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**Tissue culture and cryopreservation
of *Daucus carota* L.**

Abstract

Cryopreservation offers a viable and safe storage method for germplasm in contrast to more traditional germplasm conservation methods, but can be technically difficult and can involve the use of expensive equipment. The present study achieved the production and maintenance of callus and suspension cultures and somatic embryos of *Daucus Carota* L. for use in cryopreservation experimentation. The effects of dehydration and the use of the cryoprotectant glycerol on the cryostorability of carrot callus using an ultra-fast freeze technique was also explored. Successful cryopreservation was achieved using this technique without dehydration or cryoprotection of the callus material, but with a relatively low efficiency of 23%. Dehydrating callus material improved cryopreservation efficiency to 38%, but the highest efficiency (65%) was observed when callus was dehydrated in conjunction with cryoprotection using a glycerol solution.

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1. Introduction and literature review.

1.1 The importance of germplasm conservation.

Increasing human population size and increased needs of industry have led to escalating and indiscriminate clearing of forest and agricultural land. This has resulted in the extinction of many plant species, and it is believed that many more species face extinction. Apart from the negative impact the loss of species may have on the ecosystem itself, these extinctions result in the depletion of the naturally occurring germplasm pools of the medicinal, forest and crop plants (Sen and Giles, 1983).

Modern agricultural practices, selection breeding and land clearance have resulted in the loss of many wild species and primitive cultivars, which are the traditional sources of genetic variability (Rao, 1981). The emphasis placed on agriculture today is increased yield, which tends to result in production and cultivation of crops with restricted genetic diversity. This approach satisfies the immediate demand for food, but the consequences of a disease outbreak, pest infestation, or climatic perturbation could be devastating to a population of limited genetic variability and consequent narrow response range to challenges (Rao, 1981).

Advances in biotechnology have allowed for the production of novel transgenic plants (e.g. Monsanto's herbicide resistant cotton). The future usefulness of the transgenic product is therefore dependent on the maintenance of the genetic identity and integrity of the product in question, which could be a cell culture, organ culture or whole plant. This consideration also applies to agronomically important strains which may be prone to genetic variation (Bock and Marsh, 1988).

All the above reasons have created a situation where the availability of a reliable, affordable method of germplasm preservation is of utmost importance.

1.2 Methods of germplasm conservation/preservation.

1.2.1 Seed storage

Storage of seeds is the traditional method of germplasm preservation. However the storage of seeds is limited to those species which are said to show "orthodox" viability characteristics. Retention of viability of these seeds can be increased by storing them in sealed containers at -18°C or less, and a moisture content of between 5 and 7% (Rao, 1981). Seed storage of orthodox seed type species is, at best, effective only in the short term, and it is not without risk. Seeds of certain species deteriorate rapidly due to seed borne pathogens whilst seeds of other species are heterozygous and, therefore, not suitable for maintaining true to type genotypes (Rao, 1981). Several staple root and tuber crops, and a number of tropical and temperate fruits are vegetatively propagated. In some of these cases the material is sterile and in others gene combinations would be lost by conversion to seed (Rao, 1981).

1.2.2 Growth of plants in field banks

A number of species produce seeds that are sensitive to low humidity and temperature, and are termed "recalcitrant", in contrast to orthodox. Even if recalcitrant seeds are stored in a moist condition, their longevity is relatively short, varying from a few weeks to a few months (Bewley and Black, 1994). An example of this is the coconut (*Cocos nucifera*), with these species there is no efficient method of propagation, and the commonly used method used today for reproduction, i.e. - by suckers arising from the base of the main stem, is slow and unreliable (Dodds and Roberts, 1985).

For the plant species where seed banks are impractical or impractical, germplasm preservation is achieved by the maintenance of these plants in field gene banks. These are expensive to run and are prone to catastrophic losses due to attack by pest and pathogen, climatic disorders, natural disasters, and are in some cases dependent on political and economic stability (Dodds and Roberts, 1985).

These problems have created the need to develop alternative methods for the storage, maintenance, and conservation/preservation of germplasm, and to explore the possibilities of the creation of germplasm banks of more rare plant material (Sera and Giles, 1983). A partial solution to the problems associated with traditional methods of germplasm conservation was provided by the development of *in vitro* technology.

1.2.3 *In-vitro* growth of plant material

Based on the phenomenon of totipotency of plant cells, (i.e.- all living cells containing a normal complement of chromosomes should be capable of regenerating the entire plant), tissue culture techniques have been extensively used in cloning and rapid propagation of many economically important plant species (Bhajwani, 1990).

Plant tissue culture is the art of growing plant cells, tissues, or organs isolated from the mother plant on artificial media (George, 1993). There are two categories of tissue culture, i.e.- cultures of unorganized tissues, and cultures of organized tissues. Although the term tissue culture is commonly used to describe all kinds of *in vitro* plant cultures, strictly speaking, it should only refer to cultures of unorganized aggregates of cells (George, 1993).

The existence of higher plants depends on the organized allocation of functions to different parts which in consequence become differentiated (i.e.- modified and

specialized to enable them to perform their specific functions). Unorganized growth is seldom found in nature, but occurs fairly frequently when pieces of whole plants are cultured *in vitro*. The tissues which are then formed typically lack any recognizable structure and contain only a limited number of the many kinds of specialized and differentiated cells found in an intact plant (George, 1993).

[1] Unorganized cell cultures.

Cultures of unorganized tissues can be divided into four different types.

- 1- Callus (or tissue) cultures. This is the growth and maintenance of largely unorganized cell masses which arise from the uncoordinated and disorganized growth of small plant organs, pieces of plant tissue, or previously cultured cells.
- 2- Suspension (or cell) cultures. This is the growth of plant cells and small cell clumps, dispersed in an agitated liquid medium.
- 3- Protoplast cultures. The culture of plant cells that have been isolated without a cell wall.
- 4- Anther cultures. The culture of complete anthers containing immature pollen microspores. The objective is usually to obtain haploid plants by the formation of somatic embryos directly from the pollen, or sometimes by organogenesis via callus (George, 1993).

Germplasm conservation via unorganized cell/tissue cultures has the major disadvantage of unacceptable genetic instability usually being associated with the methodology, and consequently this is seldom the method of choice (Rao, 1981).

[2] Cultures of organized structures.

Organ culture is used as the general term for those types of Culture in which an organized form of growth can be continuously maintained. For the purposes of plant propagation the most important types of organ culture are the following:

1- Meristem cultures. These are grown from very small shoot apices, each consisting of the apical meristematic dome with or without one or two leaf primordia. The shoot apex is typically grown to give one single shoot.

2- Shoot tip, or shoot cultures. These are initiated from shoot tips, or buds, larger than the shoot apices employed to establish meristem cultures and have several leaf primordia. These shoot apices are usually cultured in such a way that each produces multiple shoots.

3- Node cultures. These are of separate lateral buds, each carried on a small piece of stem tissue; stem pieces carrying either single or multiple nodes may be cultured. Each bud is grown to provide a single shoot.

4- Isolated root cultures. This is the growth of roots, unconnected to shoots.

5- Embryo cultures. These are produced by dissecting out developing seeds or fruits from fertilized or unfertilized zygotic embryos, and culturing *in vitro* until they have grown into mature seedlings. Embryo culture is quite distinct from somatic embryogenesis.

Complete plants can also be derived through the formation of somatic embryos, which resemble the seed embryos of intact plants, and which can grow into seedlings in the

in-vitro manner. This process is called somatic embryogenesis (George, 1993), [see later].

1.3 Nutrition and growth of *in vitro* plant material

1.3.1 Culture Media

Nutrition was the earliest aspect of plant tissue culture investigated. The development of a high salt medium (Murashige and Skoog, 1962), and derivatives thereof paved the way for the culture of the vast number of plant species so far achieved (Bhajwani, 1990). In contrast to animal cell culture, plants can generally be cultured on defined media, containing 5 classes of substances, namely inorganic macro- and micro-nutrients, a carbon/energy source, a reduced form of nitrogen, some B vitamins and phytohormones to achieve growth and organized development (Bhajwani, 1990).

Plants absorb the nutrients they require from soils, almost entirely as ions (i.e. - an atom, or group of atoms, which has either a positive charge [cation], or negative charge [anion]). Macronutrients, (and most micronutrients) are provided in plant culture media in the form of salts. In weak aqueous solutions, such as plant media, each soluble salt dissociates into two ions, one positively charged, one negatively charged (George, 1993), and is therefore easily absorbed by the plant tissues

[1] Macronutrients

Calcium, magnesium and potassium are absorbed by plant cells (normally those of the root) as the respective cations (Ca^{2+} , Mg^{2+} , and K^{+}); Nitrogen is mainly absorbed in the form of ammonium (the cation, NH_4^{+}), or nitrate (the anion NO_3^{-}); phosphorus as the phosphate ions (HPO_4^{2-} and $\text{H}_2\text{PO}_4^{-}$); and sulfur as the sulfate ion (SO_4^{2-}) (George, 1993).

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The most important step in deriving a plant tissue culture medium is the selection of macronutrient ions in the correct concentration and balance. The salts normally used to provide the macroelements in tissue culture media also provide ions of the elements sodium (Na) and chloride (Cl). Because plant cells tolerate high concentrations of Na^+ and Cl^- without injury, these ions are frequently given little importance when contemplating media changes (George, 1993).

[2] Micronutrients

In addition to the macronutrient elements, plant cells require traces of certain micronutrients. Most modern culture media use the relatively concentrated mixtures found in the medium recommended by Murashige and Skoog [MS] (1962). Micronutrient elements required by all higher plant cells include iron, manganese, zinc, boron, copper, molybdenum, and chlorine. Sodium is not generally required by higher plants, but this element may be a micronutrient for cultures of halophyte tissue, plants with C_4 photosynthetic pathways, and plants with Crassulacean acid metabolism (CAM) (George, 1993). As mentioned above, sodium and chlorine are usually present in large amounts from the macronutrient salts. An iron stock solution is prepared separately because of the problem of iron solubility. Usually the iron stock is prepared in the chelated form as the sodium salt of ferric ethylenediamine tetra-acetate (Na-FeEDTA) (Dodds and Roberts, 1985).

In addition to the known micronutrients, some media contain traces of cobalt and iodine. Whilst the possible requirement of nickel, titanium, beryllium, and aluminum remain questionable (Dodds and Roberts, 1985), one unresolved problem is that even the purest chemical reagents contain traces of inorganic contaminants, and these elements constitute a hidden source of micronutrients. Agar is also a source of numerous mineral elements (Dodds and Roberts, 1985).

[3] Micro-organic supplements

Growth and morphogenesis of plant tissue cultures can be improved by small quantities of some organic nutrients. These are mainly vitamins (including some substances that are not strictly animal vitamins), amino acids and certain undefined supplements (George, 1993).

Vitamins are compounds required by animals in very small amounts as necessary auxiliary food factors. Absence from the diet leads to abnormal growth and development and an unhealthy condition. Many of the same substances are also needed by plant cells as essential intermediates or metabolic catalysts, but intact plants, unlike animals, are able to produce their own requirements. Cultured plant cells and tissues can however become deficient in some factors; growth and survival is then improved by their addition to the medium (George, 1993).

Certain authors consider that thiamin (vitamin B₁) may be the only essential vitamin for nearly all plant tissue cultures, whereas nicotinic acid (niacin) and pyridoxine (vitamin B₆) may stimulate growth. These three compounds and myo-inositol are almost always included in media - and are ingredients of MS medium (Dodds and Roberts, 1985).

Some other vitamins that have been used in plant tissue culture media include p-aminobenzoic acid (PABA or vitamin B_x), ascorbic acid (vitamin C), biotin (vitamin H), choline chloride, cyanocobalamin (vitamin B₁₂), folic acid (vitamin Bc), calcium pantothenate, and riboflavin (vitamin B₂) (Dodds and Roberts, 1985).

With the exception of glycine (aminoacetic acid), which is a component of several media, amino acids are seldom added to plant culture media.

The trend in modern plant tissue culture has been to attempt to define the constituents of a given medium and eliminate the use of crude natural extracts (e.g. yeast extract, potato extract and coconut milk). However, these undefined additives are still useful if chemically defined media fail to produce the desired results (George, 1993).

1.3.2 Carbon sources

Only a limited number of plant cell lines have been isolated which are autotrophic when cultured *in vitro*. Autotrophic cells are capable of fully supplying their own carbohydrate needs by carbon dioxide assimilation during photosynthesis. Normally, for the culture of either cells, tissues or organs, it is necessary to incorporate a carbon source into the medium. Sucrose is almost universally used (usually at a concentration of between 20 000 and 30 000mg/l) for micropropagation purposes as it is so generally utilizable by tissue cultures (George, 1993).

A partial hydrolysis of sucrose takes place during the autoclaving of media, since sucrose is heat labile. This results in a combination of sucrose, D-glucose and D-fructose (the extent being greater when the compound is dissolved together with the other medium constituents than when it is autoclaved in pure aqueous solution (George, 1993)). Such an autoclaved medium may give completely different results compared with a medium containing filter-sterilized sucrose (Dodds and Roberts, 1985).

1.3.3 Osmotica

The uptake of water by plant cells in culture is governed by the relative water potential values between the vacuolar sap and the external culture medium, which in turn is determined by the relative concentrations of dissolved substances in the external and

internal solutions, and by the pressure exerted by the restraining cell wall (Dodds and Roberts, 1985; George, 1993).

One colloidial characteristic of the gel state of agar is the imbibitional retention of water within the micelles of the gel. Carbohydrates, as well as the solutions of inorganic salts, not only function for the carbon source and other nutritive requirements in metabolism, but they also play an important role in the regulation of the external osmotic potential. Often weakly metabolized sugar, e.g., mannitol or sorbitol, is used as an external osmoticum (Dodds and Roberts, 1985).

Somatic embryogenesis can be influenced by alterations in osmoticum as can the proper development of embryos. A low osmotic potential is often (although not always) favourable (George, 1993).

1.3.4 Plant growth regulators

Some chemicals occurring naturally within plant tissues (i.e.-endogenously), have a regulatory, rather than a nutritional role in growth and development. These compounds, which are generally active at very low concentrations, are known as plant growth substances (or plant hormones). Synthetic chemicals with similar physiological activities to plant growth substances, or compounds having the ability to modify plant growth by some other means, are usually termed plant growth regulators, (the term plant growth regulator also implies that the substance has been applied exogenously - and can therefore also refer to a natural growth substance prepared through fermentation processes) (George, 1993).

The five principal classes of plant growth substance are: Auxins, cytokinins, gibberellins, ethylene and abscisic acid.

Auxins and cytokinins are by far the most important for regulating growth and morphogenesis in plant tissue and organ cultures. In these classes, synthetic regulators have been discovered with a biological activity which equals or exceeds that of the equivalent growth substances.

No chemical alternatives to the natural gibberellins or abscisic acid are commercially available, but some natural gibberellins are extracted from cultured fungi and are available for use as exogenous regulators. However, several classes of chemicals, which are highly effective in blocking the synthesis of gibberellins within the plant are very effective growth regulators. They are usually termed anti-gibberellins or growth retardants.

Exogenous ethylene can be used as a growth regulator, but, being a gas, it is difficult to administer and control the available concentration.

Growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators supplied in the medium, and the growth substances produced endogenously. The effects of growth regulators are generally not absolute and specific - the responses of cells, tissues and organs *in vitro* can vary according to cultural conditions, the type of explant and the plant genotype (George, 1993).

[1] Auxins

These are very widely used in micropropagation and are incorporated into nutrient media to promote the growth of callus, cell suspensions or organs and to regulate morphogenesis, especially in conjunction with cytokinins (George, 1993).

Compounds are called auxins if they are capable of controlling various distinctive processes such as cell growth and cell elongation. Auxins are capable of initiating cell

division and are involved in the formation of meristems giving rise to either unorganized tissue, or defined organs. In organized tissue, auxins are responsible for the maintenance of apical dominance (George, 1993).

The most commonly detected natural auxin is IAA (3-indolyl-acetic acid), which is formed from the amino acid L-tryptophan (L-tryptophan can act as an auxin replacement in some plants) (George, 1993).

Auxins are thought to promote the growth of plant tissues in two ways. Firstly, by inducing the secretion of hydrogen ions into and through the cell wall. Potassium ions are taken into the cell to counteract the electrogenic export of H^+ ions (protons) and this has the effect of decreasing the water potential of the cell so water enters and the cell expands. Secondly, binding of auxin to the cell membrane may lead to lipid breakdown and acidification of the cell wall increasing its extensability. The first explanation however is now commonly accepted as a more favourable explanation for the rapid growth stimulating effect of auxin (George, 1993).

The second mechanism by which auxins are thought to promote growth is by an effect on RNA metabolism, possibly by inducing transcription of specific mRNA molecules, which are thought to encode proteins which are required for sustained growth (George, 1993).

Applied auxins seem to be capable of erasing the genetically programmed physiology of whole plant tissues, which had previously determined their differentiated state. Cells which respond to auxin revert to a dedifferentiated state and begin to divide (how this reprogramming is achieved is not completely understood) (George, 1993).

The auxins most commonly used in tissue cultures are: 2,4-D (2,4-dichlorophenoxyacetic acid); IBA (3-indolylbutyric acid); NAA (1-naphthalene acetic

acid) which are synthetic; and IAA (indolyl acetic acid), which is natural. Together with cytokinins, 2,4-D is used primarily for callus induction and suspension cultures, being replaced by NAA and IBA when morphogenesis is required. NAA and IBA are favored auxins for shoot culture. The process of somatic embryogenesis is often initiated in media containing high levels of auxins (especially 2,4-D), but embryos do not develop further until the auxin concentration is reduced (George, 1993).

[2]Cytokinins

Cytokinins are often used in conjunction with auxins to stimulate cell division and morphogenesis in tissue cultures. When added to shoot cultures, these compounds overcome apical dominance and release lateral buds from this dominance, therefore in this respect, they appear to have an opposite effect to endogenous auxins (George, 1993). Kinetin was the first cytokinin to be isolated, although this does not appear to be a naturally occurring cytokinin, but is believed to have been formed in the original isolates by structural rearrangement of another compound. To date at least 25 natural cytokinin plant growth substances that are structurally related to kinetin have been identified. Two such compounds routinely used in plant tissue work are zeatin (4-hydroxy-3-methyl-trans-2-butenylaminopurine), of which only the trans configuration has cytokinin activity, and 2-iP (N^6 (2-isopentyl)adenine). There are several chemical analogs of natural cytokinins, apart from kinetin, that are highly active as cytokinins. Along with kinetin, most commonly used are BAP (6-benzylaminopurine), and PBA (6-(benzylamino)-9-(2-tetrahydropyranyl)-9H-purine).

The mode of action of cytokinins is uncertain, but in tissue cultures, their effects appear to be necessary in plant cell division. Callus proliferation from the tissues of most dicotyledonous plants is usually thought to require the presence of both an auxin and a cytokinin. A low concentration of cytokinin (typically 0,5 - 2,5 μ M) is often added to the media for the induction of embryogenic callus, especially in broad leaved

plants. There is however, some evidence to suggest that cytokinins may inhibit embryogenesis in monocots. In fact the presence of endogenous cytokinins may be responsible for the inability to obtain embryogenesis in some genotypes. Cytokinins are very effective in promoting direct or indirect shoot initiation and are routinely used in commercial micropropagation.

1.4 Initiation and maintenance of callus, suspension cultures, and somatic embryos

1.4.1 Callus.

A callus, consisting of an amorphous mass of loosely arranged thin walled parenchyma cells, arising from the proliferating cells of the parent tissue, can sometimes be induced to form as a result of wounding, (e.g. at the cut end of a stem or root). Callus can also be induced by certain microorganisms (e.g. as a result from infection by *Agrobacterium rhizogenesis*). Callus formation has been observed to occur naturally in gymnosperms, ferns mosses and liverworts.

In vitro initiation of callus can be achieved by placing small pieces of the whole plant (explants) onto a growth supporting medium under sterile conditions. Under the stimulus of endogenous growth substances or growth regulating chemicals added to the medium, the metabolism of cells is changed and they begin active division. During this process, cell differentiation and specialization, which may have been occurring in the intact plant, are reversed, and the explant gives rise to new tissue which is composed of the meristematic and (mostly) unspecialised cell types typical of callus (George, 1993).

Many parts of a whole plant may have an ultimate potential to proliferate *in vitro*, but it is frequently found that callus cultures are more easily established from some organs than others. Young meristematic tissues are most suitable, but meristematic areas in older plants, such as the cambium, can give rise to callus. The choice of tissues from which callus can be started is greatest in dicotyledonous species (George, 1993).

The general growth characteristics of a callus involve a complex relationship between the plant material used to initiate the callus, the composition of the medium, and the environmental conditions during the incubation period. Establishment of a callus from the explant can be divided roughly into three developmental stages: induction, cell division, and differentiation. During the initial induction phase metabolism is stimulated as the cells prepare for division. The length of this phase depends mainly on the physiological status of the explant cells as well as the cultural conditions. Subsequently, there is a phase of active cell division as the explant cells revert to a meristematic or de-differentiated state. The third phase involves the appearance of cellular differentiation, and the expression of certain metabolic pathways that lead to the formation of secondary products (Dodds and Roberts, 1985).

In regard to callus anatomy, there is considerable variability in the extent and type of cellular differentiation. A homogeneous callus consisting entirely of parenchyma is rarely found. Cytodifferentiation occurs in the form of tracheary elements, sieve elements, suberized cells, secretory cells, and trichomes. Small nests of dividing cells form meristemoids, or vascular nodules, that may become centers for the formation of shoot apices, root primordial or incipient embryos. Vascular nodules typically consist of discrete zones of xylem and phloem separated by a cambium. The location of the nodules within the callus can be modified by altering the composition of the medium (Dodds and Roberts, 1985).

The callus formed on an original explant is called the primary callus. Secondary callus cultures are initiated from pieces of tissue dissected from primary callus. Subculture can therefore be continued over time (sometimes over a number of years), but the longer callus is maintained, the greater is the risk that the cells will suffer genetic change (George, 1993).

Generally callus tissue is not of one kind. Strains of callus differing in appearance, colour, degree of compaction and morphogenic potential commonly arise from a single explant. Some callus growths are heavily lignified and hard in texture, whereas others break easily into small fragments. Fragile growths that readily separate are termed friable cultures, and this type is usually selected for initiating suspension cultures (Dodds and Roberts, 1985).

The first successful prolonged cultures of experimentally induced callus were achieved almost simultaneously in 1939 in the laboratories of Gautheriet in Paris, Nobecourt in Grenoble, and White in Princeton. These cultures were originally derived from the explants of cambial tissue of carrot and tobacco (Dodds and Roberts, 1985).

The most important characteristic of callus, from a functional viewpoint is that this abnormal growth has the potential to develop normal roots, shoots and embryoids that can form plants (Dodds and Roberts, 1985).

1.4.2 Suspension culture

Cell, or suspension cultures are usually initiated by placing an inoculum of friable callus in liquid medium. Using agitation, single cells break off and, by division, form cell chains and clumps which fracture again to give individual cells and small cell groups (George, 1993).

A suspension culture originates with a random critical event - in which some cells undergo a transition in metabolism and growth, to produce a cell line. Some of the characteristics of a cell line include: a high degree of cell separation; homogenous cell morphology; distinct nuclei and dense cytoplasm; starch granules; relatively few trachery elements; doubling times of 24 - 72 hrs; loss of totipotency; hormone habituation and increased ploidy levels (King, 1980). Suspension cultures vary considerably in the expression of these and other cell line characteristics.

Plant cells have a natural tendency to adhere together, and this makes it impossible to obtain suspensions which consist of only dispersed single cells. The proportion and size of small cell aggregates varies according to plant variety and the medium in which the culture is grown (George, 1993).

The degree of cell dispersion in cultures is particularly susceptible to the concentration of growth regulators in the culture medium. For instance, auxin increases the specific activity of enzymes which bring about the dissolution of the middle lamella of plant cell walls. Thus by using a relatively high concentration of an auxin and a low concentration of a cytokinin growth regulator in the culture medium, it is usually possible to increase cell dispersion. However, high auxin levels will also ensure that the cells remain undifferentiated, and this is not always desirable (Narayanswami, 1977).

The growth of plant cells is more rapid in cell suspension than in callus cultures and is also more readily controlled because the culture medium can be easily amended or changed. Organs can be induced to develop in cell suspensions: root and shoot initiation usual commences in cell aggregates, whereas somatic embryos may arise from single cells (George, 1993).

Many different methods of suspension culture have been developed, and generally fall into two main types: 1.] Batch cultures, in which cells are nurtured in a fixed volume of medium until growth ceases, and 2.] Continuous cultures in which cell growth is maintained by continuous replacement of sterile nutrient media. All techniques utilize some method of agitating the culture medium to ensure cell dispersion and adequate gas exchange (George, 1993).

To initiate a suspension culture, approximately 2 - 3g of callus is required to serve as the inoculum, per 100ml suspension medium. When the plant material is first placed in the medium, there is a lag period prior to any visible signs of cell division. Thereafter there is an exponential rise in cell number, which usually becomes linear, followed by a gradual decrease in the cell division rate. Finally the cells enter a stationary or non-dividing stage. In order to maintain the viability of the cells, the culture should be subcultured early during the stationary phase, or during the phase of decelerating cellular division (Dodds and Roberts, 1985).

1.4.3 Somatic embryogenesis

New organs such as shoots and roots can be induced to form on cultured plant tissue, in a process known as morphogenesis. It is also possible to obtain embryos that are structurally similar to the embryos found in true seeds, from cells or tissues of the plant body. These are known as somatic embryos and develop via the process of somatic embryogenesis.

Morphogenetic meristems (from which organs or somatic embryos form) can theoretically occur in either of two distinct ways: 1.] from the differentiated cells of a newly transferred piece of whole plant tissue, without the proliferation of undifferentiated tissue; and 2.] from the unspecialised, unorganized and dedifferentiated cells of callus tissues and suspension cultures. These two methods of

morphogenesis are described as direct and indirect, respectively. In practice, however it is not always possible to distinguish between the two methods (Hicks, 1980).

Embryos can be distinguished from adventitious shoots because they are bipolar, having both a root and a shoot apex and cotyledon (or a coleoptile and a scutellum in monocotyledons) or a similar venation to that found in zygotic embryos of the species in question. Somatic embryos nearly always arise superficially on callus and, having no vascular connection with the underlying parental tissue, are detached easily from the surrounding cells (George, 1993).

It is generally agreed that somatic embryos are formed from cells which are typically meristematic and are therefore unlike the characteristic vacuolated parenchymatous cells found in callus and suspension cultures. These embryogenic cells have dense cytoplasm and large nuclei and often contain many starch grains (McWilliam *et al.*, 1974). It has however not yet been resolved whether embryogenic cells, and hence somatic embryos, always originate from single determined cells, or if they sometimes have a multicellular origin (George, 1993).

Somatic embryogenesis was first observed in suspension cultures of carrot (*Daucus carota* L. (Steward *et al.*, 1958). In that species the process requires some callus proliferation before embryos arise from induced embryogenic cells within the callus (Hicks, 1980). Although individual carrot cells are totipotent and therefore carry all the genetic templates necessary for the development of the whole plant, isolated single cells do not generally become transformed into embryos by repeated divisions. Embryoids are initiated in carrot callus from superficial clumps of cells associated with highly vacuolated cells that do not take part in embryogenesis (Dodds and Roberts, 1985).

Each developing embryoid passes through the sequential stages of embryo formation, that is, the globular, heart and torpedo stages. Two critical events are involved in the early programming of this process - the induction of cytodifferentiation of the proembryoid cells, and the unfolding of the developmental sequence by these proembryoid cells. Although a given culture may differentiate these embryogenic cells, their further development may be blocked by an imbalance of chemicals in the culture medium. Abnormalities known as embryonal budding and embryogenic clump formation may occur, if differentiated embryogenic cells are subjected to relatively high levels of auxin. Consequently, two distinctly different types of media may be required, one medium for the initiation of the embryogenic cells, and another for the subsequent development of the cells into embryoids. The induction medium must contain an auxin, whilst the second medium generally consists of a mixture with a lower concentration of the same auxin, or lacking auxin (Dodds and Roberts, 1985).

Suspension cultures of carrot maintained in a liquid medium (MS), containing the auxin 2,4- D, continue to grow as undifferentiated callus cells, but when subcultured into the same medium without auxin, differentiation begins and embryoids develop (McWilliam *et al.*, 1974). It appears that the most important chemical factors involved in the carrot induction medium are auxin and reduced nitrogen. The role of cytokinins in embryogenesis is somewhat obscure because of conflicting results - although zeatin (0,1 μ M) stimulates embryogenesis in carrot cell suspensions during the auxin free subculture, the process is inhibited by the addition of either kinetin or benzylaminopurine (Dodds and Roberts, 1985).

Supplementing the medium with activated charcoal has facilitated embryogenesis in several species, including carrot where the addition of 1% w/v charcoal resulted in embryoid development where previous auxin depletion had failed to produce the desired results. This may be due to adsorption of a variety of substances inhibitory to

embryogenesis, in particular phenylacetic acid and benzoic acid derivatives by the charcoal (Drew, 1979).

In general, embryogenesis occurs most readily in short term cultures, and this ability decreases with increasing duration of culture. Embryoid formation begins in carrot cultures about 4 - 6 weeks after isolation of the tissues, and an optimum embryogenic potential is reached after about 15 weeks. After the embryogenic potential has apparently been lost following 36 weeks *in vitro*, the carrot cultures can once again be induced to produce embryoids by transfer to an appropriate medium (Smith and Street, 1974).

1.4.4 Strategies for extending culture preservation times.

Germplasm conservation using tissue culture usually involves the culture of shoots or whole plantlets. The plants cannot be maintained in culture indefinitely, as after a period of time the material will have to be subcultured. Subculturing becomes necessary when the physical size of the plant becomes too great for its container, or when toxic metabolites, resulting from the growth of the plant in a sealed container, becomes detrimental to the growth. After a period of time the medium will become exhausted, or will dry out, which also necessitates replacement of the medium. The interval (or passage) between subcultures primarily depends on the rate at which the culture is grown. The interests of germplasm conservation are best served if the rate of growth is minimized (George, 1993). This is because each subculturing introduces a risk of contaminating the stock, and the labour costs are reduced by increasing the interval time between subcultures.

The interval time between subculturing can be extended by making use of two approaches, viz., - using growth retarding compounds, or applying growth retarding

conditions. A variety of growth retarding chemicals, for example maleic hydrazide or abscisic acid (ABA), have been used to reduce the growth rate of *in vitro* plantlets.

Alternatively if the tissue cultures are grown at reduced temperature, (above freezing) for example 1 -9 ° C, the growth rate and aging can be significantly slowed but not stopped. This however is not possible for all species (Bhajwani, 1990). A similar effect can sometimes be achieved by providing low light conditions, low oxygen conditions, or simulating water stress by adding osmotically active, but otherwise inert compounds into the artificial medium, (e.g. polyethylene glycol or manitol).

This approach can reduce the labour related costs associated with subculturing, although one problem may exist in that over time the stressful conditions may select for particular strains better equipped to tolerate the stresses, and thereby defeating the object of germplasm preservation. This method of germplasm storage is considered a short term solution, as cultures usually lose viability after a time characteristic to the species, with 10 years being the longest culture time achieved so far (Bock and Marsh, 1988). Additionally, some risk of loss must accrue at each subculturing operation and during each storage interval. For truly satisfactory long term storage, methods are needed that eliminate renewal operations by suspending growth (Bock and Marsh, 1988), and this is one of the aims of the present experimentation.

1.5 Cryopreservation

The freeze preservation of plant cells and tissue is a relatively recent approach to germplasm conservation which may provide the means for long term and stable germplasm storage. The principle underlying the freeze preservation basically involves bringing the cells to a state of inactive metabolism or "suspended animation" by subjecting the cells/tissue to super low temperatures, i.e. - at or near the temperature of liquid nitrogen (-196° C). This is known as "cryopreservation" (Sen and Giles, 1983).

At this temperature, cell division is completely arrested and metabolic activities immobilized.

The chances of successfully freezing, and then thawing biological tissue whilst still retaining viability appear to be inversely related to the size of the tissue specimen. This fact makes the successful freeze preservation of cell suspensions or cell cultures the easiest to achieve. However, suspension cultured plant cells or callus are less than ideal material for the purpose of germplasm preservation because of genetic changes which may occur during growth before and after freezing. For this reason, the emphasis has shifted to the storage of shoot apices, asexual embryos, and young plantlets (Bhajwani, 1990).

Dry seeds remain dormant because living cells become metabolically inert when deprived of water in the liquid phase. Biochemical reactions which are dependent on the presence of water are then suspended, especially if the seeds are also stored at low temperature. In theory, the withdrawal of water should permit plant tissues other than seeds and zygotic embryos, to be stored (George, 1993).

Nearly all the damage that may result from the transfer of cells to very low temperatures, or subsequent thawing, is a consequence of their water content, or, more specifically, intracellular ice crystal formation during cooling and thawing. A possible exception is the damage caused by interference with biochemical or biophysical pathways during temperature reduction (George, 1993).

Intracellular ice formation occurs at random temperatures during cooling and, in some systems, at random times in an isothermal system (Pitt, 1992; Macfarlane *et al.*, 1992). The possible mechanisms by which intracellular ice formation can occur are homogenous nucleation, heterogeneous nucleation, or seeding by the extracellular ice. Homogenous nucleation of ice is the spontaneous generation of new crystal nuclei in

the liquid without the assistance of a foreign surface. Heterogeneous nucleation on the other hand relies on the presence of intracellular nucleating agents and this can be any biomolecule. The molecular basis for seeding by the extracellular ice is not well understood, but it has been proposed that the intracellular ice formation occurs as a consequence of ice crystal growth through aqueous channels in the plasma membrane.

Living cells are seriously damaged by the internal formation of ice crystals which rupture cell membranes, notably the plasmalemma and tonoplast. The main objective of cryopreservation protocols therefore must be to change the physical state of water within cells to a frozen, glass like condition (which will not cause membrane damage) without crystallization or excessive cellular dehydration. The transformation of water from a liquid to a solid glass like phase is called vitrification (George, 1993).

1.5.1 Slow/stepwise freezing

For a given set of cell characteristics there exist a range of cooling rates above which intracellular ice formation becomes probable. At lower cooling rates, a different mechanism of injury, often ascribed to cell dehydration, becomes predominant. Hence there exists an optimum rate of cooling for cell survival (cooling rates far above this optimum rate can also be sufficient to avoid lethal ice crystal formation - see ultra fast freezing). The development, therefore, of a conventional cryopreservation procedure for a given cell type includes a determination of the optimum cooling rate which minimizes the intracellular ice formation (Pitt, 1992).

The response to the rate of freezing is dependent on the size of the sample being frozen, and great variation in response is shown by different species. To date, slow cooling (bringing about protective dehydration), has been the most successful approach to cryoprotection. This involves a freezing program with cooling rates of between 0.5° and 2.0° C/min to between -30° C and -40°C, and holding at that

temperature for between 10 - 60min, before plunging into liquid nitrogen. There is little evidence to suggest that the rate of freezing below -40°C has any effect on the success of the procedure (Vasil, 1984).

One of the problems with the slow freezing approach is that expensive equipment is required, and a universal freezing protocol does not exist for all species - requiring new protocols to be developed with each species to be stored. Additionally, not all plant species can be stored using the slow freezing approach - for example, recalcitrant seed embryos which are wet and desiccation sensitive (Berjak *et al.*, in press)

Other freezing methods include rapid stepwise reductions in temperature until the tissue has reached -40°C , or rapid freezing by direct immersion into liquid nitrogen. Neither of these techniques have been as successful as the slow freezing approach.

1.5.2 Cryoprotection

At present it is very difficult to freeze biological specimens to sub-freezing temperatures without adversely affecting the viability. The utilization of chemicals with cryoprotective properties paved the way for the development of effective cryopreservation techniques, the most important of these "cryopreservants" being glycerol and dimethyl sulfoxide (DMSO) (Withers, 1979).

Substances acting as cryoprotectants are said to depress both the freezing and supercooling points of pure water, thereby reducing the amount of water available to freeze (Mycok, Watt and Berjak, 1993). Often these cryoprotectants are more effective if used in combination. Another possible advantage to using the cryoprotectant DMSO (by virtue of its free radical scavenging activity) is the protection afforded it against damage from free radicals. This may be important over

time, as free radical formation is one of the processes which can occur at the temperature of liquid nitrogen (George, 1993).

The various chemicals used as cryoprotectants have little in common except high solubility in water. Although their mode of action is not entirely clear; it is believed their action may depend on an ability both to reduce the concentration of salts within the cell, and reduce the growth of damaging ice crystals (George, 1993).

However the use of cryoprotectants has some important disadvantages, in that they can be poisonous to plant tissue (e.g. dimethyl sulphoxide), the extent of toxicity varying with the type and concentration of the cryoprotectant, and the plant species in question. Additionally, it is often necessary to remove the cryoprotectant from the tissues after retrieval from the cryogen, which complicates the procedure by adding an additional step, at a point which the plant material is very fragile (Berjak *et al.*, 1995).

1.5.3 Ultra-fast freezing

An alternative to the conventional, slow freezing, procedure has been attempted with limited success. This is rapid freezing, which is achieved by direct immersion of the plant material into liquid nitrogen, e.g. Mycock, Watt and Berjak, 1993. The theory behind this approach is that if the tissue passes through the temperature range at which lethal intracellular ice crystal damage occurs sufficiently quickly, intracellular ice crystal formation is either prevented, or only small discreet, non injurious ice crystals form - and the intracellular contents pass directly into a vitrified state (Berjak *et al.*, 1995).

The limited success of this approach may not be due to faulty logic, but because sufficient cooling rates were not achieved in the experimental work, (most commonly, cooling rates of approximately several hundred ° C were attempted). Following the

observation that the more rapidly plant tissue can be frozen for electron microscopical observation, the better is the preservation of its ultrastructure, the idea was proposed that similar cooling rates should be attempted for the rapid freeze protocols in cryopreservation, [in the region of hundreds, or even thousands of ° C per second](Berjak *et al.*, 1995)

The ultra-fast cooling rates achieved for the preparation of tissue for electron microscopy (EM) are made possible by the extremely small volumes prepared for EM. The plant material most useful in cryopreservation is usually of a significantly larger volume, and therefore the negative effects of this increased volume must be counteracted in some way. Sufficient cooling rates might be achieved if the rate of immersion into the cryogen is increased, and the insulation (provided by the vessel in which the plant material is contained), is minimized. These ends may be achieved by rapid propulsion of naked plant tissue into the cryogen (Berjak *et al.*, 1995).

Aside from rate of cooling, another very important variable to consider concerning the survival of plant material subject to sub-zero temperatures, is the moisture content. Previous research involving direct immersion of plantlets into liquid nitrogen has shown that the desiccation of plantlets was very important. The extent of desiccation was also critical, with certain limits of moisture, above or below which the plant will not survive (Bajaj, 1987; Mycock *et al.* 1993).

2. Aims and objectives

2.1 Aims

The aims of this project were the familiarization with the initiation and culture of callus, cell suspensions and somatic embryos, and the development of a rapid freeze protocol for the preservation of carrot (*Daucus carota*) germplasm.

2.2 Objectives

The objectives underlying this study were:

- 1] Initiate and maintain a callus culture of carrot.
- 2] Initiate and maintain a suspension culture of carrot.
- 3] Initiate and maintain a culture of somatic embryos of carrot.
- 4] Determine water content of carrot callus
- 5] Preserve carrot germplasm by ultra fast freezing, (to -195 degrees C).
- 6] Recover viable germplasm by fast thawing, (to 23 degrees C).

3. Materials and Methods

3.1 Initiation and maintenance of callus culture

Callus was induced from a primary explant excised from the taproot of carrot (*Daucus carota*). The explant was derived from the vicinity of the vascular cambium - as it has been demonstrated that derivatives of the cambium divide vigorously to form extensive callus (Dodds and Roberts, 1985).

All materials and solutions (where possible), were sterilized in the autoclave machine before use (at 121° C and 1.2 Kpi), and sterile laboratory technique was employed throughout the duration of the experiment, to minimize the possibility of infection.

The callus initiating medium was based on that described by Murashige and Skoog (MS medium), in 1962. The MS basal salts and vitamins were supplemented with indolacetic acid (IAA) at 10mg/l; Kinetin at 0.1mg/l; Sucrose at 3% w/v; and agar at 0.8% w/v (pH = 5.7).

The carrot (from which the external 1-2mm of tissue was removed), was sliced transversely, into slices approximately 1,0cm thick, which were then transferred into a beaker containing sterile, double distilled water. A 300cm³ Hypochlorite solution (prepared by adding 40%v/v commercial bleach to sterile double distilled water. - to result in a 0,5%NaOCL solution) was added to the beaker for 10min. The slices were then rinsed for approximately 30s in 3 separate rinse beakers containing 300cm³ sterile double distilled water to remove any NaOCL from the tissue. Short cylinders of tissue were removed from the cambial zone of the slices with a sterile cork borer, (0,5cm diameter). These cylinders were temporarily stored in sterile double distilled water. The final explant preparation step involved trimming and discarding approximately

2mm of tissue from each end of the cylinders (using sterile, flamed forceps and scalpel), and the slicing of the remaining cylinder into three pieces approximately 2mm thick. These explants, measuring approximately 0,5mm in diameter, and 2mm thick were placed in sterile double distilled water for temporary storage while the rest of the explants were being prepared. The explants were rinsed once more in sterile double distilled water before being placed on the surface of sterile Whatman no1 filter paper - where the bottom and top surface of each explant was blotted. The explants were then immediately transferred to the surface of the culture medium (one explant per tube). The culture tubes were then sealed and placed in the tissue culture room, where they were maintained in the dark at 25° C.

The callus was grown for a minimum of 4 - 6 weeks, as it has previously been determined that this is the minimum culture time necessary for the callus to develop the potential for embryogenesis (Reinert *et al.*, 1971).

To optimize callus formation, and minimize bacterial and fungal contamination, this basic protocol was modified to assess the effects of :

- 1] Treatment of the mother plant material with antibiotic.
- 2] Growth of the explants on callus initiating medium supplemented with antibiotic.
- 3] Increasing the duration of surface sterilization time
- 4] Increasing the concentration of sodium hyperchlorite during surface sterilization.

These assessments were carried out in a series of separate experiments, designed to individually assess the parameters introduced above.

3.1.1 Experiment 1.

To assess the effects of extending the surface sterilization procedure, as well as treating the explants with antibiotic, material prepared according to the original

protocol (Dodds and Roberts, 1985), (culture group 1), was compared with cultures having the following modifications: Culture group 2- The surface sterilization step was extended from 10 min to 20min. Culture group 3- After the external 1-2mm of tissue was removed from the taproot. The plant material was immersed in a solution of distilled water containing streptomycin at 10mg/l for 1hr, after which the standard protocol was followed.

These three cultures were compared with each other, and differences in callus initiation, sterile callus initiation, and callus growth rate were assessed at 14 and 28 days.

3.1.2 Experiment 2

In order to assess the effects of the antibiotic streptomycin on callus growth, a comparison of sterile callus initiation and callus growth rate was made between culture group 3 and culture group 4. In the latter (culture group 4) the explants were grown on callus initiating medium supplemented with 10mg/l streptomycin.

3.1.3 Experiment 3.

The same comparison of sterile callus initiation and callus growth rate was made between culture group's 3 and cultures group's 5,6 and 7. These cultures were grown to assess the effects of increasing exposure time to, and concentration of sodium hyperchlorite. Culture group 5 was exactly the same as culture group 3, except for the surface sterilization treatment being extended to 20min. Culture group 6 was surface sterilized in 1% sodium hyperchlorite for 10min, whilst culture group 7 was surface sterilized in 1% sodium hyperchlorite for 20min.

All comparisons made of callus growth were carried out 28 days after the initiation of the cultures the time at which the cultures are believed to be competent for somatic embryogenesis, (Reinert *et al* 1971). These comparisons were made according to a calculated "growth index". The largest cultures were considered optimum in terms of the parameters explored, and were awarded a value of 3 (corresponding to 100%). Those cultures which exhibited no callus formation were considered failed cultures and were assigned a value of 0 - contaminated cultures were not considered in assessing callus growth. Callus cultures which were relatively small or medium at the time of measurement (i.e. - 28 days), were assigned values of 1 and 2 respectively. The values were totaled for each culture group, and expressed as a percentage (or callus culture growth index). All cultures were assessed at the same time.

The results obtained from callus culture experiments 1,2, and 3, expressed in terms of percentage sterile callus initiation and the numerical representation of growth (i.e. the growth index) were then represented in graphical form. The data was evaluated for significance using a student's t-test, with a confidence level of 95% ($p = 0.05$).

After 4 weeks of growth, the largest, healthiest, and most friable cultures were selected, and either sub-cultured out, or used to initiate suspension cultures.

3.2 Initiation and Maintenance of suspension culture

The initiation of a cell suspension culture required a relatively large amount of callus to serve as the inoculum, for example 2 - 3g for 100cm³ suspension medium (Helgeson 1979). The callus obtained from the previous step served as the inoculum for the suspension culture.

Carrot cell suspensions were initiated on an M.S. basal medium supplemented with the auxin 2,4,D, 1,0mg/l and sucrose, 3.0g/l (pH = 5.7). Exogenous cytokinin was not required.

The suspension culture was prepared by adding 25ml suspension medium into 125ml Erlenmeyer flasks. The callus was removed from the culture tubes with forceps and transferred to a Petri dish containing Whatman no1 filter paper. The callus blocks were trimmed with a sterile scalpel, and only the young actively growing callus was used for the inoculum. Each flask received an inoculum of about 500 - 750mg of callus in order to ensure the initiation of the culture. Suspension medium was introduced into the flasks which were then weighed. The flasks were weighed again once the inoculum had been introduced, so that the exact weight of inoculum could be determined while maintaining sterility. The inoculated flasks were placed on a rotary shaker at a speed of 100rpm, under light conditions at 25° C.

After suspension culture initiation, a daily assessment of cell number was made using a haemocytometer. Cell separation was assisted with a chromium trioxide solution - one volume of the cell suspension, (care was taken to remove any residual inoculum or larger clumps of cells by filtering the aliquot of cells through a nylon filter, pore size 100 μ m), was combined with 2 volumes of 8% aqueous solution of chromium trioxide. The solution was heated to 70° C for 5 min, and was further macerated by repeated pumping through the orifice of a Pasteur pipette once the solution had cooled. The macerate was then placed on a haemocytometer for counting. The results were used to prepare a growth curve of cell number vs. time, which could then be used to assess the optimum times for sub-culturing of the suspension culture, as well as the optimum time for initiation of somatic embryogenesis. According to George, (1993), this corresponds to the progressive deceleration phase of growth for sub-culturing, and the exponential phase of growth for somatic embryogenesis.

Prior to subculture or initiation of somatic embryogenesis, the suspension culture was filtered through an industrial nylon filter, (100 μ m pore size) to remove the residual inoculum and larger clumps of cells.

Samples of callus and suspension cultures were examined using phase contrast, and Namarski interference microscopy so that the cells could be evaluated for average cell sizes, relative density of cytoplasm and overall morphology of the cells in culture.

3.3 Somatic embryogenesis

Petri dishes, in which somatic embryos were grown, were prepared to accept the cell suspension. The embryo generation medium comprised of MS basic salts and vitamins supplemented with zeatin, 0.2mg/l; sucrose, 2% w/v; and agar, 1% w/v [pH = 5.7], (Dodds and Roberts, 1985). This medium contained no endogenous source of auxin.

Aliquots of 2ml carrot suspension culture were added to the surface of the medium in the Petri dishes. The dishes were sealed with parafilm and left to incubate at 25 ° C, in the dark.

Observations were made on the developing embryos at weekly intervals, using the dissecting microscope and representative photographs taken of each developmental stage, progressing from the induced embryogenic cells to the globular/early heart shape stage.

The embryogenic potential of the suspension cultures was determined 6 weeks after initiation of embryogenesis by visual inspection of the embryoids. The number of embryoids present was determined by placing the Petri dish over a black card with 1cm² grid lines to aid in counting. This information was used to calculate the average

embryogenic potential per ml. of suspension culture, which in turn was used for expressing the potential per gram of callus used for suspension culture initiation.

Somatic embryogenesis in carrot can also be induced directly from callus. This was attempted so as to contrast the viability of embryogenesis directly from the callus with embryogenesis from suspension cultures. Healthy blocks of callus weighing approximately 500mg were transferred onto fresh medium, consisting of the same ingredients as callus induction medium, except with no auxin. This callus was left to incubate under the same conditions as the previous callus culture. The amount of embryoids formed was determined visually, and scored as the amount of embryos formed per gram of callus.

3.4 Cryopreservation.

3.4.1 Determination of water content and establishment of drying curves.

Blocks of callus (5 - 10mg) were trimmed from the stock callus culture, these were then left to dry, in the laminar flow hood. The sample weights were determined at 10 minute intervals, and the loss in weight at each interval was considered equivalent to the water lost during dehydration. These callus blocks were then placed in a convection oven at 80° C for 24 hrs (after which time the tissue was devoid of all freezable water). The callus was then weighed again, and the difference in weight from the hydrated callus, (i.e.- the weight of 100% of the freezable water), was determined. The percentage water loss due to dehydration can then be worked out and a drying curve set up.

3.4.2 Cryopreservation of callus without prior treatment (control).

Blocks of callus (5-10mg) were trimmed from the stock callus culture, which had been placed on the non-absorbent surface of a sterile perspex disposable Petri dish lid, which served as the cutting platform.

Immediately after separation, the individual callus blocks were placed inside wire mesh baskets and thrust into the liquid nitrogen cryogen. The wire mesh baskets allowed for instant exposure to the cryogen, and the baskets further assisted in the maintenance of a steep temperature gradient by acting as a heat sink, (because of the heat conducting nature of the metal baskets), for the insulating layer which would tend to form around the tissue (Mycock *et al.* 1991).

The callus was left in the cryogen for 5 minutes, after which time it was transferred directly into liquid callus initiating medium (identical to the M.S. based medium used in callus culture, without agar) for thawing. The thawing medium was maintained at 25 degrees C, and was subject to vigorous agitation during the thawing process.

After 60 seconds in the thawing medium, the callus samples were removed from the wire mesh baskets and placed on Whatman no.1 filter paper, to remove the adhering layer of thawing medium, and were then transferred onto solid callus culture medium, in tissue culture tubes.

The callus was then placed for incubation at 25° C, in the dark.

To control for the effects of cryopreservation, additional callus blocks were treated in exactly the same way as described above except for the 5 minute immersion in liquid nitrogen.

This experiment, as with all subsequent cryopreservation experiments, was performed on 25 individual callus blocks and repeated twice.

3.4.3 Dehydration and Cryopreservation of callus.

Callus blocks were prepared as described above. Prior to immersion in the cryogen, the callus blocks were placed on a drying platform (again the non absorbent lid of a perspex petri dish), which was placed in front of the air filter of the laminar flow bench. The air flow rate was maintained at a constant rate.

Three separate dehydrating procedures were performed, at 10 minute, 20 minute, and 30 minute exposure times. After dehydration, the callus was cryopreserved and cultured out as described above. Again, the experimental controls differed only in that they were not frozen in liquid nitrogen.

3.4.4. Cryoprotection, dehydration and Cryopreservation of callus.

This experiment was carried out in the same way as the dehydration and cryopreservation experiment except that prior to immersion in liquid nitrogen, the callus blocks were soaked in a cryoprotectant of glycerol. Thus the effects of cryoprotection with glycerol, as well as the effects of dehydration after treatment with glycerol could be assessed.

The treatment in glycerol was carried out in two steps : The callus was first soaked in an aqueous solution of 5% w/v glycerol for 15 minutes, followed by a second soak of 15 minutes in an aqueous solution of 10%w/v glycerol.

3.4.5 Assessment of Cryopreservation Experiments.

Continuous observations were made on the cryopreserved material, as well as the controls over a 4 week period. The controls were assessed for percentage survival and the time taken for the control callus blocks to double in volume - to provide a reference for the effects of cryopreservation on regenerating callus growth rate.

3.4.6 Data collection.

The cryopreservation experiments were all terminated after 4 weeks. At this point all cultures were assessed visually for callus growth, those samples which had definitely resumed growth were scored as having successfully survived the cryopreservation procedure (or, in the case of controls, survived the isolation procedure) - and this was expressed as a percentage survival.

Since the other samples may have survived cryopreservation, but not yet resumed growth, (or had not yet grown enough for visual detection), these samples were subject to a determination of cell survival using the Triphenyltetrazolium Chloride method (Towill and Mazur, 1975). This test is based on the fact that living cells produce formazan as a result of the reduction of triphenyltetrazolium chloride - resulting in the release of a pink colour. The tetrazolium reagents accept electrons from the electron transport chain of the mitochondria; resulting in conversion of these oxidation reduction indicators being converted formazan precipitates. There is a close correlation between the amount of formazan produced and the percentage of viable cells in the sample (Dodds and Roberts, 1985). This test was performed separately on explants which had been determined as having survived the cryoprotection (above), as well as those explants which were either dead or in lag phase. Explants from each separate cryopreservation experiment were pooled and then subject to the test.

The procedure involved the following steps:

- 1] Place 50 mg of tissue sample per 1ml solution of 0.6% triphenyl tetrazolium chloride in a buffer containing 0.05M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and 0.05M KH_2PO_4 .
- 2] Incubate for 15h at 30 degrees C.
- 3] The TCC solution is drained off and the tissue washed with distilled water.
- 4] The tissue is then extracted with 2 volumes of ethanol (95%) in a water bath at 80 degrees for 5 min.
- 5] The extract is then cooled and diluted to 3 original tissue volumes with 95% ethanol.
- 6] The absorbency (of the formazan) is measured with a spectrophotometer set at 530nm. The amount of formazan produced by the frozen cells is expressed as a percentage (survival) of formazan produced by the control (unfrozen) callus.

4. Results and Discussion

4.1 Callus culture initiation and maintenance.

4.1.1 Callus initiation.

The first culture to be initiated was conducted according to the published protocol of Dodds and Roberts, (1985) and was completely unsuccessful. There was no indication of callus formation after two weeks, and 100% bacterial contamination (observable after approximately 1 week). The freshness of the mother plant material used in that experiment could not be guaranteed, with the possibility that the plant material was senescent, consequently the results of this culture group were disregarded. In all subsequent experiments, only material which had been harvested on the day of culturing was utilized.

A second culture (culture group 1) was initiated, also according to the published protocol of Dodds and Roberts, (1985). However problems with bacterial contamination persisted, and therefore in an attempt to improve the sterility of the cultures, the effect of increasing the exposure time to sodium hypochlorite (culture group 2), and the effect of soaking the mother plant material in streptomycin at 10mg/l for 1hr (culture group 3) were compared with the unchanged protocol in terms of callus initiation (experiment 1).

[1] Experiment 1

The results of experiment 1 are summarized in figure 1.

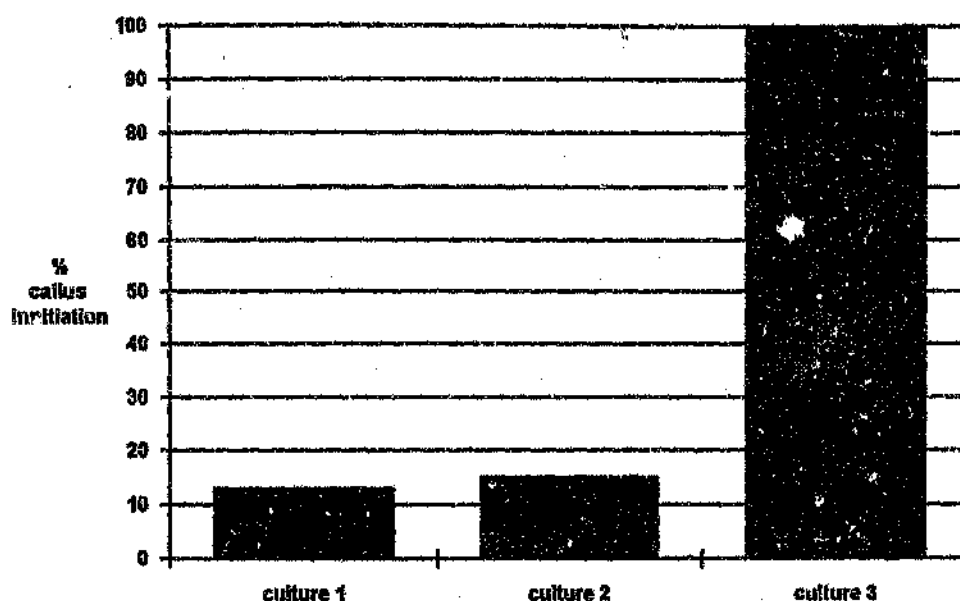


Figure 1. Percentage callus initiation from carrot root material

culture group 1: Standard, unaltered protocol (Dodds and Roberts, 1985)

culture group 2: Surface sterilization time extended to 20min, 0,5% sodium hyperchlorite.

culture group 3: 1hr pre soak in streptomycin at 10mg/ml.

* indicates significant difference to previous culture group ($p = 0,05$)

After 14 days, there was evidence of callus formation in 15% of culture group 1, this figure was not significantly different from culture 2 (17%), in which mother plant material had been exposed to sodium hyperchlorite for an additional 10 minutes. However, a marked improvement in the percentage callus development was observed in culture group 3, with 100% of the explants showing signs of callus initiation. Bacterial contamination still presented a large problem. Contamination developed in culture groups 1 and 2 along with the initiating callus, which can be seen in plate 1, (iii) - where callus formation can be seen in the infected culture. A more meaningful

consideration in the present experimentation would be of sterile callus initiation. The three culture groups were therefore compared in terms of sterile callus initiation, fig. 2.

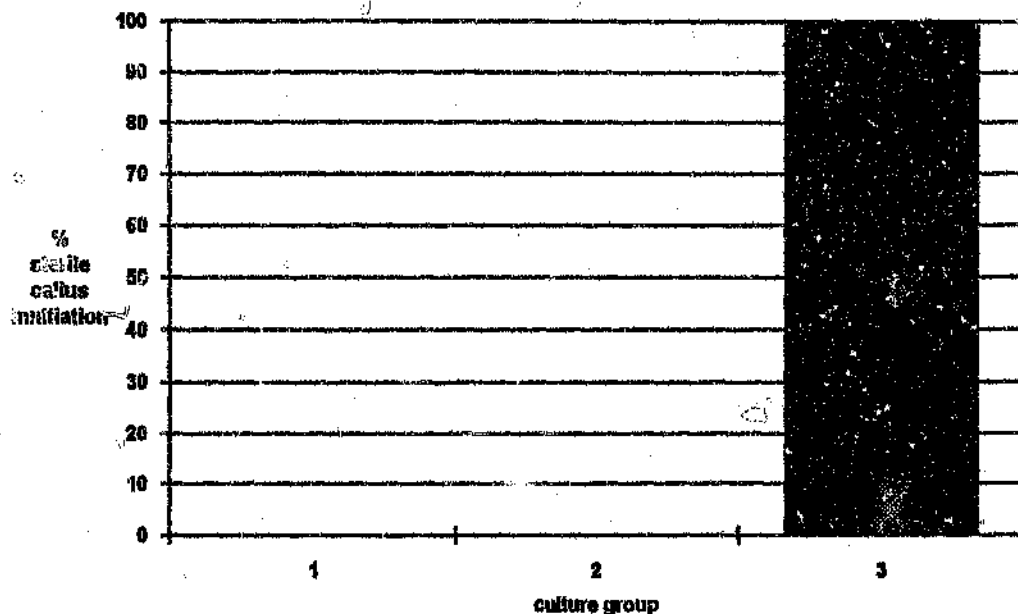


Figure 2. Percentage sterile callus initiation from carrot root material.

culture group 1: Standard, unaltered protocol, (Dodds and Roberts, 1985)

culture group 2: Surface sterilization time extended to 20min, 0.5% sodium hyperchlorite.

culture group 3: 1hr pre-soak in streptomycin at 10mg/ml.

* Indicates significant difference to previous culture group ($p = 0.05$)

It was evident that increasing the exposure time of the mother plant material to sodium hyperchlorite did not confer any advantage to achieving sterile callus, with neither culture groups 1 nor 2 yielding any usable callus.

Treatment with Streptomycin was very successful in terms of the initiation of sterile callus, with the optimum result being observed.

[2] Post contamination sterilization

An attempt was therefore made to eradicate the bacterial infection in those cultures in which callus initiation had occurred by soaking the explants in 0.5% sodium hyperchlorite for 5 minutes. However the callus cultures were very fragile, (with very thin cell walls), and this treatment proved too harsh for the explants, all of which died. This suggested that prevention of bacterial contamination, rather than cure is a favourable approach to yielding sterile callus.

[3] Pre contamination sterilization.

It was observed that the bacterial infections were almost certainly a result of an endogenous bacterium resident in the plant material, since it occurred in 100% of culture groups 1 and 2, and had identical growth characteristics and morphology, (see plate 1, iii and iv). An *ex vitro* contamination could not account for the observed results, especially in light of the results of group 3.

Another argument for the contamination arising from endogenous bacteria, was the failure of sodium hyperchlorite (a very effective surface sterilant) to eradicate the contamination, whereas a 1 hour soak in streptomycin was sufficient for the antibiotic to penetrate the plant material, and resulted in 100% sterility.

[4] Experiment 2.

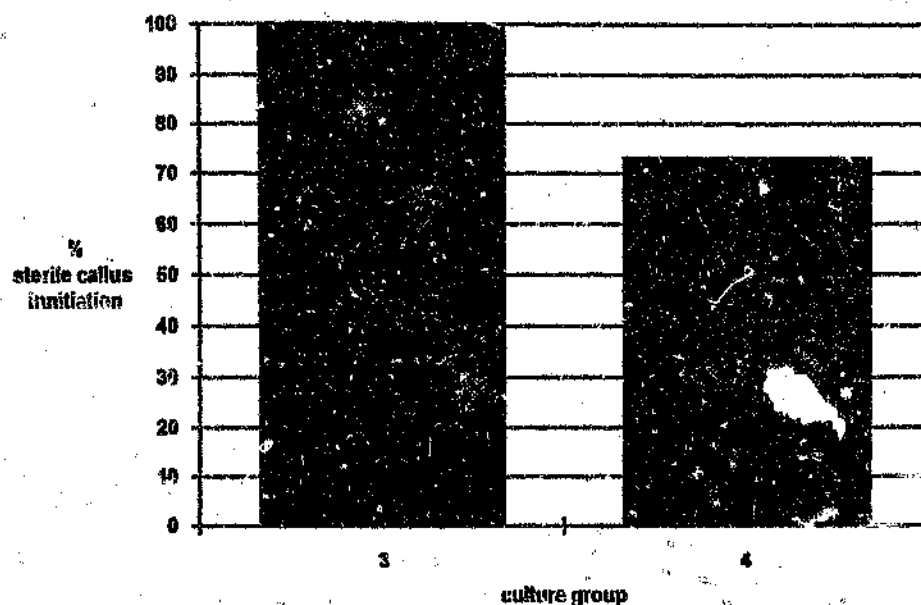


Figure 3. Percentage sterile callus formation from carrot root material.

culture group 3: 1hr pre soak in streptomycin (10mg/ml)

culture group 4: 1hr streptomycin soak and grown on streptomycin supplemented medium (10mg/ml)

* indicates significant difference to previous culture group ($p = 0.05$)

The streptomycin supplemented medium resulted in a number of apparently failed cultures at day 14 after initiation, (25% of the culture group) [fig.3]. It is believed that this was not due to contamination (bacterial or fungal), but most likely due to the growth suppressant effects of the antibiotic on the plant material. This phenomenon has been observed in a number of other species (George, 1993). Supplementing the medium with streptomycin, in terms of callus initiation, was therefore deleterious to the production of sterile callus. To test whether streptomycin supplemented medium conferred any other advantages (e.g. assisting in the maintenance of sterility), further observations through time would be necessary.

[5.] Experiment 3.

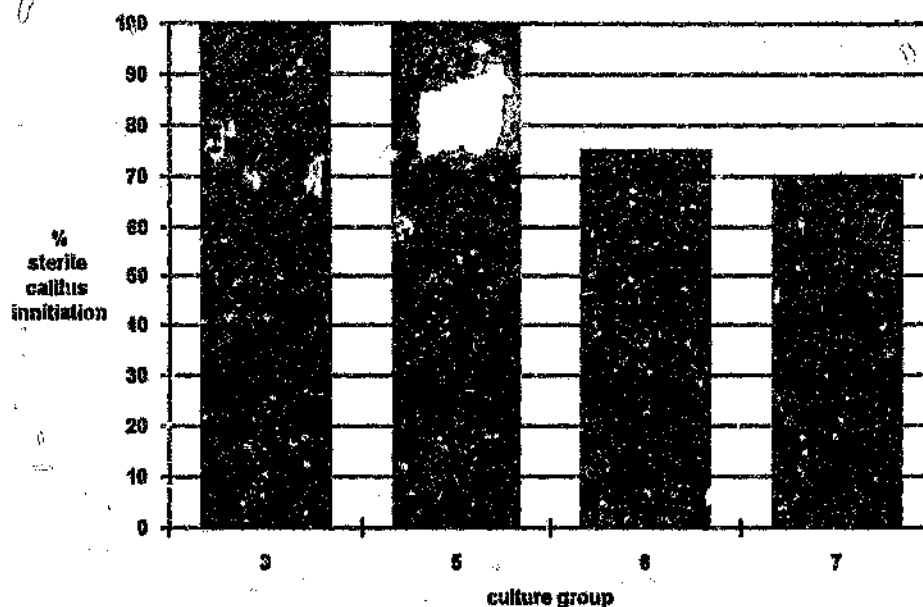


Figure 4. Percentage sterile callus initiation from carrot root material.

culture group 3: 1hr pre-soak in streptomycin at 10mg/ml.

culture group 5: 1hr streptomycin pre-soak, 20min exposure to sodium hyperchlorite at 0.5%.

culture group 6: 1hr streptomycin pre-soak, 1% sodium hyperchlorite.

culture group 7: 1hr streptomycin pre-soak, 20min exposure to 1% sodium hyperchlorite.

* indicates significant difference to previous culture group ($p = 0.05$)

Increasing the time that the plant material was exposed to sodium hyperchlorite had no significant effect on sterile callus initiation, (fig. 4). However, the increase in

concentration did exert a negative effect, demonstrating the plant tissues sensitivity to higher concentrations of sodium hypochlorite (fig. 4).

4.1.2 Callus growth.

In terms of suspension culture initiation the most important consideration in terms of the final goal of the callus experiment is the amount of callus formed after 4 weeks of culture.

The effects of the protocol changes performed in experiments 2 and 3, expressed in terms of the callus growth index, are summarized in Figs 5 and 6.

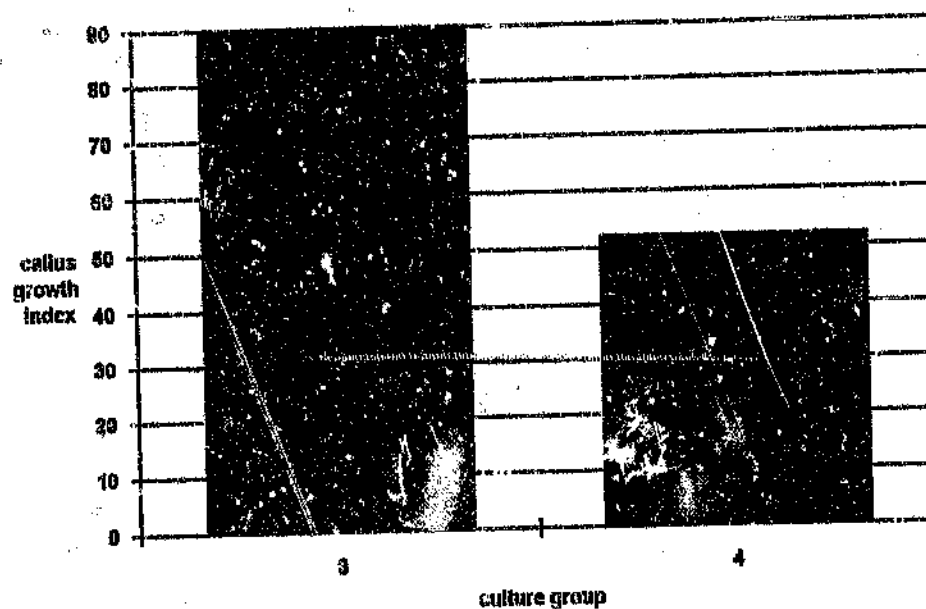


Figure 5. Callus growth index of sterile callus from carrot root material.

culture group 3: 1hr pre-soak in streptomycin at 10mg/ml

culture group 4: 1hr streptomycin soak, grown on streptomycin supplemented medium (10mg/ml)

* indicates significant decrease to previous culture group ($p = 0.05$)

Supplementation of the callus medium with 10mg/ml of streptomycin conferred no additional advantage in maintaining sterility, as no additional infections were observed (Fig. 5). However as with the negative effect in callus initiation, the antibiotic exerted a negative effect on callus growth rate (Fig. 5).

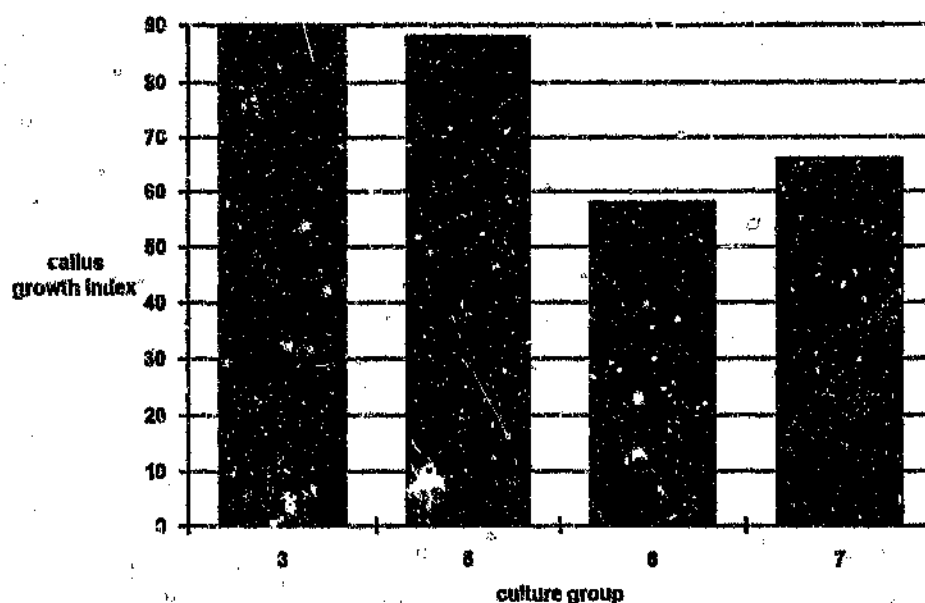


Figure 6. Callus growth index of sterile callus from carrot root material.

culture group 3: 1hr pre-soak in streptomycin at 10mg/ml.

culture group 5: 1hr streptomycin pre-soak and 20min exposure to 0.5% sodium hyperchlorite.

culture group 6: 1hr streptomycin pre-soak and 1% sodium hyperchlorite.

culture group 7: 1hr streptomycin pre-soak and 20min exposure to 1% sodium hyperchlorite.

* Indicates significant difference to previous culture group ($p < 0.05$)

Increasing the surface sterilization time from 10 to 20 minutes had no significant effect on the growth of the callus cultures, in both the standard concentration of 0.5%, and the increased concentration of 1% (Fig. 6). However, the increase in sodium hyperchlorite concentration had a negative effect on callus growth, again demonstrating the sensitivity of the plant material to the sterilant (Fig. 6).

The most successful approach to obtaining sterility was therefore coupling exposure of the plant material to an antibiotic (and allowing the antibiotic to saturate the plant material), with the surface sterilization procedure. The surface sterilization procedure outlined in the standard protocol was sufficient, and any increase in the concentration of sodium hyperchlorite was in fact deleterious to the initiation and growth of callus. Plant material was completely sterilized by this procedure, and any further exposure to streptomycin proved to be disadvantageous.

It was interesting to note that the original protocol was completely successful for Reinert *et al.* (1971), but was totally unsuccessful presently. A possible explanation for this was that the contaminants those authors had to combat were all surface contaminants, whereas the plant material used presently, (grown in the warmer southern hemispheric climate as opposed to that found in England), had a different contaminant profile and/or endogenous (internal) contaminants.

The growth of a representative culture from culture group 3 was recorded photographically at weekly intervals. This was the most successful culture group in terms of number of sterile cultures initiated, as well as the amount of callus formed at the end of 4 weeks of culture. Plate 2, ii - v represent the best callus yields achieved in this series of experiments at weeks 1 - 4 respectively. Plate 2, i and ii are of the same culture (i.e. at week 1 - after which time callus formation is only just visible around the edges of the explant), and is included to demonstrate the original explant size (both

height and circumference), when comparing the amount of callus formation through time.

At week 4 (plate 2, v) there was little or no senescent callus present on the cultures, which allowed for the entire culture to be used for subculturing (1 : 4). The callus cultures were maintained by subculturing out every 4 weeks thereafter.

A callus consisting wholly of uniform parenchyma cells is witnessed only rarely (Reinert and Bajaj, 1977). The cells in a callus may consist of a variety of cell types, sizes and shapes, differing in their vacuolation, cell contents and wall thickening. Plate 3, v demonstrates some cytodifferentiation (tracheal element formation) which occurred in one of the cultures, although generally the callus was parenchymous in type (plate 3, v and vi). The average cell size (largest per cell diameter measurement) was 60 μ m (SD = 11 μ m).

Achieving optimum results in tissue culture is often a matter of optimizing numerous parameters (George, 1993). To manipulate each parameter individually can be extremely time consuming, the present experimentation on callus culture was done simultaneously. However, in hindsight it can be seen that culture group 3 achieved adequate results, such as to render all further experimentation redundant.

4.2 Suspension culture initiation and maintenance.

Although suspension cultures are initiated from supposedly sterile material, the rate of callus growth on solid culture medium can in some cases be high enough to exert growth suppressive effects on trace bacterial and fungal contaminants. Upon initiation of a suspension culture, these growth suppressive effects are removed - and contamination can ensue. This, coupled with the risk of introducing contaminants when initiating the cultures, makes suspension cultures especially prone to infection.

Of the 30 cultures initiated, 4 were lost to bacterial contamination and 7 due to fungal contamination, (Fig. 7).

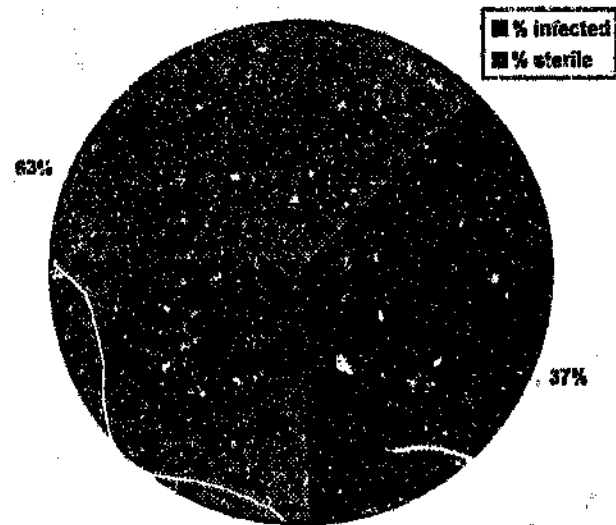


Figure 7. Percentage infection in suspension cultures

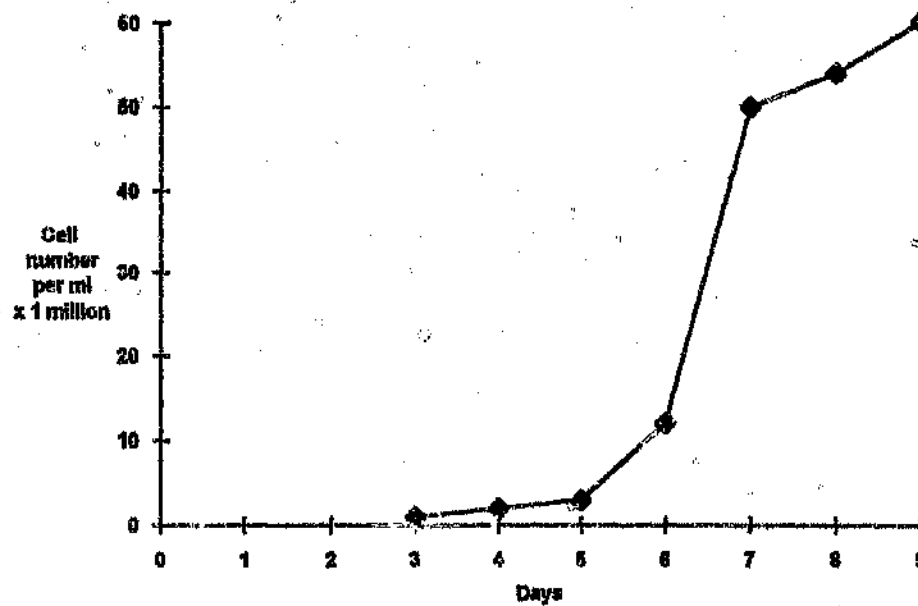


Figure 8. Cell number of carrot suspension culture vs. time.

As is true of most cell cultures the growth of the carrot suspension culture followed a sigmoidal pattern (Fig. 8). It was determined that the optimum subculturing time was

7 days after culture initiation, and the time at which somatic embryogenesis should be initiated was after 6 days, (corresponding to the deceleration of growth phase, and the exponential increase phase of growth respectively).

The microscopic evaluation of the suspension culture revealed a high percentage of cells in various stages of division (see plate 3, ii - iv). Micrographs were taken of a 5 day old culture, which had just entered the exponential stage of growth, at which time a peak in mitosis would be observed. Cell morphology was extremely variable, consisting of spherical, slipper shaped, gourd shaped and oval cells, (see plate 3, i - iv). These differences had no correlation to the stage of the cell cycle, which can be seen in plate 3, iii and iv, showing late mitosis. The cell size was also variable, with the diameter per cell ranging from 30 μ m to 120 μ m (the average cell size was 70 μ m, SD 14 μ m).

4.3 Somatic embryogenesis.

Suspension cultures were initiated from 28, 35, 42 and 49 days of callus culture. These suspensions were then used to inoculate plates for the initiation of somatic embryos, (somatic embryogenesis was always initiated from freshly initiated suspension cultures, at day 6). No somatic embryogenesis was observed from the 4 or 5 week old callus (8 plates each were inoculated). However, of the 8 plates initiated from 6 week old callus, two demonstrated somatic embryogenesis, but in all twenty plates initiated from 7 week old callus, somatic embryogenesis was observed. Reinert *et al.*, (1971) observed that callus only became competent for somatic embryogenesis between 4 and 6 weeks after initiation of callus cultures from fresh carrot material. The present observations are therefore in agreement with these authors, albeit the results were far more favorable after 6 weeks of callus culture, and in light of this it is recommended that callus culture times of at least 7 weeks are used for initiating somatic embryogenesis. It was interesting to note that both failed and successful cultures could be initiated

from the same 42 day old suspension culture. This observation cannot be explained since there were no apparent differences between cultures. The average number of embryoids observed from the 6 week old callus was relatively low when compared to the plates initiated from 7 week old callus, table 1.

Table 1. Success of embryogenesis vs. time.

Callus culture age (weeks)	successful embryogenesis (success/plates initiated)	average no of embryoid per successful plate
4 weeks	0/8	-
5 weeks	0/8	-
6 weeks	2/8	1. = 4)
7 weeks	20/20	24 (SD = 10)

From the average weight of inoculum for the suspension cultures (620mg) it could be calculated that the average number of embryoids formed per of 7 week old callus inoculum was calculated to be 483. This result demonstrated the power of somatic embryogenesis via suspension culture for plant reproduction. However, there were very large deviations in the number of embryoids observed per plate. There was nevertheless an even and complete range of developmental stages of somatic embryogenesis. It should be noted that this experiment was designed simply to obtain somatic embryos for the next intended stage of the experiment (cryopreservation), and to evaluate the amount of embryos formed per gram of callus inoculum. The observed variations had not been anticipated regarding callus age on somatic embryogenesis, and therefore statistical evaluation of the effects of callus inoculum age on the success of somatic embryogenesis was not conducted. It may be that more favourable and consistent results will be observed for callus inoculum older than 7 weeks, but this will have to be the subject of another appropriately designed experiment.

The process of somatic embryogenesis goes through stages reminiscent of in vivo zygotic embryogenesis, although there is no one single manner of progressing from the first embryogenically determined cell to the later stages of embryogenesis (Bhajwani and Razdan 1983). In some cases, somatic embryogenesis was preceded by the development of a suspensor (plate 4, i - vi), and in other cases no evidence of a suspensor was present, (plate 4, vii - ix). Plate 4 i - iii show developing suspensors of varying lengths, Plate 4, iv demonstrates the first anticlinal division which later results in formation of the pre-globular and early globular embryo, shown in Plate 4, v and vi. Plate 4, vii - ix show developing embryos lacking any suspensors, demonstrating pre-globular, early heart shape, and early torpedo stage respectively (after Wetheral, 1978).

Somatic embryogenesis directly from callus was also initiated to contrast the rate of embryogenesis and amount of embryoids formed per gram of callus with the results obtained via suspension culture. The results from that experiment were not intended for cryopreservation work, as there was insufficient time to grow up the volume of callus required to produce a usable amount of embryos. The average amount of embryos formed per gram of callus was 18, and should be contrasted to 483 embryos per gram when initiating from suspension culture. However, the speed of development of somatic embryos via suspension culture was slower than from callus. Such differences were not detectable in previously published work (Bhajwani and Razdan, 1983). If similar speeds of development were achieved, approximately 25 times the amount of plantlets per gram of callus inoculum can be generated from suspension cultures.

4.4 Cryopreservation.

4.4.1. Percentage Survival , visual determination.

The initial intention of the present research was experimentation on the cryostorage of both callus and somatic embryos. However, in view of the project deadline, the rate of somatic embryogenesis was too slow for this work to be included in this project. Although somatic embryogenesis was successful after 6 weeks in culture, there were very few embryoids at the intended developmental stage (heart shape stage). Reinert *et al.*, (1971) reported the presence of the more advanced torpedo stage embryoids after 3 weeks of incubation under identical conditions. The reason for this discrepancy in rate of embryogenesis is therefore difficult to determine.

The overall aim of conserving any germplasm is ultimately the production of whole plants after a period of conservation. In terms of micropropagation somatic embryogenesis often yields higher multiplication rates than other methods, and the embryoids produced are easy to handle in terms of cryopreservation. Conversely, indirect somatic embryogenesis (via callus) has the disadvantage of somaclonal variation, which can yield genetic variability. It is also possible that the cryopreservation process may select for an undesirable genotype, although this would be a more unlikely problem when working with the genetically more stable somatic embryos.

Somatic embryos however, would be far more difficult than callus to successfully cryopreserve, as they consist of organized tissues which would be far less tolerant of a significant proportion of damage/death of the tissues due to freezing. However there are many plant species for which cryopreservation of zygotic embryos is the only alternative for long term, reliable germplasm conservation (see earlier). The successful

cryopreservation of somatic embryos of orthodox plant species would provide a model for developing a germplasm preservation protocol using cryopreservation for these recalcitrant plant species (Mycock, Watt and Berjak, 1991). For this reason, a cryopreservation of somatic embryos of carrot would have relevance, even if it is not the most efficient method of germplasm conservation for this specific species.

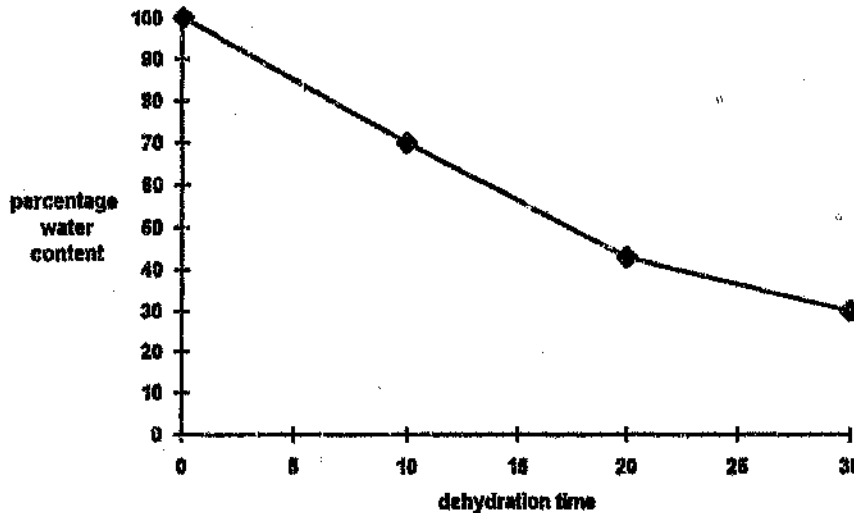


Figure 9. Drying curve for callus from carrot root material.

Determinations on the effects of dehydration were performed on untreated callus as well as callus soaked in the cryoprotectant of glycerol (figure 9). No significant difference was observed between these two determinations ($p = 0.05$), consequently the standard curve demonstrated above was constructed using a pool of both sets of data.

The results of the cryopreservation experiments are summarized in the following tables:

Table 2. Cryopreserved, no cryoprotectant

Dry time	0 minutes	10 minutes	20 minutes	30 minutes
% survival	23	30	38	32
% survival (control)	100	100	98	95
%ave. h2O content	100	70	43	28

Table 3 Cryopreserved, with cryoprotectant

Dry time	0 minutes	10 minutes	20 minutes	30 minutes
% survival	46	59	56	65
% survival (control)	96	90	86	72
% ave. h2O content	100	74	41	31

The two potential sources of cell damage during cryopreservation are the formation of large ice crystals inside the cell, leading to the rupture of organelles and the cell itself, and the intracellular concentration of solutes increasing to toxic levels. Cells may also suffer the loss of vital solutes through leakage during the freezing process, (Bhajwani and Razdan, 1977).

Dehydration, both in unprotected as well as cryoprotected callus led to an improvement in percentage survival, although the results suggest that the optimum dehydration levels differ depending on whether cryoprotectant has been used or not. Dehydration of the plant material before or during freezing results in an increase in the concentration of solutes before the freezing of the protoplasm occurs, and cryoprotectants offer protection from this toxic solution effect (Bhujwani and Razdan, 1977). This protection afforded by the glycerol may be one explanation as to why

cryoprotected callus withstood increased levels of dehydration. The cryoprotectant effect due to prevention of large ice crystal formation may be an independent protective effect, (Bhajwani and Razdan, 1977).

The uncryoprotected, undehydrated callus performed poorly next to the other experiments, suggesting that the freezing rate was still too slow to disclude dehydration and cryoprotection treatments. The simplification of the cryoprotection protocol through abandoning these treatments may still be possible if even faster freezing rates (and therefore a faster transition of cellular water into the vitrified state), were achieved. Some possible approaches to this end would be subfreezing liquid nitrogen to temperatures just above solid nitrogen (about -210°C) by maintaining the nitrogen under a vacuum just prior to immersion of the plant material. Faster freezing rates would also be provided if a method for increasing the speed of introduction into the cryogen was devised, such as forcibly projecting the sample in the cryogen (Wesley-Smith et al., 1992). A steeper temperature gradient could also be provided by using liquid helium (about -230°C), although the expense of this material defeats some of the object of revising the cryopreservation protocol.

Dehydrating the callus for 20 min or more (corresponding to 57% dehydration) had an adverse effect on survival of the control samples. Increasing the dehydration time from 20 to 30 min also lowered the rate of survival of the cryopreserved material, indicating that 20min was close to optimum. However, in the case of cryopreserving with cryoprotection, dehydration up to 30 min (69% dehydrated) provided the highest percentage survival achieved in all the experiments - however dehydration to this point also resulted in a very low survival rate for the control (just slightly above the frozen samples). It is highly unlikely that the cryopreservation treatment will increase the percentage survival observed in callus subject to the glycerol exposure and dehydration, and so 70% dehydration must be close to the optimum dehydration level.

The pre soak in glycerol had a significantly favorable effect on the percentage survival of frozen callus (Table 3). Historically, the most successful cryoprotectant has been dimethyl sulphoxide (DMSO), however there are serious problems associated with this compound - at higher concentrations it reduces respiration and inhibits RNA and protein synthesis of the material to be cryopreserved, it can also be toxic to the experimenter (Reinert and Bajaj, 1977). It is often necessary to pre-chill the DMSO solution before exposing the plant material, (as exposure of cells to the cryoprotectant at room temperature adversely affects their viability), and rinse the cryoprotectant off the tissue. However, both these additional steps put the tissue under increased stress (Reinert and Bajaj, 1977).

Glycerol has all the advantages of DMSO as a cryoprotectant, in that it has a relatively low molecular weight, is easily miscible with the aqueous solvent, and permeates effectively into the cells. Further it is non toxic and does not need to be washed from the cells, and it was for these reasons that glycerol was used in the present experimentation.

DMSO has been used in the achievement of a 70% survival rate for carrot cell suspensions (Bhajwani 1990), frozen using a slow freeze technique, and this is comparable with the present result of 65% (a difference I could not test for significance as I lacked details of his work).

The rate of callus growth in those cryopreserved cultures in which regeneration was seen to occur was very slow in comparison with the controls. The volume of callus of the controls doubled after approximately 10 days (glycerol treatment or drying had no effect on this), whereas at the time the experiment was terminated (4 weeks after cryopreservation), none of the cryopreserved cultures had doubled in volume, with the largest increase in volume estimated to be approximately 20%. The new callus growth was generally localized to a very small point on the culture. This is because

cryopreservation affects different parts of the tissue in different ways, and much of the callus was left in lag repair phase (or was dead), and so comparing the time taken for a change in volume between the cryopreserved cultures and the controls did not prove to be a viable method of regenerated callus growth rate. It would be more desirable to compare the growth rates of isolated regenerated callus with control callus of the same volume.

4.4.2 TTC viability test.

Table 4. TTC viability test - uncryoprotected and cryoprotected callus.

sample:	0 min R	0 min L	10min R	10min L	20min R	20min L	30minR	30min L
uncryoprotected*	9.4%	4.2%	12.7%	8.6%	11.1%	3.5%	13.0%	6.6%
cryoprotected*	10.3%	11.4%	17.6%	1.6%	38.0%	14.5%	24.9%	11.6%

* Values refer to the amount of formazan produced, expressed as a percentage of the control (unfrozen) callus.

- The letters R and L in the sample row refer to pooled recovered cultures, (R) and pooled cultures which are either dead or in the lag phase, (L).

The results of the TTC test indicate that for each separate cryopreservation treatment, some callus tissue was able to produce formazan precipitates. This is important, as this suggests that if the individual explants were allowed more time to recover, a higher rate of survival may be observed for each different treatment.

The results obtained in this experiment indicate the amount of formazan produced by pooled samples, and therefore these values cannot be compared to the percentage survival of the explants from tables 2 and 3, since varying amounts of tissue per explant may have survived, and this has not been accounted for. The test does suggest

however, that a higher proportion of tissue survived in the cryoprotected samples as opposed to the uncryoprotected samples.

The staining method cannot be used in determining the regenerating ability of plant material immediately after thawing, as a false positive result may be observed from cells showing a weak staining, but which might eventually die in cultures. It should also be borne in mind that the same may be true of cells showing viability at the four week point presently tested. Ideally the experiment should be left to run until one can be certain that the plant tissue has either regenerated or died. After thawing, the cells may be partially injured, or in a state of cold shock, and do not divide but still retain some physiological activity i.e. respiratory and enzymatic. In such cultures a low survival rate, and a relatively long post thaw lag phase can be observed, and during this period, the TTC test not detect potential survivors. Therefore any data on the viability of cells based on the staining reaction alone would be insufficient and misleading. Bhajwani and Razdan (1983), reported such a case in which the TTC test gave a negative result, and cultures started to grow and divide after a lag period of between 4 and 6 months. This also suggests that the percentage survival figures quoted above might have been improved if the experiment were left to continue.

It must be noted that the overall aims of this project were, firstly the initiation and maintenance of callus and suspension cultures, and the production of somatic embryos - and secondly to successfully cryopreserve *Daucus Carota* using a fast freeze technique.

The parameters tested in the callus culture were done so only to establish a sterile culture with which to proceed on to the next stage of the project - less than optimum results would have been accepted. Those parameters tested in the experiments on dehydration and cryopreservation, and cryopreservation with cryoprotection were conducted in the hope of achieving a successful cryopreservation with the simplest

procedure possible. Further work would be required to optimize the protocol for the highest yield in percentage survival. The experiments should be considered preliminary in achieving that goal - but importantly serve as an indicator on which aspects of the cryopreservation procedure would warrant further attention, and which would not. It would also be interesting to assess the effects of cryoprotection with varying concentrations of glycerol and varying exposure time, the present optimum being exposure to 10% glycerol for 30mins.

The problem with work of this nature is that there are many parameters which should be tested, and which can be very time consuming. Although previously published work can serve as a guideline, there appears to be no relationships in how different plant groups (or even different species in the same genera) respond to these many variables. For this reason, each set of parameters must be re-tested on each new plant species for which a cryopreservation procedure is to be determined. This is another reason for developing the simplest protocol possible (i.e. ultra fast freezing with no cryoprotection).

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Plate 1.

- 1.] Freshly explanted culture, showing relative diameter of culture tube and volume of callus culture media for culture requiring subculture every 4 weeks.
- 2.] Explant *in vitro* after 1 week incubation, showing early callus formation around the edges of the explant.
- 3.] Infected callus explant after 1 week incubation, showing characteristic bacterial infection spreading around the base of the explant. Despite infection, callus formation can still be observed on the explant.
- 4.] Infected callus culture after 2 weeks incubation, with the medium invaded by the bacteria. At this point callus was irretrievably lost, with attempts at surface sterilization of contaminated callus with sodium hyperchlorite being futile.



Plate 2.

Scalebars consistent with last marked bar unless otherwise indicated.

1 and 2.] Callus explant at 1 week of incubation, showing early callus formation and relative size of explant to callus growth at later stages.

3.] Callus explant at 2 weeks of incubation.

4.] Callus explant at 3 weeks of incubation.

5.] Callus explant at 4 weeks of incubation. At this point the callus showed no signs of senescence or localized dehydration, and the entire culture could be used for subculture or initiation of suspension cultures.

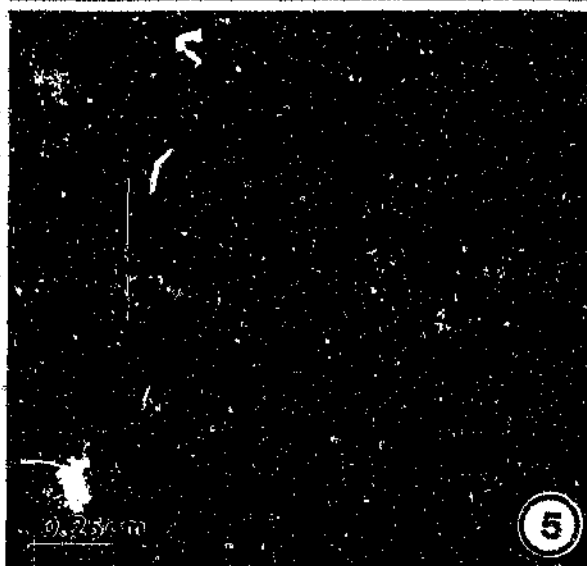
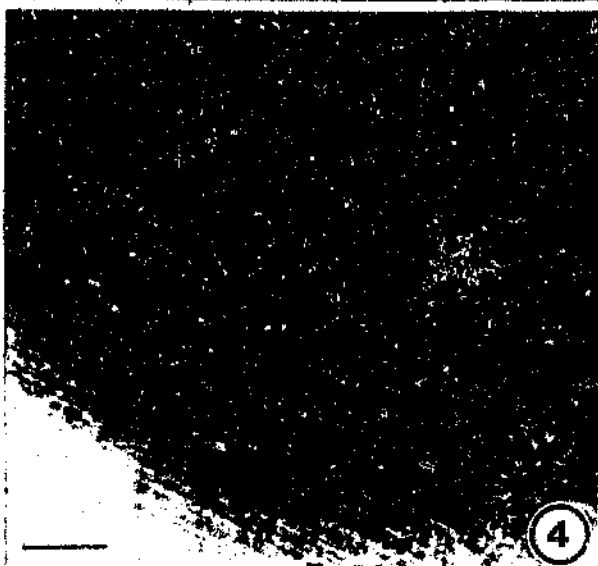
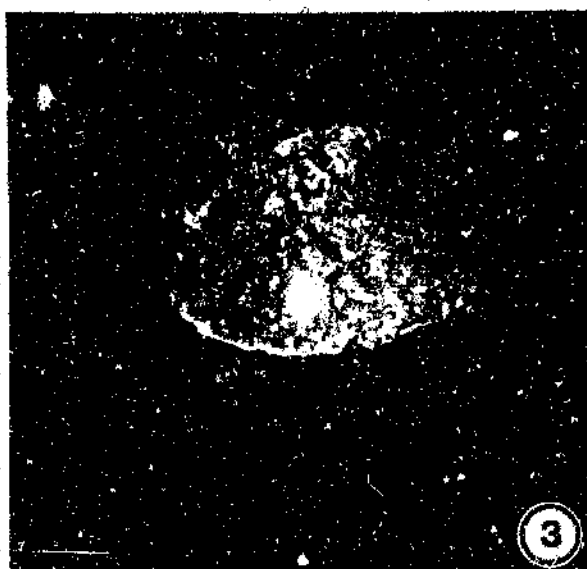
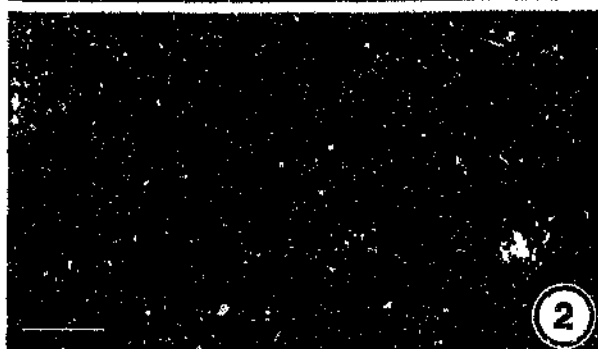


Plate 3.

Scalebars consistent with last marked bar unless otherwise indicated.

1 - 4.] Namarski interference microscopy, of a representative sample of cells in a suspension of 5 days old, showing variation in morphology and size. At this point in the culture, a high proportion of cells were in various stages of mitosis, and cell number was increasing exponentially. Cell morphology and size had no correlation with cell cycle phase (iii and iv).

5.] Light phase microscopy of callus showing cytodifferentiation, and cellular morphology of callus. Callus tissue contains cells of a more consistent size and shape than cells in suspension.

6.] Namarski interference microscopy of a squash preparation of callus.

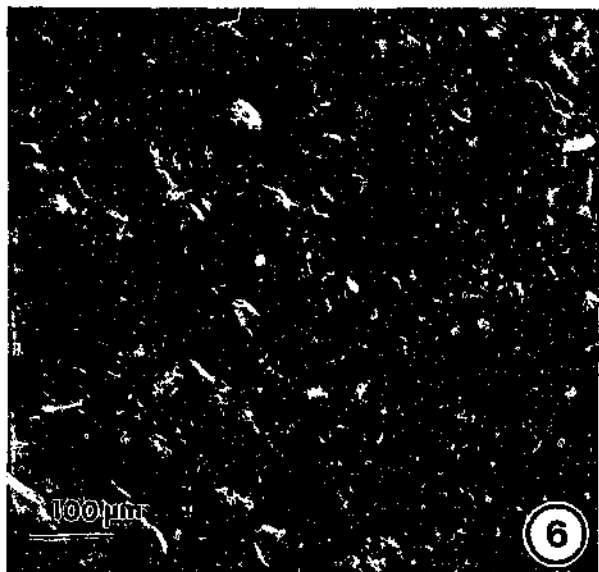
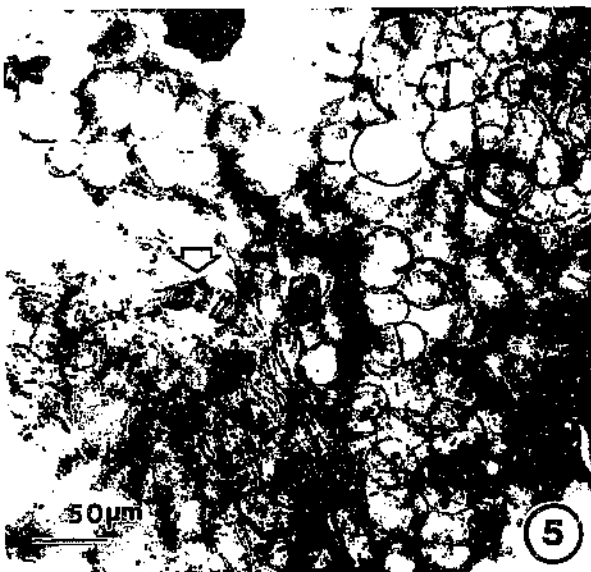
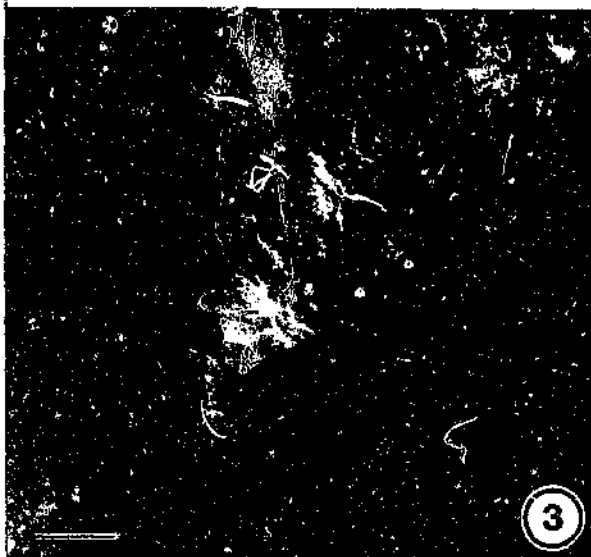


Plate 4.

Scalebars consistent with last marked bar.

1 - 3.] Developing suspensors of varying length.

4.] First anticlinal division, and start of embryo formation.

5.] Preglobular stage.

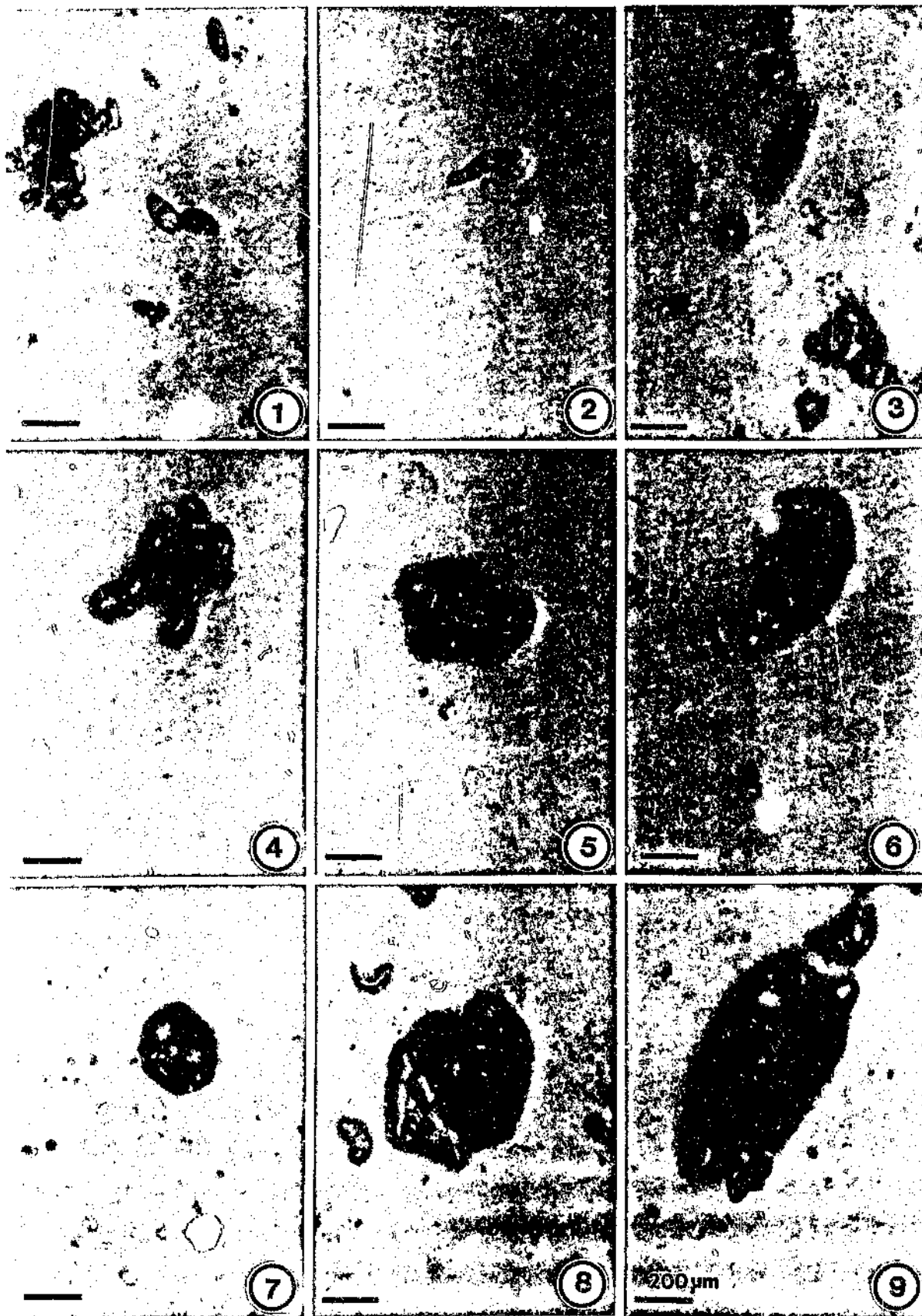
6.] Early globular stage.

7.] Preglobular stage, no suspensor visible.

8.] Early heart stage.

9.] Early torpedo stage.







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Name of thesis: Tissue culture and cryopreservation of *Daucus carota*

PUBLISHER:

University of the Witwatersrand, Johannesburg

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