plated at a density of 0.5 x 10^5 cells ml^{-1} onto the surface of Lam (1977b) cell plating medium (Table 6) and incubated at 25 $\pm$ 1°C and 16 hours photoperiod with a light intensity of 250 lux. After 4 weeks, when calluses reached the size of 2 - 3 mm in diameter, the light intensity was increased to 1000 lux. After 2 weeks, when calluses had become light green, they were transferred singly either to the shoot regeneration medium developed by Lam (1977b) (Med. RD), or to the medium developed by Bokelmann and Roest (1983) (Med. RE, Table 13).

Shoot regeneration started 6 weeks after transferring calluses to medium RE and 8 weeks to medium RD in both Desiree and Maris Piper. When shoots were 1 - 2 cm long, they were excised and rooted on shoot culture medium NA.

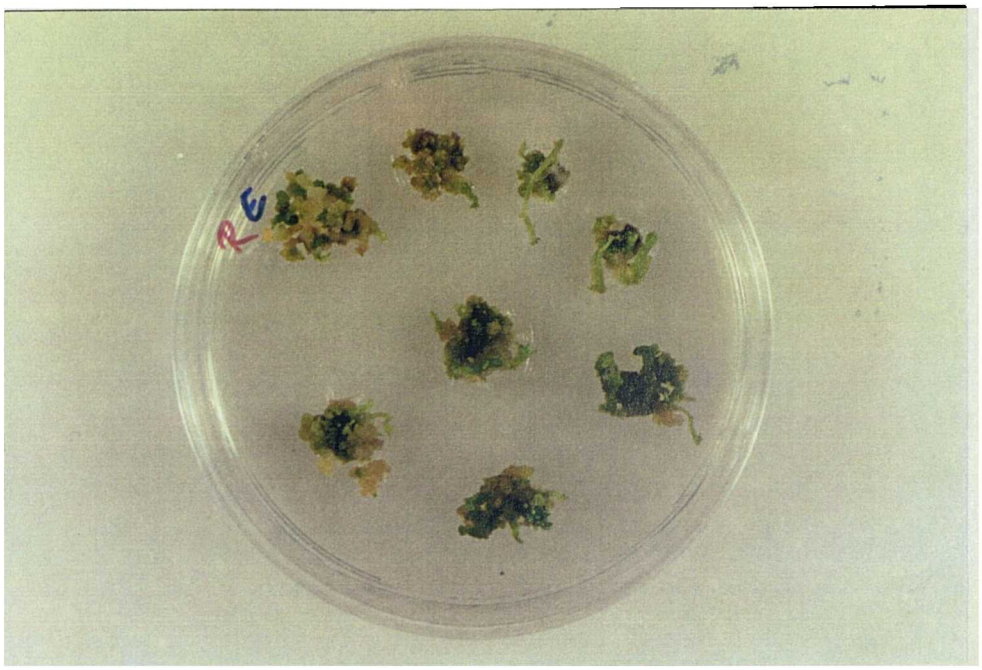


Fig. 49. Shoot initiation from cv. Maris Piper calluses produced from stem-derived suspension-cultured cells, cultured on shoot regeneration medium of Bokelmann and Roest (1983). Photographed after 8 weeks. (Petri dish Ø 90 mm)

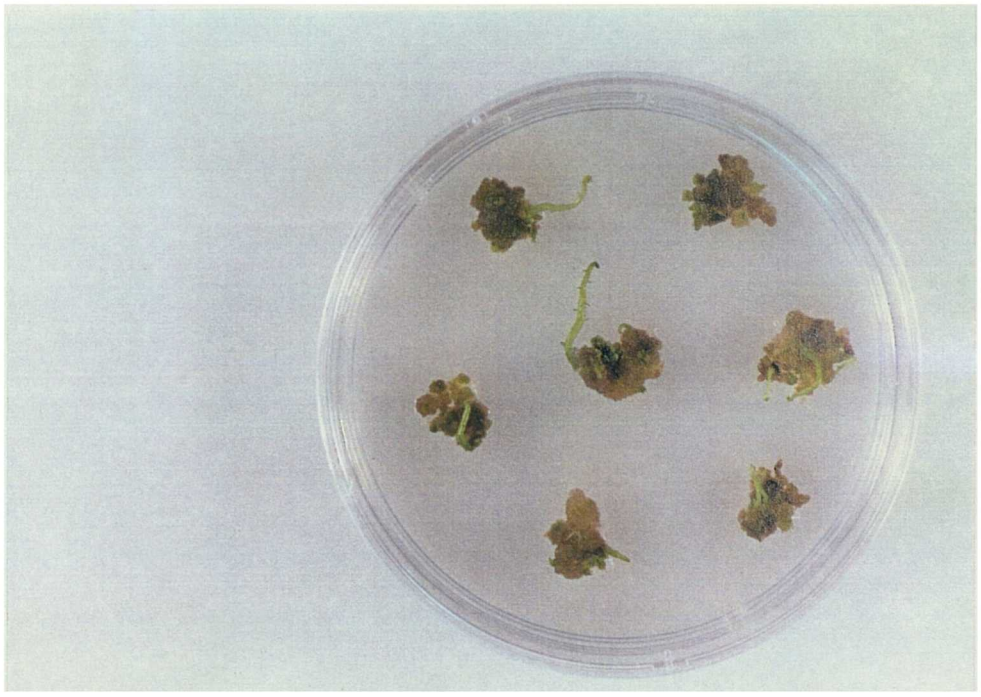


Fig. 50. Shoot initiation from cv. Desiree calluses produced from stem-derived suspension-cultured cells, cultured on shoot regeneration medium of Bokelmann and Roest (1983). Photographed after 8 weeks. (Petri dish Ø 90 mm)

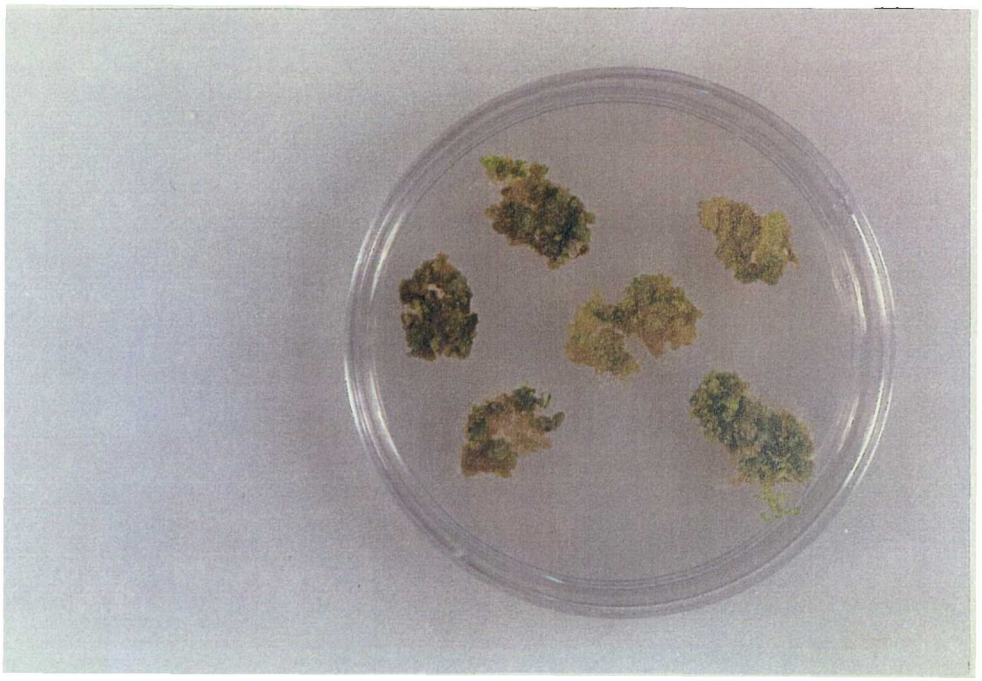


Fig. 51. Shoot initiation from cv. Maris Piper calluses produced from stem-derived suspension-cultured cells, cultured on shoot regeneration medium of Lam (1977b). Photographed after 10 weeks. (Petri dish Ø 90 mm)

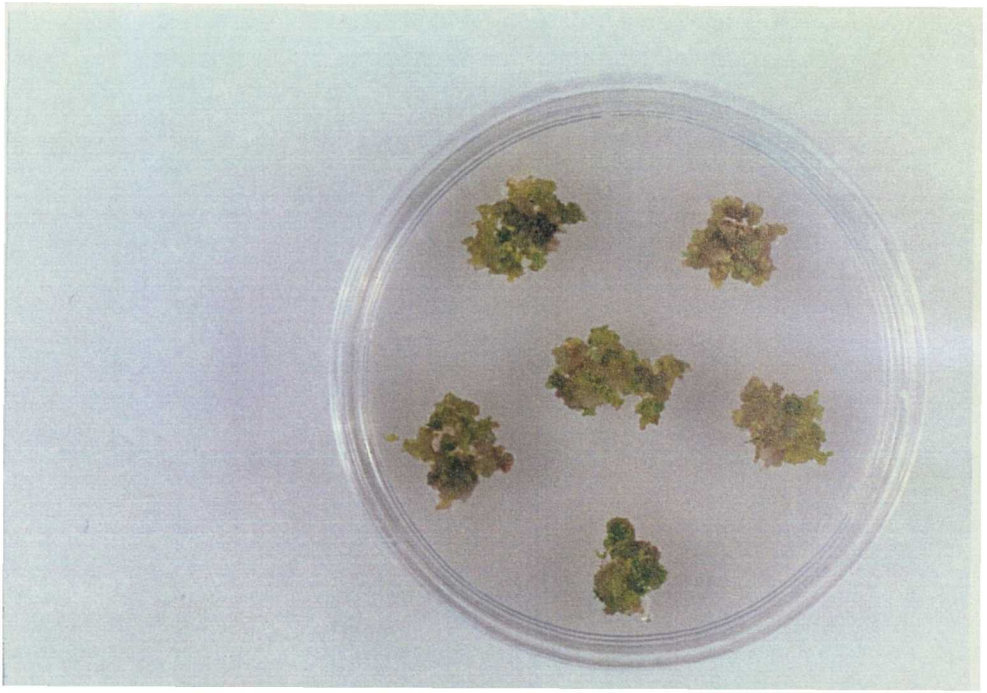


Fig. 52. Shoot initiation from cv. Desiree calluses produced from stem-derived suspension-cultured cells, cultured on shoot regeneration medium of Lam (1977b). Photographed after 10 weeks. (Petri dish Ø 90 mm)

3.6.5 From protoplast-derived calluses

Protoplast-derived calluses were also transferred either to medium RD or medium RE for shoot regeneration. The first shoot was produced from mesophyll cell protoplast-derived callus of Maris Piper on medium RE. Calluses derived from mesophyll cell protoplasts differentiated about 2 weeks earlier than calluses derived from cultured-cell protoplasts. Shoot regeneration was also 2 weeks earlier from the calluses cultured on regeneration medium RE compared with medium RD. Shoot regeneration on medium RE started after 6 weeks from mesophyll cell protoplast-derived calluses and after 8 weeks from cultured-cell protoplast-derived calluses in both Desiree and Maris Piper. Shoot regeneration from mesophyll cell protoplast-derived calluses was also observed after 8 weeks of culture on medium RD in both Desiree and Maris Piper but it took 10 weeks from cultured-cell protoplast-derived calluses. Regenerated shoots 1 - 2 cm long were removed and transferred to shoot culture medium NA for rooting.

After 3 weeks, when the plantlets had developed root systems, they were transferred to small plastic pots containing sterilized compost. These pots were covered with transparent plastic containers to maintain high humidity around the plantlets. After 2 weeks the plantlets were transferred to pots containing soil mix (John Innes No. 2) and moved to a greenhouse under non-sterile conditions.

3.7 *In vitro* tuberization

In *S. tuberosum* cvs. Desiree and Maris Piper nodal segments cultured on medium TC, microtuber initiation started after 3 days of transferring the cultures to 20°C in an 8 hours photoperiod with a low light intensity (250 lux). This was followed by transfer

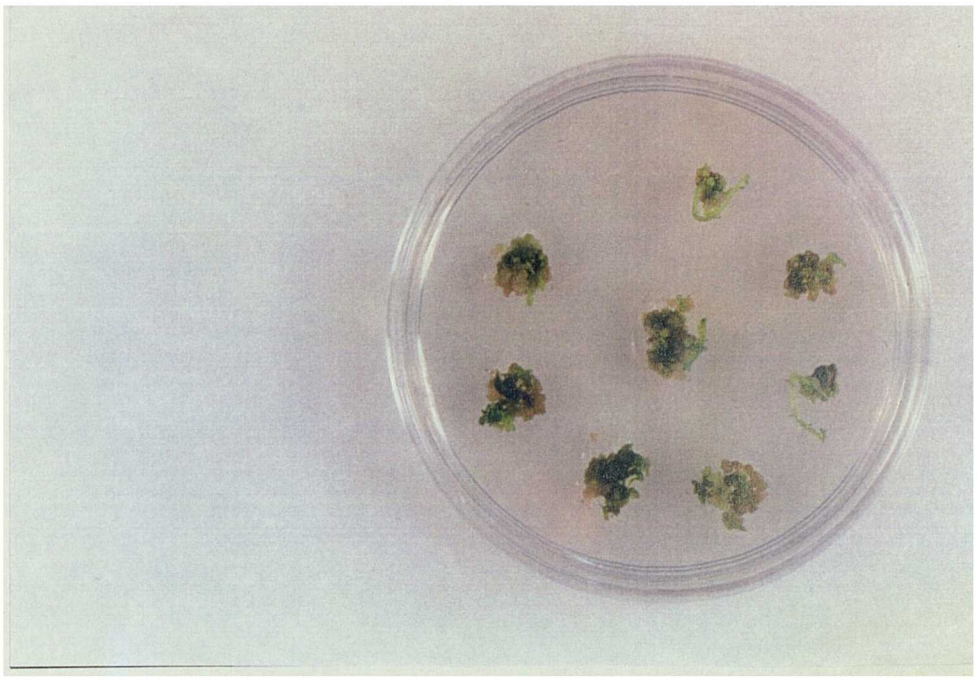


Fig. 53. Shoot initiation from mesophyll cell protoplast-derived calluses of cv. Desiree on shoot regeneration medium of Bokelmann and Roest (1983). Photographed after 8 weeks of culture. (Petri dish Ø 90 mm)

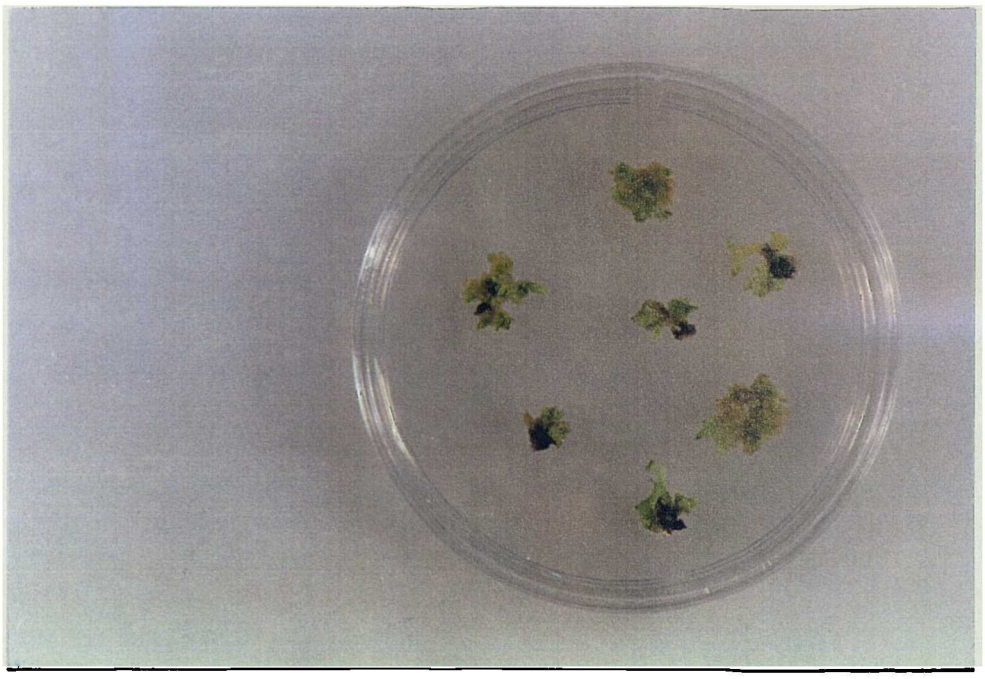


Fig. 54. Shoot initiation from mesophyll cell protoplast-derived calluses of cv. Maris Piper on shoot regeneration medium of Bokelmann and Roest (1983). Photographed after 8 weeks of culture. (Petri dish Ø 90 mm)

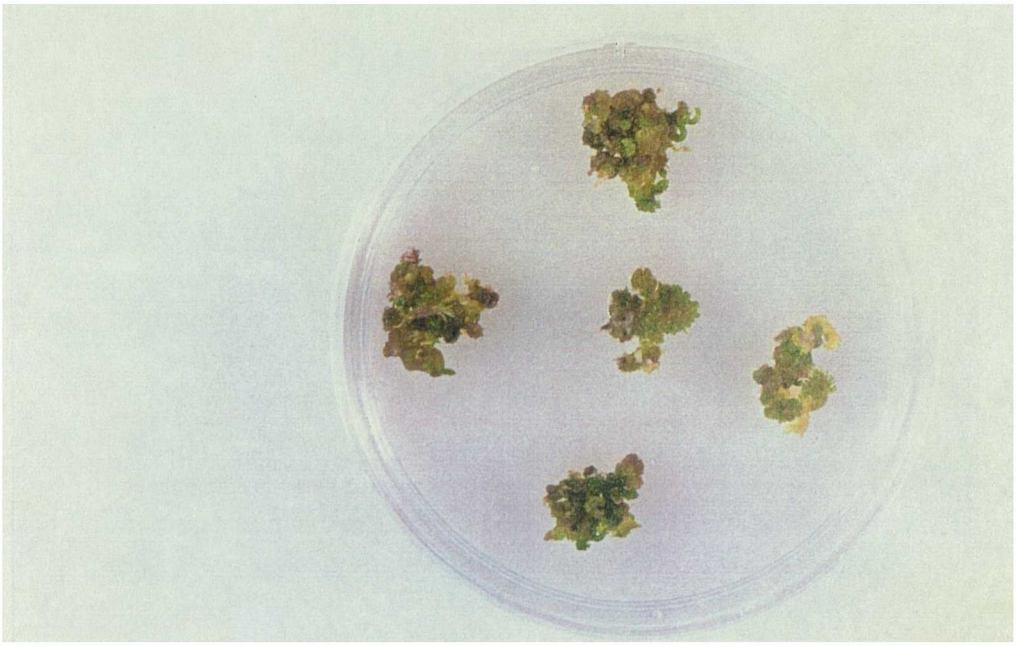


Fig. 55. Shoot initiation from mesophyll cell protoplast-derived calluses of cv. Desiree on shoot regeneration medium of Lam (1977b). Photographed after 10 weeks of culture. (Petri dish Ø 90 mm)

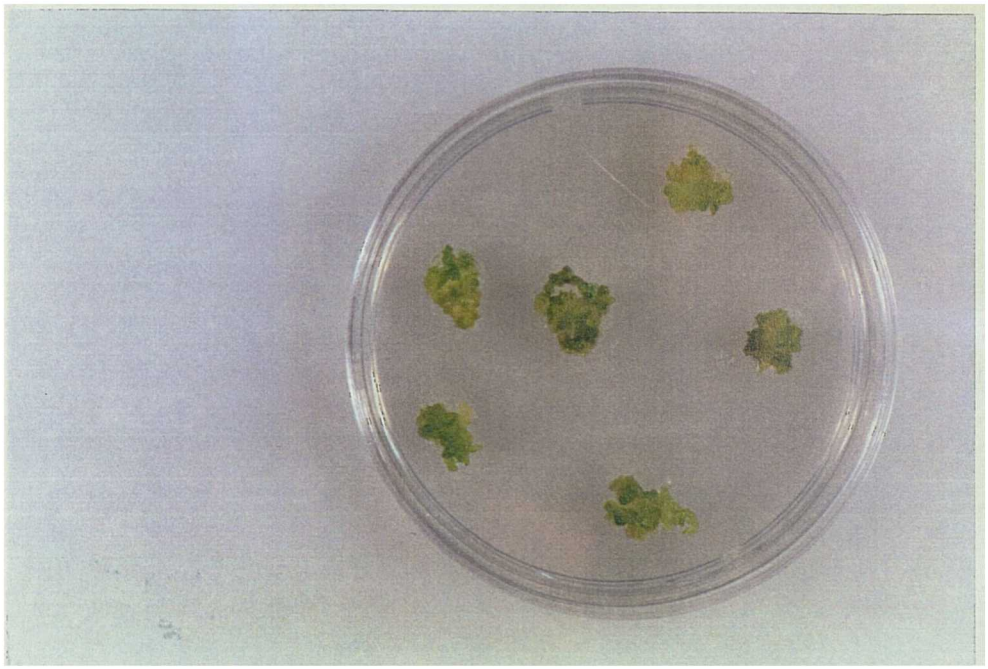


Fig. 56. Shoot initiation from mesophyll cell protoplast-derived calluses of cv. Maris Piper on shoot regeneration medium of Lam (1977b). Photographed after 10 weeks of culture. (Petri dish Ø 90 mm)

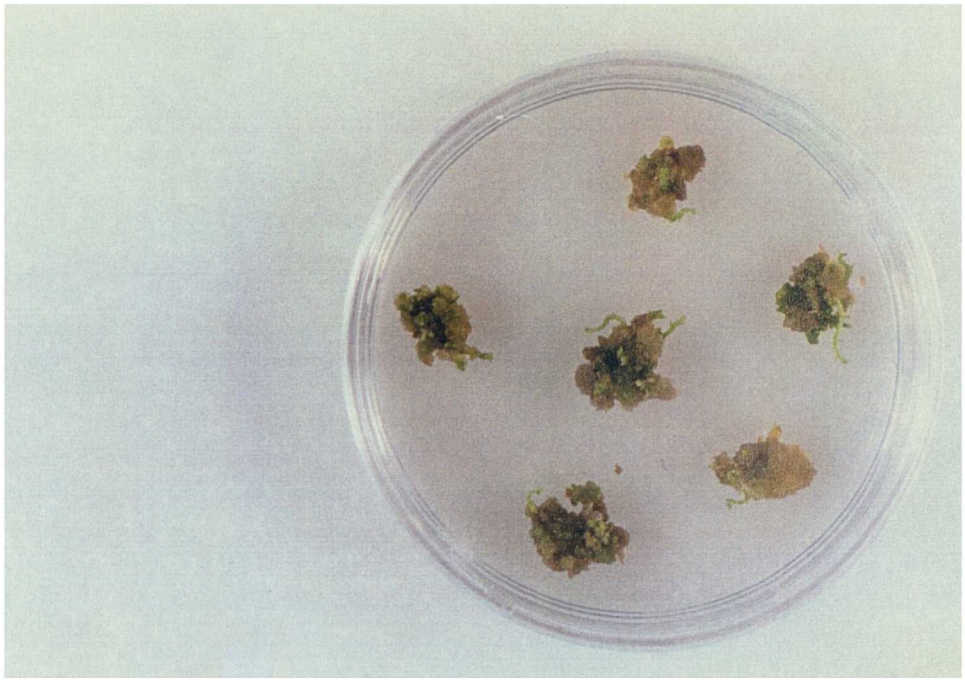


Fig. 57. Shoot regeneration from cultured-cell protoplast-derived calluses of cv. Desiree on shoot regeneration medium of Bokelmann and Roest (1983). Photographed after 11 weeks of culture. (Petri dish Ø 90 mm)

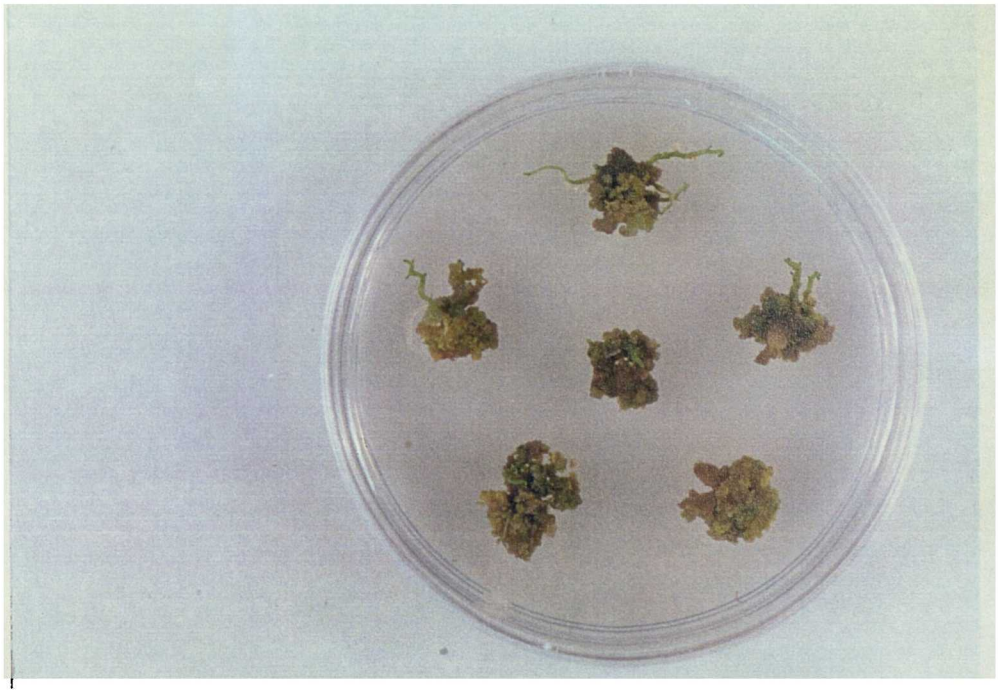


Fig. 58. Shoot regeneration from cultured-cell protoplast-derived calluses of cv. Maris Piper on shoot regeneration medium of Lam (1977b). Photographed after 13 weeks of culture. (Petri dish Ø 90 mm)

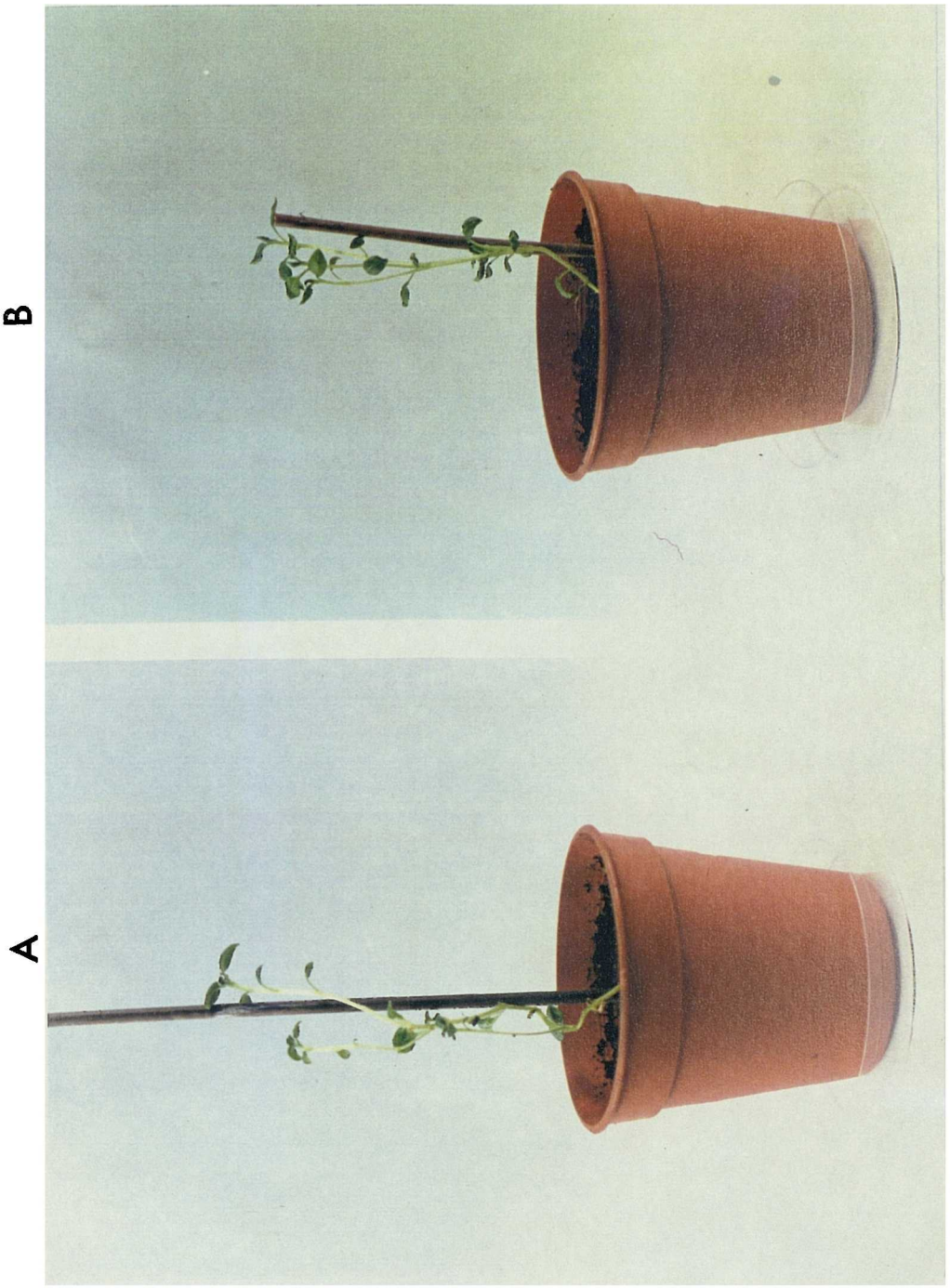


Fig. 59. Maris Piper plantlets regenerated from protoplasts and transferred to sterilized compost.

A. Regenerated from mesophyll cell protoplast.

B. Regenerated from cultured cell protoplast.

to medium TB, in which Desiree and Maris Piper nodal segments took 7 and 10 days respectively after transferring the cultures to tuber-inducing conditions. In medium TA, tuber initiation was delayed compared with other media (Table 31).

In *S. commersonii* and *S. acaule*, the time taken to initiate microtubers was considerably longer compared with the cultivars of *S. tuberosum*. No tuber formation was observed in *S. acaule* nodal segments cultured on medium TA. In general, medium TC was more efficient for microtuber induction, followed by medium TB, while medium TA proved to be unsuitable for early induction of microtubers.

Vegetative growth of cultures was evaluated on the basis of shoot height (stem length) over a 12 week period. All the four types tested produced considerably taller shoots (6.75 - 12.6 cm) on medium TA (Table 32) in which no growth regulator was added. On medium TB, which contained 5 mg l^{-1} BAP, smaller shoots were developed compared with medium TA. The least shoot height was recorded on medium TC which contained 5 mg l^{-1} BAP and 0.5 mg l^{-1} 2,4-D. All the nodal segments cultured on medium TC formed callus, and most of these formed stolons and tubers without producing any shoots. Some nodal segments on medium TC (especially of Desiree and Maris Piper) produced only callus, these produced neither shoots nor tubers. On medium TB, some nodal segments also produced callus in smaller amounts along with shoots and tubers. On medium TA, no callus formation was observed.

Only four segments, each containing an axillary bud, were transferred to each culture jar. Some segments failed to produce microtubers, while some produced more than one

Table 31. Time (days) required for tuber initiation after transferring the nodal segments to tuber-inducing media.

Potato type	Med. TA	Med. TB	Med. TC
Desiree	29.00 ± 1.22	21.00 ± 1.41	17.25 ± 0.43
Maris Piper	34.75 ± 1.78	24.00 ± 2.55	17.00 ± 0.71
S. acaule	n.o*	61.00 ± 5.48	59.00 ± 4.90
S. commersonii	40.75 ± 3.63	48.00 ± 3.60	40.75 ± 1.78

Data represent means ± SD of 4 replicates

* Tuberization did not occur even after 12 weeks of culture

Table 32. Shoot height (cm) of plantlets 12 weeks after transferring the nodal segments to tuber-inducing media.

Potato type	Med. TA	Med. TB	Med. TC
Desiree	7.98 ± 0.87	2.28 ± 0.29	0.91 ± 0.13
Maris Piper	7.92 ± 0.85	2.60 ± 0.83	0.83 ± 0.27
S. acaule	6.75 ± 1.27	1.78 ± 0.66	1.30 ± 0.49
S. commersonii	12.60 ± 0.83	4.45 ± 1.63	2.75 ± 0.59

Data represent means ± SD of 4 replicates

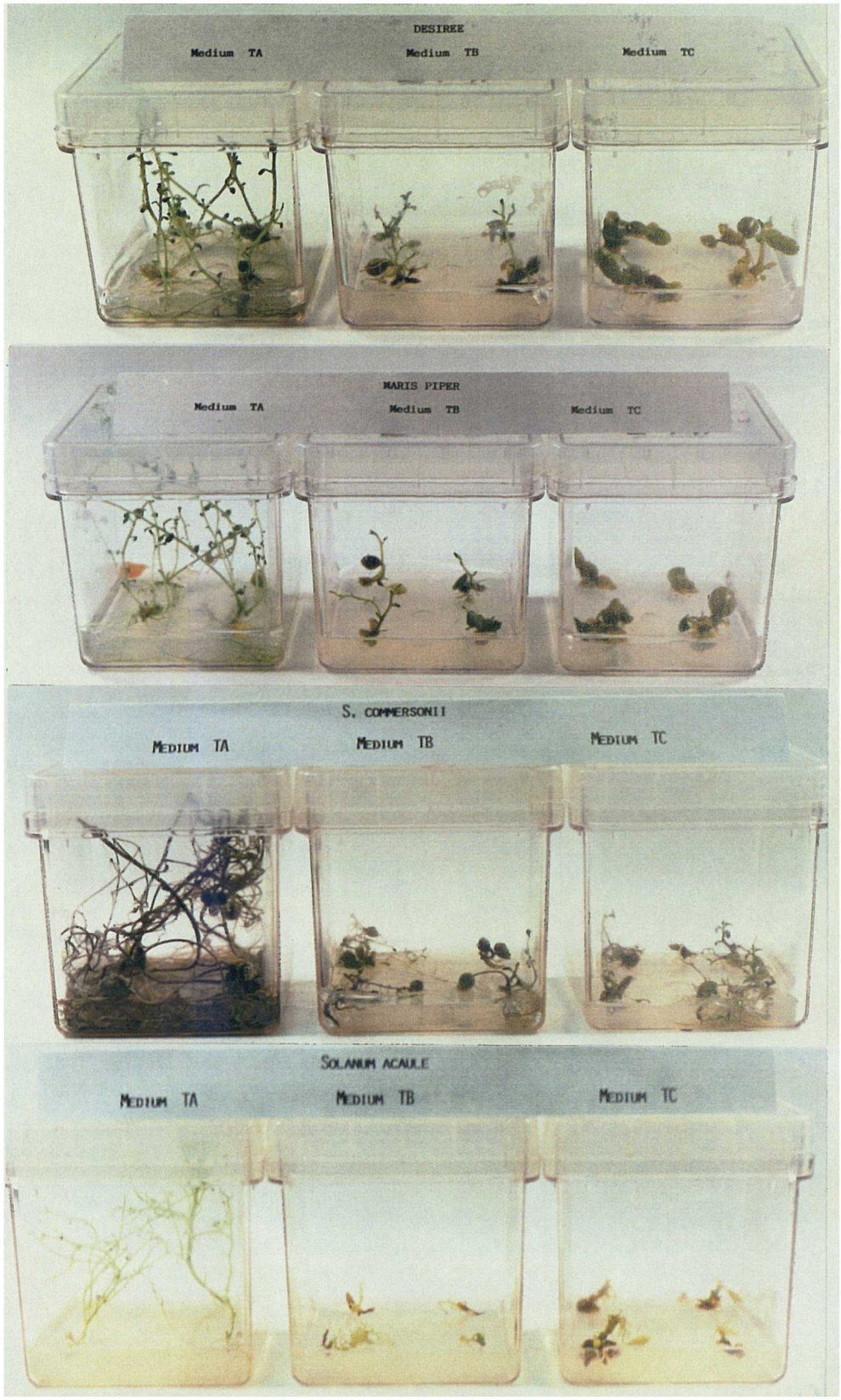


Fig. 60. *In vitro* tuberization in potato. Photographed after 12 weeks of culturing nodal segments on tuber-inducing media.

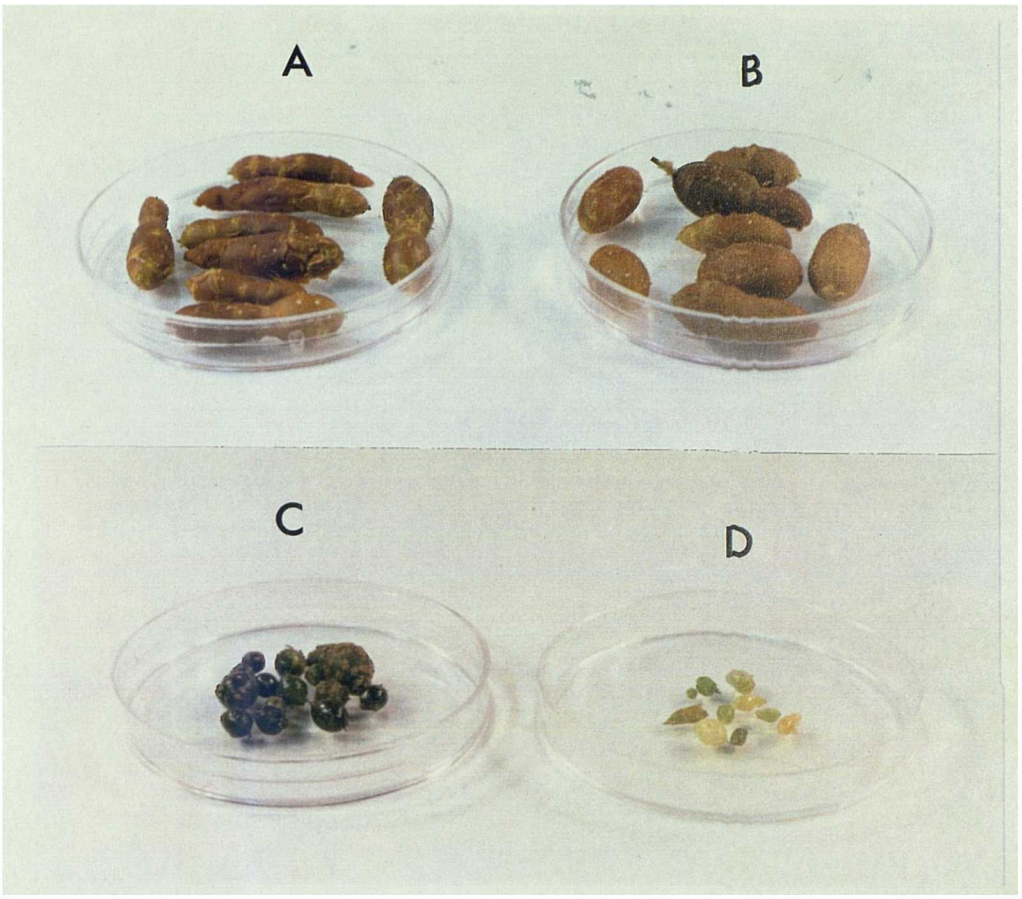


Fig. 61. *In vitro*-produced microtubers. (Petri dish Ø 55 mm)

A. Desiree

B. Maris Piper

C. *S. commersonii*

D. *S. acaule*.

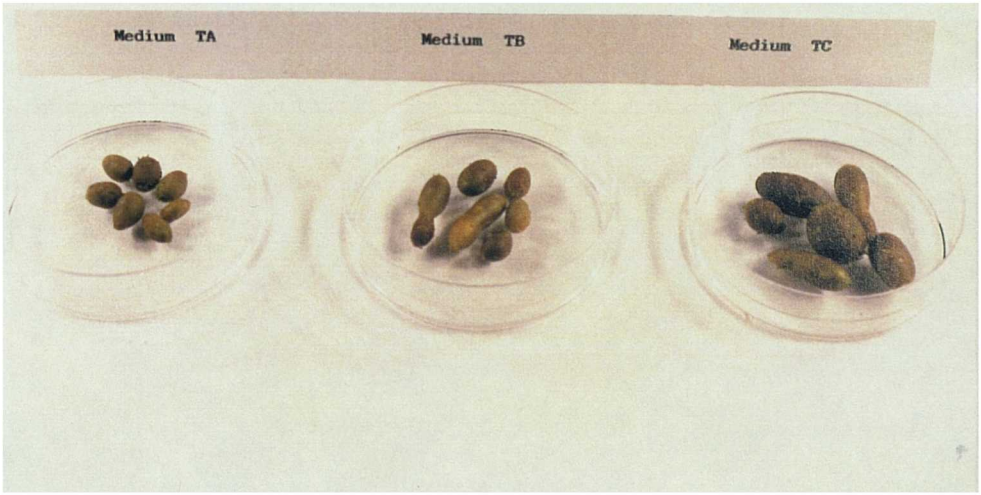


Fig. 62. Effect of medium on tuber size of *in vitro* produced microtubers of Maris Piper. (Petri dish Ø 50 mm)

microtuber. Therefore, data were recorded as the number of tubers produced/culture jar over a period of 12 weeks (Table 33). Maximum number of tubers (9.5/culture jar) was produced in *S. commersonii* on medium TA, while no tubers formed on the same medium in *S. acaule*. In all other cultures, the number of tubers produced per jar ranged from 3 - 5.25 (Table 33).

In *S. tuberosum* cvs. Desiree and Maris Piper, the highest average weights/tuber were obtained when produced on medium TC, followed by on medium TB, while medium TA resulted in lower tuber weights. Although medium TA failed to induce microtubers in *S. acaule* cultures, but this resulted in higher average weight/tuber of *S. commersonii* compared with medium TA and medium TB. When *S. acaule* and *S. commersonii* were either produced on medium TB or medium TC, their average weights on these media were almost equal (Table 34).

It can be concluded that medium TC proved better for *in vitro* tuberization in *S. tuberosum* cvs. Desiree and Maris Piper and medium TA for *S. commersonii*. Despite some tuberization, none of the media tested proved efficient for *in vitro* microtuber production in *S. acaule*. Furthermore, microtubers of cv. Desiree produced on these media had a very different morphology from the normal commercial tubers, while those of Maris Piper were more normal-shaped but had red skin colour. The skin colours of microtubers of *S. commersonii* and *S. acaule* were dark green and light green respectively (Fig. 61).

Microtubers were also produced from stems of some plantlets in Desiree and Maris

Table 33. No. of microtubers produced/culture jar 12 weeks after transferring the nodal segments to tuber-inducing media.

Potato type	Med. TA	Med. TB	Med. TC
Desiree	5.25 ± 0.83	4.00 ± 0.71	5.25 ± 0.83
Maris Piper	5.00 ± 1.22	4.00 ± 0.71	4.75 ± 0.43
S. acaule	0.00* ± 0.00	3.00 ± 0.71	4.00 ± 0.71
S. commersonii	9.50 ± 2.06	4.25 ± 0.83	5.25 ± 0.83

Data represent means ± SD of 4 replicates

* Tuberization did not occur even after 12 weeks of culture

Table 34. Average weight/tuber (mg) harvested 12 weeks after transferring the nodal segments to tuber inducing-media.

Potato type	Med. TA	Med. TB	Med. TC
Desiree	144.98 ± 32.69	192.17 ± 33.28	375.35 ± 48.84
Maris Piper	148.92 ± 41.88	183.92 ± 35.54	422.75 ± 56.46
S. acaule	n.o*	22.88 ± 1.83	22.82 ± 1.05
S. commersonii	61.47 ± 6.14	50.71 ± 1.57	51.47 ± 1.90

Data represent means ± SD of 4 replicates

* Tuberization did not occur even after 12 weeks of culture

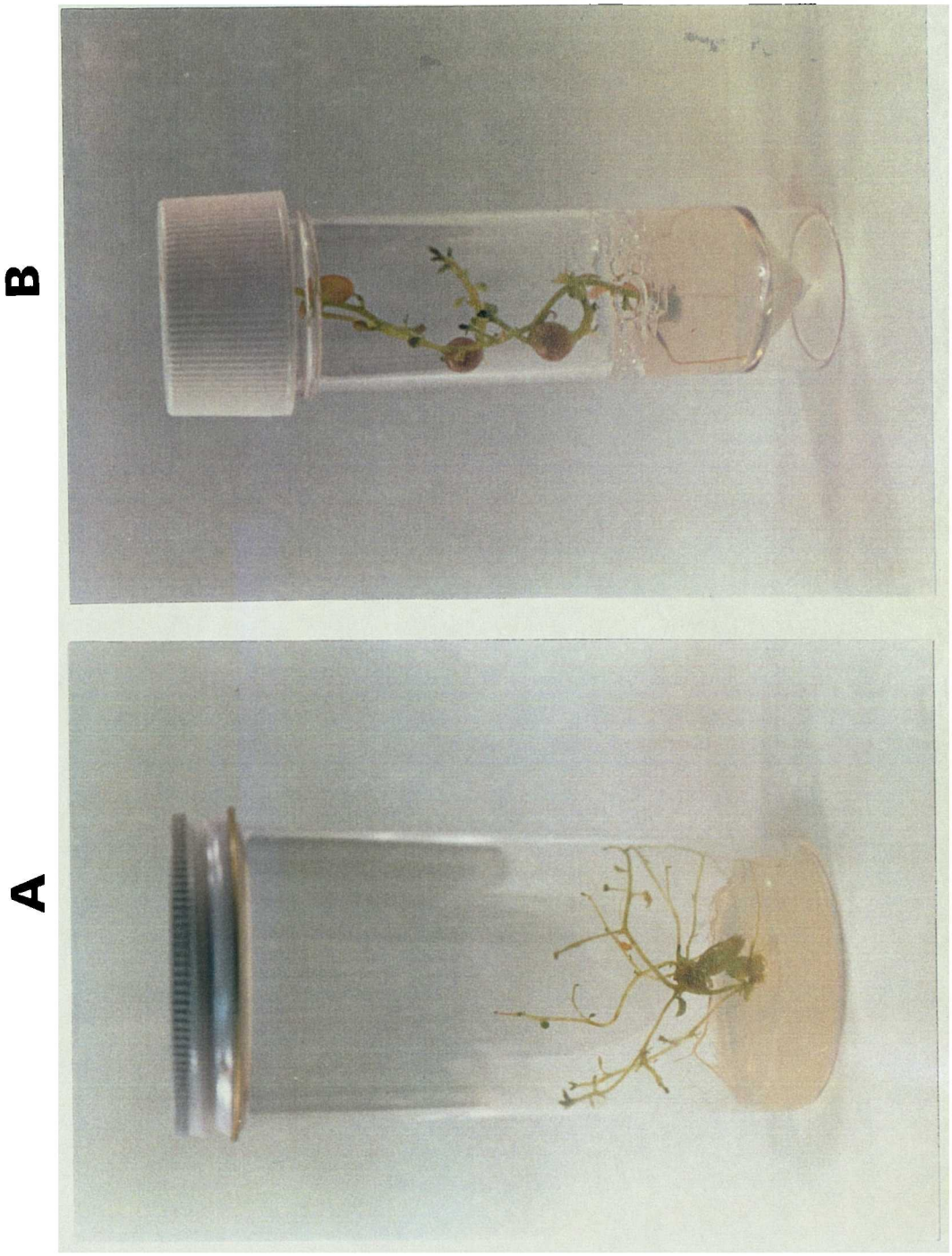


Fig. 63. Potato axillary bud cultures on medium developed by Espinoza *et al.* (1986), producing microtubers. Photographed after 12 weeks.

A. Desiree B. Maris Piper

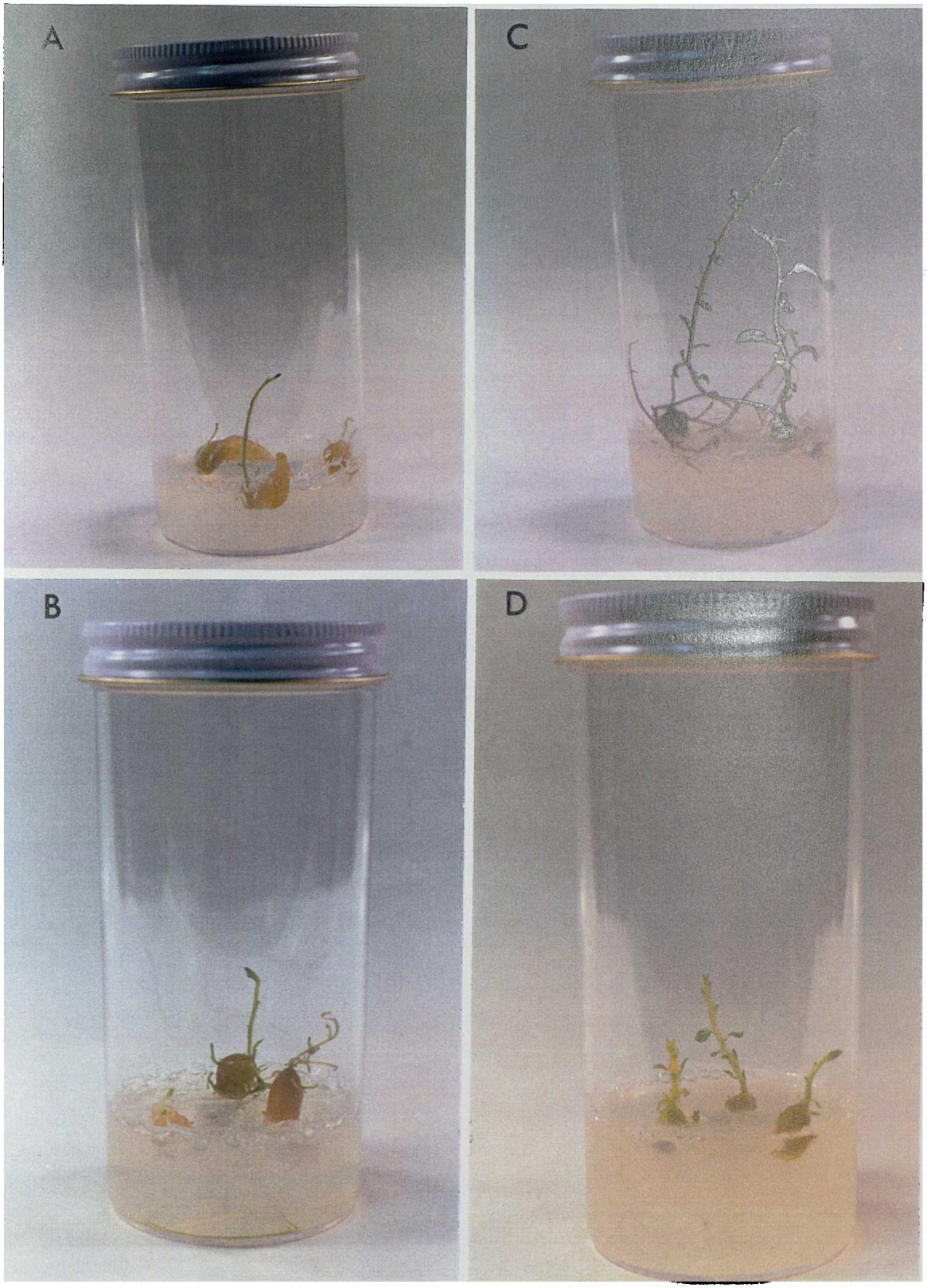


Fig. 64. Shoot production from microtubers grown *in vitro*, cultured on shoot culture medium of Espinoza *et al.* (1986). Photographed after 2 weeks.

A. Desiree B. M. Piper C. *S. commersonii* D. *S. acaule*

Piper cultures after 8 weeks of subculturing axillary buds on shoot culture media NA. No microtuber formation was observed in the cultures of *S. commersonii* and *S. acaule*. Some of the microtubers sprouted in the growing cultures, giving new shoots (Fig. 63). When freshly harvested microtubers were cultured on shoot culture medium NA, these produced shoots (Fig. 64) probably showing that these were not deeply dormant.

3.8 *In vitro* selection

Hydroxyproline resistant lines have been reported possessing increased frost tolerance (van Swaaij *et al.* 1986, 1987a & b; Tantau and Dorffling, 1991). Therefore, attempts were made in the present study to select hyp-resistant cell lines from potato tissue cultures and to determine their degree of frost tolerance. For this purpose organized tissue (axillary buds), unorganized tissue (callus), and cells (suspension-cultured cells) were used.

3.8.1 Experiments with axillary buds

3.8.1.1 Growth of axillary buds on medium containing hydroxyproline

Nodal cuttings of *S. tuberosum* cvs. Desiree and Maris Piper, and wild species *S. commersonii* and *S. acaule,* each containing an axillary bud, were prepared from sterile *in vitro* shoot cultures and placed on the shoot culture medium of Espinoza *et al.* (1986) (Med. NA) in batches, each containing a different concentration of hyp (hydroxyproline). The concentrations used were 20 mM and 30 mM, and a control was also included. Four replications were prepared.

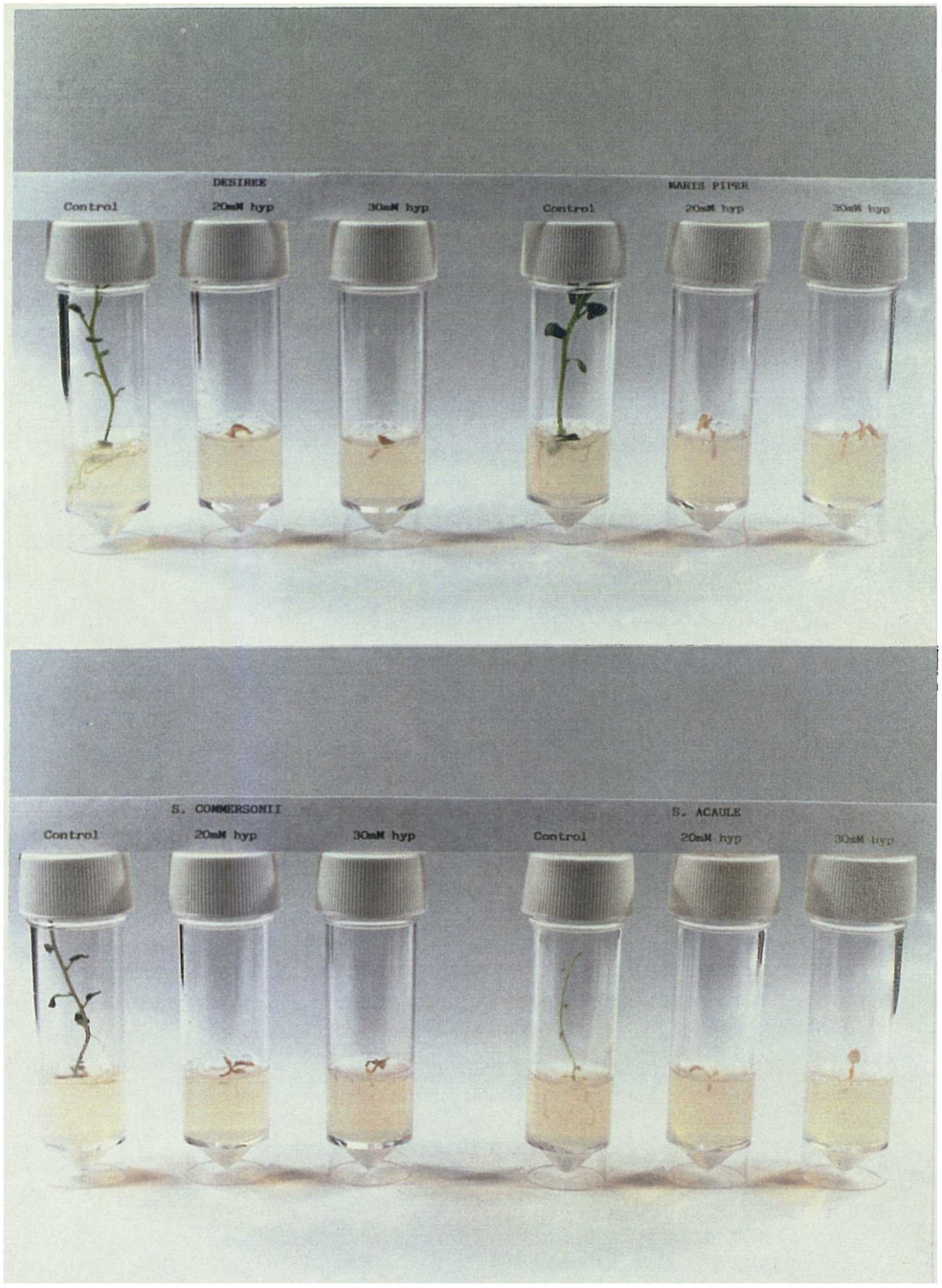


Fig. 65. Growth of potato axillary buds subcultured on shoot culture medium of Espinoza *et al.* (1986) (Med. NA) as control, and on the same medium with 20 and 30 mM hydroxyproline. Photographed after 4 weeks.

Table 35. General description of growth of potato axillary buds on shoot culture medium (Med. NA) as control, and containing different concentrations of hydroxyproline. The figures are a subjective assessment of growth on a scale 1 - 10 (see footnote).

Potato type	Hydroxyproline concentration			
	Control	10 mM	20 mM	30 mM
Desiree				
Bud No. 1	8	1	1	1
2	7	1	1	1
3	5	1	1	1
4	4	2	1	1
Maris Piper				
1	4	2	1	1
2	6	1	1	1
3	4	2	1	1
4	7	1	1	1
S. commersonii				
1	4	2	1	1
2	7	2	1	1
3	8	1	1	1
4	5	2	1	1
S. acaule				
1	4	3	1	1
2	5	3	1	1
3	7	1	1	1
4	6	1	3	1

Subjective growth assessment:
1. Dead necrotic 2. Green but no growth 3. Slow growth 4. Below average growth 5. Average growth (-) 6. Average growth (+) 7. Above av. growth 8. Good growth 9. Very good growth 10. Rapid growth.

Table 36. Survival (%) of potato axillary buds on shoot culture medium (Med. NA) as control, and on the same medium containing different concentrations of hydroxyproline.

Potato type	Hydroxyproline concentration			
	Control	10 mM	20 mM	30 mM
Desiree	100	0	0	0
Maris Piper	100	0	0	0
S. commersonii	100	0	0	0
S. acaule	100	50	25	0

Table 37. Potato plantlet shoot growth (cm) during 4 weeks after transferring nodal cuttings to shoot culture medium (Med. NA) as control, and on the same medium containing different concentrations of hydroxyproline (10, 20 and 30 mM).

Days of culture	Desiree				Maris Piper			
	Control	10 mM	20 mM	30 mM	Control	10 mM	20 mM	30 mM
7	1.87	0.00	0.00	0.00	1.92	0.00	0.00	0.00
14	3.07	0.00	0.00	0.00	2.90	0.00	0.00	0.00
21	4.37	0.00	0.00	0.00	3.77	0.00	0.00	0.00
28	5.70	0.00	0.00	0.00	4.37	0.00	0.00	0.00
Days of culture	*S.commersonii*				*S. acaule*			
	Control	10 mM	20 mM	30 mM	Control	10 mM	20 mM	30 mM
7	1.72	0.00	0.00	0.00	1.05	0.30	0.20	0.00
14	3.72	0.00	0.00	0.00	1.77	0.55	0.30	0.00
21	6.00	0.00	0.00	0.00	2.57	0.60	0.30	0.00
28	6.57	0.00	0.00	0.00	3.20	0.80	0.30	0.00

Four replicates. Data represent means of surviving replicates

All the axillary buds on control medium grew well. None of the tissues placed on medium containing 20 or 30 mM hyp survived, and axillary buds became necrotic and died (Fig. 65). The experiment was therefore repeated and a hyp concentration of 10 mM was also included. The media in this second experiment therefore contained; control or no hyp, 10 mM, 20 mM and 30 mM hyp. Growth assessments were made during a period of 4 weeks. General descriptions of growth on the basis of a subjective assessment are given in Table 35, and measurements of shoot height recorded at 7 day intervals are presented in Table 37.

After 4 weeks of culture, it was found that axillary buds grew well on medium without hyp. On medium with 10 mM hyp, some axillary buds became necrotic and died while others remained green but without any growth. However, some buds of *S. acaule* succeeded in growing, but very slowly (Table 37). On medium with 20 or 30 mM hyp, all the axillary buds became necrotic and died with the exception of few axillary buds of *S. acaule* which showed a little growth on medium containing 20 mM hyp. Survival of axillary buds on media containing hyp is given in Table 36. After 4 weeks, shoots produced on media containing hyp were subcultured onto fresh media of the same hyp concentrations. Upon subculture, these failed to grow and died.

3.8.1.2 Growth of axillary buds on media containing proline or mannitol

Increase in frost tolerance in potato has been reported when proline was applied exogenously to the shoot cultures (van Swaaij *et al.* 1985) or cultured cells (Hellergren and Li, 1981). To investigate whether increase in frost tolerance is due to the involvement of this amino acid in a frost tolerance mechanism, or due to the stress

Table 38. Potato plantlet shoot growth (cm) during 4 weeks after transferring nodal cuttings to shoot culture medium (Med. NA) as control, and to the same medium containing 0.05 M proline or 0.05 M mannitol.

Days of culture	Desiree			Maris Piper			*S. commersonii*			*S. acaule*		
	Control	Proline	Mannitol	Control	Proline	Mannitol	Control	Proline	Mannitol	Control	Proline	Mannitol
7	1.60 ±0.31	0.72 ±0.22	1.37 ±0.37	1.32 ±0.40	0.70 ±0.27	1.20 ±0.32	1.67 ±0.33	1.02 ±0.08	1.40 ±0.29	1.07 ±0.18	0.70 ±0.07	1.00 ±0.28
14	2.67 ±0.63	1.25 ±0.48	2.12 ±0.71	2.32 ±0.40	1.22 ±0.39	1.75 ±0.36	3.42 ±0.35	2.07 ±0.15	2.25 ±0.35	2.55 ±0.66	1.42 ±0.23	1.65 ±0.33
21	3.20 ±0.63	1.60 ±0.58	2.50 ±0.89	2.95 ±0.56	1.60 ±0.49	2.25 ±0.50	4.42 ±0.35	3.00 ±0.23	3.27 ±0.65	3.30 ±0.82	1.92 ±0.19	2.17 ±0.19
28	4.32 ±1.02	1.82 ±0.65	2.82 ±0.90	3.90 ±0.46	1.80 ±0.52	2.70 ±0.54	5.57 ±0.54	4.47 ±0.30	4.62 ±0.97	3.62 ±0.73	2.10 ±0.16	2.77 ±0.11

Data represent means ± SD of 4 replicates

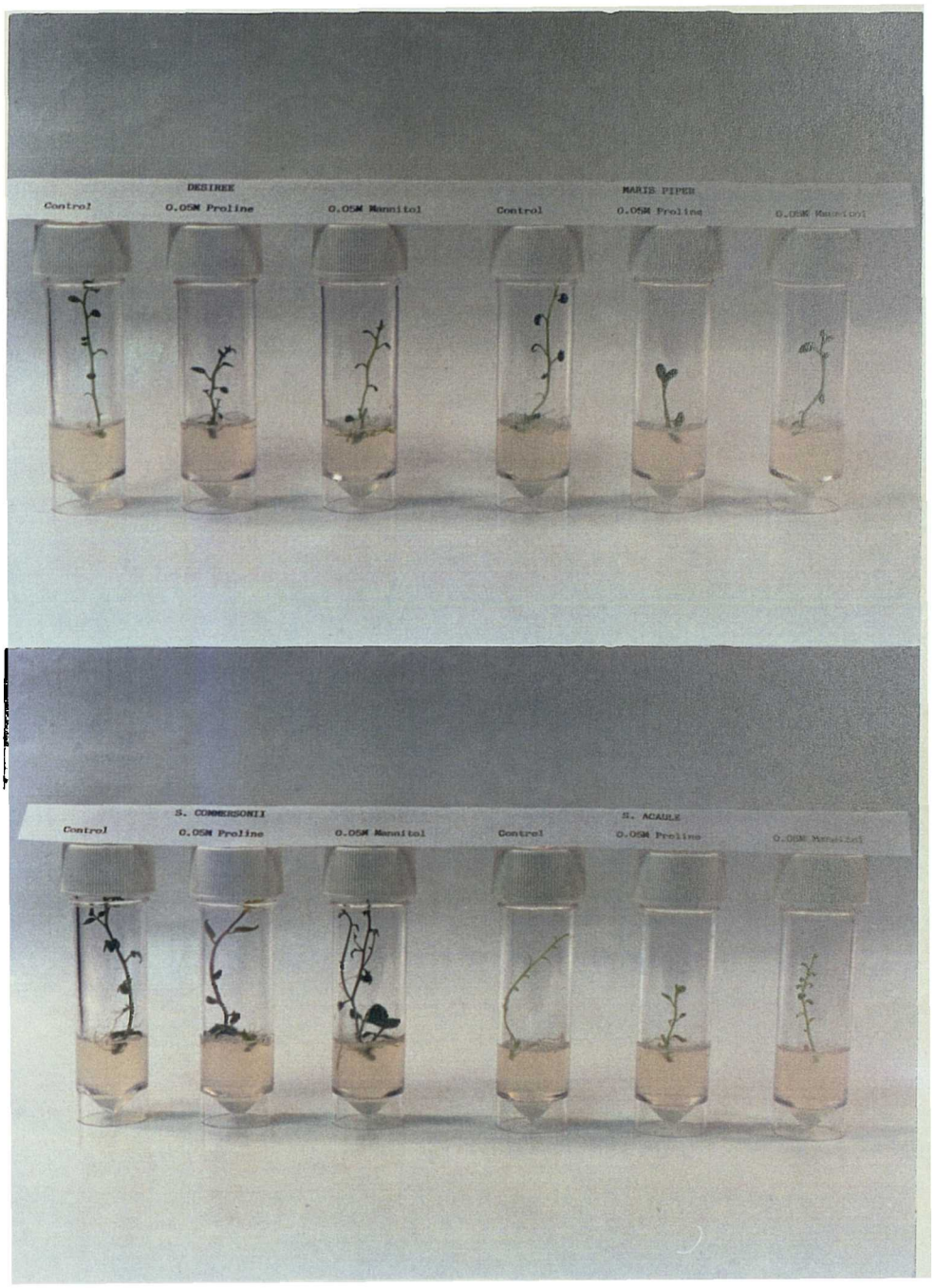


Fig. 66. Growth of potato axillary buds subcultured on shoot culture medium of Espinoza *et al.* (1986) (Med. NA) as control, and on the same medium supplemented with 0.05 M proline or mannitol. Photographed after 4 weeks.

produced by proline, nodal cuttings were transferred to shoot culture medium NA as control, and also to the same medium containing 0.05 M proline or 0.05 M mannitol. Growth of cultures was assessed during 4 weeks on the basis of shoot height, and the results are presented in Table 38.

All the axillary buds transferred to the control medium grew well, while on medium containing 0.05 M proline or mannitol, growth was reduced (Fig. 66). Reduction in growth was greater on medium containing 0.05 M proline compared with medium containing 0.05 M mannitol. Bleaching of leaves was also observed in *S. commersonii* cultures on medium containing 0.05 M proline.

3.8.1.3 Effect of exogenously-applied proline on frost tolerance of potato leaves

Four weeks after transferring nodal cuttings to shoot culture medium NA, and on the same medium containing 0.05 M proline or mannitol, leaves were excised from shoots in culture and their degree of frost tolerance was determined (see Materials and Methods). Values of frost killing-temperatures (°C) are given in Table 39.

The effect of mannitol in the medium on frost tolerance of leaves was almost nil, while exogenously-applied proline to the shoot cultures resulted in about 0.8 to 1.2° lower FKT values (see Section 2.14.2) compared with controls in *Solanum* species.

3.8.1.4 Effect of exogenously-applied proline on hyp tolerance

Shoots produced on shoot culture medium NA containing 0.05 M proline were excised, subdivided into nodal cuttings each containing an axillary bud, and

Table 39. Effect of exogenously-applied proline and mannitol on frost-killing temperatures (°C) of potato leaves. Frost tolerance was determined (see Section 2.14.2) after four weeks of culture of nodal cuttings on shoot culture medium (Med. NA) containing 0.05 M proline or mannitol.

Potato type	Control	0.05 M Proline	0.05 M Mannitol
Desiree	-2.8	-3.7	-3.0
Maris Piper	-2.9	-3.8	-2.8
S. commersonii	-4.4	-5.6	-4.4
S. acaule	-5.8	-6.6	-6.0

Table 40. Survival (%) of potato axillary buds on shoot culture medium (Med. NA) as control, and on the same medium containing different concentrations of hydroxyproline. Nodal cuttings, each containing an axillary bud, were taken from plantlets grown on shoot culture medium (Med. NA) containing 0.05 M proline and subcultured onto these media.

Potato type	Hydroxyproline concentration			
	Control	10 mM	20 mM	30 mM
Desiree	100	25	0	0
Maris Piper	100	25	50	100
S. commersonii	100	25	25	25
S. acaule	100	25	25	0

Table 41. Potato plantlet shoot growth (cm) during 4 weeks, after subculturing nodal cuttings on shoot culture medium (Med. NA) as control, and on the same medium containing hydroxyproline (10, 20 and 30 mM). These cuttings were taken from plantlets growing on shoot culture medium (Med. NA) containing 0.05 M proline.

Days of culture	Desiree				Maris Piper			
	Control	10 mM	20 mM	30 mM	Control	10 mM	20 mM	30 mM
7	1.62	0.45	0.00	0.00	1.30	0.40	0.25	0.25
14	4.02	0.90	0.00	0.00	2.15	0.60	0.45	0.42
21	5.17	0.90	0.00	0.00	3.45	0.70	0.55	0.52
28	6.32	0.90	0.00	0.00	3.75	0.90	0.65	0.62
Days of culture	*S.commersonii*				*S. acaule*			
	Control	10 mM	20 mM	30 mM	Control	10 mM	20 mM	30 mM
7	1.52	0.20	0.27	0.10	0.72	0.30	0.40	0.00
14	4.02	0.50	0.36	0.20	1.67	0.40	0.60	0.00
21	5.70	0.50	0.40	0.20	2.00	0.60	0.60	0.00
28	6.62	0.60	0.40	0.20	2.52	0.70	0.70	0.00

Four replicates. Data represent means of surviving replicates

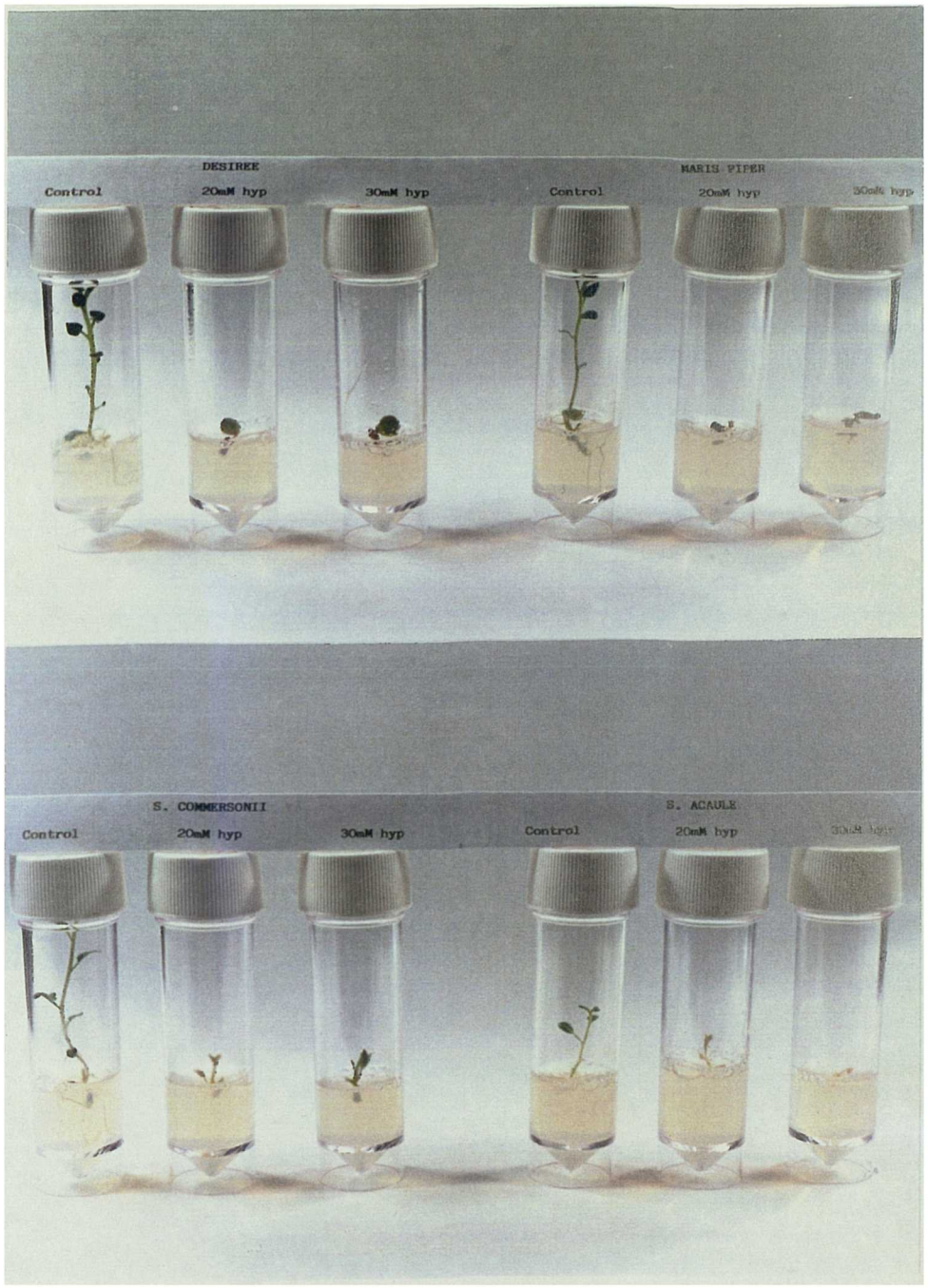


Fig. 67. Growth of potato axillary buds taken from shoots produced on shoot culture medium of Espinoza *et al.* (1986) (Med. NA) supplemented with 0.05 M proline, and subcultured onto shoot culture medium NA as control, and onto the same medium with 20 and 30 mM hydroxyproline. Photographed after 4 weeks.

subcultured onto shoot culture medium NA containing 10, 20 or 30 mM hyp. A control medium without hyp was also included to compare the growth. The per cent survival of axillary buds on medium containing different concentrations of hyp is given in Table 40. Growth of cultures was evaluated over a period of 4 weeks on the basis of shoot height of surviving axillary buds and the results are presented in Table 41.

The first sign of hyp toxicity observed was inhibition of root growth followed by inhibition of shoot growth (Fig. 67). Although some of the axillary buds succeeded in growing on hyp-contained medium, their growth was strongly inhibited and none of the shoots produced was more than 0.9 cm in height.

3.8.1.5 Selection of hyp-resistant variants from axillary bud cultures

Shoots produced on medium containing hyp were subcultured onto fresh medium with the same concentration of hyp. Further subcultures were made at 4 - 6 week intervals by subdividing the shoots into nodal cuttings, each containing at least one axillary bud. Upon subculture axillary buds started dying and after 2 - 3 subcultures, all the cultures were dead and attempts to select variants from organised tissue proved unsuccessful.

3.8.2 Experiments with callus

3.8.2.1 The growth of callus on media containing hyp

Samples of *S. tuberosum* cvs. Desiree and Maris Piper leaf calluses subcultured on solidified MS medium plus 3 mg l^{-1} 2,4-D were weighed and subcultured onto the fresh medium of same composition but in different batches containing a range of hyp concentrations. The hyp concentrations added to the medium were 10, 20 and 30 mM.

Table 42. Changes in fresh weights of *S. tuberosum* cvs. Desiree and Maris Piper leaf calluses on MS medium plus 3 mg l^{-1} 2,4-D as control, and on the same medium with a range of hydroxyproline concentrations (10, 20 and 30 mM).

Days of culture	Desiree				Maris Piper			
	Control	10 mM	20 mM	30 mM	Control	10 mM	20 mM	30 mM
0	225.5 ±11.7	226.0 ±7.8	226.5 ±10.4	225.0 ±14.2	225.5 ±7.9	225.5 ±10.2	225.2 ±2.5	224.5 ±8.4
7	565.5 ±28.9	262.2 ±15.6	238.2 ±18.6	223.7 ±18.6	501.0 ±54.5	240.7 ±16.6	239.2 ±4.3	223.7 ±12.0
14	1123.2 ±56.0	222.5 ±12.5	207.7 ±19.2	168.5 ±13.9	982.0 ±63.8	199.2 ±23.7	196.2 ±30.4	197.2 ±14.6
21	2267.0 ±81.8	203.0 ±17.1	153.0 ±9.8	143.0 ±14.9	1898.2 ±132.9	169.5 ±22.9	156.7 ±24.8	150.2 ±10.1
28	2583.7 ±112.5	159.5 ±31.4	141.2 ±9.6	140.0 ± 19.0	2261.7 ±98.9	154.2 ±17.8	142.5 ±25.2	138.2 ±7.0

Data represent means ± SD of 4 replicates

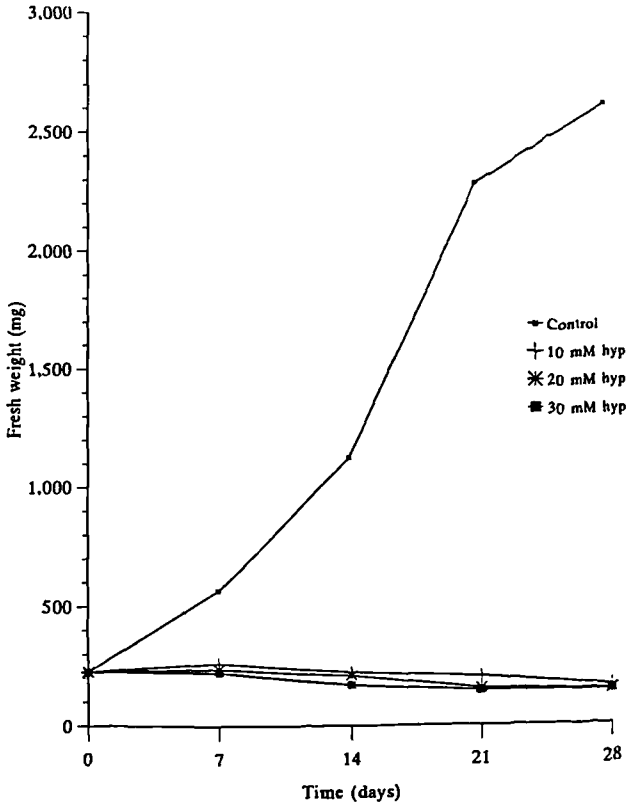


Fig. 68. Growth of *S. tuberosum* cv. Desiree leaf callus on MS medium + 3 mg/l 2,4-D as control, and on this medium with a range of hydroxyproline concentrations.

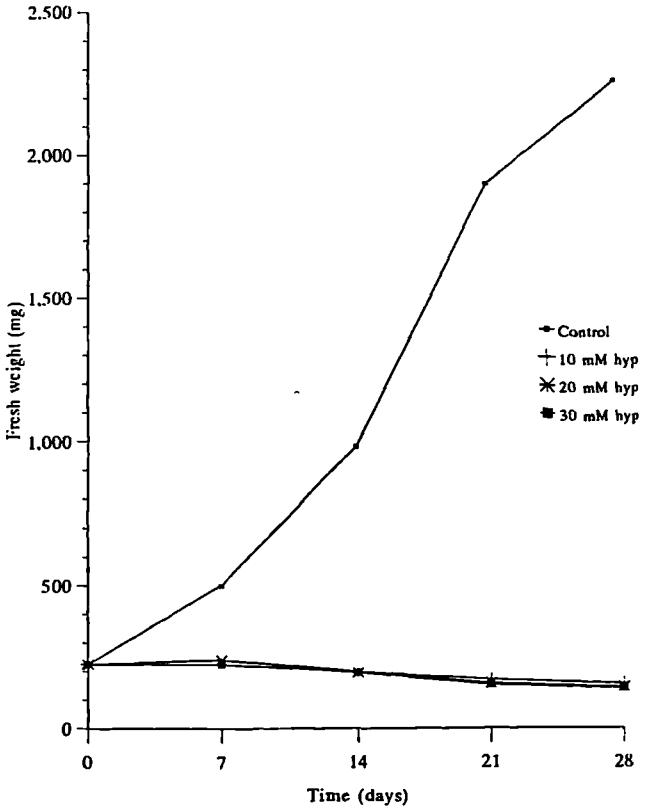


Fig. 69. Growth of *S. tuberosum* cv. Maris Piper leaf callus on MS medium + 3 mg/l 2,4-D as control, and on this medium with a range of hydroxyproline concentrations.

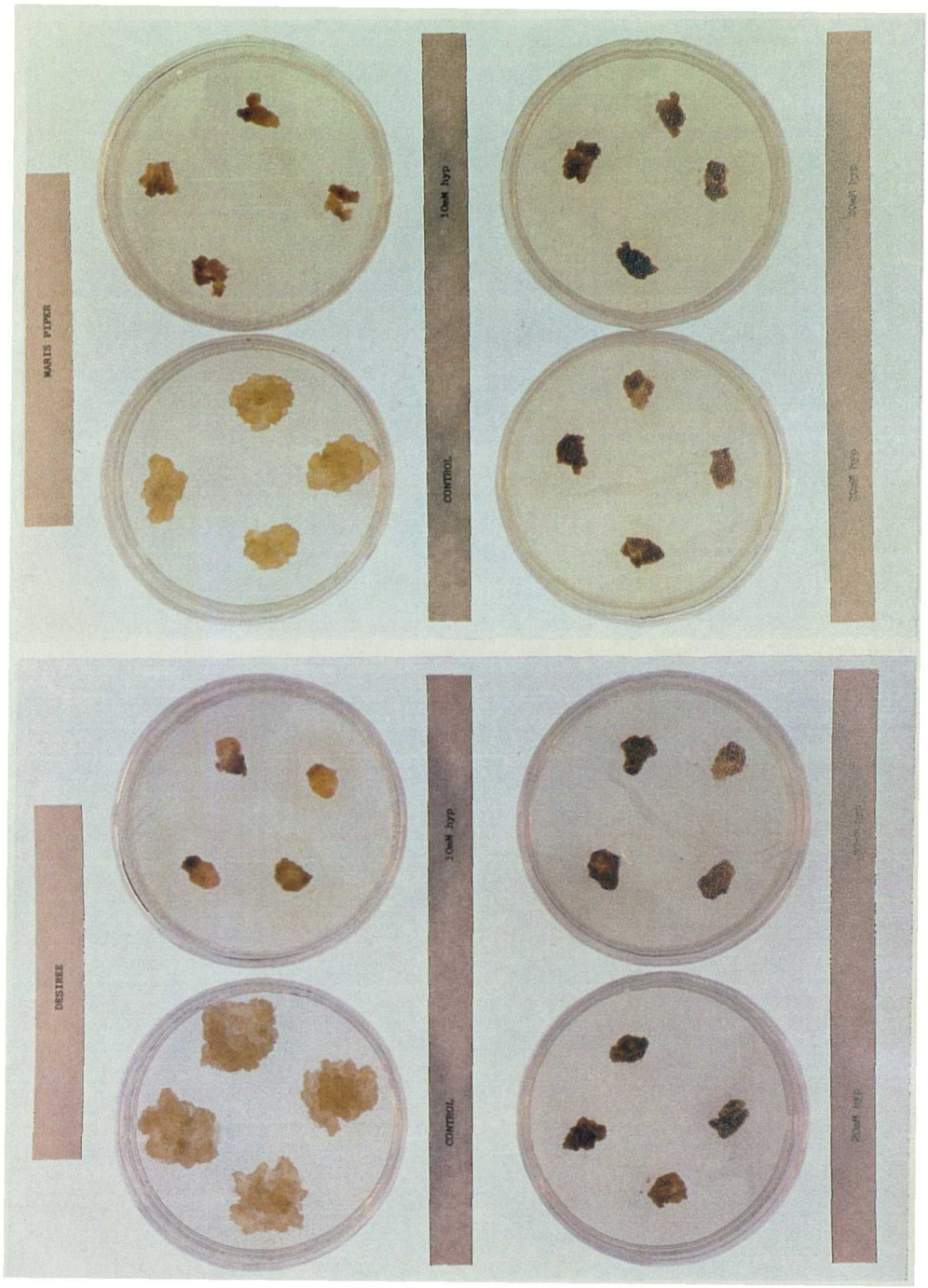


Fig. 70. *S. tuberosum* cvs. Desiree and Maris Piper leaf calluses, subcultured on MS medium + 3 mg l^{-1} 2,4-D as control, and on the same medium with 10, 20 and 30 mM hydroxyproline. Photographed after 3 weeks. (Petri dish Ø 90 mm)

A control medium without hyp was also included and 4 replicates were prepared for each batch of medium. All the cultures were weighed at 7 day intervals and growth of cultures was assessed on the basis of changes in their fresh weights. Results are summarized in Table 42 and growth curves of Desiree and Maris Piper calluses for each hyp concentration are plotted against time in Fig. 68 and 69.

Although after 7 days of culture, there was a slight increase in fresh weights of calluses cultured on medium containing 10 or 20 mM hyp, a reduction in fresh weights occurred later. Calluses grown on medium containing 30 mM hyp did not show any increases in their fresh weights. After 28 days of culture, almost all the calluses (except some variant cells in a few calluses) grown on medium containing hyp changed in colour from creamy white to yellow or brown and were apparently dead, while calluses grown on control medium followed a normal pattern of growth. Fig. 70 shows examples of the growth of Desiree and Maris Piper calluses on control and media containing different hyp concentrations.

3.8.2.2 Selection of hyp-resistant lines from calluses

When callus cultures on media containing various hyp concentrations (10, 20 and 30 mM) were subcultured onto the fresh media of same composition, growth was observed in parts of some callus pieces (Fig. 71). Dead calluses were discarded and only growing calluses were selected. It could be possible that few variant cells, perhaps mutants, were hyp-resistant and were able to grow on hyp-containing media. Each sector of growing cells was treated as a variant cell line, subcultured separately, and maintained through subcultures onto fresh medium with the same concentration

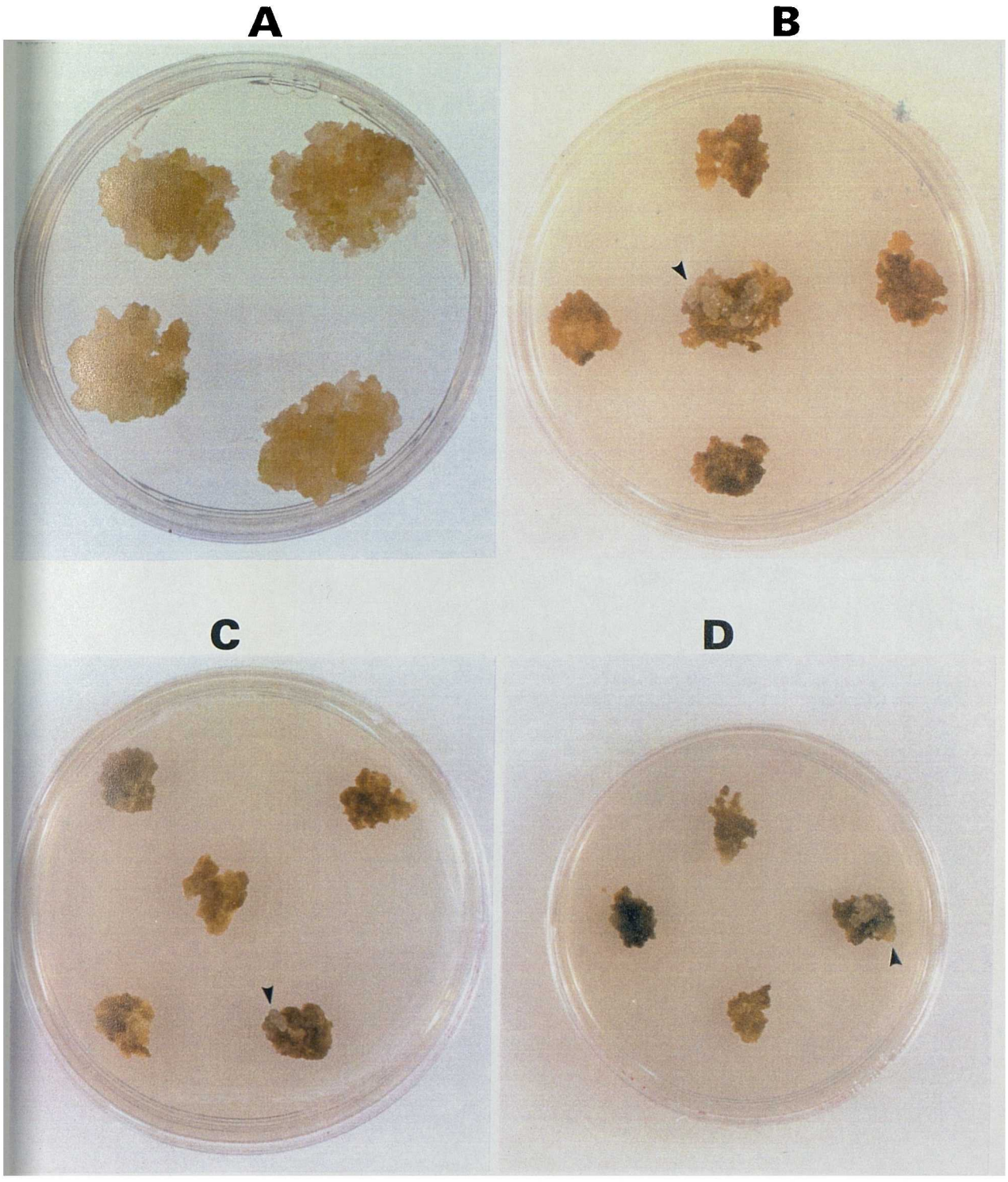
Fig. 71. Selection of hyp-resistant lines from callus cultures. Resistance could be seen as sectorial growth from some callus pieces (arrows). Photographed after 4 weeks of subculture. (Petri dish Ø 90 mm)

A. Maris Piper leaf calluses subcultured onto the MS medium + 3 mg l^{-1} 2,4-D.

B. Desiree leaf calluses subcultured onto the MS medium + 3 mg l^{-1} 2,4-D and 10 mM hydroxyproline.

C. Desiree leaf calluses subcultured onto the MS medium + 3 mg l^{-1} 2,4-D and 20 mM hydroxyproline.

D. Maris Piper leaf calluses subcultured onto the MS medium + 3 mg l^{-1} 2,4-D and 30 mM hydroxyproline.


A
B
C
D

of hyp. Some of these cell lines still failed to grow when subcultured onto fresh media, and among the cell lines which were able to grow, some grew faster than others. Those which became necrotic or were unable to grow, were discarded and only areas of tissue showing growth were subcultured onto the fresh media. After 6 subcultures the following cell lines had become established.

	hyp concentrations		
Potato cultivar	10 mM	20 mM	30 mM
Desiree	D10-1	D20-1	D30-1
	D10-2	D20-2	
	D10-3	D20-3	
Maris Piper	M10-1	M20-1	
		M20-2	

3.8.2.3 Growth of callus on medium containing proline or mannitol

To investigate the effects of proline on callus growth and its frost tolerance, 0.05 M proline, or 0.05 M mannitol, was added to the nutrient medium. Samples of Desiree and Maris Piper calluses were weighed and transferred to MS medium plus 3 mg l^{-1} 2,4-D as control, and to the same medium containing 0.05 M proline or 0.05 M mannitol. To assess the callus growth, fresh weight measurements were made after 7 and 14 days of culture on these media. Results are presented in Table 43, and Fig. 72 shows the fresh weights of calluses on these media after 14 days.

Growth of both Desiree and Maris Piper callus was reduced on media containing proline or mannitol compared with control, and the reduction was greater on medium

Table 43. Changes in fresh weights of *S. tuberosum* cvs. Desiree and Maris Piper leaf calluses on MS medium containing 3 mg l^{-1} 2,4-D as control, and on the same medium supplemented with 0.05 M proline or 0.05 M mannitol.

Days of culture	Desiree			Maris Piper		
	Control	Proline	Mannitol	Control	Proline	Mannitol
0	224.25 ±13.17	225.25 ±6.06	224.50 ±8.29	223.00 ±10.79	227.25 ±6.36	231.25 ±6.26
7	582.50 ±51.85	413.00 ±8.15	462.75 ±37.81	517.00 ±13.21	466.00 ±19.95	482.50 ±66.73
14	1155.00 ±126.84	678.00 ±59.23	732.25 ±84.78	1083.00 ±155.06	750.50 ±32.17	815.75 ±21.03

Data represent means ± SD of 4 replicates

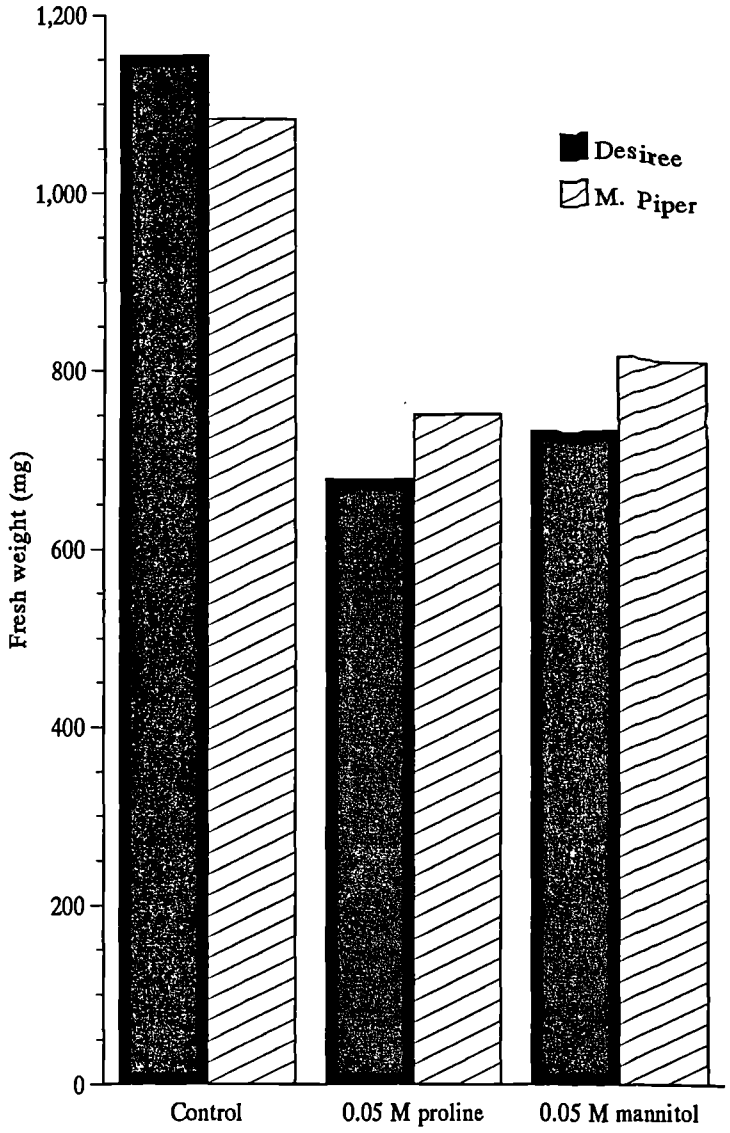


Fig. 72. Fresh weights (mg) of *S. tuberosum* cvs. Desiree and M. Piper leaf calluses on MS medium + 3mg/l 2,4-D as control, and on this medium with 0.05 M proline or 0.05 M mannitol, after 2 weeks of culture. All original explants were approx. 225 mg in weight.

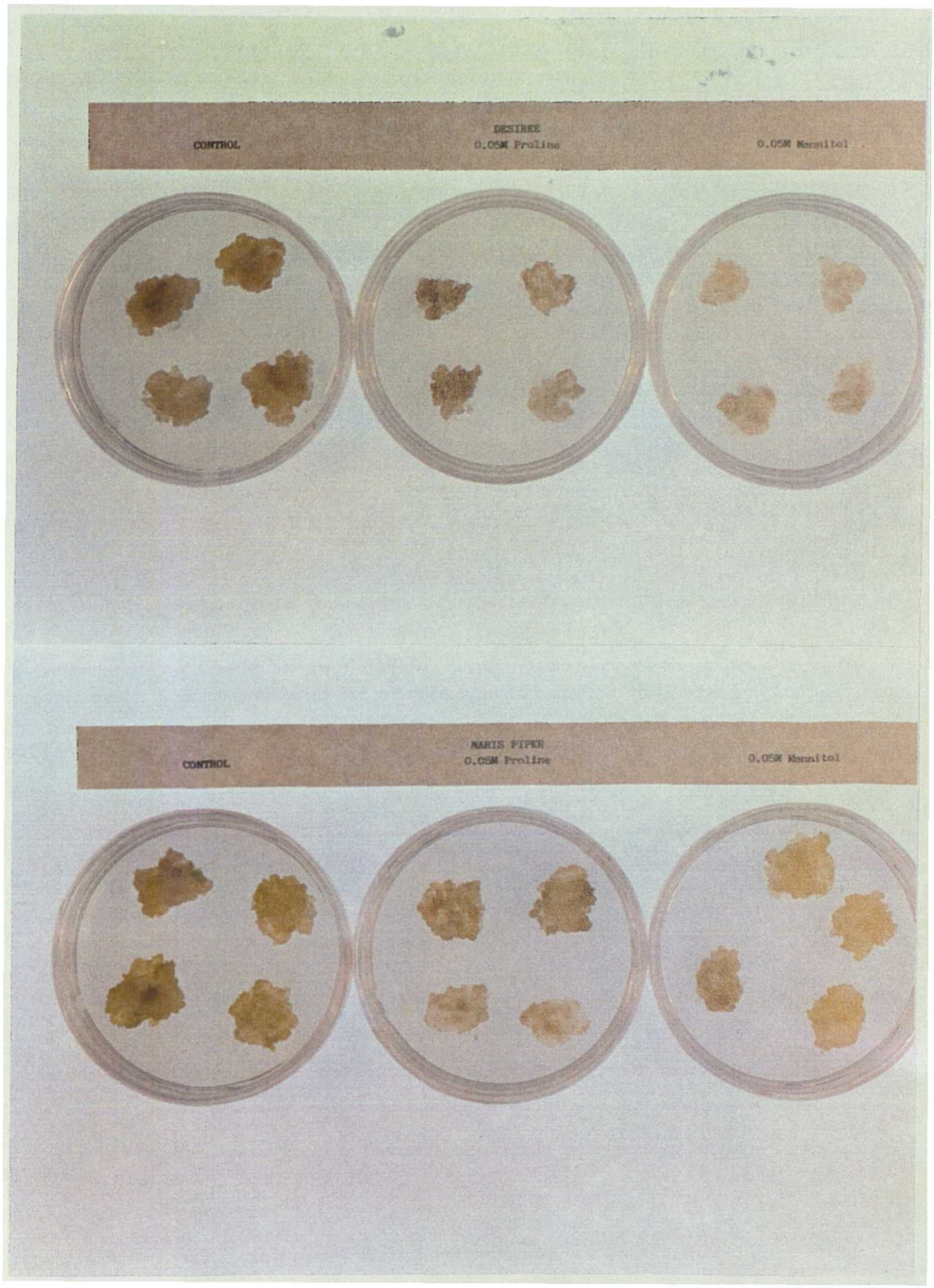


Fig. 73. *S. tuberosum* cvs. Desiree and Maris Piper leaf calluses, subcultured on MS medium + 3 mg l^{-1} 2,4-D as control, and on the same medium with 0.05 M proline or mannitol. Photographed after 2 weeks. (Petri dish Ø 90 mm)

Table 44. Effect of exogenously-applied proline and mannitol on frost-killing temperatures (°C) of *S. tuberosum* leaf calluses. Frost tolerance was determined (see Section 2.14.2) two weeks after subculturing the calluses onto MS medium plus 3 mg l^{-1} 2,4-D as control, and onto the same medium containing 0.05 M proline or 0.05 M mannitol.

Treatment	Desiree	Maris Piper
Control	-2.8	-2.8
0.05 M Proline	-3.8	-3.9
0.05 M Mannitol	-2.9	-2.8

containing proline than on that containing mannitol. Moreover, Maris Piper calluses proved to be more tolerant to stress produced by proline or mannitol, and reduction in their fresh weights was less on these media compared with Desiree calluses.

3.8.2.4 Effect of proline on frost tolerance of potato callus

After 2 weeks of callus growth on control medium and medium containing proline or mannitol, pieces of callus were removed and their frost tolerance was determined (see Materials and Methods). Frost killing-temperature values (°C) are given in Table 44. Addition of mannitol to the medium did not affect the frost tolerance of calluses, while addition of proline to the medium resulted in an increased frost tolerance of callus cells. FKT values (see Section 2.14.2) were 1.0 - 1.1 degrees lower when calluses were grown on medium containing proline compared with the control.

3.8.2.5 Effect of exogenously-applied proline on hyp tolerance of calluses

After 2 weeks of growth on medium containing 0.05 M proline, calluses were subcultured onto media containing 10, 20 or 30 mM hyp. A control medium without hyp was also included and calluses were also subcultured on this medium for comparison of growth. Callus growth was recorded over a period of 4 weeks on the basis of their fresh weight measurements. Results are given in Table 45, and growth curves of the calluses of cvs. Desiree and Maris Piper are plotted against time in Fig. 74 and 75.

Growth of calluses of both Desiree and Maris Piper was markedly reduced due to the hyp present in the solidified nutrient medium. As the hyp concentration increased,

Table 45. Changes in fresh weights of *S. tuberosum* cvs. Desiree and Maris Piper leaf calluses subcultured on MS medium plus 3 mg l^{-1} 2,4-D as control, and on the same medium with a range of hydroxyproline concentrations (10 - 30 mM). Calluses were first grown for 2 weeks on MS medium containing 3 mg l^{-1} 2,4-D and 0.05 M proline, and then subcultured onto the above media.

Days of culture	Desiree				Maris Piper			
	Control	10 mM	20 mM	30 mM	Control	10 mM	20 mM	30 mM
0	224.7 ±5.9	224.5 ±10.2	224.2 ±9.6	222.0 ±12.5	225.0 ±7.4	224.2 ±5.6	223.2 ±10.3	225.2 ±7.0
7	559.9 ±38.3	356.2 ±14.1	354.2 ±26.7	357.0 ±24.9	503.5 ±42.3	332.7 ±19.5	302.7 ±20.7	317.7 ±30.4
14	1136.2 ±76.0	530.5 ±37.5	482.0 ±31.7	475.7 ±29.4	981.0 ±63.4	508.2 ±36.0	495.5 ±55.0	497.0 ±39.5
21	2189.7 ±140.1	692.7 ±49.6	636.2 ±88.4	565.5 ±40.2	1847.0 ±101.6	667.2 ±45.2	665.7 ±21.3	620.5 ±87.3
28	2628.7 ±181.1	924.2 ±91.8	726.0 ±82.4	650.0 ±32.6	2178.7 ±139.8	828.7 ±50.7	671.7 ±65.9	645.5 ±82.4

Data represent means ± SD of 4 replicates

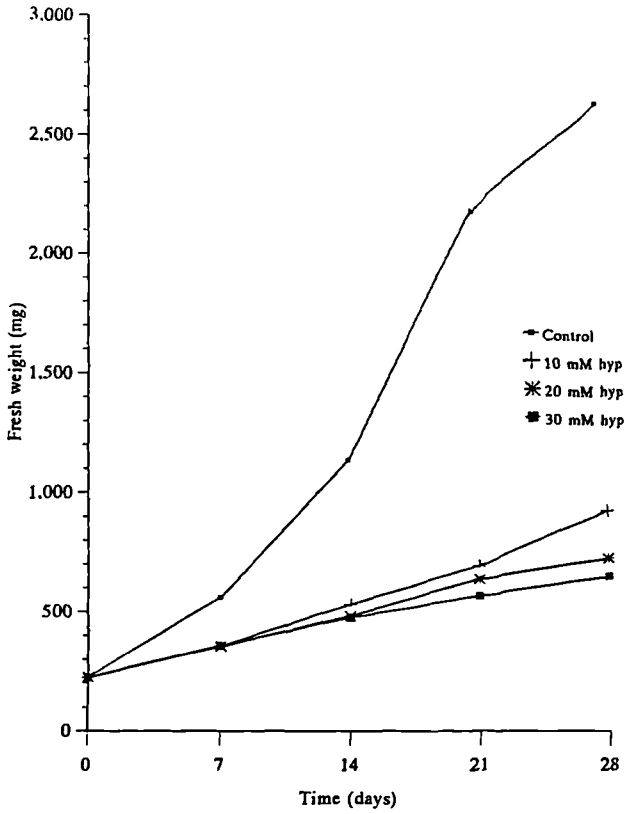


Fig. 74. Growth of *S. tuberosum* cv. Desiree leaf callus on MS medium + 3 mg/l 2,4-D with a range of hydroxyproline concentrations. Subcultured after growing for 2 weeks on medium containing 0.05 M proline.

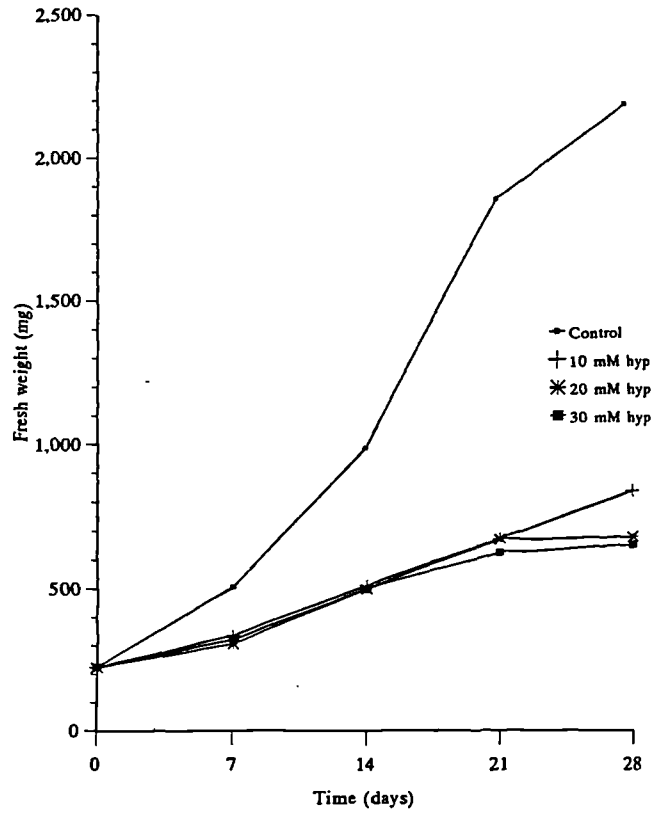


Fig. 75. Growth of *S. tuberosum* cv. M. Piper leaf callus on MS medium + 3 mg/l 2,4-D with a range of hydroxyproline concentrations. Subcultured after growing for 2 weeks on medium containing 0.05 M proline.

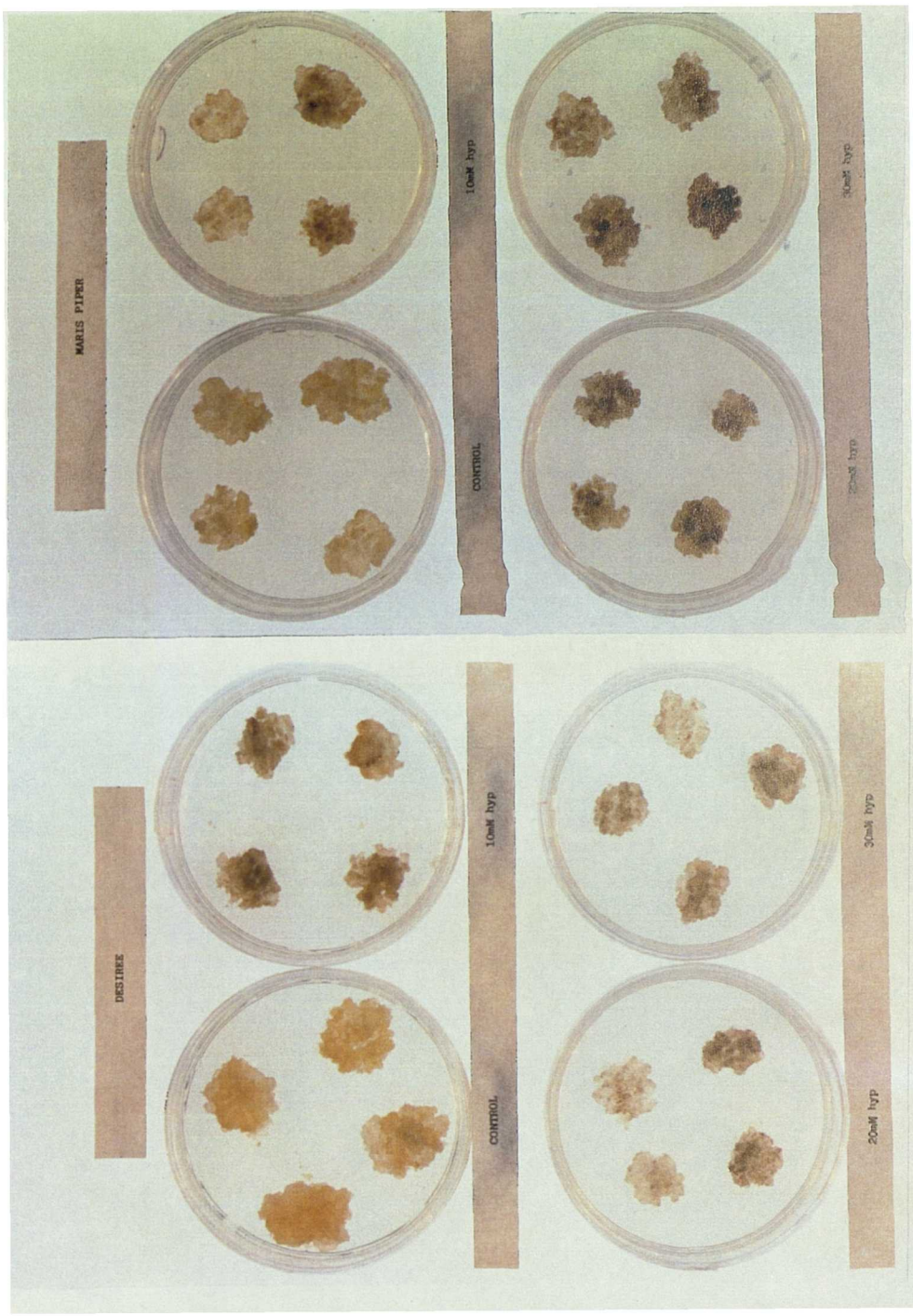


Fig. 76. *S. tuberosum* cvs. Desiree and Maris Piper leaf calluses, grown on MS medium + 3 mg l^{-1} 2,4-D + 0.05 M proline for 2 weeks and subcultured onto MS medium + 3 mg l^{-1} 2,4-D as control, and onto the same medium with 10, 20 and 30 mM hydroxyproline. Photographed after 4 weeks. (Petri dish Ø 90 mm)

growth of calluses became reduced in both Desiree and Maris Piper calluses. Fig. 76 shows the growth of Desiree and Maris Piper calluses on the medium containing a range of hyp concentrations.

3.8.2.6 Selection of hyp-resistant lines from calluses grown on medium containing proline

Callus cultures on medium with various hyp concentrations were subcultured up to 4 passages at 4 week intervals. Growth of calluses was dramatically reduced after each subculture, some of the calluses became necrotic and died. Only growing calluses were subcultured onto fresh medium of the same composition. After 4 subcultures, most of the calluses were dead except some sectors which probably arose from variant cells tolerant to hyp. These sectors were subcultured individually onto fresh media of same compositions. After 4 further subcultures, only a few lines were still growing well. These were selected, maintained through subcultures, and were denoted as follows.

	hyp concentration		
Potato cultivar	10 mM	20 mM	30 mM
Desiree	D10-4	D20-4	D30-2
	D10-5	D20-5	D30-3
	D10-6		
	D10-7		
Maris Piper	M10-2	M20-3	M30-1
	M10-3		
	M10-4		

3.8.3 Experiments with suspension-cultured cells

3.8.3.1 Selection of *hyp-resistant cell lines from suspension-cultured cells*

A. Direct Selection

When leaf-derived cell suspensions of *S. tuberosum* cvs. Desiree and Maris Piper were plated on Lam (1977b) cell plating medium containing 5 or 10 mM hyp, growth of cells was completely inhibited. From 40 petri dishes prepared (10 for each hyp concentration per cultivar), a few cells were able to grow to form cell colonies. After 6 weeks, these colonies were transferred onto fresh medium containing the same concentration of hyp. Upon subculture, some colonies failed to survive, but those which grew well were subcultured at 4 week intervals onto fresh medium. After 4 subcultures the following hyp-resistant cell lines were obtained.

	Hyp concentration	
Potato cultivar	5 mM	10 mM
Desiree		D10-8
		D10-9
		D10-11
Maris Piper	M5-1	M10-5

The frequency of hyp-resistant cell lines was calculated using the following formula.

$$\text{Frequency of resistant cell lines} = \frac{\text{No. of hyp-resistant cell lines}}{\text{Total number of cells plated}}$$

Out of 2×10^7 cells plated, only five colonies were found to be hyp-resistant and had been selected. Therefore the frequency of hyp-resistant cell lines was only 0.25×10^{-6}.

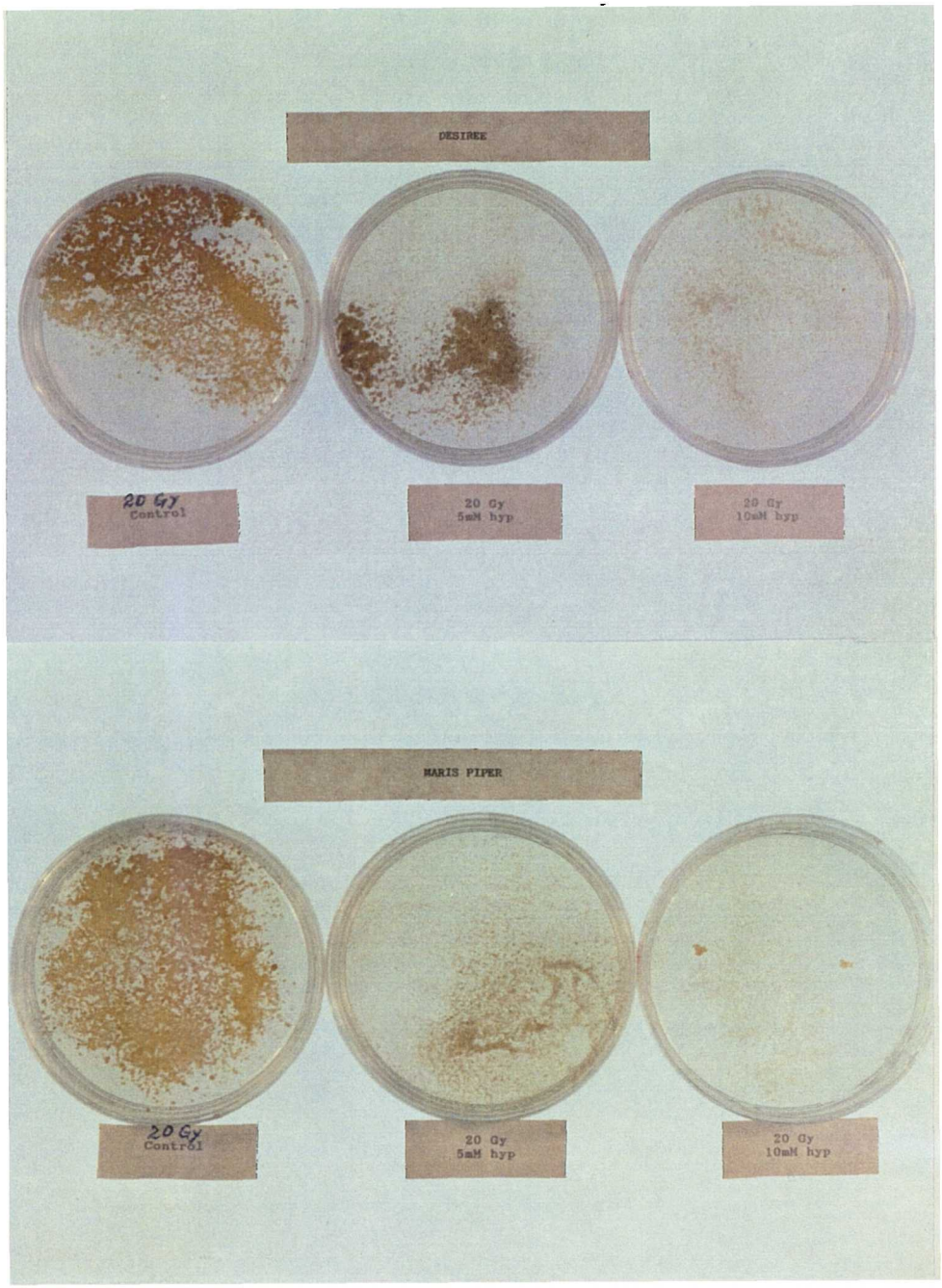


Fig. 77. *S. tuberosum* cvs. Desiree and Maris Piper suspension-cultured cells, irradiated to a dose of 20 Gy and plated on Lam (1977b) cell plating medium as control, and on the same medium with 5 and 10 mM hydroxyproline. Photographed after 4 weeks. (Petri dish Ø 90 mm)

B. Selection after mutagenic treatment

Mutagenic treatment was carried out with gamma rays using a low dose of 20 Gy that was not found to affect the cell viability or growth after plating. Irradiated cell suspensions of *S. tuberosum* cvs. Desiree and Maris Piper were plated on Lam (1977b) cell plating medium containing 5 or 10 mM hyp at a plating density of 0.5 x 10^6 cells ml^{-1}. After 6 weeks only a few colonies were found growing and were transferred onto MS medium containing the same concentration of hyp. A total of seven colonies, four on medium containing 5 mM hyp and three on medium containing 10 mM hyp were found growing, and hence the frequency of hyp-resistant colonies recorded was 0.35 x 10^{-6}. Hyp-resistant colonies were denoted as follows.

	Hyp concentration	
Potato cultivar	5 mM	10 mM
Desiree	D5-1	D10-12
	D5-2	
Maris Piper	M5-2	M10-6
	M5-3	M10-7

C. Selection after freezing treatment

Leaf-derived cell suspensions of *S. tuberosum* cvs. Desiree and Maris Piper were frozen to -6°C, held at this temperature for at least one hour, raised to 4°C and plated on solidified Lam (1977b) cell plating medium. At least 10 petri dishes were prepared for each Desiree and Maris Piper. Growth of cells was inhibited at an early stage, but later small cell colonies started to grow. Eight colonies were isolated and four of these

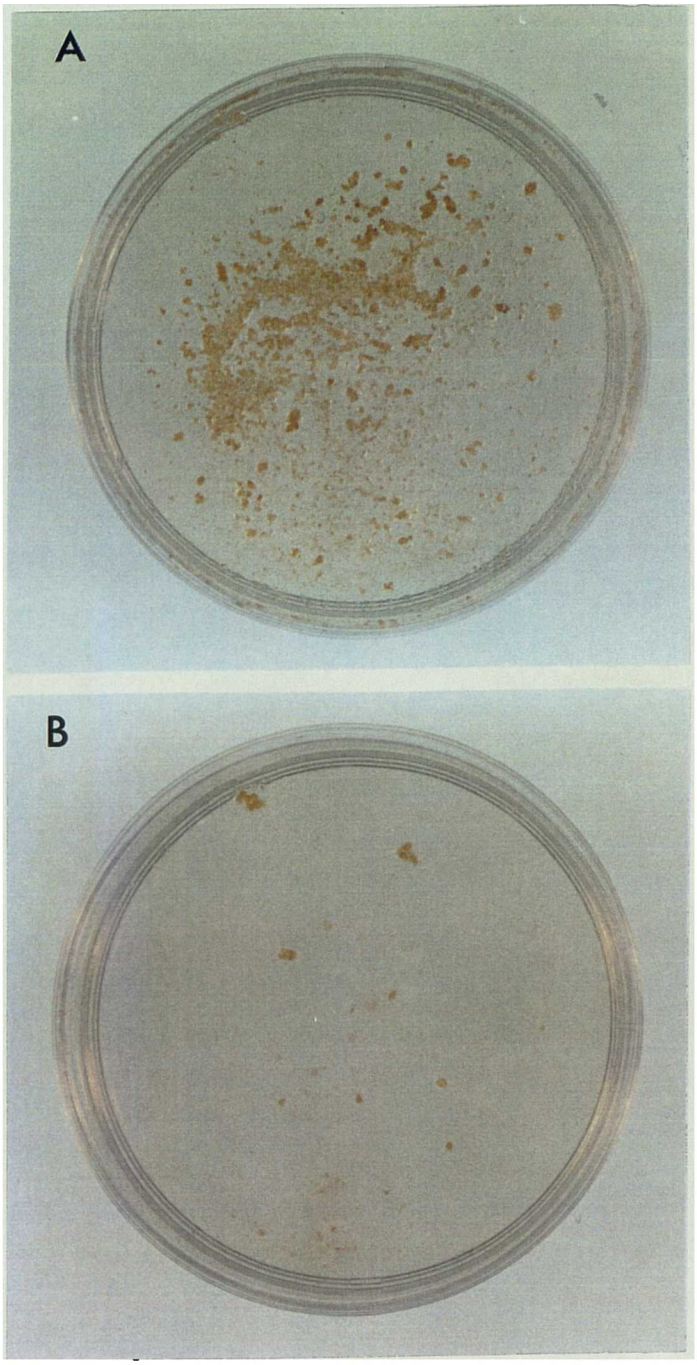


Fig. 78. Growth of *S. tuberosum* cv. Desiree suspension-cultured cells, plated on Lam (1977b) cell plating medium. Photographed after 4 weeks. (Petri dish Ø 90 mm)

A. Cell suspensions (untreated), plated on this medium.

B. Cell suspensions, frozen to -6°C and then plated.

were transferred to medium containing 5 mM hyp, while the other 4 were transferred to medium containing 10 mM hyp. Only two colonies survived and these were denoted as follows.

	Hyp concentration	
Potato cultivar	5 mM	10 mM
Desiree	D5-3	
Maris Piper		M10-8

The frequency of hyp-resistant lines was only 0.2×10^{-6}.

3.8.4 Frost tolerance of selected cell lines

When hyp-resistant cell lines were well-established, 15 of them were used for determination of frost tolerance by using the TTC-viability assay of Towill and Mazur (1975) (see Materials and Methods). FKT values (°C) of these hyp-resistant lines and their controls are given in Table 46.

Most of the fifteen hyp-resistant lines tested for frost tolerance showed higher frost tolerance than non-selected controls (Table 46). Two had nearly identical FKT values while two lines showed less frost tolerance compared to controls. Among the eight lines selected from Desiree callus, six were found better, with lower FKT (°C) values, than control, while among the seven lines selected from Maris Piper, five were better than control with greater frost tolerance. Two lines, D20-1 and D30-1 were found to be more frost-tolerant than others and therefore were used for further work.

Table 46. Frost-killing temperatures (°C) of selected hyp-resistant cell lines and non-selected controls. Frost tolerance was determined (see Section 2.14.2) using the TTC-viability assay of Towill and Mazur (1975).

Genotype/cell line	FKT (°C)	Genotype/cell line	FKT (°C)
Desiree (control)	-2.8	M. Piper (control)	-2.9
D5-1	-3.2	M5-1	-2.7
D5-3	-2.8	M5-2	-3.5
D10-5	-3.7	M10-4	-3.1
D10-8	-2.6	M10-6	-3.9
D20-1	-4.2	M20-2	-2.9
D20-2	-4.0	M20-3	-4.1
D30-1	-4.5	M30-1	-3.5
D30-3	-3.4		

Table 47. Changes in fresh weights of hyp-resistant calluses transferred to the normal (hyp-free) medium compared with similar calluses transferred from hyp-containing media to hyp-containing media.

Time (days)	D20-1		D30-1	
	20 mM hyp maintained on 20 mM hyp	20 mM hyp transferred to hyp-free	30 mM hyp maintained on 30 mM hyp	30 mM hyp transferred to hyp-free
0	200 ± 12.35	200 ± 10.25	200 ± 9.00	200 ± 7.38
7	276 ± 26.24	319 ± 35.31	285 ± 23.79	294 ± 28.35
14	543 ± 48.22	653 ± 60.71	521 ± 35.41	576 ± 39.29
21	971 ± 87.67	1197 ± 102.26	923 ± 78.78	1132 ± 98.12
28	1463 ± 85.89	1603 ± 107.42	1311 ± 94.90	1574 ± 92.46

Data represent means ± SD of 4 replicates

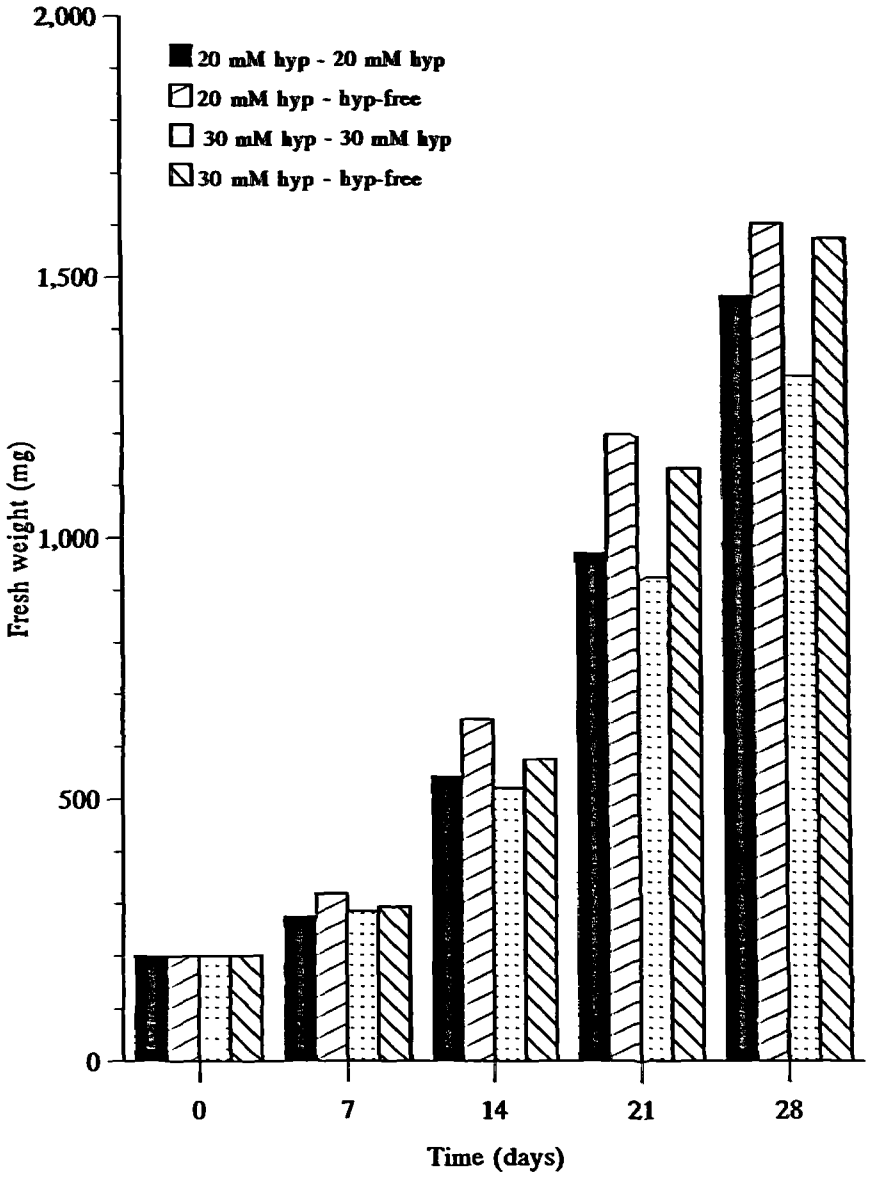


Fig. 79. Growth of hyp-resistant calluses on hyp-containing media, and when transferred to hyp-free medium.

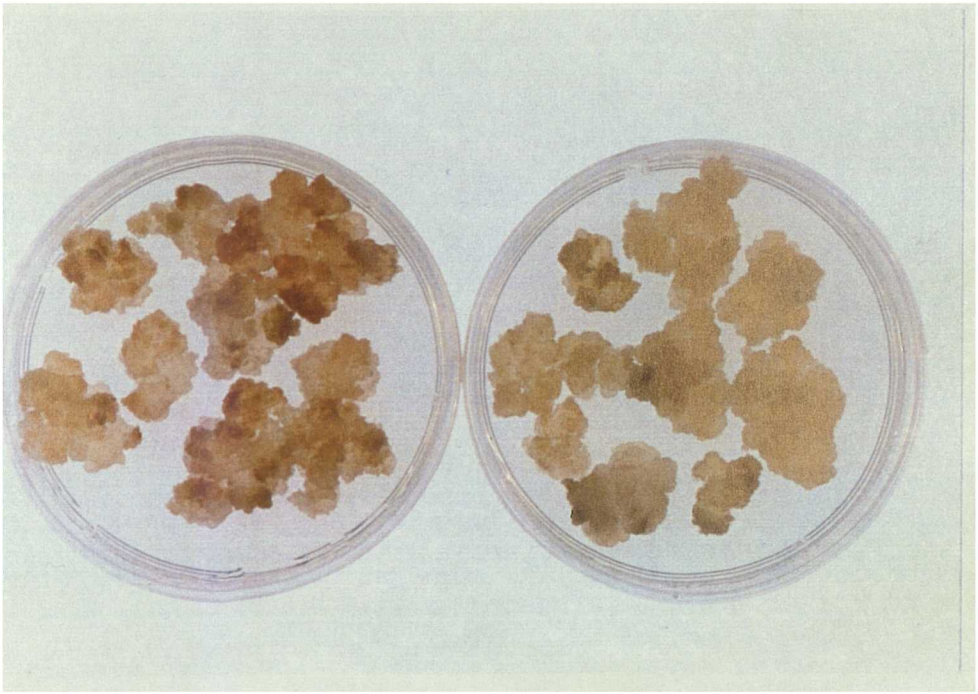


Fig. 80. Growth of hyp-resistant cell line D20-1 callus on MS medium + 3 mg l^{-1} 2,4-D + 20 mM hydroxyproline (left), and on the same medium but without hydroxyproline (right). Photographed after 4 weeks. (Petri dish Ø 90 mm)

3.8.5 Stability of hyp-resistant cell lines

3.8.5.1 Through subculturing on hyp-free medium

To discover whether the hydroxyproline tolerance in the selected cell lines on hyp-containing media could be permanent or just a temporary adjustment to cellular metabolism, the two lines D20-1 and D30-1, which had been selected to grow on media containing hyp, were transferred to the normal (hyp-free) medium.

The growth of D20-1 cell line on medium containing 20 mM hyp and that of D30-1 on medium containing 30 mM hyp, and when both were transferred to hyp-free medium are given in Table 47. Fig. 79 shows that hyp-resistant lines (D20-1 and D30-1) selected on media containing hyp by subculturing through several passages with 4 week intervals in each subculture, when transferred to the normal (hyp-free) medium grew better compared with those maintained throughout on hyp-containing medium. After 2 subcultures on normal medium, the degree of frost tolerance of these lines was determined, the FKT values obtained were -4.0°C for D20-1 and -4.5°C for D30-1. It appears that line D30-1 had maintained its frost tolerance level, while the frost tolerance of D20-1 was decreased and FKT value changed from -4.2°C to -4.0°C.

The callus cultures which had been transferred to the normal medium were subcultured twice on the normal medium and then taken back to the hyp-containing media. The growth of these cultures was then compared with those maintained on hyp-containing media throughout. All cultures, including controls and transfers, were subcultured at the usual intervals of time onto fresh media. The results are given in Table 48.

Table 48. Changes in fresh weights of hyp-resistant calluses transferred to the normal (hyp-free) medium and returned to hyp-containing media, compared with similar calluses transferred throughout from hyp-containing media to hyp-containing media.

Time (days)	D20-1		D30-1	
	20 mM hyp - 20 mM hyp	20 mM hyp - hyp-free - 20 mM hyp	30 mM hyp - 30 mM hyp	30 mM hyp - hyp-free - 30 mM hyp
0	200 ± 11.51	200 ± 10.79	200 ± 8.66	200 ± 10.89
7	308 ± 22.42	254 ± 24.18	296 ± 30.35	263 ± 33.76
14	602 ± 37.43	421 ± 45.47	482 ± 48.31	407 ± 38.94
21	1064 ± 68.96	793 ± 82.16	824 ± 73.46	673 ± 79.36
28	1494 ± 116.15	1132 ± 123.11	1256 ± 118.09	1051 ± 101.07

Data represent means ± SD of 4 replicates

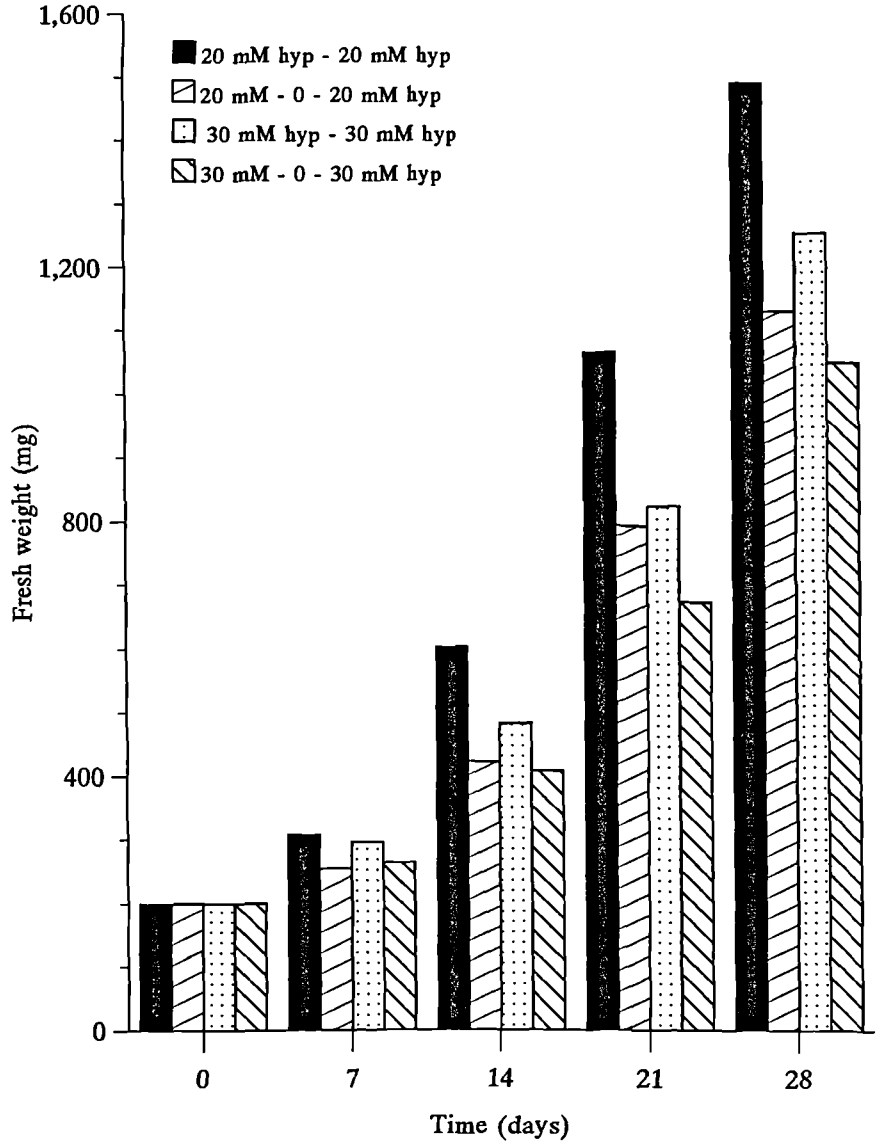


Fig. 81. Growth of hyp-resistant calluses maintained throughout on hyp-containing media, and of similar calluses when returned to hyp-containing media after 2 subcultures on hyp-free medium.

The reactions to changes in the composition of the nutrient media shown by the cell lines are illustrated in Fig. 81. When the hyp-resistant lines were subcultured onto normal medium and then taken back to hyp-containing media, their growth was depressed and the increases in their fresh weights were lower compared with those maintained throughout on hyp-containing media, and also compared with their growth during two passages on normal medium.

3.8.5.2 Through plant regeneration from hyp-resistant cell lines

Attempts were made to regenerate plantlets from hyp-resistant cell lines D20-1 and D30-1 maintained throughout on hyp-containing media. Growing calluses of these lines were transferred onto the shoot regeneration medium of Iapichino *et al.* (1991) (Med. RB). Upon transfer to the regeneration conditions, most of the calluses became hard and compact, some showed necrosis and died, and only a few callus pieces remained friable. After every 4 weeks, surviving calluses were transferred onto fresh regeneration medium. Within 16 weeks, all the calluses of D30-1 line had become brown, necrotic and died. However, after about 20 weeks from transfer to regeneration medium, dark green nodules appeared from the lower sides of some of the calluses of line D20-1 (Fig. 82). The first shoot appeared 24 weeks after transfer of calluses from hyp-containing medium to hyp-free regeneration medium. About 1 - 2 cm-long regenerated shoots were transferred to the shoot culture medium of Espinoza *et al.* (1986) (Med. NA) for rooting.

Regenerated shoots exhibited wide morphological variation. Abnormal types such as; shoots with very small internodes and rosette growth, shoots with narrow leaves, and

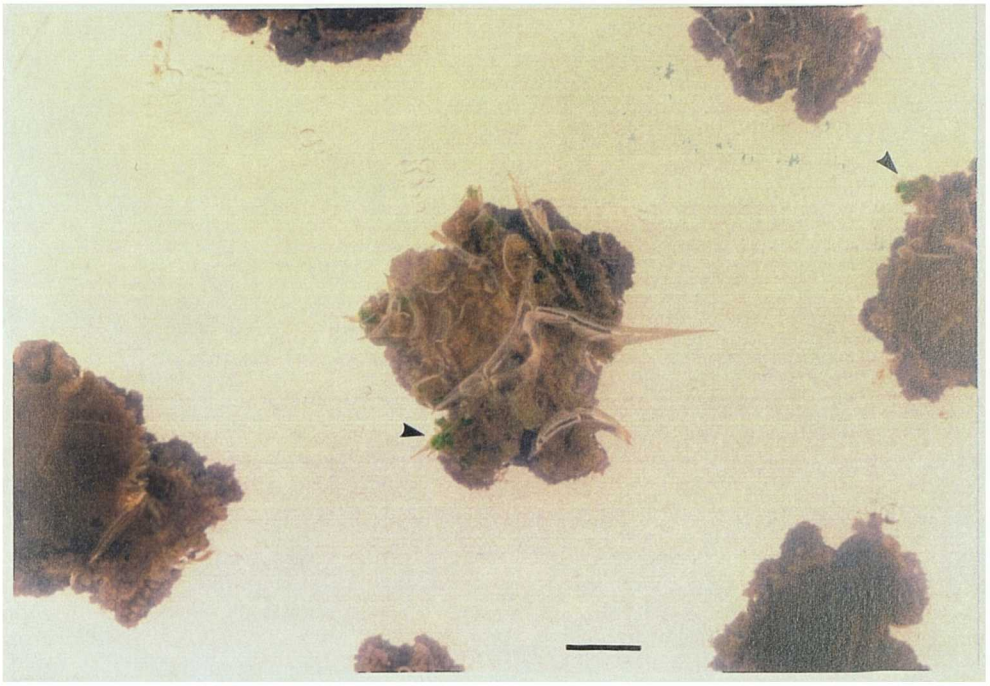


Fig. 82. Shoot primordia (arrow) arising from calluses of hyp-resistant cell line D20-1 on shoot regeneration medium of Iapichino *et al.* (1991) (Med. RB). Photographed after 22 weeks. (Scale bar = 5 mm)

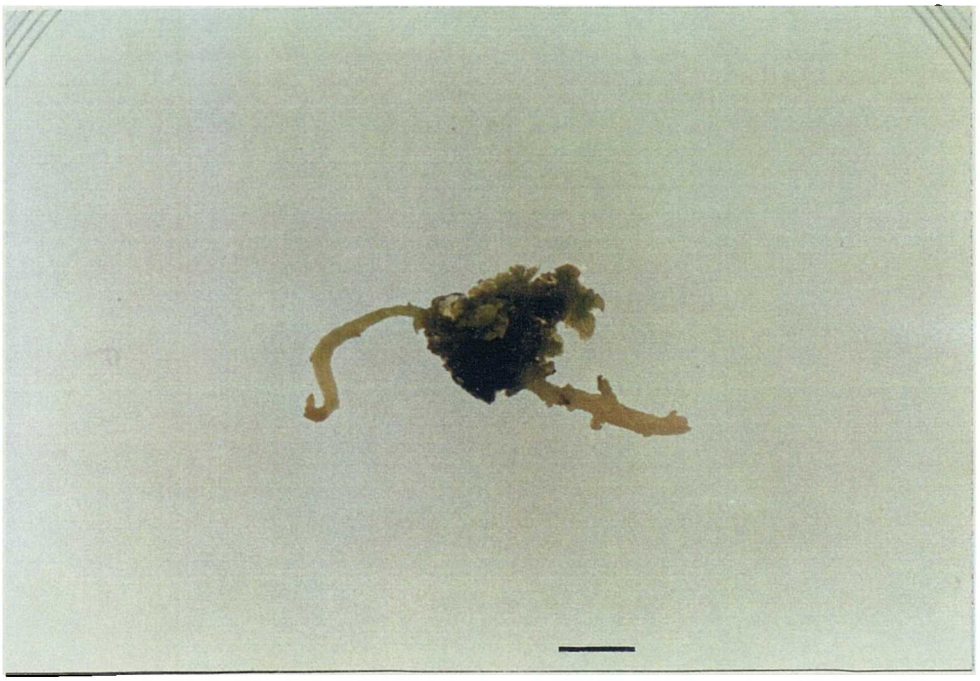


Fig. 83. Shoot regeneration from callus of hyp-resistant cell line D20-1 on shoot regeneration medium of Iapichino *et al.* (1991) (Med. RB). Photographed after 26 weeks. (Scale bar = 5 mm)

Fig. 84. Morphology of shoots regenerated from callus of hyp-resistant cell line D20-1.

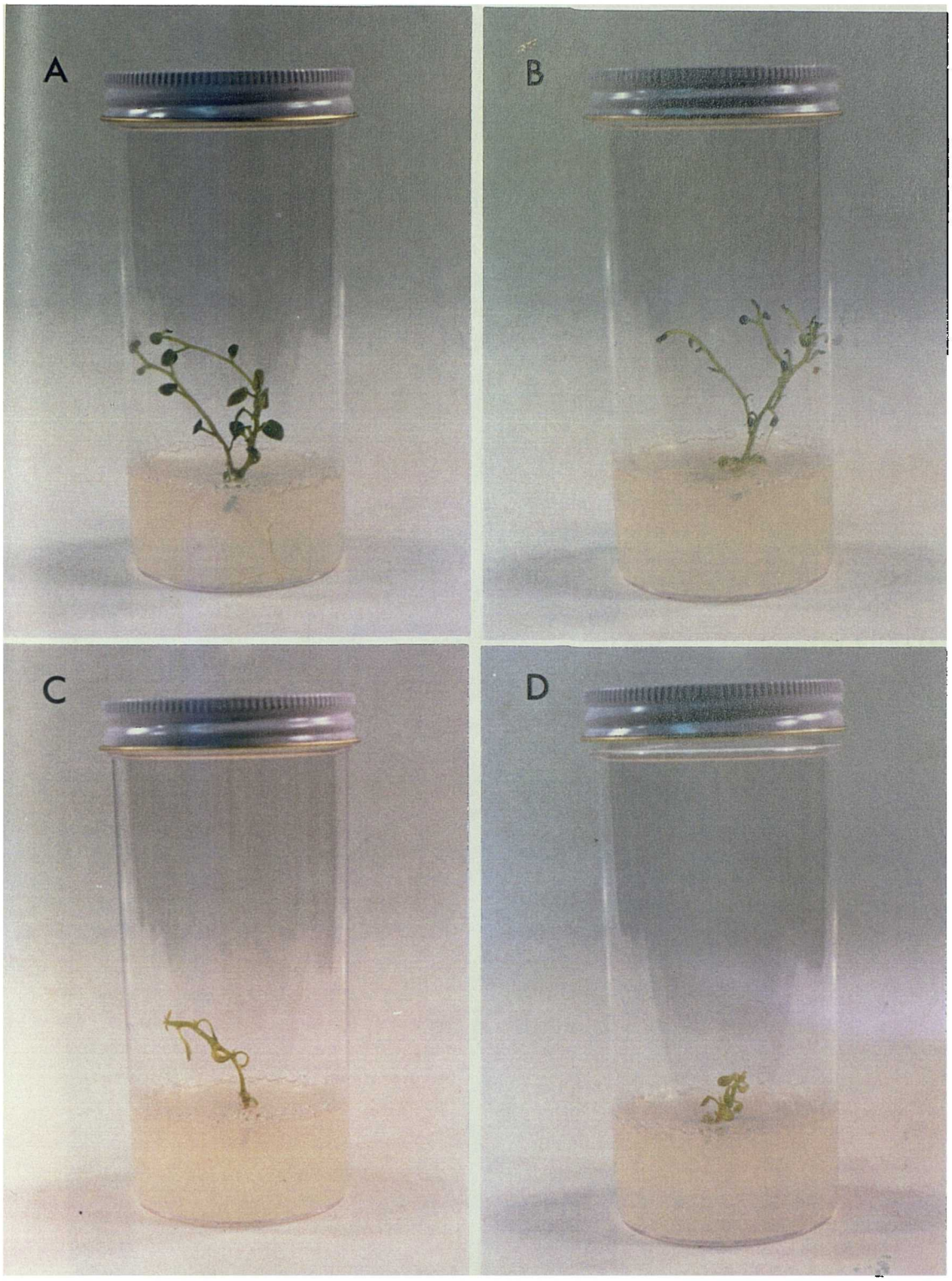


Fig. 85. Examples of shoots regenerated from calluses of hyp-resistant cell line D20-1 and transferred to shoot culture medium of Espinoza *et al.* (1986) (Med. NA) for rooting. Photographed after 2 weeks.

A & B. Roots which were produced can be seen.

C & D. Plantlets, of poor quality, on which no roots were produced.

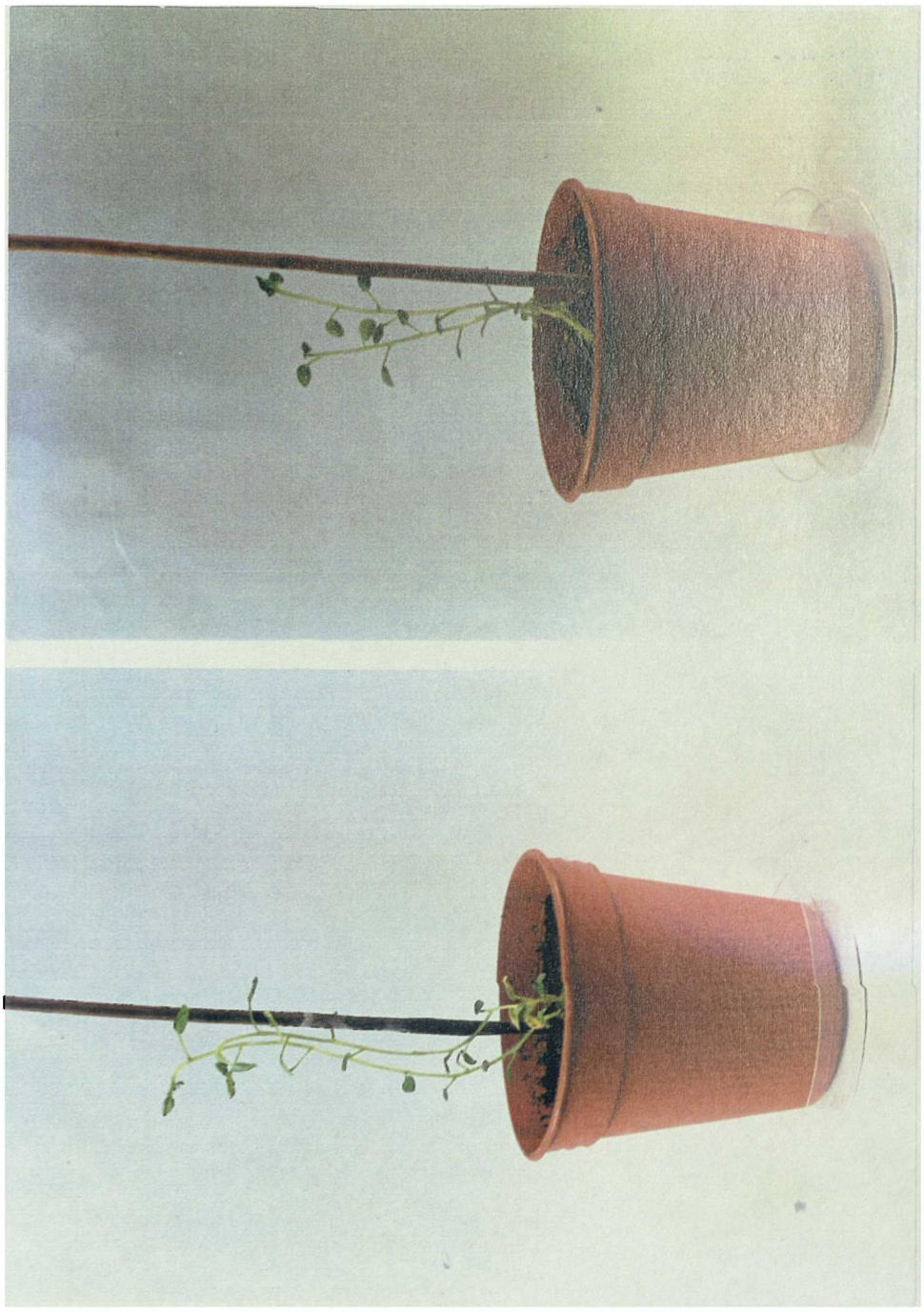


Fig. 86. Plantlets of hyp-resistant line D20-1, transferred to sterilized compost. Photographed after 3 weeks of growth on compost.

shoots with bleached leaves could be differentiated (Fig. 84). None of these abnormal types formed roots upon transfer to the shoot culture medium NA (Fig. 85), but they remained alive even 12 weeks after transfer. Those shoots that formed roots in shoot culture medium NA were transferred to sterilized compost in pots (Fig. 86) and moved to a greenhouse.

To investigate any frost tolerance trait retained by plantlets from cell line D20-1, the degree of frost tolerance of leaves from some of the regenerated plants was determined. FKT values of leaves ranged between -3.4°C and -3.8°C, which is still 0.6 to 1.0°C lower than the control, non-treated plants, but if compared with the frost tolerance of the callus (-4.2°C) from which these plantlets were regenerated, frost tolerance was decreased.

3.9 Cytology of callus cells

Cellular structure of callus cells was investigated a); to compare any ultrastructural differences between frost-sensitive and frost-tolerant *Solanum* species, and between normal and a selected frost-tolerant line, and b); to observe frost damage to the callus cells after subjecting them to freezing temperatures.

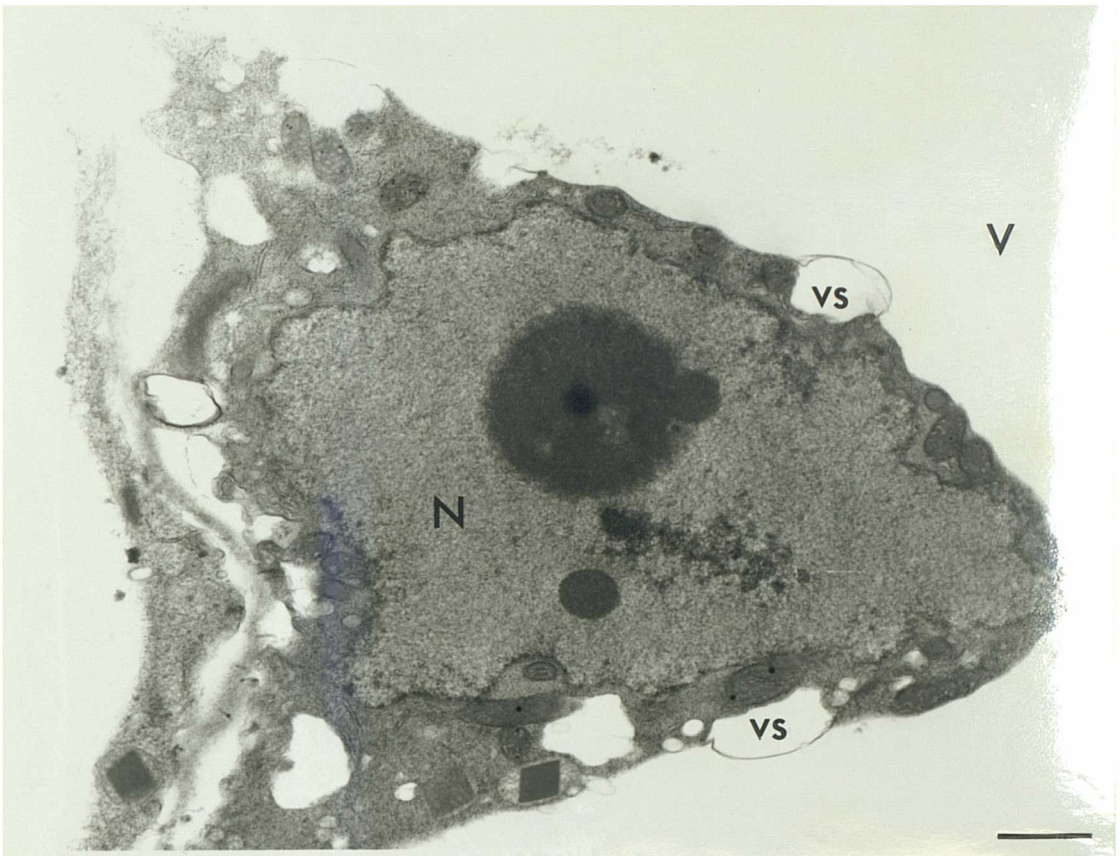
3.9.1 *S. tuberosum* cv. Desiree

Commercial cultivars of potato are frost-sensitive. For cytological investigations, actively-growing callus of *S. tuberosum*, the cv. Desiree was used. It is evident that callus cells contain a very large vacuole (V, Fig. 87 and 89) which occupies the major volume of the cell. The nucleus lies to one side of the cell surrounded by a thin layer

Figures 87 to 92 show examples of the structure of callus cells of *S. tuberosum* cv. Desiree.

Fig. 87. The nucleus (N) is contained within a thin layer of cytoplasm. Vesicles (vs) appear to be discharging into the vacuole (V). (Scale bar = 1 μm)

Fig. 88. General view of nucleus showing a nucleolus with a lightly-stained area, possibly nuclear vacuole. (Scale bar = 2 μm)


V
VS
N
VS

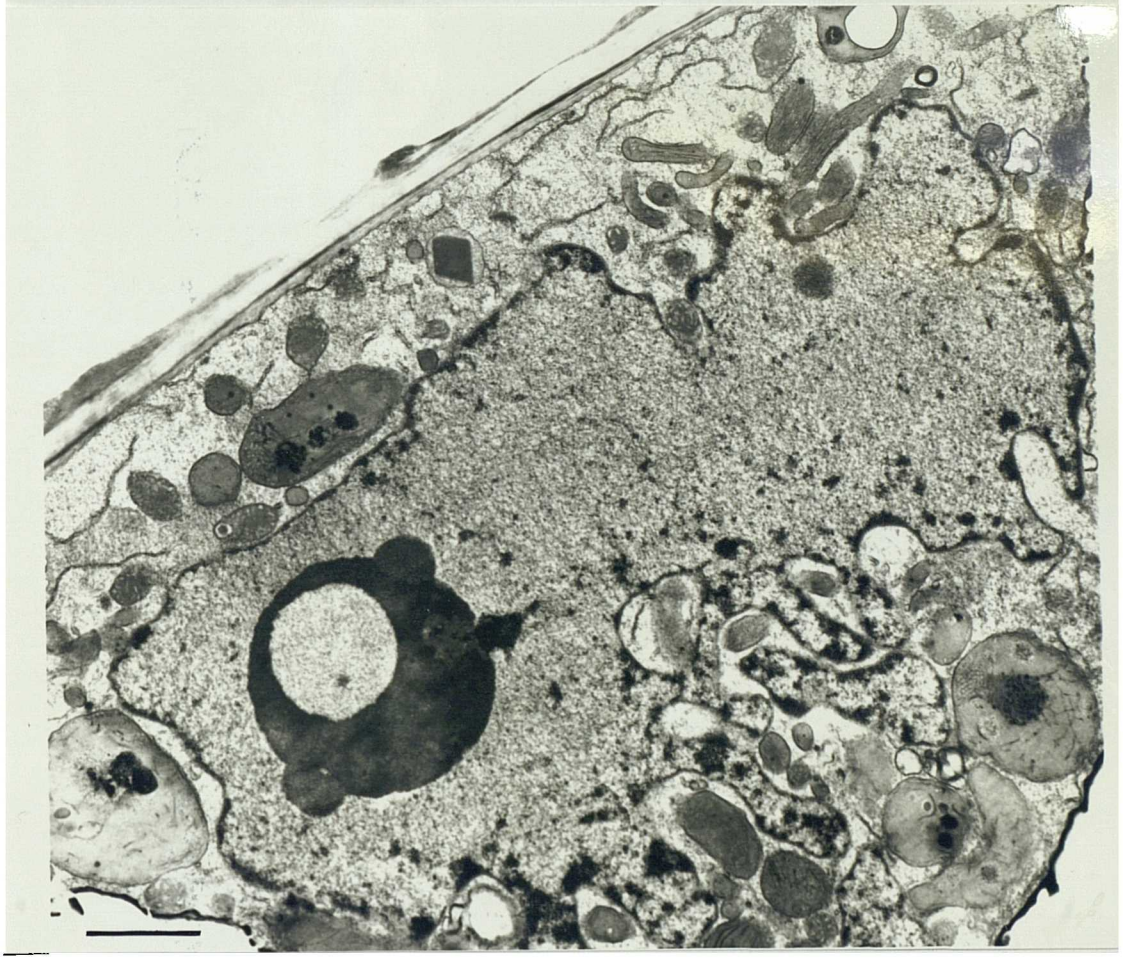
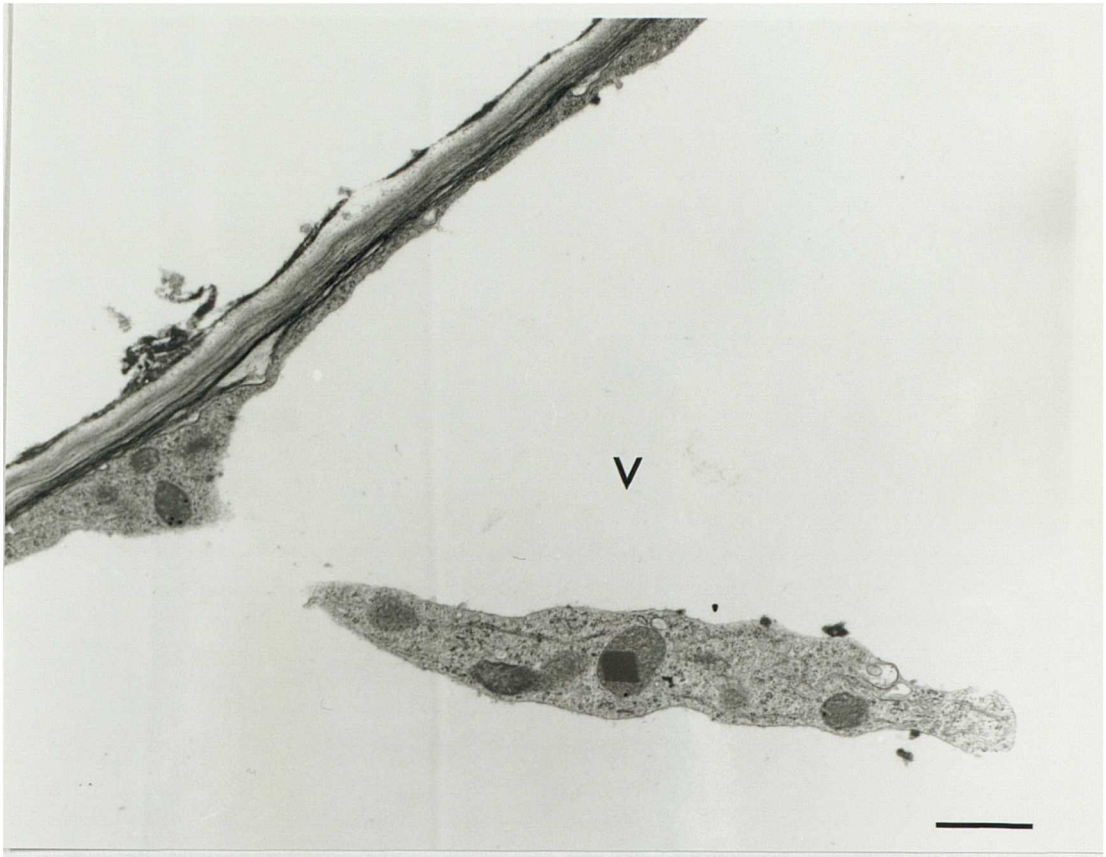


Fig. 89. Section along part of a cytoplasmic strand passing across the vacuole (V). A microbody containing a protein crystal is prominent in the cytoplasm. (Scale bar = 1 μm)

Fig. 90. A part of cell showing the nuclear envelope (ne). Plastids (p), mitochondria (m), dictyosome (d) and ribosomes (r) in the cytoplasm. (Scale bar = 0.2 μm)


V

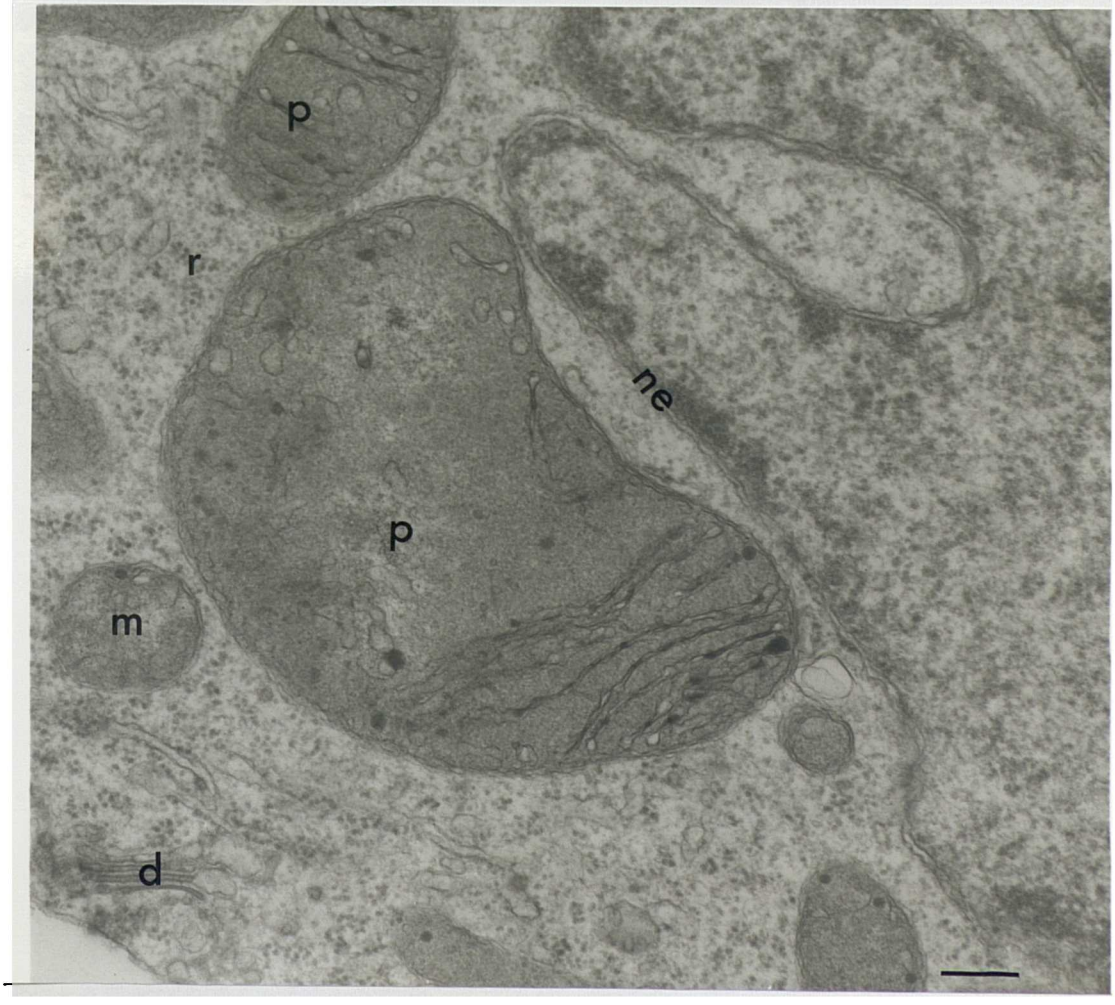
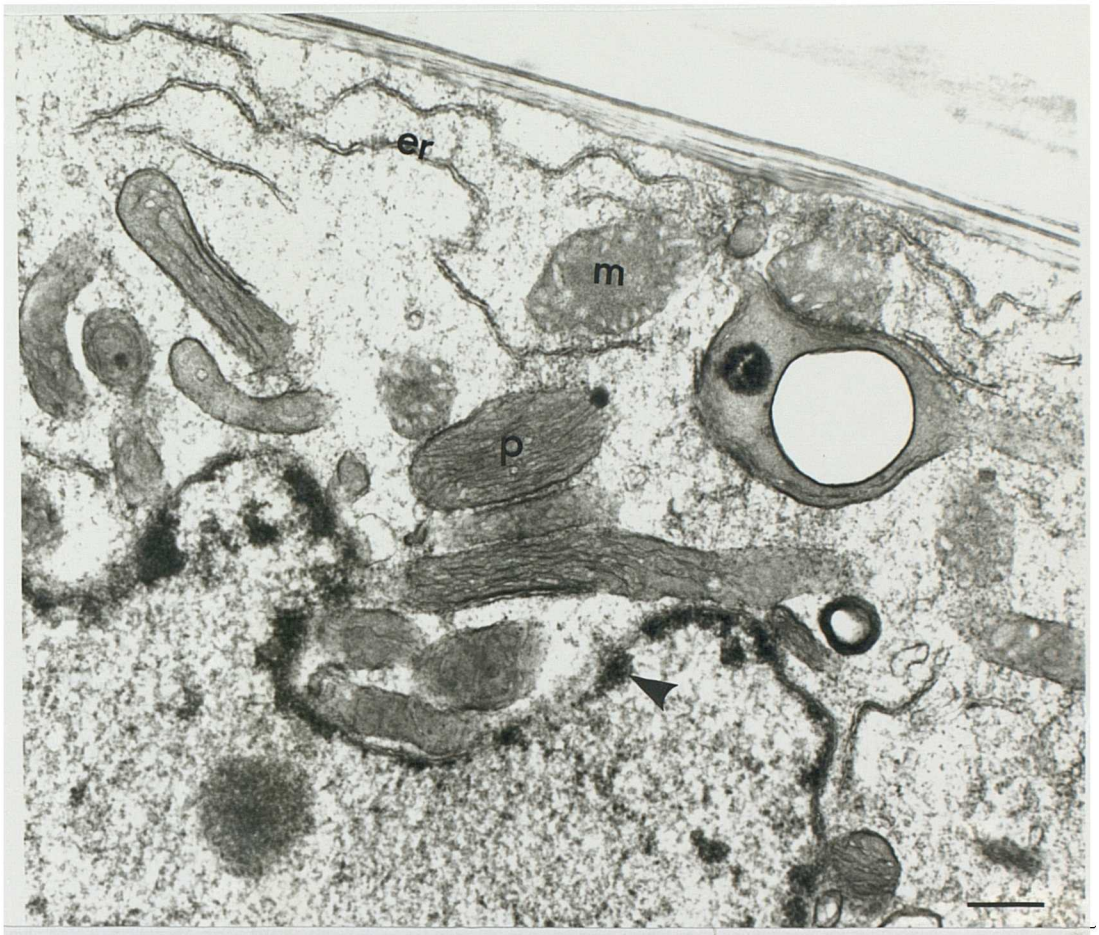
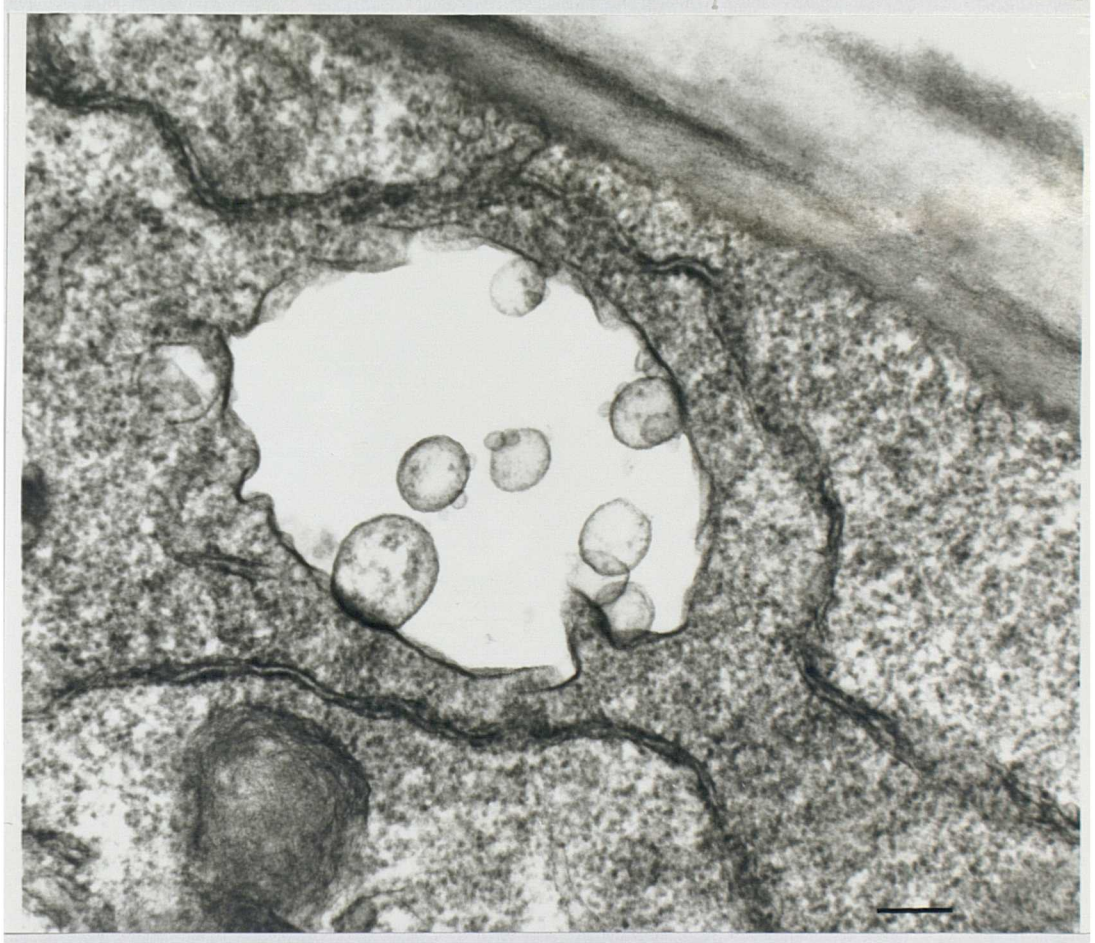

p
r
ne
p
m
d

Fig. 91. View of cytoplasm containing mitochondria (m), plastids (p) and endoplasmic reticulum (er). The association of chromatin with the nuclear envelope can be seen (arrow). (Scale bar = 0.5 μm)

Fig. 92. A small cytoplasmic vacuole with material apparently passing inwards enclosed by membrane as small vesicles. (Scale bar = 0.2 μm)


er
m
p



of cytoplasm, although some pockets of more extensive cytoplasm occur in the vicinity of the nucleus. The size of nucleus appears quite large with usually one nucleolus in view. The nucleus is bounded by the nuclear envelope comprised of two unit membranes (Fig. 90). The shape of the nucleus appears quite variable, from a simple sphere to many-lobed (Fig. 88). The shape and size of nucleus seen would also vary with the plane through which the sections were cut.

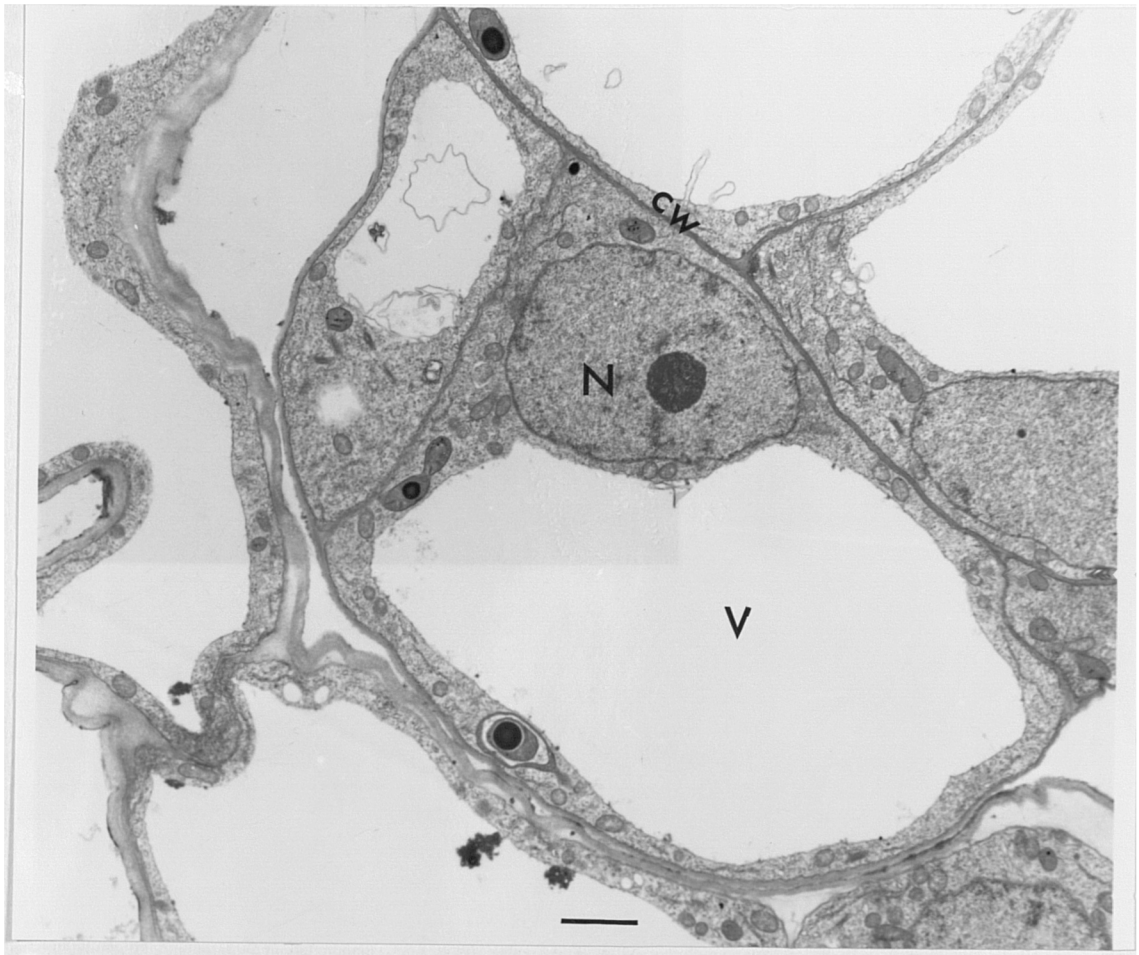
Most of the nuclear volume is occupied by chromatin which forms a dispersed network of material distributed throughout the nuclear sap, with many dense aggregations, especially associated with the nuclear envelope (Fig. 91). The shape of the nucleolus is usually spherical but in the nucleus of some cells, a lobe-shaped nucleolus can be seen (Fig. 88). The nucleolus often contains one or more electron-transparent regions and a dense granular zone. Some nucleoli also contain an area with very lightly-staining properties (Fig. 88) which might be nucleolar vacuoles, regarded as a sign of an active nucleolus. In many sections the nucleolar organizer can be seen (arrow, Fig. 88), as the place of connection with the chromatin material.

The cytoplasm is enclosed by a plasma membrane which is surrounded by the cell wall. The cytoplasm contains different types of organelles, and among these plastids, mitochondria and microbodies are most prominent. Plastids are usually bigger than mitochondria, and their internal membrane systems are sometimes extensive but not well organized (Fig. 90 and 91). The mitochondrion appears to consist of an outer membrane enclosing a inner membrane which is folded into deep invaginations called cristae (Fig. 91). The shape of the mitochondria may appear circular or elliptical

Figures 93 to 96 show examples of the structure of callus cells of *S. commersonii*.

Fig. 93. A general view of a callus cell showing the cell wall (cw), cytoplasm containing different organelles, nucleus (N) with a nucleolus, and vacuole (V). (Scale bar = 2 μm)

Fig. 94. A general view of a nucleus with chromatin surrounded by nuclear envelope (ne). Different organelles are present in the cytoplasm. (Scale bar = 1 μm)


cw
N
V

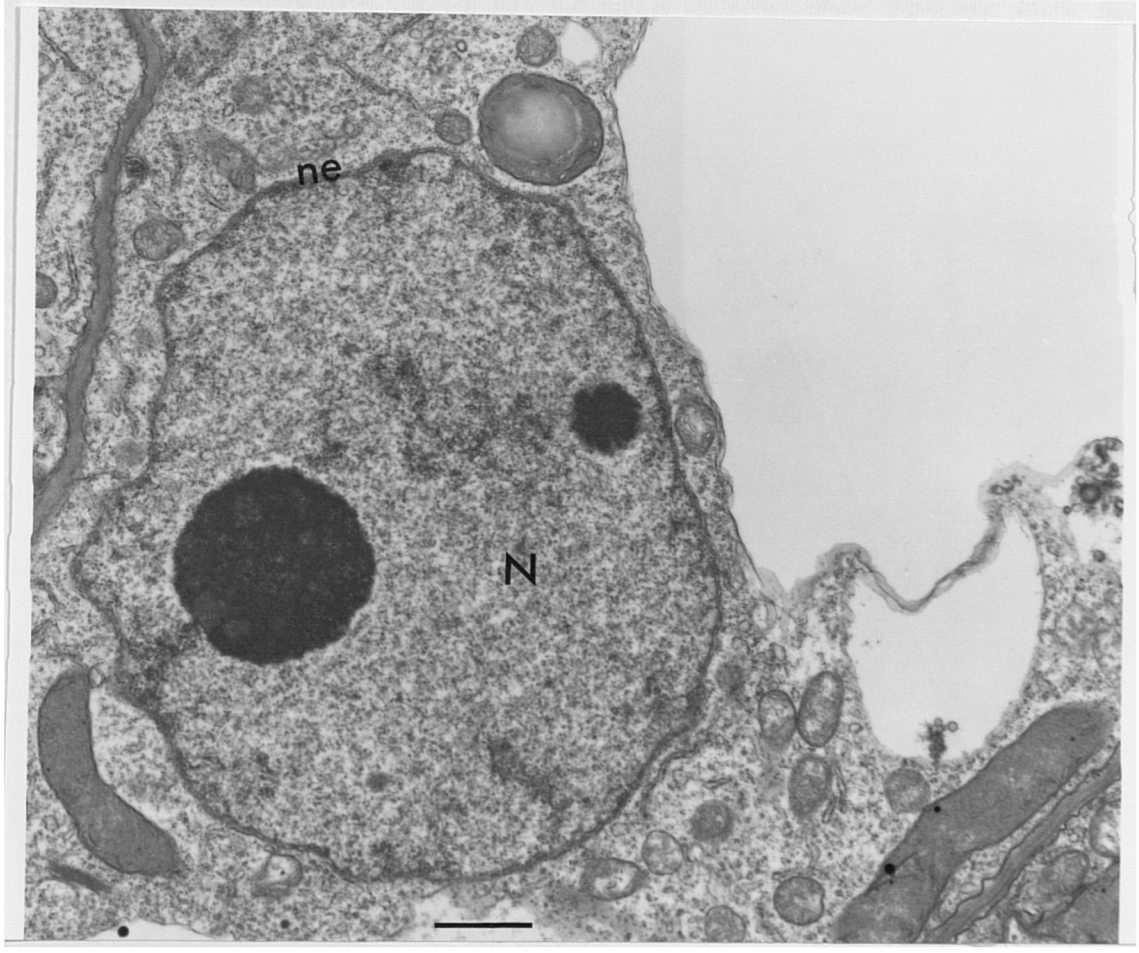
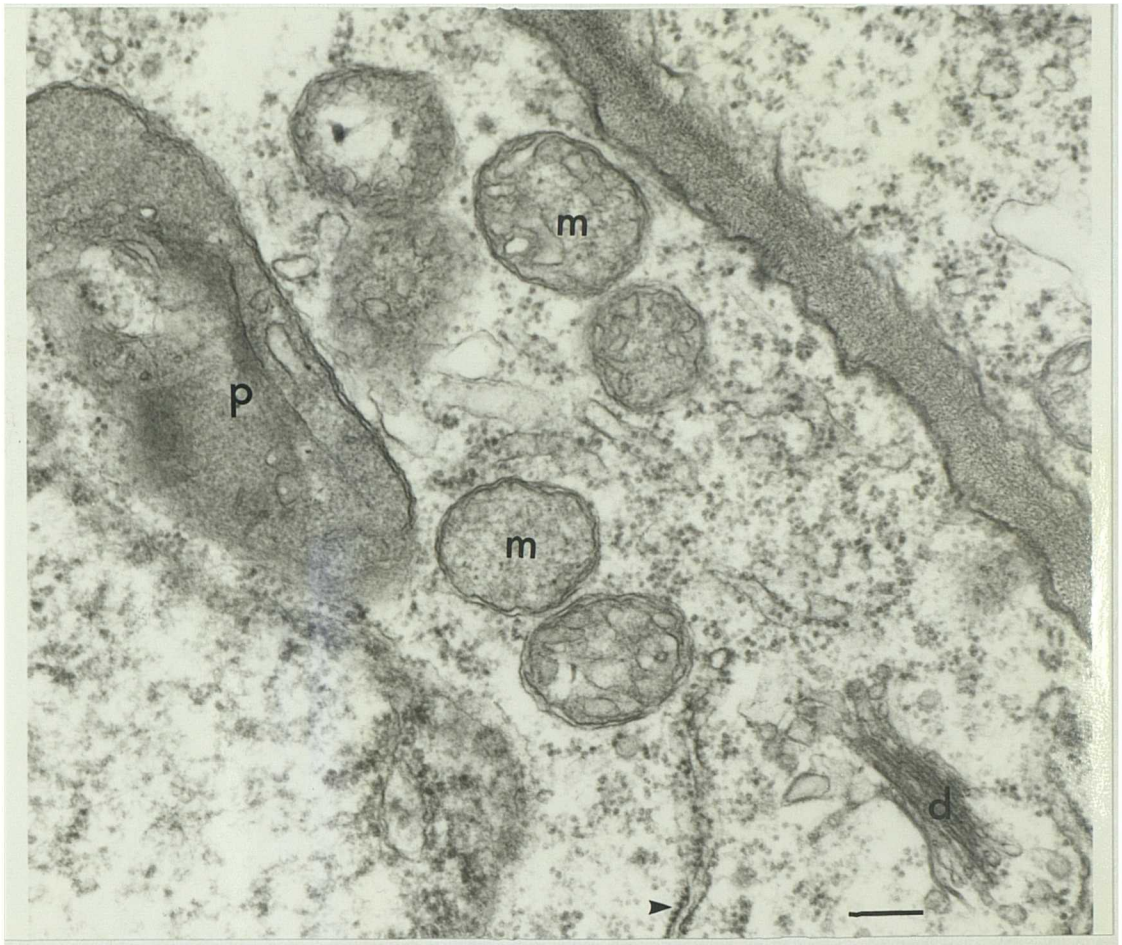
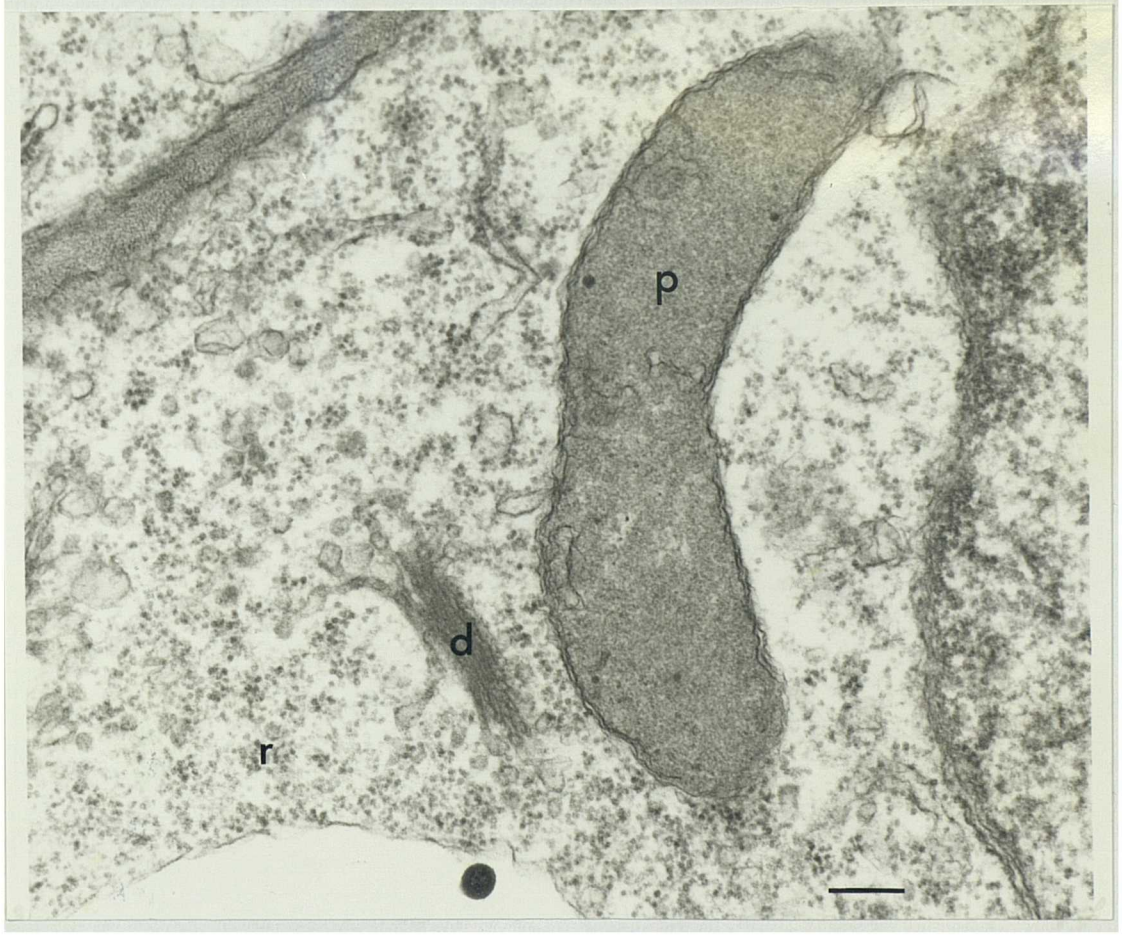

ne
N

Fig. 95. An area of cytoplasm in a *S. commersonii* callus cell showing plastid (p), mitochondria (m) and dictyosome (d). Endoplasmic reticulum can be seen to possess associated ribosomes (arrow). (Scale bar = 0.2 μm)

Fig. 96. An elongated plastid (p) in the cytoplasm. Dictyosome (d) and ribosomes (r) can also be seen. (Scale bar = 0.2 μm)

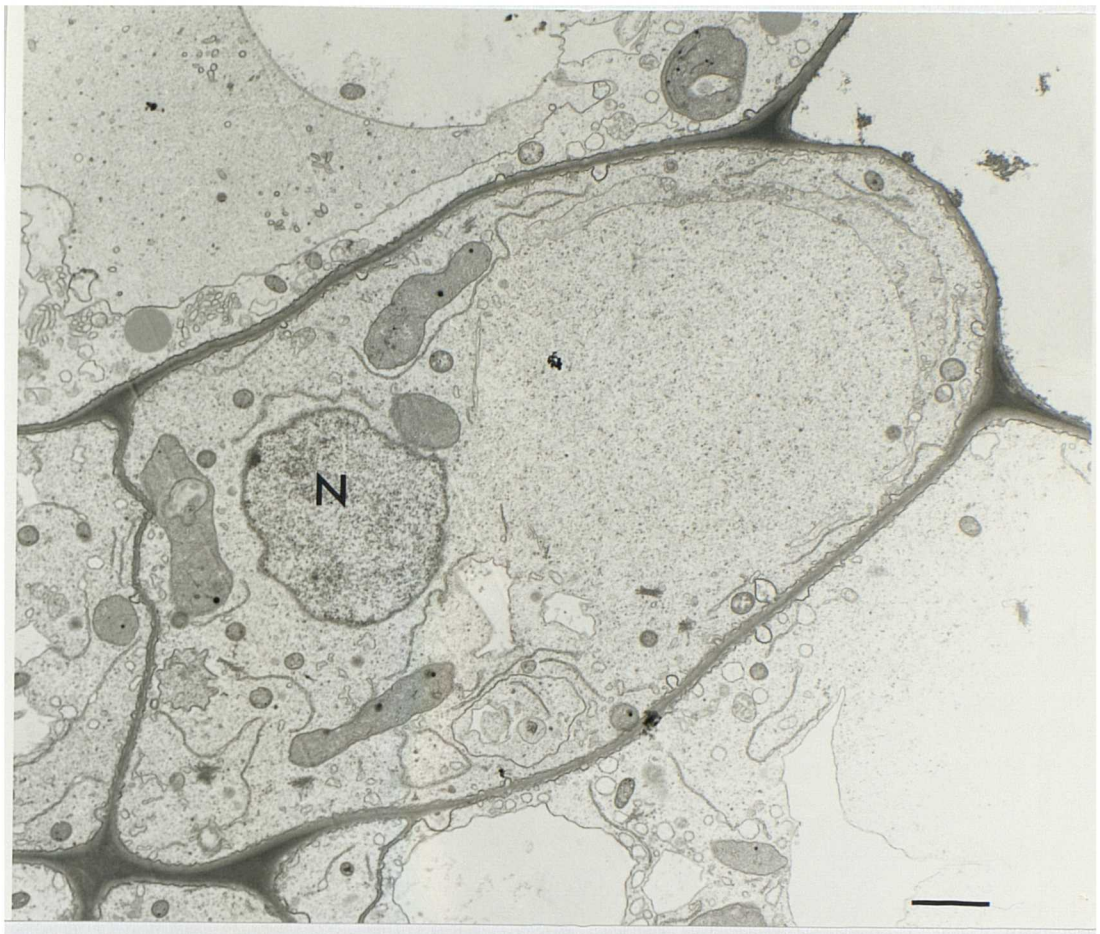

m
p
m
d

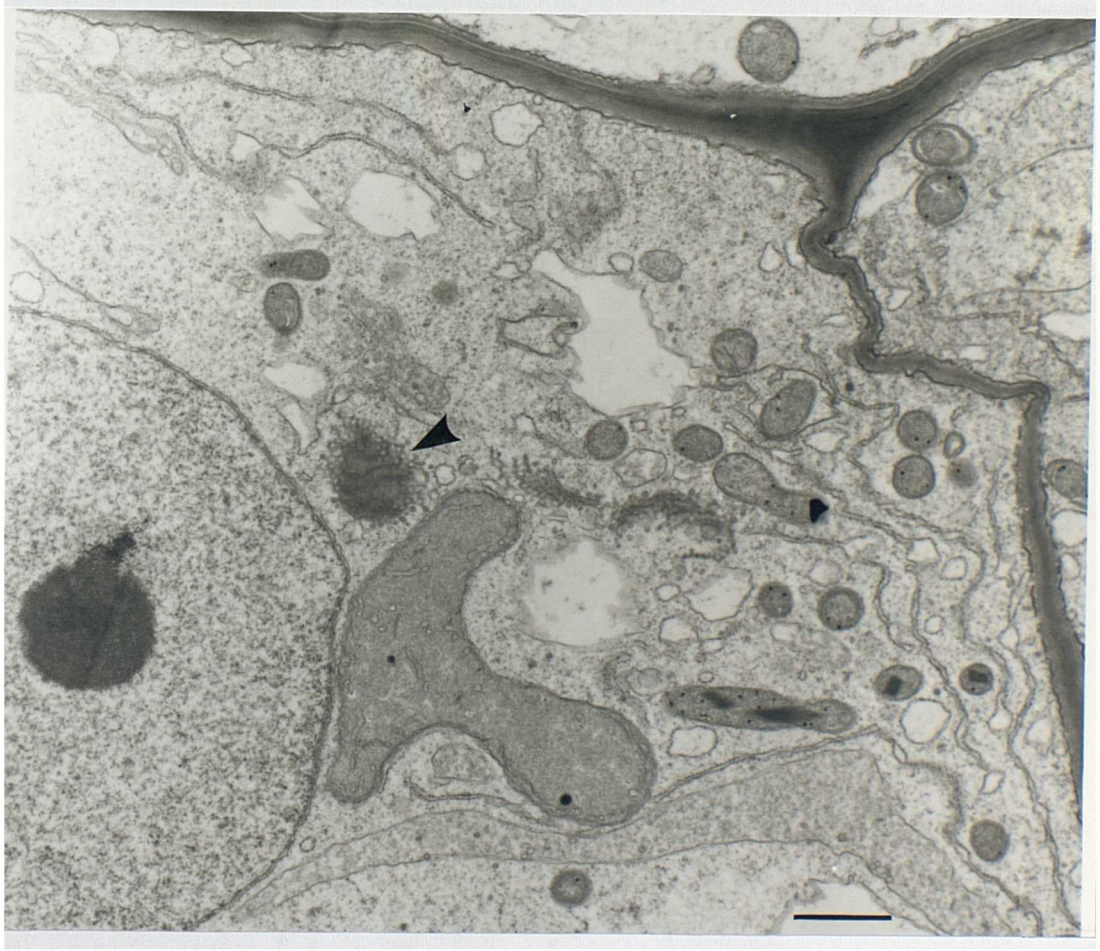

p
d
r

Figures 97 to 100 show examples of the structure of callus cells of *S. acaule*.

Fig. 97. General view of a callus cell surrounded by a cell wall, showing cytoplasm and nucleus (N). Most of the organelles are confined to the periphery of the cell. In the cytoplasm, plastids of unusual shapes are prominent. (Scale bar = 2 μm)

Fig. 98. Part of a nucleus with nuclear envelope, chromatin, and a nucleolus with nucleolar organizer. In the cytoplasm three dictyosomes are present, one of which is seen in face view (arrow). (Scale bar = 1 μm)


N



according to the plane of the section. Some microbodies, or peroxisomes, containing crystalline granules, are also present in the cytoplasm (Fig. 87 and 89). The paracrystalline granules are probably of protein, and are commonly found in the cells of storage tissue (Burgess, 1985).

In the cytoplasm, the apparently randomly-dispersed paired membrane sheets (endoplasmic reticulum) which permeate the cytoplasm as a series of flattened cisternae are seen (Fig. 91 and 92). Dictyosomes are not common but when found consisted of a series of flattened membraneous sacs, with peripheral vesicles (Fig. 90). Ribosomes, arranged in groups called polysomes also occur in the cytoplasm (Fig. 90). In cytoplasm various vesicles (Fig. 92) and some vacuolated organelles are also present (Fig. 91).

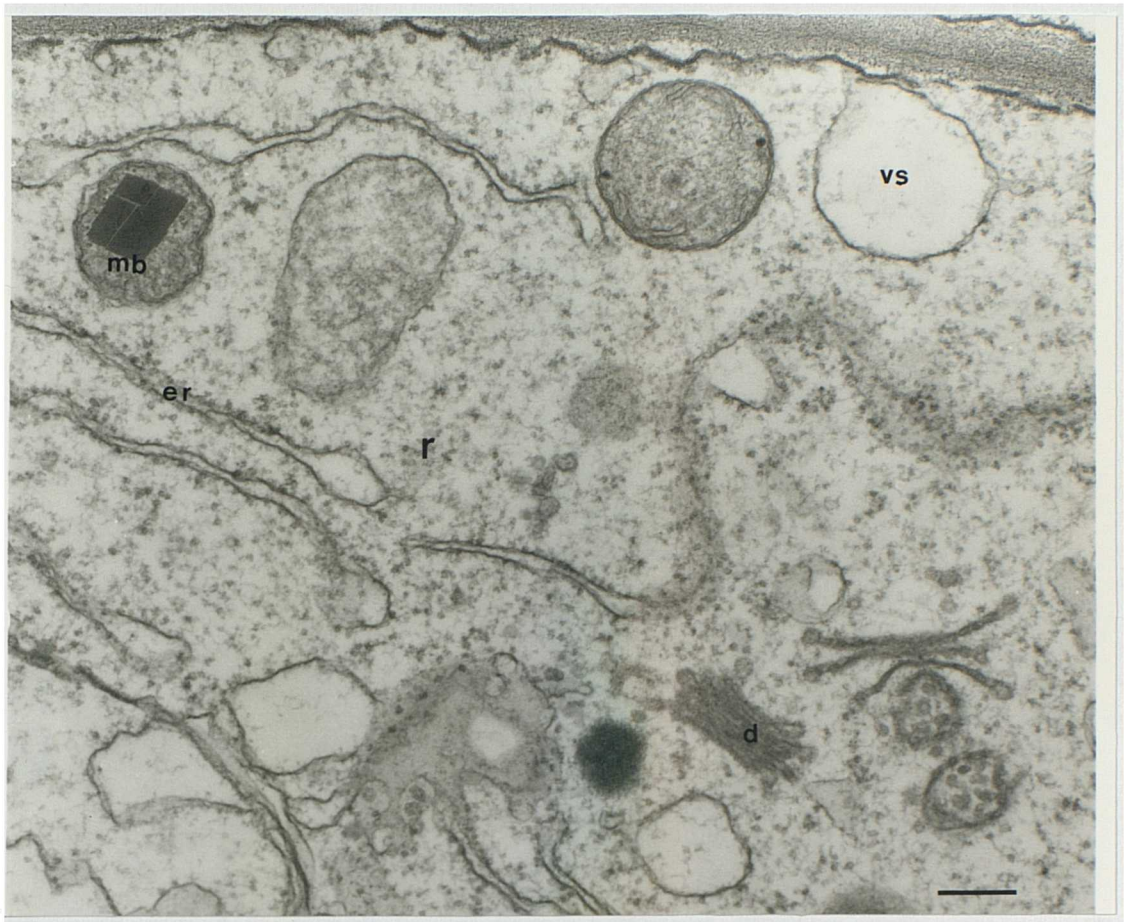
The vacuole appears to be quite large and separated from the cytoplasm by a membrane, the tonoplast. A few vesicles (labelled vs) can also be seen (Fig. 87) at the surface of the tonoplast apparently passing into the vacuole.

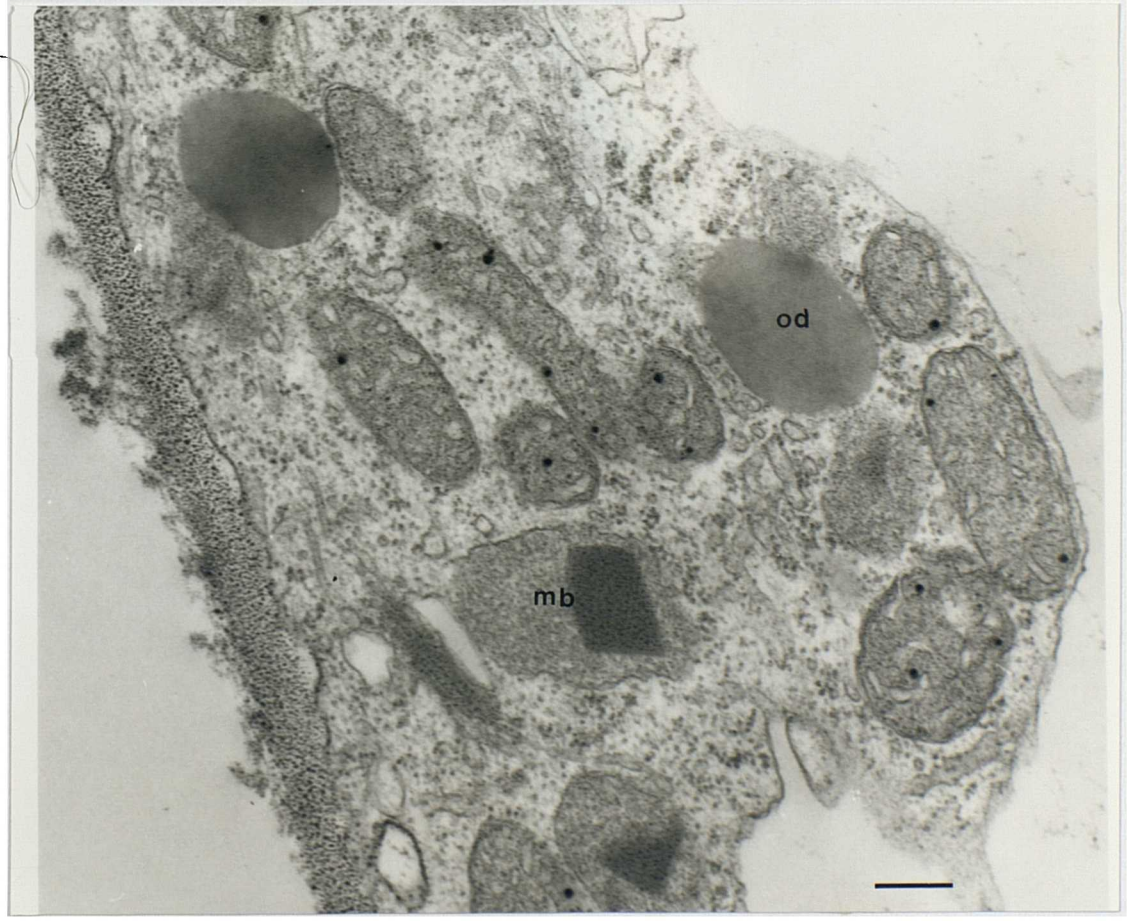
3.9.2 *S. commersonii*

The callus cells of *S. commersonii* (a frost-tolerant species) present a similar structure with a complete range of cellular organelles (Fig. 93 to 96). The most prominent cellular organelles, after the nucleus, are the plastids and mitochondria (Fig. 95). Some plastids, usually larger in size, also contain starch grains which are darkly-stained in the photographs (Fig. 93).

Fig. 99. A part of cytoplasm of *S. acaule* callus cell showing swollen endoplasmic reticulum (er), vesicles (vs), microbody (mb), and dictyosome (d). Ribosomes (r) as polysomes can be seen in the background of cytoplasm, and attached to the ER. (scale bar = 0.2 μm)

Fig. 100. A part of peripheral cytoplasm adjacent to the cell wall, showing the plasma membrane, microbodies containing protein crystals (mb) and oil droplets (od). (Scale bar = 0.2 μm)

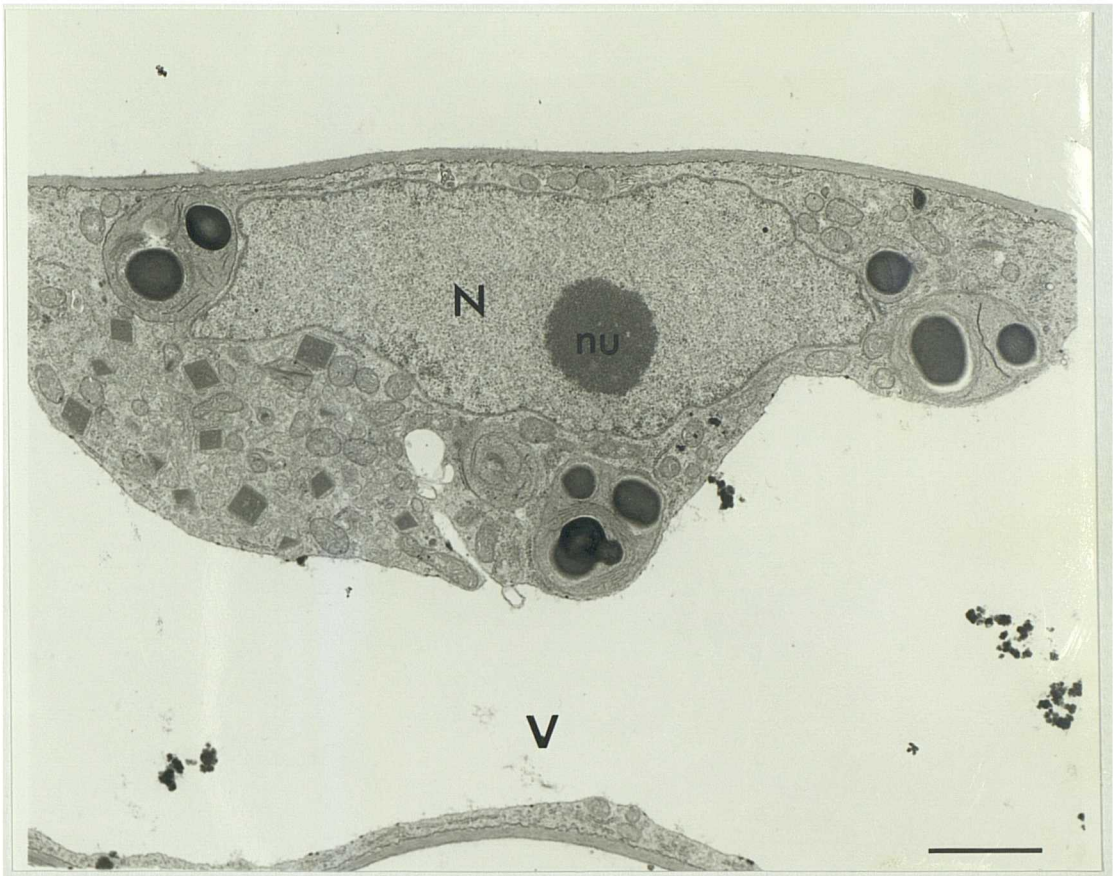

vs
mb
er
r
d


od
mb

Figures 101 to 104 show examples of the structure of callus cells of frost-tolerant line (D20-1), selected from Desiree callus.

Fig. 101. Nucleus (N) lying in the vacuole (V), surrounded by cytoplasm. The nucleolus (nu) and network of chromatin can be seen in the nucleus. (Scale bar = 2 μm)

Fig. 102. Part of Fig. 101 at a higher magnification, showing a large plastid containing starch grains, various microbodies containing protein crystals, and many mitochondria, some of which are very elongated. (Scale bar = 0.5 μm)


N
nu
V

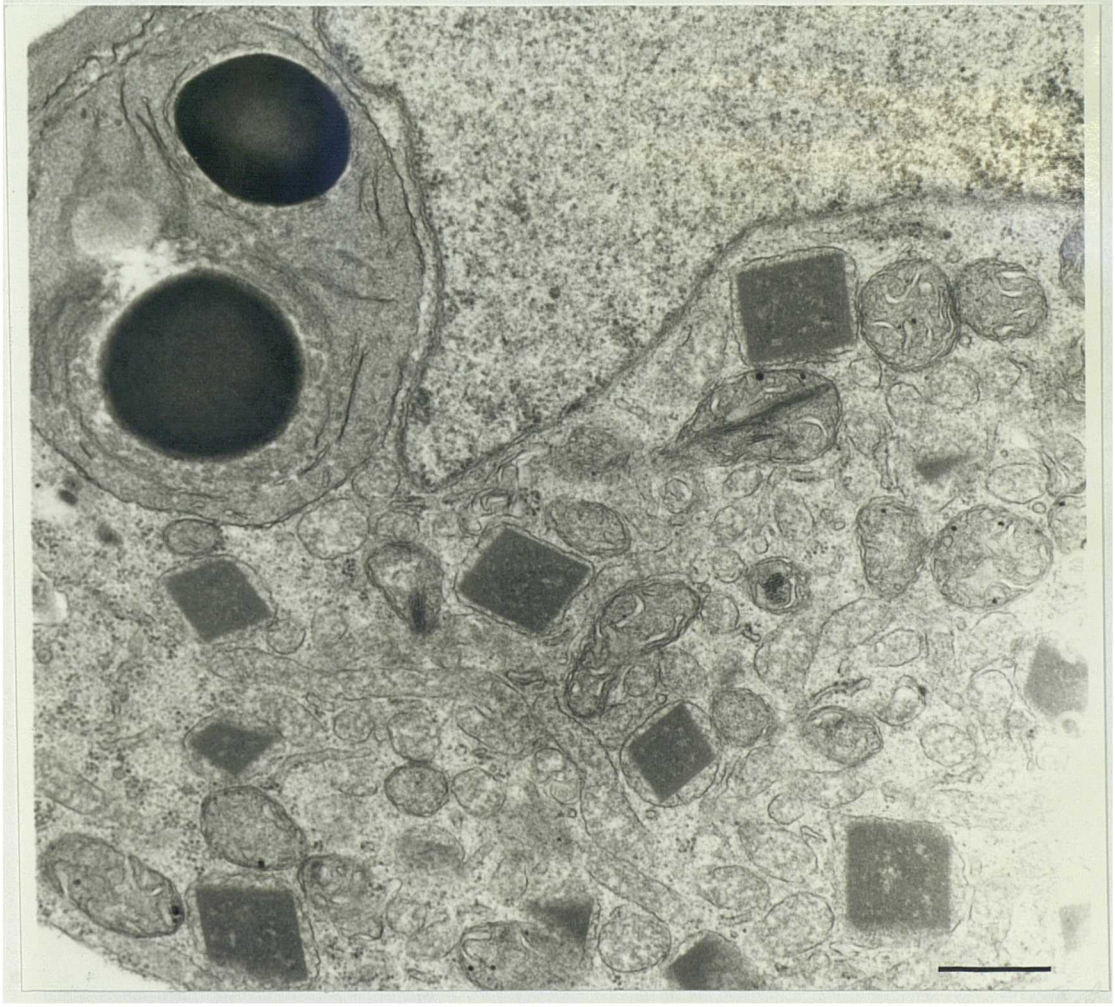
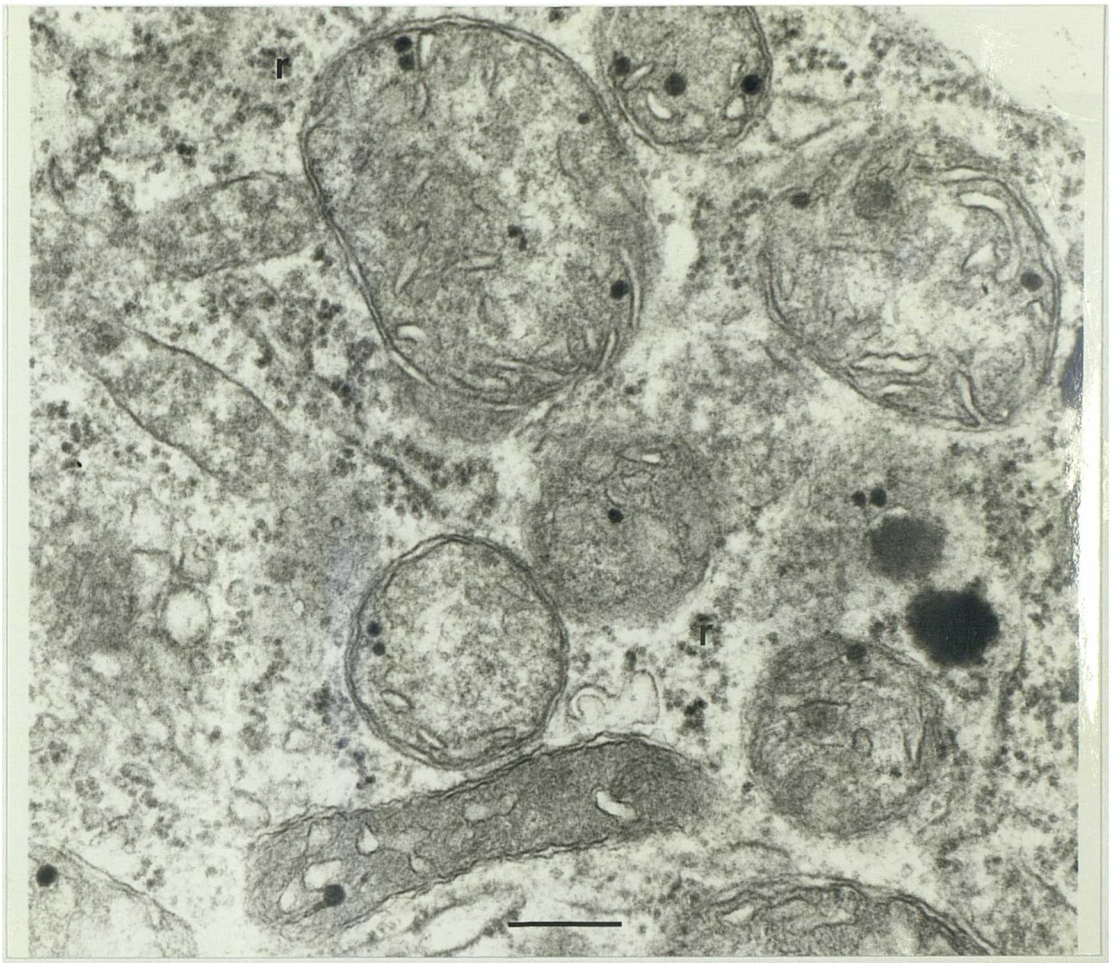
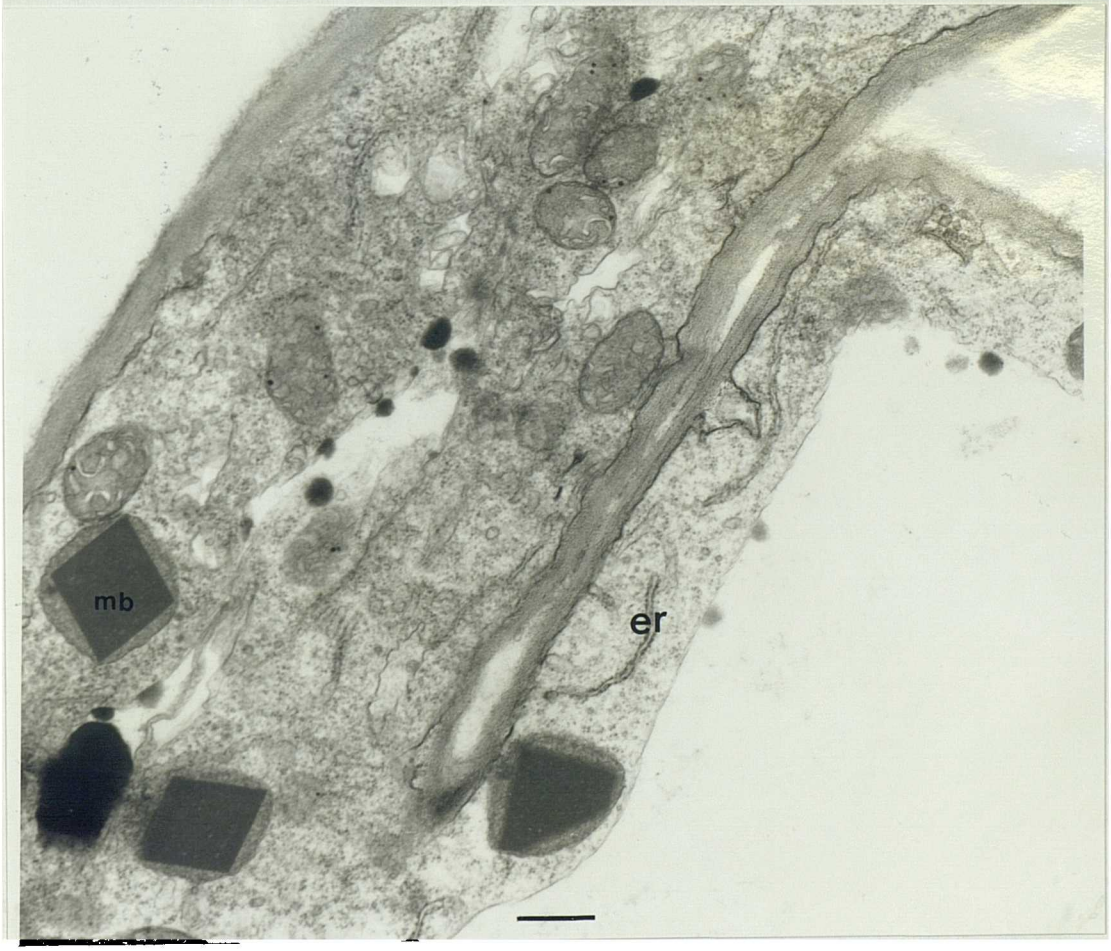


Fig. 103. A part of the cytoplasm from frost-tolerant Desiree callus (D20-1) showing mitochondria. Ribosomes (r) as polysomes can be seen in the background. (Scale bar = 0.2 μm)

Fig. 104. A part of cytoplasm showing endoplasmic reticulum (er), and microbodies containing protein crystals (mb). (Scale bar = 0.5 μm)


r
r


mb
er

3.9.3 *S. acaule*

The cells of *S. acaule* (also a frost-tolerant species) appear to contain more cytoplasmic contents compared with Desiree callus cells possibly because the callus cells are smaller, or the callus is younger. Cell vacuoles are less prominent and organelles such as plastids and mitochondria are confined to the periphery of the cytoplasm (Fig. 97). Besides regular and cup-shaped plastids, many unusual-shaped plastids are also present (Fig. 97 and 98). Microbodies containing protein crystals, dictyosomes and some oil droplets can also be seen in the cytoplasm (Fig. 100). In some cells, the endoplasmic reticulum appears to be swollen (Fig. 98 and 99).

3.9.4 Frost-tolerant callus (D20-1)

The callus of frost-tolerant line (D20-1) selected from Desiree callus was also fixed for cytological studies. The main difference noticed is the presence of an increased number of microbodies containing protein crystals in the cytoplasm of the cells of frost-tolerant callus (Fig. 102 and 104). Normal-shaped plastids, many mitochondria and microbodies (Fig. 103), and also some large plastids containing starch grains (Fig. 101), can be seen similar to the callus cells of *S. commersonii*. In the vacuole, there are also some discharged or accumulated substances which are darkly-stained in the photograph (Fig. 101).

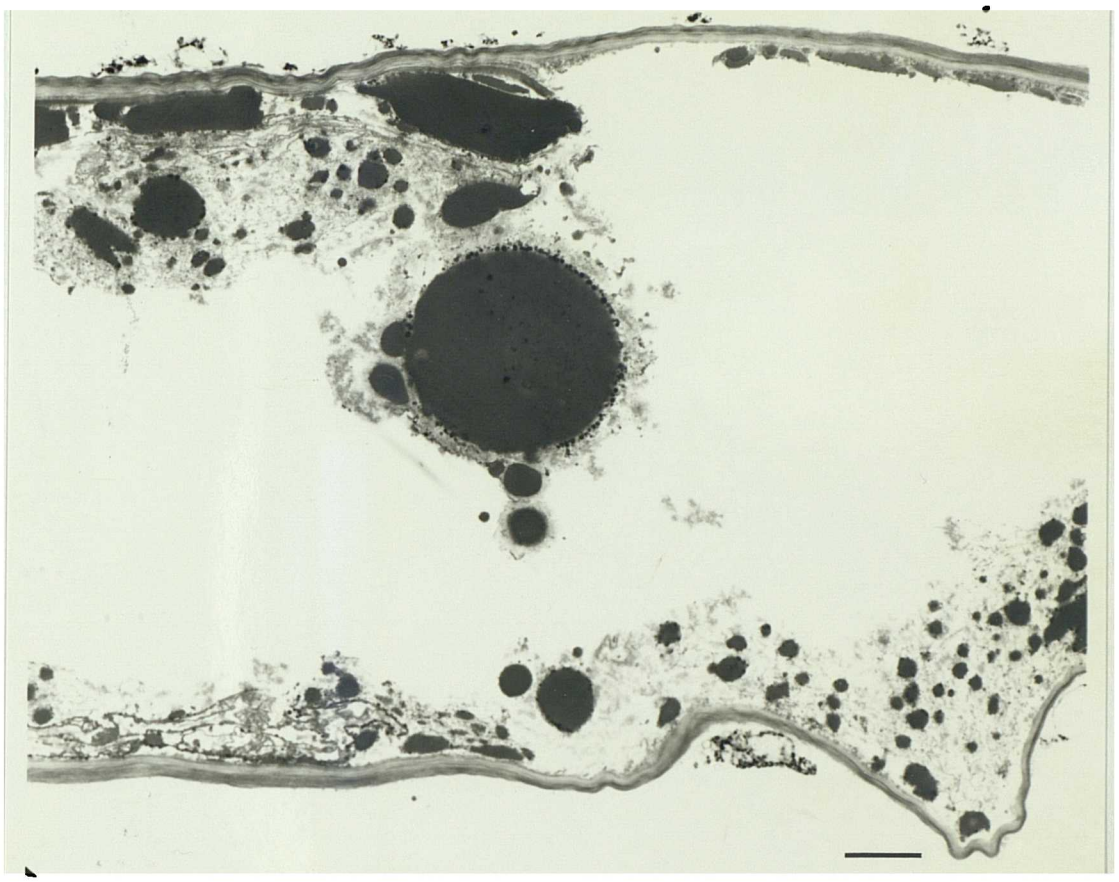
3.10 Effect of freezing on callus cells

To examine the cellular damage due to freezing temperatures, callus pieces of *S. tuberosum* cv. Desiree were frozen to -3, -6, and -10°C at a rate of 2°C h^{-1}. Samples were exposed to each test temperature for at least 1 hour before they were fixed for

Figures 105 to 108 show examples of cellular damage to the structure of *S. tuberosum* cv. Desiree callus cells, frozen to -3°C.

Fig. 105. Part of a cell bounded by cell wall, showing disorganized cytoplasm. (Scale bar = 2 μm)

Fig. 106. A part of cytoplasm showing organelles with indistinct internal membranes. Ribosomes are not distributed in a normal fashion and fibrils (possibly protein) can also be seen. (Scale bar = 1 μm)



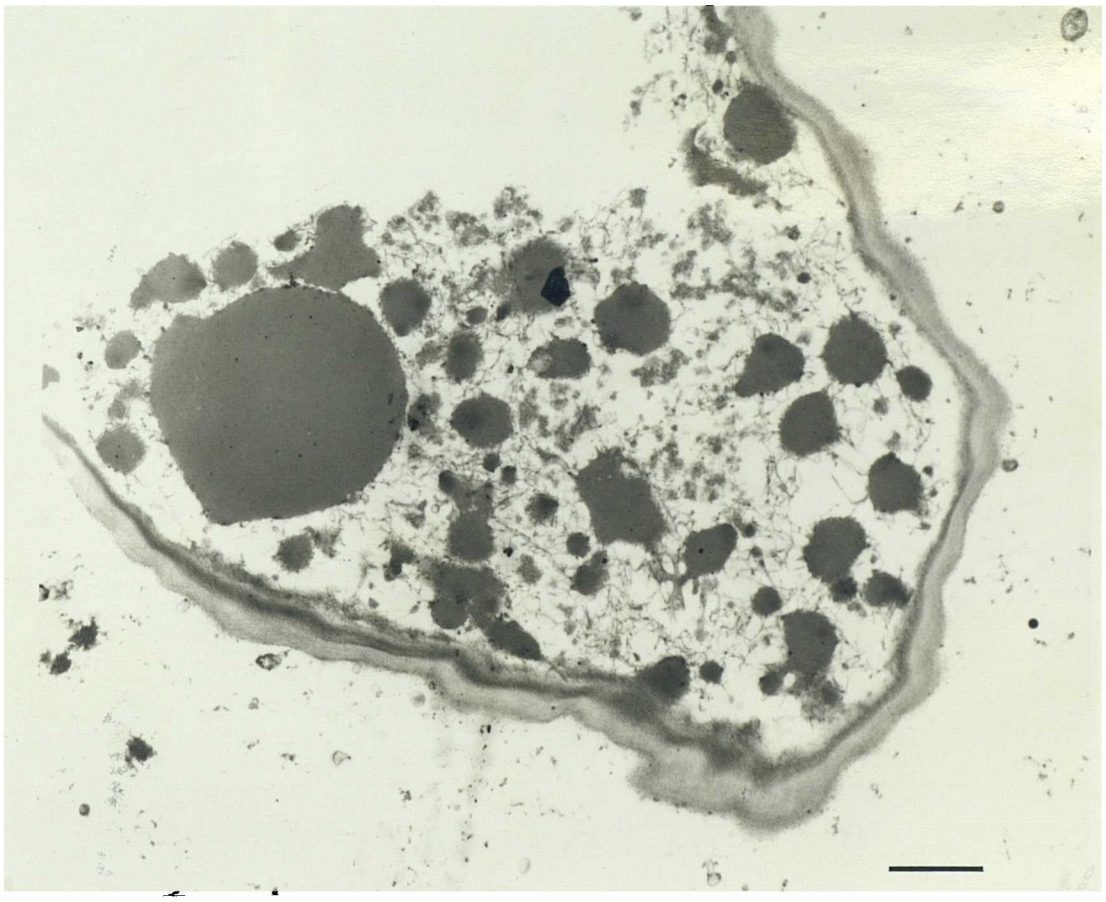
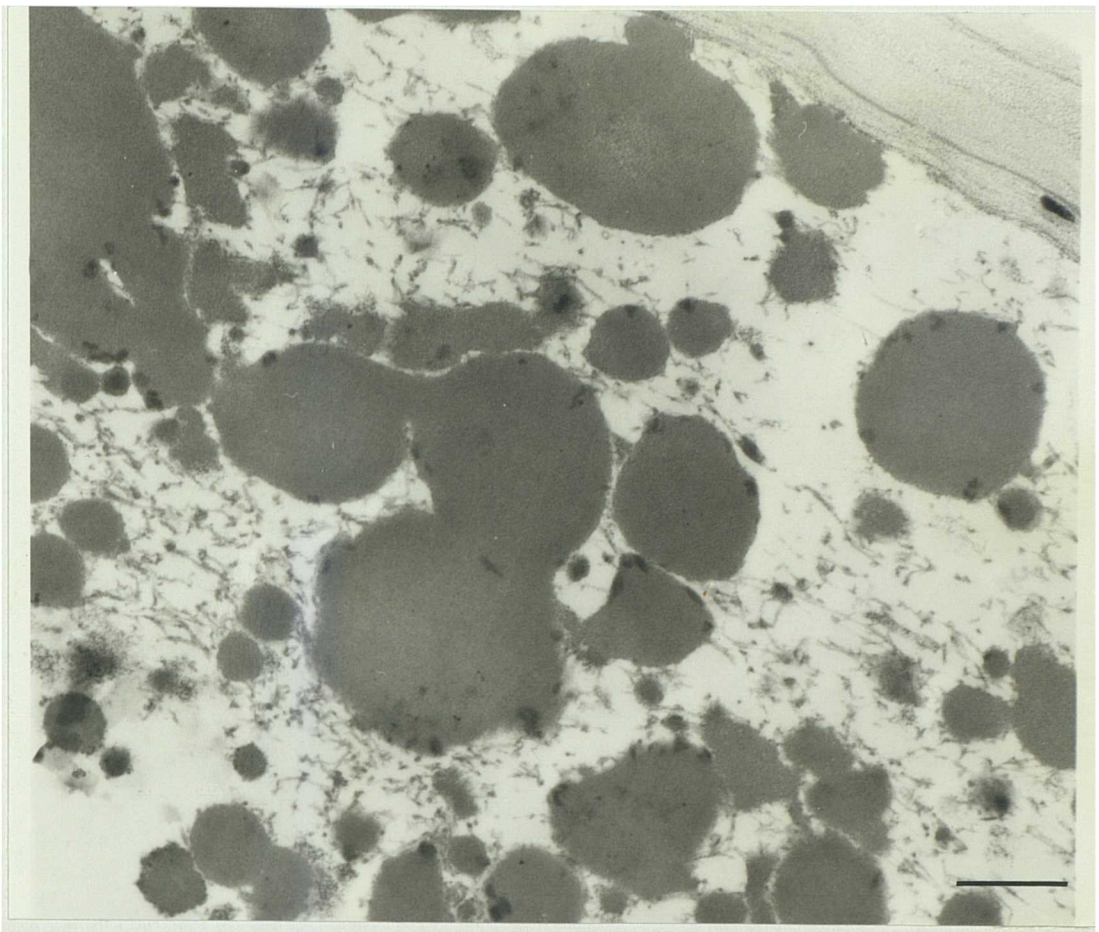
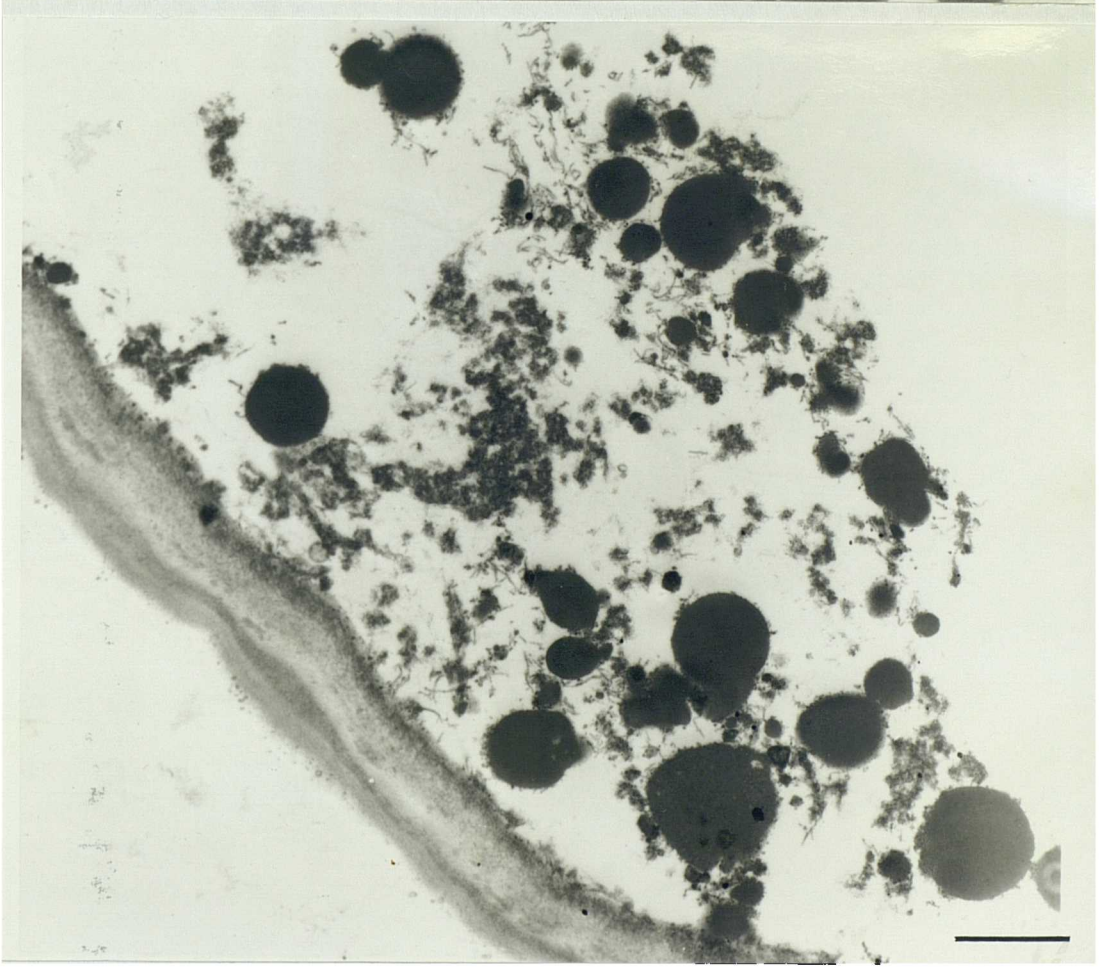


Fig. 107. An enlarged view of organelles in the cytoplasm of a cell subjected to -3°C. Some organelles appear to be swollen. (Scale bar = 0.5 μm)

Fig. 108. An enlarged view of a part of cell wall and disorganized cytoplasm. (Scale bar = 2 μm)





microscopy. Freezing damage was examined in the electron microscope.

A. Callus cells subjected to -3°C

The cells are regularly-shaped but the cytoplasm within presents a disorganized appearance. In addition, the cytoplasmic background in the electron micrographs appears to be changed in texture (Fig. 106), and ribosomes or polysomes are not distributed in the cytoplasm in a normal fashion. Endoplasmic reticulum has also been affected and some fibrillar material has appeared in the cytoplasm, possibly coagulated protein. Most of the major organelles such as plastids and mitochondria appear more swollen and darkly-stained (Fig. 107) than those in normal, unfrozen cells. In plastids, the internal membranes are indistinct but their envelope membranes seem intact (Fig. 108). Plasma membranes seem to be intact, but the tonoplast appear to be ruptured (Fig. 105).

B. Callus cells subjected to -6°C

In the cells frozen to the lower temperature of -6°C, the cell walls and cytoplasm appear distorted and collapsed, but organelles can still be distinguished (Fig. 109). Plastids in some cells still could be recognized due to the presence of osmiophilic globules (Fig. 110). In some cells both the plasma membrane and tonoplast seem to be ruptured (Fig. 112). The nucleus also appears to be damaged, with flocculated chromatin (Fig. 110).

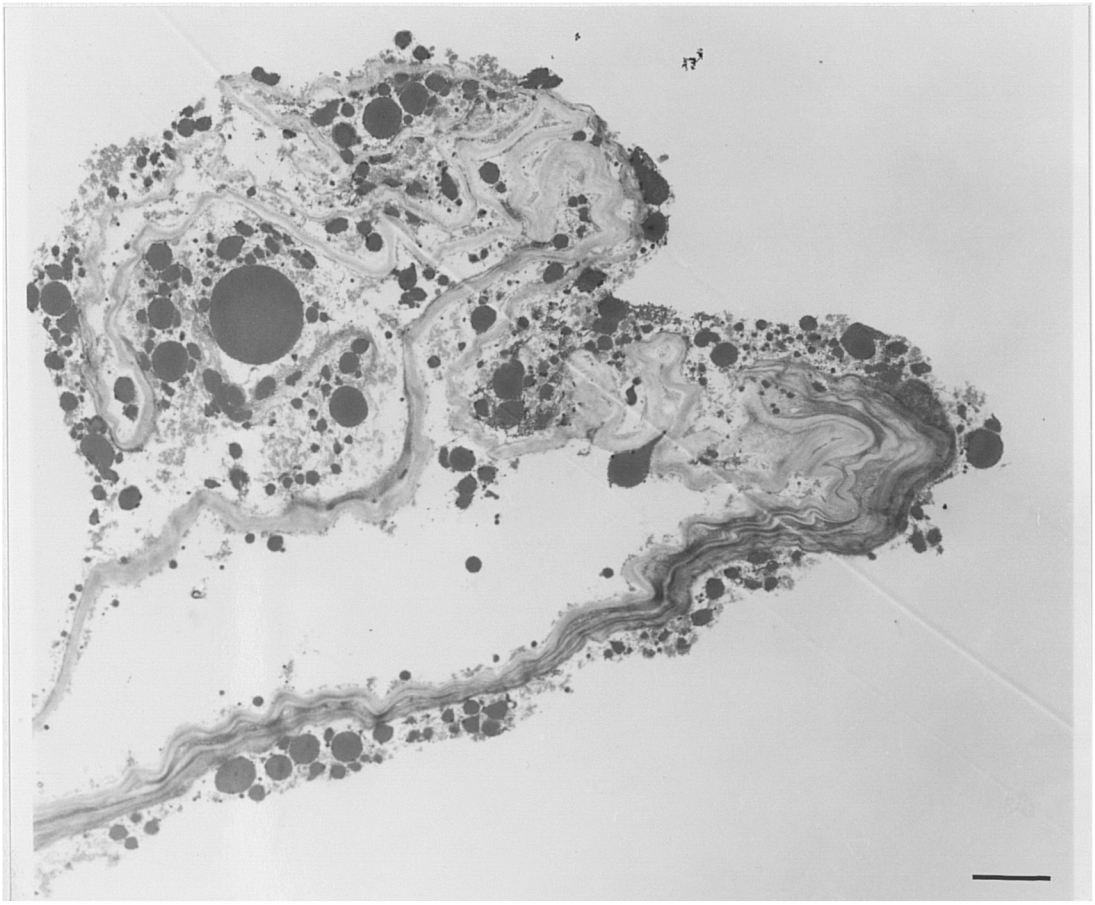
C. Callus cells subjected to -10°C

Exposure of callus cells to the lowest freezing temperature of -10°C resulted in severe

Figures 109 to 112 show examples of cellular damage to the structure of *S. tuberosum* cv. Desiree callus cells, after freezing to -6°C.

Fig. 109. A general view of distorted cells showing severe freezing injury. (Scale bar = 2 μm)

Fig. 110. A part of cytoplasm surrounded by plasma membrane and cell wall. A damaged nucleus (N) can also be seen. (Scale bar = 2 μm)



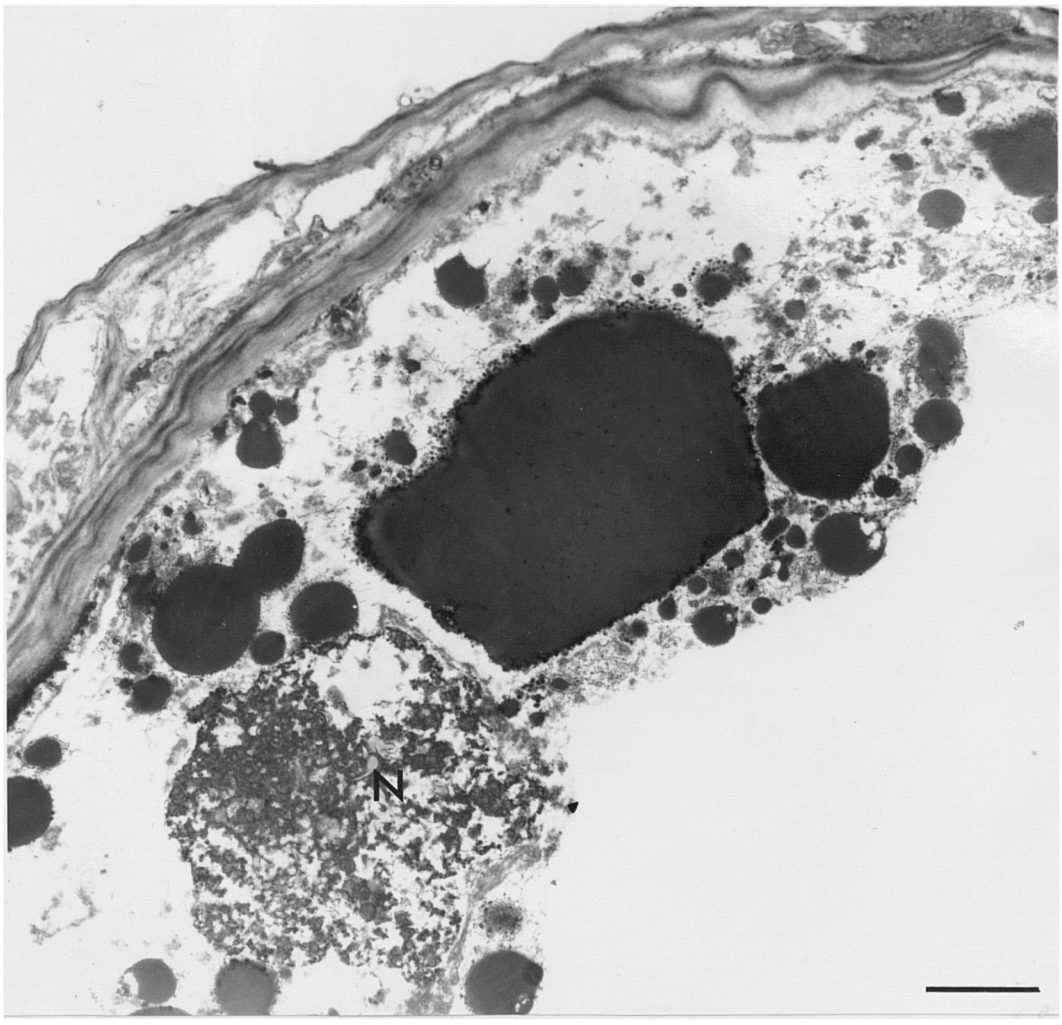
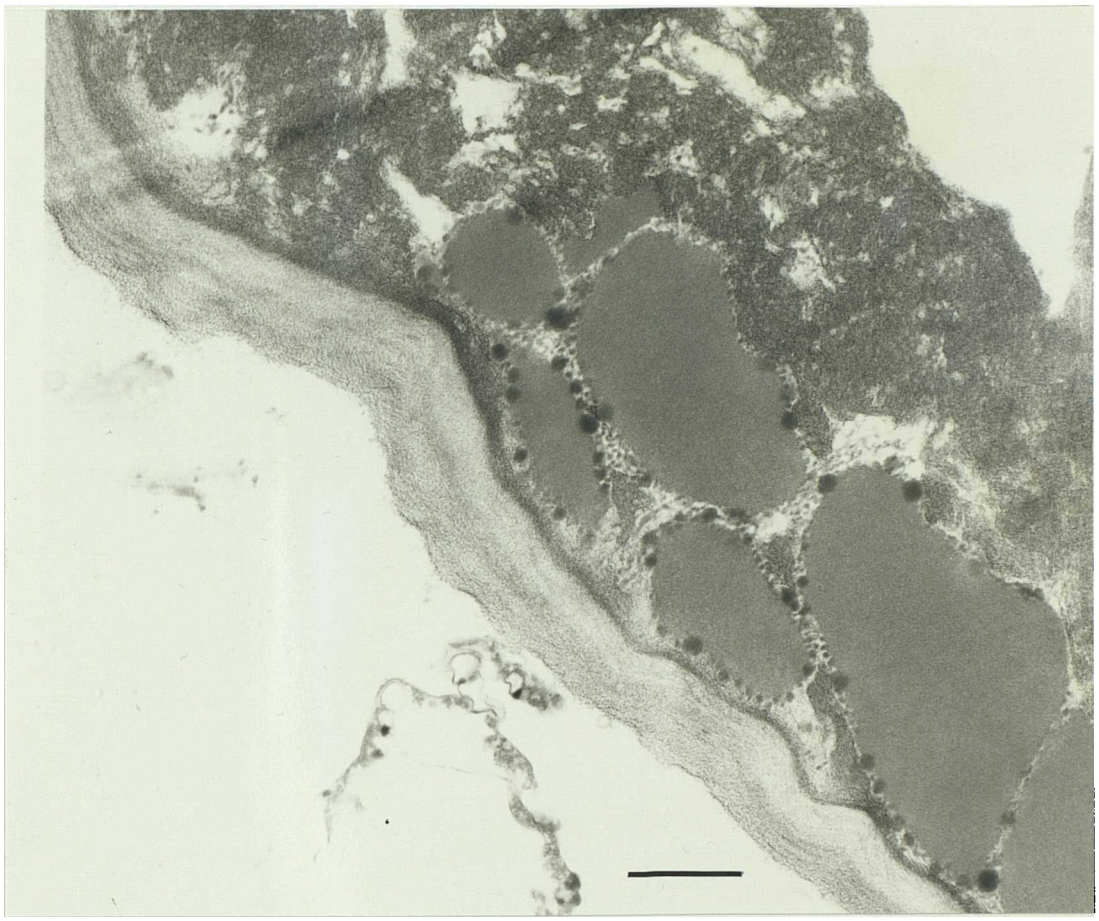
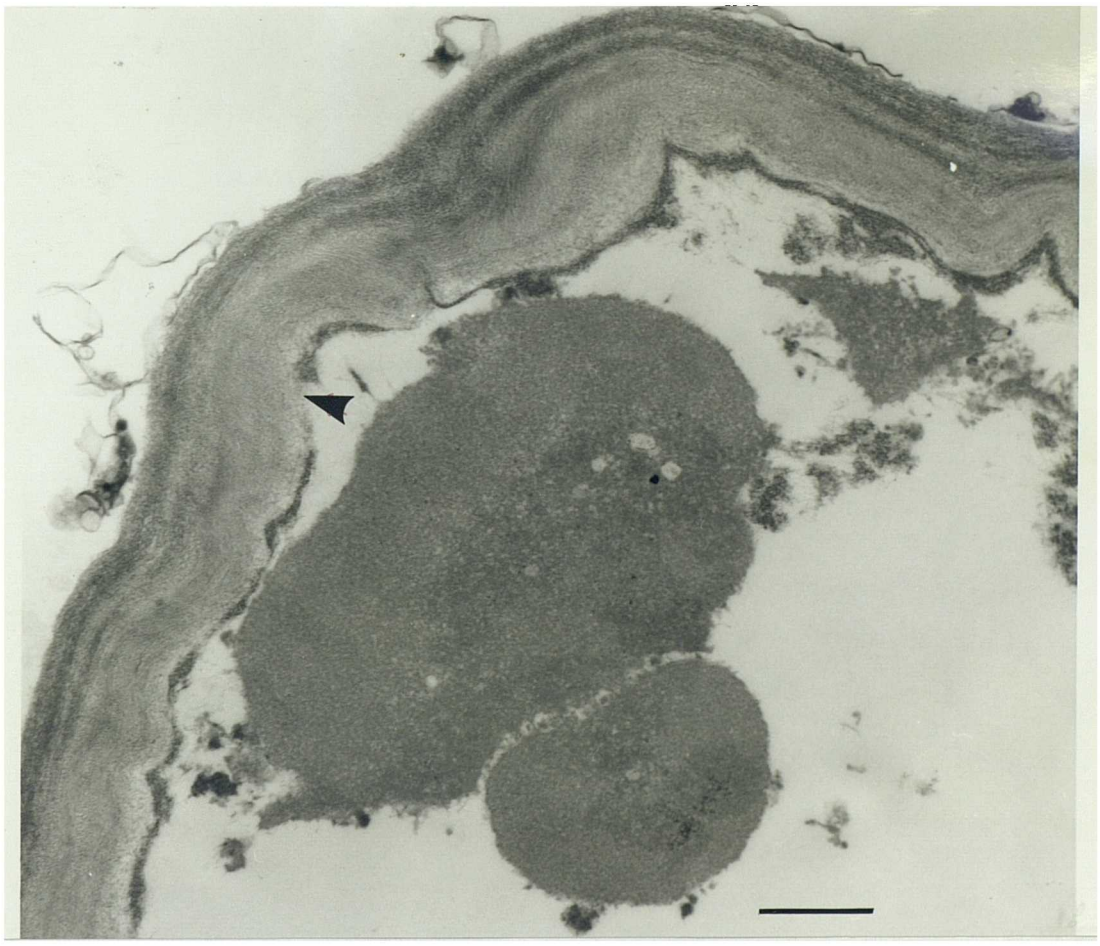

N

Fig. 111. A part of distorted cytoplasm, in which identification of organelles is difficult. (Scale bar = 0.5 μm)

Fig. 112. A part of a cell wall and plasma membrane. The plasma membrane appears to be discontinuous (arrow). (Scale bar = 0.5 μm)



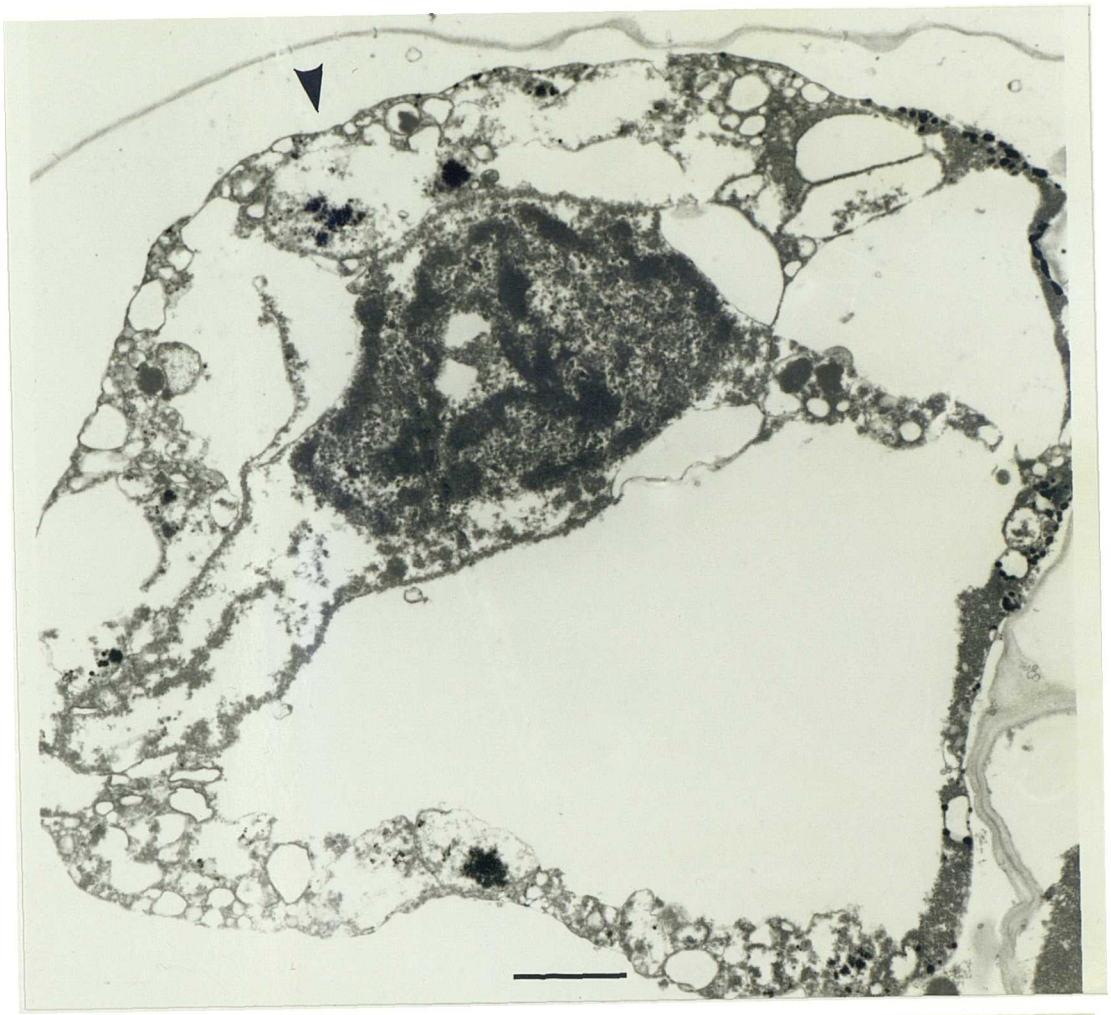


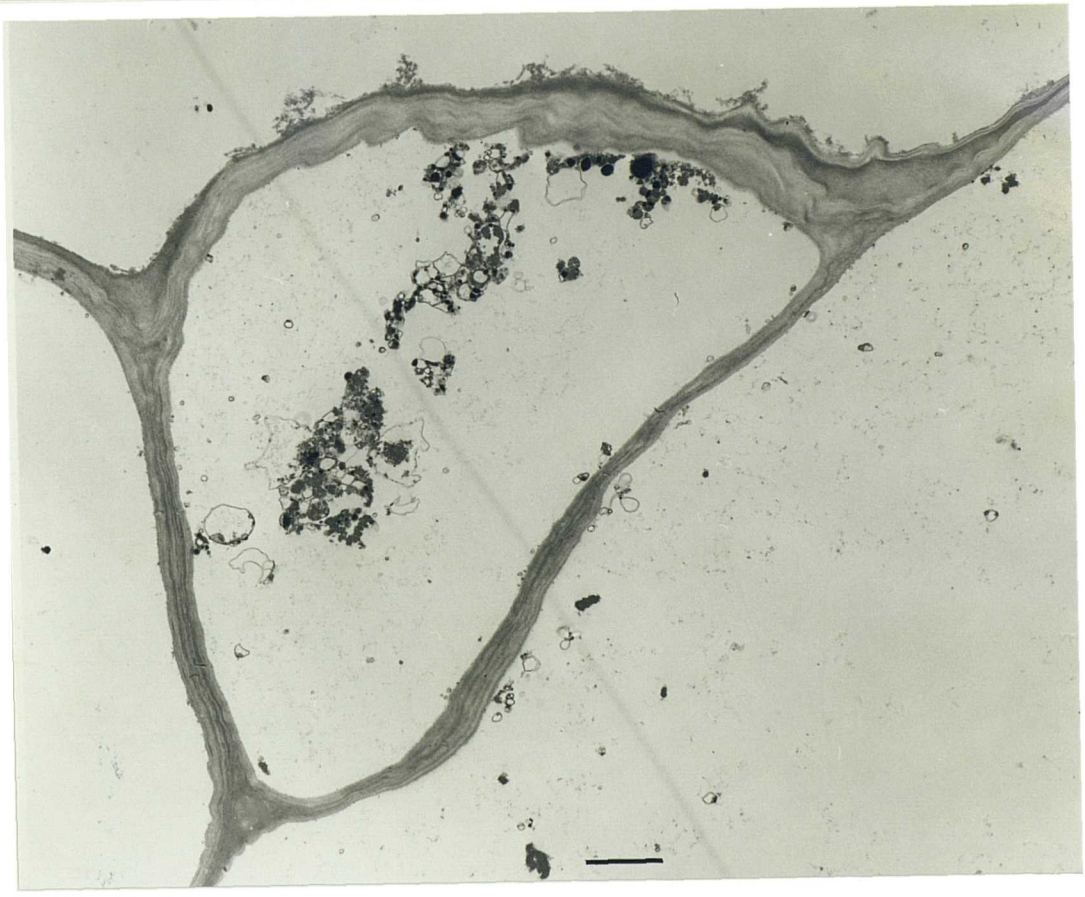
injury to the cells (Fig. 113 to 116). The cytoplasm appears to be in a highly disorganized state, with a flocculated appearance (Fig. 116). Severe vesiculation can be seen in some cases (Fig. 115). Many vesicles of varying sizes have appeared in the cytoplasm, which are probably the result of swelling or disintegration of the endoplasmic reticulum system. In some cells the only organelles which could be identified were plastids (Fig. 113). The damage is so severe in certain cases that no organelles can be distinguished (Fig. 116). Some cells with intact cell walls, appear empty or with little, highly-damaged cytoplasmic material (Fig. 114). The nucleus also appeared to be disorganized. "Frost plasmolysis" could also be seen in some cells (Fig. 113).

Figures 113 to 116 show examples of cellular damage to the structure of *S. tuberosum* cv. Desiree callus cells, frozen to -10°C.

Fig. 113. A general view of freeze-damaged cell, showing plasmolysis (arrow). (Scale bar = 2 μm)

Fig. 114. A severely-injured cell, showing only fragments of cytoplasm remaining. (Scale bar = 2 μm)





4. DISCUSSION

4.1 Shoot cultures and plant multiplication

During vegetative growth, the potato produces a succession of nodes each with a leaf and its associated axillary bud. If a nodal cutting, including an axillary bud and about 5 mm of stem, is grown on a synthetic medium, the axillary bud will develop into a complete plantlet. This technique of single node culture is mainly used for rapid *in vitro* shoot multiplication. Nodal cuttings can be obtained from *in vitro*-derived shoots, greenhouse-grown plants or tuber sprouts. The culture medium could be either growth regulator-free (Hussey and Stacey, 1981; Bokelmann and Roest, 1983; Tavazza and Ancora, 1986) or containing GA_3 (Roca *et al.*, 1978; Espinoza *et al.*, 1986). The medium used for micropropagation can be important, for example when using the shoot material for later protoplast isolation, the medium has marked effects on the number of protoplasts recovered and their subsequent plating efficiencies (Shepard and Totten, 1977; Foulger and Jones, 1986). In the present work, the aim was to establish *in vitro* shoot cultures to use for the isolation of protoplasts, and therefore nodal cuttings were cultured on two media, namely that developed by Espinoza *et al.* (1986) (Med. NA) containing 0.25 mg l^{-1} GA_3, and that developed by Shahin (1984) (Med. NB), which does not contain any growth regulator. Growth was more rapid on the medium of Espinoza *et al.* (1986) compared with the medium of Shahin (1984), probably due to the presence of GA_3 in the medium which stimulates cell division and/or cell enlargement.

Among the types of potato used, the growth from the axillary buds of *S. commersonii*

was most vigorous followed by the *S. tuberosum* cultivars, while growth from *S. acaule* axillary buds was poor. Of the two *S. tuberosum* cultivars, the growth from Desiree axillary buds was better than from those of Maris Piper. These differences were probably due to specific nutritional and hormonal requirements in the medium. Variability in growth of axillary buds has already been reported among potato genotypes (Al-Wareh *et al.*, 1989; Caligari and Powell, 1989; Coleman *et al.*, 1990).

Lack of ventilation during shoot growth in closed vessels can lead to dramatic modifications of gaseous composition (Cournac *et al.*, 1991). Volatile products such as ethylene can accumulate and inhibit growth. During shoot multiplication, if culture jars were tightly closed, shoot morphology became stoloniferous-like, with small leaves, or shoots with numerous partially-developed leaves and with adventitious roots, all of which are most likely to be the result of ethylene accumulation (Hussey and Stacey, 1981; Wang and Hu, 1985). The effect of ethylene has implications for the choice of culture vessel used both for experimental studies, and for commercial micropropagation systems. Therefore, culture vessels should be used with covers which allow the maximum gaseous exchange, at the same time preserving aseptic conditions.

4.2 Callus culture

4.2.1 Callus induction and growth

It is now well established that on a suitably defined medium and under controlled conditions, pieces of organized tissue of almost any plant can proliferate into an unorganized mass of cells called callus. When an organized tissue is injured, viable

cells at the site of injury start to divide as a "wound response" and when the wound is sealed up, the cell divisions cease. If, however, wound tissues are isolated from the plant, and maintained on a synthetic nutrient medium, the natural regulatory mechanism which limits the extent of cell divisions can be over-ridden, and callus proliferation will continue as long as nutrient and growth regulators are supplied (Lindsey and Jones, 1989).

Callus has been successfully induced from tuber tissue in many laboratories since 1951 when the work of Steward and Caplin was published. Since then many reports have been published on callus induction and growth from explants taken from different organs of potato. Patrascu (1981) reported the use of 2,4-D and NAA in concentrations ranging from 2 - 10 mg l^{-1} for callus initiation from stem explants of 10 cultivars of *S. tuberosum* and found that the best medium for callus initiation was one containing 2 mg l^{-1} 2,4-D. Chung and Sim (1986) initiated callus successfully from tuber discs with a concentration of 3 mg l^{-1} of 2,4-D in four potato cultivars. In the present studies, callus was induced from tuber tissue of *S. tuberosum*, internodal explants of *S. tuberosum* and *S. acaule*, on Murashige and Skoog (1962) basal medium with an added concentration of 3 mg l^{-1} of 2,4-D. For induction of callus from leaf explants of *S. tuberosum* and *S. commersonii*, 0.3 mg l^{-1} kinetin was also added in this medium.

The apparent lack of an obvious lag phase in the growth curves (Figures 9, 10 and 11) is probably because no weighing was done between days 0 and 7. It would be expected that a lag phase would occur early in the growth of the cultures. Growth curves show

that there was a decrease in the growth rate of callus after 28 days.

4.2.2 Tissue response to callusing

If an explant is isolated from meristematic cells (shoot meristem and cambium), it starts to proliferate quickly because cells divide immediately without de-differentiation, while explants taken from differentiated cells (e.g. tuber and leaf tissue) must de-differentiate before cell division can occur. The length of the induction phase depends mainly on the physiological status of the explant as well as the culture conditions.

In the present work, stem explants proliferated more rapidly than either leaf or tuber explants. Callus initiation from stem explants started after 4 - 5 days of placing on nutrient medium and from tuber explants after 5 - 7 days of culture. Callus initiation from leaf explants was even slower (8 - 10 days). This was probably due to the highly differentiated state of leaf tissue.

2,4-D at a concentration of 3 mg l^{-1} proved quite sufficient for callus induction from both stem and tuber explants. 2,4-D alone failed to proliferate callus from leaf explants, and the addition of kinetin (0.3 mg l^{-1}) was found necessary. This was possibly due to the difference in the levels of endogenous growth hormones.

4.2.3 Genotypic response to callus growth

A great variation exists in response to tissue culture among different genotypes. This fact causes special difficulties in vegetatively-propagated plants, as a cultivar usually represents one genotype. Differences among cultivars have been recorded regarding

the consistency, colour and growth rate of callus (Patrascu, 1981). Gavinlertvatana and Li (1980) observed differences in callus formation from leaf explants of different potato genotypes. Chandra *et al.* (1981) reported differences in callus growth from dihaploid and tetraploid genotypes. The growth of callus was significantly more in Kufri Jyoti (a tetraploid) as compared with PH-255 (a dihaploid of Kufri Jyoti). In contrast to this, Fish and Jones (1988) obtained significantly more callus from dihaploid genotypes, but these were not the same cultivars as in the previous case. They also noticed variations in callus growth among dihaploids derived from the same tetraploid. Not only the callus growth varied among potato cultivars, but differences also existed in callus-forming stage and morphological pattern (Chung and Sim, 1986).

Generally, in all types of explant (stem, leaf and tuber) in the present work, callus formation was most rapid in Desiree, followed by Maris Piper. Initiation of callus from stem and leaf explants of *S. acaule* and *S. commersonii* was slow and resulted in compact-textured callus. After 3-4 subcultures (every 4 weeks), callus texture gradually improved and rapidly-growing, friable callus cultures were established. These results indicate that tuber-bearing *Solanum* species, or even the different commercial cultivars of *S. tuberosum* may have different nutritional requirements for proliferation and callus growth.

4.2.4 Effect of physical state of culture medium on callus growth

Differences in callus growth could occur due to the agar used for solidifying the culture medium. Agar decreases the ease of availability of nutrients as well as the outward loss of waste products. In the present work, liquid medium under membrane

rafts gave better results and established callus rapidly. However, the membrane rafts were not so practical in use as they caused some difficulties during fresh weight measurements and subculture. The membrane rafts themselves were very fragile and easily damaged. The present results agree with those of previous workers (Hamilton *et al.*, 1985; Hamilton *et al.*, 1986; Adelberg *et al.*, 1989).

Although callus proliferation proceeded at a faster rate on liquid medium as compared to agar-solidified medium, the callus produced on liquid medium was softer, watery and fragile in texture. Some increase in callus fresh weight seemed due merely to higher water content.

4.3 Cell suspension cultures

Plant growth, defined as irreversible increase in volume accompanied by an increase in dry weight (Ryu *et al.*, 1990), occurs by cell expansion that can also be accompanied by cell division. Under suitable growth conditions, plant cells increase in size and ultimately divide, with the formation of two cells capable of further growth. There are several methods of determining the growth rates of plant cells, these include fresh cell weight, dry cell weight, packed cell volume, cell counting, mitotic index, protein content, nucleic acid content, and pH measurements. In the present studies measurements of packed cell volume and cell counting were used for demonstrating the growth of suspension cultures.

For cell suspension cultures, mostly medium of the same composition as that used for callus growth has been employed, but without agar (Anstis and Northcote, 1973; De

Vries and Bokelmann, 1986; Tan *et al.*, 1987; Tavazza *et al.*, 1988), and some workers used this medium with slight modifications in the composition and/or concentrations (Lam, 1977b; Austin and Cassells, 1983; Handley and Sink, 1985; Chung and Sim, 1986). For better growth of cell suspensions, Lam (1977b) suggested full concentrations of MS major elements without NH_4NO_3 and 1/5 the concentration of MS minor elements. On the other hand Chung and Sim (1986) found full strength of MS minor salts to be optimal for growth of cell suspensions. Full concentration of MS minor inorganic salts has frequently been used in liquid media for growth of cell suspensions (Handley and Sink, 1985; De Vries and Bokelmann, 1986; Lindeque *et al.*, 1991; Gerard *et al.*, 1991). In the present work, medium of the same composition (but without agar) used for callus growth was used for tuber callus-derived cell suspensions, but for suspension cultures derived from stem callus, the medium of Lam (1977b) was used, in which the concentrations of MS minor elements were reduced to 1/2. These media were found to be efficient for supporting the growth of cell suspensions of different origins.

During incubation in nutrient medium, the amount of cell material increases for a limited period of time until the culture reaches a level of maximum yield of cell material. If it is subcultured at this point, a similar pattern of growth and yield will be repeated. In the present studies, suspension cultures initiated from the actively-growing callus showed growth patterns similar to those reported by other workers. There was a lag phase of about 2 days followed by a period of active growth for 14 days. After 16 days the cultures entered a growth declining phase.

A suspension culture consists of cells and cell aggregates dispersed and growing in an agitated liquid medium. Tuber callus-derived cell suspensions in MS medium frequently produced cell aggregates and cell clumps, while in stem callus-derived cell suspensions in Lam (1977b) medium, the frequency of cell aggregates was lower, and mostly single cells were observed. Formation of cell aggregates and large cell clumps in cell suspension cultures of *S. tuberosum* has been reported by several workers (e.g. Bajaj and Dionne, 1967; Chung and Sim, 1986). Bajaj and Dionne (1967) observed cell clumps in the cell suspension cultures of *S. tuberosum* cv. Green Mountain. Because their cell suspensions were not filtered to remove these cell clumps, upon subculture they developed into large callus masses. In their experiments the best dissociation into single cells was obtained when MS medium was supplemented with 2,4-D (0.2 mg l^{-1}), Kinetin (0.2 mg l^{-1}) and yeast extract (1000 mg l^{-1}). In the present work, when 2,4-D alone was used, cell aggregates and cell clumps were produced but when 2,4-D was used in combination with kinetin (Lam, 1977b medium), the frequency of single cells was high. Growth rate, colour, texture and friability of the original callus also influences the dissociation of cells into single cells and small cell aggregates. In the present work, although friable calluses were used for initiation of cell suspensions, these were of different origin, which might have influenced dissociation in the agitated liquid medium. Tan *et al.* (1987) also reported genotypic effects, in which a higher frequency of larger cell aggregates occurred in cell suspension of *S. lycopersicoides* than in those of *Lycopersicon pennellii* grown in the same medium.

4.4 Protoplast isolation

Leaf mesophyll cells and suspension-cultured cells are the most common sources for protoplast isolation and have been used in many different investigations. In *Solanum* species, leaf mesophyll cells have been used more frequently than any other source (Prakash *et al.*, 1985; Haberlach *et al.*, 1985; Tavazza and Ancora, 1986; Briza and Machova, 1991). For the isolation of non-green protoplasts, *in vitro*-cultured cells are an excellent source. They do not need sterilization, respond better to *in vitro* culture conditions and require a lower quantity of cell-separating enzyme. However, different genotypes respond differently to the protoplast isolation methods for these two starting materials.

In the present studies, the method of Haberlach *et al.* (1985) was used to isolate mesophyll cell protoplasts from leaves of greenhouse-grown plants, and also from *in vitro* shoot cultures. Plant growth conditions and physiological state have been found to be critical for potato protoplast yield and their subsequent growth (Shepard, 1980). Generally, leaves from 40 - 60 day old plants and from positions 4 - 7 from the shoot apex are considered most suitable in potato for higher protoplast yield (Prakash *et al.*, 1985). In the present studies, leaves from *S. tuberosum* plants grown in a greenhouse at 25°C under 12/12 hours light and dark cycle gave consistently reasonable yields of viable protoplasts. The protoplasts were isolated from plants aged 45, 52 and 59 days. The responses of the two cultivars were not the same, with the highest yields occurring in cv. Desiree (3.73×10^5) at 52 days, and in Maris Piper (4.99×10^5) at 59 days. Overall, leaves from the cv. Maris Piper resulted in higher yields than those from the cv. Desiree.

Conversely, the yield of protoplasts from *in vitro* shoot cultures also varied, being highest in Desiree (2.5×10^6 g^{-1} tissue fresh weight) compared with *S. commersonii* and Maris Piper. In *S. tuberosum*, higher yields were obtained from *in vitro* shoot cultures compared with the leaves of greenhouse-grown plants from equivalent amounts of tissue. This was probably because leaves of *in vitro* shoot cultures have very thin cuticles with less resistance to penetration of enzymes into the leaf tissue.

Differences among potato genotypes in protoplast production have been reported many times (e.g. Prakash *et al.*, 1985; Haberlach *et al.*, 1985; Tavazza and Ancora, 1986; Radke and Grun, 1986; Foulger and Jones, 1986; Taylor and Secor, 1988; Feher *et al.*, 1989; Xu *et al.*, 1991).

The most important factors for the liberation of protoplasts from cultured cells are their physiological state, the kind and concentration of degrading enzymes, and the incubation period. In the present work, 5 to 7-days old cell suspensions were used and three enzyme compositions were tested.

Protoplast yields varied depending upon the enzyme composition and incubation period. In both Desiree and Maris Piper, the yields of protoplasts were higher when cells were incubated in enzyme mixture I, containing 1% cellulase Onozuka and 0.1% Pectolyase Y-23, for 3 hours. The lowest protoplast yields were obtained with 2% cellulase Onozuka and 1% Macerozyme R-10 (enzyme mixture II) and an incubation period of 5 hours. However, when concentrations of cellulase Onozuka and Macerozyme R-10 were increased to 3 and 2% respectively (enzyme mixture III) and

incubation period extended to 12 hours, the yields of protoplasts increased 2.5 to 3-fold. This was still lower than the yield with enzyme mixture I (Table 30) and also a lowered protoplast viability resulted. Similar variations in the influence of enzyme composition on protoplast yields have been reported by several workers (e.g. Handley and Sink, 1985; Chung and Sim, 1986; Tavazza *et al.*, 1988).

In the present work, Pectolyase Y-23 proved a powerful macerating agent, and in combination with cellulase Onozuka released a large number of protoplasts. Similar results were reported by Nagata and Ishii (1979) for protoplast isolation from mesophyll cells of pea.

4.5 Protoplast culture

For the culture of *S. tuberosum* mesophyll protoplasts, the media developed by Haberlach *et al.* (1985) were used. These are modifications of the basal composition described by Lam (1977b), which included the inorganic salts of Murashige and Skoog (1962), and organic addenda of Nitsch and Nitsch (1969).

Each time protoplasts were isolated from leaves of greenhouse-grown plants, they were found to become contaminated after a few days of culture. This was probably because the plants were grown under non-sterile conditions, and contamination of the growing plants by microorganisms is an important technical problem in protoplast experiments. Sterilization techniques failed to eliminate all the contamination.

Calluses were successfully produced from protoplasts of both Desiree and Maris Piper

when these were isolated from *in vitro* shoot cultures. Leaves were already aseptic and adapted to *in vitro* conditions. However, protoplasts isolated from *in vitro* shoot cultures of *S. commersonii* failed to form colonies upon culture. Recently, Cardi *et al.* (1990) have reported on protoplast isolation, culture and plantlet regeneration from protoplasts of *S. commersonii* but they used V-KM medium (Bokelmann and Roest, 1983) for protoplast culture. Different genotypes most probably have specific nutritional or hormonal requirements to initiate, and/or sustain, division of their protoplasts necessary for colony formation.

For protoplast culture, modified MS medium (MS medium without NH_4NO_3) has been frequently used by several workers (e.g. Handley and Sink, 1985; De Vries and Bokelmann, 1986; Kikuta *et al.*, 1986; Tavazza and Ancora, 1986; Tavazza *et al.*, 1988), while some workers used V-KM medium (Binding *et al.*, 1978; Bokelmann and Roest, 1983; Feher *et al.*, 1989; Cardi *et al.*, 1990). Both of these media have been shown to generate calluses from potato protoplasts. In the present work for the culture of cultured-cell protoplasts, MS-KM medium, a modification of V-KM medium (Bokelmann and Roest, 1983) was used, in which the inorganic salts of V-47 medium (Binding, 1974) were replaced with MS inorganic salts. Hence this medium was composed of inorganic salts of modified MS medium and the organic salts of V-KM medium. Modified MS inorganic salts are commercially available as powder mix.

Although calluses were obtained from cell suspension-derived protoplasts of both cultivars of *S. tuberosum*, no useful comparison of their growth, plating efficiency and colony formation can be made with that of mesophyll protoplasts because they were

of different origin, and different media and conditions were used for their culture.

4.6 Plant regeneration

The potato, like other Solanaceous genera, shows considerable regeneration activity in culture, and adventitious shoots can be produced both directly from organ cultures and from callus under appropriate conditions.

Organogenesis is highly dependent on the interaction between naturally-occurring endogenous growth hormones and exogenous growth regulators added to the culture medium. Depending upon the genotype, the origin of the explant and the culture conditions, it is often necessary to alter the composition and/or concentration of growth regulators in the culture medium. Generally a low ratio of auxin to cytokinin is required for adventitious shoot development.

A. From tuber explants

In the present studies, three media developed by Jarret *et al.* (1980a), Iapichino *et al.* (1991), and Ahloowalia (1982) were used to regenerate shoots from tuber explants of *S. tuberosum* cvs. Desiree and Maris Piper. Shoots arose from small protuberances of tissue formed around the edges of the explant four weeks after transfer but the time required for shoot initiation varied with the medium used. Although shoot regeneration was earlier on the medium of Ahloowalia (1982), the number of shoots produced was higher on the medium of Iapichino *et al.* (1991). No differences in the time required for shoot initiation were observed between the explants of Desiree and Maris Piper. The medium of Jarret *et al.* (1980a), which was developed for shoot regeneration from

tuber explants, proved unsuccessful in the present work, while the media of Iapichino *et al.* (1991) and Ahloowalia (1982), developed for shoot regeneration from internodal explants and callus respectively, proved to be successful. Similar results were reported by Wheeler *et al.* (1985), who compared the regeneration media of Jarret *et al.* (1980a), Shepard (1980) (C/D media), and of Roest and Bokelmann (1976) for shoot regeneration from tuber explants of 12 cultivars of *S. tuberosum*, including Desiree and Maris Piper. These media were developed for shoot regeneration from tuber explants, rachis explants and from protoplast-derived calluses respectively. A limited success was obtained in 7 cultivars on the C/D media of Shepard (1980). A few explants gave only one or two shoots and none of the media induced profuse shoot formation.

In the present work, both media containing zeatin (those of Iapichino *et al.*, 1991, and Ahloowalia, 1982) proved successful, while the medium containing BAP (Jarret *et al.*, 1980a) failed to generate shoots from tuber explants, which indicates that zeatin is more effective than BAP. Similar results have been reported by Shermann and Bevan (1988) in tuber explants of *S. tuberosum* cultivars. Lam (1975) induced adventitious shoots from cultured tuber explants on a medium containing 0.8 mg l^{-1} kinetin and 0.4 mg l^{-1} BAP. In a later experiment, he found that the addition of zeatin resulted in the formation of fully-developed shoots (Lam, 1977a). Kikuta and Okazawa (1982) also obtained shoots from tuber explants using 0.5 mg l^{-1} zeatin and 0.1 mg l^{-1} IAA in the medium.

B. From stem explants

Explants cultured on the medium of Jarret *et al.* (1980a), containing 3 mg l^{-1} BAP, 0.3 mg l^{-1} IAA and 0.5 mg l^{-1} GA_3, first produced callus and shoots were later generated (in *S. tuberosum* only) from that callus. On the other hand on the medium of Iapichino *et al.* (1991), containing 2 mg l^{-1} zeatin and 1 mg l^{-1} IAA, multiple shoots arose from swellings on tissues with a minimum amount of callus formation at the cut edges of the explants. Shoot regeneration (when this occurred) from internodal explants was 3 - 4 weeks earlier on the medium of Iapichino *et al.* (1991) than on the medium of Jarret *et al.* (1980a). Several workers have reported BAP to be an effective growth regulator in stimulating organogenesis in different *S. tuberosum* cultivars (e.g. Lam, 1975; Webb *et al.*, 1983). In the present work, medium containing zeatin was more effective than that containing BAP. Similar findings have been reported on stem and leaf explants of dihaploid clones of *S. tuberosum* (Pett and Tiemann, 1987) and of *S. commersonii* (Iapichino *et al.*, 1991).

Shoots were regenerated from internodal explants of *S. commersonii* only on the medium of Iapichino *et al.* (1991), while internodal explants of *S. acaule* failed even to survive on either of the media tested. Therefore a satisfactory protocol should have to be developed before any experimental work could be performed with *S. acaule*.

Fish and Jones (1988) compared tissue culture responses between related tetraploid and dihaploid potato genotypes on the basis of shoot formation from leaf explants using the shoot regeneration medium developed by Karp *et al.* (1984). They observed large differences in tissue culture response between tetraploid and dihaploid genotypes and

even among dihaploid genotypes derived from the same tetraploid parent. Other workers have also reported genotypic differences for regeneration ability from explant cultures of potato clones (Wheeler *et al.*, 1985; Kaburu M'Ribu and Veilleux, 1990; Cardi *et al.*, 1992).

Due to their morphological differences, the explants do not represent identical tissues, and therefore a direct comparison of explants (tuber and stem) may not be appropriate. It is, however, important that various tissues can be used as explants for shoot regeneration.

C. From callus cultures

Desiree and Maris Piper tuber calluses were subcultured onto the shoot regeneration media of Jarret *et al.* (1980a), Iapichino *et al.* (1991) and Ahloowalia (1982). No signs of shoot initiation were observed on regeneration media even after 26 weeks of culture, when the experiment was discarded. This is probably because the callus used was two years old and had been maintained through routine subcultures, and had lost its morphogenic ability (Lam, 1977b; Bhojwani and Razdan, 1983; Pierik, 1987).

Shoot regeneration from leaf calluses of both Desiree and Maris Piper was achieved on all the three media tested, viz., the medium of Iapichino *et al.* (1991), of Lam (1977b), and of Ahloowalia (1982). Shoots were initiated earlier from the calluses subcultured on the medium of Iapichino *et al.* (1991) followed by that of Lam (1977b), while it took the longest time on the medium of Ahloowalia (1982). This was probably due to the concentrations of cytokinin used, which were 2 mg l^{-1} zeatin; 0.2 mg l^{-1}

kinetin plus 0.5 mg l^{-1} BAP and 0.5 mg l^{-1} zeatin; and 1 mg l^{-1} zeatin in a different auxin to cytokinin ratio; respectively in the above media.

In the present studies, shoot regeneration was also 1 - 2 weeks earlier from Maris Piper callus than from Desiree callus which indicates that cultivars may vary in their response to shoot regeneration.

D. From suspension-cultured cells

Plantlets were regenerated from stem callus-derived cell suspension cultures of the cvs. Desiree and Maris Piper. Cell suspensions were plated onto the cell plating medium of Lam (1977b) to regenerate the calluses from cultured cells. After 4 weeks, when calluses were 2 - 3 mm in diameter, instead of transferring them to a second medium with a reduced concentration of NAA for greening (Lam, 1977b; Austin and Cassells, 1983; Lindeque *et al.*, 1991), they were kept on the same medium but the light intensity was increased from 250 lux to 1000 lux. This proved quite useful and calluses became light green, and were then transferred to the media for shoot regeneration. The media chosen for use in present work were.

a) Medium of Lam (1977b), developed for shoot regeneration from tuber callus-derived cell suspensions of cv. Superior.

b) Medium of Bokelmann and Roest (1983), developed for shoot regeneration from protoplast-derived calluses of cv. Bintje.

Both media were found to be efficient in shoot regeneration from calluses derived from suspension-cultured cells of stem origin. The medium of Lam (1977b) has been

successfully used by some workers (Austin and Cassells, 1983; Lindeque *et al.*, 1991) for shoot regeneration from stem callus-derived cell suspensions. Shoot regeneration was 2 weeks earlier from the calluses subcultured on the medium of Bokelmann and Roest (1983) than from those subcultured on the medium of Lam (1977b), probably because these media contained different growth regulators in different concentrations. Cytokinins could possibly act through transfer RNA, and the medium of Bokelmann and Roest (1983) contained zeatin riboside which was probably readily available to the plant cells.

E. From protoplast-derived calluses

Two shoot regeneration media, viz., that of Lam (1977b) and of Bokelmann and Roest (1983), which proved successful for shoot regeneration from calluses derived from suspension-cultured cells, were used for shoot regeneration from protoplast-derived calluses. Shoot initiation was about 2 weeks earlier from calluses subcultured onto the medium of Bokelmann and Roest (1983) than those subcultured onto Lam (1977b) medium, as occurred in the case of shoot regeneration from calluses derived from suspension-cultured cells.

Shoot regeneration was also about 2 weeks earlier from calluses derived from mesophyll protoplasts than from calluses derived from suspension-cell protoplasts. This was probably because cell suspensions were maintained in a medium which contained 2,4-D (2 mg l^{-1}), which affected the morphogenic ability of cells resulting in delayed differentiation. The results of the present study are in agreement with those of Tan *et al.* (1987), who noticed differences in shoot regeneration between leaf mesophyll

protoplasts and cell suspension-derived protoplasts of *S. lycopersicoides* and *Lycopersicon pennellii*.

4.7 *In vitro* tuberization

It is possible to induce tubers from axillary buds under appropriate stimuli, and there is general agreement that the stimulus leading to tuberization is hormonal in nature, but is affected by other factors including photoperiod, temperature, nitrogen level, and also applied growth substances. Addition of a cytokinin to the medium promotes stolon formation *in vitro* followed by tuberization (Hussey and Stacey, 1984). Several workers (Tovar *et al.*, 1985; Estrada *et al.*, 1986; Lentini and Earle, 1991) used 5 mg l^{-1} BAP in combination with 500 mg l^{-1} CCC to induce tuberization from a range of potato genotypes, while Rosell *et al.* (1987) used 2 mg l^{-1} BAP and 2 mg l^{-1} NAA in combination with 100 mg l^{-1} CCC. Wang and Hu (1982) did not add any growth-inhibiting substance to the medium but used a higher concentration of BAP (10 mg l^{-1}). Kwiatkowski *et al.* (1988) used kinetin 10 mg l^{-1} with IAA 0.1 mg l^{-1} to induce microtubers *in vitro* from single nodal cuttings of 43 potato clones and two wild species (*S. microdontom and S. pinnatisectum*).

The methods adopted by most of the workers utilize either a cytokinin together with CCC, or cytokinin alone but at a higher concentration. In the present study no growth-inhibiting substance was used. When 5 mg l^{-1} BAP was added to the MS medium plus 80 g l^{-1} sucrose (Med. TB), this reduced shoot growth in all the potato types tested, speeded up the tuberization process and increased individual tuber weight in *S. tuberosum* and *S. acaule*, but reduced tuber number in *S. tuberosum* and *S.*

commersonii. When 0.5 mg l^{-1} 2,4-D was also added to this medium (Med. TC) this further reduced vegetative growth of all potato types, enhanced the tuberization process with a slight improvement in tuber number in *S. tuberosum* and *S. acaule*, and increased tuber weight in *S. tuberosum*. *S. acaule* only produced microtubers when BAP was added to the medium, no microtubers were produced from *S. acaule* nodal segments on medium lacking BAP (Med. TA). In contrast, in *S. commersonii* tuber number and average tuber weight were higher when no growth regulator was added to the medium (Med. TA). This confirmed that *in vitro* tuberization could be achieved without using growth regulators, at least in some genotypes. This is in conformity with the results of Garner and Blake (1989) who achieved reliable microtuber production by culturing nodal explants of *S. tuberosum* cvs. Pentland Javelin and Maris Piper without addition of growth regulating substances.

The effect of auxins (2,4-D, IAA, NAA) on *in vitro* tuberization depends upon their concentrations used. Lower concentrations ($\leq$ 1 mg l^{-1}) induce tuberization, while higher concentrations are less effective and even inhibitory (Wang and Hu, 1985). In the present study, addition of 2,4-D (0.5 mg l^{-1}) to the medium with BAP (5 mg l^{-1}) gave the best results in *S. tuberosum* cultures. Mangat *et al.* (1984) also reported increased numbers of tubers on nodal segments cultured *in vitro* on media containing low concentrations of 2,4-D (0.022 - 0.22 mg l^{-1}). In their experiment higher concentrations (2.2 mg l^{-1} or above) resulted in the formation of stolons rather than microtubers and only a few small tubers were formed. In the present study, a low concentration of 2,4-D (0.5 mg l^{-1}) was used and microtuber formation consistently occurred at a rate of one microtuber per explanted node. In some instances two or

more microtubers were formed on each nodal explant, suggesting that further general improvement may be possible.

Tuber initiation occurred earlier in *S. tuberosum*, followed by *S. commersonii*, while *S. acaule* took a considerably longer time to form tubers. Tuberization was earlier in *S. tuberosum* cv. Desiree compared to Maris Piper. These results are in accordance with the findings of Jones *et al.* (1989) who noticed marked genotype dependency for the induction of microtubers *in vitro*. In their experiments, tuberization was also earlier in Desiree than Maris Piper.

Some morphological differences were also noticed in *in vitro*-produced microtubers compared with field-produced tubers. These differences were probably due to the carry-over effects of growth regulators used in the medium to induce tuberization. Another factor could be the artificial environmental conditions used for tuber induction. *In vitro*-produced microtubers in this work also had either only shallow, or a short period of dormancy. Upon transfer to the shoot culture medium of Espinoza *et al.* (1986), they sprouted. Tuber dormancy is supposedly related to the hormonal balance within the tuber. Media constituents, especially growth regulating substances used for tuber induction, play a regulatory role in controlling tuber dormancy. In the present experiment, BAP and 2,4-D were used and no growth-inhibiting substance was added to the tuber-inducing media. The added growth regulators might have affected the physiological state of the produced microtubers. Harvey *et al.* (1991) also observed premature sprouting of microtubers *in vitro* when lower concentrations of ancymidol or paclobutrazol were used in the tuber-inducing medium, while higher concentrations

reduced premature sprouting. Neither ancymidol nor paclobutrazol prevented sprouting when microtubers were removed from plantlets and placed in conditions favourable for growth. Environmental conditions during tuber initiation also play a critical role in tuber dormancy. Microtubers induced under 8 h photoperiod had a shorter dormancy period than when induced in complete darkness (Tovar *et al.*, 1985; Estrada *et al.*, 1986). This could also be one reason for the short dormancy period because the microtubers in the present work were induced under an 8 h photoperiod. Normal plantlets could be derived from these microtubers, and these, therefore, could play a useful role in the production of seed tubers.

Microtubers were also produced from the stems of the plantlets on the shoot culture medium of Espinoza *et al.* (1986), which contained 0.25 mg l^{-1} GA_3 and 2 mg l^{-1} Ca-pantothenic acid, after 8 weeks of culture under a 16 h photoperiod. Gibberellins promote vegetative growth and inhibit tuberization and tuber development (Bottini *et al.*, 1981; Koda and Okazawa, 1983; Hussey and Stacey, 1984). In the present work, the medium also contained Ca-pantothenic acid, which may have counteracted the inhibitory effect of GA_3 on tuberization.

In general, continuous darkness or short photoperiod induces tuberization (Wang and Hu, 1982; Charles and Rossignol, 1992), but conflicting results have also been reported on the effect of photoperiod on microtuber development. The work of Hussey and Stacey (1981) suggested beneficial effects of 16 or 24 h photoperiods on tuberization over 8 h photoperiod. Later they reported that short photoperiods had no enhancing effect on the number of microtubers produced after 16 weeks on a medium

without cytokinin (Hussey and Stacey, 1984). Garner and Blake (1989) found that complete darkness or short photoperiod promoted an initial acceleration in the rate of microtuber formation but there was no discernible effect on the number of microtubers produced. Wang and Hu (1985) suggested that a short photoperiod of low light intensity or complete darkness should be used and cytokinin added to the medium to stimulate the tuberization process. On the other hand a long photoperiod at a relatively high light intensity should be used if cytokinins are not added in the medium. Thus, formation of microtubers on plantlets grown on a medium without cytokinin and maintained under 16 h photoperiod with a light intensity of 1000 lux is in accordance with these findings. The reason for such a light requirements is obscure.

During micropropagation, when the culture vessels were tightly closed, shoot morphology became altered, indicating an accumulation of ethylene in the vessels, but tuberization still occurred in *S. tuberosum* cultures. The role of ethylene on tuberization of potato is controversial. Some believe it induces tuberization while others feel it inhibits tuberization. Gracia-Torres and Gomez-Campo (1973) reported that application of ethrel (an ethylene-releasing agent) led to an increase in the frequency of tuberization of potato sprouts *in vitro*. Other workers, nevertheless, found that ethylene was not only ineffective in inducing tuberization (Koda and Okazawa, 1983) but actually inhibited the process (Wang and Hu, 1985; Mingo-Castel *et al.* 1976). Hussey and Stacey (1984) found that sealing the culture vessels had an overall strongly inhibitory effect on tuberization and the inhibition was greater in short days than in continuous light. These results indicate that ethylene affects tuberization but the mode of action is uncertain.

4.8 *In vitro* selection

4.8.1 Selection for hyp-resistance

A. From axillary bud cultures

The main aim of *in vitro* selection is to produce plants with modified genotypes. If selection is made from unorganized/undifferentiated cultures, the ability to regenerate plantlets from selected cells is essential. This is frequently difficult with established callus or suspension cultures, which may have lost their morphogenic ability if maintained for long periods. Secondly there is no guarantee that selection made for a particular characteristic at the cellular level will also be expressed in plants regenerated from those cultures (Dix, 1977; Templeton-Somers *et al.*, 1981). One way of bypassing these difficulties is to make selections with fully or partially differentiated tissue. The possible systems could be axillary bud or meristem cultures, highly organized explants (e.g. leaf segments) or somatic and androgenetic embryos (Dix, 1986).

Using an adventitious technique van Harten *et al.* (1981) obtained a wide range of mutant forms with a very low rate of chimerism from X-irradiated (22.5 - 27.5 Gy) leaf explants of *S. tuberosum* cv. Desiree. They also observed mutations in shoots derived from the control, non-irradiated explants. Sonnino *et al.* (1984) also detected a range of mutations in plants produced from axillary bud cultures of cv. Desiree, initiated from gamma-irradiated (30 Gy) *in vitro* plantlets. Most of the mutants were also found to be apparently homogeneous and no variation was observed in the control plants. Changes in ploidy level through shoot regeneration from leaf, stem and tuber explants of potato clones has also been reported by several workers (e.g. Karp *et al.*,

1984; Wheeler *et al.*, 1985; Kaburu M'Ribu and Veilleux, 1990; Cardi *et al.*, 1992).

In the present work, no mutagen was used, and nodal cuttings, each containing its associated axillary bud, were cultured on a medium containing a range of hyp (hydroxyproline) concentrations. Although attempts to select for hyp-resistance from axillary bud cultures failed, it gave a general idea about hyp concentrations which could then be used for selection from callus cultures and suspension-cultured cells. The major drawback of using differentiated cultures such as axillary buds is the non-occurrence of somaclonal variation. The only possibility is if a chimera is already present in an axillary bud, that will show variation, but the chances are rare.

B. From callus cultures

Callus is considered to be the simplest material to which to apply selection pressure and has been used to select numerous variant cell lines. Resistance selection in callus cultures is straightforward to perform. Callus pieces are placed on a selective medium and resistance is observed as sectorial growth from the cultured callus pieces which may predominantly consist of dead or growth-inhibited cells. Any new growth can be excised and subcultured. A number of amino acid analogue resistant lines have been selected in this way e.g. azetidine-2-carboxylic acid (a proline analogue) resistant lines of tobacco (Dridze *et al.*, 1991).

In the present work, calluses of *S. tuberosum* cvs. Desiree and Maris Piper were subcultured onto MS medium plus 3 mg l^{-1} 2,4-D in separate batches containing hyp concentrations of 10, 20 and 30 mM. The growing sectors of the calluses on these

media were excised and subcultured onto fresh media of same composition. After several passages a number of hyp-resistant lines were established.

It has long been established that unorganized tissue cultures tend to be cytogenetically unstable. Changes in both number and structure of chromosomes have been observed, and these increase as the culture period is prolonged. Thus, tissue culture-induced, or somaclonal, variation can be exploited and selection for a desirable trait made.

C. From plated cells of suspension cultures

The advantages of using suspension-cultured cell are that the single cells and small aggregates of cells are exposed to selection pressure, individual colonies can be selected, and a large population of cells can be screened out with minimum effort and space requirements. Moreover cells are in good contact with the selection medium, which is a problem with callus or organized tissues. Therefore resistant colonies can be selected by incorporating toxic levels of selective agents into a plating medium, or by exposing the plates to appropriate environmental conditions such as low temperature (Dix and Street, 1976).

In the present studies, suspension-cultured cells of *S. tuberosum* cvs. Desiree and Maris Piper were plated on Lam (1977b) cell plating medium containing 5 or 10 mM hyp and several hyp-resistant colonies were isolated with a spontaneous frequency of 0.25×10^{-6}. When cells were irradiated with gamma rays at a dose of 20 Gy before plating, the frequency of resistant colonies recorded was 0.35×10^{-6} showing a slight improvement. Low doses of gamma radiation tend to increase the rate of mutations in

plant cell cultures without discernable damage to the cell structure. Although mutations are random, sometimes mutagenic treatment may yield some desirable changes in the genome which can be selected and exploited. The present study is at variance with early findings of van Swaaij *et al.* (1986) who reported a frequency of 2.9×10^{-6} for hyp-resistant cell colonies which was not increased when mutagenic treatment with ENU (10 or 50 μM) was applied.

Hyp-resistant lines have been reported to possess increased frost tolerance (van Swaaij *et al.*, 1986, 1987a & b: Tantau and Dorffling, 1991). To test whether directly selected frost-tolerant lines are also hyp-resistant, suspension-cultured cells were exposed to a freezing temperature of -6°C (see Materials and Methods, Section 2.14.1 C) and plated on Lam (1977b) cell plating medium. Eight cell lines were established and these were transferred to hyp-containing media. Only two lines grew well, showing resistance to hyp, while the other six failed to survive on the selective medium. This suggests that frost tolerance and hyp resistance might be linked, at least in some variants.

4.8.2 Effect of proline on growth and frost tolerance of cultures

When proline was applied exogenously to the axillary bud cultures of *Solanum* species, it reduced their growth. Similar results were obtained with callus cultures of *S. tuberosum* cvs. Desiree and Maris Piper. As far we are aware, the only report published on the effects of proline on the growth of *in vitro* cultures of *Solanum* is that of Hellergren and Li (1981), who studied the mode of action of proline on freezing tolerance in *S. tuberosum*, and also reported that when cells were frozen and grown

on a medium containing 0.17 M proline, the growth of cells was slow. In the present work, both axillary bud and callus cultures showed reduced growth rates when grown on a medium containing 0.05 M proline.

Exogenously-applied proline also resulted in increased frost tolerance of leaves and callus cultures. This was probably due to the accumulation of proline within the cells, and its role in protection against frost is documented (van Swaaij *et al.*, 1985 & 1986; Dobslaw and Bielika, 1988; Tantau and Dorffling, 1991). It has been suggested that proline has a membrane-stabilizing effect during the stress and protects against denaturation by freeze-induced dehydration (Hellergren and Li, 1981). Increase in frost tolerance in leaves of shoot cultures of *S. andigena* (van Swaaij *et al.*, 1985) and suspension-cultured cells of *S. tuberosum* (Hellergren and Li, 1981) has already been reported after application of 0.05 M proline to shoot cultures and 0.43 M proline to the cell suspension cultures. The results of the present study are therefore in accordance with previous findings.

To find out whether the increase in frost tolerance is due to the osmotic effect of proline (0.05 M) added to the medium, or whether some sort of physiological mechanism was involved, 0.05 M mannitol was added in separate cultures. Addition of mannitol to the medium reduced the growth of cultures to a lower extent but did not result in an increase in frost tolerance. This indicates that increased frost tolerance was not due merely to an osmotic effect and makes an involvement of proline in the mechanism of frost tolerance more likely.

4.8.3 Selection for frost tolerance

Tissue culture techniques can be used as convenient aids for selection procedures. One approach relies on the direct use of the *in vitro* cultures for selection, and other is the use of cultures for rapid multiplication and the production of plantlets for testing in the greenhouse or the field.

In the present studies, described above, hyp-resistant lines were selected from cell cultures of *S. tuberosum* and then evaluated for frost tolerance. Most of the selected hyp-resistant lines showed an increased degree of frost tolerance compared with non-selected controls. A few lines which were found to be hyp-resistant did not appear to be frost-tolerant, and showed lower or equal degrees of frost tolerance to non-selected controls. The increased frost tolerance in other hyp-resistant cell lines was probably due to elevated proline contents within these lines. It is hypothesised that these lines were able to convert some of the hydroxyproline into proline. On the other hand, in those lines which were not frost-tolerant but were hyp-resistant, the mechanism might be different. It is assumed that in these lines hyp-resistance was due to inhibition of the uptake of hydroxyproline from the medium.

In hyp-resistant lines, proline occurs in different cellular compartments: cytoplasm and/or vacuole. Proline accumulated in the cytoplasm has direct stabilizing effects on cytoplasmic membranes and proteins. If proline is accumulated in the vacuole, this stabilizing effect will be reduced, irrespective of the total proline concentration in the cells. Fricke and Pahlich (1990) have reported changes in the proline pools between cytoplasm and vacuole. It can therefore be hypothesised that the cell lines which did

not show any increase in frost tolerance had accumulated proline in their vacuoles. Further research should be carried out to determine the cause of differences in frost tolerance among hyp-resistant cell lines, including total proline contents and in compartments.

The results of the present selection studies are in agreement with results obtained by van Swaaij *et al.* (1986 & 1987a) with cell cultures of potato, and by Tantau and Dorffling (1991) with cell cultures of wheat. They selected several hyp-resistant cell lines from cell cultures, which also showed increased frost tolerance. Results of these studies demonstrate that *in vitro* selection for resistance to hyp makes it possible to isolate cell lines with increased frost tolerance. It is of course essential that such selected lines should be able to be regenerated into plants, and that frost tolerance should be expressed at the whole plant level, and further, that it should be genetically transmittable. Plant regeneration has already been reported in previous studies (van Swaaij *et al.*, 1986 & 1987a) and has been achieved in the present work.

4.8.4 Plantlet regeneration from frost-tolerant cell lines

Efforts to regenerate plantlets from two frost-tolerant cell lines proved partially successful, one of the two tolerant lines was able to be regenerated into plantlets while the other failed. This was probably due to the loss of morphogenic ability of this line because callus had been more than 18 months in culture. Further, the shoots regenerated from the remaining line displayed phenotypic variability. The observed variations could possibly be due to somaclonal variation, while phenotypic alterations due to the pleiotropic effects of hyp on chromosomal structure cannot be ruled out.

4.8.5 Stability of selected cell lines

A. Hyp-resistant cell lines

When two hyp-resistant cell lines were subcultured on non-selective (hyp-free) medium, their growth, estimated on fresh weight basis, increased. After two passages on non-selective medium, when callus of these lines was brought back to the selective medium their growth rate again decreased and their recorded growth was less compared to similar calluses which were maintained throughout on selective medium. This indicates that hyp-resistance of these lines was decreased upon transfer to non-selective medium. Similar results have been reported in hyp-resistant lines selected from cell cultures of potato by van Swaaij *et al.* (1986 & 1987a) , and wheat by Tantau and Dorffling (1991). It may be possible that growth on a non-selective medium was accompanied by a decrease in the proline levels within cells. As the proline contents of these lines were not measured, this assumption remains speculative.

This also suggests that hyp-resistance was probably either due to the part conversion of hydroxyproline into proline by variant cells, or due to a physiological adaptation of these cells to the hyp-stress, but the mechanism is not yet known. Enzyme activity converting free hydroxyproline into proline has already been shown in carrot slices (Varner, 1980).

B. Frost-tolerant cell lines

The plants regenerated from the frost-tolerant callus still possessed a lower level of frost tolerance in their leaves than was shown by the callus from which the plants were regenerated. However, their tolerance was still greater than that of control, non-

selected plants. Therefore, the selected trait appeared to be partially stable. As frost tolerance was maintained through plant regeneration, this suggests that this trait could be a result of genetic rather than epigenetic change, but the question remains as to why the degree of frost tolerance was lowered. This could probably be due to the instability of genomes in the cell culture systems (somaclonal variation).

4.9 Cytology of *Solanum* callus cells

Almost all callus cultures are derived from tissues composed of *vacuolated cells. Two* main types of vacuolated cells are used for callus induction; those of vascular cambium, which are already in a state of active division, and parenchyma cells which may be induced to divide. Rapid division within tissue results in callus formation, during which large of masses of highly vacuolated cells are formed. Considerable changes can occur in cellular structure due to activation of callus induction and development. Established callus might produce a range of cell form and structure characteristic of their mode of growth (Yeoman and Street, 1977).

In the present study, the cytology of callus cells of *Solanum* species was investigated to compare the cellular structure of frost-sensitive (*S. tuberosum* cv. Desiree) with frost-tolerant species (*S. commersonii* and *S. acaule*), and a frost-tolerant cell line (D20-1) selected from unorganized cultures of *S. tuberosum*. It is possible that cells of frost-tolerant species or lines may present a different cellular organization.

The callus cells of *Solanum* species possessed a normal range of cellular organelles, and their structure and arrangement suggested that the cells were metabolically active.

In the cells of *S. tuberosum* and *S. acaule*, very little starch was seen in the plastids, and this suggests that the cells were fixed while in an active state of growth, where food would be used in metabolism rather than being stored. However, in the cells of *S. commersonii* and in the frost-tolerant callus of Desiree starch grains were present in the plastids. The plastids which contained starch grains appeared bigger in size than others without starch grains.

It proved very difficult to demonstrate any significant cytological differences between the callus cells of frost-sensitive and frost-tolerant species. Studies have been made to locate ultrastructural differences in the leaf mesophyll cells of frost-sensitive and frost-tolerant *Solanum* species (Chen *et al.*, 1977), but we are not aware of any work published on ultrastructural differences in cultured cells.

Chen *et al.* (1977) reported that the cell walls of leaf mesophyll cells of *S. acaule* were about twice as thick as those of *S. tuberosum* cv. Red Pontiac, and that the chloroplasts of *S. acaule* contained a higher number of osmiophilic globules than the chloroplasts of *S. tuberosum*. These differences could not be demonstrated in the callus cells in the present work, possibly because of their active state of growth. In the cells of *S. acaule*, vacuoles were smaller and most of the cellular volume was filled with cytoplasm, with the cellular organelles arranged at the periphery of the cytoplasm suggestive of cells dividing. However, callus cells of the frost-tolerant line (D20-1) selected from Desiree callus differed in the number of protein crystal-containing microbodies, and in the starch grains present in the plastids. It is tempting to suggest that the presence of an increased number of microbodies containing protein crystals

suggests the involvement of protein in frost tolerance. However, as this was not noticed in the callus cells of the frost-tolerant wild species, the mechanism of frost tolerance could be different if this were so. Moreover, in the vacuoles of the frost-tolerant callus cells only, some darkly-stained spots were seen in the photographs that could be possibly due to substances such as proline, which had been over produced and/or accumulated in the cell vacuoles.

4.10 Freezing damage to the cell structure

A freezing temperature of -3°C is regarded as a frost-killing temperature for *S. tuberosum*. When callus cells were frozen to this temperature, organelles appeared swollen and were probably non-functional. Plasma membranes still seemed to be intact, whereas the tonoplasts appeared damaged. The damage to cellular structure appeared irreversible and hence, at -3°C *S. tuberosum* cells are killed. Similar damage has been reported by Palta and Li (1982) in the cells of *S. tuberosum* leaflets frozen to -3.5°C. In their experiment, cells frozen to -3°C appeared to be normal and similar to the control (unfrozen). Probably this difference is due to the different nature of cells (mesophyll origin and callus origin) and to the freezing techniques used. On the other hand it could be due to the variability for frost tolerance within the species *S. tuberosum*. Plasma membranes appeared intact in their experiment, as also in the present study, which indicates that at this temperature freezing injury was not caused by physical rupture of plasma membranes, but these might have become denaturated.

In the present study, when cells were frozen to a temperature of -6°C, the damage became severe and plasma membranes were observed to be ruptured. At -10°C the

damage became so severe that in some cells organelles could no longer be recognized. Many vesicles also appeared in the cytoplasm in some cells. These vesicles did not appear to be discharged into the vacuole, probably due to the damaged state of the tonoplasts and the prevention of normal cellular activities. Moreover, the cytoplasm became fragmented and again no fragments were found in the vacuole. The temperature range (control to -10°C) used in the present study is now considered to be too wide, and it would be interesting to examine the cellular damage after exposing the cells more gradually to a range between 0°C and -3°C.

4.11 Conclusion

Tissues and cells of *Solanum* species can successfully be cultured *in vitro* on suitably-defined media. Protoplasts can be isolated from leaves and suspension-cultured cells and regenerated into plants. Microtubers can be induced *in vitro* on cultured stem segments of *Solanum* species. Somaclonal variation can be exploited for the selection of hyp-resistant and frost-tolerant cell lines. Hyp-resistance and frost tolerance could be linked and might have a common basis, at least in some selected lines. Most of the hyp-resistant lines also show increased levels of frost tolerance. It is possible to regenerate some of the resistant lines into plants which possess slightly increased frost tolerance in their leaves compared with normal, control plants. Cells from a selected frost-tolerant line contained a higher number of protein-containing microbodies compared with normal callus cells derived from the same cultivar.

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