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IN VITRO MORPHOGENESIS FROM
TISSUES, CELLS AND PROTOPLASTS
OF
SOLANUM MELONGENA

by

© STEPHEN CLAIR GLEDDIE, B.Sc.

A thesis submitted to the Faculty
of Graduate Studies and Research
in partial fulfillment of the
requirements for the degree of
Master of Science

Department of Biology
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September, 1981

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ABSTRACT

The hormonal, physiological and nutritional parameters influencing morphogenesis, in cultured tissues, cells, and protoplasts of Solanum melongena were examined. Leaf explants cultured with 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) or 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) showed considerable callus growth while those cultured with indole-3-acetic acid (IAA) or phenylacetic acid (PAA) gave no proliferative response. Somatic embryogenesis in leaf explants and cultured cells required exogenous NAA. The optimal embryogenic response was obtained at 10 mg/L and contrary to findings with other species, this concentration of auxin did not inhibit embryo maturation to the cotyledonous stage. The other auxins tested failed to induce somatic embryogenesis. All of the cytokinins tested inhibited NAA-induced somatic embryogenesis.

Embryogenesis was under control of the nitrogen composition of the basal medium. NH_4^+ was essential and organic nitrogen could not be substituted. An optimal $\text{NO}_3^-/\text{NH}_4^+$ ratio of 2/1 was established with the total nitrogen content at 60 mM. Although each of seven cultivars used was embryogenic, there were significant quantitative differences in their capacities to form embryos. Somatic embryos excised from tissue cultures and cultured on auxin-free medium gave rise to fertile plants when planted in

soil and transferred to a greenhouse.

Protoplasts isolated from embryogenic cell suspensions were cultured and shown to undergo reformation of cell walls, cell division and formation of cell colonies. Protoplast-derived cells lacked embryogenic potential but plant regeneration was achieved through cytokinin-specific shoot organogenesis.

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with very few reports appearing in the literature prior to 1960.

A variety of plant tissues or organs have been shown to undergo somatic embryogenesis when cultured in vitro. Raghavan (1976) reported examples of embryogenic callus derived from explants of stems, hypocotyls, mesocotyls, petioles, shoot apices, floral buds, and root tissues. In addition, somatic embryogenesis has been observed in cultured callus cells derived from leaf lamina (Rao et al. 1973), root phloem (Halperin and Wetherell 1964), and cotyledons (Ratnamba and Chopra 1974). Tisserat et al. (1979) have reviewed the literature to January 1978 and recorded 32 Angiosperm families (81 genera and 132 species) which have been reported to produce somatic embryos from tissues cultured in vitro. Reports since that date have added several species to this list and reconfirmed preliminary observations (Table 1).

Embryogenesis in vitro has been most thoroughly studied with tissues and cells derived from carrot (Daucus carota). Among the first observations of plant regeneration from cultured carrot cells were those by Levine (1947, 1950). When auxin-treated, root-derived callus was transferred to a medium lacking auxin, differentiation occurred and plantlets with normal leaves and independent root systems could be isolated from the tissue mass. Steward et al. (1958) and Reinert (1958) provided evidence that this process resembled zygotic embryogenesis. Steward et al. (1958) described the pattern of development from spherical masses of cultured cells called "proembryos" through the stages of development into whole plants. Halperin and Wetherell (1964) were able to trace the sequence of events back to proembryos of 10 cells or fewer and their cytological examinations revealed that exposure of callus to 2,4-D in the absence of other growth regulators

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7

LIST OF ABBREVIATIONS

ABA	-	abscisic acid
ALAR	-	succinic acid 2,2-methylhydrazide
BAP	-	6-benzylamino purine
CCC	-	2-chloroethyl trimethylammonium chloride
2,4-D	-	2,4-dichlorophenoxyacetic acid
GA ₃	-	gibberellic acid
IAA	-	indole-3-acetic acid
IBA	-	indolebutyric acid
2iP	-	2-isopentyladenine
MCPA	-	2-methyl-4 chlorophenoxyacetic acid
NAA	-	1-naphthaleneacetic acid
NOA	-	2-naphthoxyacetic acid
OCPA	-	o-chlorophenoxyacetic acid
PAA	-	phenylacetic acid
PCIB	-	p-chlorophenoxyisobutyric acid
PCPA	-	p-chlorophenoxyacetic acid
POA	-	phenoxyacetic acid
2,4,5-T	-	2,4,5-trichlorophenoxyacetic acid
2,4,6-T	-	2,4,6-trichlorophenoxyacetic acid
TIBA	-	2,3,5-triiodobenzoic acid

INTRODUCTION

Plant tissue culture techniques have great potential to serve as a useful adjunct to conventional plant breeding and for the genetic modification of crop plants. These techniques allow for in vitro induction of mutations, selection of mutant cell lines and the characterization of regenerated mutant plants. In addition, with the ability to regenerate plants from protoplasts the potential for wide parasexual or somatic hybridization arises. Regeneration of hybrid plants following protoplast fusion has been demonstrated in several genera and the technique has demonstrated potential benefits for crop improvement (see Keller et al. 1981).

In order to fully realize the genetic potential of tissue culture techniques it is essential that intact plants can be recovered from cultured tissues and cells. Morphogenesis in cell cultures may follow either one of two development processes, known as embryogenesis and organogenesis. Embryogenesis is a bipolar morphogenic pattern of development with the simultaneous differentiation of a root and shoot axis as opposed to organogenesis which is the unipolar differentiation of either a shoot, root or other organ. Although these developmental processes have led to plant regeneration in a wide array of species (Street 1979; Evans et al. 1981), reliable methods of plant regeneration from cultured cells of many economically important crops are not available.

In Solanum melongena L. (eggplant) previous studies have

demonstrated that intact plants may be recovered from tissue cultures via embryogenesis (Yamada et al. 1967; Matsuoka and Hinata 1979) or organogenesis (Kamata and Rao 1978). These workers did not clearly define the parameters which influenced the morphogenic responses nor did they report successful cell or protoplast isolation and subsequent culture.

The objective of the present study was to develop reliable procedures for plant regeneration in tissue cultures of eggplant. Emphasis has been placed on the induction of embryogenesis in leaf explants, cell suspensions and protoplast-derived cultures. An efficient bioassay has been developed to evaluate the parameters which influence this process in callus and cell suspension cultures. The investigation included identification of the conditions required for protoplast isolation, culture and subsequent plant regeneration.

LITERATURE REVIEW

A. Somatic Embryogenesis — Historical Perspective

Embryogenesis generally refers to the process of embryo development subsequent to fertilization. Asexual embryogenesis as opposed to zygotic embryogenesis is the process of initiation of complete plant embryos from cells that are not immediate products of gametic fusion. The process has been termed somatic embryogenesis, polyembryony, adventive embryogenesis, and sporophytic embryony. More specific terms referring to the origin of embryos such as microspore embryogenesis (androgenesis) and nucellar embryogenesis have also been employed. Throughout this review the term "somatic embryogenesis" will be used and applied to non-gametophytic, non-zygotic plant cells which undergo embryo development. The process of embryogenesis in gametophytic tissues (e.g. microspores) will not be given detailed consideration.

Webber (1940) credits Leeuwenhoek as being the first to report naturally occurring polyembryony in orange seeds in 1719. Since then multiple embryos have been shown to originate from nucellar, antipodal and synergid cells as well as from the endosperm and the integuments of the seeds of 239 Angiosperm species (Tisserat et al. 1979). Although naturally occurring asexual embryogenesis has been confined to intra-ovular structures it has served to increase awareness of the potential totipotency of somatic cells. Asexual embryogenesis in vitro, on the other hand, is a rather recently observed phenomenon

with very few reports appearing in the literature prior to 1960.

A variety of plant tissues or organs have been shown to undergo somatic embryogenesis when cultured in vitro. Raghavan (1976) reported examples of embryogenic callus derived from explants of stems, hypocotyls, mesocotyls, petioles, shoot apices, floral buds, and root tissues. In addition, somatic embryogenesis has been observed in cultured callus cells derived from leaf lamina (Rao et al. 1973), root phloem (Halperin and Wetherell 1964), and cotyledons (Ratnamba and Chopra 1974). Tisserat et al. (1979) have reviewed the literature to January 1978 and recorded 32 Angiosperm families (81 genera and 132 species) which have been reported to produce somatic embryos from tissues cultured in vitro. Reports since that date have added several species to this list and reconfirmed preliminary observations (Table 1).

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TABLE 1. Recent reports of in vitro somatic embryogenesis in
Angiosperm species

<u>Apium graveolens</u>	Al-Abta and Collin 1979
" "	Zee and Wu 1980
" "	Al-Abta and Collin 1978
<u>Brassica campestris</u>	Bhattacharya and Sen 1980
<u>Brassica napus</u>	Thomas et al. 1976
" "	Keller and Armstrong 1977
<u>Brassica oleracea</u>	Pareek and Chandra 1978
<u>Carica stipulata</u>	Litz and Conover 1980
<u>Citrus sinensis</u>	Kochba and Spiegel-Roy 1977
" "	Kochba et al. 1978 a,b
<u>Coffea arabica</u>	Söndahl and Sharp 1977
<u>Coffea canephora</u>	Nassuth et al. 1980
<u>Daucus carota</u>	Sung et al. 1979
" "	Fridborg et al. 1978
" "	Fujimura and Komamine 1980 a,b
" "	Kamada and Harada 1979
<u>Gossypium klotzschianum</u>	Price and Smith 1979
<u>Ipoemoca batatas</u>	Hwang et al. 1980
<u>Lycopersicon peruvianum</u>	Zapata and Sink 1981
<u>Medicago sativa</u>	Johnson et al. 1981

continued...

TABLE 1, continued

<u>Medicago sativa</u>	Kao and Michayluk 1980
" "	Dos Santos <u>et al.</u> 1980
<u>Nicotiana sylvestris</u>	Facciotti and Pilet 1979
<u>Nicotiana tabacum</u>	Lörz <u>et al.</u> 1977
<u>Panax ginseng</u>	Chang and Hsing 1980
<u>Paulownia tomentosa</u>	Radojevic 1979
<u>Pennisetum americanum</u>	Vasil and Vasil 1980
	Vasil and Vasil 1981
<u>Petroselinum hortense</u>	Masuda <u>et al.</u> 1977
<u>Perilla frutescens</u>	Tanimoto and Harada 1980
<u>Phoenix dactylifera</u>	Reynolds and Murashige 1979
<u>Pimpinella anisum</u>	Huber <u>et al.</u> 1978
<u>Santalum album</u>	Bapat and Rao 1979
<u>Solanum melongena</u>	Matsuoka and Hinata 1979
<u>Sorghum bicolor</u>	Brettell <u>et al.</u> 1980
<u>Thalictrum urbaini</u>	Yang and Chang 1980
<u>Theobroma cacao</u>	Pence <u>et al.</u> 1980
<u>Trifolium pratense</u>	Phillips and Collins 1980
<u>Vitis vinifera</u>	Mullins and Srinivasan 1976

promoted cellular division characteristic of incipient embryogenesis. Somatic embryogenesis has been observed in carrot callus (Halperin and Wetherell 1964), cell suspensions (Steward et al. 1958), and protoplast-derived cells (Grambow et al. 1972).

B. Origin and Early Development of Somatic Embryos

An early suggestion of Steward et al. (1958) was that physical isolation of cells is a requirement for expression of in vitro somatic embryogenesis in carrot cell suspensions. This view does not, however, appear to be valid. Observations from many species have shown that embryogenesis can proceed directly from plant tissues without the necessity for cellular isolation or extensive cell divisions, e.g. nucellar polyembryony (Haccius 1978), androgenesis from microspores (Nitsch and Nitsch 1969) and direct embryogenesis from epidermal cells (Konar et al. 1972). By comparison isolated single cells (Button and Botha 1975) or cultured protoplasts (Kameya and Uchimiya 1972; Dorion et al. 1975; Poirier-Hamon et al. 1975) generally undergo division leading to the development of large, unorganized multicellular aggregates from which embryos subsequently differentiate.

The morphological development of somatic carrot embryos has been reported in several studies. McWilliam et al. (1974) have revealed that in carrot cell suspensions embryos are initiated at or near the surface of cellular aggregates and then released from these embryogenic clumps into the medium as free-floating structures. These workers traced the sequence of segmentations in single, superficial cells of embryogenic clumps. They observed that in the early stages of embryogeny, the cleavage pattern of somatic Daucus embryos differs from that of zygotic Daucus embryos described by Borthwick (1931). Suspensors,

when present in somatic embryos, were composed of a single or double file of two or three cells (McWilliam et al. 1974). The cleavage pattern of zygotic embryos appears to be much more uniform with suspensors consistently composed of linear single files of seven cells (Borthwick 1931). Somatic embryogenesis closely resembled zygotic embryogenesis during the later stages of development (heart-shaped, cotyledonous and mature embryos). Fujimura and Komamine (1980a) made serial observations of embryogenesis in carrot cell suspensions using cell clusters that divided synchronously. Through filtration only cell clusters of 30-47 μ m diameter were observed. Cell division occurred slowly and cell volumes remained constant throughout the cell clusters for the first three days. After three or four days of culture, rapid cell division occurred at a particular locus on the cell cluster. The volume of the cells became smaller at this locus and the shape of this locus became spherical as the entire cluster developed into a globular embryo. Later stages of culture revealed heart-shaped, torpedo-shaped and mature embryos.

Embryos from epidermal cells of Ranunculus sceleratus showed typical embryo development of the Crucifer type with the exception that the suspensor was either reduced or absent (Konar et al. 1972). A single epidermal cell was followed through a transverse division forming basal and terminal cells. The basal cell sometimes gave rise to a suspensor by one or more transverse divisions. The terminal cell divided longitudinally and subsequent transverse divisions gave rise to a globular embryo. At the 16 cell stage a dermatogen was formed by the epidermal cell layer. The walls of the embryogenic epidermal cells were rich in plasmodesmata but as

the globular embryos matured the cytoplasmic continuity of the basal cell with the other epidermal cells was broken.

The development and organization of somatic embryos in Brassica oleracea leaf callus has been described by Pareek and Chandra (1978). The embryos developed from superficial cells on the callus mass which initially divided several times without appreciable cell enlargement. Young globular embryos varied widely in shape from spherical to ellipsoidal and in many cases a 3- or 4-celled suspensor was seen. All stages of embryogeny were observed including mature cotyledonous embryos of the Crucifer type.

C. Factors Influencing Somatic Embryogenesis

1. Auxins

Excised plant tissues and organs are usually cultured on a medium supplemented with auxins and cytokinins to promote cell division and growth. This medium is termed the primary culture medium and with a ratio of auxin to cytokinin near unity it usually promotes the growth of callus consisting of undifferentiated parenchyma-like cells. When excised plant tissues are cultured on a medium supplemented with a high cytokinin to auxin ratio shoot organogenesis often results while a high auxin to cytokinin ratio usually promotes root organogenesis (Skoog and Miller 1957). Somatic embryogenesis does not normally occur in the presence of these hormones.

There have been a few reports of embryogenesis in tissues cultured entirely in the absence of growth regulators, e.g. parsley endosperm (Masuda et al. 1977), zygotic embryo callus of Theobroma cacao (Pense et al. 1980), cotyledons of Ilex aquifolium (Hu and Sussex 1971) and anthers of certain Solanaceous species (Tomes and

Collins 1976). In most cases, however, induction of embryogenesis requires prior cultures on auxin-containing medium (with or without cytokinins) followed by transfer to auxin-free medium. It appears that auxin is necessary to establish embryogenic capacity but at the same time exogenous auxin inhibits embryo development (Kohlenbach 1978). This scheme of in vitro culture on auxin-containing primary medium has been successfully applied to a variety of species: D. carota (Halperin and Wetherell 1964), Carum carvi (Ammirato 1974), Santalum album (Rao 1965), Petroselinum hortense (Vasil and Hildebrandt 1966), Phoenix dactylifera (Reynolds and Murashige 1979), Pennisetum americanum (Vasil and Vasil 1981), Perilla frutescens (Tanimato and Harada 1980).

Evans et al. (1981) have reviewed the literature and conclude that auxins are most often added to the primary culture medium as the sole growth regulator, with 2,4-D being most frequently used followed by NAA, IAA and NOA. The secondary medium is usually free of growth regulators or in some cases contains reduced levels of auxin and/or other growth regulators. Since systematic evaluations of these requirements are seldom performed, it is difficult to draw conclusions about the efficacy of specific growth regulators.

D. carota is the species most often studied with regard to somatic embryogenesis. Carrot requires 2,4-D in the primary culture medium to stimulate cell division and the initiation of "embryogenic centers"; removal of 2,4-D from the secondary culture medium permits embryo development (Halperin and Wetherell 1964, 1965). Somatic embryogenesis in carrot cell suspensions has been synchronized to a degree by sequential filtration of cells through nylon screens

(Fujimura and Komamine 1975). Cell clusters of uniform size were rinsed free of residual 2,4-D and cultured in various secondary media. Somatic embryogenesis was observed in 50% of the inoculated cell clusters placed in hormone-free secondary medium. The number of embryos formed was not significantly affected by 2,4-D from 10^{-12} to 10^{-10} M in the secondary medium but above 10^{-10} M it inhibited somatic embryogenesis. Sung *et al.* (1979) cultured carrot tissues in a liquid cell suspension medium supplemented with 0.1 mg/L 2,4-D. At this concentration 2,4-D inhibited embryo differentiation at the earliest stages and promoted callus-like growth of parenchymal cells. At a 2,4-D concentration of 0.04 mg/L in the liquid medium some globular embryos were produced but their subsequent development was arrested. Continued culture in this medium promoted a loss of differentiation and these globular embryoids proliferated as callus masses. Only when 2,4-D was removed from the culture medium entirely did embryo development continue.

In some species the embryogenic development of cultured cells is auxin-specific. Sangwan and Harada (1975) tested the ability of growth regulators to promote callus growth, organogenesis and somatic embryogenesis in Antirrhinum majus stem explants. The auxins IAA and NAA induced limited callus development and abundant root organogenesis, while 2,4-D induced soft, friable callus and occasional embryogenesis. The combination of coconut milk and NOA induced friable green callus and numerous embryos which developed into plantlets upon transfer to hormone-free medium. Tanimoto and Harada (1980), however, have shown that leaf discs of P. frutescens cultured on NOA-supplemented medium produced very few somatic embryos while those cultured on 2,4-D

produced numerous embryos.

Citrus sinensis has provided a model system for the study of somatic embryogenesis in vitro. Ovular ~~callus~~ from the 'Shamouti' orange became habituated, i.e. lost the growth requirement for exogenous auxin and cytokinin, yet it maintained a steady level of embryogenesis (Kochba and Button 1974). Embryogenesis was stimulated by ageing of the callus or sucrose starvation prior to subculture. The auxins IAA and NAA inhibited embryogenesis at concentrations of 0.001 to 10 mg/L although callus growth was stimulated by low concentrations of either auxin. The inhibitors of auxin synthesis, 5-hydroxy-nitrobenzyl-bromide and 7-aza-indole both greatly stimulated embryogenesis as did the anti-cytokinin 8-aza-guanine. Embryogenesis was markedly stimulated by ABA, the ethylene analogue 2-chlorethyl-phosphonic acid (ethephon), and the inhibitors of GA₃ synthesis CCC, and succinic acid 2,2-methylhydrazide (Kochba et al. 1978a). Presumably the endogenous growth substances in this habituated culture continuously induce embryogenic cells but at the same time prevent further embryonic development (as they do when supplied to non-habituated cultures). The inhibitors lower the effective endogenous growth regulator concentration allowing expression of the embryogenic capacity. In non-habituated carrot cultures, however, the antiauxins 2,4,6-T, and PCIB inhibited embryogenesis when included in the secondary culture medium (Fujimura and Komamine 1979b). These observations would indicate that unlike Citrus callus, which has sufficient endogenous auxin levels and can initiate embryos in the presence of antiauxins, carrot callus requires auxin at very low concentrations to support somatic embryogenesis.

The metabolism of ^{14}C -labelled 2,4-D in embryogenic carrot and non-embryogenic soybean cell cultures differs dramatically (Montague *et al.* 1981). Soybean cells converted most of the exogenously supplied 2,4-D to amino acid conjugates while carrot cells contained primarily free 2,4-D. After a long term exposure to 2,4-D and subsequent transfer to hormone-free medium, carrot cells released much more 2,4-D than did soybean cells. The retention of 2,4-D by soybean cells was shown to be a time-dependent metabolic conversion to 2,4-D aspartic and 2,4-D-glutamic acids. This suggests that the retention of a stable 2,4-D metabolite in soybean cells is responsible for their failure to form embryos in hormone-free medium. Epstein *et al.* (1977) also found that ^{14}C -labelled IAA was conjugated to aspartate by habituated Citrus callus. This stable auxin metabolite may be responsible for the inhibition of embryogenesis by exogenously supplied auxins in this species.

2. Cytokinins

The effect of cytokinins in the induction of somatic embryogenesis is less clear than that of auxins. Rao *et al.* (1973) found that the inclusion of cytokinins with 2,4-D in the primary medium inhibited the induction of embryos on Petunia. Fujimura and Komamine (1980b), however, were able to raise the frequency of somatic embryogenesis in carrot with the addition of zeatin to the secondary medium. The cytokinins BAP and kinetin were inhibitory and yet 2iP was shown to promote embryogenesis in the secondary medium (Sung *et al.* 1979).

Excised, immature barley zygotic embryos cultured on a medium containing kinetin as the sole growth regulator frequently developed embryo-like outgrowths (Norstog 1970). These embryos appeared on

the side of the zygotic embryo opposite the scutellum and consisted of a definite root-shoot axis and a coleoptile-like first leaf. Bapat and Rao (1979) were able to induce somatic embryogenesis on hypocotyl segments of Sandalwood which were supplied with BAP as the sole growth regulator. Brettell et al. (1980) observed somatic embryogenesis and plant regeneration from cultured immature inflorescences of Sorghum which were cultured on medium supplemented with kinetin and BAP at high concentrations and 2,4-D at low concentration. Mullins and Srinivasan (1976) have reported that grapevine ovules require high levels of both NOA and BAP in the primary medium for the induction of embryogenic callus.

To induce embryogenic callus, proliferation cytokinins are often supplied in the primary culture medium at low concentrations with auxins at higher concentration (Litz and Conover 1980; Phillips and Collins 1980; Sita et al. 1980; Pareek and Chandra 1978; Reynolds and Murashige 1979; Price and Smith 1979; Yang and Chang 1980). However, a definite requirement for high cytokinin to auxin ratios in the primary and secondary media has been observed for embryo induction on leaf explants of Coffea arabica (Söndahl and Sharp 1977). The presence of both a cytokinin and an auxin were essential for callus proliferation with kinetin and 2,4-D being the best growth regulators. High frequencies of somatic embryos were observed on media which contained high kinetin concentrations in conjunction with either NAA, IBA or 2,4-D at low concentrations. The specific auxin supplied was not critical, however these workers did not determine if other cytokinins were also able to promote high frequency embryogenesis.

3. Other growth regulators

Banerjee and Gupta (1976) devised a bioassay to test the influence of growth regulators on embryogenesis in leaf explant calli of Nigella sativa. Growth and embryogenesis of the callus cultures was dependent upon coconut milk in the medium, however, proembryonic masses failed to mature unless coconut milk was replaced by casein hydrolysate. Embryo maturation in Nigella explants was also inhibited by auxins and cytokinins which suggests that the naturally-occurring growth regulators in coconut milk are responsible for the suppression of the embryogenic development. Steward et al. (1958) and Sussex and Frei (1968) also stimulated embryogenesis in carrot cells by the addition of coconut milk in primary medium followed by its removal in the secondary medium of carrot cultures. Kameya and Uchimiya (1972), however, promoted embryogenesis in carrot protoplast-derived cells by increasing the coconut milk concentration from 1% in primary medium to 5% in secondary medium.

GA₃ has been shown to inhibit embryogenesis in habituated Citrus cultures (Kochba et al. 1978a) while it has been successfully employed in the secondary medium for somatic embryogenesis of grapevine (Mullins and Srinivasan 1976).

Tisserat et al. (1979) have concluded that "no addendum of growth regulating substances has been shown to promote asexual embryogenesis directly. Any positive influence by auxin or cytokinin.... can be attributable to the beneficial action on callus development..." This statement is perhaps an oversimplification of the role played by growth regulators due to the lack of detailed, systematic studies demonstrating the growth regulator specificity of somatic embryogenesis.

4. Nitrogen

Halperin and Wetherell (1965) studied the relative effectiveness of various nitrogen sources in stimulating embryogenesis in callus cultures of carrot. In the absence of ammonium ions, calli derived from root phloem would not form embryos when the 2,4-D content of the medium was reduced. Phloem-derived callus would grow indefinitely with nitrate as the sole nitrogen source, the optimal nitrate concentration was 60 mM. Addition of as little as 5 mM ammonium to this medium stimulated embryogenesis. Microscopic examination of callus grown exclusively on nitrate revealed only single cells and a virtual absence of multicellular filaments, aggregates and proembryos which were observed in ammonium supplemented callus. These authors concluded that the requirement for ammonium must be met at an early stage of embryonic development and that reduced nitrogen in the form of glutamine, asparagine or coconut milk could not be substituted for ammonium.

In contrast to the findings of Halperin and Wetherell (1965), Reinert et al. (1967) found no qualitative difference between the capacity of oxidized and reduced nitrogen to induce embryogenesis in carrot cultures but reported that the total amount of nitrogen in the medium was critical for the induction of embryogenic callus. Medium supplemented with 3 mM nitrate nitrogen was not sufficient to induce somatic embryos. When the nitrate content was raised to 15, 24 or 44 mM somatic embryogenesis was stimulated even though this medium did not contain reduced nitrogen. Embryogenesis also occurred if 40 mM glutamine or 60 mM total nitrogen was supplied as a mixture of amino acids (Edamin). Wetherell and Dougall (1976) also observed

somatic embryogenesis in a carrot culture with several organic nitrogenous compounds as the sole nitrogen source. Casein hydrolysate, glutamine and alanine at either 15 or 30 mM total nitrogen concentration all supported similar frequencies of somatic embryogenesis.

The metabolism of nitrogenous compounds significantly affects the pH of the culture medium and influences embryogenesis (Wetherell and Dougall 1976). Carrot cell suspension cultures grown in a medium supplied with 5-95 mM KNO_3 would undergo embryogenesis when as little as 0.1 mM NH_4Cl was added. By monitoring at various intervals it was observed that high NH_4/NO_3 ratios present unfavorable pH conditions for growth and embryogenesis. With glutamine or alanine as the sole nitrogen source the medium pH rose above 6.0, and embryogenesis was not totally suppressed. Further evidence that the pH of the culture medium is a critical factor supporting somatic embryogenesis has been presented by Dougall and Verma (1978). If carrot cell suspensions were grown in unbuffered medium and NH_4^+ was supplied as the sole nitrogen source, then the pH of the medium dropped as the cells metabolized NH_4^+ suppressing both growth and embryogenesis. When a continuous titration was used to maintain the pH near 5.8 embryogenesis and growth occurred in cell suspensions with NH_4^+ as the sole nitrogen source.

5. Monovalent cations

A potassium requirement for growth and embryogenesis in carrot cell suspension cultures was established by Brown et al. (1976). Maximum growth was obtained with very low K^+ concentrations (1 mM), while optimal embryogenesis required higher concentrations (20 mM). The specific role for K^+ remains unclear since at 20 mM it inhibited

growth of the cell suspensions. With sulfate as the counterion the optimal concentration of K^+ for embryogenesis was narrow (0.3 mM to 1.0 mM) and the results were variable, with poor embryo morphology. Growth and embryogenesis were severely depressed when phosphate was used as the counterion at levels greater than 1 mM. Chloride, however, was not an inhibitory counterion. A correlation between tissue K^+ and embryo number could be shown with cultures grown on glutamine as the sole nitrogen source but not when other nitrogen sources were supplied. Neither Na^+ nor NH_4^+ could replace the requirement for K^+ but Na^+ actually suppressed growth and embryogenesis in the range from 1 to 30 mM (Brown et al. 1976). The origin of the plant tissues which are cultured in vitro may affect the inhibition of embryogenesis by Na^+ . NaCl when added to the callus induction and culture medium suppressed embryogenesis in cultured carrot phloem and xylem tissues but did not affect cambial tissue explants (Komizerko and Khretonova 1973).

6. Basal medium

The basal media formulations used in plant tissue culture differ most in their nitrogen and potassium contents (Appendix, Tables 1-6). Most workers choose a medium for its ability to support undifferentiated callus growth on tissue explants and then may or may not perform systematic evaluations of the inorganic salt concentrations required for somatic embryogenesis. Somatic embryogenesis has been observed in Daucus (Reinert et al. 1967) and in Brassica (Bhattacharya and Sen 1980) on SH medium (Appendix, Table 5) and in Pimpinella (Huber et al. 1978) on B_5 medium (Appendix, Table 2), however, Evans et al. (1981) conclude that the majority of workers to date

have used MS medium to achieve somatic embryogenesis. MS medium (Appendix, Table 1) has substantial concentrations of reduced nitrogen and potassium and no sodium. The inorganic salt requirements are near optimal for somatic embryogenesis as work with embryogenic carrot cultures has shown, yet this medium was originally designed for tobacco pith callus growth (Murashige and Skoog 1962).

7. Sugars

Sucrose and glucose are commonly added to tissue culture media as carbon sources, however, their effects on morphogenesis have only been measured in a few cases (Wright and Northcote 1972; Minocha and Halperin 1974). Verma and Dougall (1977) studied the effect of sugars on growth and somatic embryogenesis in vitro. Carrot cell suspensions were transferred from medium with 2,4-D to hormone-free medium supplemented with test carbohydrates at 2% (w/v). Arabinose, xylose, ribose, rhamnose, sorbose, sorbitol, mannitol, myoinositol, tagatose, phytic acid and glucuronic acid did not support measurable growth or embryogenesis. The tissues grew satisfactorily on fructose, glucose, mannose, maltose, or sucrose but grew only after a marked delay on galactose, raffinose or stachyose. It was not possible to demonstrate a separation of growth and embryogenesis since embryogenesis was observed on every medium containing a sugar that supported growth. Although galactose is considered to be toxic to some plant species (de Kock et al. 1979) it supported growth and embryogenesis of carrot cells and was found by Kochba et al. (1978b) to stimulate somatic embryogenesis in Citrus cultures. Experiments with precursors of IAA indicated that galactose possibly inhibited the conversion of indoleacetaldehyde to IAA (Kochba et al. (1978b) An alternative

explanation for the unusual action of galactose on Citrus cultures is that sugar starvation promotes somatic embryogenesis since Kochba and Button (1974) reported that both ageing and preculture on sugar-free medium stimulated somatic embryogenesis.

The majority of tissue culture media employ sucrose concentrations from 2 to 3% (w/v). Modifications have been made for the culture of and induction of embryogenesis in specialized tissues and cells. Microspore embryogenesis in cultured anthers has been stimulated by high (10% w/v) sucrose concentrations (Keller et al. 1975; Sharp et al. 1971). On the other hand, carrot root protoplasts have been cultured in a medium lacking sugar (Kameya and Uchimiya 1972) and the resultant cells were subsequently shown to be embryogenic.

8. Culture environment

The physical culture conditions required for somatic embryogenesis from cell and explant cultures have usually not been systematically studied. Some workers culture explants in complete darkness (Chang and Hsing 1980), or in continuous light to achieve somatic embryogenesis (Rao et al. 1973). Culture temperatures usually range from 25°C (Thomas et al. 1972) to 27°C (Thomas et al. 1977). Somatic embryogenesis is possible in some species from both callus and liquid cultures although Pence et al. (1980) observed that liquid medium promoted a higher frequency of embryogenesis in T. cacao cultures.

Kessell and Carr (1972) were able to stimulate somatic embryogenesis in suspension cultures of carrot by lowering the level of dissolved oxygen (DO_2). Kessell et al. (1977) demonstrated that lowering the DO_2 below a critical level led to an increase in the level of cyanide sensitive respiration and the cellular ATP content.

Increased ATP content of the cells could also be achieved by adding adenosine to the medium. Irrespective of the manner in which the cellular level of ATP was raised, more embryos were produced than in control cultures. Other workers have reported a stimulation of microspore embryogenesis when the anthers were subjected to reduced atmospheric pressure for 10 to 90 minutes (Imamura and Harada 1980).

9. Sources of explants

In many cases explant source does not affect the frequency of somatic embryogenesis. Both storage root and hypocotyl explants of carrot produce embryos when transferred from a medium containing 2,4-D to an auxin-free medium (Steward et al. 1958; Kamada and Harada 1979). Rao et al. (1973) found that both leaf discs and internodal stem segments of Petunia responded to the same medium by producing embryos. Hypocotyl or cotyledon segments of Anethum graveolens (Ratnamba and Chopra 1974) and leaf or petiole explants of Thalictrum urbani produced embryos in vitro (Yang and Chang 1980).

Explants from different sources may, however, have different hormonal requirements for the induction of embryogenesis. Stem explants of Apium graveolens required a high auxin to cytokinin ratio for embryogenesis (Al-Abta and Collin 1979) while leaf and petiole explants gave an optimal embryogenic response with an even ratio (Williams and Collin 1976; Zee and Wu 1980). The influence that the explant source exerts on the embryogenic response has also been demonstrated by Radojevic (1979) in Paulownia tomentosa. Ovular callus required 2,4-D and kinetin-supplemented medium while zygotic embryo callus required IAA and kinetin for embryogenesis.

D. Cytology, Histology and Biochemistry of Somatic Embryogenesis

Sussex (1972) examined the cytological responses in a carrot cell suspension which was synchronously triggered to initiate somatic embryos by the removal of coconut milk. Initial changes included increased staining of the cytoplasm (ribosomes), a decrease in vacuolation, and increases in nuclear size and the number of nucleoli. Cells grown in coconut milk rarely deviated from the uninucleolate condition whereas those in medium lacking coconut milk showed a tendency to become multinucleolate with up to 19 nucleoli observed in one nucleus. The increase in nuclear area by lobing suggested increased nuclear activity.

Histological analysis of somatic embryogeny in R. sceleratus hypocotyl explants has shown that epidermal cells give rise directly to somatic embryos (Konar et al. 1972). These highly cytoplasmic epidermal cells underwent several cell divisions leading to the development of small cellular aggregates and eventually globular embryos. Up to the globular stage the cells remained densely cytoplasmic, full of ribosomes and contained a centrally located nucleus. As development proceeded to the torpedo stage the more superficial cells of the embryo expanded and became vacuolated, while the central core of cells remained small, cytoplasmic and meristematic. Lipid-rich spherosomes containing a protein stroma were particularly prominent at this time.

Studies on somatic embryogenesis in Coffea species revealed that C. canephora stem explants cultured on a medium containing auxin formed callus initials in all parenchymatic tissues between the epidermis and the vascular cambium. The epidermis, pericyclic fibres,

vascular cambium, xylem or pith parenchyma did not contain any callus initials. Subsequently proembryo-like structures arose from the parenchymal callus, which appeared to contain localized pockets of meristematic activity (Nassuth et al. 1980). Söndahl et al. (1979) reported that leaf explants of C. arabica cultured on a medium supplemented with 2,4-D gave rise to two distinct populations of callus cells; one composed of greatly elongated cells and the other consisting of small spherical cells. The elongated cells were predominant in primary callus in the presence of 2,4-D while the second population of embryogenic cells became prevalent after 2,4-D was withdrawn from the medium. This embryogenic callus became friable and white and was characterized by uniform, small, spherical, cytoplasm rich cells whose daughter cells remained associated as a coherent mass and eventually differentiated into globular embryos.

Zee et al. (1979) demonstrated the accumulation of starch granules in cells of petiole explants of Chinese celery as early as four days from culture on a medium supplemented with 2,4-D. Cells surrounding the vascular bundles began to divide rapidly, giving rise to a layer of meristematic cells such that by day six the vascular bundles were encircled by several layers of highly cytoplasmic, meristematic cells. From day ten, the development of proembryos from these meristematic cells was apparent, with the greatest number of proembryos always forming on the phloem side of the vascular bundles.

When stem explants of Petunia were cultured on 2,4-D medium somatic embryos arose from meristematic groups of cambial cells dense with starch granules. When explants were cultured on the same medium supplemented with BAP no embryos were observed and the cambial cells were non-meristematic and devoid of starch granules (Handro

et al. 1973).

Gregor et al. (1974) revealed limited but distinct differences in the non-histone chromosomal proteins of carrot cells grown in primary and secondary media. Cells 'induced' to differentiate embryos on 2,4-D-free medium were lacking a group of low molecular weight non-histone proteins while no changes were detectable in the histone proteins. These 'induced' cells were shown to have higher chromatin template activity than 'control' cells grown in 2,4-D (Matsumoto et al. 1975).

The synthesis of proteins and RNA in carrot cell cultures increased within 2-4 h after removal of 2,4-D from the medium (Sengupta and Raghavan 1980). Since the rates of synthesis of both macromolecules increased simultaneously relations between the two processes were not clear. However, since 2,4-D is known to non-specifically stimulate the synthesis of nucleic acids and proteins, its removal from the medium might be expected to reduce the rate of synthesis of these macromolecules. The possibility that a higher turnover rate of macromolecules is characteristic of embryogenesis has been supported by Fujimura et al. (1980). Using a synchronous culture of carrot cells they established that cells which are withdrawn from auxin-containing medium show increased ^{14}C -uridine and ^3H -leucine incorporation. However, the total RNA and protein content of cells grown either with or without auxin remained constant suggesting that qualitative changes in macromolecular synthesis occur yet quantitative changes cannot be detected. Zee et al. (1979) have attempted to detect changes in the soluble protein composition of embryogenic celery explants by SDS electrophoresis. They observed

changes in protein banding patterns with time as callus and embryos developed on 2,4-D containing medium. However, since the embryogenic cells consist of a small percentage of the total cell number and 2,4-D is known to rapidly and non-specifically stimulate protein synthesis *in vitro* (Setterfield *et al.* 1978) the accuracy of such an assay for early biochemical events involved in embryogenesis may be questioned.

E. Regeneration of Plants from Somatic Embryos

The early stages of embryogeny *in vivo* are characterized by heterotrophic growth of the embryo at the expense of the surrounding endosperm tissue. It is only at the bilaterally symmetrical stage and the beginning of cotyledonary development that the embryo begins autotrophic development. Early workers were able to culture fully mature zygotic Datura embryos on a strictly inorganic medium while immature (heart-stage) zygotic embryos required IAA, yeast extract, coconut milk and high (12-18% w/v) sucrose concentrations (Van Overbeek *et al.* 1942). Culture of immature zygotic embryos on medium with high osmotic potential imitated the ovular environment and prevented cell elongation which led to precocious germination. Raghavan and Torrey (1963) successfully cultured globular zygotic Capsella embryos at reduced osmotic potential on a semi-solid medium supplemented with vitamins, IAA, kinetin, adenine sulfate and 2% (w/v) sucrose. Recent progress in zygotic embryo culture has been reviewed (Raghavan 1980) and has led to applications such as the rescue of inviable hybrid embryos, overcoming seed dormancy, and shortening the breeding cycle of plants.

Few studies have reported the nutritional requirements of somatic embryos. Cultured Daucus embryos were shown to have specific sucrose

requirements (Neuman and De Garcia 1974). When 2% sucrose was supplied the embryos only developed roots, however, 1% sucrose supported normal germination of the shoot and root axis.

Cultured somatic embryos are prone to developmental abnormalities such as malformed cotyledons and adventive embryos forming on their hypocotyls; zeatin and GA_3 both were found to increase the frequency of aberrantly formed embryos while ABA suppressed abnormal development (Ammirato 1977). Somatic embryos of Eschscholzia californica seldom develop into normal plantlets when cultured in vitro (Kavathekar et al. 1978). By suggesting that embryos which failed to develop into normal plantlets behaved as dormant embryos, these workers studied environmental as well as chemical factors which are known to release dormancy in vivo. Chilling treatments, auxins, GA_3 and cytokinins, however, did not stimulate normal development of plantlets.

The difficulties encountered in somatic embryo culture may relate to the unusual culture conditions necessary to stimulate somatic embryogenesis in vitro. The addition of auxins to the induction medium may stimulate the production of inhibitors such as phenolic compounds (Fridborg et al. 1978). It has been shown that high levels of sucrose required for induction of embryogenesis may also have deleterious effects on normal embryo germination (Sharp et al. 1971; Keller et al. 1975; Keller and Armstrong 1977).

F. Morphogenesis in Protoplast-Derived Cultures

The methods for protoplast isolation and culture have been successfully applied to a variety of species (see reviews by Cove 1979; Butenko 1979; Vasil et al. 1979). Callus formation and unipolar root and shoot organogenesis usually occur after the transfer

of protoplast-derived cell colonies to agar-solidified medium. Induction of morphogenesis is usually achieved through manipulation of cytokinin/auxin ratios in a manner similar to that developed for tissue explants, callus and cell cultures. However, the number of plant species in which somatic embryogenesis from protoplast-derived cultures has been observed is relatively low (Table 2).

Somatic embryogenesis in protoplast cultures was first reported in carrot by Kameya and Uchimiya (1972) and Grambow *et al.* (1972). The two groups used vastly different culture conditions to achieve embryogenesis. Kameya and Uchimiya (1972) isolated protoplasts from carrot root slices and cultured the protoplasts in weak-salt medium lacking NH_4^+ and sugar and supplemented with 0.1 mg/L 2,4-D and 1% coconut milk. For embryogenesis, cell colonies were transferred to the same basal medium free of 2,4-D and supplemented with 5% coconut milk or 1 mg/L kinetin. These colonies formed large multicellular aggregates from which 5 to 30 embryos arose with eight weeks. Grambow *et al.* (1972) isolated carrot protoplasts from cell suspension cultures and cultured them in B_5 medium (Appendix, Table 2) supplemented with 0.1 mg/L 2,4-D. The protoplast-derived cell colonies were not transferred to fresh medium but were supplied with several dilutions of hormone-free B_5 medium. Within two weeks of culture the cell clusters produced embryos from which plants could be regenerated.

Studies on embryogenesis in alfalfa protoplast cultures revealed plant to plant variation in the ability of populations of protoplasts to divide and form embryos (Kao and Michayluk 1980). The protoplasts obtained from different plants may require modified culture conditions

TABLE 2. Reports of somatic embryogenesis from plant protoplast
cultures

Species	References
<u>Antirrhinum majus</u>	Poirier-Hamon and Rao 1974; Prat and Poirier-Hamon 1975
<u>Asparagus officinalis</u>	Bui Dang Ha <u>et al.</u> 1975
<u>Atropa belladonna</u>	Gosch <u>et al.</u> 1975
<u>Citrus sinensis</u>	Vardi <u>et al.</u> 1975
<u>Daucus carota</u>	Grambow <u>et al.</u> 1972; Kameya and Uchimiya 1972; Dudits <u>et al.</u> 1976
<u>Lycopersicon peruvianum</u>	Zapata and Sink 1981
<u>Medicago sativa</u>	Kao and Michayluk 1980; Dos Santos <u>et al.</u> 1980; Johnson <u>et al.</u> 1981
<u>Nicotiana glauca</u>	Facciotti and Pilet 1979
<u>Nicotiana tabacum</u>	Lörz <u>et al.</u> 1977
<u>Pennisetum americanum</u>	Vasil and Vasil 1980
<u>Ranunculus sceleratus</u>	Dorion <u>et al.</u> 1975

to regenerate cell walls, divide and form embryos in culture. Evidence from alfalfa cell suspensions supports this view. Suspension cultures from one plant only formed embryoids in the presence of 1 mg/L 2,4-D and 0.1 mg/L zeatin-riboside while suspension cultures derived from another plant only formed embryoids in the presence of 1 mg/L NAA and 0.1 mg/L BAP (Kao and Michayluk 1980). Further evidence that genotype is important in M. sativa protoplast culture has been provided by Johnson et al. (1981). These workers were unable to regenerate protoplast-derived cell colonies by embryogenesis in the liquid culture medium used by Kao and Michayluk (1980). It was possible to select culture conditions which induced somatic embryogenesis on petiole explant callus and to modify this medium for leaf mesophyll protoplast cultures. Protoplast-derived cell colonies required 21 days of culture in 2,4-D (0.2 mg/L)-supplemented medium prior to transfer to hormone-free medium for the induction of embryogenesis. A study by Dos Santos et al. (1980) revealed that yet another set of culture conditions were required to achieve division of alfalfa leaf mesophyll protoplasts. These workers were unable to obtain protoplast regeneration in liquid medium unless the medium and protoplasts were cultured over filter paper discs placed on agar-solidified medium. Embryogenesis was only possible if the protoplast-derived cells were cultured with a high cytokinin to auxin ratio (0.5 mg/L BAP and 0.05 mg/L NAA).

The highly specific growth regulator requirements for protoplast-derived cell colonies has been demonstrated by Lörz et al. (1977). Mesophyll protoplasts of Nicotiana tabacum were cultured in a basal medium supplemented with 2,000 combinations of auxins and cytokinins in a systematic study. Proembryo-like structures were observed in

liquid culture medium supplied with PCPA and kinetin or Dicamba and BAP. These proembryos only gave rise to callus if they remained in the same medium; transfer to Wood and Braun (1961) medium allowed normal embryo development.

Vardi et al. (1975) were only able to achieve somatic embryogenesis in protoplast-derived cells of Citrus if the cells were subjected to X-ray irradiation. Protoplasts formed a cell wall and divided but unless they were irradiated they did not demonstrate any morphogenic capacity.

Thomas et al. (1976) observed that haploid microspore-derived plants from B. napus gave rise in culture to large numbers of somatic embryos on their stems. Leaf mesophyll protoplasts derived from the same plants were also cultured but it was not possible to achieve somatic embryogenesis from these cells. After X-ray irradiation shoot and root organogenesis but not embryogenesis occurred in these protoplast-derived cells.

A comparative study of protoplast regeneration of 77 species of dicotyledonous plants has been performed by Binding et al. (1981). Under identical experimental conditions mitosis was observed in 54 species, and shoots or whole plants were regenerated in 21 of these species, however, somatic embryogenesis was not observed.

G. Morphogenesis in Tissue Cultures of Solanum melongena

Yamada et al. (1967) reported the initiation of callus from zygotic embryos of the eggplant cultivars Sinkro and Kantoao. In response to low NAA concentrations (0.01 to 0.001 mg/L) callus from Sinkro but not Kantoao produced somatic embryos within four weeks of culture. Callus of Kantoao did respond to medium supplemented with

IAA and kinetin by producing shoot primordia which developed into normal shoots which could be rooted.

Kamat and Rao (1978) observed the formation of shoot buds on excised hypocotyl segments of eggplant cultured on medium supplemented with IAA. Buds could be transferred to the greenhouse but the frequency of bud regeneration was low (4-5 buds per cm hypocotyl segment). The auxins NAA and NOA induced rooting and callus formation, respectively, but totally suppressed bud regeneration. The influence of growth regulators on the process of shoot bud regeneration was regarded as critical and the authors concluded that the auxins differed significantly in their influence on morphogenesis since 2,4-D was unable to induce callus growth on hypocotyl explants. Shoot bud formation was not a function of exogenous cytokinin activity since IAA alone could induce shoot organogenesis.

Matsuoka and Hinata (1979) have observed several patterns of regeneration from hypocotyl explants of eggplant depending on the NAA concentration in the medium. Medium lacking growth regulators supported shoot organogenesis on the explants and BAP enhanced this response. Low concentrations of NAA (0.016 mg/L) stimulated root organogenesis while callus was produced at 0.8 mg/L NAA. Elevated NAA levels (8 mg/L) induced embryogenesis after six weeks. These embryos developed into normal plants when transferred to hormone-free medium. Fourteen commercial cultivars were tested and all formed embryos but genotypic variation was observed in the optimal level of NAA. The importance of genotype in morphogenesis is further reflected by the unsuccessful attempts to regenerate plants from eggplant callus by Fassuliotis (1975). To date, there are no reports in the literature concerning growth or morphogenesis in eggplant cell suspension or protoplast-derived cultures.

MATERIALS AND METHODS

A. Plant Material

Seeds of Solanum melongena L (eggplant) were obtained from Stokes Seeds Ltd., St. Catharines, Ontario. The cultivar Imperial Black Beauty was used throughout, except in one experiment in which the influence of genotype on somatic embryogenesis was tested. The seeds were germinated in a soil:peat:sand mix (3:1:1) and grown in flats in a greenhouse maintained on a day/night temperature cycle of 25/15°C. A 16 h photoperiod with average illumination of 300 microeinsteins ($\mu\text{E m}^{-2}\text{sec}^{-1}$) was supplied by fluorescent and incandescent lamps. The plants were watered daily and fed a 20:20:20 (N:P:K) liquid nutrient solution twice per week.

B. Preparation of Media

The medium used in most studies was that of Murashige and Skoog (1962) as modified by Douglas et al. (1981), hereafter referred to as MS-D medium (Appendix, Table 1). Several other media were compared to MS-D. These include B₅ (Gamborg et al. 1968; Appendix, Table 2), NT (Nagata and Takebe 1971; Appendix, Table 3), M₅₁C (Furner et al. 1978; Appendix, Table 4) and SH medium (Schenk and Hildebrandt 1972; Appendix, Table 5). For solidification, 0.8% (w/v) Difco bacto-agar was added. All solid media were adjusted to pH 5.8 with 0.1N KOH prior to autoclaving.

A number of growth regulators were added to the various media tested. Prior to use, stock solutions of the auxins NAA, 2,4-D, 2,4,5-T,

PAA, IAA and the growth regulators ABA, TIBA and GA₃, 100 mg were prepared by dissolving the crystalline compound in 2-5 mL absolute alcohol and made up to one liter with distilled water. The cytokinins zeatin, kinetin, BAP and 2iP were prepared by dissolving 100 mg of crystalline compound in 2-5 mL 12N HCl at 60°C and diluting to one liter with distilled H₂O. Growth regulator stocks were maintained in a refrigerator (1°C) for a maximum of six months prior to the preparation of fresh solutions. Coconut milk was collected from ripe coconuts, heated to 80°C, filtered and frozen prior to use. All growth regulators were added to the medium prior to autoclaving except IAA and ABA which were filter-sterilized and added to autoclaved medium after cooling.

C. Culture of Explants

The first and second leaves were excised from 25 day-old plants and surface-sterilized for 15 min in 7% (w/v) calcium hypochlorite followed by five rinses in sterile demineralized water. Using a cork borer and scalpel, 200 mm² discs were cut from the leaf lamina and placed on solid medium. A total of 25 explants were cut from the leaf lamina and placed on solid medium. A total of 25 explants were cut for each treatment in each experiment and all experiments were repeated at least once. Except for experiments on effects of temperature and light intensity the explants were cultured at 25°C under continuous fluorescent light (30 $\mu\text{E m}^{-2}\text{sec}^{-1}$). The effect of a range of culture conditions and modifications of media on somatic embryogenesis and shoot organogenesis in leaf explant cultures was studied. The conditions used in these experiments are detailed in the Results section.

D. Cell Suspensions

Cell suspension cultures were initiated from primary callus growing on leaf explants cultured on MS-D + 10 mg/L NAA. Four grams of callus tissue were transferred into 125 mL Ehrlenmeyer flasks containing 33 mL of liquid MS-D supplemented with either 0.5 or 2.0 mg/L 2,4-D or 10 mg/L NAA. The pH of the liquid medium was adjusted to 5.5 with 0.1N KOH prior to autoclaving. The cultures were maintained on a gyrotory shaker at 150 rpm at 25°C in continuous light ($30 \mu\text{E m}^{-2}\text{sec}^{-1}$). Every sixth day 5–10 mL of suspension were transferred into 33 mL of fresh medium.

For studies on the induction of embryogenesis cells collected by filtration on 125 μm mesh nylon filters and rinsed with 50 mL of hormone-free MS-D medium. Cell colonies about 2 mm in diameter (117 ± 15 mg fr. wt.) were selected and plated on various solid media (see Results). The mean number of cells contained in these colonies was calculated by transferring a colony into 5 mL of enzyme solution (see section F below) and counting the resulting protoplasts on a hemocytometer. The mean number of cells per colony was $7.5 \pm 0.3 \times 10^5$.

E. Scoring and Culture of Somatic Embryos

Leaf explant cultures were evaluated for embryogenesis under a dissecting microscope after 30 days of culture. The total number of chlorophyllous heart-shaped, cotyledonous or torpedo-shaped embryos formed per explant was counted. The embryo frequency (f) was calculated as the mean number of embryos per leaf explant based on 25 explants per treatment from each of two separate experiments. Embryo frequency from plated cell colonies from suspension cultures was scored after 60 days of culture.

For continued culture, somatic embryos were excised from the callus and transferred to various embryo-culture media as described in Results. Except for an experiment on the effect of light and temperature embryos were cultured at 25°C under continuous fluorescent light ($30 \mu\text{E m}^{-2}\text{sec}^{-1}$). When these embryos developed normal leaves and roots they were transferred to peat pellets (Jiffy-7) and maintained in a mist chamber for 10-14 days prior to potting and maintenance in a greenhouse. The survival of cultured embryos was calculated as the percentage of embryos cultured initially which showed normal shoot and root development after four weeks.

F. Isolation and Culture of Protoplasts

For isolation of protoplasts, cells were filtered from suspension cultures using 125 μm mesh nylon filters and approximately 2 g (fr. wt.) cells were placed in 10 mL of enzyme solution in 100 x 15 mm plastic petri dishes. The dishes were sealed with parafilm and incubated on a reciprocating shaker (30 strokes/min) at 25°C in light ($25 \mu\text{E m}^{-2}\text{sec}^{-1}$). The enzymes used were Driselase (Kyowa Hakko Kogyo Co. Ltd., Tokyo), Rhozyme HP 150 (Rohm and Haas, Philadelphia) and 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 and centrifuged for 12 min at 1500 $\times g$ to remove insoluble material. A final solution containing 1% Driselase, 1% Rhozyme and 0.1% onozuka in 0.35 M mannitol was prepared (pH adjusted to 5.8 with 0.1N KOH) and sterilized by sequential filtration through 1.2, 0.4 and 0.2 μm filters.

Following incubation in enzymes the protoplasts were separated

from undigested tissue 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 (Appendix, Table 3), MS-D (Appendix, Table 1), or SH-2 medium (Schenk and Hoffmann 1979; Appendix, Table 6), modified by addition of 0.35 M glucose or 0.35 M mannitol, reduction of sucrose concentration to 1% (w/v) and omission of agar. Protoplasts were cultured in 50–200 μL drops or as liquid layers in 60 $\times$ 15 mm Falcon petri dishes, sealed with parafilm and incubated at 25°C at low light intensity (7 $\mu\text{E m}^{-2}\text{sec}^{-1}$). Plating efficiency was defined as the average percentage of cells undergoing cell division based on five independent drops or liquid layer cultures. After two weeks, the protoplast cultures were moved to higher light intensity (30 $\mu\text{E m}^{-2}\text{sec}^{-1}$). At three weeks, each culture drop was supplemented with 50 μL of protoplast culture medium, with or without osmotic stabilizer, while liquid layer cultures were supplemented with 2–3 mL of culture medium. After six weeks protoplast-derived colonies were transferred to various regeneration media (see Results) in attempts to stimulate embryogenesis or organogenesis. Regenerated plantlets were transferred to peat pellets (Jiffy-7) and maintained in a mist chamber for 10–14 days prior to potting and maintenance in a greenhouse.

RESULTS

A. Callus Growth on Leaf Explants

1. Effect of auxins

To test the effects of auxins on callus induction, leaf explants were cultured on MS-D supplemented with NAA, 2,4-D, 2,4,5-T, IAA or PAA (Fig. 1). When explants were cultured on MS-D free of auxin or any other growth regulator neither callus growth nor morphogenic responses were observed. Explants cultured with 0.1 to 10 mg/L NAA, 2,4-D or 2,4,5-T showed callus growth after 20 days while those cultured with IAA or PAA gave little or no proliferative response. Callus was initiated near the veins and edges of the explant but eventually spread over the entire surface (Figs. 2, 3). NAA at 0.1 mg/L stimulated rooting from the explant edges and the callus surface (Fig. 2). Callus growth was increased and rooting decreased at 1.0 mg/L NAA (Fig. 3). At 10 mg/L NAA there was abundant callus proliferation and little rooting (Fig. 4) with the greatest fresh weight gain occurring between day 15 and day 30 (Fig. 5). NAA induced callus was friable and white while that formed on 2,4-D and 2,4,5-T was hard and yellow-brown.

2. Effect of cytokinins

Leaf explants cultured on medium supplemented only with cytokinins formed very little callus. A synergistic effect on callus growth resulted when 1.0 mg/L of any cytokinin, (kinetin, zeatin, BAP or 2ip) was added to MS-D + 10 mg/L NAA. Callus which developed on these media was hard, green and proliferated rapidly.

FIGURE 1. Leaf explant of S. melongena cultured on agar-solidified medium for 1 day.

FIGURE 2. Root initials and callus growth on a leaf explant cultured on MS-D + 0.1 mg/L NAA for 30 days.

FIGURE 3. Callus, root hairs, and somatic embryo (arrow) development on a leaf explant cultured on MS + 1.0 mg/L NAA for 30 days.

FIGURE 4. Developing somatic embryos (arrows) on the callus surface of an explant cultured on MS-D + 10.0 mg/L NAA for 30 days.

FIGURE 5. The relationship between leaf explant fresh weight and ~~time~~ ⁱⁿ culture. Explants were cultured on MS-D + 10 mg/L NAA.

B. Somatic Embryogenesis on Leaf Explants

1. Effect of growth regulators

The auxins IAA, 2,4-D, 2,4,5-T, PAA and NAA were added to MS-D at 0.1-10 mg/L to test their effects on embryogenesis. With the exception of 2,4-D at 1.0 mg/L, NAA was the only auxin capable of inducing embryogenesis (Table 3). NAA, however, consistently stimulated much higher frequencies of somatic embryogenesis. The optimal NAA concentration was 10 mg/L although embryogenesis was observed across the range 0.1 to 25 mg/L (Table 4). Within 15 days of culture on medium containing 10 mg/L NAA, many small green organized centers of cells were visible on the callus surface (Fig. 6). The number of embryos formed increased dramatically between days 21 to 30 (Fig. 10) with many reaching the cotyledonous stage of development (Fig. 7). When cotyledonous embryos were excised (Fig. 8) and recultured on hormone-free medium they germinated and developed into plantlets which could be potted and transferred to the greenhouse (Fig. 9).

To test the effect of cytokinins on somatic embryogenesis, MS-D medium containing 10 mg/L NAA was supplemented with kinetin, zeatin, BAP or 2iP at a range of concentrations (0.1 to 10 mg/L). All cytokinins strongly inhibited the embryogenic response while stimulating callus growth (Table 5).

ABA (0.1 to 10 mg/L) was found to inhibit embryogenesis when added to MS-D + 10 mg/L NAA (Table 6). The embryos produced on ABA-supplemented medium were morphologically similar to those produced on ABA-free medium.

TABLE 3. Effect of auxins on somatic embryogenesis in
leaf explant cultures of Solanum melongena

Auxin	Conc. (mg/L)	f	S.E.
None	-	0	-
IAA	0.1	0	-
	1	0	-
	10	0	-
2,4-D	0.1	0	-
	1	0.1	0.03
	10	0	-
2,4,5-T	0.1	0	-
	1	0	-
	10	0	-
PAA	0.1	0	-
	1	0	-
	10	0	-
NAA	0.1	0.4	0.02
	1	2.5	0.08
	10	21.3	1.2

Note: Auxins were supplied in MS-D medium solidified with 0.8% agar.

f = embryo frequency as represented by the mean number of embryos per leaf explant (200 mm²) based on 50 explants from two independent experiments.

S.E. = standard error.

TABLE 4. Effect of NAA concentration on somatic
embryogenesis in leaf explant cultures of S. melongena

NAA conc. (mg/L)	f	S.E.
1	2.5	0.4
5	9.9	1.0
10	19.2	1.9
15	0	-
25	0.1	-

Note: NAA added to MS-D medium.

f = embryo frequency as described in Table 3.

S.E. = standard error.

FIGURE 6. A 24 day-old explant cultured on MS-D + 10 mg/L NAA. Arrows denote developing embryos on the callus, with some root initials (R) and remnants of the original leaf explant (L).

FIGURE 7. A 30 day-old callus mass on MS-D + 10 mg/L NAA with a developing somatic embryo displaying expanding cotyledons (arrow).

FIGURE 8. Excised somatic embryo prior to culture on embryo culture medium.

FIGURE 9. Mature eggplant derived from a somatic embryo after 60 days of greenhouse growth.

FIGURE 10. The relationship of embryo yield with age of explant culture. Explants were cultured on MS-D + 10 mg/L NAA.

TABLE 5. Effect of cytokinins on somatic embryogenesis in
leaf explant cultures of S. melongena

Cytokinin	Conc. (mg/L)	f	S.E.
None	-	23.1	1.7
Kinetin	0.1	5.1	0.9
	1	0	-
	10	0	-
BAP	0.1	1.0	0.2
	1	0	-
	10	0	-
Zeatin	0.1	0.1	0.1
	1	0	-
	10	0	-
2iP	0.1	0.2	0.1
	1	0	-
	10	0	-

Note: Cytokinins were added to MS-D medium + 10 mg/L NAA.
f = embryo frequency as described in Table 3.
S.E. = standard error.

TABLE 6. Effect of abscisic acid on somatic embryogenesis
in leaf explant cultures of *S. melongena*

ABA conc. (mg/L)	NAA conc. (mg/L)	f	S.E.
0.1	-	0	-
1	-	0	-
10	-	0	-
-	10	32.0	2.5
0.1	10	17.9	3.0
1	10	6.5	2.9
10	10	0.1	0.02

Note: ABA and NAA were added to MS-D medium.
f = embryo frequency as described in Table 3.
S.E. = standard error.

2. Effect of genotype

The frequency of embryo induction on the callus surface was strongly influenced by the genotype of the donor plant. Leaf explants from each of seven commercial eggplant cultivars tested underwent embryogenesis but the highest frequency occurred in Imperial Black Beauty (Table 7).

3. Effect of basal medium

Five different nutrient-salt formulations were tested for their ability to support embryogenesis in *S. melongena* leaf explants. MS-D and M₅₁C media were superior to B₅, NT and SH media (Table 8).

4. Effect of nitrogen

The various media used in the experiment described above differed significantly in nitrogen content (Table 8). B₅ contains a total NH_4^+ concentration of 2 mM while MS-D contains 20.6 mM total NH_4^+ . The critical role of NH_4^+ in somatic embryogenesis was demonstrated in a test in which the elevation of NH_4^+ concentration in B₅ to 20.6 mM greatly stimulated embryo yield (Table 9). It was not possible to obtain somatic embryogenesis with either NH_4^+ or NO_3^- as the sole source of nitrogen in the medium (Table 9, see also Table 11). M₅₁C medium also contains low concentrations of NH_4^+ , however, this is supplemented with organic nitrogen in the form of casein hydrolysate and glutamine. Organic sources of reduced nitrogen were added to nitrogen-free MS-D medium and assayed for their ability to induce somatic embryogenesis. Sixty mM nitrogen in the form of casamino acids or casein hydrolysate was ineffective in the induction of somatic embryogenesis (Table 10). However, lower concentrations of organic nitrogen

TABLE 7. Effect of genotype on somatic embryogenesis
in leaf explant cultures of S. melongena

Cultivar	f	S.E.
Imperial Black Beauty	26.2	1.3
Burpee Hybrid	14.8	1.6
Black Bell	11.8	1.3
Jersey King	9.6	1.2
French Imperial	8.6	1.0
Dusky	5.6	1.0
Blacknite	5.6	0.7

Note: MS-D + 10 mg/L NAA

f = embryo frequency as described in Table 3.

S.E. = standard error

TABLE 8. Comparison of basal media for induction of somatic embryogenesis in leaf explant cultures of S. melongena

Medium	Ion conc. (mM)			Organic nitrogen (mM)			Total nitrogen (mM)	f	S.E.
	Total NH ₄ ⁺	Total NO ₃ ⁻	Total K ⁺	Casein H	Glutamine				
MS (Murashige and Skoog 1962)	20.6	39.4	20.1	-	-	60	20.4	1.4	
M ₅₁ C (Furner <u>et al.</u> 1978)	5	24.8	21.8	2.2	4.0	36	15.2	1.2	
NT (Nagata and Takebe 1971)	10.3	19.7	14.4	-	-	30	5.9	0.5	
B ₅ (Gamborg <u>et al.</u> 1968)	2	35.8	24.8	-	-	26.8	0.9	0.1	
SH (Schenk and Hildebrandt 1972)	2.6	24.8	24.8	-	-	27.4	0	0	

Note: All media supplemented with 10 mg/L NAA.
f = embryo frequency as described in Table 3.
S.E. = standard error.

TABLE 9. Effect of NH_4^+ and NO_3^- concentration on somatic embryogenesis in leaf explant cultures of *S. melongena*

Medium	ion conc. (mM)		f	S.E.
	NO_3^-	NH_4^+		
B ₅	24.8	2	6.2	1.1
B ₅ modified	43.4	20.6	18.5	2.8
MS-D	39.4	20.6	20.8	1.6
MS-D modified	0	10.3	0	-
	0	20.6	0	-
	0	41.2	0	-
	0	82.4	0	-
	19.7	0	0	-
	39.4	0	0	-
	78.8	0	0	-
	157.6	0	0	-

Note: KNO_3 and NH_4NO_3 were utilized in the modified media containing 10 mg/L NAA.

f = embryo frequency as described in Table 3.

S.E. = Standard error.

TABLE 10. Effect of organic nitrogen sources on somatic embryogenesis in leaf explant cultures of *S. melongena*

Supplement	Total N Conc. (mM)	f
None	-	0
Casamino acids	7	3.3
" "	60	0
Casein hydrolysate	11	3.2
" "	60	0
Glutamine	13.5	0
γ -amino butyric acid	9.6	0
Casamino acids	7	
+ Casein hydrolysate	11	
+ Glutamine	13.5	
+ γ -amino butyric acid	<u>9.6</u>	
Total (N)	41.1	12.2

Note: NH_4NO_3 and KNO_3 were omitted from MS-D + 10 mg/L NAA.
 f = embryo frequency as described in Table 3.
 All supplements were added at the concentrations indicated except casamino acids and casein hydrolysate whose total nitrogen contents were based on a 10% nitrogen content of casamino acids and a 15.8% nitrogen content of casein hydrolysate.

(7 mM casamino acids and 11 mM casein hydrolysate) stimulated a low embryogenic response. Glutamine and γ -aminobutyric acid were ineffective nitrogen sources but a definite synergism was observed between all the organic nitrogen sources used at low concentrations (Table 10).

An experiment was performed to evaluate the importance of the NO_3^- : NH_4^+ ratio in the induction of embryos with the total nitrogen maintained at the MS-D level (60 mM). There was a significant effect on the frequency of embryogenesis as the ratios changed from 16:1 to 1:16 (Table 11). A NO_3^- : NH_4^+ ratio of 2:1 was optimal while low ratios were more inhibitory to callus growth and embryogenesis than were high ratios.

The total nitrogen content of the induction medium was critical for the induction of somatic embryogenesis. In an experiment with the nitrate: ammonium ratio maintained at 2:1, the optimal total nitrogen content was 60–90 mM (Table 12). However, when lower nitrate:ammonium ratios were employed (e.g. 1:1) the optimal range of total nitrogen changed [49–128 mM (Table 13)].

5. Effect of monovalent cations

In order to assess the influence of potassium-ion concentration on somatic embryogenesis, MS-D medium was prepared with 60 mM total nitrogen (20.6 mM NH_4NO_3 and 18.8 mM NaNO_3) and potassium was supplied as KCl across the concentration range from 1.25 to 70 mM. Potassium did not significantly influence somatic embryogenesis in the range from 1.25 to 50 mM while above 50 mM it slightly suppressed the embryogenic response (Table 14).

The effect of sodium ions on somatic embryogenesis was tested by supplementing MS-D with 0–100 mM NaCl. Sodium did not affect embryogenesis when present at concentrations of less than 40 mM but above this concentration it was inhibitory (Table 15).

TABLE 11. Effect of nitrate:ammonium ratio on somatic embryogenesis
in leaf explant cultures of *S. melongena*

Conc. (mM)		Ratio	f	S.E.
KNO ₃	NH ₄ citrate			
0	60		0	-
3.52	56.5	1:16	0	
6.6	53.4	1:8	0.9	0.5
12	48	1:4	5.1	2.0
20	40	1:2	16.5	2.4
30	30	1:1	16.1	2.0
40	20	2:1	26.1	2.3
48	12	4:1	16.6	2.7
53.4	6.6	8:1	8.1	3.0
56.5	3.5	16:1	7.4	2.4
60	0	-	0.5	0.3

Note: NH₄NO₃ was omitted and KNO₃ and NH₄ citrate were supplied in MS-D + 10 mg/L NAA. Total N was 60 mM.

f = embryo frequency as described in Table 3.

S.E. = standard error.

TABLE 12. Effect of the total nitrogen concentration with a constant $\text{NO}_3^-:\text{NH}_4^+$ ratio on somatic embryogenesis in leaf explant cultures of S. melongena

Total N (mM)	f	S.E.
15	2.8	1.1
30	16.8	1.7
60	19.5	2.1
90	19.9	2.0
120	2.4	0.9
150	0	-
180	0	-

Note: NH_4NO_3 and KNO_3 were omitted and NaNO_3 and NH_4 citrate were supplied in MS-D + 10 mg/L NAA at an $\text{NO}_3^-:\text{NH}_4^+$ ratio of 2:1. Potassium was supplied as KCl at 20 mM.
 f = embryo frequency as described in Table 3.
 S.E. = Standard error.

TABLE 13. Effect of the total nitrogen concentration on somatic embryogenesis in leaf explant cultures of *S. melongena*

Conc. (mM)				
NO_3^-	NH_4^+	Total (N)	f	S.E.
9	15	24	7.6	3.4
9	31	40	10.5	3.3
18	31	49	20.9	2.2
38	20	58	11.6	1.2
38	31	69	18.3	2.3
18	62	80	20.3	2.0
38	62	100	20.8	1.9
66	62	128	20.2	1.8
66	124	190	7.3	3.0

Note: NH_4NO_3 was omitted and KNO_3 and NH_4Cl were supplied in MS-D + 10 mg/L NAA.

f = embryo frequency as described in Table 3.

S.E. = Standard error

TABLE 14. Effect of potassium concentration on somatic embryogenesis in leaf explant cultures of S. melongena

K^+ (mM)	f	S.E.
1.25	18.2	1.7
2	21.5	2.6
5	21.7	3.0
10	21.1	2.4
20	21.2	2.1
30	19.0	2.4
40	16.5	1.9
50	19.5	2.1
60	13.2	1.4
70	11.0	1.2

Note: KCl added to MS-D medium with 60 mM total nitrogen (20.6 mM NH_4NO_3 and 18.8 mM $NaNO_3$ + 10 mg/L NAA.
 f = embryo frequency as described in Table 3.
 S.E. = Standard error.

TABLE 15. Effect of sodium on somatic embryogenesis in leaf explant cultures of S. melongena

Na ⁺ (mM)	f	S.E.
0	20.1	1.4
1	19.8	1.8
10	20.5	2.1
20	19.1	2.1
40	17.5	2.2
60	16.1	1.5
80	12.3	2.3
100	11.7	1.6

Note: NaCl added to MS-D + 10 mg/L NAA.
 f = embryo frequency as described in Table 3.
 S.E. = Standard error.

6. Effect of sugars

A study of the effect of sucrose concentration on embryogenesis revealed that maximal response occurred at 2% (w/v), which is equivalent to 60 mM sucrose. Both elevated and reduced sucrose levels (10 and 0.5% w/v) markedly inhibited embryogenesis (Table 16).

Various sugars were added to MS-D at 60 mM (the optimal sucrose concentration) to test their ability to induce somatic embryogenesis. Embryos were induced at high frequencies in the presence of sucrose or glucose while fructose was only partially effective (Table 17). Neither callus growth nor somatic embryogenesis occurred in the presence of galactose, raffinose or xylose.

In order to determine if inhibition of embryogenesis at high sucrose concentrations was due to osmotic effects, the effect of osmotic potential on embryo formation by supplementing MS-D with mannitol from 0 to 0.6 M was evaluated. A clear osmotic inhibition of somatic embryogenesis was observed between 0.2–0.4 M mannitol (Table 18).

7. Effect of culture conditions

The culture temperature and light requirements for somatic embryogenesis were tested by culturing explants in complete darkness or under fluorescent light ($30 \mu\text{E m}^{-2}\text{sec}^{-1}$) at 25 and 30°C. Light was absolutely essential for somatic embryogenesis although some callus developed in the dark. At 30°C callus growth and somatic embryogenesis occurred earlier than at 25°C, and the frequency of embryos formed was higher (Table 19).

The orientation of the explants on the surface of the medium was not critical for somatic embryogenesis (Table 20). Callus proliferation and the frequency of somatic embryogenesis was the same if the upper or lower leaf surface was placed in contact with the medium. Following

TABLE 16. Effect of sucrose concentration on somatic embryogenesis in leaf explant cultures of S. melongena

Sucrose conc. (% w/v)	f	S.E.
0.5	2.2	2.8
1	11.2	1.9
2	20.7	3.0
5	10.0	1.5
10	1.8	0.6

Note: MS-D + 10 mg/L NAA.

f = embryo frequency as described in Table 3.

S.E. = standard error.

TABLE 17. Effect of sugar source on somatic embryogenesis
in leaf explant cultures of S. melongena

Sugar (60 mM)	f	S.E.
sucrose	24.0	2.1
glucose	19.2	1.7
fructose	10.5	1.6
galactose	0	-
raffinose	0	-
xylose	0	-

Note: All sugars supplied in MS-D + 10 mg/L NAA.
f = embryo frequency as described in Table 3.
S.E. = standard error.

TABLE 18. Effect of osmotic potential on somatic
embryogenesis in leaf explant cultures of S. melongena

Mannitol conc. (M)	f	S.E.
0	22.6	2.0
0.05	20.2	2.4
0.1	18.0	1.7
0.2	13.8	2.3
0.4	0.2	0.1
0.6	0	-

Note: Mannitol was added to MS-D + 10 mg NAA.
f = embryo frequency as described in Table 3.
S.E. = standard error

TABLE 19. Effect of light and temperature on somatic embryogenesis in leaf explant cultures of S. melongena

Temp. (°C)	Light intensity ($\mu\text{E m}^{-2}\text{sec}^{-1}$)	f	S.E.
25	0	0	-
25	30	20.8	2.2
30	0	0	-
30	30	27.2	1.9

Note: MS-D medium + 10 mg/L NAA.

f = embryo frequency as described in Table 3.

S.E. = standard error.

TABLE 20. Effect of explant orientation on somatic embryogenesis in leaf explant cultures of S. melongena

Leaf surface in contact with agar	f	S.E.
Upper	28.4	1.9
Lower	26.6	2.7

Note: MS-D + 10 mg/L NAA.

f = embryo frequency as described in Table 3.

S.E. = Standard error.

a 30 min vacuum pretreatment excised leaf discs lost the ability to form somatic embryos but not the ability to form callus (Table 21).

C. Culture of Somatic Embryos

1. Effect of osmotic potential

Cotyledonous somatic embryos on 30 day-old leaf explant cultures could be excised and germinated on agar medium. Surviving embryos were defined as those which displayed root and shoot regeneration after 30 days; approximately 75% of surviving embryos developed into normal plantlets that could be transferred to soil. Cultured embryos often failed to germinate a normal dicotyledonous shoot or normal roots. Callus growth and root hair initiation occurred on the radicals of some embryos while others developed multiple, fused or no cotyledons. The osmotic potential of the embryo culture medium was varied by the addition of 0 to 0.3 M mannitol. Increased osmotic potential resulted in decreased embryo survival. When 0.3 M mannitol was supplied to MS-D medium the survival of cultured embryos was one-half as great as when mannitol was absent (Table 22).

2. Effect of growth regulators

The cytokinin kinetin, the anti-auxin TIBA, and the growth promoter coconut milk were tested separately for their abilities to promote survival of cultured somatic embryos (Table 23). Kinetin was inhibitory to embryo survival due to the adverse effect it had on root development. TIBA and coconut milk had no significant effect on embryo survival.

The inhibitor ABA was added to the embryo induction medium and those embryos which were induced were excised from the callus and cultured on hormone-free MS-D medium. ABA in the induction medium did not promote higher embryo survival (Table 24).

TABLE 21. Effect of reduced pressure pretreatment on somatic
embryogenesis in leaf explant cultures of S. melongena

Air pressure	f	S.E.
Atmospheric	21.5	2.4
0.5 Atmospheric (30 min)	0	0

Note: MS-D + 10 mg/L NAA.

f = embryo frequency as described in Table 3.

S.E. = standard error

TABLE 22. Effect of osmotic potential on cultured somatic embryos of S. melongena

Mannitol conc. (M)	Survival (%)
0	45
0.1	35
0.2	30
0.3	23

Note: Somatic embryos were excised from callus initiated on MS-D + 10 mg/L NAA. Survival of embryos is represented by the % of 125 embryos initially cultured which showed normal root and shoot development by 30 days. Embryo culture medium was MS-D with 1% (w/v) sucrose.

TABLE 23. Effect of various growth regulators on
cultured somatic embryos of S. melongena

Regulator	Conc.	Survival (%)
Kinetin	0 mg/L	34
	1 "	23
	2 "	10
TIBA	0 mg/L	13
	0.1 "	15
	2 "	15
Coconut milk	0 % (v/v)	34
	1 "	0
	5 "	18
	10 "	10

Note: All somatic embryos excised from callus initiated on MS-D + 10 mg/L NAA. Survival of embryos as described in Table 22, based on 30 embryos per treatment. All embryos cultured on MS-D with 1% (w/v) sucrose.

TABLE 24. Effect of abscisic acid (ABA)
in the induction medium on cultured
somatic embryos of S. melongena

ABA conc. (mg/L)	Survival (%)
0	10
0.1	10
1	6

Note: All somatic embryos excised from callus initiated on MS-D + 10 mg/L NAA supplemented with various amounts of ABA. Survival of embryos as described in Table 22 based on 40 embryos per treatment. All embryos cultured on MS-D with 1% (w/v) sucrose.

3. Effect of culture conditions

Activated charcoal increased the survival of cultured embryos at a very narrow concentration range in the order of 1%, w/v, (Table 25). This additive promoted vigorous root development but often was detrimental to normal shoot development.

Culture temperature influenced embryo survival between 15 and 35°C. A linear relationship was established between the temperatures from 15 to 30°C and embryo survival with 30°C being the best temperature (Table 26).

Tests on the effect of basal nutrient salt concentration on embryo survival revealed that the optimal concentration was full-strength or one-half strength MS-D while more dilute concentrations were inferior (Table 27).

The age of the callus from which the embryos were excised did not critically influence embryo survival, however, wide fluctuations in embryo survival in different experiments may be due to the morphological abnormalities described above (section C.1).

D. Shoot Organogenesis on Leaf Explants

When leaf explants were cultured on MS-D medium, supplemented with a cytokinin (kinetin, zeatin, BAP or 2iP) at various concentrations callus growth was negligible, yet shoot primordia were formed on the upper epidermis of the explant after 10 days of culture (Fig. 11). Kinetin and BAP were the most effective cytokinins tested for the induction of shoot primordia (Table 28). The primordia formed on kinetin-supplemented media were unique because they often matured to shoots bearing normal leaves and occasionally roots were formed (Fig. 12). Plants were regenerated from the shoot primordia induced by all of the cytokinins, however, those induced by kinetin were most successfully rooted and

TABLE 25. Effect of activated charcoal on
cultured somatic embryos of S. melongena

Activated charcoal conc. % (w/v)	Survival (%)
0	16
0.5	10
1.0	24
1.5	8

Note: All somatic embryos excised from
callus initiated on MS-D + 10 mg/L NAA.
Survival of embryos as described in Table 22
based on 75 embryos per treatment. Embryo
culture medium was B5 with 1% (w/v) sucrose.

TABLE 26. Effect of temperature on cultured
somatic embryos of S. melongena

Temperature (°C)	Survival (%)
15	16
25	21
30	46
35	14

Note: All somatic embryos excised from callus cultures initiated on MS-D + 10 mg/L NAA. Survival of embryos as described in Table 22. based on 35 embryos per treatment. Embryo culture medium was MS-D with 1% (w/v) sucrose.

TABLE 27. Effect of concentration of basal MS-D medium on cultured somatic embryos of S. melongena

Medium	Strength	Survival (%)
MS-D	1 X	51
"	1/2 X	57
"	1/4 X	26
"	1/8 X	8

Note: All somatic embryos excised from callus initiated on MS-D + 10 mg/L NAA. Survival of embryos as described in Table 22 based on 100 embryos per treatment. All culture media supplemented with 1% (w/v) sucrose.

FIGURE 11. Leaf explant cultured for 14 days on MS-D + 1 mg/L BAP. Arrows denote shoot primordia at various stages of development. Leaf tissue (L) shows little callus proliferation (C).

FIGURE 12. Leaf explant (L) cultured for 30 days on MS-D + 2 mg/L kinetin. Shoots (arrows) show hypocotyl elongation and leaf expansion with some root organogenesis (R).

TABLE 28. Effect of cytokinin type and concentration on shoot organogenesis in leaf explant cultures of *S. melongena*

Cytokinin	Conc. (mg/L)	f	S.E.
None	-	0	-
Kinetin	0.5	0	-
	2	2.3	0.8
	5	4.0	1.1
	10	4.0	1.0
BAP	0.1	0	-
	1	2.4	1.0
	5	2.3	0.2
	10	5.3	1.1
	20	1.2	0.3
Zeatin	0.5	2.3	0.7
	5	2.7	0.8
	10	1.9	1.0
2iP	0.5	1.8	0.4
	5	2.5	0.6
	10	2.5	0.6

Note: Cytokinins supplied in MS-D medium lacking auxin.
 f = mean number of shoots per explant (200 mm²) based on 50 leaf explants in two independent experiments.
 S.E. = standard error.

transferred to the greenhouse.

The orientation of the cultured leaf explants on cytokinin medium was critical for the organogenic response. Explants cultured with the upper leaf epidermis in contact with the agar produced more shoots than those with the lower epidermis contacting the medium (Table 29). IAA supplied alone at 1.0 mg/L induced shoot primordia on leaf explants at very low frequencies (3 shoots in 50 explants); however, plants could not be regenerated from these primordia.

E. Growth and Morphogenesis of Cell Suspensions

Liquid cell suspension cultures were easily initiated from the soft, friable primary callus of eggplant cultured on MS-D + 10 mg/L NAA. Cell suspensions were grown in this medium or MS-D + 0.5 mg/L 2,4-D or MS-D + 2.0 mg/L 2,4-D. Cell suspensions grew equally well at either level of 2,4-D but grew at a considerably slower rate in MS-D + 10 mg/L NAA (Fig. 13). In addition to the different rates of growth, the cell suspension morphology was different on the two auxins (Figs. 14, 15). Cells grown in 2,4-D formed a fine, granular cell suspension of single cells and small clusters of cells, while cells grown in NAA formed large, multicellular aggregates. Somatic embryogeny was inhibited by 2,4-D (Fig. 16) while it was promoted by NAA (Fig. 16) yet cells grown in the presence of 2,4-D were often organized into small groups of densely cytoplasmic cells. Microscopic observations revealed very early stages of embryogenesis in the cell suspensions grown in 2,4-D, however, these proembryos never developed beyond the globular stages (Figs. 18-20). Suspensors were present on some proembryos (Figs. 18, 19) while globular embryos often had a distinct dermatogen (Fig. 20). The multicellular aggregates formed in NAA-supplemented medium were often the sources of numerous embryos

TABLE 29. Effect of explant orientation on shoot organogenesis²
 in leaf explant cultures of S. melongena

Leaf surface in contact with agar	Hormone supplement	f	S.E.
Upper	1 mg/L BAP	4.8	0.7
Lower	"	1.2	0.4
Upper	10 mg/L BAP	7.7	1.0
Lower	"	2.3	0.4

Note: BAP added to MS-D medium.

f = shoot frequency as described in Table 28.

S.E. = Standard error.

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OF / DE

FIGURE 13. The relationship between cell suspension growth (fresh weight) and the length of incubation. 250 mg of cells were incubated in 33 mL of MS-D supplemented with 0.5 mg/L 2,4-D (Δ), 2 mg/L 2,4-D (o) or 10 mg/L NAA ($\square$).

FIGURE 14. S. melongena cell suspension grown in liquid MS-D +
0.5 mg/L 2,4-D.

FIGURE 15. Cell suspension grown in liquid MD-D + 10 mg/L NAA.

FIGURE 16. The same cells as in Figure 14 transferred to a petri dish.

FIGURE 17. The same cells as in Figure 15 transferred to a petri dish.

FIGURE 18. Small colonies from the cell suspension shown in Figure 14.
Arrow indicates an early globular embryo with a large
suspensor.

FIGURE 19. A globular embryo from the cell suspension shown in Figure 14.
The suspensor is composed of one large cell and several smaller
cells (arrow).

FIGURE 20. An advanced globular embryo from the cell suspension shown in
Figure 14. A dermatogen layer of large non-meristematic cells
has enveloped the inner meristematic zone. The suspensor
region has been damaged (arrow).

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which continued development to the cotyledonous stages.

In order to assay for the ability of auxin to promote somatic embryogenesis from cell colonies, colonies grown in 2,4-D were transferred to agar-solidified medium supplemented with either 2,4-D or NAA. NAA promoted somatic embryogenesis from small cell aggregates and single cells, whereas no response was observed with 2,4-D or hormone-free medium (Table 30). The highest embryo yields were obtained at 10 mg/L NAA.

The effect of cytokinins added to MS-D + 10 mg/L NAA on embryogenesis from cell colonies is shown in Table 31. All cytokinins tested inhibited somatic embryogenesis without inhibiting callus growth.

Various sugars were supplied in MS-D + 10 mg/L NAA to test for their abilities to induce somatic embryos from cell colonies. Sucrose and glucose gave somatic embryogenesis while fructose, galactose, raffinose and xylose only supported undifferentiated cell growth (Table 32).

All cytokinins tested were able to promote shoot organogenesis from isolated cell colonies at concentrations from 0.5 to 10 mg/L (Table 33). Shoot primordia were not formed if cytokinins were omitted from the medium or if zeatin was supplied at low concentrations (0.5 mg/L). At high concentrations (5–10 mg/L) kinetin stimulated the highest frequency of shoot primordia which were also morphologically more developed than shoot primordia formed by the other cytokinins.

F. Protoplast Isolation and Culture

Cells from suspension cultures could be treated with enzymes for 12–24 h to release protoplasts. The yield of protoplasts per gram fr. wt. of cells was measured after filtering the protoplasts through nylon mesh screens, and was found to be directly proportional to the length of enzyme treatment. A 12 h digestion yielded an average 30×10^6 protoplasts

TABLE 30. Effect of auxins on somatic embryogenesis in cell colonies from suspension cultures of S. melongena

Auxin	Conc. (mg/L)	f	S.E.
None	-	0	-
NAA	0.1	0.4	0.1
	1	2.1	0.5
	10	5.5	1.3
2,4-D	0.1	0	-
	1	0	-
	10	0	-

Note: Cell suspensions grown in liquid MS-D + 0.5 mg/L 2,4-D. Cells were taken from suspension 3 days after transfer. Auxins were supplied in MS-D medium solidified with 0.8% agar.

f = embryo frequency as represented by the mean number of embryos per cell colony based on 25 colonies per treatment.

S.E. = Standard error.

TABLE 31. Effect of cytokinins supplied with NAA on somatic embryogenesis in cell colonies from suspension cultures of S. melongena

Cytokinin	Conc. (mg/L)	f	S.E.
None	-	1.5	0.4
Kinetin	0.1	0.5	0.2
	1	0.3	0.1
	10	0	-
BAP	0.1	1.1	0.3
	1	0.5	0.3
	10	0	-
Zeatin	0.1	1.5	0.4
	1	0.1	0.1
	10	0	-
2iP	0.1	0.8	0.4
	1	0.2	0.2
	10	0	-

Note: Cell suspensions grown in liquid MS-D + 0.5 mg/L 2,4-D. Cells taken from suspension 3 days after transfer to fresh medium. Cytokinins were supplied in MS-D + 10 mg/L NAA solidified with 0.8% agar.

f = embryo frequency as described in Table 30.

S.E. = Standard error.

TABLE 32. Effect of various sugars on somatic
embryogenesis in cell colonies from suspension
cultures of S. melongena

Sugar	f	S.E.
Sucrose	1.8	1.0
Glucose	1.9	0.44
Fructose	0	-
Galactose	0	-
Raffinose	0	-
Xylose	0	-

Note: Cell suspensions grown in liquid MS-D + 0.5 mg/L 2,4-D. Cells were taken from suspension 3 days after transfer. All sugars supplied at 2% (w/v) in MS-D + 10 mg/L NAA solidified with 0.8% agar.

f = embryo frequency as described in Table 30.

S.E. = Standard error.

TABLE 33. Effect of cytokinins on shoot organogenesis in cell colonies from suspension cultures of *S. melongena*

Cytokinin	Conc. (mg/L)	f	S.E.
None	-	0	-
Kinetin	- 0.5	0.1	0.01
	2	0.2	0.1
	5	0.8	0.4
	10	1.2	0.4
BAP	0.5	0.4	0.1
	5	0.1	0.3
	10	0.2	0.2
Zeatin	0.5	0	-
	5	0.6	0.2
	10	1.2	0.3
ZiP	0.5	0.1	0.3
	5	0.4	0.3
	10	0.4	0.3

Note: Cell suspension grown in liquid MS-D + 0.5 mg/L 2,4-D. Cells were taken from suspension 3 days after transfer to fresh medium. Cytokinins were supplied in MS-D medium solidified with 0.8% agar.

f = the mean number of shoots per colony based on 25 colonies per treatment.

S.E. = Standard error.

per g fr. wt. and a 24 h digestion yielded an average 12×10^7 protoplasts per g fr. wt. Protoplasts were small and densely cytoplasmic (Fig. 21). For culture the protoplasts were diluted to a final density of 1×10^4 to 1×10^6 per mL of medium, with optimal plating efficiency occurring at 1×10^5 per mL. Protoplast elongation corresponding with cell wall reformation was evident after two days of culture in NT or MS-D media (Fig. 22). Greatly elongated cells were occasionally observed after seven days of culture (Fig. 23). These long cells did not subsequently divide. After 10–14 days of culture, the first division of protoplast-derived cells occurred (Fig. 24). The second division in protoplast-derived cultures grown in NT medium supplemented with 3 mg/L NAA and 1 mg/L BAP was seen 16–20 days after protoplast isolation (Fig. 25).

The plating efficiency was measured after 14 days of culture in different media. No divisions were observed in SH-2 medium supplemented with 3 mg/L NAA and 1 mg/L BAP. A plating efficiency of 1–4% was obtained when the protoplasts were cultured in MS-D medium supplemented with 3 mg/L NAA and 1 mg/L BAP. NT medium was the best medium tested supporting plating efficiencies up to 30%. The following modifications to the NT protoplast culture medium failed to increase plating efficiency above 30%: substitution of 0.35 M mannitol for 0.35 M glucose, omission of BAP, substitution of kinetin or zeatin for BAP, substitution of either IAA or 2,4-D for NAA, partial solidification of the medium with 0.4% agar, and dispensing the protoplasts in liquid NT medium onto filter paper supported on agar solidified NT medium.

Protoplast-derived cell colonies (~50 mg) were transferred to a variety of agar-solidified regeneration media six weeks after protoplast isolation. The regeneration media consisted of MS-D supplemented with

FIGURE 21. Freshly isolated protoplasts of *S. melongena* prior to culture.

FIGURE 22. Protoplasts after 24 h of culture in liquid NT + 3 mg/L NAA and 0.5 mg/L BAP showing change of shape.

FIGURE 23. Protoplast-derived cells which have changed shape and reformed cell walls after 1 week of culture in NT + 3 mg/L NAA and 0.5 mg/L BAP.

FIGURE 24. The first division of small protoplast-derived cells (arrow) after 12 days of culture in NT + 3 mg/L NAA and 0.5 mg/L BAP.

FIGURE 25. The second division of protoplast-derived cells (arrows) after 16 days of culture in NT + 3 mg/L NAA and 0.5 mg/L BAP.

FIGURE 26. Shoot regeneration in protoplast-derived cell colonies 3 weeks after transfer to regeneration medium (MS-D + 2 mg/L kinetin).

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various auxins and cytokinins but with no osmotic stabilizer (Table 34) and included the additions of 10 mg/L NAA and 5 mg/L kinetin which have previously been shown to stimulate embryogenesis (Tables 3, 29) and organogenesis (Tables 27, 32), respectively. Somatic embryogenesis was not observed from protoplast-derived cell colonies on any medium. Very infrequently shoot primordia were produced after 10 weeks on colonies cultured on MS-D + 2 mg/L kinetin (9 primordia/50 colonies). These shoot primordia elongated (Fig. 26) and when excised from the callus they were rooted on hormone-free B₅ medium supplemented with 1% sucrose. Two regenerates have been transferred to peat pellets (Jiffy-7) in a mist chamber and after 14 days were planted in soil in the greenhouse.

TABLE 34. Auxin-cytokinin combinations used in tests for regeneration of *S. melongena* protoplast-derived cells

Auxin	Conc (mg/L)	Cytokinin	Conc. (mg/L)
NAA	0.1	-	-
"	1.0	-	-
"	5.0	-	-
"	10.0	-	-
"	15.0	-	-
"	25.0	-	-
"	1.0	BAP	0.1
"	1.0	"	1.0
"	1.0	"	2.0
"	0.1	"	2.0
"	0.1	"	10.0
"	1.0	"	10.0
"	2.0	"	2.0
-	-	"	1.0
-	-	"	2.0
-	-	"	10.0
-	-	Kinetin	0.5
-	-	"	1.0
-	-	"	5.0
-	-	"	10.0
IAA	1.0	"	3.0
"	0.1	"	2.0
"	0.1	"	0.3
"	0.5	"	2.0

continued...

Table 34, cont'd.

Auxin	Conc. (mg/L)	Cytokinin	Conc. (mg/L)
NAA	0.5	kinetin	2.0
"	0.1	"	2.0
-	-	Zeatin	0.1
-	-	"	1.0
-	-	"	2.0
-	-	-	10.0
2,4-D	0.05	-	-
"	0.1	-	-
"	0.5	-	-
"	1.0	-	-
"	2.0	-	-
"	0.1	Kinetin	2.0
"	0.2	"	2.0
"	0.5	"	2.0

Note: All hormones were added to agar solidified MS-D medium supplemented with 2% sucrose. Colonies were cultured for 16 weeks.

DISCUSSION

A. Morphogenesis in Tissue and Cell Cultures

Solanum melongena leaf explants cultured on media containing different auxins gave markedly different responses. Callus proliferation was observed on leaf explants which were supplied with either 2,4-D, 2,4,5-T or NAA but a very high frequency of induction and development of chlorophyllous embryos was only apparent on those explants which were cultured on NAA. In addition to stimulating embryogenesis, NAA also supported embryo maturation up to the cotyledonous stage. Somatic embryos on NAA medium were observed in globular, heart-shaped, and mature torpedo-shaped stages resembling normal zygotic cotyledonous embryos. NAA stimulated somatic embryogenesis across a wide range of concentrations with the relatively high concentration of 10 mg/L being optimal. Matsuoka and Hinata (1979) also observed stimulatory effects of high concentrations of NAA on embryogenesis in eggplant hypocotyl explants. They reported that somatic embryogenesis was promoted by 8 mg/L NAA and that this concentration of NAA was not detrimental for embryo maturation to the cotyledonous stage.

The action of NAA in embryogenesis in eggplant is fundamentally different from the role of auxins in other species where auxins generally inhibit embryo development beyond the globular stage. Auxins have been shown to inhibit maturation of somatic embryos of carrot (Halperin and Wetherell 1964; Fujimura and Komamine 1975; Sung et al. 1979), Nigella (Banerjee and Gupta 1976), celery (Al-Abta and Collin

1978), Antirrhinum (Sangwan and Harada 1975), Brassica (Pareek and Chandra 1978) and Citrus (Kochba and Spiegel-Roy 1977). For this reason, somatic embryogenesis and subsequent plant regeneration most often requires that cells or tissue explants be supplied with auxins for a short duration followed by removal or reduction of the auxin levels in the medium. Eggplant embryogeny is not adversely affected by exogenous auxin and removal of auxin is only required for later development of mature embryos to plantlets.

The novel response of cultured eggplant cells was also observed with embryogenesis in cell suspension cultures. When cells were grown in liquid medium supplemented with 2,4-D mature embryos were never produced but with NAA somatic embryo induction and maturation was stimulated. The growth rate of cells in the two media differed considerably with 2,4-D supporting the greatest rate of fresh weight gain. Microscopic observations revealed that cells in both 2,4-D and NAA supplemented media were often very small, densely cytoplasmic and organized into small multicellular aggregates. Proembryonic masses, and very immature globular proembryos were also observed in both media. Some of these embryos displayed normal globular morphology with multicellular suspensors and a distinct epidermal cell layer of 'dermatogen'. The proembryos from 2,4-D medium could not, however, be germinated when transferred to hormone-free medium suggesting that 2,4-D is capable of stimulating embryogenic cell aggregates but also exerts permanent inhibitory influences on development of embryos beyond the globular stage. As with leaf explants, NAA stimulated embryogenesis in cell suspensions and promoted development to the cotyledonous stages.

Further evidence for the stimulatory effects of NAA on somatic embryo development is provided from experiments on somatic embryogenesis

from cell colonies. Cell suspension colonies which were grown in 0.5 mg/L 2,4-D and transferred to agar-solidified medium lacking auxin or containing a range of 2,4-D concentrations showed no embryogenic response. Colonies transferred to NAA-supplemented medium did produce somatic embryos. The transfer of cells from 2,4-D supplemented medium to 2,4-D-free medium has been shown to induce embryogenesis in many genera including: Daucus (Halperin and Wetherell 1964), Petunia (Rao et al. 1973), Sorghum (Thomas et al. 1977), Apium (Al-Abta and Collin 1978), Paulownia (Radojevic 1978), Phoenix (Reynolds and Murashige 1979), and Pennisetum (Vasil and Vasil 1981).

These experiments have demonstrated a unique response of eggplant somatic cells to the exogeneous supply of the auxin NAA. Although quantitatively the various auxins gave different rates of callus growth on explants or in suspensions the induction of somatic embryos was only achieved with NAA. The fact that a 100-fold concentration range of the other auxins failed to promote embryogenesis suggests that the action of NAA in embryogenesis is qualitatively unique. Although Hicks (1965) was unable to display qualitative differences in the abilities of 26 auxins to promote cell growth, auxins have previously displayed quantitative differences in many bioassays which have allowed conclusions regarding their structure-activity relationships (Schneider and Wightman 1978). Of the non-phenoxyacetic acid derivatives used in the present study, PAA, IAA and NAA, only NAA induced callus formation while both phenoxy derivatives 2,4-D and 2,4,5-T promoted massive callus growth. It is interesting to note that PAA was the only auxin which did not promote callus growth nor any morphogenic response and yet it was the closest auxin structurally to NAA. Therefore, it can be suggested that the naphthalene moiety is an essential component of the auxin NAA and that

the phenyl moiety can not be substituted. The auxin-specificity in somatic embryogenesis may rest at the site of intracellular receptors and auxin metabolism (Street 1979) or at the nuclear level by direct action on nuclear activity and gene expression (Dudits et al. 1979). Only detailed metabolic and histological analysis can resolve these questions.

The pronounced auxin-specificity for somatic embryogenesis observed in eggplant has not been demonstrated through systematic studies in cultured explants of many other species. The induction of somatic embryogenesis on stem explants of snapdragon has been demonstrated to be a specific response to NOA and coconut milk, while the auxins IAA, NAA, and 2,4-D were ineffective (Sangwan and Harada 1975). Carrot hypocotyl explants produced somatic embryos when supplied with either IAA, NOA, NAA, 2,4-D, 2,4,5-T, MCPA, or PCPA but not POA or OCPA (Kamada and Harada 1979).

Somatic embryogenesis in cultures of eggplant supplied with NAA was inhibited by the four cytokinins tested, kinetin, zeatin, BAP and 2iP. Matsuoka and Hinata (1979) also observed inhibition of embryogenesis on hypocotyls of eggplant by BAP. Similarly, embryogenesis in Petunia (Rao et al. 1973), Nigella (Banerjee and Gupta 1976), Citrus (Kochba and Spiegel-Roy 1977), Daucus (Kamada and Harada 1979), Panax (Chang and Hsing 1980) and Petroselinum (Tanimoto and Harada 1980) was inhibited by cytokinins. Cytokinins have, however, been implicated as a necessary factor for somatic embryogenesis in some species (Norstog 1970; Mullins and Srinivasan 1976; Söndahl and Sharp 1977), and have been added to auxin-supplemented primary media without inhibiting embryogenesis in others (Litz and Conover 1980; Phillips and Collins 1980; Sita et al. 1980).

Cytokinins stimulated shoot organogenesis when supplied as the sole growth regulator to eggplant leaf explants or cell colonies but the response was not cytokinin-specific. Shoot primordia were never produced on hormone-free medium, however, the auxin IAA at 1 mg/L did stimulate low frequency shoot organogenesis. Matsuoka and Hinata (1979) using different cultivars observed that hypocotyl explants of eggplant produced shoot primordia when cultured on hormone-free medium while BAP enhanced the organogenic response. Kamata and Rao (1978) reported induction of shoot primordia on hypocotyl explants by either of the cytokinins BAP, zeatin, kinetin or 6-benzyl-9-tetrahydropyran adenine and by the auxin IAA.

ABA added with 10 mg/L NAA reduced somatic embryogenesis in leaf explants of eggplant without altering the morphology of the embryos produced. ABA has been shown to inhibit embryogenesis in carrot-cell suspension cultures (Kamada and Harada 1979), to stimulate embryogenesis in Citrus (Kochba et al. 1978a) and shoot organogenesis in explants of Begonia leaf (Heidi 1968) and sweet potato tuber (Yamaguchi and Nagajima 1974) and to not influence shoot formation on tobacco callus (Thorpe and Meier 1973). ABA also suppressed developmental and morphological abnormalities that occur in NAA-induced embryos in caraway cell cultures (Ammirato 1974, 1977). These highly variable results defy explanation in physiological terms at the present time.

The genotype of S. melongena markedly influenced the frequency of NAA-induced somatic embryos in leaf explants. Although a non-embryogenic variety was not found the possibility exists that if more unrelated genotypes were evaluated and other conditions used it would be possible. The importance of genotype was also shown in a study by

Yamada et al. (1967) who isolated embryogenic callus from S. melongena var. Sinkro but obtained non-embryogenic callus from the variety Kantroao. Matsuoka and Hinata (1979) have observed varietal differences in both embryogenesis and shoot organogenesis on hypocotyl explants of 14 cultivars of eggplant. In general, those varieties with a high potential for embryogenesis had a low potential for shoot and root organogenesis and vice versa. The fact that Fassuliotis (1975) was unable to regenerate explants of eggplant from tissue cultures and that Kamata and Rao (1978) only observed callus formation and rooting of hypocotyls on NAA-containing medium suggests that genotype is critical for in vitro morphogenic responses in this species. The wide differences in embryogenic responses reported here allow for the possibility of analysis of the genotypic basis of embryogenesis through breeding experiments.

The genotype of donor plants has been implicated as a factor controlling somatic embryogenesis in Medicago sativa (Johnson et al. 1981), microspore embryogenesis in many species including Solanum tuberosum (Jacobsen and Sopory 1978) and in vitro shoot organogenesis in Lycopersicon esculentum (Behki and Leslie 1976). Vasil and Vasil (1981), however, have reported that somatic embryogenesis in callus cultures of 40 Pennisetum americanum cultivars, P. purpureum, and Panicum maximum does not appear to show any genotypic dependence.

An experiment comparing basal media revealed that MS-D medium was best for somatic embryogenesis in eggplant. Previous successful studies of morphogenesis in eggplant have all used MS medium (Yamada et al. 1967; Kamata and Rao 1978; Matsuoka and Hinata 1979). This medium has also been the most often used for studies of somatic

embryogenesis in other species (Evans et al. 1981) although systematic evaluations of the effect of basal medium on somatic embryogenesis have not often been carried out.

The importance of NH_4^+ on somatic embryogenesis in eggplant was demonstrated when total nitrogen content of the medium was maintained at 60 mM and the effect of varying the NH_4^+ content on somatic embryogenesis was determined. It is clear that media with low NH_4^+ content do not support high frequency somatic embryogenesis in eggplant even though the total nitrogen content of the media is not limiting. It has been reported that somatic embryogenesis can occur on media lacking ammonium (Reinert et al. 1967; Bhattacharya and Sen 1980) and on media with low ammonium contents (Huber et al. 1978; Halperin and Wetherell 1965), however, media with high concentrations of ammonium salts were not compared in these studies.

Organic nitrogen sources can satisfy the apparent requirement for NH_4^+ by embryogenic carrot cells (Wetherell and Dougall 1976) but S. melongena leaf discs could not be stimulated to form a high frequency of somatic embryos with organic nitrogen supplements. When a total nitrogen concentration of 60 mM was supplied as either casamino acids or casein hydrolysate the leaf explants failed to proliferate callus or form somatic embryos, however, at lower concentrations these reduced nitrogen sources stimulated a low frequency of somatic embryogenesis.

S. melongena leaf explants produced callus and somatic embryos across a range of $\text{NO}_3^-/\text{NH}_4^+$ ratios but high $\text{NO}_3^-/\text{NH}_4^+$ ratios were more favourable to embryogenesis. Evidence from embryogenic carrot cultures indicates that low $\text{NO}_3^-/\text{NH}_4^+$ ratios present unfavourable conditions for growth and embryogenesis (Wetherell and Dougall 1976).

The toxic effects of NH_4^+ on the growth of non-embryogenic cell cultures has also been established and is due to lowered pH of the medium as the cells metabolize NH_4^+ (Gamborg and Shyluk 1970). If compensation was made for the pH changes by continuous titration carrot cell culture growth and embryogenesis was observed on medium supplemented with NH_4^+ as the sole nitrogen source (Dougall and Verma 1978).

It was not possible to increase the frequency of somatic embryogenesis by increasing total nitrogen content of MS-D medium. Al-Abta and Collin (1979) increased the total nitrogen content of MS medium by a factor of 2, 3 or 4 and found that in all cases the frequency of somatic embryogenesis in celery suspension cultures decreased.

Somatic embryogenesis in leaf explants of eggplant did not require more than 1.25 mM K^+ in the medium while previous work has shown that embryogenesis in carrot cultures requires 20 mM K^+ (Wetherell and Dougall 1976). On the other hand, concentrations of K^+ above 20 mM were inhibitory to carrot cell growth and embryogenesis but somatic embryogenesis in eggplant was not inhibited by K^+ concentrations of 50 mM. KCl was supplied to MS-D medium modified with 20 mM NH_4NO_3 and 18 mM NaNO_3 as the nitrogen sources. Since MS-D medium lacks Na^+ (Appendix, Table 1) it was necessary to test the effect of Na^+ added to MS-D medium on the frequency of somatic embryogenesis. When 20 mM Na^+ was added to MS-D medium no change in frequency of somatic embryogenesis was observed. These results suggest that the monovalent cations K^+ and Na^+ did not significantly influence the frequency of somatic embryogenesis in eggplant leaf cultures. For this reason these cations were not critical factors when they were used as the counterions for NO_3^- in experiments varying the nitrogen content of MS-D.

The media formulation MS devised by Murashige and Skoog (1962) proved to be the best combination of inorganic salts for somatic embryogenesis. This medium was originally devised to support the maximum growth of callus from pith of tobacco which is a plant in the same family as eggplant. By an analysis of the aqueous leaf extracts of tobacco leaves, and systematic substitutions of inorganic salts, these workers arrived at the levels present in MS medium. This tedious evaluation of the physiologically active concentration of nutrients present in an intact Solanaceous plant has not been repeated by other workers in any other plant species. The present study indicates, however, that the levels of inorganic salts present in a differentiating plant organ are near optimal for induction of morphogenesis in vitro.

Since most workers do not perform detailed evaluations of the nutritional and hormonal requirements for plant cells cultured in vitro, the significance of many failures to induce morphogenesis must be questioned. Biological activity of any growth regulator not only varies with concentration but also with the milieu in which it is placed. Development of new media for families other than Solanaceae through analysis of mineral constituents of differentiated tissues may be necessary before morphogenesis is routine.

Experiments with various sugars demonstrated that any sugar which will support callus proliferation in eggplant will also stimulate somatic embryogenesis. The sugars, sucrose, glucose and fructose all supported callus proliferation and embryogenesis on leaf explant cultures while galactose, raffinose and xylose proved ineffective in inducing either callus growth or somatic embryogenesis. Galactose

and the galactose-yielding sugar raffinose have been shown to stimulate somatic embryogenesis in habituated Citrus cultures (Kochba et al. 1978b). Although galactose is thought to inhibit the synthesis of IAA (Kochba et al. 1978b), this result is not unusual since it has been shown that other anti-auxins also stimulated embryogenesis in these cultures (Kochba and Spiegel-Roy 1977).

The total sucrose concentration of MS-D medium was found to be a critical factor in somatic embryogenesis (Table 17). Since the optimal sucrose level was 0.06 M, and 0.15 M reduced the frequency of embryogenesis, the possibility that the higher sucrose concentration may present unfavourable osmotic conditions in the medium was investigated. The metabolically inert sugar-alcohol mannitol was added to MS-D supplemented with 0.06 M sucrose to determine the effect of the osmotic potential of the medium on somatic embryogenesis. Medium containing 0.06 M sucrose plus 0.1 M mannitol did not adversely affect the frequency of somatic embryos. This result suggests that the reduced frequency of somatic embryos observed on medium containing 0.15 M sucrose was not due to osmotic potential alone but to another effect of elevated sucrose concentration. The effect of osmotic potential on somatic embryogenesis was apparent at 0.2 M mannitol and therefore the drastic reduction in the frequency of embryogenesis at 0.3 M sucrose may be due to osmotic effects. Most tissue culture media employ sucrose concentrations which vary from 0.03 M to 0.12 M for studies on morphogenesis yet no reports of the specific effects of sucrose concentration or osmotic potential on somatic embryogenesis exist. Rapeseed requires elevated sucrose levels (0.3 M) for production of microspore embryos and this requirement cannot be met by substituting metabolically inert

compounds or other disaccharides (Lacapra 1977). Reduced sucrose levels (0.015–0.03 M) have stimulated shoot organogenesis in callus cultures of tobacco (Barg and Umiel 1977) and Brown et al. (1979) have demonstrated a dual role of sucrose as osmoticum and energy source during shoot organogenesis in tobacco callus.

Although callus growth was independent of lighting conditions, light was an essential component of the culture environment for somatic embryogenesis in eggplant leaf callus. Elevated culture temperatures also stimulated growth and increased the frequency of somatic embryogenesis. Sopory and Maheshwari (1976) have previously shown that microspore embryogenesis in Datura increases linearly with temperature from 22°C to 30°C. The 30°C temperature treatments which stimulated microspore embryogenesis in B. napus have been replaced in part by short duration treatments at even higher temperatures, i.e. 35 or 40°C (Keller and Armstrong 1978). Such treatments might also be beneficial for eggplant.

Orientation of the leaf explant on the agar surface did not affect the frequency of somatic embryogenesis or the growth of callus but shoot organogenesis was critically affected by explant orientation. Since callus proliferation preceded somatic embryogenesis the orientation of the explants had no effect on somatic embryo initiation which occurred within callus cells. Shoots on the other hand did not emerge from callus cells but rather from primordia formed on the upper epidermis. The higher frequency of shoot induction with the upper epidermis in contact with the agar surface suggests diffusion of nutrients and/or cytokinins within tissues is a potential limiting factor in explant cultures. Ross and Thorpe (1973) provided evidence

for the physiological gradients present in callus cultures which form shoots preferentially on their lower halves. Orientation of stem explants on agar medium has influenced the frequency of shoot organogenesis in B. napus (Stringam 1977) and eggplant (Kamat and Rao 1978). In contrast, Davies and Dale (1979) reported that cytokinin-induced shoot organogenesis on excised leaf explants of Solanum laciniatum was unaffected by explant orientation. Plant regeneration from leaf explants has been reported in many species and yet the effects of orientation have not been discussed, suggesting that orientation is not critical in most instances.

Although reduced oxygen levels in liquid cultures have stimulated somatic embryogenesis (Kessell et al. 1977) and reduced atmospheric pressure has stimulated microspore embryogenesis (Imamura and Harada 1980), a reduced atmospheric pressure pretreatment totally inhibited somatic embryogenesis in eggplant.

B. Culture of Somatic Embryos

The frequency of successful plant regeneration from eggplant somatic embryos was highly variable and always less than 50%. This is in agreement, however, with previous reports of somatic embryo culture (Brettell et al. 1980). It may be that culture of somatic embryos requires nutritional hormonal and physical conditions that vary according to the developmental stages of the embryo. Also trauma resulting from embryo excision and transfer from induction medium to culture medium may account for low success rates. Many of the problems encountered in somatic embryo culture have also been observed and are not yet resolved in the culture of immature zygotic embryos (Raghavan 1976).

An important factor bearing on somatic embryo culture is the wide range of developmental and morphological stages present in any one callus or suspension culture. The culture of embryos that have been induced in vitro has often resulted in low yields of normal plants (Ammirato 1974, 1977; Keller and Armstrong 1977; Brettell et al. 1980). Among the developmental abnormalities observed in eggplant somatic embryos was necrosis of the shoot apex, callus proliferation on the embryo, and uncoordinated organ growth. Since a high concentration of NAA was required to induce embryogenesis, the abnormal growth of excised embryos when transferred to hormone-free medium may be due to carryover of residual auxin. The inhibitor of auxin transport TIBA was added to the embryo culture medium to prevent callus growth and aid normal embryo development but failed to influence either process. Ammirato (1974, 1977) has reported that ABA treatments controlled developmental abnormalities associated with NAA-induced caraway embryos, however, eggplant embryos which were induced on medium containing ABA did not differ in morphology or survival from those induced on medium lacking ABA.

Activated charcoal has been used as a medium additive to stimulate somatic embryogenesis in carrot cell cultures (Fridborg and Eriksson 1975) and it has been demonstrated that the beneficial effect of this treatment is due to adsorption of PAA and derivatives of benzoic acid which accumulate in the medium and may exert negative effects on differentiation (Fridborg et al. 1978). When activated charcoal was added to eggplant embryo-culture medium no stimulation of embryo germination was apparent. Since the length of time the somatic embryos were left on the NAA-induction medium also had no

effect on subsequent development the reason for low frequency embryo survival may be due to reasons other than the accumulation of inhibitors.

Survival of eggplant embryos increased linearly with culture temperature to 30°C. Norstog and Klein (1972) have demonstrated that elevated culture temperatures controlled the precocious germination of cultured barley embryos.

Although dilute concentrations of MS-D medium failed to support normal eggplant embryo differentiation, at full-strength MS-D salts may be toxic to embryos undergoing later development and autotrophic growth. Monnier (1978) described the different toxic effects of nutrient solutions on embryo survival of zygotic Capsella embryos. In general, non-toxic media are not able to induce normal differentiation of globular embryos of this species. Those media capable of supporting organ differentiation are high-salt media which also prove to be toxic to subsequent embryo survival.

C. Morphogenesis in Protoplast Cultures

Isolation of protoplasts from embryogenic suspension cells of eggplant and their subsequent culture was possible with procedures previously applied to Nicotiana species (Douglas et al. 1981). The modified NT medium was satisfactory for rapid formation of cell colonies and prolific callus growth of protoplast-derived cells. However, these cell colonies completely lacked embryogenic capacity when transferred to media which promoted somatic embryogenesis in non-protoplasted cell suspensions. Similar loss of embryogenic capacity in protoplast-derived cells has been reported for Brassica (Thomas et al. 1976) and for Citrus (Vardi et al. 1975).

The lack of success in achieving somatic embryogenesis from protoplast-derived cells may be related to harmful side-effects of the wall-degrading enzymes used to isolate protoplasts, osmotic conditions of protoplast preparation, disruption of membrane or cytoskeleton elements during wall removal and/or abnormal reformation of the cell wall.

Plasmolysis has two major consequences for the plant-cell plasma membrane. It breaks links between the outer surface of the membrane and the cell wall and reduces the surface area of lipid bilayer available to accommodate membrane bound proteins. Freeze-fracture studies of plasmolyzed potato callus cells and enzymatically isolated protoplasts failed to reveal membrane surface differences between their membranes (Wilkinson and Northcote 1980). Both inner membrane surfaces contained two-dimensional hexagonal arrays of proteins which are not observed in unplasmolyzed cells. Plasmolysis has also been shown to decrease the rate of incorporation of labelled precursor into RNAs and proteins of tobacco protoplasts (Premecz *et al.* 1978) yet the rates of O_2 exchange and CO_2 fixation by mesophyll protoplasts of tobacco were similar to those of the original leaf tissues (Morris and Thain 1980).

The wall-degrading enzymes used to isolate protoplasts have not been highly purified and are known to contain different enzyme activities, including phosphatidase, which may have adverse affects on membrane proteins (Kao 1978).

Plasma membrane-wall connections are broken by the mechanical stresses of plasmolysis but the fate of cytoplasmic-membrane connections inside the protoplasts is unknown. It is conceivable

that plasmolysis and reduced atmospheric pressure pretreatments both disrupted cytoskeleton-membrane connections as well as membrane-wall associations. Reestablishment of such connections during protoplast culture may not have the order necessary for integrated morphogenic development.

Removal of the cell wall undoubtedly breaks all plasmodesmata of embryogenic cells. Embryogenic epidermal cells of Ranunculus have pronounced plasmodesmatic connections with adjacent cells (Konar et al. 1972) as have embryogenic suspension culture cells of carrot (McWilliam et al. 1974). If the regenerated cells from protoplasts cannot reform similar intercellular connections then this may be the reason for loss of embryogenic competence. Since it is not known if isolated protoplasts reform a cell wall identical to their original wall these remain as further areas of investigation.

Preliminary studies with embryogenic cell aggregates of eggplant indicated that embryogenesis was not suppressed by plasmolysis alone or in conjunction with short enzyme digests in which the wall was not completely removed. However, in the multicellular aggregates of the suspension culture uniformity of these treatments can be questioned. The small, highly cytoplasmic cells in the center of many aggregates may not have been fully affected. Plasmodesmata may not have been broken or upon osmotic swelling normal intercellular cytoplasmic connections may have reformed.

Although there are several reports of somatic embryogenesis in protoplast-derived cells, the validity of the findings is open to question in some cases. Fujimura and Komamine (1980a) clearly

demonstrated that very small cell aggregates of carrot, capable of passing through nylon mesh with 47 μm pore sizes, can give rise to somatic embryos. Yet some workers have attempted to separate protoplasts from residual undigested tissues, cells and cell aggregates by using filters of much larger pore size. Quite possibly embryogenic cell aggregates could have contaminated the protoplasts and given rise to the embryos observed. Some typical pore sizes used: 500 μm (Dos Santos et al. 1980), 100 μm (Facciotti and Pilet 1979; Kameya and Uchimiya 1972), 60 μm (Kao and Michayluk 1980), 50 μm (Vasil and Vasil 1980), 40 μm (Gosch et al. 1975), 30 μm (Vardi et al. 1975), glass wool of undefined pore size (Johnson et al. 1981), and no filtration at all (Zapata and Sink 1981; Lörz et al. 1977). Although Vardi et al. (1975) used a pore size considerably smaller than 47 μm to filter Citrus protoplasts other workers have macerated Citrus callus and filtered single cells with walls through 30 μm pores and demonstrated that these cells underwent embryogenesis (Button and Botha 1975). Discontinuous gradient centrifugation has been used to separate cells and cellular debris from protoplast preparations (Piwowarczyk 1979), however, somatic embryogenesis has not been demonstrated from any highly purified protoplast preparations. This presents the possibility that embryogenesis is not possible from protoplast-derived cells of any species. Unequivocal embryo formation from protoplast-derived cells could be achieved through production of hybrid embryos following protoplast fusion. Although somatic hybrid plants have been produced from protoplasts by organogenesis (Keller et al. 1981), embryogenesis in hybrid protoplast cultures remains to be demonstrated.

Another possible limitation on embryogenesis from protoplast-derived cells may result from culture conditions for embryogenesis in explants and cells being vastly different than those required of protoplast-derived cells. The present study and those by Thomas *et al.* (1976) and Vardi *et al.* (1975) have demonstrated a lack of success in achieving embryos from protoplast-derived cells using procedures which were successful for unprotoplasted cells and tissues. Considerable work remains on embryogenesis in protoplast-derived cells, particularly as an important application is plant regeneration following protoplast fusion, or genetic transformation.

Plant regeneration was achieved in eggplant protoplast-derived cells through induction of shoot organogenesis and subsequent rooting of the induced shoots. The only cytokinin to induce shoot organogenesis on protoplast-derived callus was kinetin although all cytokinins tested (kinetin, zeatin, BAP, 2iP) were able to induce shoots on leaf explants and suspension-derived cell colonies. Again this indicates that the protoplast-derived cells have noticeably different morphogenic capacity than the starting cell suspensions. The fact that morphogenesis in protoplast-derived cells is possible, however, gives hope that appropriate culture conditions for embryogenesis may be found. Nevertheless, it has been demonstrated for the first time that plants of eggplant can be regenerated from protoplasts, a prerequisite for somatic hybridization with this species.

CONCLUSIONS

This study has shown the various morphogenic responses possible by eggplant tissues, cells and protoplasts when cultured in vitro. Many physiological factors were shown to influence these responses.

An efficient bioassay was established to study the factors critical for somatic embryogenesis.

Most auxins promoted callus growth on leaf explants but explants supplied with NAA displayed a propensity for somatic embryogenesis. All growth regulators tested in association with NAA were detrimental to the embryogenic response. Somatic embryogenesis in eggplant tissues and cells was dependent upon exogenous NAA and no growth regulator tested could mimic this requirement.

Other species have not demonstrated a requirement for high concentrations of NAA which suggests somatic embryogenesis in eggplant is unique. This response is under genotypic control due to the quantitative results obtained with seven eggplant cultivars.

The composition of the basal medium was important since nitrogen content, monovalent cations, sugar content and osmotic potential all influenced the frequency of embryogenesis.

Although high frequencies of embryos were consistently produced on explants or from cell colonies, embryo culture usually only results in 30-40% successful plant regeneration. Further progress in the area of in vitro embryo culture may result in wide spread applications

of somatic embryogenesis. Valuable genotypes and sexually non-reproductive plants may be asexually propagated. High-frequency embryogenesis is also desirable for in vitro mutation selection and regeneration from cultured tissues or single cells.

Cell suspension cultures were good sources of viable protoplasts. Protoplast-derived cells and non-protoplasted cells differed in their responses to hormonal stimuli. Cytokinin-specific shoot organogenesis was possible from protoplast-derived cells. These shoots rooted and formed normal plants, displaying the prerequisite progression of single cell to whole plant necessary for somatic hybridization or genetic transformation.

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TABLE 6. Nutrient composition of SH-2 medium

Component		Conc. (mg/L)
Macronutrients	KNO_3	2,000
	$\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$	25
	Iron (Sequestrene Fe-330, Geigy)	40
Micronutrients	H_3BO_3	10
	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	10
	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25
	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025
Vitamins	Nicotinic acid	1
	Thiamine.HCl	10
	Pyridoxine.HCl	1
	Myo-inositol	100
Sucrose		2000

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APPENDIX I

TABLE I. Nutrient composition of MS-D 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 B₅ medium

Component		Conc. (mg/L)
Macronutrients	NaH ₂ PO ₄ ·H ₂ O	150
	KNO ₃	2500
	(NH ₄) ₂ SO ₄	134
	MgSO ₄ ·7H ₂ O	250
	CaCl ₂ ·2H ₂ O	750
	Iron (Sequestrene Fe-330, Geigy)	40
Micronutrients	MnSO ₄ ·H ₂ O	10
	H ₃ BO ₃	3
	ZnSO ₄ ·7H ₂ O	2
	Na ₂ MoO ₄ ·2H ₂ O	0.25
	CuSO ₄ ·5H ₂ O	0.025
	CoCl ₂ ·6H ₂ O	0.025
	KI	0.83
Vitamins	Nicotinic acid	1
	Thiamine.HCl	10
	Pyridoxine.HCl	1
	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 M₅₁C medium

Component		Conc. (mg/L)
Macronutrients	KNO ₃	2,000
	NH ₄ NO ₃	400
	MgSO ₄ ·7H ₂ O	370
	CaCl ₂ ·2H ₂ O	300
	KH ₂ PO ₄	275
	Fe-EDTA	40
Micronutrients	H ₃ BO ₃	3
	MnSO ₄ ·H ₂ O	10
	ZnSO ₄ ·7H ₂ O	2
	Na ₂ MoO ₄ ·2H ₂ O	0.25
	CuSO ₄ ·5H ₂ O	0.025
	CaCl ₂ ·6H ₂ O	0.025
	KI	0.83
Vitamins	Nicotinic acid	1
	Thiamine.HCl	10
	Pyridoxine.HCl	1
	Myo-inositol	100
Organic Nitrogen	Glutamine	300
	Casein hydrolysate	200
Sucrose		2000

TABLE 5. 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 6. Nutrient composition of SH-2 medium

Component		Conc. (mg/L)
Macronutrients	KNO_3	2,000
	$\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$	25
	Iron (Sequestrene Fe-330, Geigy)	40
Micronutrients	H_3BO_3	10
	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	10
	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25
	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025
Vitamins	Nicotinic acid	1
	Thiamine.HCl	10
	Pyridoxine.HCl	1
	Myo-inositol	100
Sucrose		2000

END

1 2 10 9 18 4

FIN