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PLANT REGENERATION AND SOMATIC HYBRIDIZATION

IN THE GENUS SOLANUM

by

STEPHEN CLAIR GLEDDIE, B.Sc., M.Sc.

A thesis submitted to the Faculty of
Graduate Studies and Research in the partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

Department of Biology

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Ottawa, Ontario

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ABSTRACT

Cell suspension cultures of eggplant (Solanum melongena L.) were used as a source of protoplasts for regeneration and somatic hybridization studies. Greater yields of protoplasts were obtained from cells grown in 2,4,-D than in NAA. The efficiency of cell division following protoplast isolation and culture was optimized at 25% while the optimal plating efficiency was 1%. Protoplast division and colony formation occurred in several media; however, somatic embryogenesis occurred in protoplast-derived cell colonies following 20-40 days of culture in media containing 1 mg/L 2,4-D. Plant regeneration was achieved by plating isolated embryos on cytokinin-supplemented medium to promote further cycles of somatic embryogenesis and shoot organogenesis.

Explant cultures were established in: Solanum sisymbriifolium, S. aviculare, S. gilo, S. torvum, S. aculeatissimum, S. khasianum, and S. macrocarpon. Plants could be regenerated via shoot organogenesis on cytokinin medium in all cases except in S. khasianum. Rapidly-growing cell suspensions in MS medium + 2 mg/L 2,4-D were explored as sources of protoplasts in these species. All cell suspensions were capable of proliferation as callus masses, but neither organogenesis nor embryogenesis were observed.

Protoplasts were isolated from cell suspensions of these species and cultured in the same medium used for eggplant protoplast culture. Cell divisions were observed in S. aviculare, S. sisymbriifolium, and occasionally in S. gilo but not in the other species. Plant regeneration was obtained only in S. aviculare protoplast-derived colonies. Solanum sisymbriifolium protoplast cultures, on the other hand, divided with low efficiencies and plants could not be regenerated.

Protoplasts of 6-azauracil (AU) resistant eggplant lines were fused by PEG treatment with those of S. sisymbriifolium to create somatic hybrids between these sexually-incompatible species. Following fusion, colonies were selected which were

capable of growth in medium containing 1 mM AB. S. sisymbriifolium cells could not grow in this medium and were eliminated. After 42 days the resulting presumptive hybrids were placed on medium containing zeatin which had favoured the production of purple shoots on S. sisymbriifolium tissue explants. Explants of eggplant only turned green on this medium and could be easily distinguished from those of S. sisymbriifolium. A total of 40 purple colonies which were selected were subsequently shown to be somatic hybrids. Plants were regenerated from 29 hybrid colonies. These regenerants flowered in the greenhouse but were male-sterile.

Morphological traits intermediate to the parental species were observed on each hybrid. These included: flower colour, leaf shape, and trichome type and density. The hybrid plants had reduced growth habit and did not display any hybrid vigour, although they flowered more than either parent.

Cytogenetic analysis revealed that all hybrids were aneuploids but their chromosome numbers were close to the sum of the parents. Cytogenetic analysis could not distinguish between the chromosomes of the two species however the variable chromosome numbers may have accounted for the extreme variation in phenotype between the regenerants.

Biochemical analysis revealed that both nuclear genes for several isoenzymes were expressed among the hybrids. In addition, isoelectric focussing of the large subunit of Rubisco provided evidence that each hybrid expressed only S. sisymbriifolium chloroplasts.

Preliminary testing of these hybrids for their reactions to infection with the Southern root-knot nematode suggests they have inherited this desirable trait from S. sisymbriifolium however attempts to backcross the hybrids with eggplant have thus far failed.

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INTRODUCTION

Solanum melongena L. (eggplant) has reportedly been cultivated for over 1500 years in Asia before being introduced to Europe by Arabic people during the dark ages (Khan 1979). Eggplant is a slow-growing vegetable cultivated year-round in tropical climates while in temperate latitudes it is transplanted to the field after the danger of frost has passed. It is widely used as a medicinal plant in addition to being an important vegetable in many diets. Some medicinal properties of eggplant tissues and extracts include the treatment of diabetes, asthma, cholera, bronchitis, and dysuria while fruit and leaves are reported to lower blood cholesterol levels (Khan 1979). Although commercial production of eggplant is limited in North America (8-10 thousand acres), annual world-wide production in 1981 amounted to 4.6 million metric tonnes with China being the world's largest producer (FAO Production Yearbook 1981).

Although there are several diseases and pests of eggplant (see Table 1), the most serious are the soil-borne pathogens, particularly Verticillium wilt and bacterial wilt as well as nematode infestations. These pathogens are so severe in Japan that more than 20% of commercially-grown eggplants are grafted onto resistant root stocks (Yamakawa 1982). Grafting is not an ideal solution to disease problems, however, since grafted plants may break under pressure and become more susceptible to physiological disorders such as magnesium-deficiency. In addition to lowering productivity, Mg-deficient eggplants are susceptible to tobacco mosaic virus (TMV)

TABLE 1. Pests of eggplant and sources of resistance

Pest	Resistant species	Reference(s)
Two spotted spidermite (<u>Tetranychus urticae</u>)	<u>Solanum macrocarpon</u> L.	Schaff <u>et al.</u> 1982
Carminc spidermite (<u>T. cinnabarinus</u>)	<u>S. mammosum</u> L. <u>S. pseudocapsicum</u> L. <u>S. sisymbriifolium</u> Lam.	Schalk <u>et al.</u> 1975
Fruit and shoot borer (<u>Leucinodes orbanalis</u>)	<u>S. integrifolium</u> Poir. <u>S. khasianum</u> L. <u>S. hispidum</u> Pers. <u>S. sisymbriifolium</u> Lam.	Khan <u>et al.</u> 1978 Sharma <u>et al.</u> 1980
Little leaf (mycoplasma)	<u>S. hispidum</u> Pers. <u>S. integrifolium</u> Poir	Rao 1980 Khan <u>et al.</u> 1978
Eggplant mosaic virus (<u>Tymovirus</u>)	<u>S. hispidum</u> Pers.	Rao 1980
Bacterial wilt (<u>Pseudomonas solanacearum</u>)	<u>S. torvum</u> Swartz <u>S. integrifolium</u> Poir.	Yamakawa 1982
Fungal wilt (<u>Verticillium dahliae</u> , <u>V. albo-atrum</u>)	<u>S. sisymbriifolium</u> Lam.	Fassuliotis and Dukes 1972
Root-knot nematodes (<u>Meloidogyne incognita</u> , <u>M. javanica</u>)	<u>S. sisymbriifolium</u> Lam.	Fassuliotis and Dukes 1972

infections (Seaker et al. 1982).

An effective solution to the disease and nematode problems would be to transfer resistance from wild Solanum species into eggplant (see Table 1). Many attempts have been made to sexually hybridize eggplant with wild Solanum species (see Table 2), but sterile or partially sterile progeny usually result and backcrossing and/or selfing of hybrids has been unsuccessful.

Somatic hybridization has been proposed as a novel method for the genetic modification of plants because natural barriers to sexual crossing can be bypassed. Somatic hybridization depends upon the successful integration of several specific technical procedures. These include: (i) isolation of sufficient quantities of viable protoplasts, (ii) induction of protoplast fusion, (iii) post-fusion protoplast culture to stimulate wall regeneration and cell division, (iv) selection of hybrid cells, calli or meristems, (v) regeneration of plants and (vi) validation of the hybrid nature of regenerants.

The present study was designed to explore the potential application of somatic hybridization between eggplant and related wild Solanaceous species. The specific objectives of the work were as follows:

- to develop tissue, cell, and protoplast manipulations of eggplant and related wild Solanum species sufficiently to warrant their use in somatic hybridization experiments
- to achieve somatic hybridization between sexually-incompatible species
- to develop methods for the selection of hybrid cells
- to regenerate hybrid plants which could serve as a genetic bridge for transfer of disease resistance into the cultivated eggplant.

TABLE 2. Recent hybrids between eggplant and wild Solanum species

Hybrid	Results	Reference(s)
<u>S. melongena</u> L. x <u>S. macrocarpon</u> L.	partially fertile F_1 s	Schaff et al. 1982
<u>S. gilo</u> Raddi x <u>S. melongena</u> L.	sterile F_1 s	Omidiji 1981
<u>S. integrifolium</u> Poir. x <u>S. melongena</u> L.	sterile F_1 s	Rao and Baksh 1981 Kirti and Rao 1982
<u>S. melongena</u> L. x <u>S. inolicum</u> L.	F_4 progeny obtained	Rao and Kumar 1980
<u>S. melongena</u> x <u>S. khasianum</u> L.	F_1 obtained by embryo rescue, F_2 obtained	Sharma et al. 1980
<u>S. melongena</u> L. x <u>S. hispidum</u> Pers.	sterile F_1 s	Rao 1980
<u>S. melongena</u> L. x <u>S. torvum</u> Sw.	sterile F_1 s	McCammon and Honma 1982

LITERATURE REVIEW

A. Protoplast Isolation

The first description of isolated protoplasts was by Klercker in 1892, who physically removed the plant cell-wall from the protoplasm (Cocking 1972). When cells of most angiosperms are plasmolyzed in salt or sugar solutions, it is sometimes possible to cut through the cell-wall without damaging the cytoplasm and release the intact protoplast. This procedure has provided protoplasts of onion bulb scale, beet tissue, radish epidermis, and cucumber mesocarp (Cocking 1972).

Enzymatic digestion of plant tissue to release protoplasts was first demonstrated by Cocking (1960), and since that report many potent cell-wall degrading enzymes have become commercially available (Davey 1983). These generally fall into three categories: cellulases, hemicellulases, and pectinases. Cellulases are usually essential for protoplast isolation and are often used in combination with pectinases and hemicellulases for dicotyledonous species. Graminaceous leaf protoplasts are most efficiently isolated with combinations of cellulases, xylanases, and pectinases (Ishii and Mogi 1983). Commercial enzyme preparations often contain contaminating enzymes, including deleterious types such as phosphatidase (Kao 1978) and proteases. Gel filtration has been used to desalt crude enzyme mixtures (dos Santos *et al.* 1980; Slabas *et al.* 1980) as has dialysis (Gresshoff 1980). The advantages of these procedures have not been conclusively demonstrated; many laboratories produce viable

protoplasts without enzyme purification. Desalting and lyophilization have in one case led to decreased protoplast yield without improved stability (Cassells and Barlass 1976), perhaps due to an increase in the concentration of high molecular weight toxic substances.

Plant cells are normally protected from osmotic swelling and lysis in hypotonic conditions by the cell wall. Protection of protoplasts is accomplished by dissolving wall-degrading enzymes in isotonic or slightly hypertonic solutions of sugars, sugar alcohols, or salts. In some cases, divalent cations are added to increase membrane stability (Rose 1980) while potassium dextran sulphate (Takebe et al. 1968) and polyamines (Galston et al. 1978) have been added to counteract the effect of toxic factors (ribonucleases) which may be released from cells during wall digestion. Tween 80, a detergent, has been added to the enzyme solution to enhance the release of mesophyll protoplasts (Lang and Kohlenbach 1982).

A range of enzyme concentrations from 0.025% to 5% (w/v) have been used in protoplast isolation (de la Roche et al. 1977; Nagata and Ishii 1983). The concentrations used depend upon tissue source, incubation time and temperature and the season. Generally the pH of the enzyme solution is 5.5–6.0 and in some cases buffers are added for stability (Kao and Michayluk 1975). Incubation temperatures of 20–27°C are common but extremes such as 2°C (de la Roche et al. 1977) and 36°C (Othman and Paranjothy 1980) have been used. The time required for protoplast isolation is highly variable and dependent upon plant species, tissue sources, pH, enzyme concentrations and types and temperature. Although some tissues yield protoplasts

in 30 min (Nagata and Ishii 1983), other cells require up to 24 hr (Kao et al. 1974).

Protoplasts have been isolated from a wide variety of tissue and cell types including leaves, shoots, roots, fruits, root nodules, hypocotyls, coleoptiles, microspores, and tissue cultures (Vasil and Vasil 1980). Leaf tissues and cell suspension cultures have been used as protoplast sources in most fusion experiments because they are easy to obtain in large quantities. Leaf protoplasts have been produced in a two-step method which employs a pectinase to release cells from mesophyll tissue followed by treatment with cellulase to convert the cells into protoplasts (Takebe et al. 1968). A single step involving a mixture of several enzymes is employed by most investigators although solutions of different enzyme concentrations can be used in sequence (Kantha et al. 1974; Gamborg et al. 1975). Some workers discard the initial enzyme fraction which contains cellular debris and dead protoplasts and replace it with a fresh enzyme solution. Tissue taken directly from plants requires surface-sterilization although a technique has been devised to isolate sterile protoplast populations from unsterilized tissues (Wilson et al. 1980). Shoot cultures propagated in vitro serve as a ready source of mesophyll protoplasts eliminating the problem of contamination.

Several procedural modifications may increase the yield of protoplasts. These include peeling the leaf epidermis (Zapata et al. 1977), brushing leaves with carborundum to expose mesophyll cells (Hughes et al. 1978), cutting the leaves into thin strips to facilitate enzyme entry (Huber and Edwards 1975), cutting hypocotyls into small pieces (Glimelius 1984), forcing the enzyme into intercellular spaces through evacuation (Chin and Scott 1979) and

agitation of enzymes and tissues (Grun and Chu 1978).

Many factors have been shown to affect the yield, stability and the subsequent capacity for regeneration of protoplasts. The physiological conditions of donor plants or cells prior to enzyme treatment have a marked influence on protoplast yield and/or stability (Table 3). Cells, shoots or plantlets grown aseptically in vitro under controlled conditions give more consistent yields of protoplasts (Oelck et al. 1982) than plants in greenhouse or field conditions. Variations in the isolation process have been devised to improve the yield and stability of protoplasts for a variety of species (Table 4).

Following digestion of the cell-walls protoplasts must be removed from the enzymes, cell debris and toxic substances released from the donor tissue. In most cases, protoplasts can be purified by passage through stainless steel or nylon filters followed by centrifugation at low speed (100 xg). In some cases, protoplasts have been washed by flotation in concentrated osmotica such as mannitol (Gatenby and Cocking 1977), sucrose (Shahin and Shepard 1980), Ficoll (Lin 1980), or Percoll (Adams and Townsend 1983). Discontinuous gradient centrifugation and two-phase separation techniques are reported to be effective in removing cell debris and contaminating organelles (Piwowarczyk 1979; Slabas et al. 1980). An anti-galactan-sepharose conjugate has been used to bind cellular debris (Keller and Stone 1978).

B. Protoplast Culture

Following isolation and purification protoplasts are suspended in suitable media and maintained in culture where they may respond by reforming a cell wall and undergoing cell division (Fowke and Gamborg 1980): Maximizing the frequency of protoplasts

TABLE 3. Influence of physiological conditions and/or pretreatments
of donor tissue on protoplast yield

Condition	Reference(s)
Season	Cassells and Cocker 1982
Donor plant genotype	Crowder <u>et al.</u> 1979
Donor plant (leaf) age	Bhojwani and White 1982; Engler and Grogan 1983
Light intensity received by donor plants	Cassells and Barlass 1978; Saxena <u>et al.</u> 1978
Photoperiod received by donor plants	Hughes <u>et al.</u> 1978
Humidity levels during growth of donors	Hughes <u>et al.</u> 1978; Tal and Watts 1979
Calcium and nitrogen supplementation of donor plants	Cassells and Cocker 1982
Growth rate of donor cells (tissues)	Kao and Michayluk 1980; Hackmah and von Arnold 1983
Concentration and source of nitrogen in donor cell culture medium	Wallin <u>et al.</u> 1977; Fukunaga and King 1978
Auxin type and/or conc. in donor cell culture medium	Douglas <u>et al.</u> 1981a; Wallin <u>et al.</u> 1977
Dark pregrowth or pretreatment	Hughes <u>et al.</u> 1978
Low temp. pretreatment	Shepard and Totten 1977
Preculture on low sucrose	Simmonds <u>et al.</u> 1979
Pretreatment with sulphhydryl agents	Senn and Pilet 1980
Pretreatment with cycloheximide and kinetin	Kaur-Sawhney <u>et al.</u> 1977
Pretreatment with gibberellin	Landgren 1981

TABLE 4. Influence of isolation conditions on protoplast yield

Condition	Reference(s)
Surface sterilization method	Lin 1980
Enzyme type	Lin 1980; Opatrny <u>et al.</u> 1980; Vardi <u>et al.</u> 1982
Enzyme concentration	Cassells and Barlass 1976; Burger and Hackett 1982
Vacuum infiltration of enzymes	Chin and Scott 1979
Depth (volume) of enzyme solution	Hughes <u>et al.</u> 1978
Agitation rate during isolation	Chin and Scott 1978
Temperature	Huber and Edwards 1975
pH	Huber and Edwards 1975; Chin and Scott 1979
Composition, concentration of osmoticum	Hughes <u>et al.</u> 1978; Smolenskaya and Raïdugina 1981
Addition of divalent cations to enzyme	Rose 1980; Simmonds <u>et al.</u> 1979
Addition of polyamines to the enzyme	Galston <u>et al.</u> 1978
Treatment with reducing agents	Schmidt and Poole 1980
Addition of potassium dextran sulphate to enzymes	Passiatore and Sink 1981

that divide to form cell colonies is critical to successful somatic hybridization studies. Many factors have been shown to influence the viability and subsequent plating efficiency (~~are~~ the proportion of protoplasts which form cell colonies). These include physiology of the donor tissue prior to protoplast isolation, procedures used in the isolation process, composition of the culture medium and environmental conditions used for culture (Tables 5 and 6).

The nutrient requirements of isolated protoplasts are very similar to those of cultured cells and tissues with the exception that protoplasts require an external osmoticum. The other essential components are source of reduced carbon (sugar) which may also serve as the osmoticum, inorganic salts, vitamins and growth regulators. Most species require an auxin and many also require a cytokinin, for wall regeneration and cell division. Many modifications of media have been made which significantly influence plating efficiency (see Table 6). In some cases, conditioned medium obtained from cell suspension cultures has beneficially supplemented protoplast media (Durand 1979). Nurse cultures consisting of irradiated cells (Cella and Galun 1980) albino cells (Menczel et al. 1978) or auxotrophic mutant protoplasts (Hein et al. 1983) have been used to increase the plating efficiency in low density cultures.

Protoplasts are commonly suspended in liquid medium and plated as either droplets or thin layers in petri dishes. Microdrop techniques permit the culture of small numbers of protoplasts (Kao 1977) and multiple drop arrays are convenient to test large numbers of modified media (Potrykus et al. 1979). Protoplasts have been embedded in agar and in some cases divisions only occurred in solid medium. The addition of activated charcoal has improved

TABLE 5. Influence of donor physiology and protoplast isolation conditions on viability and plating efficiency

Condition or factor	Reference(s)
Donor plant age	Chupeau <u>et al.</u> 1978; Dos Santos <u>et al.</u> 1980
Age of <u>in vitro</u> grown shoots	Van Slogteren <u>et al.</u> 1980; Oelck <u>et al.</u> 1982
Donor leaf age	Johnson <u>et al.</u> 1980
Donor cell growth rate	Strauss and Potrykus 1980; Potrykus <u>et al.</u> 1979a
Donor genotype	Kao and Michayluk 1980; Mulhbach 1980
Donor growth temperature	Tal and Watts 1979; Saxena <u>et al.</u> 1982
Light intensity during growth	Negrutiu and Mousseau 1980; Ulrich <u>et al.</u> 1980
Photoperiod during donor growth	Shepard and Totten 1977
Humidity level during donor growth	Tal and Watts 1979
Polyphenol oxidase level of donors	Schenck and Hoffmann 1979
Composition of medium for <u>in vitro</u> growth of donors	Saxena <u>et al.</u> 1982
Insecticide treatment of donor plants	Ulrich <u>et al.</u> 1980
Preculture of donor tissue explants	Gatenby and Cocking 1977
Low temperature pretreatment	Shepard and Totten 1977
Ethylene release during isolation	Cassells <u>et al.</u> 1980
Length of plasmolysis	Pental <u>et al.</u> 1982
Length of incubation in enzyme	Chin and Scott 1979; Passiatore and Sink 1981

TABLE 6. Influence of medium composition and environmental factors
on viability and plating efficiency in protoplast fusion

Condition or factor	Reference(s)
Source of water	Watts <u>et al.</u> 1974
Osmoticum concentration	Xu <u>et al.</u> 1981; Takeuchi and Komamine 1982
Osmoticum composition	Vardi <u>et al.</u> 1982; Van Slogteren <u>et al.</u> 1980; Xu <u>et al.</u> 1981
Physical state of medium	Dos Santos <u>et al.</u> 1980; Xu <u>et al.</u> 1981
Type of solidifying agent	Engler and Grogan 1983; Adaohambanásco and Roscoe 1982
Activated charcoal	Sugawara <u>et al.</u> 1983
Mineral salt composition	Mulbach 1980; Schenk and Hoffmann 1979
Calcium concentration	Von Arnold and Eriksson 1977
Zinc concentration	Von Arnold and Eriksson 1977
Ammonium concentration	Von Arnold and Eriksson 1977; Zapata and Sink 1981
Addition of auxin	Takeuchi and Komamine 1982
Addition of cytokinin	Opatrný <u>et al.</u> 1980; Von Arnold and Eriksson 1977
Addition of amino acids, sugars, organic acids, or vitamins	Kao and Michayluk 1975; Negrutiu and Mousseau 1980
Addition of polyamines	Kaur-Sawhney <u>et al.</u> 1980
Addition of concanavalin A	Eriksson <u>et al.</u> 1978
Addition of sodium succinate	Engler and Grogan 1983
Age of nurse tissue	Cella and Galun 1980
Medium pH	Bharal and Rashid 1980
Culture temperature	Bhojwani and White 1982; Engler and Grogan 1983; Bidney and Shepard 1980
Light quality	Passiatore and Sink 1981
Protoplast density	Strauss and Potrykus 1980; Nomura <u>et al.</u> 1983

division efficiency in some cases (Sugawara et al. 1983). Protoplasts have also been immobilized in calcium alginate (Adaohambanasco and Roscoe 1982), and agarose (Adams and Townsend 1983). In some cases, protoplast cultures are transferred from liquid to agar medium after short culture periods while alternatively agarose blocks are placed into liquid medium in order to sustain growth (Shillito et al. 1983). Layering of protoplast suspensions on filter paper placed over agar medium has led to improved plating efficiency in some species (Dos Santos et al. 1980). Quadrant dishes which physically separate protoplasts and conditioned medium have been used successfully for the culture of Solanum tuberosum protoplasts (Bidney and Shepard 1980).

After a culture period of several days to several weeks protoplast cultures require addition of fresh medium, usually with reduced osmoticum. Once protoplast-derived cell colonies are visible to the naked eye they are usually transferred to solid medium for continued growth or induction of plant regeneration.

Plants have been regenerated from protoplasts in a large number of species (Table 7). It should be noted that with some of the major crop species, especially the cereals, plant regeneration has thus far been very difficult to achieve. Cell division has been reported in protoplast cultures of barley (Koblitz 1976), corn (Potrykus et al. 1979) and sorghum (Brar et al. 1980), however, morphogenesis has not been induced. Recently plantlets (but not fertile plants) have been regenerated from protoplasts derived from cell suspension cultures of Pennisetum and Panicum (Vasil and Vasil 1980; Lu et al. 1981). Since morphogenic cultures have been described for several cereal species (Ozias-Akins and Vasil 1982;

TABLE 7. Species in which plants have been regenerated from
protoplasts

Species	Reference(s)
<u>Arabidopsis thaliana</u>	Binding <u>et al.</u> 1981
<u>Asparagus officinalis</u>	Bui-Dang-Ha <u>et al.</u> 1975
<u>Atropa belladonna</u>	Lörz and Potrykus 1979
<u>Brassica napus</u> , <u>B. oleracea</u>	Lu <u>et al.</u> 1982; Xu <u>et al.</u> 1982
<u>Bromus inermis</u>	Kao <u>et al.</u> 1973
<u>Capsicum annuum</u>	Saxena <u>et al.</u> 1981
<u>Cichorium</u> (2 species)	Binding <u>et al.</u> 1981; Crepy <u>et al.</u> 1982
<u>Citrus sinensis</u>	Vardi <u>et al.</u> 1975
<u>Datura</u> (3 species)	Schieder 1977
<u>Daucus carota</u>	Dudits <u>et al.</u> 1976
<u>Euphorbia lathyris</u>	Redenbaugh <u>et al.</u> 1982
<u>Gaillardia grandiflora</u>	Binding <u>et al.</u> 1981
<u>Helianthus annuus</u>	Binding <u>et al.</u> 1981
<u>Hyoscyamus muticus</u>	Wernicke and Thomas 1980
<u>Lactuca sativa</u>	Engler and Grogan 1982
<u>Linum usitatissimum</u>	Barakat and Cocking 1983
<u>Lotus corniculatis</u>	Ahuja <u>et al.</u> 1983
<u>Lycopersicon esculentum</u> , <u>L. peruvianum</u>	Zapata and Sink 1981; Morgan and Cocking 1982
<u>Medicago sativa</u> , <u>M. glutinosa</u> , <u>M. coerulea</u>	Johnson <u>et al.</u> 1981; Arcioni <u>et al.</u> 1982
<u>Nemesia strumosa</u>	Hess and Leipoldt 1979
<u>Nicotiana</u> (19 species and 2 interspecific hybrids)	Passiatore and Sink; Bourgin <u>et al.</u> 1979; Evans 1979

cont'd...

TABLE 7, cont'd.

Species	Reference(s)
<u>Nigella arvensis</u>	Binding <u>et al.</u> 1981
<u>Panicum maximum</u>	Lu <u>et al.</u> 1981
<u>Pennisetum americanum</u> , <u>P. purpureum</u>	Vasil and Vasil 1980; Vasil <u>et al.</u> 1983
<u>Petunia</u> (6 species and 1 interspecific hybrid)	Sink and Power 1977
<u>Ranunculus scleratus</u>	Dorion <u>et al.</u> 1975
<u>Rehmannia glutinosa</u>	Xu and Davey 1983
<u>Reseda luteola</u>	Binding <u>et al.</u> 1981
<u>Salpiglossis sinuata</u>	Boyes <u>et al.</u> 1980
<u>Senecio</u> (4 species)	Binding <u>et al.</u> 1981
<u>Solanum</u> (8 species and 1 interspecific hybrid)	Grun and Chu 1978; Shepard and Totten 1977; Saxena <u>et al.</u> 1982; Binding <u>et al.</u> 1981
<u>Trifolium repens</u>	Gresshoff 1980; Ahuja <u>et al.</u> 1983
<u>Trigonella</u> (2 species)	Shekhawat and Galston 1983

Jones and Dale 1982) future efforts with cereal protoplast cultures will undoubtedly concentrate on morphogenic cell suspensions. Culture of legume protoplasts has also received considerable attention recently. The grain legumes have proven difficult for induction of morphogenesis but several forage legumes have provided protoplasts capable of plant regeneration (Atanassov and Brown 1984; Shekhawat and Galston 1983; Ahuja et al. 1983).

Several members of the genus Solanum have been successfully regenerated from leaf mesophyll protoplasts, although cell suspension-derived protoplasts have not been regenerated to date. These species include S. tuberosum (Thomas et al. 1982), S. brevidens (Nelson et al. 1983), S. khasianum (Kowalczyk et al. 1983), S. nigrum (Nehls 1978), S. dulcamara (Binding and Nehls 1977), S. luteum (Binding et al. 1981) and S. melongena (Jia and Potrykus 1981).

In many cases protoplast-derived calli have markedly reduced morphogenic capacity compared to the tissues originally used for protoplast isolation. It is not clear if reduced morphogenic capacity is related to enzymatic disruption of tissue organization, or to other irreversible effects during isolation and culture.

C. Protoplast Fusion

Currently the method of choice for protoplast fusion in most laboratories involves agglutination of protoplasts in the presence of polyethylene glycol (PEG) followed by fusion during elution with culture medium (Kao and Michayluk 1974). Other fusogens have been identified, including alkaline calcium solutions (Keller and Melchers 1973), dextran (Kameya 1979), polyvinyl alcohol (Nagata 1978), synthetic phospholipids (Nagata et al. 1979) and electrical stimulation (Zimmermann and Scheurich 1981). None of these has yet

been demonstrated to be superior to PEG.

Electrofusion chambers are small and limited numbers of protoplasts can be used although this limitation may be circumvented by continuous flow fusion chambers (Bates et al. 1983). While the long-term effects of electric pulses on the ability of plant protoplasts to form colonies and regenerate plants have not been adequately studied, this technique has been used successfully for fusion of yeast protoplasts (Halfmann et al. 1983). A desirable feature of electrofusion is elimination of post-fusion washing. The advantages of the PEG technique include no additional equipment and easy treatment of large numbers of protoplasts. If adequate numbers of protoplasts can be fused, then the toxic effects of PEG are not critical and may help in the selection of heterokaryons and hybrid cells. In general, mesophyll protoplasts are more sensitive to PEG treatment and in fusion experiments with tissue culture-derived protoplasts, the majority of one species can therefore be eliminated (Gleba et al. 1982).

PEG may be used in a variety of ways including treating protoplast monolayers on coverslips or petri dishes (Kao et al. 1974) or suspensions in centrifuge tubes (Douglas et al. 1981b). The duration of fusogen treatment varies from 10–15 min depending upon the concentration and molecular weight of PEG. Solutions of 25–45% (w/v) of PEG (MW 1540–6000) are commonly used. Following this incubation the fusogen is usually slowly diluted with culture medium. Modifications of the PEG fusion technique include dilution with alkaline calcium solutions (Kao et al. 1974) and use of dilute PEG solutions buffered at high pH (Schieder 1978). The rate and frequency of fusion are influenced by the initial condition of

the protoplasts, the types of fusion process used and other factors associated with the procedure (Keller et al., 1982; Lazar 1983).

Subsequent to fusion protoplasts are washed and maintained according to methods described for protoplast culture (Section C). Post-fusion treatments such as temporary exposure to elevated calcium levels have been used to increase stability (Keller and Melchers 1973) while lower calcium and pH have been used to reduce agglutination and facilitate micromanipulation (Hein et al. 1983). Heterokaryon survival is influenced by the condition of the donor cells or tissues and methods used for fusion and culture.

D. Identification and Selection of Hybrid Cells and/or Plants

The availability of procedures for identifying and selecting hybrids is an essential aspect of successful somatic hybridization. Selection may be carried out immediately after fusion, during protoplast culture or during various stages of plant regeneration. The techniques used to select hybrid cells, colonies or plants are all variants of three main methods: cytological recognition, metabolic and/or genetic complementation and the recognition of morphological traits in regenerated plants. These procedures have been successfully employed in the production of a number of interspecific and intergeneric hybrid plants (Tables 8, 9).

(1) Cytological identification

Cytological selection systems depend upon the ability to identify and distinguish by microscopic observation, two parental populations of protoplasts. For example, protoplasts isolated from cells grown in suspension culture are characterized by dense cytoplasmic strands and lack of colour. Protoplasts isolated

TABLE 8. Interspecific somatic hybrid plants produced by protoplast fusion

Combination	Reference(s)
<u>Brassica oleracea</u> + <u>B. campestris</u>	Schenck and Robbelen 1982
<u>Datura innoxia</u> + <u>D. discolor</u>	Schieder 1978
<u>D. candida</u>	" 1980
<u>D. sanguinea</u>	" 1980
<u>D. stramonium</u>	" 1978
<u>D. quercifolia</u>	" 1982
<u>Daucus carota</u> + <u>D. capillifolius</u>	Kameya et al. 1981
<u>Medicago sativa</u> + <u>M. falcata</u>	Teoule 1983
<u>Nicotiana tabacum</u> + <u>N. alata</u>	Nagao 1979
<u>N. glutinosa</u>	Uchimiya 1982
<u>N. knightiana</u>	Menczel et al. 1981
<u>N. glauca</u>	Evans et al. 1980
<u>N. nesophila</u>	Flick and Evans 1982
<u>N. otophora</u>	Evans et al. 1983
<u>N. plumbaginifolia</u>	Sidorov et al. 1981
<u>N. repanda</u>	Nagao 1982
<u>N. rustica</u>	Douglas et al. 1983b
<u>N. stocktonii</u>	Evans et al. 1983
<u>N. sylvestris</u>	" 1983
<u>N. glauca</u> + <u>N. langsдорфii</u>	Chupeau et al. 1978
<u>N. knightiana</u> + <u>N. sylvestris</u>	Menczel et al. 1978
<u>N. rustica</u> + <u>N. sylvestris</u>	Gleddie et al. 1983b
<u>Petunia parodii</u> + <u>P. hybrida</u>	Itoh and Futsuhara 1983
<u>P. inflata</u>	Power et al. 1979
<u>P. parviflora</u>	" 1980
<u>Solanum tuberosum</u> + <u>S. chacoense</u>	Butenko and Kuchko 1979

TABLE 9. Intergeneric somatic hybrid plants produced by
protoplast fusion

Combination	Reference(s)
<u>Atropa belladonna</u> + <u>Petunia hybrida</u>	Gosch and Reinert 1978
" " + <u>Nicotiana chinensis</u>	Gleba <u>et al.</u> 1982
<u>Datura innoxia</u> + <u>Atropa belladonna</u>	Krumbiegel and Schieder 1979
<u>Daucus carota</u> + <u>Aegopodium podagraria</u>	Dudits <u>et al.</u> 1979
" " + <u>Petroselinum hortense</u>	" " 1980
<u>Hyoscyamus muticus</u> + <u>Nicotiana tabacum</u>	Jia <u>et al.</u> 1983
<u>Lycopersicon esculentum</u> + <u>Solanum tuberosum</u>	Melchers <u>et al.</u> 1978
<u>Nicotiana tabacum</u> + <u>Salpiglossis sinuata</u>	Nagao 1982
<u>Arabidopsis thaliana</u> + <u>Brassica campestris</u>	Hoffmann and Adachi 1981

from leaf mesophyll or flower petals, on the other hand, are usually vacuolated and brightly pigmented. For 24-48 hr subsequent to the fusion of cell culture-derived soybean protoplasts with N. glauca mesophyll protoplasts, heterokaryons consisting of chloroplast-rich and chloroplast-free zones could be visually identified (Wetter and Kao 1980). Similar procedures have been used to isolate heterokaryons of Arabidopsis thaliana + Brassica campestris (Gleba and Hoffmann 1980) and Nicotiana tabacum + N. knightiana (Menczel et al. 1981).

Staining protoplast populations with specific vital fluorescent probes which can be detected in fusion products provides another method of heterokaryon identification (Redenbaugh et al. 1982). The fusion of protoplasts from species producing alkaloids with those producing phenolics in their cell vacuoles results in a recognizable precipitation reaction (Constabel et al. 1980) which could also be used to identify heterokaryons.

Once heterokaryons are recognized, they must be separated from the parental protoplasts. Several workers have used micropipettes for the manual isolation of visually-identified heterokaryons (Pattnaik et al. 1982), or microdrop culture dishes to physically separate heterokaryons in distinct culture drops (Wetter and Kao 1980). Recent progress in the culture and regeneration of isolated, individual protoplasts has been made by placing microdrops under a thin layer of mineral oil (Koop et al. 1983) or into nurse cultures of protoplasts prepared from auxotrophic mutants (Hein et al. 1983).

Automated sorting of heterokaryons may be possible using fluorescence activated cell sorters (Redenbaugh et al. 1982) but regeneration of somatic hybrid calli or plants from cells selected

in this manner has not yet been reported. Cell sorting eliminates the need for manual isolation of heterokaryons and their subsequent culture with nurse cells since up to 2,000 protoplasts per hour can be sorted. Flow cytometry may also be applicable to both mesophyll and cell culture-derived protoplasts since several non-destructive techniques for vitally staining various protoplasts are available (Meadows and Potrykus 1981).

(2) Biochemical and/or genetic complementation

A variety of selection schemes based on complementation have proven successful. One of the earliest and most common selection schemes involved the use of non-allelic, chlorophyll-deficient mutants which complemented each other leading to chlorophyll production in hybrid cultures and/or plants. Melchers and Labib (1974) were able to select intraspecific tobacco hybrids by the complementation of two light-sensitive, chlorophyll-deficient strains. Similar approaches have been used to select interspecific somatic hybrids of N. rustica + N. tabacum (Douglas et al. 1981b), and intraspecific hybrids of Datura innoxia (Schieder 1977). The semi-dominant albino sulfur mutant (Su) of tobacco has been successfully complemented by wild-type lines of other Nicotiana species to produce interspecific hybrid plants (Evans et al. 1980, 1983). This selection scheme is based on the fact that regenerating homozygous (Su/Su) shoots appear white, shoots of wild-type species appear dark green and hybrid shoots are identifiable on the basis of light green colour. A disadvantage of this selection system is that several months are required for shoot morphogenesis and identification of hybrids. A system based on the use of the chlorophyll-deficient mutant 'chlorotica' of N. rustica has allowed

early selection of hybrid colonies resulting from fusion with wild-type N. sylvestris (Gleddie et al. 1983b).

Other non-allelic complementing systems include mutant tobacco cell lines deficient in nitrate reductase which have produced nitrate-utilizing hybrid colonies and plants (Glimelius et al. 1978; Lazar et al. 1983). Intergeneric somatic hybrids between Nicotiana and Hyoscyamus have been produced by complementation between auxotrophic mutants which individually required reduced nitrogen and nicotinamide (Jia et al. 1983).

A variation of complementation selection used the ability of heterokaryons to grow and regenerate on a medium which is non-permissive to the parental species. This approach does not require specific mutants but depends upon physiological differences between parental and hybrid cultures. Unfortunately, this depends upon the availability of sexually-produced hybrids to devise a selective medium. Calli of N. glauca + N. langsdorffii somatic hybrids were isolated on the basis of their ability to grow on hormone-free medium while the parental cells required auxin (Chupeau et al. 1978). Power et al. (1977) developed a medium in which only the somatic hybrids of Petunia hybrida + P. parodii underwent cell division. Toxic substances released from protoplast cultures of Brassica oleracea and B. campestris inhibited their growth and regeneration but permitted the selective regeneration of hybrids (Schenck and Robbelen 1982). In many cases, it has been noted that although the parental cell colonies could grow on the regeneration medium, hybrid colonies grew more vigorously (Douglas et al. 1981b; Gleddie et al. 1983b).

Nehls (1978) fused protoplasts of Solanum nigrum and Petunia hybrida which had been pretreated with the metabolic inhibitors diethylpyrocarbonate and iodoacetate, respectively. These inhibitors prevented cell division in the parental lines but allowed the selection of one heterokaryon colony. This method requires testing to establish concentrations of inhibitors which will block growth of parental cells without destroying their potential for fusion and complementation in heterokaryons. Biochemical inactivation has been successfully used as a selection scheme when combined with irradiation of the other parental species (Sidorov et al. 1981). Irradiation or biochemical inactivation of protoplasts has been used to establish unidirectional transfer of organelles from the inactivated species into another, untreated species. This technique is most efficient when selectable markers such as antibiotic resistance are located on the organelles to be transferred (Maliga et al. 1982; Menczel et al. 1983).

The fusion of protoplasts of parental lines each differing in resistance to a different compound should result in the formation of dual resistance in hybrids if resistance is expressed as a dominant or co-dominant trait. White and Vasil (1979) selected intraspecific somatic hybrid callus of N. sylvestris cells resistant to 5-methyltryptophan and S-2-aminoethylcysteine. Cell lines of Daucus carota resistant to the same amino-acid analogues have also been used to produce intraspecific hybrids (Harms et al. 1981), and intergeneric hybrid colonies between D. carota and N. tabacum (Hauptmann et al. 1983).

(3) Recognition of morphological traits

Several selections of somatic hybrid plants have been made

after plants were regenerated. Tomato + potato hybrids were initially selected on the basis of abnormal plant morphology (Melchers et al. 1978). Cytoplasmic hybrids or 'cybrids' produced through protoplast fusion have been identified on the basis of floral morphology (Izhar and Power 1979) or a combination of both floral and leaf morphology (Belliard et al. 1979). Chlorophyll-deficient mutants have been fused with wild-type strains and somatic hybrids were identified on the basis of the morphological traits of regenerated green plants (Krumbiegel and Schieder 1979). Vegetative and floral characteristics which were intermediate to those of the parental plants allowed the identification of a somatic hybrid between Medicago sativa and M. falcata (Teoule 1983). In cases where morphological markers were inconclusive, it has been necessary to carry out biochemical analysis of all regenerated plants to confirm hybridization (Uchimiya 1982). Selection at the stage of the regenerated plants is less efficient than other methods since it occurs long after the fusion experiment. Furthermore, because of space requirements, selection at this level greatly restricts the number of fusion products that can be screened.

E. Characterization of Hybrid Cells and Plants

Following the selection of putative hybrid cells or colonies, it is essential that methods be developed to confirm the hybridity of the material and thus eliminate parental lines which may have escaped the selection pressure. A variety of techniques have been applied to the characterization of hybrid cells, colonies, or plants, including: cytological, morphological, and biochemical analysis.

(1) Morphological analysis

Morphological traits intermediate to those of the parents are

usually expressed in somatic hybrids. This is especially true of floral and leaf characteristics of hybrids in Datura (Schieder 1978), Nicotiana (Gleddie et al. 1983b) and Petunia (Itoh and Futsuhara 1983). Tomato + potato somatic hybrids were shown to have intermediate sensitivity to chilling temperatures (Smillie et al. 1979). Hybrid vigour has resulted in increased plant height and/or leaf area in somatic hybrids of Nicotiana (Douglas et al. 1981c; Nagao 1979), Datura (Schieder 1978) and Solanum (Butenko and Kuchko 1979).

Novel morphological traits have been detected in several parasexual hybrids. Nicotiana rustica + N. tabacum hybrids expressed leaf mottling similar to that due to magnesium-deficiency and often failed to form roots (Douglas et al. 1981c). Several other Nicotiana somatic hybrid combinations have failed to root or only a small percentage of the total number of plants could be induced to root (Gleddie et al. 1981b; Nagao 1982). Tomato + potato hybrid shoots also failed to root and had to be grafted to tomato stocks (Melchers et al. 1978). In addition to vegetative abnormalities, abnormal floral development including the failure to flower has been reported (Schieder 1980) although intergeneric hybrids between Nicotiana and Hyocymus have flowered and set seed (Jia et al. 1983). These morphological abnormalities may be due to an altered regulation of hormone metabolism. In the genus Nicotiana it is common for certain sexual hybrids to form tumours which are also due to altered metabolism of growth regulators.

(2) Cytogenetic analysis

Cytogenetic analyses have been undertaken as a method of demonstrating or confirming the existence of somatic hybrid cells. Such analyses are especially important for hybrid cell cultures in

which morphogenesis cannot be induced (Wetter and Kao 1980; Nehls 1978). However, the parental mitotic chromosomes must be distinguishable on the basis of size or other features for cytological evaluation to be effective. In certain Nicotiana fusion products the presence of hybrid interphase nuclei could be demonstrated through the identification of parental heterochromatic blocks (Maliga et al. 1978). Nagl and Hoffmann (1980) were able to provide confirmatory evidence of the hybrid nature of Arabidopsis + Brassica fusion products through electron microscopic morphometry of their chromatin.

Fusion of protoplasts from actively-dividing cell cultures with those isolated from mitotically-inactive leaf cells results in the induction of mitosis in the leaf nuclei (Kao et al. 1974). Fusion of mitotic protoplasts with interphase protoplasts leads to premature chromosome condensation and pulverization in the interphase nuclei (Szabados and Dudits 1980). In most heterokaryons, however, the nuclear cycles are generally synchronized, the chromosomes are aligned on a common metaphase plate and are surrounded by a common nuclear membrane at the end of the first mitotic division (Kao et al. 1974). Hybrid nuclear formation need not always occur during the first cell division as shown by Binding and Nehls (1978) who reported the persistence of the heterokaryotic condition after mitosis.

The nuclear behaviour of hybrid cells during prolonged periods of culture has been followed in a number of cases. Constabel et al. (1977) observed that the parental genomes in Vicia hajastana + Glycine max hybrids would enter metaphase with a slight asynchrony and would assemble on the metaphase plate as distinct groups rather than in a mixed or integrated fashion. These hybrid lines retained

most of the parental genomes during the first four weeks of culture. In the case of V. faba + P. hybrida most of the chromosomes of one of the parental species were lost during the first 50 weeks of culture, however, some of both parental chromosomes were still present in some lines after two years of culture (Binding and Nehls 1978). Kao reported that many Nicotiana chromosomes were lost from N. glauca + G. max hybrid lines in the first six months of culture during which mitotic abnormalities such as chromosome bridges, fragments and lagging chromosomes were detected. The system appeared to stabilize since three-year-old cultures still retained Nicotiana chromosomes although they had undergone recognizable morphological changes (Wetter and Kao 1980). Cytological abnormalities including chromosomal bridges, fragments, and rings were detected in Arabidopsis + Brassica hybrid cells (Gleba and Hoffmann 1980). Again, an eventual cytological stabilization might be expected since both parental chromosome types were still present after 15 months of culture. In Atropa + Nicotiana hybrid cells few cytological abnormalities were detected and chromosomes of both parents were retained in most clones for more than one year (Gleba et al. 1982).

Preferential loss of chromosomes from one partner in a fusion combination has been demonstrated for soybean + N. glauca (Kao 1977; Wetter and Kao 1980), soybean + N. tabacum (Chien et al. 1982), D. carota + Petroselinum hortense (Dudits et al. 1980), and D. innoxia + Atropa belladonna hybrids (Schieder 1982). Among the possible causes for the asymmetric loss of chromosomes in somatic hybrids, unequal doubling times of the parental cells may be the most significant. Asynchronous division of the two genomes may cause lagging chromosomes and chromosome breaks (Kao 1977; Chien et

al. 1982). It is important to note that chromosome elimination from hybrid plants is not restricted to somatic hybrids, but also occurs in certain sexual crosses (Kasha and Kao 1970).

Cytological instability apparently persists during morphogenesis in a number of somatic hybrid systems and has resulted in regenerants with variable chromosome numbers. Douglas *et al.* (1981c) found that the chromosome number of *N. rustica* + *N. tabacum* ranged from 68–96 with only one plant having the amphidiploid number of 96. Cytological instability, including the development of hypo- and hyperaneuploidy has also been reported in other *Nicotiana* interspecific somatic hybrids (Chupeau *et al.* 1978; Nagao 1979; Maliga *et al.* 1978) in interspecific somatic hybrids of *Petunia* (Power *et al.* 1980) and *Datura* (Schieder 1980) and intergeneric hybrids of *Atropa* + *Datura* (Krumbiegel and Schieder 1979), *Arabidopsis* + *Brassica* (Gleba and Hoffmann 1980) and *Lycopersicon* + *Solanum* (Melchers *et al.* 1978). Aneuploid chromosome numbers have even been observed in intraspecific somatic hybrids of tobacco (Glimelius *et al.* 1978). On the other hand, somatic hybrid combinations in which all the regenerants had the expected amphiploid chromosome number or the occasional loss or gain of only one or a few chromosomes have also been identified (Evans *et al.* 1980; Gleddie *et al.* 1983b).

It is possible that there is increasing chromosomal instability as evolutionary distance between the parental species increases, however, other factors must also be involved since wide ranges of chromosome numbers and variations in cytological abnormalities have been found in some interspecific hybrids (Douglas *et al.* 1981c). The length of time in which hybrids were grown as callus prior to morphogenesis may be significant since more cytological abnormalities

are likely to develop in callus. Rapid plant regeneration subsequent to fusion would favour cytological stability since it has been established that plant cells in culture can become polyploid or aneuploid (Krikorian et al. 1984). Therefore the use of cell suspensions as sources of protoplasts may predispose heterokaryons to cytological variation and instability. Protoplast fusion and culture and plant regeneration techniques including medium composition might also be significant factors influencing stability. This may explain the differences in chromosome number for N. glauca + N. langsdorffii hybrids observed by Carlson et al. (1972) and Chupeau et al. (1978).

Cytological instabilities apparently persist within the plant body of certain parasexual hybrids. Chimeras with varying shades of leaf and petal colour were observed in N. rustica + N. tabacum somatic hybrids (Douglas et al. 1981c). Intra-plant segregation for distinct leaf and flower traits was also reported in N. tabacum + N. knightiana hybrids (Menczel et al. 1981). Cytological variation including mixoploidy and the appearance of multinucleates could be observed in different cells of the same root tips in some of these hybrids. The mixoploid condition has been identified in intraspecific N. tabacum hybrids (Glimelius et al. 1978).

Interactions between parental genomes following fusion may result in chromosomal rearrangements, including translocations, inversions or deletions which can go undetected cytologically. Chromosome banding techniques or genetic markers may be used to reveal such cryptic cytogenetic alterations. Several interspecific Nicotiana somatic hybrids have been produced with the homozygous sulfur mutant as one parent (Evans et al. 1980, 1983). When

protoplasts of this chlorophyll-deficient line are fused with wild-type protoplasts, hybrid colonies are selected on the basis of their light-green colour. Occasionally, double spots, which are areas of white and dark green, are found on the leaves of plants regenerated from such heterozygous hybrids. These areas are thought to be the result of mitotic crossing-over. It is conceivable that this mechanism may account for the vast morphological variation observed in somatic hybrid plants with similar chromosome numbers (Gleddie et al. 1983b).

(3) Biochemical analysis

Isozyme analysis by polyacrylamide electrophoresis has revealed the presence of both parental isozyme bands in extracts from the presumed hybrids, providing confirmatory evidence for the production of somatic hybrids (Douglas et al. 1981c; Gleddie et al. 1983b). However, the electrophoretic isozyme bands from hybrid calli or plants are not always a simple addition of parental bands. Certain parental bands were absent in the isozyme analysis of some interspecific hybrid tobacco plants (Douglas et al. 1981c), possibly as the result of absence of certain parental chromosomes in the hybrids. In G. max + N. glauca calli there was a loss of isozyme bands over a six month culture period paralleling a loss of Nicotiana chromosomes during that period (Wetter 1977). In N. rustica + N. tabacum somatic hybrids, new bands not present in either parent have been detected (Douglas et al. 1981c). The presence of such isozyme bands might relate to the derepression of certain genes as a result of genome interactions. With some enzymes, such as aspartate amino-transferase, new bands with migration patterns intermediate between the parental lines were detected. Such bands most likely indicate hybrid isozymes

resulting from interaction of parental polypeptide subunits. In N. rustica + N. tabacum, analysis of aspartate amino-transferase isozymes revealed the presence of a hybrid band in the somatic hybrids, but not in the sexual hybrids, indicating that the cytoplasm may play a role in regulating enzyme synthesis or assembly (Douglas et al. 1983c).

RUBPCase, the chief protein constituent of chloroplasts and a key enzyme in photosynthesis, consists of two polypeptide subunits. The peptides of the small subunit are coded by nuclear genes whereas the large subunit is encoded by the plastid genome. Isoelectric focussing of the subunit peptide chains has revealed species differences which provide a tool for characterizing plastid inheritance in hybrids. Studies with Nicotiana somatic hybrids have shown that small subunit peptides of both parental species are present while the large subunit consisted of only one parental type of peptide (Douglas et al. 1981d; Menczel et al. 1981). Evidence for mixed plastid populations or for plastid recombination has thus far not been obtained. Segregation of plastid types within cells apparently takes place soon after protoplast fusion, although some hybrid calli are three-dimensional mosaics of cells containing different chloroplast type (Akada and Hirai 1983). This segregation process may occur in callus colonies prior to regeneration but can extend into the regenerated plants since chimeras with respect to plastid type have been identified (Chen et al. 1977; Iwai et al. 1981). In some cases such as potato + tomato hybrids (Poulsen et al. 1980), the segregation process appears to be random with equal numbers of plants bearing the two plastid types. In other combinations a specific plastid type may be preferentially retained. Analysis of

14 N. rustica + N. tabacum hybrids revealed that 11 carried N. rustica plastids (Douglas et al. 1981d). In this case the pre-dominance of hybrids with N. rustica plastids may have been related to the method of hybrid selection which was based on early greening of protoplast-derived colonies, a trait associated specifically with N. rustica. This is supported by the finding that only N. rustica plastids were detected in somatic hybrids with wild-type N. sylvestris (Gleddie et al. 1983b).

Electrophoretic analysis of restriction enzyme digests of organelle DNA provides a sensitive method of identifying organelle genomes in somatic hybrids (Gatenby et al. 1980). Nicotiana parasexual hybrids possessed only one parental type of plastid DNA indicating that plastid recombination did not occur (Belliard et al. 1979).

Various antibiotics can be used as chloroplast markers because resistance is coded for by the chloroplast genome. Tentoxin, streptomycin and lincomycin are examples of antibiotics used to characterize organelle inheritance in hybrid plants (Menczel et al. 1983; Cseplo and Maliga 1982).

Among the markers used to characterize mitochondrial inheritance among somatic hybrids, DNA restriction fragments and cytoplasmic male sterility are the most common. There is considerable evidence for intermolecular mitochondrial recombination since somatic hybrid mitochondrial genomes are composed of DNA fragments derived from both parents in novel combinations (Belliard et al. 1979; Galun et al. 1982; Pelletier et al. 1983). Future methods of hybrid characterization and analysis may take advantage of recent developments in molecular biology. There is evidence that species-specific

highly repetitive nuclear DNA probes can be used to verify the presence of both genomes (Uchimiya et al. 1983). This method is based upon the isolation of repeated DNA which is specific for the species from which it came. This is a means of conclusively identifying the presence of DNA from both partners after fusion, which may be valuable in cases where cytological, morphological and biochemical evidence is inconclusive.

Secondary metabolites which have been used to characterize somatic hybrids include: alkaloids (Nagao 1982), volatile compounds (Ninnemann and Juttner 1981), and pigment content and ratios (Dudits et al. 1979).

F. Agricultural Applications of Protoplast Fusion

In order to be agriculturally useful somatic hybrids must possess and be capable of transferring traits of interest to plant breeders and growers. These might include disease, herbicide, or stress tolerance, and/or improved plant or fruit structure, yield or quality. Although conventional plant breeding has been successfully achieving these goals, the value of protoplast fusion is that it may serve as an additional tool for the breeder to create novel germplasm. The unique mixture of organelle and nuclear genomes created when protoplasts are fused ensures considerably more genetic variation than could be obtained by sexual hybridization. Cytoplasmic traits of value for crop improvement include herbicide tolerance which is encoded by the chloroplast genome in Brassica and Solanum (Steinback et al. 1981), Helminthosporium maydis resistance coded by the mitochondrial genome in Zea and cytoplasmic male sterility (CMS) which is a mitochondrial trait of many species (Levings and Pring 1976).

7

Recently somatic hybridization has been successfully used to produce plants containing foreign cytoplasm from different genera (Pelletier et al. 1983). Cytoplasmic male-sterile (CMS) lines combining the Brassica napus nucleus within the Raphanus sativa cytoplasm have potential value for hybrid rapeseed production. These alloplasmic lines can be obtained by sexual crosses and continued backcrosses with the same male parent, however, they suffer from developmental abnormalities such as chlorophyll-deficiency (Grun 1976). Protoplast fusion of these chlorotic, alloplasmic plants with B. napus lines containing functional chloroplasts or with atrazine-resistant B. campestris protoplasts has successfully combined the radish mitochondria (coding for CMS) with functional chloroplasts of B. napus or B. campestris and the B. napus genome (Pelletier et al. 1983). Because the mitochondria and chloroplasts are maternally-inherited organelles, it would have been impossible to obtain such hybrid cytoplasm by sexual means.

Another application of somatic hybridization is in transfer of nuclear traits from wild species into cultivated species. This relatively crude approach to genetic engineering has several advantages over the molecular approach. The gene(s) to be transferred need not be identified or isolated, cloned in vectors or expressed under the influence of artificial promoters after transformation. Groups of interacting polygenes may also be manipulated which is very difficult by molecular techniques. The disadvantage to fusion is that often many undesirable traits (including sterility) are also transferred to hybrids necessitating extensive backcrossing and selection to segregate desired traits.

Somatic hybrids between cultivated tobacco and disease-resistant wild relatives have recently been described (Evans et al. 1981; Uchimiya 1982). Nicotiana glutinosa is resistant to tobacco mosaic virus (TMV) and the hypersensitive-resistant response was also observed in the somatic hybrids, but the backcrosses have not yet been described (Uchimiya 1982). Nicotiana nesophila is another TMV-resistant species which has been hybridized somatically with tobacco but not backcrossed (Evans et al. 1982). Solanum chacoense is a diploid species which has been hybridized somatically with S. tuberosum (Butenko et al. 1982). Although the parental species were both susceptible to potato virus Y, the single somatic hybrid plant regenerated was resistant.

In most previous examples of protoplast fusion high degrees of sterility and genetic and phenotypic variability have precluded the practical use of the resulting hybrids in plant breeding. Recently, however, N. tabacum + N. rustica somatic hybrids produced by Douglas et al. (1981b) have been successfully used in a tobacco breeding program. These somatic hybrids were successfully backcrossed with pollen from a commercial tobacco cultivar and following recurrent selection and several backcrosses the resulting plants were field evaluated (Pandeya et al. 1984). Eight genotypes were identified as immune to blue mold (Peronospora tabacina) and in 1984 several morphologically desirable lines and 94 backcrossed and selfed new breeding lines of tobacco were under field trials. These results indicate that superior cultivars can be generated which combine several traits from wild species into cultivated ones following diligent backcrossing and field evaluations.

C H A P T E R 1

• "SOMATIC EMBRYOGENESIS AND PLANT REGENERATION

from

CULTURED PROTOPLASTS OF SOLANUM MELONGENA L. (EGGPLANT)"

INTRODUCTION

In order to successfully develop somatic hybridization and other genetic manipulations in crop plants it is essential to establish reliable sources of viable protoplasts and methods for their culture and plant regeneration. Although protoplasts may be derived from a wide variety of tissues and cells (Keller et al. 1982) two commonly-reported sources are leaf mesophyll and cell suspension cultures. Cell suspensions are advantageous because they provide sterile cells grown under precisely-defined environmental conditions in large quantities. Furthermore, protoplasts of these cells are already adapted to in vitro culture conditions. Numerous studies on the culture of cell suspension-derived protoplasts have been reported (Douglas et al. 1981a; Arcioni et al. 1982; Tewes et al. 1984) and such protoplasts have also been successfully used in somatic hybridization studies (Douglas et al. 1981b; Gleddie et al. 1983b).

Plant regeneration from cell suspension and protoplast cultures may follow one of two developmental processes, known as embryogenesis and organogenesis. Embryogenesis is a bipolar morphogenic pattern of development leading to the simultaneous differentiation of a root and shoot axis while organogenesis is the unipolar differentiation of either a root or a shoot. Embryogenesis has been reported in protoplast cultures derived from cell suspensions of Medicago sativa (Arcioni et al. 1982).

Daucus carota (Grambow et al. 1972), Pennisetum americanum (Vasil and Vasil 1980), and Atropa belladonna (Gösch et al. 1975) whereas organogenesis has been reported in Nicotiana species (Douglas et al. 1981a), Solanum phureja (Schumann et al. 1980), Datura innoxia (Furner et al. 1978), Browallia viscosa (Power and Berry 1979), and Arabidopsis thaliana (Xuan and Menczel 1980).

There have been several reports of successful plantlet (Bhatt and Fassuliotis 1981; Saxena et al. 1981) and mature plant (Jia and Potrykus 1981) regeneration from isolated mesophyll protoplasts of eggplant (Solanum melongena L.). The present study has investigated the use of cell suspension cultures as sources of protoplasts in eggplant, since high-frequency plant regeneration from these cultures has been demonstrated via embryogenesis and organogenesis (Gleddie et al. 1983a). It was suggested by Jia and Potrykus (1981) that there was need for optimization of the plating efficiency and induction of buds on colonies regenerated from eggplant protoplasts. The present study has attempted to provide such optimization and to investigate regeneration of somatic embryos from isolated protoplasts and protoplast-derived colonies.

MATERIALS AND METHODS

Seeds of Solanum melongena L. cv. 'Imperial Black Beauty' were obtained from Stokes Seeds Ltd., St. Catharines, Ont., and germinated in soil in a greenhouse. Plants were maintained at a day/night temperature of 25/15°C under a 16-hr photoperiod ($360 \mu\text{E m}^{-2}\text{sec}^{-1}$) supplemented with fluorescent lights. The plants were watered daily and fed a 20:20:20 (N:P:K) liquid nutrient solution twice weekly.

To establish callus cultures, leaves of 25-day-old plants were excised and surface-sterilized in 0.7% (w/v) calcium hypochlorite solution followed by five rinses in sterile distilled water. Using a sterile cork borer and scalpel, 200 mm^2 disks were cut from the leaf lamina. These were plated on agar-solidified callus induction medium which was a modified Murashige and Skoog (1962) medium (MS, Appendix, Table 1) supplemented with 10 mg/L naphthaleneacetic acid (NAA). Two disks were cultured per 20 x 60 mm plastic petri plates (containing 15 mL medium) which were sealed with parafilm and incubated at 25°C under continuous fluorescent light ($30 \mu\text{E m}^{-2}\text{sec}^{-1}$).

Cell suspension cultures were initiated by placing 3–4 g of callus derived from the leaf disks into 125 mL Erlenmeyer flasks containing 33 mL of liquid MS medium and agitating on a gyratory shaker at 150 rpm at 25°C under continuous fluorescent light ($30 \mu\text{E m}^{-2}\text{sec}^{-1}$). The medium was supplemented with 10 mg/L NAA or 2 mg/L 2,4-D and the pH was adjusted to 5.5 with 0.1 N KOH prior to autoclaving. Every fifth day 5–10 mL of suspension culture

was transferred into 33 mL of fresh medium.

For studies of morphogenesis from unprotoplasted cells, suspension cultures three days after transfer were filtered through 125 μ m mesh nylon filters. The cells collected on the filters were thoroughly rinsed in hormone-free liquid MS medium. Cell colonies (100–150 mg fr. wt. each) were selected with sterile forceps and transferred to various agar-solidified media. The mean number of cells in these colonies was determined by transferring a colony into 5 mL of enzyme solution (described below) and counting the liberated protoplasts in a hemocytometer after 24-hr incubation. The mean number of cells per 117 mg colony was estimated at $7.5 \pm 0.3 \times 10^5$. The frequency of embryos or shoots formed per colony was determined under a dissecting microscope after 60 days of culture, based on 25 colonies per treatment.

For protoplast isolation, cells were filtered from liquid suspension culture using 125 μ m mesh nylon filters, and approximately 2 g (fr. wt.) of cells were placed in 15 mL of enzyme solution in 100 x 15 mm plastic petri dishes. The dishes were sealed with parafilm and incubated for 6–8 hours on a reciprocating shaker (30 strokes/min) at 25°C in low light ($7 \mu\text{E m}^{-2}\text{sec}^{-1}$). The enzymes used were Driselase (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan), Rhozyme HP 150 (Rohm and Haas, Philadelphia, U.S.A.), and Cellulase Onozuka R-10 (Kinki Yakult Mfg. Co. Ltd., Nishinomiya, Japan). Individual 10% (w/v) solutions of each enzyme were prepared in 0.35 M mannitol. A final enzyme solution containing 1% Driselase, 1% Rhozyme, and 1% Cellulase in 0.35 M mannitol was prepared (pH adjusted to 5.8 with 0.1 N KOH) and sterilized by sequential filtration through 1.2, 0.4 and 0.2 μ m filters.

Following incubation in enzymes, the protoplasts were separated from undigested tissues and cells by successive passages through 125 and 44 μm mesh nylon filters. They were then washed three times in culture medium by centrifugation (100 $\times g$, 4 min). The protoplast culture medium was either NT (Nagata and Takebe 1971), MS, SH (Schenck and Hoffmann 1979), or Kao [1977 (Appendix, Tables 1-4)] each modified by the addition of 0.35 M glucose, 0.05 M sucrose, 3 mg/L NAA and 1 mg/L BAP with or without 1 mg/L 2,4-D and the omission of agar. Protoplasts ($1 \times 10^4/\text{mL}$) were cultured in 50-100 μL drops or as thin-liquid layers (1 mL) in 60 x 15 mm plastic petri dishes, sealed with parafilm and incubated at 25°C in low light ($7 \mu\text{E m}^{-2}\text{sec}^{-1}$), in a humid environment provided within sealed plastic boxes containing 40 mL of water in a 50 mL beaker.

The cell-division efficiency was defined as the frequency of cell divisions (%) after 14 days of culture. This was measured as an average of five independent drops or liquid layers by counting the protoplasts in the field of view of an inverted microscope (160X) in five separate dishes (5 x 80 protoplasts observed per treatment). At this time the dishes were moved to a higher light intensity ($30 \mu\text{E m}^{-2}\text{sec}^{-1}$) and after three weeks each culture drop was supplemented with 50 μL of the same culture medium also containing a reduced osmoticum of 0.15 M glucose. Liquid layers were also supplemented with 0.5 mL of this medium at this time. After five weeks of culture protoplast-derived colonies were supplied with 0.5 mL culture medium lacking glucose.

The plating efficiency was defined as frequency (%) of macroscopic colony formation compared to total number of protoplasts plated after 42 days of culture. At this time, cultures which

contained microcolonies or embryos in the late globular stages of development were transferred to agar-solidified Kao medium supplemented with 0.1 mg/L 2,4-D, and 1 mg/L of either zeatin, kinetin or BAP. Embryos or green meristematic regions were excised from these colonies and plated on hormone-free B₅ medium (Gamborg et al. 1968), however, direct regeneration of plants was not observed. Excised embryos and green meristematic colonies were therefore cultured on Kao medium supplemented with 1 or 5 mg/L of kinetin, zeatin or BAP. By repeated subculture on the same medium, these green meristematic regions of protoplast-derived colonies regenerated normal shoots which were rooted on hormone-free B₅ medium. Plantlets were transferred to peat pellets (Jiffy -7) in a mist chamber for 2-3 weeks prior to planting in soil in a greenhouse..

RESULTS

Leaf explants which were cultured on medium containing 10 mg/L NAA produced a rapidly-growing, friable, white callus which also formed somatic embryos after 18- 30 days. When this callus was placed into liquid medium containing 10 mg/L NAA, a rapidly-growing cell suspension was established. This cell suspension formed somatic embryos which developed to the cotyledonary stage in liquid medium and regenerated plants upon transfer to agar solidified medium as previously reported (Gleddie et al. 1983a).

Cell suspensions were also initiated by placing callus of the same origin into liquid medium containing 0.5, 1.0, or 2.0 mg/L 2,4-D, however, 2mg/L 2,4-D cell suspensions grew fastest and were used in subsequent experiments. The growth rates of cells in either 2,4-D or NAA were similar, however, dramatic morphogenic differences were observed in these cell cultures. Cells which were maintained in 2,4-D medium consisted of small aggregates and single cells, while suspensions maintained in NAA medium were more heterogenous and had many large aggregates and embryogenic centers. Although somatic embryogenesis was never observed in cells maintained in the presence of 2,4-D, it was possible to transfer cells from 2,4-D medium into NAA medium and induce embryogenesis. The embryogenic potential of cells maintained in 2 mg/L 2,4-D medium for over two years was verified by taking aliquots of these cells and transferring them into NAA-supplemented medium. The frequency of embryogenesis among these transferred cells decreased with increasing age of the cultures over the two-year period in which observations were made (Table 10). The results indicate, however, that either

cell suspension type (NAA or 2,4-D supplemented) could undergo morphogenesis with reasonable frequency after at least 12 months and would, therefore, be good sources of protoplasts for subsequent studies.

Since the auxin source supplied to the progenitor cell suspension culture has been shown to influence yield and subsequent regenerative capacity of protoplasts in other studies (Douglas *et al.* 1981a), a comparison was made between protoplasts derived from cell suspension maintained on MS medium with two different auxins (2 mg/L 2,4-D or 10 mg/L NAA). Numerous qualitative and quantitative differences between the two populations of protoplasts were observed (Figs. 1-4, Table 11). The yield of protoplasts from a rapidly-growing cell suspension maintained in 2,4-D medium was 1×10^7 per g fr. wt. after a 12-hr enzyme digestion, while NAA-grown cells yielded only 1×10^5 protoplasts per g fr. wt. Many cell aggregates in the NAA cell suspension were relatively intact with undigested walls even after 24-hr enzyme treatment. Other major differences between these protoplast populations included their size and degree of vacuolation. Cells grown in 2,4-D gave protoplasts of more uniform size (25-30 μ m diameter) with abundant cytoplasmic strands (Fig. 1). Cells maintained in NAA on the other hand, released much more heterogeneous population of protoplasts. Although the majority of these protoplasts was very small (10 μ m diameter) and highly vacuolated, occasionally very large protoplasts were also formed (Fig. 2).

After 10 days culture in liquid NT medium the division efficiency of protoplasts derived from 2,4-D or NAA-grown cells was very different (Table 11). Protoplasts from 2,4-D grown cells

had a high frequency of cell wall reformation and showed a division frequency of 25% (Table 11). A large number of these cells grew into visible colonies (Fig. 3); an average of 129 colonies was observed per dish with a plating efficiency of 1.29%. Protoplasts from NAA-grown cells had only a 0.5% division efficiency and very few cells continued to grow into visible colonies resulting in a plating efficiency of 0.002% (Table 11; Fig. 4). As a result of these observations cell suspensions maintained in 2 mg/L 2,4-D were used as sources of protoplasts in all subsequent experiments.

A variety of media which have been reported to induce morphogenic responses in cultures of protoplasts were compared for their ability to promote cell division and embryogenesis (Table 12). When NT medium was supplemented with 3 mg/L NAA and 1 mg/L BAP the efficiency of division of cultured protoplasts was 25%. When MS basal medium was supplemented with the same hormones, the efficiency of division decreased to 4% while SH medium failed to support division of eggplant protoplasts. There was little difference in the division efficiency supported by NT or Kao media (Table 12).

The following modifications failed to increase the division efficiency of the protoplasts above 25%: substitution of 0.35 M mannitol for 0.35 M glucose, embedding the protoplasts in semi-solid medium containing 0.4% (w/v) agar or 0.4% (w/v) agarose, floating the protoplasts in medium over Ficoll, or dispensing the protoplasts in liquid NT medium onto sterile filter paper supported on agar-solidified medium.

The growth regulators which were used to optimize protoplast division and colony formation in NT medium were NAA at 3 mg/L and BAP at 1 mg/L. However, embryogenesis was never observed in this medium. The first modification of this medium in attempts to achieve embryogenesis, consisted of reducing and removing BAP since cytokinins strongly suppress embryogenesis in unprotoplasted cells of this species (Gleddie *et al.* 1983a). The division efficiency and plating efficiency were greatly reduced in the absence of BAP (6.2% and 0.057%, respectively) while an intermediate concentration of BAP (0.5 mg/L) was also detrimental to the division efficiency (13%) and the plating efficiency (0.041%). Although other cytokinins were not tested, there was definitely a beneficial effect of 1 mg/L BAP supplied to NT medium. Medium without this cytokinin or with concentrations less than 1 mg/L promoted drastic elongation of a high proportion of the protoplast-derived cells, some of which exceeded 20 μ m in length, however, these cells seldom divided (Fig. 5).

Addition of 1 mg/L 2,4-D to either NT or Kao medium (already containing 3 mg/L NAA and 1 mg/L BAP) had significant morphogenic effects (Table 12). The division frequency was not significantly increased in these conditions, but direct formation of somatic embryos in the protoplast culture was observed (Figs. 6-10). The first cell division occurred after seven days of culture while the second occurred after 10-14 days. Rarely, protoplasts underwent an asymmetrical second division (Fig. 7) which led to the formation of proembryo-like structures. Globular, heart-shaped, and torpedo-shaped embryos were observed in liquid medium after 25-40 days of culture (Figs. 8-10).

Efforts to quantify embryogenesis in protoplast cultures were hampered by the low proportion (2-5%) of microcolonies which formed proembryos or embryos (2-5 proembryos per dish). The vast majority of the embryogenic microcolonies were indistinguishable at this time, from non-embryogenic microcolonies generated on 2,4-D-free medium. The well-defined stages of embryogeny were only observed in liquid NT or Kao medium supplemented with 1.0 mg/L 2,4-D. If 1.0 mg/L 2,4-D was replaced by 0.1 mg/L 2,4-D somatic embryos were not observed, however, other concentrations of 2,4-D were not tested. If NAA was removed (i.e. NT or Kao medium + 1.0 mg/L 2,4-D and 1.0 mg/L BAP) then the frequency of embryogenesis was reduced to 0.2 % of the microcolonies which were observed. Unprotoplasted cells of eggplant respond to 10 mg/L NAA by undergoing somatic embryogenesis (Gleddie *et al.* 1983a), and therefore high concentration (10.0 mg/L) NAA was tested in NT or Kao medium, however, the efficiency of division was greatly reduced (0.8% and 0).

The cell colonies produced in 2,4-D-supplemented NT or Kao media were similar to those formed in suspension cultures of non-protoplasted cells containing 10 mg/L NAA which are also embryogenic. These consisted of many very small cytoplasmic-rich cells in small aggregates (Fig. 11). After 42 days of culture such protoplast-derived colonies (containing proembryos) were transferred to agar-solidified Kao or MS medium containing a variety of growth regulators. Only those colonies which were transferred to Kao or MS medium containing 0.1 mg/L 2,4-D and 1 mg/L of either BAP, zeatin or kinetin responded by producing embryos (Fig. 12). The mean number of normal, bipolar, embryos produced was one per 60 colonies plated, however, many green, "meristematic structures"

which could not be readily identified as embryos but were more "shoot-like" in appearance were also produced in 5% of the colonies. When somatic embryos or "shoot-like" structures were excised and plated on hormone-free B₅ medium they failed to regenerate directly into plants. When they were transferred to MS medium containing 1-5 mg/L of either BAP, zeatin, or kinetin, they responded by producing either shoots or embryos from their hypocotyls (Fig. 13). Often, successive transfers (up to five) on these media ultimately stimulated plant regeneration (Figs. 14-15).

At best the efficiency of plant regeneration from excised embryos was low (22% of embryos obtained can regenerate plants). However, the procedures are capable of generating many cell colonies from protoplasts and give approximately one mature flowering plant per 10^6 protoplasts plated.

DISCUSSION

It has been previously demonstrated that cell suspensions of eggplant maintained in NAA-supplemented medium were embryogenic, while those in 2,4-D medium were not (Gleddie et al. 1983a). The present study has shown that the progenitor cell suspension has a dramatic influence on the protoplasts which are isolated from it (Table 11). In addition to liberating more protoplasts, the 2,4-D-supplemented cells provided a homogenous population of cytoplasmic protoplasts that divided with an efficiency of 25% under optimal conditions. The embryogenic cell suspension maintained in NAA-supplemented medium, however, responded to enzyme treatment by producing fewer protoplasts, with reduced division and plating efficiencies. Cells of N. rustica cv. chlorotica which were grown on 2,4-D-supplemented medium liberated 50% more protoplasts than those grown in the presence of NAA (Douglas, et al. 1981a), and similar results have been reported for Haplopappas gracilis (Wallin et al. 1977).

The very small vacuolated protoplasts which were obtained from NAA-grown suspensions of eggplant (Fig. 2) displayed the same poor response to culture as has been reported for carrot protoplasts isolated from somatic embryos (Nomura et al. 1983). It appears that superior quality protoplasts, with higher capacities for sustained divisions, can be isolated from rapidly growing cell suspensions and that cell suspensions undergoing embryogenesis or differentiation or cell enlargement may be less desirable sources of protoplasts. In protoplast cultures of eggplant which

supported high division frequencies there was an almost total lack of 'giant cells' (Fig. 6). In carrot protoplast populations, giant cells were formed under non-regenerating and non-embryogenic conditions, while they were infrequently found under conditions which stimulated embryogenesis (Nomura et al. 1983).

In this study, it was observed that the basal medium employed for culture of protoplasts had a pronounced effect on protoplast division (Table 12). NT and Kao media were clearly superior to MS and SH media. The main difference between these media is in their total nitrogen content and composition (Appendix, Tables 1-4). The frequency of division of protoplasts was optimal in media, Kao or NT, with intermediate NH_4^+ content (Table 12). SH medium, which lacks NH_4^+ , did not support protoplast division while MS medium may have too much NH_4^+ for optimal protoplast growth. The inability of MS medium to support protoplast division with high efficiency (Table 12) may be due to a toxic effect of NH_4^+ on the division of cells plated at low densities, since after 42 days of culture in NT or Kao medium, protoplast-derived microcolonies were successfully transferred to agar-solidified MS medium. Mesophyll-derived protoplasts of eggplant were also successfully cultured in Kao medium (Bhatt and Fassuliotis 1981; Saxena et al. 1981) or DPD medium, which is also a low NH_4^+ (3.375 mM) medium (Jia and Potrykus 1981). The apparent sensitivity of Solanum protoplasts to ammonium ions was also reported by Grun and Chu (1978).

Previous work with leaf explants and cell suspensions of eggplant has demonstrated that the nitrogen content of the

medium was critical for embryogenesis of non-protoplasted cells (Gleddie et al. 1983a). It was shown that the optimal frequency of embryogenesis occurred in MS medium and that NT and Kao media were inferior for embryogenesis.

The osmotic environment of the medium in this study was supplied by 0.35 M glucose since it was previously determined to be optimal (Gleddie, MSc. Thesis 1981), however, substitution of 0.35 M mannitol did not greatly affect the division frequency. A mixture of glucose and sucrose (Bhatt and Fassuliotis, 1981) or 0.3–0.5 M mannitol (Saxena et al. 1981; Jia and Potrykus 1981) have been successfully used which indicates that the osmotic component of the medium is not a critical requirement with this species.

The addition of 1 mg/L 2,4-D to several different basal media stimulated a slightly higher frequency of division among eggplant protoplasts although it was not essential for division (Table 12). It has been reported that 2,4-D is essential for division of S. nigrum (Nehls 1978) and S. dulcamara (Binding and Nehls 1977) protoplasts. The most significant effect of 2,4-D in the protoplast culture medium of eggplant was, however, that it promoted the development of cell colonies into "promembryo-like" distinct embryogenic cell masses. These protoplast colonies contained cells in various stages of embryogeny (Figs. 8–10) which are identical to those in unprotoplasted cells maintained in the presence of high concentrations of NAA (Gleddie et al. 1983a). In this previous work, however, the formation of somatic embryos from unprotoplasted cells in liquid medium was shown to specifically

require NAA and not 2,4-D. In protoplast-derived cells studied here the situation was reversed and 2,4-D was essential for embryogenesis. The cytokinin requirement of protoplast-derived cells and non-protoplasted cells was also different. BAP was required in the protoplast culture medium for high division efficiencies and consequently for high plating efficiencies, whereas BAP was extremely inhibitory of NAA-induced embryogenesis of non-protoplasted cells (Gleddie *et al.* 1983a). Cytokinins play a significant role in the plant regeneration process from protoplast-derived colonies and proembryos since mature, cotyledonous, green embryos were never observed in protoplast cultures. It was only after the protoplast-derived microcalli containing proembryos, or immature embryos, were transferred to agar-solidified MS or Kao media with 1 mg/L cytokinin and a reduced auxin (0.1 mg/L 2,4-D) that mature green embryos were observed. Unprotoplasted cells, by contrast, formed fully differentiated, chlorophyllous embryos in the presence of NAA alone (Gleddie *et al.* 1981a).

Comparison of the relative efficiencies of somatic embryogenesis by plating unprotoplasted cells on NAA medium (Table 10) or by plating isolated protoplasts in 2,4-D medium (Table 12) showed that the two were similar. From a 12-month-old suspension, one somatic embryo was formed by 7×10^5 unprotoplasted cells plated on 10 mg/L NAA (Table 10). From 1×10^4 protoplasts, however, an average of two embryos were formed (Table 12).

The results in this study indicate that cells and isolated protoplasts of a species can differ in their optimal conditions for somatic embryogenesis. The results of Vardi et al. (1975) with Citrus and Thomas et al. (1976) with Brassica cells and protoplasts have demonstrated that the conditions which favour embryogenesis from tissues and cells do not apply to cells derived from protoplasts. It is possible that medium composition and other procedures used to isolate and culture protoplasts may alter cellular factors which affect embryogenesis in unprotoplasted cells. Impurities in the enzymes used to isolate protoplasts, high osmotic conditions and their effects on membrane and cytoskeleton and/or abnormal cell-wall reformation after protoplasting are all possible reasons for the overall altered response to growth regulators which stimulate embryogenesis.

The frequency of conversion of somatic embryos into mature plants was disappointingly low (approximately one in four embryos could be regenerated by repeated culture on cytokinin-containing medium). Nevertheless, it should be possible to use the eggplant cell suspension protoplast system in studies of somatic hybridization if efficient means to select hybrid cells or colonies could be devised.

TABLE 10. Effect of culture age on somatic
embryogenesis in cell colonies from
suspension cultures of S. melongena L.

Culture age (months)	f
6	2.1
12	1.2
24	0.01

Note: Cell suspensions were maintained in liquid MS + 2 mg/L 2,4-D. Cells were plated on MS + 10 mg/L NAA medium solidified with agar.

f = embryo frequency as represented by the mean number of embryos per colony ($7.5 \pm 0.3 \times 10^5$ cells) based on 25 colonies

TABLE 11. Effect of auxin in cell suspension-culture medium on protoplast isolation and culture.

Observations	Suspension grown in:	
	2,4-D (2 mg/L)	NAA (10 mg/L)
Protoplast yield	1×10^7	1×10^5
Cytoplasmic strands	present	absent
Mean diameter	28 μ m	12 μ m
Division efficiency (%)	25%	0.5%
Plating efficiency (%)	1.29%	0.002%

Note: Suspensions were maintained in MS medium + the auxins indicated for six months. Yield was measured as the number of protoplasts liberated per g fresh weight in an enzyme solution (1% Driselase, 1% Rhozyme, 1% Cellulase in 0.35 M mannitol) after a 12 hr. digestion.

Division efficiency was measured after 10 days of culture in NT medium supplemented with 3 mg/L NAA and 1 mg/L BAP.

Plating efficiency was calculated after 45 days.

TABLE 12. Effect of various media and 2,4-D on protoplast culture and somatic embryogenesis in *S. melongena* L.

Medium	2,4-D (1 mg/L)	Nitrogen Content		Division efficiency %	Plating efficiency %	Embryos/100 microcalli
		NH ₄ ⁺ (mM)	NO ₃ ⁻ (mM)			
MS	-	20.6	39.4	4	0.09	0
"	+	20.6	39.4	8	0.034	0
NT	-	10.3	18.2	22	1.11	0
"	+	10.3	18.2	27	1.31	1.7
Kao	-	7.5	26.3	24	1.17	0
"	+	7.5	26.3	30	1.24	1.9
SH	-	0	19.8	0	0	0
"	+	0	19.8	0	0	0

Note: All media were supplied with 3 mg/L NAA + 1 mg/L BAP.
Division frequency was measured after 14 days of culture.
Protoplast cultures were observed after 45 days of culture in liquid medium for embryogenesis.

FIGURE 1. Freshly isolated protoplasts of eggplant from a six-month old cell suspension maintained in MS + 2 mg/L 2,4-D. Bar represents 10 μ m.

FIGURE 2. Freshly isolated protoplasts of eggplant from a six-month old cell suspension maintained in MS + 10 mg/L NAA. Bar represent 10 μ m.

FIGURE 3. Protoplast-derived cells forming 4 and 8 cell colonies after 20 days of culture. Original protoplasts were isolated from a cell suspension maintained in MS + 2 mg/L 2,4-D. Bar represents 20 μ m.

FIGURE 4. Protoplast-derived cells elongating and dividing after 20 days of culture. Original protoplasts were isolated from a cell suspension maintained in MS + 10 mg/L NAA. Bar represents 20 μ m.

FIGURE 5. Protoplast-derived cells from a 2,4-D-grown cell suspension after 20 days of culture in medium lacking BAP. Note the greatly elongated cell. Bar represent 10 μ m.

FIGURE 6. A thin layer protoplast culture of eggplant after 7 days in NT medium containing 1 mg/L 2,4-D, 3 mg/L NAA and 1 mg/L BAP.

FIGURE 7. An asymmetrical first division of protoplasts in this medium often resulted in more cell divisions occurring in one-half of the small colony. Protoplast culture in same medium as Fig. 6 after 14 days.

FIGURE 8. Protoplast-derived cell colony in medium containing 2,4-D after 21 days of culture. Note the globular bipolar nature of the colony.

FIGURE 9. Protoplast-derived cell colony after 35 days in medium containing 2,4-D. The embryonic structure is bipolar and has rudimentary cotyledons. This 'heart-shaped' embryo is composed of numerous small, cytoplasmically dense cells.

FIGURE 10. Protoplast-derived colony after 52 days of culture in medium containing 2,4-D. This pro-embryo is very similar in shape to somatic or zygotic embryos that approach maturity.

FIGURE 11. The typical yield of protoplast-derived cell colonies of eggplant plated at 10^4 /dish in Kao medium + 1 mg/L 2,4-D, 3 mg/L NAA and 1 mg/L BAP. Approximately 100 microcolonies have formed (Plating efficiency = $100/10^4 = 1\%$) after 6 weeks in liquid medium.

FIGURE 12. Protoplast-derived colonies of eggplant after plating on agar solidified Kao medium + 0.1 mg/L 2,4-D and 1 mg/L kinetin for 4 weeks. Note the developing somatic embryos (arrows).

FIGURE 13. Isolated somatic embryos from protoplasts of eggplant after 30 days of culture on Kao medium + 0.1 mg/L 2,4-D, 1 mg/L kinetin. Note the dark green apex and white radicle of the embryos.

FIGURE 14. An isolated somatic embryo after 29 days of culture on hormone-free B_5 medium. Note the callus formation, root elongation but failure of apex to differentiate. This embryo did not produce a plant.

FIGURE 15. A regenerated eggplant from an isolated protoplast-derived embryo 16 weeks after protoplast culture.

CHAPTER 2

"THE CULTURE OF EXCISED TISSUES, CELL SUSPENSIONS
AND PROTOPLASTS OF SEVERAL WILD SOLANUM SPECIES".

INTRODUCTION

In order to successfully employ a species in somatic hybridization studies it is essential to obtain information on conditions necessary for in vitro culture and totipotency of cultured cells, tissues and protoplasts. In the genus Solanum tissue culture studies have been undertaken with the cultivated species S. tuberosum (Jacobsen et al. 1983), and S. melongena (Gleddie et al. 1983a) and with several wild species including S. dulcamara and S. nigrum (Zenkteler 1972) and S. laciniatum (Chandler and Dodds 1983). Protoplast culture and plant regeneration protocols have also been established for S. tuberosum (Shepard and Totten 1977), S. dulcamara (Binding and Nehls 1977), S. nigrum (Nehls 1978), an S. phureja x S. chacoense F₁ hybrid (Grun and Chu 1978), S. phureja (Schumann et al. 1980), S. luteum (Binding et al. 1981), S. brevidens (Nelson et al. 1983), and S. khasianum (Kowalczyk et al. 1983). Valuable information has been obtained through: a) culturing tissue explants and developing methods for inducing morphogenesis, b) establishing cell suspension cultures which can be tested for their growth characteristics and morphogenic capacity and which can serve as a source of protoplasts, c) establishing conditions for isolation and culture of protoplasts which optimize plating and plant regeneration processes.

The objectives of the present study were to develop cell and protoplast culture techniques in several wild Solanum species for use in somatic hybridization studies with eggplant, Solanum melongena L. Several of these species have been identified as possessing desirable traits such as resistance to pests and pathogens of eggplant (see Table 1). Because cell suspension cultures have been successfully used in other somatic hybridization experiments (Douglas et al. 1981b; Gleddie et al. 1983b) the approach taken was to develop cell-suspension cultures of these species.

MATERIALS AND METHODS

Seeds of Solanum aviculare Forst. f. [U.S.D.A. Plant Introduction (PI #280049)], S. aculeatissimum Jacq. (PI #183949), S. gilo Raddi (PI #194166), S. khasianum Clarke (Syn. S. viarum Dunal, PI #331063), and S. sisymbriifolium Lam. (PI #331140, 337597, 358311, 381291) were obtained from the U.S.D.A. Regional Plant Introduction Center, Experiment, GA. Seeds of S. torvum Swartz. were obtained from Dr. K. McCammon, Michigan State University and seeds of S. macrocarpon (Garden #0133) and S. sisymbriifolium Lam (Garden #S. 136) from Dr. S. Marsh, University Botanical Gardens, Birmingham, U.K. Plants were grown in soil in a greenhouse and maintained under a 16 hr photoperiod ($360 \mu\text{E m}^{-2} \text{sec}^{-1}$) with a day:night temperature of 25:15°C. They were watered twice daily and fed commercial fertilizer solution 20:20:20 (N:P:K) twice weekly.

The young expanding leaves and stem internodes of 4-16 week-old plants were used as explant sources. These tissues were surface-sterilized in a 0.7% (w/v) calcium hypochlorite solution for 15 min followed by five rinses in sterile water. Leaf explants were prepared with a 16 mm diameter cork borer. Stem internodes were cut into 1 cm lengths and cut once transversely. Four stem segments or two leaf explants were cultured per 60 x 20 mm plastic petri dish containing 15 mL of solidified medium (0.8% Difco agar). The medium used in all explant and cell suspension cultures was that of Murashige and Skoog (1962) as modified by Douglas et al. (1981a). The medium was supplemented with 0.1-5.0 mg/L of either

of the auxins or cytokinins tested. The auxins tested for their capacity to induce callus proliferation, root initiation or embryogenesis were: 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 1-naphthaleneacetic acid (NAA), 2-naphthoxyacetic acid (NOA), indole-3-acetic acid (IAA), or indole-3-propionic acid (IPA). The cytokinins tested for their capacity to promote shoot organogenesis were: kinetin, zeatin, BAP, 6-benzylaminopurine riboside (BARI), and 2-isopentyladenine (2iP).

Cultures were maintained at 25°C under continuous fluorescent light ($30 \mu\text{E m}^{-2} \text{sec}^{-1}$) for 30 days at which time they were evaluated for embryogenesis or organogenesis under a dissecting microscope. The mean numbers of shoots, shoot primordia and roots per explant were recorded.

Cell suspensions were initiated from the most rapidly-growing callus cultures in each species by the methods described in Chapter 1. Cell suspensions were initiated and maintained only in MS medium containing 2.0 mg/L 2,4-D. Tests for the induction of morphogenesis in cell colonies isolated from these cell suspensions were performed by collecting cells on 125 μm mesh nylon filters. They were rinsed in 50 mL of hormone-free MS medium and cell colonies about 2mm diameter (100-200mg fr.wt.) were selected and plated on agar-solidified MS medium. The growth regulators added to this medium individually at concentrations of 0.1-5.0mg/L were the auxins 2,4-D, 2,4,5-T, NAA, NOA, IAA, and IPA or the cytokinins kinetin, zeatin, BAP, BARI, and 2iP. The morphogenic response was observed after 60 days of culture.

Protoplasts were isolated from each cell suspension after six months of culture according to the same protocol described for eggplant protoplast isolation (Chapter 1). The basal medium used for protoplast culture was either NT, MS or Kao, each modified with 0.35 M glucose and 0.03 M sucrose added as osmoticum and 3 mg/L NAA, 1 mg/L 2,4-D and 1 mg/L BAP supplied as growth regulators. The dilution of the osmoticum, and transfer of microcalli to agar-solidified medium were performed as for eggplant protoplast cultures. The microcalli were placed on MS medium supplemented with either BAP or kinetin or zeatin at 1 or 5 mg/L. Shoot primordia which formed on these calli were transferred to MS medium supplemented with 1 mg/L zeatin for shoot elongation. Excised shoots were rooted on hormone-free B₅ medium.

RESULTS

1. Explant Culture

Proliferating callus cultures formed on stem explants of Solanum sisymbriifolium cultured on auxin-supplemented medium (Table 13, Fig. 16). Callus formed on stem explants only in the presence of exogenous 2,4-D, 2,4,5-T and NAA but not with NOA, IAA or IPA. Callus was never observed on leaf explants regardless of the auxin type or concentration (Table 13). Root initiation occurred regularly in these cultures both from callus and directly from organized explant tissue (Fig. 16) but, unlike with S. melongena, auxins did not promote somatic embryogenesis. Since a high concentration of NAA (10 mg/L) promoted the highest frequency of embryogenesis in eggplant callus, this concentration of NAA was tested on leaf and stem explants of this species, however, somatic embryogenesis was never observed in S. sisymbriifolium or the other species tested in this study. A rapidly-growing callus of S. sisymbriifolium was used to establish a cell suspension culture which was used in further protoplast experiments.

The effect of cytokinins on leaf and stem explants of S. sisymbriifolium was dramatic. Very high frequency shoot organogenesis was observed on leaf explants cultured without auxin in the presence of either zeatin, BAP or BARI while kinetin and 2iP were much less effective (Table 14, Figs. 18, 19). Leaf explants were much more responsive, while stem explants often failed to respond (Fig. 17, Table 14). The shoot primordia initiated on BAP, BARI or zeatin were dark purple due to

anthocyanins but as the shoots elongated the purple colour became less pronounced (see Chapter III, Fig. 69). These shoots could be excised from the cultures and rooted on hormone-free medium (Fig. 20) or maintained on cytokinin-supplemented medium as proliferating shoot cultures (Fig. 21). All excised shoots of S. sisymbriifolium rooted and after 2-3 weeks they were transferred to soil and maintained in the greenhouse until they matured and set seed.

With S. aviculare all auxins tested except NOA were capable of promoting prolific callus growth on both stem internodes and leaf explants (Table 15, Fig. 22). Low concentrations of either NAA (0.1 mg/L), IAA (1.0 mg/L) or IPA (1.0 mg/L) very efficiently induced roots on leaf explants (Fig. 24). Rapidly proliferating callus derived from leaf explants cultured on 2,4-D was used to initiate cell suspension cultures.

When cytokinins were employed as the sole growth regulator with explants of S. aviculare, numerous dark purple shoots and meristematic centres were formed (Fig. 23). Stem internodes only rarely produced multiple primordia, while leaf explants often produced multiple primordia, under a broad range of cytokinin concentrations (Table 16). Regenerated shoots of S. aviculare were easily rooted on hormone-free medium and fertile plants regenerated (Fig. 25, 26).

Due to the limited number of plants of S. gilo available, it was not possible to survey a wide range of media for their influence on callus initiation and shoot regeneration. Leaf explants were suitable for callus induction with exogenous

2,4-D, NAA or NOA in the medium (Table 17, Figs. 27, 28). Callus also formed in medium containing zeatin or BAP at low concentrations (0.1 and 1.0 mg/L). It was compact and green yet totally lacked shoot primordia (Fig. 29, Table 18). When the concentration of zeatin was increased to 5.0 mg/L occasionally shoots were formed (Fig. 28, Table 18) which were rooted in vitro (Fig. 30) and raised to fertile plants (Fig. 31).

Solanum aculeatissimum leaf disks which were cultured on either cytokinin or auxin-supplemented medium failed to respond and usually turned brown (Figs. 32, 33). Very occasionally root organogenesis was observed in leaf explants of this species (Fig. 32). Stem segments were more responsive and when cultured on auxin-supplemented medium, rapidly-growing callus cultures were established (Table 19). Once again the synthetic auxins 2,4-D, 2,4,5-T, and NOA were superior for establishing and maintaining callus cultures.

The frequency of shoots on stem internodes of S. aculeatissimum in the presence of cytokinins was very low (Fig. 34) but all of the cytokinins tested except 2iP was capable of promoting some differentiation (Table 20). On cytokinin-supplemented medium it was possible to maintain small morphogenic explants which continuously proliferated new shoots. These shoots were rooted and resulting young plantlets were spiny as were the mature regenerated plants (Figs. 34-36).

Massive callus proliferation was observed on leaf explants of S. torvum cultured on either auxin- or cytokinin-supplemented medium (Figs. 37, 38). The auxins 2,4-D and 2,4,5-T were

especially efficient promoters of massive, friable white callus which was very similar to embryogenic eggplant callus. However, neither embryogenesis nor root induction were observed on S. torvum leaf or stem explant cultures (Table 21). Stems of S. torvum plants were very woody and internode explants responded poorly to both auxins and cytokinins. For this reason the survey of auxins and cytokinins was restricted to leaf explants.

Each of the cytokinins tested in addition to promoting callus growth, efficiently induced shoots on S. torvum leaf explants (Table 22, Fig. 38). A high concentration of kinetin (5.0 mg/L) was the most efficient (Table 23). Although these shoots could be rooted and transferred to soil, plants did not set seed in the greenhouse.

Leaf explants of S. macrocarpon responded to low auxin concentrations by producing numerous root hairs and limited callus (Fig. 39). It was not possible to establish rapidly-growing callus of this species. Shoots were occasionally regenerated on medium supplemented with 5 mg/L BAP (Fig. 40), however, the other cytokinins failed to promote either callus growth or shoot organogenesis.

Solanum khasianum was the least responsive species in vitro and most of the explants placed on either auxin- or cytokinin-supplemented medium displayed necrosis after two weeks. Nevertheless, it was possible to establish callus on 2,4-D-supplemented medium (Fig. 41) and this was used to initiate a cell suspension culture. Very infrequently shoot organogenesis was promoted by 1.0 mg/L BAP (Fig. 42), however, plants were not established.

2. Suspension Cultures

After the initiation of callus cultures of each species, attempts were made to establish cell suspension cultures. By placing rapidly-growing friable cultures into liquid MS medium supplemented with 2 mg/L 2,4-D, rapidly-growing suspensions of S. aviculare, S. sisymbriifolium, S. gilo, S. torvum and slow-growing suspensions of S. khasianum and S. aculeatissimum were obtained (Table 23). Solanum macrocarpon was not established in liquid medium. The rapidly-growing cell suspensions had doubling times of 2-3 days and were transferred to fresh medium every fifth day. The slow-growing suspensions were transferred every 10 days. Cell suspensions of all species produced phenolic compounds which caused the medium to turn dark brown or black if the cells were not transferred regularly. This was a reversible phenomenon since cell suspensions which were not transferred on time could become normal and translucent after repeated subculture. Production of phenolic substances was not limited to cells in liquid medium since callus cultures showed the same brown appearance with increasing age.

After six months of growth in liquid medium morphogenic competence of each cell suspension culture was evaluated: When cell colonies of each species were filtered and plated on agar-solidified MS medium with 0.1-5.0 mg/L of each cytokinin or auxin which was evaluated for explant culture, only undifferentiated callus was observed. All cell suspensions failed to show morphogenic competence, even under the optimal conditions for shoot organogenesis established with primary callus cultures for each particular species. Most cell colonies on cytokinin-supplemented medium proliferated as

light-green undifferentiated callus, those on auxin turned brown or dark brown after several weeks.

3. Protoplast Culture

Protoplasts were isolated from each rapidly-growing suspension culture in the mid-log phase of growth (day 3 from transfer). Yield of protoplasts was drastically different for each species (Table 24). This is not unexpected since the enzyme solution was optimized for eggplant protoplast isolation. Incubation of S. aviculare or S. sisymbriifolium cells for 6-8 hours produced good yields of viable protoplasts while S. gilo cells required 18 hours. Protoplast preparations from rapidly-growing cell suspensions were the least contaminated with cellular debris while S. aculeatissimum and S. khasianum protoplasts were heavily contaminated with debris (Figs. 57, 58).

Protoplasts of S. aviculare could be isolated in large numbers (1×10^6 /g fr. wt.), relatively free of cellular debris, after an 8-hr digestion in enzymes. Cell wall reformation occurred within 24 hr in Kao medium (Fig. 43) and the first cell divisions were observed after 48 hr (Fig. 44). After 3 days of culture 50% of the protoplasts divided (Fig. 45) and the dividing cells often formed long chains by subsequent divisions (Figs. 46, 47). After 28 days of culture in liquid medium an average of 633 microcalli were produced per 10^4 protoplasts plated. This 6.3% plating efficiency was much higher than the plating efficiency of eggplant (1.6%) even though the protoplast culture medium was optimized for eggplant. When these cell colonies were isolated

from liquid medium and plated on agar-solidified regeneration medium (MS + BAP or kinetin or zeatin at 1 or 5 mg/L) an unexpected morphogenic response occurred. Nearly 80% of colonies responded by producing shoot meristems after 4 weeks (Fig. 48). These meristems were very dark purple and easily visible in the white callus (Figs. 48, 50). Shoot elongation and rooting was achieved after subsequent transfer to low cytokinin medium (0.1 mg/L) for a further 2-6 weeks (Figs. 49, 50). From the sample of 50 plants which were regenerated and planted in soil, normal phenotypes were observed and most plants flowered and set seed.

Protoplasts isolated from S. sisymbriifolium cell suspensions were much larger and richer in cytoplasm than those of S. aviculare (Fig. 51). The first sign of cell wall reformation was observed after 48 hr of culture (Fig. 53) and was accompanied by the dramatic elongation of 30% of the protoplasts. Occasionally, up to 1% of the protoplasts of S. sisymbriifolium were capable of division in Kao medium supplemented with 1 mg/L 2,4-D and 1 mg/L BAP (Fig. 52). Although the greatly elongated cells very rarely divided, they appeared to survive in this medium for several weeks (Fig. 54). Small cell colonies were observed after 3 weeks of culture (Fig. 55) and although they were slow-growing, it was possible to transfer microcalli onto agar-solidified medium after 5 weeks of culture. Fourteen percent of the microcalli transferred onto regeneration medium were capable of forming callus cultures but all modifications of media tested failed to promote shoot organogenesis or embryogenesis (Fig. 56).

Protoplast-division and sustained colony formation was only observed in species, S. sisymbriifolium and S. aviculare. A comparison between several protoplast culture media (Kao, NT and MS) revealed that the highest plating efficiency with both species was in Kao medium. The plating efficiency of S. aviculare protoplasts was 6.3% in Kao, 0.07% in MS, and 0.0055% in NT while the values for S. sisymbriifolium were 0.064% in Kao, 0.0033% in MS and 0.016% in NT medium.

Protoplasts of S. khasianum and S. aculeatissimum were not capable of cell division in the only medium tested [Kao medium (Table 24, Figs, 57, 58)]. Solanum gilo protoplasts were isolated only after a digestion period of 18 hr in enzyme solution. These protoplasts were small and uniform and although occasional division was observed in Kao medium (Table 24, Fig. 59), colonies and callus could not be regenerated. Solanum torvum protoplasts were incapable of cell division after they had reformed cell walls in Kao medium.

DISCUSSION

Although the process of cell proliferation and shoot organogenesis is very common throughout the plant kingdom, each species has specific nutritional and hormonal requirements. The MS culture medium devised for Nicotiana callus cultures (Murashige and Skoog 1962) has proven successful for proliferation of many other Solanaceous species, including eggplant (Gleddie et al. 1983a), tomato (Seeni and Gnanam 1981) and potato (Jacobsen et al. 1983). Modified MS medium was also suitable for supporting callus growth, shoot organogenesis and plant regeneration in six Solanum species in this study.

Distinct differences in in vitro response were observed between explants from different organs of the same species and between different species. For example, leaf explants of S. sisymbriifolium produced prolific roots but not callus on auxin-containing medium yet on the same medium stem explants efficiently proliferated callus (Table 13). Stems of S. aculeatissimum were also superior to leaves as proliferative explants on auxin-supplemented medium. Solanum aviculare, on the other hand, produced rapidly-growing calli on all auxins tested from both stem and leaf explants with no significant difference in frequency of callus induction (15-30%) between the sources of explants (Table 15). Callus cultures were established in each species with reasonable efficiencies and degrees of reliability. Other workers have noted a pronounced effect of explant source on initiation of callus cultures in Solanaceous

species, including Digitalis (Tewes et al. 1982), Lycopersicon (Locy 1983) and S. melongena (Alicchio et al. 1982).

In this study it was possible to induce shoot formation and regenerate plants of each species surveyed using cultured explants of either leaf or stem origin. Shoot induction required exogenous cytokinin and there were significant differences in some cases between cytokinin types and concentrations which promoted the highest frequencies of shoot primordia. Each of the cytokinins tested promoted shoot regeneration from leaf explants of S. sisymbriifolium yet kinetin and BARI were ineffective for stem explants (Table 14). Alternatively BARI at 5.0 mg/L promoted the highest frequency of shoots on leaf explants (27 shoots/explant) while stem explants were totally unresponsive to this cytokinin (Table 14).

A similar result was observed in explants of S. aviculare where BARI was ineffective with stem explants but promoted shoot primordia on leaf explants (Table 16). A specific cytokinin requirement for shoot induction was observed in leaf explants of S. gilo although only two cytokinins were tested (Table 18).

Kut and Evans (1982) tested the in vitro response of eight wild tomato and two related Solanum species on a variety of media. There were significant differences in shoot production between leaf explants of Lycopersicon pimpinellifolium cultured on the eight different media while L. chilense produced approximately equal numbers of shoots on all cytokinins. Eggplant leaf explants have also been shown to produce nearly equal numbers of shoots on a variety of different cytokinins (Gleddie et al. 1983a).

Cytokinins have been supplied as the sole growth regulator to promote shoot organogenesis and plant regeneration from other members of the genus Solanum including: S. laciniatum (Chandler and ~~eds~~ 1983), S. dulcamara and S. nigrum (Zenkteler 1972), and S. melongena (Gledde et al. 1983a). Of the species employed in the present study, only S. sisymbriifolium, S. khasianum and S. aculeatissimum have been previously used for in vitro studies. The study by Fassuliotis (1975) revealed that callus could be initiated on stem parenchyma of S. sisymbriifolium by supplying both 2,4-D and IAA to the medium. Callus tissue which was placed on medium supplemented with 2iP underwent shoot organogenesis and plant regeneration. In the present study it was noted that other cytokinins (especially zeatin and BARI) promoted numerous shoot primordia on leaf explants of this species (Table 14) and that these young primordia accumulated anthocyanin. Such results underscore the importance of studying a variety of growth regulators across a range of concentrations.

The medicinal plant S. khasianum has also been previously manipulated in vitro (Bhatt et al. 1979; Kowalczyk et al. 1983). Both of these studies found that explant culture in darkness was essential for cytokinin-induced shoot organogenesis. Although S. khasianum was the most recalcitrant species in the present survey, it was possible to generate both callus and very rare (1 shoot/23 explants) shoot organogenesis in the light. The necrosis of leaf explants which were cultured in the light (Fig. 42) was also observed on shoot-forming cultures in dark (Kowalczyk et al. 1983).

2

A previous report described the initiation of callus cultures of S. aculeatissimum from young seedlings for the purpose of studying glycoalkaloid production in vitro (Kadkade and Madrid, 1977). In the present study mature leaves were found to be inferior sources of explants but stem internodes, responded on both callus-induction and shoot-induction media.

Regenerated plants of S. aviculare, S. sisymbriifolium and S. gilo set seed in the greenhouse while regenerated flowering plants of S. aculeatissimum, S. torvum and S. khasianum did not; hand pollination was not attempted. It is likely that lack of fertility was due to self-incompatibility mechanisms rather than genetic alterations which occurred during in vitro culture because seed-derived plants were also infertile in the greenhouse.

Cell suspensions have been described as valuable sources of protoplasts since they are sources of large quantities of sterile material well adapted to growth in liquid medium (see Literature Review). For this reason, and due to previous successful somatic hybridization studies using cell suspension protoplasts (Douglas et al. 1981b; Gleddie et al. 1983a) it was considered important to establish cell suspension cultures of the wild Solanum species as a prelude to protoplast and somatic hybridization studies. Although only a limited variety of media and auxin types and concentrations were tested, it was possible to establish rapidly-growing S. aviculare, S. sisymbriifolium, S. gilo and S. torvum cell suspensions. The complete lack of plant regeneration from established cell suspensions after six months culture was disappointing yet not uncommon for the Solanaceae. Long-term

tomato callus lines lost the capacity for shoot regeneration while normal plantlets were regenerated from younger callus lines (Meredith 1979). These results were very different than were observed with eggplant cell suspensions which still regenerated somatic embryos after two years in culture (Chapter I, Table 10).

Protoplast isolation, culture, division of cells and colony formation was successful in the present study with S. aviculare, S. sisymbriifolium and S. gilo. Successful plant regeneration was achieved in protoplast-derived colonies of S. aviculare even though the cell suspension was not morphogenic. When protoplast-derived colonies of this species were plated on zeatin or BAP-supplemented regeneration medium, they formed numerous dark purple meristems (Fig. 48). This was extremely common in these cell colonies (80%) and very reminiscent of the original tissue explants which also regenerated purple shoots on cytokinin-supplemented medium (Fig. 19). Previous cases of successful morphogenesis from protoplasts which were isolated from non-morphogenic cell suspensions have not been reported. Unprotoplasted and protoplast-derived cells of S. melongena also responded very differently to the same medium (Chapter I).

Although the cell suspension-derived protoplasts of S. sisymbriifolium underwent wall regeneration, cell elongation and several cell divisions, the plating efficiency of this species was extremely low (Table 25). Despite the limited number of cell colonies available it was possible to screen several media for their capacity to regenerate plants. Unfortunately, plant regeneration was unsuccessful.

The lack of sustained divisions in protoplast cultures of S. khasianum and S. aculeatissimum and the few divisions observed in S. gilo protoplasts may have been due to nutritional and/or hormonal inadequacies in the medium modifications tested. Kao's medium was successful for eggplant protoplast culture and the plating efficiency of calli was 1-5% when glucose (0.35 M) was the osmoticum. Mesophyll protoplasts of S. khasianum have been regenerated to plants in the same medium (Kao) using sucrose or mannitol as osmoticum while protoplast stability was not maintained in the presence of glucose or sorbitol osmotica (Kowalczyk et al. 1983). It is therefore possible that major modifications to the osmoticum, growth regulators and nutrient composition of the medium could result in successful protoplast division and plant regeneration from these species.

In order to successfully regenerate somatic hybrid plants it is not always essential that the starting populations of protoplasts be morphogenic. In the case of S. tuberosum and L. esculentum somatic hybrids produced by Melchers et al. (1978) the tomato mesophyll protoplasts were not capable of regeneration yet hybrid plants were recovered. Brassica oleracea and B. campestris hybrid colonies were also morphogenic while the starting populations of both species were not (Schenck and Röbbelen 1982). Although eggplant protoplasts were capable of successful plant regeneration while the wild species S. sisymbriifolium which carries important agronomic traits is not, a somatic hybrid combination between these species proved to be capable of plant regeneration (Chapter III).

TABLE 13. Effect of auxins on morphogenesis in leaf and stem explants of *S. sisymbriifolium*

Auxin	Conc. (mg/L)	In vitro response		% response *	
		C	R	C	R
<u>Leaf explants</u>					
2,4-D	0.1	-	+	0	50
2,4,5-T	0.1	-	+	0	10
NAA	5.0	-	+	0	72
NOA	0.1, 1.0, 5.0	-	-	0	0
IAA	0.1, 1.0, 5.0	-	-	0	0
IPA	0.1, 1.0, 5.0	-	-	0	0
<u>Stem explants</u>					
2,4-D	0.1	+	-	11	0
	1.0	+	+	100	50
	5.0	+	-	100	0
2,4,5-T	0.1	-	-	0	0
	1.0	-	+	0	100
	5.0	+	-	50	0
NAA	0.1	+	+	80	10
	1.0	+	+	42	5
	5.0	+	+	20	4
NOA	0.1, 1.0, 5.0	-	-	0	0
IAA	0.1	-	-	0	0
	1.0	-	+	0	4
	5.0	-	+	0	3
IPA	0.1, 1.0, 5.0	-	-	0	0

Note: Cultures were scored for morphogenesis after 30 days.

C = callus proliferation

R = root initiation

* percent of 25 explants which gave the indicated in vitro responses.

TABLE 14. Effect of cytokinins on shoot organogenesis in leaf and stem explants of *S. sisymbriifolium*

Cytokinin	Conc. (mg/L)	f	% response*
<u>Leaf explants</u>			
Kinetin	0.1	0	0
	1.0	2.0	28
	5.0	1.0	54
Zeatin	0.1	1.0	4
	1.0	17.5	92
	5.0	16.0	88
BAP	0.1	0	0
	1.0	5.0	56
	5.0	10.1	48
BARI	0.1	0	0
	1.0	2.0	24
	5.0	27.0	84
2iP	0.1	0	0
	1.0	1.0	4
	5.0	3.0	12
<u>Stem explants</u>			
Kinetin	0.1, 1.0, 5.0	0	0
Zeatin	0.1	0	0
	1.0	5.0	32
	5.0	5.0	16
BAP	0.1	0	0
	1.0	1.0	20
	5.0	2.4	8
BARI	0.1, 1.0, 5.0	0	0
2iP	1.0	1.0	90

Note: Cultures were scored for shoot organogenesis after 30 days. f represents the mean number of shoots per responding explant. * percent of 25 explants which gave shoot organogenesis.

TABLE 15. Effect of auxins on morphogenesis in leaf and stem explants of *S. aviculare*

Auxin	Conc. (mg/L)	In vitro response		% response	
		C	R	C	R
<u>Leaf explants</u>					
2,4-D	0.1	+	-	20	0
	1.0	+	-	32	0
	5.0	+	-	26	0
2,4,5-T	0.1	+	-	24	0
	1.0	+	-	12	0
	5.0	+	-	20	0
NAA	0.1	-	+	8	8
	1.0	+	-	22	0
	5.0	+	-	26	0
NOA	0.1	+	-	22	0
	1.0	+	-	40	0
	5.0	+	-	28	0
IAA	0.1	-	-	0	0
	1.0	-	+	0	56
	5.0	+	+	12	8
IPA	1.0	-	+	0	24
<u>Stem explants</u>					
2,4-D	1.0	+	-	12	0
2,4,5-T	0.1	+	-	8	0
	1.0	+	-	16	0
NAA	1.0	+	-	22	0
	5.0	+	-	20	0
NOA	0.1, 1.0, 5.0	-	-	0	0
IAA	5.0	+	-	24	0
IPA	5.0	+	-	16	0

Note: Cultures were scored and data is recorded as in Table 13.

TABLE 16. Effect of cytokinins on shoot organogenesis in leaf and stem explants of S. aviculare

Cytokinin	Conc. (mg/L)	f	% response
<u>Leaf explants</u>			
Kinetin	0.1	0	0
	1.0	0	0
	5.0	4	50
Zeatin	0.1	2.2	96
	1.0	8.8	92
	5.0	5.3	76
BAP	0.1	1.6	12
	1.0	6.8	76
	5.0	19.8	88
BARI	0.1	6.9	52
	1.0	6.6	80
	5.0	9.0	84
2iP	0.1	0	0
	1.0	3	28
	5.0	4	60
<u>Stem explants</u>			
Kinetin	0.1	3	88
	1.0	1.3	56
	5.0	1.3	64
Zeatin	0.1	1.3	36
	1.0	1.6	44
	5.0	1.0	52
BAP	0.1	1.6	20
	1.0	2.0	8
	5.0	0	0
BARI	0.1, 1.0, 5.0	-	0
2iP	0.1	0	0
	1.0	1.6	12
	5.0	1.0	4

Note: Cultures were scored and data is recorded as in Table 14.

TABLE 17. Effect of auxins on morphogenesis in leaf explants
of S. gilo.

Auxin	Conc. (mg/L)	<u>In vitro</u> response		% response	
		C	R	C	R
NAA	0.1	-	-	0	0
	1.0	+	-	100	0
	6.0	+	-	70	0
NOA	0.1	+	-	10	0
	1.0	+	-	100	0
	5.0	+	-	70	0
2,4-D	0.1	+	-	36	0
	1.0	+	-	100	0
	5.0	+	-	100	0

Note: Cultures were scored and data is recorded as in Table 13.

TABLE 18. Effect of cytokinins on shoot organogenesis in leaf explants of S. gilo

Cytokinin	Conc. (mg/L)	f	% response
BAP	0.1	0	0
	1.0	0	0
	1.0	0	0
	5.0	0	0
Zeatin	0.1	0	0
	1.0	0	0
	5.0	1.0	12

Note: Cultures (10 per treatment) were scored and data is recorded as in Table 14.

TABLE 19. Effect of auxins on morphogenesis in stem explants
of S. aculeatissimum

Auxin	Conc. (mg/L)	<u>In vitro</u> response		% response	
		C	R	C	R
2,4-D	0.1	+	+	50	24
	1.0	+	-	100	0
	5.0	+	+	88	4
2,4,5-T	0.1	-	-	0	0
	1.0	+	-	100	0
	5.0	+	-	100	0
NAA	0.1	-	+	0	12
	1.0	-	+	0	100
NOA	0.1	+	+	8	8
	1.0	-	+	0	36
	5.0	+	-	76	0
IAA	0.1	-	+	0	8
	1.0	-	+	0	32
	5.0	-	-	0	0

Note: Cultures were scored and data is recorded as in Table 13.

TABLE 20. Effect of cytokinins on shoot organogenesis in stem explants of S. aculeatissimum

Cytokinin	Conc. (mg/L)	f	% response
Kinetin	0.1	1.0	4
	1.0	1.0	4
	5.0	2.6	20
Zeatin	0.1	1.3	4
	1.0	3.0	12
	5.0	1.6	12
BAP	0.1	3	8
	1.0	0	0
	5.0	1.0	4
BARI	0.1	2.6	8
	1.0	1.0	4
	5.0	0	-
2iP	0.1	-	0
	1.0	-	0
	5.0	-	0

Note: Cultures were scored and data is recorded as in Table 14.

TABLE 21. Effect of auxins on morphogenesis in leaf explants
of S. torvum

Auxin	Conc. (mg/L)	<u>In vitro</u> <u>response</u>		% response	
		C	R	C	R
2,4-D	0.1	-	-	0	0
	1.0	-	-	0	0
	5.0	+	-	88	0
2,4,5-T	0.1	+	-	76	0
	1.0	+	+	72	4
	5.0	+	-	100	0
NAA	0.1	+	-	36	0
	1.0	+	-	88	0
	5.0	+	-	20	0
NOA	0.1	+	-	4	0
	1.0	+	-	20	0
	5.0	+	-	68	0
IAA	0.1	-	-	0	0
	1.0	-	-	0	0
	5.0	-	+	0	16
IPA	0.1	-	-	0	0
	1.0	-	-	0	0
	5.0	-	-	0	0

Note: Cultures were scored and data is recorded as in Table 13.

TABLE-22. Effect of cytokinins on shoot organogenesis in leaf explants of S. torvum

Cytokinin	Conc. (mg/L)	f	% response
Kinetin	0.1	4.5	92
	1.0	12.0	84
	5.0	15.5	76
Zeatin	0.1	3.5	68
	1.0	2	12
	5.0	3.6	32
BAP	0.1	0	0
	1.0	0	0
	5.0	3	16
BARI	0.1	0	0
	1.0	0	0
	5.0	2.6	20
2iP	0.1	0	0
	1.0	1.0	4
	5.0	.3	48

Note: Cultures were scored and data is recorded as in Table 14.

TABLE 23. Characteristics of cell suspension cultures of wild Solanum species which showed maximum growth in liquid MS medium with 2 mg/L 2,4-D.

Species	Primary explant	Callus-induction hormones (mg/L)	Time between transfers (days)	Response on shoot-induction medium
<u>S. aviculare</u>	leaf	1.0 2,4-D	5	callus growth
<u>S. sisymbriifolium</u>	stem	5.0 NAA	4	very little callus growth
<u>S. gilo</u>	leaf	5.0 BAP	5	none
<u>S. aculeatissimum</u>	stem	1.0 2,4-D	10	"
<u>S. torvum</u>	leaf	5.0 2,4,5-T	4	very good callus growth
<u>S. khasianum</u>	stem	1.0 2,4-D	10	none

Note: The most rapidly-growing and friable-appearing callus cultures on MS medium were chosen to inoculate the liquid culture medium.
 Cell suspension culture and maintenance were performed as for eggplant (see Chapter I, Materials and Methods).
 Shoot-induction medium consisted of agar-solidified MS supplemented with 0.1-5.0 mg/L of BAP, BARI, zeatin or kinetin.

TABLE 24. Isolation and culture of protoplasts of several wild Solanum species

Species	Yield # protoplast/ g fr. wt.	Wall regeneration (days)	Time of 1st division (days)	Division frequency at day 7	Plating efficiency
<u>S. aviculare</u>	1×10^6	1	2	60%	6.3 %
<u>S. sisymbriifolium</u>	4.5×10^5	2	3	7.5%	0.06%
<u>S. torvum</u>	2×10^5	3	-	-	0
<u>S. aculeatissimum</u>	1×10^5	5-7	-	-	0
<u>S. gilo</u>	1×10^5	4	6	0.5%	0
<u>S. khasianum</u>	1×10^5	4-6	-	-	0

Note: Cell suspensions used for protoplast isolation were maintained in MS medium + 2 mg/L 2,4-D. Protoplasts were cultured in Kao medium + 1 mg/L 2,4-D, 3 mg/L NAA and 1 mg/L BAP. Values given are means from 5 isolation experiments for S. aviculare and S. sisymbriifolium and 2 experiments for the other species.

Plating efficiency is the percentage of protoplasts forming microcalli at 42 days.

FIGURE 16. Stem explants of S. sisymbriifolium cultured on MS medium supplemented with 1.0 mg/L 2,4-D. Note in addition to callus formation about 50% of the explants responded by producing roots.

FIGURE 17. Stem explants of S. sisymbriifolium cultured on MS medium supplemented with 1.0 mg/L kinetin, showing complete lack of organogenesis.

FIGURE 18. Leaf explants of S. sisymbriifolium cultured on MS medium supplemented with 5 mg/L zeatin for 18 days. Note the proliferation of shoot primordia.

FIGURE 19. Close-up of a leaf explant of S. sisymbriifolium cultured on MS + 1 mg/L zeatin for 28 days. Note the elongating shoot primordia.

FIGURE 20. Excised shoot primordia of S. sisymbriifolium which was cultured on hormone-free B₅ medium for the induction of roots. Note the spines on the small leaves.

FIGURE 21. Meristematic leaf explants of S. sisymbriifolium which were maintained on shoot-induction medium containing 1 mg/L zeatin.

FIGURE 22. Stem explants of S. aviculare cultured on MS medium containing 1 mg/L NAA.

FIGURE 23. Leaf explants of S. aviculare cultured on MS medium containing 5 mg/L BAP. Note the prolific shoot production and elongation of these shoots.

FIGURE 24. Leaf explants of S. aviculare cultured on MS medium supplemented with 1 mg/L IAA. Note the prolific root induction but lack of callus production.

FIGURE 25. Excised shoot of S. aviculare cultured on hormone-free B₅ medium for root initiation. Note the serrated leaves of the young shoot.

FIGURE 26. Mature plant which set seed in the greenhouse which was regenerated from a leaf explant of S. aviculare.

FIGURE 27. Leaf explant of S. gilo which was cultured on MS containing 5 mg/L NAA.

FIGURE 28. Leaf explants of S. gilo which were cultured on MS medium containing 5.0 mg/L zeatin. Note the small organized, meristematic centres surrounding the leaf explant.

FIGURE 29. Leaf explants of S. gilo which were cultured on MS medium containing 1 mg/L zeatin. Note the prolific callus production in the absence of auxins, yet lack of shoot organogenesis.

FIGURE 30. Three excised meristematic areas from a leaf explant cultured on 5.0 mg/L zeatin. These meristems elongated and rooted on hormone-free medium.

FIGURE 31. A mature plant of S. gilo which set seed in the greenhouse after regeneration from a leaf explant.

FIGURE 32. Leaf explants of S. aculeatissimum which were cultured for 25 days on MS medium containing 1 mg/L 2,4-D. Note the lack of callus proliferation but the sporadic induction of roots.

FIGURE 33. Leaf explant of S. aculeatissimum which was cultured on MS medium containing 1 mg/L BAP.

FIGURE 34. Stem explants of S. aculeatissimum which were cultured on MS medium containing 5.0 mg/L kinetin. Note the infrequent production of shoots.

FIGURE 35. Excised shoots of S. aculeatissimum which were cultured on MS medium containing 1 mg/L kinetin to promote elongation and maintain the green colour.

FIGURE 36. Excised shoot of S. aculeatissimum which was cultured on hormone-free medium. Note the spines and leaf hairs.

FIGURE 37. Leaf explants of S. torvum cultured on MS medium containing 1 mg/L NAA. Note the proliferating white, friable callus on the circumference of the leaf explant.

FIGURE 38. Leaf explants of S. torvum cultured on MS medium containing 1.0 mg/L kinetin. Note the proliferation of callus in addition to shoot organogenesis.

FIGURE 39. Leaf explants of S. macrocarpon cultured on 1.0 mg/L IAA. Note the proliferation of roots and root hairs, with very little callus proliferation.

FIGURE 40. Leaf explant of S. macrocarpon cultured on MS medium containing 5 mg/L BAP which responded by producing occasional shoot organogenesis.

FIGURE 41. Leaf explant cultures of S. khasianum cultured in MS medium containing 1 mg/L 2,4-D. Note the moderately-growing callus on the explant.

FIGURE 42. Leaf explants of S. khasianum cultured in the 1 mg/L BAP which turned brown, showed necrotic regions on upper and lower surfaces, but did not undergo shoot organogenesis.

FIGURE 43. Protoplasts of S. aviculare after 24 hr of culture in Kao medium containing 3 mg/L NAA, 1 mg/L 2,4-D, and 1 mg/L BAP. The oblong shape of these cells is characteristic of new formed cell walls on the protoplasts.

FIGURE 44. The first division of S. aviculare protoplast-derived cells cultured in the same medium as Fig. 43 after 48 hr of culture.

FIGURE 45. The second division of protoplast-derived cells of S. aviculare. Note the cells divide in long files.

FIGURE 46. The 3rd division of long files of S. aviculare cells after 1 week of culture.

FIGURE 47. After 10 days of culture small colonies and cell aggregates of S. aviculare protoplast origin were formed.

FIGURE 48. Small protoplast-derived microcalli of S. aviculare after 3 weeks of culture on agar-solidified MS medium containing 1 mg/L zeatin. Note the formation of approximately 1 shoot bud per colony.

FIGURE 49. A protoplast-derived shoot of S. aviculare which has elongated on hormone-free B₅ medium.

FIGURE 50. The elongation of a shoot meristem on a protoplast-derived microcallus of S. aviculare. Note the dark deep colour (purple) to the shoot primordia formed on the white callus.

FIGURE 51. An isolated protoplast of S. sisymbriifolium from a cell suspension culture. Note the characteristic cytoplasmic strands.

FIGURE 52. The first division of S. sisymbriifolium protoplasts after 10 days of culture in Kao medium + 3 mg/L NAA, 1 mg/L 2,4-D, and 1 mg/L BAP.

FIGURE 53. The drastic elongation of S. sisymbriifolium cells 3 days after the isolation and culture of protoplasts.

FIGURE 54. A division of an elongated cell in a protoplast culture of S. sisymbriifolium 15 days after the isolation of the protoplasts.

FIGURE 55. A slow-growing, 3 week-old protoplast-derived cell colony of S. sisymbriifolium. Note the starch granules and plastids.

FIGURE 56. A cell colony of S. sisymbriifolium protoplast-origin after 1 month of growth on agar-solidified medium containing cytokinin. Note the lack of shoot organogenesis.

FIGURE 57. Protoplasts of S. khasianum cultured for 10 days on Kao medium. Note the lack of cell divisions.

FIGURE 58. Protoplasts of S. aculeatissimum after 7 days of culture in Kao medium. Note the elongation and change of shape but also the absence of cell division.

FIGURE 59. A protoplast-derived population of S. gilo cells. Note the occasional division but lack of colony formation after 14 days.

CHAPTER 3

"SOMATIC HYBRIDIZATION BETWEEN SOLANUM MELONGENA L.

and

SOLANUM SISYMBRIIFOLIUM LAM."

INTRODUCTION

Solanum species have thus far been used to a limited extent in somatic hybridizations. Melchers et al. (1978) obtained somatic hybrid plants between dihaploid S. tuberosum and Lycopersicon esculentum and subsequently the genetic segregation of chilling resistance among the hybrids was reported (Smillie et al. 1979). The same species combination has recently been recreated by Shepard et al. (1983) using a tetraploid S. tuberosum cultivar. In both cases the resulting hybrids were sterile and backcrosses to either parent could not be made. Butenko (1982) reported the regeneration of a single plant of the somatic hybrid S. tuberosum + S. chacoense. This hybrid line displayed resistance to potato virus Y even though both parents were susceptible. Although sexual hybridization between these species was also successful, the reaction of these hybrids to the virus was not studied. Several somatic hybrid plants between haploid S. tuberosum and an atrazine-resistant biotype of S. nigrum have also been regenerated and the atrazine tolerance trait, which is chloroplast encoded, was inherited in some clones (Binding et al. 1982).

The non-tuberous Solanum species have not previously been utilized in somatic hybridization although they constitute numerically the vast majority of the genus including economically-important members such as eggplant. Several desirable agronomic traits have been identified in the South American species S. sisymbriifolium Lam., including resistance to root-knot nematodes (Fassuliotis and Bhatt 1982),

and carmine spider mites (Schalk et al. 1975). Resistance to these pests is unknown in eggplant (S. melongena L.) germplasm and several unsuccessful attempts to cross these species sexually have been made (Fassuliotis 1975; Seck 1983). Protoplast fusion offers the possibility of bypassing complex sexual barriers between species. Because it has been possible to develop methodology for protoplast isolation and culture in both S. melongena and S. sisymbriifolium (Chapters 1, 2), as well as to achieve plant regeneration in S. melongena, it was feasible to consider the somatic hybridization of these species. Therefore, this study was undertaken to create, select, regenerate and characterize somatic hybrid plants between S. melongena and S. sisymbriifolium with the long term objective of transferring the desired traits into eggplant.

MATERIALS AND METHODS

A suspension culture of S. sisymbriifolium (Garden No. S136) isolated from a stem callus culture was maintained in liquid MS medium supplemented with 2 mg/L 2,4-D, and used for protoplast isolation (Chapter 2). Two cell suspensions (AU^R_8 and AU^R_{10}) of eggplant cv. 'Imperial Black Beauty' (isolated by D. F. Brown, Bishop's University, Lennoxville, Quebec) which were capable of growth in the presence of 6-azauracil (AU) were grown in the same medium.

The growth rates of each suspension culture was measured in the presence and absence of 6-azauracil by filtering out large cell aggregates on a 1,000 μ m nylon filter. The small cell aggregates were then suspended in liquid medium at a density of 0.2 g fr. wt./mL. One-half mL of this suspension was pipetted onto sterile filter paper (Whatman No. 1, 5.5 cm diameter) placed on agar-solidified Kao (1977) medium containing 2 mg/L 2,4-D and various concentrations of filter-sterilized AU. The fresh weight of cells on filter paper was measured every second day for three weeks.

Protoplasts of both species were isolated according to methods described in Chapter 1. Protoplasts were washed and cultured with or without 1 mM AU, in liquid Kao medium supplemented with 1 mg/L 2,4-D, 3 mg/L NAA, 1 mg/L BAP.

For fusion, 10^6 protoplasts of each species were mixed in a 16 x 125 mm glass tube and centrifuged at 100 xg for 5 min. In one experiment, protoplasts of AU^R_8 were treated for 15 min with 5 mM iodoacetic acid followed by three rinses in Kao medium prior to

mixing. Following mixing, the protoplasts were pelleted, the medium was decanted and the protoplasts resuspended in 0.5 mL of culture medium. One mL of 50% (w/v) polyethylene glycol PEG, MW1540 solution containing 0.1 M glucose, 0.7 mM KH_2PO_4 , and 10.5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, pH 5.8 was slowly added down the side of the tube. Following a 15 min incubation, the PEG solution was diluted successively with one-quarter, one-half, and one mL of Kao medium at 5 min intervals. The protoplasts were sedimented by centrifugation at 100 xg for 3 min and resuspended in 5 mL of Kao medium for one hour. A total of four fusion experiments were performed.

For post-fusion culture, the protoplasts were resuspended in Kao medium containing 1 mg/L 2,4-D, 3 mg/L NAA and 1 mg/L BAP, with or without 1 mM AU at a density of 10^5 per mL and dispensed as one mL thin layers in 15 x 60 mm Falcon plastic petri dishes. The dishes were sealed with parafilm and incubated in a water-saturated environment inside plastic containers under low light ($7 \mu\text{E m}^{-2}\text{sec}^{-1}$) at 25°C. Efficiency of protoplast division was determined by scoring one hundred protoplasts per dish, in five separate dishes per treatment, after 7 days of culture. After 14 days of culture, the dishes were transferred to a higher light intensity ($30 \mu\text{E m}^{-2}\text{sec}^{-1}$) and 7 days later each dish was supplied 0.5 mL of Kao medium supplemented with 1 mM AU and 0.1 M glucose. At this time the dishes were removed from the plastic boxes and maintained thereafter in the same conditions at 70% R.H.

Six weeks post-fusion macroscopic cell colonies were counted, isolated with forceps, and placed onto Kao medium lacking glucose and AU but supplemented with 0.8% Difco agar, 2% (w/v) sucrose, 0.1 mg/L 2,4-D, and 1 mg/L BAP. The colonies grew rapidly on this

medium and after 14–28 days they were transferred to MS or Kao medium lacking 2,4-D but supplemented with a range of cytokinin types and concentrations.

Selected colonies which turned purple on MS or Kao media supplemented with 1 mg/L zeatin or 1 mg/L ~~BAP~~ or 5 mg/L BAP were excised and transferred to MS medium supplemented with 1 mg/L zeatin for induction of shoot organogenesis. Once regenerated shoots had developed one or two normal leaves, they were transferred to agar-solidified hormone-free MS or B₅ medium. Rooted shoots were assigned line numbers, transferred to 7 cm diameter plastic pots containing sterilized vermiculite; and maintained in a growth chamber with 70% R.H. with a 25:20°C (day:night) temperature under a 16-hr daylight ($30 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$) supplied by fluorescent lights. When the plants had developed to the 5–6 leaf stage with adequate roots, they were transplanted to 13 cm diameter pots containing a soil:peat:sand mixture (1:2:1). Plants of the parental species S. sisymbriifolium and S. melongena regenerated directly from leaf explant cultures were maintained under the same conditions in the greenhouse.

Morphological data from all plants was collected after two months of greenhouse growth. During this period the plants were also examined cytologically. Excised root tips were pretreated in water for 24 hr at 0°C, fixed for 24 hr in acetic acid: ethanol (1:3) and stained in Snow's solution (Snow, 1963).

Analysis of isoenzymes was performed on the three or four healthy young leaves of regenerated plants after 1–3 months of greenhouse growth. 0.5 g of material was ground with a glass rod in a test tube with 1.3 mL 0.2 M Tris HCl buffer, pH 8.5, containing 1 M sucrose and 5 mg/mL DL-dithiothreitol (DTT). The homogenate was

microfuged (138,000 xg for 5 min) and protein content of the supernatant determined by a modified Lowry technique (Wetter 1977).

Polyacrylamide gel electrophoresis was carried out by placing samples containing 75 µg protein (in the sucrose solution) and a trace of bromophenol blue on a 2.5% stacking slab gel. The separating gel was a 5% polyacrylamide and the alkaline buffer that described by Davis (1964). The gels were run at 20 watts (constant power) until the bromophenol blue had migrated to within 1 cm of the bottom (usually 120–180 min). Aspartate aminotransferase (AAT) isozymes were detected by a modified technique which used L-cystine sulfinic acid in place of L-aspartic acid as the substrate (Yagi *et al.* 1981). The substrate and staining solutions for alanyl aminopeptidase (AAP), 6-phosphogluconate dehydrogenase (6-PGDH), leucine aminopeptidase (LAP), and peroxidase were as described by Wetter and Dyck (1983).

Crystallization of ribulose 1, 5 biphosphate carboxylase/oxygenase (Rubisco) from eggplant leaves by the method described by Lowe (1977) was unsuccessful and, as a substitute, the protein was prepared from Nicotiana tabacum leaves. Fifty grams leaf material were ground in 40 mL of extraction buffer containing 0.5 M Tris HCl pH 7.8, 0.2 M NaCl, 0.01 M MgCl₂, 0.005 M EDTA and 0.1 M β mercaptoethanol. After squeezing through four layers of cheesecloth and heating to 40°C for 10 min, the extract was centrifuged for 1 hr at 30,000 xg. Seventy-five mL of extract were desalted on a Sephadex G-50 column and 10 mL fractions were collected. Rubisco was crystallized at 4°C in the presence of 5 mM NaHCO₃ and 0.5 mM MgCl₂ with a yield of 287 mg. Polyclonal antisera against the crystalline Rubisco was prepared in rabbits by D. R. Bundle, National Research Council, Ottawa.

Rubisco was isolated from the leaves of eight randomly selected hybrid plants and the parental species by following the methods described by Wetter and Dyck (1983). This involved extracting 0.5 g leaf material with 1 mL Gray's buffer and centrifuging the homogenate (48,000 xg for 30 min). The supernatant was immunoprecipitated with 0.3 mL anti-Rubisco rabbit serum. After incubation the antibody-Rubisco complex was centrifuged and washed twice with a borate-saline solution containing 0.0025 M borax, 0.04 M boric acid, and 0.12 M NaCl pH adjusted to 7.3. The precipitate was solubilized, carboxymethylated and passed through a Sephadex G-25 column. Isoelectric focussing was carried out on a slab gel according to the methods described by Wetter and Dyck (1983).

To study the inheritance of the embryogenic and organogenic in vitro responses among the hybrids, and to test for their levels of AU-resistance, leaf explants of some somatic hybrids were cultured as described in Chapter 1. Cell suspensions of these hybrid lines were isolated as described in Chapter 1, and tested for AU-resistance as described above. Preliminary measurements of uracil phosphoribosyl-transferase (UPRTase) activity among hybrids and parental lines were performed according to Jones (1984).

RESULTS

Wild-type eggplant cells, two cell lines of eggplant selected after EMS-treatment, which were azauracil-resistant and a cell suspension of S. sisymbriifolium were tested for the ability to grow on agar-solidified medium containing various concentrations of 6-azauracil (AU). The AU-resistant eggplant cell cultures, AU^R₈ and AU^R₁₀, were able to grow equally well on either 0.5 or 1.0 mM AU at about one-half the rate they grew on AU-free medium (Figs. 60, 61). Wild-type eggplant cells, by comparison, grew better on control medium and were severely inhibited in the presence of 1 mM AU (Fig. 62). Cells of S. sisymbriifolium, on the other hand, were not severely affected by 0.5 mM AU in the culture medium, while 1 mM was strongly inhibitory (Fig. 63).

This information indicated it was possible to distinguish between AU^R₈ or AU^R₁₀ and S. sisymbriifolium cells growing in AU after 20 days. Even though the wild-type S. sisymbriifolium cells were capable of limited growth in media containing AU and AU^R₁₀ cells grew slower in the presence of AU, it was possible to see clear differences between these cell lines after 21 days in 1 mM AU (Figs. 64, 65).

Protoplasts of AU^R₈ and AU^R₁₀, obtained at yields of 6×10^5 and 8×10^5 per g fr. wt. were cultured in the presence and absence of 1 mM AU. They were compared with S. sisymbriifolium protoplasts cultured in similar conditions. All three populations were capable of cell-wall reformation within three days of culture. The first division of AU^R₈ and AU^R₁₀ protoplasts occurred after seven

days of culture in the presence or absence of AU and the frequency of division and plating efficiency were not adversely affected by 1 mM AU (Table 25). After 42 days in liquid medium, it was possible to visually identify the colonies of AU^R8 and AU^R10 and transfer them to agar medium (Fig. 66).

The first division of S. sisymbriifolium protoplasts was noted after four days in medium lacking AU (Table 25). The division efficiency after 7 days was 5% and after 30 days a plating efficiency of 0.045% was recorded. In 0.5 mM AU the division efficiency was also 5%, while the plating efficiency was 0.063%. In 1 mM AU, however, neither divisions, nor colony formation were observed (Fig. 67, Table 26).

Based on the different in vitro responses between eggplant and S. sisymbriifolium it was possible to devise a hybrid cell selection scheme. Hybridization would be between AU^R lines of eggplant and wild-type S. sisymbriifolium and following fusion treatment protoplasts would be cultured with 1 mM AU. Unfused and homofused S. sisymbriifolium protoplasts would fail to grow. Parental eggplant protoplasts could grow but, when transferred to medium supplemented with zeatin, would only produce white or green colonies. On the zeatin medium hybrid colonies would also grow but might produce purple shoot primordia as observed with S. sisymbriifolium cultures on this medium (Fig. 69). Due to the distinct green appearance of eggplant cultures on this medium (Fig. 68), it was theorized that cell colonies growing in the presence of 1 mM AU and producing anthocyanin would be somatic hybrids.

Following three fusion experiments between AU^R10 and S. sisymbriifolium protoplasts, numerous cell colonies were regenerated.

The average frequency of division among PEG-treated protoplasts was 14% after 14 days and an average of 26 colonies per dish was recovered after 42 days of continuous culture in 1 mM AU (Fig. 70). This represented a plating efficiency of 0.026%. Using unfused protoplasts, no S. sisymbriifolium cell colonies were recovered under these conditions while plating efficiency was 0.046% with AU^R10. From a mixture of both species without PEG added the plating efficiency was 0.039%. At 42 days of culture the suspensions containing colonies were layered on agar-solidified Kao medium containing 0.1 mg/L 2,4-D and 1 mg/L zeatin. Of the total estimated 740 colonies present after PEG treatment from three experiments, 250 showed rapidly-growing, organized, white callus in two weeks but no purple regions or shoot primordia. These colonies were then placed onto agar-solidified MS medium containing 1 mg/L zeatin as the sole growth regulator in an attempt to induce shoots. Within three weeks 37 colonies showed distinct localized purple areas (Fig. 72). Cell colonies which resulted from a mixture of two species without PEG fusion treatment often turned green on this medium (a trait specific for eggplant) but gave no purple primordia (Fig. 71).

Shoots were regenerated from each of the 37 purple colonies (Fig. 73) and to date 26 separate lines have been rooted and established in soil. In addition, one successful fusion experiment was conducted between protoplasts of S. sisymbriifolium and those of AU^R8 which were inactivated with iodoacetic acid. From this fusion, 14 colonies were recovered and three of those produced purple meristems. Plants were regenerated from each of these three colonies.

Morphological characteristics of the plants regenerated from purple colonies were compared with those of plants of eggplant and S. sisymbriifolium regenerated directly from leaf explant cultures (Table 26). Due to difficulties in rooting some hybrid lines and the death of others after transplantation, morphological data on most plants was gathered at various times ranging from 1-2 years after the initial fusion experiment. The somatic hybrid plants were less vigorous and more fragile than the parental species. The height of each hybrid was considerably less than either parental species (Table 26, Fig. 74). Due in part to variation among the hybrid lines in shoot growth rates, frequency of root induction and vigour after transplantation to vermiculite, there was always a range of morphological variation seen among the hybrids (Fig. 75).

Morphological characteristics intermediate in the regenerants between distinct differences in eggplant and S. sisymbriifolium (Figs. 76, 77) provided preliminary evidence supporting the theory that the selected regenerants were hybrids. The most striking difference between the parental species was in their leaves. Eggplant has oval leaves which are pubescent but not spiny. Solanum sisymbriifolium leaves, on the other hand, have pinnately-lobed leaves, and many 0.4 and 1.0 cm long spines along their adaxial and abaxial surfaces (Figs. 78, 79). A range of morphological variation was observed amongst the leaves of each regenerant as well as between the regenerated lines (Fig. 79). Although the hybrids occasionally produced spines on their leaves or petioles, in most respects, including shape, the leaf morphology more closely resembled that of eggplant (Figs. 78, 79). In one case a leaf was formed which was parted (Fig. 82), however, the pinnate separation of lobes was

never observed. Many hybrid leaves showed signs of variegation, retarded development of one lateral half of the leaf created by the mid-vein, and extreme variation in blade and petiole size (Fig. 79). The leaves of eggplant and S. sisymbriifolium plants regenerated from tissue culture were, by contrast, very uniform.

A second morphological distinction between eggplant and S. sisymbriifolium was in flower colour, shape and structure (Fig. 80). Eggplant had dark purple flowers composed of seven fused petals, with average of seven anthers per plant (variability exists even in seed plants of this cultivar), and was heterostylous (long-styled flowers set seed, short-styled flowers do not). Flowers of S. sisymbriifolium, on the other hand, had five white fused petals, five anthers and long styles. The flowers of S. sisymbriifolium plants turned light purple with increasing age but never became as dark as eggplant flowers. The somatic hybrids all flowered very prematurely and consequently many, small flowers were formed (Table 26). The majority of the hybrid flowers were light purple (Fig. 80) although some plants had predominantly white flowers. Floral abnormalities amongst the regenerated hybrids included the appearance of separate petals in every hybrid flower although neither parental species had this trait. There was extreme variation in the number of petals per flower, the observed range being four to ten and the variation amongst flowers on any one regenerant was also great (Fig. 81). Although most hybrid flowers produced a normal style and stigma, very few produced any anthers (Fig. 81). Among all of the flowers observed on hybrid regenerants, none had anthers containing pollen. In addition to exhibiting severe sterility the anthers which developed often showed other abnormalities such as

fusion to petals; in one case, the anthers all developed into new flower buds (Fig. 81).

The phenotypic variability observed in floral, leaf and root characteristics (Fig. 82, 83) was paralleled by cytogenetic variation in the regenerated hybrids. Cytogenetic analysis of the S. sisymbriifolium, AU^R10, and AU^R8 cell cultures, indicated that these cells were predominantly diploid ($2n = 24$), although many aneuploid and polyploid cells were observed. Since it has not been possible to regenerate plants from the cell lines used for fusions, cytogenetic comparisons were made between eggplant and S. sisymbriifolium plants regenerated from explant cultures (Fig. 84). In both cases, plants were normal diploids ($2n = 24$). The somatic hybrid plants, however, were often mixoploids composed of cells with different chromosome numbers (Table 26). The majority of the regenerants were predominantly hypo-aneuploids with chromosome numbers ranging from $2n = 38$ to 48 between different plants (Figs. 85-87). The greatest variation observed in one plant was line 5 which had cells ranging from $2n = 38$ to 45 in the same root tip. Similar morphology of eggplant and S. sisymbriifolium chromosomes precluded the identification of specific chromosomes among the hybrids.

Biochemical analysis of the regenerated hybrids was also used to establish hybridity and, in addition, to attempt to define the pattern of chloroplast inheritance among the hybrids. Electrophoretic separation of leucine aminopeptidase, alanyl aminopeptidase, 6-phosphogluconate dehydrogenase and aspartate aminotransferase isoenzymes confirmed the hybrid nature of 13 regenerants. In each of the hybrid plants examined the nuclear genomes of both

parental species were expressed since the isoenzyme banding patterns of the hybrids resembled a mixture of the parental enzymes (Figs. 88, 89).

In the case of leucine aminopeptidase a novel band which was not present in either parent was detected in somatic hybrid line 7 (Fig. 88). To determine if this result was an artifact this enzyme was assayed in gels containing less protein and run for a longer time. The unique band still appeared. Somatic hybrid line 7 also displayed a different 6-PDH banding pattern than the mixture of the parental extracts (Fig. 89). Eggplant and S. sisymbriifolium had predominant, electrophoretically-separate bands of 6-PGDH activity (Fig. 89). Somatic hybrid line 7 contained several bands which were specific to one or the other parent yet it was missing the predominant eggplant band for 6-PGDH (Fig. 89).

Evidence for nuclear hybridization was also provided by the results of isoelectric focussing of the small subunit of Rubisco (Fig. 90). The small subunit patterns for eggplant and S. sisymbriifolium were electrophoretically distinct and both patterns appeared in the eight somatic hybrids analyzed (Fig. 90), indicating that both nuclear genes for this protein were expressed in the somatic hybrids.

Separation of the large subunits by isoelectric focussing revealed that only the S. sisymbriifolium chloroplast genome was expressed in all eight hybrids analyzed (Fig. 90). The eggplant large subunits were electrophoretically distinct from those of S. sisymbriifolium and could be clearly seen in an artificial mixture of protein extracts of the two species.

In order to assess possible inheritance of AU-resistance

among the somatic hybrids three approaches were taken. Leaf explants from three biochemically and cytogenetically confirmed hybrids were placed on shoot induction (MS + 1 mg/L zeatin) or embryo induction medium (MS + 10 mg/L NAA) with or without 0.5 mM AU. Leaf explants of wild-type eggplant and S. sisymbriifolium did not produce shoots or embryos on media supplemented with AU (Table 27). Leaf explants cultured from somatic hybrid plants 5, 7 and 16 were also inhibited by 0.5 mM AU (Table 27). Eggplant leaf explants respond to culture medium containing 10 mg/L NAA by producing numerous somatic embryos while leaf explants of S. sisymbriifolium did not (Table 27). Leaf explants of the somatic hybrids were not embryogenic under these conditions, although shoot organogenesis, which was a trait characteristic of both parental species, was observed on all hybrid and parental leaf explants (Table 27). These results suggest that somatic embryogenesis is a complex process more sensitive to genetic alterations than organogenesis.

The leaf-explant assay was inconclusive in demonstrating whether or not these somatic hybrids had inherited resistance to 6-azauracil. It is not known if AU-resistance is expressed at the whole plant level since it has not been possible to regenerate plants from AU^R8, AU^R10 or any other azauracil-resistant cell culture (D. F. Brown, unpublished). When cell suspension cultures were initiated from the somatic hybrid lines 7 and 16, it was possible to assess their reactions to AU-supplemented medium (Figs. 91, 92). Although these cell suspensions grew at a much slower rate than the AU^R10 and S. sisymbriifolium cell cultures (Figs. 62, 63) they were not inhibited as much as the wild-type cell lines in the presence of 1 mM AU

(Figs. 91, 92) indicating that they may have some 6-azauracil resistance. Since it was not possible to unequivocally assay the inheritance of AU-resistance among the hybrids at the explant, or cell suspension levels, uracil phosphoribosyltransferase (UPRT) activity, was measured. Preliminary evidence suggested that UPRTase activity was lower among AU^R lines than wild-type eggplant and S. sisymbriifolium cell lines. The hybrid lines 5 and 7 had low levels while line 16 had a high level of UPRTase activity, however, these results were not consistently reproducible.

A population of several somatic hybrids and regenerated parental species were tested (at the U.S.D.A. Vegetable Crops Lab, Charleston, SC) for their reactions to inoculations with 500 eggs per plant of the Southern root-knot nematode (Table 28). Sixty days after inoculation, galls containing many saccate females were produced on eggplant roots while S. sisymbriifolium and the somatic hybrids did not permit sexual differentiation of larvae (Fig. 96). The preliminary results indicate that the S. sisymbriifolium nematode-resistant trait was expressed in several somatic hybrids.

Solanum sisymbriifolium displays high levels of resistance to carmine spider mites, while eggplant is extremely susceptible to this pest. Since the glandular hairs of several Solanum species are responsible for conferring insect resistance (Gibson and Pickett 1983), a comparison by light and scanning electron microscopy was made of the leaf trichomes of S. sisymbriifolium, eggplant, and their somatic hybrids. Glandular hairs similar to those of S. sisymbriifolium were definitely observed on the upper and lower leaf surfaces of hybrid lines 7, 9, 12 and 16 (Figs. 93-95).

DISCUSSION

Growth of wild-type eggplant and S. sisymbriifolium cells in medium containing 6-azauracil (AU) was severely limited or non-existent while two AU^R eggplant cell lines were capable of reduced but substantial growth with this inhibitor. Resistance to AU thus became a possible selectable marker for hybrid cells. Because it is more efficient to select hybrid colonies as soon as possible after protoplast fusion, information regarding the response of cultured protoplasts to AU was gathered (Table 25). Solanum sisymbriifolium protoplasts did not undergo division and colony formation at a level of AU (1 mM) that did not seriously inhibit the division or plating efficiency of AU^R8 or AU^R10 protoplasts. The addition of 1 mM AU to the culture medium could therefore be an effective method of eliminating S. sisymbriifolium protoplasts during post-fusion culture.

Observations on cultured leaf and stem explants of S. sisymbriifolium on MS medium containing several cytokinins revealed that very young shoot primordia synthesized anthocyanins and were deep purple in colour (Chapter 2; Fig. 69). Previously it was shown that eggplant leaf explants responded to the same combination of cytokinins in MS medium by producing only green shoots (Gleddie et al. 1983a; Fig. 68). Furthermore, although protoplast-derived cell colonies were capable of undergoing embryogenesis on MS medium containing 1 mg/L zeatin, anthocyanins were never formed. These observations formed the second component

of the selection system: the possibility for distinguishing between shoot primordia of eggplant and eggplant + S. sisymbriifolium hybrids.

The two-step selection system depended on two important assumptions: (1) that AU resistance was expressed in the hybrids during protoplast culture and (2) that anthocyanin synthesis occurred in hybrid meristems during morphogenesis. These assumptions were proven correct and the selection system was successfully used in three fusion experiments. However, in these experiments large numbers of protoplast-derived cell colonies developed from the unfused or homofused AU^R 8 or AU^R 10 eggplant cells. This meant that numerous colonies (750) had to then be screened on the zeatin-supplemented medium in order to recover a few (37) hybrid colonies with purple shoot primordia.

In order to create a more efficient selection system, AU^R 8 protoplasts were pretreated with the metabolic inactivator, iodoacetic acid. In one experiment this pretreatment was successful and of only 14 colonies transferred to zeatin medium after AU selection, three turned purple. Iodoacetic acid has also been used to eliminate one parental line in other somatic hybridization studies (Sidorov et al. 1981). The use of metabolic inactivating agents to select hybrid cells was first reported in mammalian cell fusion experiments (Wright 1978). However, the difficulty with this technique in the present study was the great variation in concentration of iodoacetic acid required to inactivate different populations of AU^R protoplasts, and this method of enriching the selection system could not be repeated. Such problems may in fact be more common in studies

involving plant cells which are possibly more sensitive to these agents. There would be even more difficulty expected if two inactivating agents were employed to eliminate both parental species. This is perhaps why hybrid plants have thus far not been obtained by employing two metabolic inactivators to inactivate both parents. In one case heterokaryons were selected but cell colonies not obtained (Nelms 1978b). In another study there was a great variation between experiments in the concentration of iodoacetate needed to inactivate Nicotiana protoplasts (personal communication).

Although the hybrid purple colonies displayed 100% shoot induction, all attempts to regenerate plants from protoplasts isolated from the parental cell suspensions (AU^R 8, AU^R 10, and S. sisymbriifolium) have failed (D. F. Brown, unpublished; Chapter 2). In a sense then, organogenesis itself became a selection system for hybrid colonies. Restoration of morphogenic competence has been observed in N. rustica + N. tabacum (Douglas et al. 1981b) and N. rustica + N. sylvestris (Gleddie et al. 1983b) somatic hybrid colonies after fusion of protoplasts from cell suspensions which had greatly reduced or no morphogenic competence. Somatic hybrids of Brassica campestris and Arabidopsis thaliana have also been successfully regenerated whereas neither parent was totipotent (Gleba and Hoffmann 1979).

The hybrid purple colonies which were isolated and regenerated were not more vigorous than the green or white colonies on zeatin-supplemented medium. In other studies heterotic effects were expressed during the in vitro growth of hybrid cells and colonies between N. glauca + N. langsdorffii (Smith et al. 1976). Similar observations were made for Datura + Atropa hybrid cells (Krumbiegel and Schieder 1979) and for interspecific Nicotiana

hybrid colonies (Douglas et al. 1981b; Gleddie et al. 1983b).

Extreme difficulty encountered in rooting hybrid shoots of N. rustica + N. tabacum (Douglas et al. 1981b) and S. tuberosum + L. esculentum (Melchers et al. 1978) was not encountered in the present case. It was possible to root several shoots of each of the 26 out of 37 hybrid lines, although there were distinct differences in the ease of root induction between lines. In several cases, excessive rooting was a problem as shoots formed roots all along their stems and even above flowering branches (Fig. 83). These rooting abnormalities were also observed on plants derived from these shoots growing in the greenhouse.

Morphological criteria which were indicative of hybridization included intermediate floral and leaf characters (Figs. 79, 80). It is common for somatic hybrids of Solanaceous species to show traits intermediate between the parental species. This was the case for hybrids in Datura (Schieder 1978), Nicotiana (Evans et al. 1980; Douglas et al. 1981c), Petunia (Power et al. 1980), and Solanum (Binding et al. 1982).

Several distinct morphological traits were intermediate among the S. melongena + S. sisymbriifolium hybrids: flower colour; sepal, petal and anther numbers; leaf shape and size; number of spines on leaves and stems; frequency and type of trichomes. Intermediate trichome morphology was used as the basis for selection of putative somatic hybrids between S. tuberosum and S. nigrum (Binding et al. 1982). The S. tuberosum + S. chacoense somatic hybrid was intermediate in leaf and tuber morphology to the parental species (Butenko and Kuchko 1980), while the

S. tuberosum + L. esculentum intergenic somatic hybrids created by Shepard et al. (1983) were more similar to potato than tomato in leaf shape and growth habit. The S. tuberosum + L. esculentum somatic hybrids described by Melchers et al. (1978) were intermediate to the parental species in leaf shape and chilling responses, yet they were morphologically unique in many ways including branching habit, loss of apical dominance, and degree of flowering.

Several novel morphological traits were observed in the hybrids produced in this study. Most notable was the occurrence of separate petals on all hybrid flowers (Fig. 79) while both parents had only fused petals. Eggplant has 7 fused petals and S. sisymbriifolium has 5; although the average petal number per hybrid plant fell between these two values, there were some hybrid flowers with as many as 10 and as few as 4 petals. The flowers of eggplant form a pedicel directly on the stem while those of S. sisymbriifolium form on branches. All of the somatic hybrids formed multiple flowers on branches with none directly on the stem. The number of flowers per branch and per plant was much greater in all hybrids than in either parent. The hybrids all flowered prematurely, often while rooting in vitro which suggests alterations in endogenous growth regulator metabolism.

Despite the number of flowers produced per plant, the general growth habit of the hybrids was slow and even retarded. It was often difficult to establish young hybrids in the soil because they were susceptible to fungal infections which was also the case with the S. tuberosum + L. esculentum hybrids (Shepard et al. 1983). Many morphological characteristics including

plant height, leaf area, flower diameter and overall plant vigour were much greater among the parental species than all of the hybrid lines regenerated in this study (Table 26). The opposite situation was observed by Butenko *et al.* (1982) with the S. tuberosum + S. chacoense somatic hybrid. This plant had a more vigorous growth habit, greater leaf area, larger guard cells and pollen grains, and was more resistant to potato virus Y than either parental species. Hybrid vigour has also been reported for plant height and leaf areas among hybrids of Nicotiana (Douglas *et al.* 1981c) and Datura (Schieder 1978).

The cytogenetic analysis of the regenerated hybrid plants also provided evidence regarding their hybridity. All of the hybrids showed chromosome numbers close to the sum of the parental chromosome numbers, however it was not possible to identify the individual chromosomes of each parent. Since plants could not be regenerated from either parental cell line used for protoplasts the chromosomal stability of regenerants from them could not be assessed. A cytogenetic analysis of the parental cell suspensions revealed that they were predominantly diploid, although considerable variation in chromosome number was observed. The variation in chromosome numbers observed in the regenerated hybrids (Table 26) undoubtedly accounts for the tremendous morphological variation among the hybrid plants. Since most of the regenerated hybrids were hypoaneuploid, there may be a preferential loss of chromosomes of one parent in this hybrid combination. Preferential loss of chromosomes from one partner of a fusion combination has been documented for soybean + N. glauca (Wetter and Kao 1980), Daucus carota + Petroselinum hortense

(Dudits et al. 1980), and Datura innoxia + Atropa belladonna (Schieder 1982). A more probable cause for cytogenetic variation among the regenerants is that the sources of protoplasts were cell suspensions, each maintained in vitro for over six months prior to the fusion. In addition, the AU^R lines were treated with EMS prior to selecting for the ability to grow on AU. Cytological instability is very common among regenerated somatic hybrids, and aneuploids have even been reported for intraspecific tobacco hybrids (Glimelius 1978).

The cytogenetic abnormalities can undoubtedly be implicated in the sterility observed among the regenerants. Each hybrid line flowered, but few if any anthers were produced, and pollen was always absent. Because floral development is a complex developmental process, it is a prime target for altered gene regulation caused by cytoplasmic and nuclear fusion. Alterations to either or both parental genomes, such as cryptic chromosomal rearrangements, and mitotic recombination or cytoplasmic segregations and recombinations may be causes for phenotypic and floral irregularities. Since mitochondrial recombination has been reported to occur following protoplast fusion (Belliard et al. 1979), and cytoplasmic male sterility is a mitochondrial encoded trait (Levings and Pring 1976) it may be that the male sterility in these hybrids is due to altered mitochondrial genomes.

The lack of successful sexual hybridizations with S. melongena (Table 2) indicates that natural barriers isolate this species from others of the genus Solanum. If nuclear-cytoplasmic incompatibility is involved, then somatic hybridization may not be a means to circumvent the barrier.

Biochemical studies of 13 randomly selected hybrid lines confirmed each to be a somatic hybrid. The isozyme analysis revealed that 12 hybrids expressed the summation of the parental bands of LAP and 6-PGDH while one plant (line 7) was unique (Figs. 88, 89). This plant was missing a predominant eggplant band of 6-PGDH and expressed a novel, hybrid LAP band. This plant (line 7) was aneuploid ($2n = 45$) and it may be that loss of an eggplant chromosome accounted for the loss of the 6-PGDH band, while a subunit association may cause the novel LAP band. An alternative explanation for the novel band is that it may represent a new molecule resulting from altered post-transcriptional modifications. Among 10 *N. rustica* + *N. tabacum* somatic hybrids none exhibited a banding pattern which was simply the summation of the parental species (Douglas *et al.* 1981c). However, in the present study most isozymes assayed confirmed the hybrid nature of the regenerants, which showed enzyme patterns which were simply the summation of parental patterns. Almost all enzymes assayed were electrophoretically distinct in the parents unlike the situation with *Nicotiana* species (Douglas *et al.* 1981c; Evans *et al.* 1980). This may be due to the distant taxonomical separation between *S. melongena* and *S. sisymbriifolium*, which belong to different sub-groups of the genus *Solanum*.

Since the large and small subunits of Rubisco are plastid and nuclear-encoded, respectively, the isoelectric-focussing analysis of this enzyme provides a means to identify the expression of chloroplast and nuclear genomes in the somatic hybrids. All eight lines evaluated expressed the small subunits of both parents which provided confirmation of the nuclear

hybridization (Fig. 90). Similar results were used to confirm hybridization between several Nicotiana species (Douglas et al. 1981c; Gleddie et al. 1983b).

The large Rubisco subunit of S. sisymbriifolium was expressed in each of the eight hybrids analyzed (Fig. 90). This uniparental expression of the chloroplast genome has been previously reported for Nicotiana somatic hybrids (Evans et al. 1980; 1982; Gleddie et al. 1983b). In many cases the random segregation of parental chloroplasts has been reported among the somatic hybrids (Iwai et al. 1980; Melchers et al. 1978; Scowcroft and Larkin 1981). One possible reason for the non-random segregation of chloroplasts in this study is that somehow plastid type was linked to the selection system. Following the 2-step selection system, only purple colonies were selected and all hybrid plants were derived from these. It is conceivable that many non-purple colonies were also fusion products, but because they had S. melongena chloroplasts they failed to turn purple. The percentage of the population of hybrids which was assayed was small (8/26 plants) and this may account for the apparent uniparental inheritance of chloroplasts among these hybrids.

Isoelectric-focussing of the large subunit of Rubisco only provides information concerning gene expression. It does not definitively rule out the possibility that both chloroplast types are present in a hybrid, with only one type expressed. Restriction endonuclease digestion of chloroplast DNA however can provide conclusive evidence for the presence of two different chloroplast types, or for chloroplast recombination. It has

been used to verify the presence of only one chloroplast type among N. tabacum + N. knightiana (Menczel et al. 1981), N. debneyi + N. debneyi (Scowcroft and Larkin 1981) and S. tuberosum + L. esculentum (Schiller et al. 1982). hybrids. Chloroplast segregation and lack of recombination among somatic hybrids appears to be universal and therefore neither recombinant plastid genomes nor S. melongena chloroplasts would be expected to appear in any of these lines.

Azauracil-resistance among the somatic hybrid lines was not conclusively demonstrated even though AU was used in the selection of hybrid cells. AU-resistance is due to lowered UPRTase activity which prevents the incorporation of AU into 6-azauridine-monophosphate (Jones 1984). Azauracil-resistant plants have not been regenerated and therefore the inheritance of this trait is unknown. Possibly the novel cytoplasm created by fusion has altered or modified the expression of this trait in the hybrids.

This study has shown that large numbers of protoplasts with high plating efficiencies are not prerequisites to successful somatic hybridization. Protoplast fusion can be successful if there exists efficient means to select and regenerate hybrid cells. In this study both starting populations of protoplasts were non-morphogenic yet the hybrid colonies were capable of regeneration. Future research will continue to attempt sexual backcrosses of eggplant pollen onto these hybrids in order to transfer the apparent nematode resistant trait into eggplant. Alternatively, this might be achieved by "back-fusion" of eggplant and the somatic hybrids. All nematode-resistant

tomato varieties are the progeny of one hybrid plant between L. esculentum and L. peruvianum (Fassuliotis, per. comm.).

The single, dominant gene for nematode-resistance in tomatoes was therefore introduced by a wild relative and hopefully a similar case will occur with eggplants.

TABLE 25. Effect of 6-azauracil-supplemented medium on protoplast cultures of AU^R 8, AU^R 10 andS. sisymbriifolium

Observations	AU concentration					
	0	0.5 mM		1.0 mM		
	AU ^R 8 AU ^R 10 <u>Solanum sisymbriifolium</u>	AU ^R 8 AU ^R 10 <u>Solanum sisymbriifolium</u>	AU ^R 8 AU ^R 10 <u>Solanum sisymbriifolium</u>	AU ^R 8 AU ^R 10 <u>Solanum sisymbriifolium</u>	AU ^R 8 AU ^R 10 <u>Solanum sisymbriifolium</u>	AU ^R 8 AU ^R 10 <u>Solanum sisymbriifolium</u>
1st division (days)	7	7	4	n.t. ^a n.t.	6	7 9
Division Efficiency (%)	18	21	5	n.t. n.t.	5	15 20 0
Plating Efficiency (%)	0.35	0.51	0.045	n.t. n.t.	0.063	0.23 0.47 0

^a n.t. = not tested

TABLE 26. Morphological and cytogenetic analysis of S. melongena, S. sisymbriifolium, and their somatic hybrids

Plant	Eggplant cv.	Mature Height (cm)	Leaf* spines	Flower colour	Mean Petal no./flower	Mean No. of flowers per cyme	Mean No. of flowers per plant	Mean Corolla width (cm)	Chromosome number
Imperial Black Beauty		35	-	purple	7 (fused)	7	6	4.5	24
<u>S. sisymbriifolium</u>		60	++++	white	5	6	16	4.5	24
Somatic hybrid									
line 5		18	-	light blue	5.6	9	37	2.3	38-43
line 6		13	+	blue	5.5	6	48	2.6	43-45
line 7		17	+	light blue	6.5 (separate)	8	112	2.4	45
line 9		15	-	blue	5.5	8	64	2.1	44 (45)
line 14		20	-	white	5.4	4	22	1.2	43-45
line 15		14	-	light blue	5.8	6	42	3.2	45
line 16		16	+	white	7.0	7	88	3.4	44 (45)

Note: Plants were analyzed after being established in soil in the greenhouse for 2-6 months.

* Leaves were scored on a scale from - = no spines to ++++ = extremely spiny.

TABLE 27. The effects of AU on morphogenesis in leaf explants of eggplant, S. sisymbriifolium and their somatic hybrids

Plant	Frequency of embryogenesis		Frequency of shoot organogenesis	
	-AU	+AU	-AU	+AU
Eggplant cv.				
Imperial Black Beauty	20.0	0	3	0
<u>S. sisymbriifolium</u>	0*	0	8	0
Somatic hybrid				
line 7	0	0	5	0
16	0	0	2	0
5	0	0	2.4	0

Note: Frequency of embryogenesis and organogenesis is the mean number of embryo or shoots per 200 mm² explant AU was supplied at 1.0 mM in MS + 10 mg/L NAA (embryogenesis) or MS + 1 mg/L zeatin (organogenesis).

* Root organogenesis was observed (30% explants).

TABLE 28. Response of S. melongena, S. sisymbriifolium, and their somatic hybrids to inoculation with Meloidogyne incognita

Plant	Mean no. of galls	Mean no. of egg masses	Gall index	Reproduction index
Eggplant cv. Imperial Black Beauty	52.5	40.5	3.75	3.25
<u>S. sisymbriifolium</u>	4.0	0	1.1	0
Somatic hybrid				
line 7	2.0	1	1	0
line 12	0	0	0	0
line 14	0	0	0	0
line 15	0	0	0	0
line 16	3	0	2	0

Note: Gall and reproduction indices are 0-5 scales: 0 = no infection, 1 = 1-2 galls or egg masses, 2 = 3-10, 3 = 11-30, 4 = 30-100, and 5 = over 100.

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FIGURE 60. The relationship between cell colony formation (fresh weight) of AU^R8 cells and the duration of incubation (days) on agar-solidified MS medium + 2 mg/L 2,4-D containing various concentrations of 6-azauracil.

FIGURE 61. The relationship between cell colony formation (fresh weight) of AU^R10 cells and the duration of incubation (days) on agar-solidified MS medium + 2 mg/L 2,4-D containing various concentrations of 6-azauracil.

FIGURE 62. The relationship between cell colony formation (fresh weight) of wild-type eggplant cells and the duration of incubation (days) on agar-solidified MS medium + 2 mg/L 2,4-D containing various concentrations of 6-azauracil.

FIGURE 63. The relationship between cell colony formation (fresh weight) of S. sisymbriifolium cells and the duration of incubation (days) on agar-solidified MS medium + 2 mg/L 2,4-D containing various concentrations of 6-azauracil.

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FIGURE 64. The growth of AU^R10 cell colonies after 21 days on MS medium + 2 mg/L 2,4-D (top dish), and after 21 days on the same medium containing 1 mM 6-azauracil (bottom dish).

FIGURE 65. The growth of S. sisymbriifolium cell colonies after 21 days on MS medium + 2 mg/L 2,4-D (top dish), and after 21 days on the same medium containing 1 mM 6-azauracil (bottom dish).

FIGURE 66. Protoplast-derived cell colonies of AU^R10 cells after 28 days of culture in Kao medium + 1 mg/L 2,4-D, 3 mg/L NAA, and 1 mg/L BAP supplemented with 1 mM 6-azauracil. Note the large cell colony and smaller aggregates of living cells.

FIGURE 67. Protoplasts and cells of S. sisymbriifolium after 28 days of culture in Kao medium + 1 mg/L 2,4-D, 3 mg/L NAA, and 1 mg/L BAP supplemented with 1 mM 6-azauracil. Note the dead cells which had formed walls and the dead protoplasts without signs of wall reformation.

FIGURE 68. A leaf explant of eggplant cultured on MS medium + 3 mg/L zeatin for 20 days. Note the large shoot forming and the smaller, dark green primordia and the lack of purple primordia and shoots.

FIGURE 69. Three leaf explants of S. sisymbriifolium cultured on MS medium + 1 mg/L zeatin for 20 days. Note the formation of multiple shoot primordia which are purple.

FIGURE 70. A 42-day-old protoplast-derived cell colony following fusion of AU^R10 and S. sisymbriifolium protoplasts. The colony was in liquid Kao medium + 1 mg/L 2,4-D, 3 mg/L NAA and 1 mg/L BAP supplemented with 1 mM 6-azauracil.

FIGURE 71. Two green cell colonies resulting from a mixture (no PEG added) of AU^R10 and S. sisymbriifolium protoplasts. The colony was an agar-solidified MS-medium + 1.0 mg/L zeatin. Note the dark green buds but lack of purple buds.

FIGURE 72. Purple cell colonies resulting from fusion of AB_{10}^R and *S. sisymbriifolium* protoplasts. The colony was on agar-solidified MS medium + 1 mg/L zeatin. Note the dark purple shoot primordia on the lower callus surface and the faint purple in the cells on the upper surface.

FIGURE 73. Regenerating shoots from purple colonies on MS medium + 1 mg/L BAP. Note the shoot formation from purple areas of the callus but the elongating shoots were green.

FIGURE 74. Regenerated plants after transfer to soil. Left: eggplant cv. Imperial Black Beauty regenerated from a leaf explant culture. Right: S. sisymbriifolium regenerated from a leaf explant. Center: somatic hybrid lines 3b, 7, and 16 from left to right.

FIGURE 75. Twenty-eight different plants resulting from protoplast fusion of eggplant and S. sisymbriifolium. Note the variations in size and morphology.

FIGURE 76. A mature plant of eggplant (S. melongena) regenerated from a leaf explant callus culture.

FIGURE 77. A flowering plant of S. sisymbriifolium regenerated from a leaf explant culture.

FIGURE 78. Side view of the underside of leaves of eggplant left, somatic hybrid line 7 center, and S. sisymbriifolium right. Note the absence of spines on eggplant, the small spine on the somatic hybrid petiole (arrow) and the large and small spines on the leaf of S. sisymbriifolium.

FIGURE 79. The leaves of S. sisymbriifolium (upper left), five somatic hybrid lines, and eggplant (lower right). Note the variation in shape, pigmentation and the failure of one half of the lamina to develop (upper right). All of the hybrid petioles were larger than those of both parents.

FIGURE 80. Flowers of eggplant (left), three somatic hybrid plants (center), and S. sisymbriifolium (right). Note the parental petals are fused, while the hybrids are not.

FIGURE 81. Six flowers from the same plant of somatic hybrid line 16. Notice the variation between the petal numbers, and presence or absence of stigmas and anthers. Note the development of new floral buds from the anthers in the center flower upper row.

FIGURE 82. A leaf of somatic hybrid line 16 showing a distinct difference in the blade shape on the two sides of the lamina.

FIGURE 83. A cutting of somatic hybrid line 9 growing in vitro on hormone-free medium. Note the root proliferation along the stem.

FIGURE 84. A root tip cell of a S. sisymbriifolium plant regenerated from a tissue explant possessing the diploid chromosome number ($2n = 24$).

FIGURE 85. A root tip cell of somatic hybrid line 16 possessing 45 chromosomes.

FIGURE 86. A root tip cell of somatic hybrid line 7 possessing 43 chromosomes.

FIGURE 87. A root tip cell of somatic hybrid line 5 possessing 38 chromosomes.

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FIGURE 88. Electrophoretic banding patterns of leucine aminopeptidase isozymes of eggplant lane 1, S. sisymbriifolium lane 2, a mixture of these species lane 3, and somatic hybrid lines 3b, 5, 7, 8, 9, 10, 11, and 16. Note the hybrid lines all appear to resemble a mixture of the parental extracts with the exception of line 7 which has a novel band.

FIGURE 89. Electrophoretic banding patterns of 6-phosphogluconate dehydrogenase isozymes of: eggplant lane 1, S. sisymbriifolium lane 2, a mixture of these species lane 3, and somatic hybrid lines 3b, 5, 7, 8, 9, 10, 11, and 16. Note the hybrid lines all resemble a mixture of the parental extracts with the exception of line 7 which is missing a strong eggplant band.

FIGURE 90. Isoelectric focussing patterns of Rubisco purified from the leaves of: eggplant lane 1, S. sisymbriifolium lane 2, hybrid lines 3b, 7, 12, 13, S. sisymbriifolium lane 7, eggplant lane 8, mixture of the parental extracts lane 9. Note the hybrid lines all resemble the mixture in the area of the small subunit (SS) indicating distinct bands from each parent. The large subunit (LS) pattern of all hybrid lines is identical to that of S. sisymbriifolium.

FIGURE 91. The relationship between cell colony formation (fresh weight) of somatic hybrid line 7 cells and the duration of incubation on agar-solidified MS + 2 mg/L 2,4-D containing various concentrations of AU.

FIGURE 92. The relationship between cell colony formation (fresh weight) of somatic hybrid line 16 cells and the duration of incubation on agar-solidified MS + 2 mg/L 2,4-D containing various concentrations of AU.

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FIGURE 93. Leaf trichome shape and structure characteristic of the lower surface of an eggplant leaf.
Note the absence of glandular hairs, but high density of stellate hairs.

FIGURE 94. Leaf trichome structure including glandular hairs on the lower leaf surface of S. sisymbriifolium leaves.

FIGURE 95. Section through a leaf of somatic hybrid line 9 showing the eggplant stellate hairs (arrowhead), and S. sisymbriifolium glandular hairs (arrows).

FIGURE 96. A highly-vacuolated, sexually-differentiated, second-stage larvae of Meloidogyne incognita isolated from the root swellings on hybrid plant 16. Larvae this immature after a 60-day incubation in the vascular system are indicative of resistance to the root-knot nematode.

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SUMMARY AND CONCLUSIONS

The ability of Solanum melongena L. (eggplant) to undergo somatic embryogenesis was previously demonstrated from excised tissues and cells grown in liquid medium (Gleddie et al. 1983a). It was established that NAA at 10 mg/L promoted high frequencies of somatic embryos, which could be excised, cultured and regenerated into fertile plants. The work of others on protoplast isolation, culture and plant regeneration with this species (Jia and Potrykus 1981) demonstrated that it was a good model for in vitro manipulations.

Because eggplant is susceptible to such an array of pests and pathogens (see Table 1) and breeding for resistance is such an important means of control, it was determined that an important economic and scientific application of "tissue culture technology" would be to attempt to create somatic hybrids between eggplant and a highly disease-resistant wild species, S. sisymbriifolium Lam. The background empirical work necessary to successfully achieve such a goal was conducted with eggplant and several wild species.

The embryogenic cell suspension cultures of eggplant were explored as sources of protoplasts. It was found, however, that superior protoplasts, with higher division and plating efficiencies could be isolated from non-embryogenic 2,4-D-grown cell suspensions. Plating efficiency of these protoplasts was optimized at 1% and

although this is less than other model systems, i.e. Nicotiana species, it was suitable to warrant continued experiments designed to create novel somatic hybrids.

A novel sequence of development was observed when 2,4-D was added to the protoplast culture medium. The direct regeneration of protoplast-derived cells into somatic embryos was observed after 20-40 days of culture. This was drastically different from the case with unprotoplasted cells. Unprotoplasted cells responded to NAA at 10 mg/L but not 2,4-D (or any other auxin) by undergoing somatic embryogenesis. In addition, it was shown that the cytokinin BAP played a critical role in protoplast-derived embryogenesis whereas it was inhibitory of non-protoplast-derived embryogenesis. Another critical difference between cells prior to and after protoplasting is their optimal NH_4^+ requirement for somatic embryogenesis. It was suggested that NH_4^+ is toxic to eggplant protoplasts and therefore protoplasts require lowered NH_4^+ content.

In order to successfully employ the wild species in a somatic hybridization program, it was necessary to study regeneration from its cells, tissues, and protoplasts. These experiments were carried out with seven wild Solanum species, four of which had not been previously used for in vitro culture.

Plant regeneration from tissue cultures of six species was achieved by using leaf or stem explants and testing a variety of growth regulators at various concentrations. MS medium was demonstrated to support callus production proliferation, and shoot organogenesis when supplemented with the appropriate growth

regulators. These findings were to be expected since MS was devised for another Solanaceous species (Nicotiana tabacum).

By testing a variety of explant sources and cytokinin types it was observed that S. sisymbriifolium leaf explants regenerated purple shoots. This observation became paramount to the selection scheme later devised for hybrid cell isolation, and underscores the importance of empirically testing various growth conditions in vitro. In effect, the time spent surveying the various parameters which influenced morphogenesis in these wild Solanum species saved some considerable effort later by helping to select hybrid colonies soon after fusion had occurred.

The lack of morphogenesis from cultured protoplasts of five of the species tested was not unexpected since, prior to this work, only eight of the 2,000 Solanum species have been regenerated from protoplasts. Also, major modifications to the culture medium (which was optimized for eggplant) were not attempted, and therefore it may be possible to regenerate plants from protoplasts of these species under other conditions.

Plant regeneration was successful in Solanum aviculare, which is a native Australian species that may have considerable medicinal use as a source of important steroids. With the techniques developed in this study, it may be possible to regenerate higher alkaloid producing variants by mutagenesis or "somaclonal variation".

The 6-azauracil (AU)-resistant cell lines isolated by D. F. Brown proved to be essential for the success of this study. By using these protoplasts which are capable of growth in AU it

was possible to eliminate S. sisymbriifolium protoplasts in post-fusion cultures. The purple meristem trait of the wild species was an equally efficient selectable marker, allowing the selection of 37 hybrid colonies.

Somatic hybrid plants were regenerated from 26 different lines. Each hybrid plant displayed morphological traits intermediate between the parents. The agronomically-important traits of S. sisymbriifolium which were transferred into the hybrids were nematode-resistance and glandular hairs (which are implicated in spider-mite-resistance). The agronomically undesirable trait, spiney leaves, was not transferred. These results are very encouraging and suggest that if fertility can be restored, or if backcrossing of the somatic hybrids is successful, that nematode resistance (which is probably a single dominant gene in this species as it is in tomato) may be transferred into eggplants.

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APPENDIX I

TABLE 1. Nutrient composition of MS medium

Component		Conc. (mg/L)
Macronutrients	NH_4NO_3	1650
	KNO_3	1900
	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440
	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370
	KH_2PO_4	170
	Iron (Sequestrene Fe-330, Geigy)	40
Micronutrients	$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	22.3
	H_3BO_3	6.2
	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.6
	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25
	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025
	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025
	KI	0.83
Vitamins	Nicotinic acid	1
	Thiamine.HCl	10
	Pyridoxine.HCl	1
	Myo-inositol	100
Sucrose		2,000

TABLE 2. Nutrient composition of SH medium

Component		Conc. (mg/L)
Macronutrients	KNO_3	2500
	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	400
	$\text{NH}_4\text{H}_2\text{PO}_4$	300
	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	200
	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	15
	Na_2EDTA	20
Micronutrients	$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	10
	H_3BO_3	5
	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	1
	$\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$	0.2
	$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$	0.1
	KI	1
Vitamins	Nicotinic acid	5
	Thiamine.HCl	5
	Pyridoxine.HCl	0.5
	Myo-inositol	100
Sucrose		2000

TABLE 3. Nutrient composition of NT medium

Component		Conc. (mg/L)
Macronutrients	NH_4NO_3	825
	KNO_3	950
	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	220
	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1233
	KH_2PO_4	680
	Iron (Sequestrene Fe-330, Geigy)	40
Micronutrients	$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	22.3
	H_3BO_3	6.2
	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.6
	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25
	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025
	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025
	KI	0.83
Vitamins	Myo-inositol	100
	Thiamine.HCl	1
Sucrose		2000

TABLE 4. Nutrient composition of Kao's medium

Component		Concentration
Vitamins (mg/L)	Myo-inositol	100.0
	Nicotinamide	1.0
	Pyridoxine . HCl	1.0
	Thiamine . HCl	10.0
	D-Calcium pantothenate	0.5
	Folic acid	0.2
	p-aminobenzoic acid	0.01
	Biotin	0.005
	Choline chloride	0.5
	Riboflavin	0.1
	Ascorbic acid	1.0
	Vitamin A	0.005
	Vitamin D ₃	0.005
	Vitamin B ₁₂	0.01
Micronutrients		As in Table 1
Macronutrients (mg/L)	NH ₄ NO ₃	600.0
	KNO ₃	0
	CaCl ₂ . 2H ₂ O	600.0
	MgSO ₄ . 7H ₂ O	300.0
	KH ₂ PO ₄	170.0
	KCl	300.0
	Iron (Sequestrene-Fe 330 -Geigy)	28.0

continued...

TABLE 4 continued

Component		Concentration
Sugars (mg/L)	Sucrose	125.0
	Fructose	125.0
	Ribose	125.0
	Xylose	125.0
	Mannose	125.0
	Rhamnose	125.0
	Cellulobiose	125.0
	Sorbitol	125.0
	Mannitol	125.0
Organic acids (mg/L) (Adjusted to pH 5.5 with NH_4OH)		
	Sodium pyruvate	5.0
	Citric acid	10.0
	Malic acid	10.0
	Fumaric acid	10.0
Other supplements:		
	Vitamin-free casamino acid	125.0 mg/L
	Coconut water (from mature fruits: heated to 60°C for 30 min and filtered)	10.0 mL

END

3	1	H	1	0	H	8	5
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FIN