SOMATIC EMBRYOGENESIS OF ROSE (*Rosa* sp.)

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ABSTRACT
SOMATIC EMBRYOGENESIS OF ROSE (*Rosa* sp.)
By Tammy Lynn Estabrooks
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Somatic embryogenesis (SE) offers vast potential as a method for clonal propagation of roses. By this method, tissue from an elite plant is cultured *in vitro* and induced into an embryogenic state, in which bipolar somatic embryos are formed. The somatic embryos are capable of regenerating whole plants that are genetically identical to the plant used as the original tissue source. Although somatic embryogenesis holds several advantages over conventional means of propagation, SE technology requires improvement before use on a commercial scale. This thesis describes studies aimed at developing somatic embryogenesis technology for roses.

A suitable methodology for SE of the commercially valuable rose cultivar 'Livin' Easy' (*Rosa hybrida* L.) was developed. This is the first report of SE induction in the cultivar 'Livin' Easy'. Murashige and Skoog basal medium supported growth and promoted successful induction of embryogenic tissue when supplemented with plant growth regulators, whereas Woody Plant Medium and plant growth regulator-free medium did not. The synthetic auxin 2,4,5-trichlorophenoxyacetic acid was successful in inducing SE at higher rates and over a greater concentration range than the commonly employed 2,4-dichlorophenoxyacetic acid. This was the first report of the utilization of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) for the induction of SE in rose. Somatic embryos successfully converted into plantlets at high rates up to 95%. Embryogenic tissue occurred on 60% of the plantlets, a phenomenon referred to as recurrent somatic embryogeneis.

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2,4-D	2,4-dichlorophenoxyacetic acid
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
ABA	abscisic acid
ac	activity
ad	adventitious shoot
ca	callus
cv	cultivar
ET	embryogenic tissue
hp	hair-like projections
IEDC	induced embryogenic determined cells
MS	Murashige and Skoog
pc	procambium
PEDC	pre-embryogenic determined cells
SE	somatic embryogenesis
PET	Presumptive Embryogenic Tissue
se	somatic embryo
S.D.	standard deviation
va	vascular tissue
wh	white
WPM	Woody Plant Medium

1. INTRODUCTION

1.1 ROSES

1.1.1 The Family Rosaceae

Roses belong to the family Rosaceae, which consists of many economically important plants such as apples, cherries, strawberries, peaches, apricots and plums. Members of this family are dicotyledonous plants characterized by the presence of alternate leaves that may be simple or compound. The flowers of this family generally consist of five sepals, often fused at their bases to form a hypanthium. Five is the common number of petals in members of the family Rosaceae, however cultivated roses may vary in petal number. Pistils may be simple or compound and ovary position varies from completely superior to half or fully inferior.

The family Rosaceae is divided into four subfamilies based mainly on fruit type and development: Maloideae, Prunoideae, Spiraeoideae and Rosoideae. Maloideae has pome-type fruit and includes the economically important genera *Malus* (apple), *Pyrus* (pear) and *Sorbus* (Mountain ash). Prunoideae, including the genus *Prunus* (apricot, peach, plum and cherry), possess fruit that are drupes. Fruit of members of the family Spiraeoideae are aggregates of follicles. This family includes the genus *Spirae* (Bridal Wreath). The fruit of the subfamily Rosoideae, which contains the genus *Rosa* (rose), is an aggregate of achenes or drupelets.

Growth habits of species in the genus *Rosa* may be upright, trailing, or climbing. The leaves are deciduous and alternate in arrangement. The leaves are composed of an

odd number of leaflets; most often five although seven leaflets are typical of lines with *R. wichurana* and *R. multiflora* in their ancestry (Grant, 2001). The woody stems are generally prickly, although in some cultivars the prickles may be absent (Grant, 2001). The blooms may be solitary or corymbose with up to 100 flowers. The flowers are hermaphroditic and vary greatly in terms of shape and size. After the flowers have bloomed, the receptacles become fleshy to form the fruit of the rose termed the 'hip'. Rose hips contain the seeds of the plant, which vary in number from few to many.

1.1.2 History of Roses

Fossils of roses have been dated to be over 60 million years old (Simpson & Orgorzaly, 2001). Roses originated in Asia and were subsequently distributed throughout the temperate and subtropical regions of the Northern Hemisphere (Roberts *et al.*,1995). The reported number of species in the genus *Rosa* varies from over 120 to 250 species (Simpson & Orgorzaly, 2001; Kim *et al.,* 2003a) with the exact number of species being unknown (Rout *et al.*, 1999). An approximated 25,000 cultivars of roses have been developed through crosses between rose species (Simpson & Orgorzaly, 2001) although some have estimated as many as 40,000 cultivars have existed over the centuries (Grant, 2001). Roses are cultivated world-wide as garden plants and for cut-flower production, accounting for over $500 million of the world's cut flower market each year (Simpson & Ororzaly, 2001). Two hundred million bushes and two billion cut-flowers are sold annually across the world (Roberts *et al.,* 1995). Roses are also produced as a source of aromatic oils used in the perfume and cosmetic industry and as a source of vitamins, oils, and pigments for use in the food industry.

1.1.3 Rose Breeding

Conventionally, new varieties of roses have been bred through the hybridization of different rose species. The vast majority of rose cultivars developed have been based on only eight of the wild *Rosa* species: *R. chinensis, R. wichuraiana, R. odorata, R. moschata, R. damascena, R. multiflora, R. foetida,* and *R. rugosa* (Krüssmann, 1981). The objective of breeding programs vary, however the common goal is to produce new and improved varieties of roses with enhanced qualities such as flower form, size, color, hardiness and increased disease resistance.

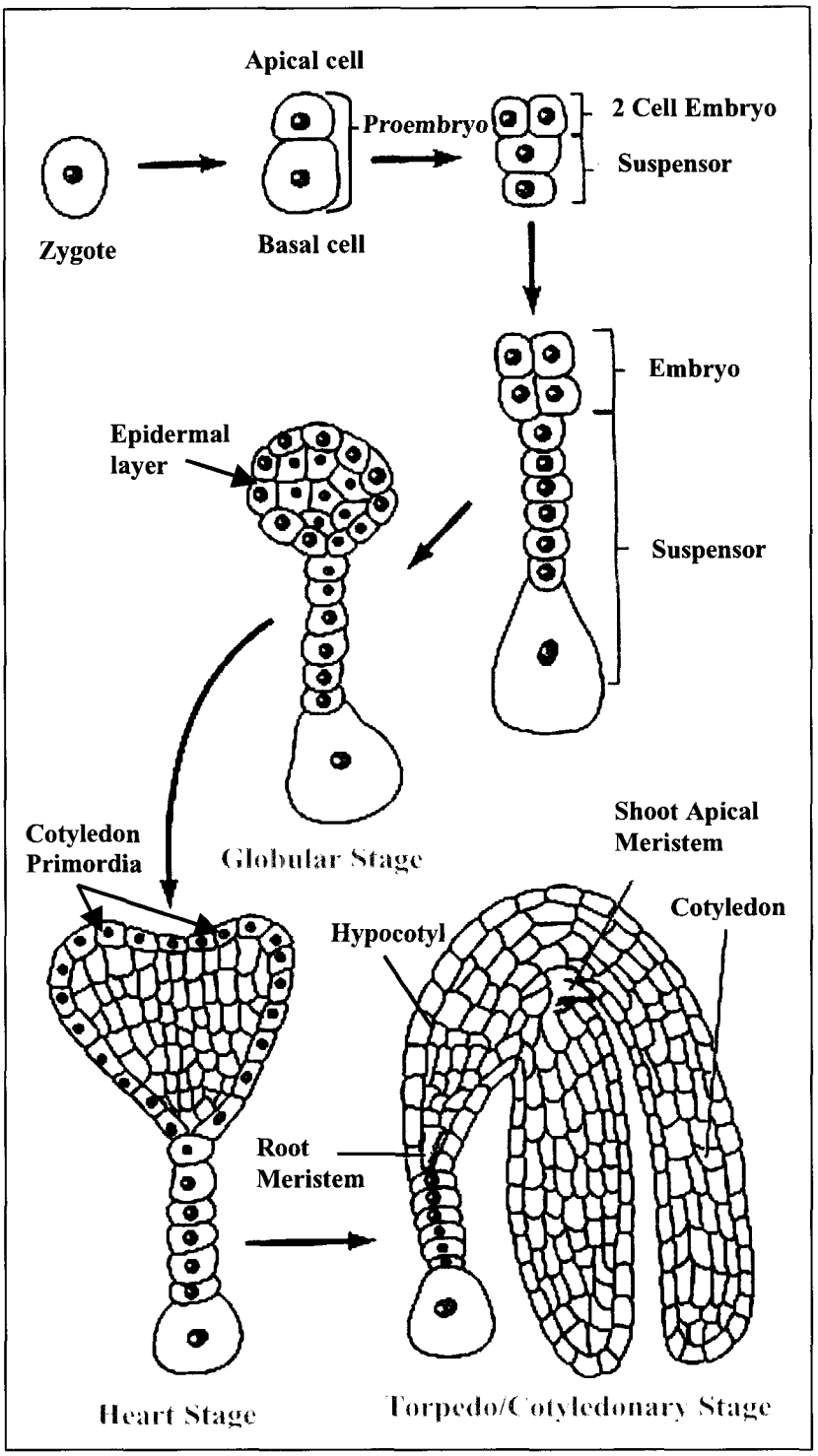
Parent selection is a key aspect to successful rose breeding. Not all roses are capable of producing seed or supplying functional pollen, while others may produce seed with poor germination capabilities. All rose flowers are hermaphroditic, containing both male and female parts, and therefore have the ability to pollinate themselves. To prevent self-pollination, the flowers of both parents are emasculated, meaning their stamens are removed. The flowers of the seed bearing parent (the female) are covered to prevent any unwanted pollen from coming into contact with the stigmas. The stamens of the male parent are kept and dried to release the pollen grains from the anthers. Once the stigmas in the female parent are in a receptive stage, evident by the secretion of a wet, glutinous substance, the pollen grains are gently applied to the surface of the stigmas. Approximately three weeks after pollination, the flower receptacles begin to enlarge. This enlarged receptacle is the fruit of the rose plant, referred to as the hip, which contains the young seed. It takes approximately three months for the rose hips to ripen. Once ripe, the seeds are removed from the hips and chilled for about six weeks to break

down the seeds' dormancy (a process called stratification). After stratification, the seeds are planted, with germination occurring approximately three to six weeks later. The seedlings are screened for desired characteristics. The time required for the release of a new cultivar of rose, from the time of pollination, is six to ten years (Dole & Wilkins, 1999). The rate of success in producing a new cultivar of rose worthy of being named is only one in every 30,000 to 40,000 seedlings (Dole & Wilkins, 1999).

1.1.4 A Closer Look at Seed Development: Zygotic Embryogenesis

As previously discussed, the seeds produced in rose breeding programs are the result of a cross between two parent plants. The success of a breeding program depends greatly on the development and germination of the seeds produced. Seed development commences with the fertilization of the female gametophyte by the pollen nuclei and continues with the development of the resulting zygotic embryo; a process referred to as zygotic embryogenesis. During pollination, the pollen tube penetrates the ovule and delivers a sperm nucleus that fuses with the egg to produce a zygote that will develop into the embryo. Another nucleus fuses with two polar nuclei of the female gametophyte to form the endosperm tissue. The endosperm tissue provides the developing embryo with nutrients. Zygotic embryogenesis in dicotyledonous plants begins when the first two divisions of the zygote occur and proceeds through characteristic stages of development including proembryo, globular, heart, torpedo and cotyledonary stages (Figure 1). A proembryo is formed by a transverse cell division to form an apical and basal cell. The basal cell forms the suspensor while the apical cell forms the embryo. The suspensor pushes the proembryo into the embryo sac cavity and transports nutrients to the

Figure 1. Diagram of characteristic stages during zygotic embryogenesis of a dicotyledonous plant (Modified from Singer, 1997).


Apical cell
Proembryo
2 Cell Embryo
Suspensor
Zygote
Basal cell
Embryo
Epidermal layer
Suspensor
Cotyledon Primordia
Globular Stage
Shoot Apical Meristem
Cotyledon
Hypocotyl
Root Meristem
Heart Stage
Torpedo/Cotyledonary Stage

proembryo. At the globular stage, three basic tissue systems can be recognized; dermal, ground, and vascular. Cotyledon primordia are evident in the heart-shaped embryo stage. These primordial elongate to form the torpedo stage embryo, which also has an apical meristem, radical, and hypocotyl.

1.1.5 Classification of Roses

Due to the complexity and large number of unnamed roses, rose breeders and nurseries classify roses based on their history, parentage and growth habits. There is not an official universal classification scheme however the majority of established rose societies in the world accept the system devised by the American Rose Society in partnership with the World Federation of Roses (http://www.ars.org). Based on this scheme, there are three main groups of roses: Species Roses, Old Garden Roses (also referred to as Antique Roses), and Modern Roses (Table 1).

1.1.5.1 The Modern Roses

Modern Roses are the common roses of the 20th century. They were the result of crosses of the European Roses and the Chinas, Teas, European and Mediterranean types, and various species roses during the 1700's and 1800's. They include several classes of roses, the following of which will be discussed here in greater detail: Hybrid Teas, Polyantha, and Floribunda roses. Hybrid Tea roses are the class of Modern roses most often associated with florists' shops. They are characterized by their large flowers generally borne one per stem. Hybrid Teas are medium to tall in habit with long stems. Examples of Hybrid Tea roses include the cultivars 'Peace' and 'Dainty Bess'.

Table 1. Classification of Roses.

Species Roses	Old Garden Roses	Modern Roses
Species	Alba	Floribunda
	Ayrshire	Grandiflora
	Bourbon	Hybrid Kordesii
	Boursalt	Hybrid Moyesii
	Centifolia	Hybrid Musk
	Damask	Hybrid Rugosa
	Hybrid Bracteata	Hybrid Wichurana
	Hybrid China	Hybrid Tea
	Hybrid Eglanteria	Large-Flowered Climber
	Hybrid Foetida	Minature
	Hybrid Gallica	Mini-Flora
	Hybrid Multiflora	Polyantha
	Hybrid Perpetual	Shrub
	Hybrid Sempervirens	
	Hybrid Setigera	
	Hybrid Soinisissima	
	Miscellaneous OGRs	
	Moss & Climbing Moss	
	Noisette	
	Portland	
	Tea & Climbing Tea	

Polyantha roses are a class of Modern roses characterized by small sized flowers borne in large clusters. The word 'polyantha' is derived from the Greek word for "many-flowered". Polyantha roses are usually compact in habit with short to medium length stems. Examples of Polyantha rose cultivars include ''Mothersday', 'Margo Koster' and 'China Doll'.

Floribunda roses were developed from crosses between Polyantha and Hybrid Tea roses. The word 'floribunda' literally means 'flowering in abundance.' Floribunda roses are characterized by their large clusters of small to medium-sized blossoms. They are compact in habit and have medium length stems. Examples of Floribunda roses include the cultivars 'Betty Prior', 'Gruss an Aachen', and 'Livin' Easy'.

1.1.6 Description of the Floribunda Rose Cultivar 'Livin' Easy'

'Livin' Easy' (*Rosa hybrida* L.) was bred in England by the hybridizer Harkness in 1992 (patent #9161) (Figure 2). This cultivar was the result of a cross between 'Southampton' and 'Remember Me'. 'Livin' Easy' is a floribunda rose, producing clusters of medium sized flowers on each stem. The flowers are an apricot/orange blend with a moderate to strong fruity fragrance. The petal count ranges from 25 to 30. The plant itself has a height of 2½' to 5' and a width of 2' to 6'.

'Livin' Easy' was selected for investigation in the present study due to its high commercial value in the rose market. 'Livin' Easy' was awarded the All American Rose Selection in 1996 and is well known for its hardiness and resistance to disease. 'Livin'

Figure 2. Photograph of rose cultivar 'Livin' Easy' (*Rosa hybrida* L.).

Easy' is also known as 'Fellowship' and 'HARwelcome'.

1.2 PROPAGATION OF ROSES

1.2.1 Traditional Methods of Rose Propagation

Grafting is a technique that has been traced back to ancient times as early as 1560 B.C. (Hartmann *et al.*, 1997). Grafting is a technique in which parts from two different plants are joined such that they unite and continue to grow as a single plant. The upper portion of the graft is termed the scion and the lower portion is referred to as the rootstock or understock. Many rose cultivars do not rapidly produce large plants suitable for sale when grown on their own root systems. As a result scions of a desired cultivar are budded onto rootstock to produce a more vigorous plant (Roberts *et al.,* 1995). *Rosa canina*, *Rosa multiflora, R. manetti,* or *Rosa* sp. Cultivar 'Dr. Huey' are some of the most common rootstocks used for roses. Budding is a form of grafting. In budding a small piece of scion bark containing a single bud is inserted beneath the bark of a rootstock via a T-shaped cut and then bound with an elastic or raffier to keep the bud in place. Budding however is a difficult method of propagation and requires considerable skill (Hamilton & Midcap, 1987). This procedure is very labour intensive, making it one of the most expensive methods of propagation (Hartmann *et al.*, 1997). It has been estimated that budding is three times more expensive than cuttings and fourteen times more costly than propagation by seed (Maynard & Bassuk, 1990). In addition to high cost, caution must be taken to prevent the spread of disease through grafting procedures. The infective agents for rose virus-induced diseases, such as Rose mosaic and Rose

rosette disease, are spread through budding. Another drawback of grafting roses is variation amongst the plants (De and Dubois, 1992).

Roses are also propagated by cuttings, hardwood and softwood. The use of rooted cuttings for clonal propagation of plants has been practiced for centuries (Cervelli & Senaratna, 1995). Softwood cuttings are generally taken from plants in spring or early summer during a growth flush when the tissue is relatively soft and succulent. Hardwood cuttings are taken from matured rose stems in autumn. Both softwood and hardwood cuttings are sometimes treated with rooting hormones to induce root formation. Cuttings are planted in a variety of formats including trenches lined with sand. Hardwood cuttings are used to propagate large quantities of the rootstock *Rosa multiflora* (Hartmann *et al.*, 1997). Rooted cutting methods can be slow, with less than 60 percent of *Rosa multiflora* hardwood cuttings developing into useable rootstocks after two years of commercial production (Davies, 1985). Lack of rooting uniformity between field location and season is another drawback (Davies, 1985). The limited availability of suitable stems also poses problems.

1.2.2 Tissue Culture of Roses

1.2.2.1 Shoot Tip Culture

Another means of propagating roses is through plant tissue culture, which is defined generally as the growth of plant cells, tissues or organs under sterile conditions in culture (*in vitro*). One technique carried out through plant tissue culture is that of shoot tip culture. Shoot tip culture was first employed for *Rosa multiflora* in 1970 (Elliott,

1970). Since then, there have been numerous reports of the *in vitro* propagation of roses via shoot tip culture (reviewed by Rout *et al.*, 1999). There are essentially four stages involved in the shoot tip culture of plants: establishment, multiplication, rooting and acclimatization. The objective of the first stage is to establish the plant in culture. The goal is to disinfect a portion of a plant successfully (referred to as an explant) and, while maintaining sterile conditions, provide an *in vitro* environment that promotes shoot production (McCown, 1986). The portion of the plant selected varies depending on the desired type of culture but nodal explants are commonly used. The nodal explant, a short piece of rose stem containing a single bud, is disinfected using chemicals, such as sodium hypochlorite, that are toxic to microorganisms but relatively nontoxic to the plant material. The explants are then placed on an artificial nutrient medium. The nutrient medium generally consists of a semisolid support (such as agar or Phytagel™), containing inorganic elements, an energy source, and vitamin supplements (Hartmann *et al.*, 1997). Plant growth regulators are also typically added to media to achieve desired growth and developmental patterns. Plant growth regulators are substances that influence growth and development of plants in a variety of ways and will be discussed in later sections.

The second stage of shoot tip culture is multiplication. The objective of this stage is to induce the explant to produce numbers of microshoots. This increase in number is achieved through repeated subculturing of the microshoot clusters onto fresh medium. At each subculture, approximately every three to four weeks, the shoots are divided to increase the total number. The third stage of shoot tip culture involves the rooting of the

explant. Rooting may be carried out *in vitro* or at the time of transplanting (*ex vitro*). *In vitro* rooting is usually achieved by adding a plant growth regulator to the medium that induces root formation. Once an explant has formed roots, it is referred to as a plantlet. *Ex vitro* rooting usually involves dipping the basal ends of each shoot into a solution containing an appropriate plant growth regulator prior to inserting the shoot into a medium such as soil.

The fourth stage of shoot tip culture involves transplanting and acclimatization of the plantlets. When plants are grown *in vitro*, the relative humidity within culture vessels is very high (up to 100%) (Johansson *et al.*, 1992). Due to this high humidity, the leaves of the plantlets have thin cuticles (lacking leaf wax) and the stomata are not functional, remaining open (Sallanon & Maziere, 1992). Furthermore, plantlets grown *in vitro* are supplied with all of the required nutrients in the culture medium and therefore are heterotrophic. The plantlets must go through an acclimatization period in which they are gradually exposed to greenhouse or outdoor conditions. During this transition period plantlets become autotrophic. They develop thicker cuticles and functional stomata capable of opening and closing. To aid in this transition, the plants are usually misted and transpiration is restricted via shading. Acclimatized plantlets can grow in normal greenhouse or field environments.

1.2.2.2 The Role of Plant Growth Regulators

Plant growth regulators are substances that, at low concentrations, regulate plant growth and differentiation. Plant growth regulators are employed in plant tissue culture

media to support and direct the developmental processes of the plant cells (Hartmann *et al.*, 1997). Plant growth regulators have been formerly referred to as plant hormones or phytohormones. Certain aspects of the term 'hormone', notably 'action at a distance from the site of synthesis', do not necessarily apply to plants and therefore the terminology was revised. The major classes of plant growth regulators include auxins, cytokinins, abscisic acid, gibberellins, and ethylene. Several other plant growth substances are known to exist, such as brassinosteroids, salicylates, and jasmonates.

Auxins are a class of plant growth regulators characterized by their ability to induce many physiological processes in plants including; cellular elongation, phototropism, apical dominance, root initiation, fruit development and abscission (Arteca, 1996). The term auxin is derived from the Greek word *auxein*; meaning to grow. Auxin production in plants occurs in actively growing tissues such as shoot meristems, leaf primordia, and developing seeds and fruits. The class auxin includes both naturally occurring compounds such as indole-3-acetic acid (IAA) as well as synthetic compounds including indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), naphthalenacetic acid (NAA), 3,6-dichloro-*o*-anisic acid (dicamba) and 4-amino-3,5,6-trichloropyridine-2-carboxylic acid (picloram).

Cytokinins are substituted adenine compounds that stimulate cell division, organ formation, chloroplast development, stomatal functioning, bud and shoot development, delay of senescence and release of lateral bud dormancy. Kinetin (6-furfurylaminopurine) was the first cytokinin isolated from autoclaved herring sperm DNA and used to induce

cell division in tobacco pith tissue (Arteca, 1996). Although kinetin is a natural compound, it is not made in plants, and is therefore usually considered a "synthetic" cytokinin . Cytokinins are believed to be synthesized in fruit and seed tissues as well as in the roots of plants where they are then translocated to the shoot regions (Arteca, 1996). 6-(4-hydroxy-3-methyl-trans-2-butenyl-amino) purine (zeatin) is a cytokinin naturally occurring in plants. Two commonly used synthetic cytokinins are 6-furfurylaminopurine (kintein) and 6-benzylaminopurine (BA).

Abscisic acid (ABA) is a naturally occurring compound in plants. ABA promotes normal embryogenesis, accumulation of seed storage proteins, dormancy and seed germination. ABA also has many inhibitory properties such as maintaining bud and seed dormancy and the slowing of cell elongation (Gasper *et al.*, 1996). ABA is synthesized in the leaves of plants, in particular in chloroplasts and other plastids.

Gibberellins are a class of plant growth regulators that stimulate cellular division, cellular elongation, seed germination, dormancy, senescence and fruit set. Gibberellins were first discovered after studies on rice plants infected by the fungus *Gibberella fujikuroi,* which caused abnormal stem elongation. Gibberellins are produced within actively growing shoots and developing seeds of plants.

Ethylene is an unsaturated hydrocarbon that promotes fruit ripening, abscission, flowering, shoot and root growth and apical dominance. Ethylene is synthesized in almost all parts of plants, especially in response to disease, damage, or senescence.

1.2.2.3 Advantages of Shoot Tip Culture

Shoot tip culture has many advantages over traditional methods of propagation. Plants produced via this system are genetically identical and thus display "phenotypic uniformity" (Kurtz *et al.*, 1991). Large volumes of disease-free plants can be rapidly produced year round without seasonal down times. Shoot tip culture may be especially useful for species that have proven difficult or impossible to propagate by traditional methods (George & Sherrington, 1984).

1.2.2.4 Disadvantages of Shoot Tip Culture

While *in vitro* shoot tip culture has the advantage of producing large numbers of genetically homogenous plants, there are several limitations associated with this method of propagation. The major disadvantage of shoot tip culture is the high initial capital and high production costs. For many plant species, the production costs are high compared to those of conventional methods such as cuttings and grafting (George & Sherrington, 1984) and are not cost competitive (Kurtz *et al.*, 1991). Labour comprises 64% of the costs involved in commercial shoot tip culture with 34% of those labour costs generated during the multiplication stage (Kurtz *et al.*, 1991).

Another drawback of shoot tip culture is that the shoots produced require subsequent rooting. The rooting process has been estimated to account for 35-75% of the total cost of shoot tip culture (Debergh & Maene, 1981). With respect to roses, difficulty has been reported in the rooting of microshoots (Ma *et al.*, 1996). Rooting capability varies, being relatively easy in *R. wichuraiana* while very difficult in *R. banksias and R.*

fortuniana (Ma *et al.*, 1996).

A substantial number of plantlets produced through shoot tip culture do not survive the transition to the greenhouse (Hazarika, 2003). Low survival rates (50-80%) have been reported for several micropropagated plants (Ma *et al.*, 1996; Fuchigami *et al.*, 1981 and Pennell, 1987). These low survival rates have obvious negative impacts on economics making this stage a major bottleneck in the propagation of many plants (Hazarika, 2003).

1.2.3 An Alternate Approach to Rose Propagation: Somatic Embryogenesis

Due to the limitations of conventional propagation methods, novel technological methods of propagation are being developed for roses. One such technology is that of the plant tissue culture process: somatic embryogenesis. Zygotic embryogenesis was summarized in section 1.1.4 and the aim of somatic embryogenesis is to induce vegetative (somatic) cells in a rose or other plant to go through the same stages as an embryo derived from an egg cell fertilized by a nucleus from a pollen tube.

1.2.3.1 The Concepts Behind Somatic Embryogenesis

Plants are multicellular organisms, composed of highly organized tissues and organs. Plant development, like that of all multicellular organisms, involves the differentiation of cells such that they "acquire distinct metabolic, structural and functional properties" (Taiz & Zeiger, 1998). Plant cells differentiate into either sexually reproductive cells or somatic (asexual) cells. Sexually reproductive cells are responsible

for the sexual production of new generations. The fusion of male and female gametes involves the recombination of genetic information. This genetic mixing results in offspring that are genetically different from the parent plants. Genetic diversity provides plants with a selective advantage helping to ensure survival of the species during environmental change by adapting to a variety of different environmental niches.

In plants, somatic or vegetative cells have two distinct functions: that of vegetative growth and that of asexual reproduction. During their lifespan, plants must continuously generate new tissues and organs. This is necessary to regenerate new organs in new growing seasons or to repair damage caused by herbaceous animals. Asexual reproduction involves the production of plants genetically identical to that of the single parent plant, a process also known as cloning. Asexual reproduction allows plants to reproduce when the costs of sexual reproduction are too high, environmental conditions are not suitable for sexual reproduction or where individuals may be isolated, in terms of distance, from other individuals. Plants are capable of asexual reproduction and regeneration of lost parts due to the totipotent nature of their cells. Many plant cells are totipotent, meaning they can differentiate into "the entire spectrum of cell types" found within the plant (Weigel & Jürgens, 2002).

For most animal cells the differentiation process is irreversible whereas plant cells have the unique ability to *de*differentiate. Once a plant has developed into a specific structure, such as a leaf, it is able to revert cells of the leaf back into an unorganized (*de*differentiated) state. Acquisition of embryogenic competence relies heavily on

dedifferentiation, "a process whereby existing transcriptional and translational profiles are erased or altered in order to allow cells to set a new development program" (Fehér *et al.*, 2003). Upon dedifferentiating, plants can access the genetic information for the entire genome and therefore regain the undifferentiated or embryonic state. Given the sessile state of plants, this ability to "adapt their programme of differentiation and growth" is an important survival mechanism (Roberts *et al.*, 2002).

The abilities of plants to reproduce asexually and plant cells to dedifferentiate have been used for plant propagation both for traditional and plant tissue culture methods. One process carried out through plant tissue culture technology is that of somatic embryogenesis (SE). SE is the development of bipolar embryos from somatic cells and tissues; bipolar meaning they possess both a shoot and a radical end like a zygotic embryo (Sharp *et al.*, 1980). The embryos produced, referred to as somatic embryos, are capable of regenerating into whole plants. The embryos are produced asexually, therefore the resulting plants are genetically identical to the tissue from which the embryos were derived. Somatic embryogenesis, in addition to being a method of vegetative propagation, can serve as an experimental tool for research into plant embryo development, which is a difficult task given the intact nature of plant seeds. Somatic embryogenesis does occur on occasion in nature (Taylor, 1967) although it is best known as a pathway used in plant tissue culture to propagate plants. Somatic embryogenesis was first reported in carrot (*Daucus carota*) in 1958 (Steward *et al.*, 1958; Reinert, 1958). The first report of somatic embryogenesis in rose was in 1988 (Douglas, 1988); however it was uncertain if the structures observed were adventitious buds or somatic embryos.

Reports of plant regeneration via SE did not emerge until the early 1990's (reviewed by Roberts *et al.*, 1995). SE has since been reported in various members of the Roseaceae family, including peach (*Prunus persica*) (Hashmi *et al.*, 1997) and apple (*Malus domestica*) (Wallin *et al.*, 1995).

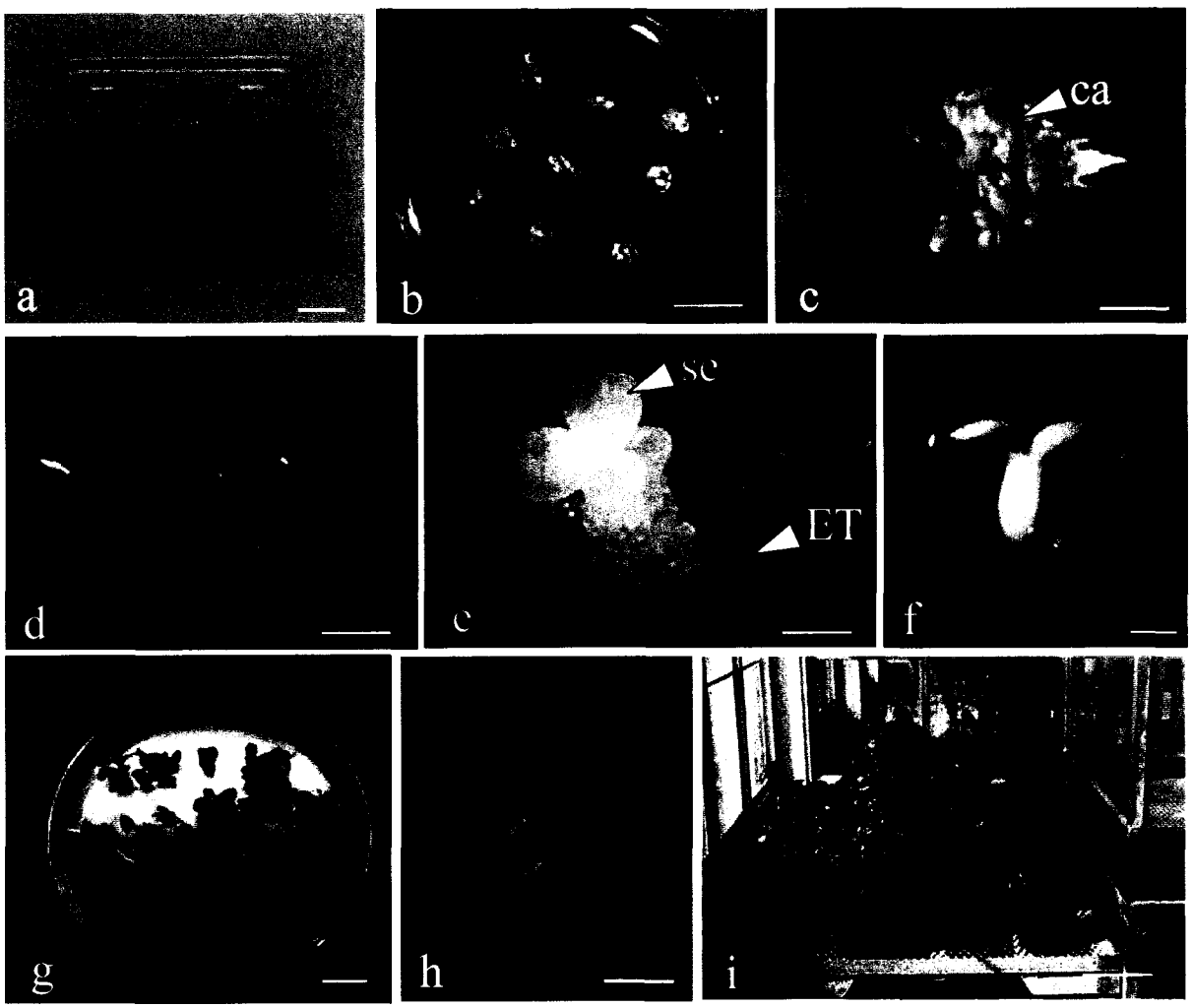
1.2.3.2 Initiation of Embryogenic Tissue

Plant cells have been classified as either "pre-embryogenic determined cells" (PEDCs) or "induced embryogenic determined cells" (IEDCs) (Sharp *et al.*, 1980; Evans *et al.*, 1981). PEDCs are genetically capable of inducing embryogenesis at the time of cultivation *in vitro*. Zygotic embryos have been reported as one source of pre-embryogenic determined cells (Merkle *et al.*, 1990). Induction of somatic embryogenesis using zygotic embryos in roses has been reported by researchers (Arene *et al.*, 1993; Kunitake *et al.*, 1993; Burger *et al.*, 1990). Zygotic embryos can be induced to form embryogenic tissue directly, a process referred to as direct somatic embryogenesis. Programs related to induced somatic embryogenic techniques often have as their objective the cloning of a mature plant after its characteristics and performance have been evaluated and found to be desirable. This is especially true of roses whose economic market is driven by consumers' demands for specific floral forms and horticultural traits such as disease resistance and winter hardiness. Zygotic embryos cannot be successfully used for this purpose since they are produced sexually through the fusion of male and female gametes. Sexual reproduction results in the zygotic embryos being genetically different from the plant that produced them. To clone a mature plant, tissues from the plant itself must be used to induce somatic embryogenesis. The tissues consist of cells

that are IEDCs and require a "genetic activation" in order to enter an embryogenic state and be capable of producing embryos. This process is known as indirect somatic embryogenesis and in roses such sources as mature leaves (Kintzios *et al.*, 1999), mature petals (Murali *et al.*, 1996), *in vitro* leaves (Ibrahim & Debergh, 2001; de Wit *et al.*, 1990), *in vitro* internodes (Rout *et al.*, 1992) and *in vitro* roots (van der Salm *et al.*, 1996) have been used.

One method of indirect somatic embryogenesis begins with a plant growing *in vitro* (Figure 3a). Portions from this plant, such as leaflets or petioles, are removed. These explants are cultured onto a callus induction medium to induce explant tissue to dedifferentiate and form callus (Figure 3 b and c). Callus is basically a mass of proliferating unorganized cells. Factors reportedly used to induce callus and subsequent embryogenic tissue include plant growth regulators (Murashige and Tisserat, 1977), stress such as starvation of the tissue (Lee *et al.,* 2001), and heat shock. Exogenously supplied auxins often play a critical role in facilitating the physiological and morphological changes required to produce a somatic embryo. Indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), and naphthalene acetic acid (NAA) are commonly used to induce SE. The exact stages and mechanisms involved in the transition of callus cells into embryogenic cells are not known, however the importance of auxins in inducing this 'switch' is very evident. Reprogrammed gene expression was evident by the synthesis of new messenger ribonucleic acid (mRNA) after exposing cells growing *in vitro* to 2,4-D (Hagen *et al.*, 1984; Van der Zaal *et al.*, 1987) (Appendix A).

Figure 3. Stages of indirect somatic embryogenesis a) *in vitro* rose shoot cultures b) plate with nine leaflet explants on callus induction medium c) leaflet with callus (ca) growth d) embryogenic tissue e) somatic embryo (se) formed from embryogenic tissue (ET) f) normal dicot somatic embryo g) petri plate containing germinants h) rose plantlet i) rose plants growing in greenhouse. a,b,g, and h Bar 1cm; c,d,e and f Bar 1mm; i Bar 100cm.


a
b
c
ca
d
e
se
ET
f
g
h
i

Embryogenic tissue can be characterized by its mucilaginous appearance and the presence of nodular structures (Figure 3 d and e). These nodular structures are early stage globular somatic embryos capable of developing into bipolar cotyledonary embryos (Figure 3 f). In the present study, the term 'embryogenic tissue' is used (rather than 'embryogenic callus') due to the fact the tissue was identified as embryogenic only after the presence of nodular structures was observed. The nodular structures, as previously stated, are actually early-stage somatic embryos. Given the fact these clusters of embryos are not comprised entirely of "an unorganized mass of cells", the term callus is no longer appropriate. The term embryogenic tissue has been previously used in the literature (Pullman *et al.*, 2003; Reidiboym-Talleux *et al.*, 1999; Murali *et al.*, 1996; and Noriega and Söndahl, 1991). It should be noted that some previous studies used the term 'embryogenic callus' to depict the equivalent of what is referred to in the present study as 'embryogenic tissue'(Castillón & Kamo, 2002; Li *et al.*, 2002; Visessuwan *et al.*, 1997; and Hsia & Korban, 1996).

To refer to tissue not yet positively identified as embryogenic tissue (ET) but displaying other characteristics of ET, such as degree of softness and mucilaginous, the term 'presumptive embryogenic tissue' was devised for the present study. In the literature, 'embryogenic callus' has been used essentially as an equivalent term but it seemed necessary to create a new term to help clarify the stages involved in indirect somatic embryogenesis.

1.2.3.3 Embryo Maturation

To elicit further development of the globular embryos, they must be subjected to a period of maturation. Maturation is a phase of growth and development required for successful germination to occur. The embryos develop and acquire nutrients, such as storage proteins and lipids, needed for germination (Roberts *et al.,* 1990). Abscisic acid (ABA), as previously mentioned, is a naturally occurring hormone that plays a role in preventing precocious germination of seeds. *In vitro*, ABA is often supplemented in the maturation medium to mimic the natural environment. ABA regulates the synthesis and deposition of storage and late embryogenesis-abundant (LEA) proteins during both somatic and zygotic embryogenesis (Dodeman *et al.*, 1997).

1.2.3.4 Cold Period

Many seeds, particularly those of the rosaceous species, will not germinate until they have been exposed to low temperatures for periods ranging from weeks up to months (Salisbury and Ross, 1992). In nature, this low temperature requirement prevents precocious germination of seeds during sub-optimal growing conditions during winter. This cold period may also be necessary for the degradation of inhibitors and the accumulation of essential growth promoters (Khan, 1977). A cold period induces a triphastic change in seeds (Khan, 1980). The first stage consists of a reduction in abscisic acid (ABA) levels followed by an increase in synthesis of cytokinin and gibberellin. The final stage, induced by cold, involves a reduction in growth regulator production. During zygotic embryo development, ABA levels peak during the maturation stage and decrease during germination. It is necessary to mimic these changes *in vitro* for efficient somatic embryo germination.

1.2.3.5 Embryo Germination and Acclimatization

Once somatic embryos have fully matured, they are transferred onto a germination medium to promote germination of the embryos into miniature plants called plantlets or germinants (Figures 3 g and h). The germination medium often contains an increased carbohydrate level. As previously discussed, plantlets growing *in vitro* are heterotrophic and not morphologically prepared to be introduced to the harsher *ex vitro* environment. A period of gradual acclimatization must be carried out in order to harden off the plantlets and allow them time to adapt to the harsher *ex vitro* conditions.

1.2.3.6 Secondary and Repetitive Somatic Embryogenesis

Unfortunately, there appears to be some confusion and discrepancies within the literature with regard to the terminology used to define secondary and repetitive somatic embryogenesis. To avoid confusion, it is necessary to state the definitions of these terms as used in the present thesis. Secondary somatic embryogenesis occurs when primary somatic embryos give rise to a second or successive cycle of somatic embryos. The term 'secondary SE' appears to be standard throughout the literature in terms of its definition and use. In an effort to be more technically correct, some authors have opted to use the term repetitive SE to refer to all cycles after the initial cycle of somatic embryogenesis. This use of the term repetitive SE would be fine except for the fact that this term is also used to describe another facet of SE. Vasic and associates (2001) defined repetitive somatic embryogenesis as successive cycles of somatic embryogenesis induced on explants of previously *regenerated* plants (Vasic *et al.*, 2001). They used the term 'repetitive SE' to describe the situation where they used leaves of plants that were the

product of somatic embryogenesis as the starting material for a new cycle of somatic embryogenesis. Confusion arises in the fact that many authors use the terms secondary SE and repetitive SE interchangeably (Merkle *et al.*, 1990). For the purpose of the present study, secondary somatic embryogenesis will refer to the production of somatic embryos from primary embryos. Repetitive somatic embryogenesis will refer to any subsequent cycles of embryos produced after secondary somatic embryogenesis. To describe somatic embryogenesis occurring from regenerated plants, the term 'recurrent somatic embryogenesis' will be used.

1.2.3.7 Advantages of Somatic Embryogenesis

Somatic embryogenesis shares many of the advantages of other forms of plant tissue culture: the production of genetically identical, disease-free plants, rapid production rates, production of high volumes, year round. In addition to these benefits, SE has many distinct economically important advantages over shoot tip culture as less space is needed and more plants can be produced (Cerveilli & Senaratna, 1995). The products of shoot tip culture are unipolar shoots, while SE produces bipolar somatic embryos, possessing both radical and shoot meristems. These bipolar embryos are capable of germinating into plantlets containing both shoots and roots: similar to that of a zygotic seed. The production of bipolar embryos greatly reduces the potential costs of production, in that it eliminates the costs of shoot multiplication and subsequent rooting procedures. Bipolar somatic embryos may also be utilized for the production of synthetic seeds. A synthetic seed typically consists of a somatic embryo enclosed within a nutritional medium and encased in an artificial seed covering such as sodium alginate or

polyoxyethylene (Gray, 1990). Synthetic seed form varies depending on the specific crop application. For example, synthetic seeds may or may not have a synthetic seed coat or may be hydrated or dehydrated (Gray & Purohit, 1991). Synthetic seeds have been developed for plants including apple rootstock M.26 (*Malus pumila* Mill.) (Brischia *et al.*, 2002); asparagus (*Asparagus officinalis* L.) (Mamiya and Sakamoto, 2001); camellia (*Camellia japonica*) (Janeiro *et al.*, 1997); and rose (*Rosa hybrida* L.) (Jayasree & Devi, 1997). Synthetic seed technology combines the convenience of seeds with the benefits of a clonal propagation system.

Somatic embryos are smaller and more uniform in size than micropropagated shoots, making them more amenable to bulk handling, mechanical sorting and automation (Cerveilli & Senaratna, 1995). Somatic embryos and embryogenic tissue can also be cryopreserved at ultra low temperatures to maintain a juvenile state during long-term field testing (Park *et al.*, 1994).

Somatic embryogenesis can also be used to develop new cultivars through somatic hybridization, which is the fusing of protoplasts (plant cells whose cellulose walls have been removed) from two cultivars or species to form a new hybrid cell. Somatic hybridization of different *Rosa* species is one means of introducing novelty or overcoming sexual incompatibility (Kim *et al.*, 2003a). It may also permit the hybridization of rose with related genera, which would greatly enhance the gene pool. Somatic embryogenesis has greatly facilitated somatic hybridization technology by providing a means for regenerating the plants from the newly generated protoplast.

Somatic hybridization followed by successful regeneration of the plants using SE has been reported in rose (Kim *et al.*, 2003a).

Genetic improvement through transformation is another method of creating new rose cultivars. Transformation can be defined as the transfer of foreign deoxyribonucleic acid (DNA) into a plant cell. SE can provide target material for genetic manipulations (Kim *et al.*, 2003b), such as in the callus or embryogenic tissue stages. The use of callus or embryogenic tissue is ideal since somatic embryogenesis arises from single cells and may be used to multiply the transformed cells. Firoozabady *et al.* (1994) used embryogenic tissue cultures of *Rosa hybrida* L. cultivar 'Royalty' in an *Agrobacterium*-mediated transformation system to produce transgenic roses.

1.2.3.8 Limitations of Somatic Embryogenesis

In comparison to conventional methods of propagation, rose SE technology is relatively new. Despite this, much progress has been made towards the development of rose SE systems, with many advantages already being seen over other propagation methods. Somatic embryogenesis offers vast potential but it requires improvement before it can be implemented on a commercial scale (Rout *et al*, 1999).

One limiting factor of rose SE is the varying capacity for somatic embryogenesis in different rose genotypes (von Arnold *et al.*, 2002). Thus, a universal protocol for SE does not yet exist. It is therefore necessary to devise and optimize protocols specifically for each cultivar, sometimes necessitating extensive experimentation. This

experimentation may result in high costs for the initial development of SE for a given rose cultivar (Cervelli & Senaratna, 1995).

A factor currently hindering SE technology in plants as a whole is somaclonal variation. Plants originating from plant tissue culture are referred to as somaclones (Larkin & Scowcroft, 1981). Genetic variation occurring amongst somaclones causes a lack of uniformity amongst the plants produced. Somaclonal variation is especially prevalent for whole plants that are regenerated from callus. It has been reported for numerous species of plants regenerated from callus, including white spruce (Isabel *et al.*, 1996), peach (Hashmi *et al.*, 1997) and ginger (Rout *et al.*, 1998). Somaclonal variation may be useful if the objective is to identify new plants with minor, yet stable, variants in desirable characteristics (Tulsieram *et al.*, 1992). In utilizing somatic embryogenesis as a clonal propagation system, somaclonal variation can be a serious drawback. As a result is necessary to monitor SE-derived plants for evidence of somaclonal variation.

Another factor hindering rose SE technology is low induction rates of embryogenic tissue (Rout *et al.*, 1999; Hsia & Korban, 1996) and low frequencies of germination (Sarasan *et al*, 2001; Rout *et al.*, 1999). There are also several rose cultivars unresponsive to routine methods employed to induce somatic embryogenesis (Kintzios *et al.*, 1999; Murali *et al.*, 1996). It is clear that to date, not enough knowledge and information has been acquired on each of the stages involved in rose SE.

1.3 OBJECTIVE OF THE PRESENT PROJECT

Somatic embryogenesis offers vast potential as a method of clonal propagation of roses but it requires improvement before use on a commercial scale (Rout *et al*, 1999). The aims of this project were to: 1) Determine a suitable method for somatic embryogenesis of 'Livin' Easy' (*Rosa hybrida* L.). 2) Gain a greater understanding of some of the factors influencing SE of 'Livin' Easy'(*Rosa hybrida* L.) and in doing so, optimize the SE system. 3) Gauge the effectiveness of the refined protocols used for 'Livin' Easy' on SE in other cultivars of rose. 4) Identify, morphologically, the different somatic embryos produced in 'Livin' Easy' via SE and assess their ability for conversion into plantlets.

2. FACTORS INFLUENCING SOMATIC EMBRYOGENESIS IN *Rosa hybrida* L. CULTIVAR 'LIVIN' EASY'

2.1 INTRODUCTION

There are several stages involved in the process of indirect somatic embryogenesis (SE): callus induction, embryogenic tissue initiation, maturation, cold treatment, germination, transplantation and acclimatization (see Chapter 1). Variables, such as media formulation, plant growth regulators, and explant type may influence somatic embryogenesis.

2.1.1 Nutrient Medium

Media formulation is a critical part of the development of protocols for plant tissue culture methods such as somatic embryogenesis (Rout *et al.*, 1998). Media must be selected that are appropriate for the particular species or cultivar and for the type of culture being undertaken. Success of somatic embryogenesis in many cultivars of rose is influenced by the nutrient media employed (de Wit *et al.*, 1990). One of the most extensively used medium for angiosperm tissue culture is that of Murashige and Skoog (Murashige and Skoog, 1962). Murashige and Skoog medium was first devised for the growth of tobacco tissue cultures. Another frequently employed medium is Woody Plant Medium (WPM) (Lloyd and McCown, 1980) which was developed for trees and shrubs such as *Betula, Kalmia, Rosa* and *Rhododendron.*

2.1.2 Auxins

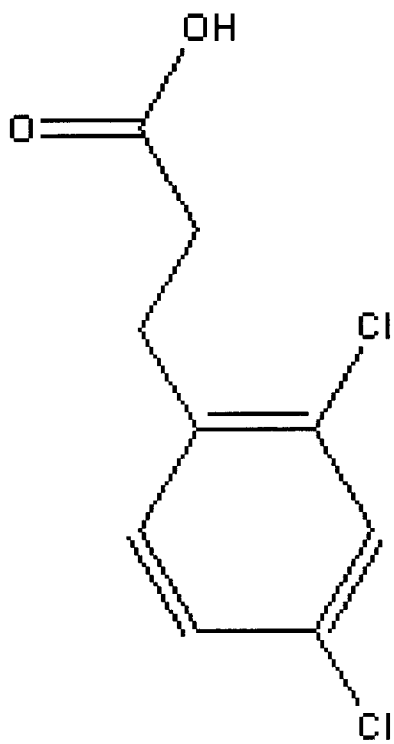
Plant growth regulators, such as auxins, play a key role in somatic embryogenesis. The synthetic auxins, 2,4-dichlorophenoxy-acetic acid (2,4-D) and 2,4,5-trichlorophen-

oxyacetic acid (2,4,5-T) are chlorinated phenoxy compounds (Figure 4). These two compounds are also used as herbicides to control many types of broadleaf weeds. 2,4-D is reported to have both cytotoxic and mutagenic effects while 2,4,5-T has cytotoxic effects but very weak, if any, mutagenic effects (Venkov *et al.*, 2000). A lower mutagenicity may be better for reducing the occurrence of somaclonal variation in regenerated plants. Both of these synthetic compounds are analogues of the natural auxin indole-3-acetic acid (IAA) (Venkov *et al.*, 2000). These synthetic analogs are much more stable than IAA, which tends to be denatured in media and rapidly metabolized within plant tissues (Gasper *et al.*, 1996). 2,4-D is the most frequently employed in embryogenic cell and tissue culture systems (Fehér *et al.*, 2003). 2,4-D has been employed in somatic embryogenesis studies on plant species including rose (Marchant *et al.*, 1996; Noriega & Söndahl, 1991; Matthews *et al.*, 1991; Roberts *et al.*, 1990). Although 2,4-D has been employed for rose somatic embryogenesis, induction rates are often low and it has proven ineffective for several cultivars (Li *et al.*, 2002). 2,4,5-T has been successfully employed in somatic embryogenesis of sweet potato (*Ipomoea batatas* L. Lam.) (Al-Mazrooei *et al.*, 1997) and chickpea (*Cicer arietinum* L.) (Sagare *et al.*, 1993) however there are no reports of its utilization in rose SE.

2.1.3 Explant Type

Explant selection is important for achieving successful induction of somatic embryogenesis. Various rose tissues have been cultured *in vitro* to test for SE response (Roberts *et al.*, 1989). Differences in response amongst explant type have been reported in studies of rose SE (Marchant *et al.*, 1996; Noriega & Söndahl, 1991; de Wit *et*

Figure 4. Chemical structures of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid.



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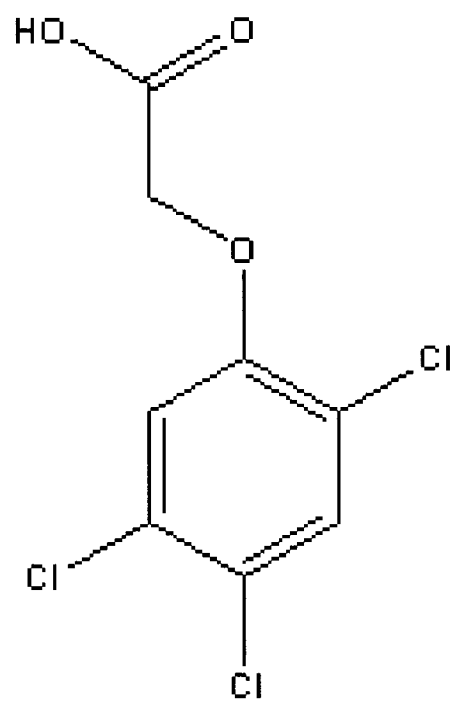


b

al., 1990). A "systematic analysis of the embryogenic potential of different explant sources" for a particular plant or cultivar may be required (Hartmann *et al.*, 1997).

2.2 OBJECTIVE

The aim of the research outlined in this chapter was to investigate the factors influencing somatic embryogenesis of the commercially valuable rose cultivar 'Livin' Easy' (*Rosa hybrida* L.); a cultivar for which no reports of somatic embryogenesis have been published. The factors studied included explant type, medium formulation and plant growth regulator type and concentration.

2.3 MATERIALS AND METHODS

2.3.1 Plant Material

Shoots of the rose cultivar 'Livin' Easy' (*Rosa hybrida* L.) were obtained from *Weeks Roses* (Upland, CA). Rooted cuttings were made from these shoots and grown in a greenhouse at Debert Tree-Breeding Centre, Debert, N.S. The rooted cuttings were collected and stored at 4°C for three days until subjected to the surface sterilization procedure. At the time of sterilization, shoots with slightly swollen buds were selected. Leaves were removed and the shoots were cut into single nodal sections approximately 4.0 cm in length. The nodal sections were rinsed with cold water and placed into a sterilized flask containing 50 ml 70% ethanol and agitated for two minutes on a rotary shaker (G10 Gyrotory® Shaker, New Brunswick Scientific, N.J., U.S.A). The nodal sections were then placed into 100ml 20% v/v solution of sodium hypochlorite containing 3-4 drops of Tween 80 and agitated for 20 minutes. The plant material was rinsed three

times in sterile distilled water, agitating for two minutes per rinse. In a laminar flow bench, one millimeter was cut from each end of the nodal sections using a sterilized scalpel. The nodal explants were placed vertically (bud up) into Phytatrays™ (Sigma) containing 100ml of ¾ strength Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) supplemented with 1.0 μM 6-benzylaminopurine (BA) (Sigma, B 3408). Media were adjusted to pH 5.7 using 1.0 N NaOH, solidified using 0.4% Phytagel™ (Sigma) and autoclaved for 20 minutes at 121°C, 104 kilopascals. The shoots were incubated at 22°C with a 16-hour photoperiod of light at 38 $\mu mol^{-2} \cdot s^{-1}$ and subcultured every four to six weeks for 16 months until being utilized for this study.

2.3.2 Callus Induction

Leaflet and petiole explants (5-7 mm in length) were aseptically excised from the previously established *in vitro* shoot cultures of 'Livin' Easy'. Four scratches, perpendicular to the midrib, were made on the underside of each leaflet using a sterilized scalpel. The wounded leaflets were placed, underside up, onto the various media (see below). Petiole explants were placed horizontally onto the media. Two different basal media were tested: MS (Murashige & Skoog, 1962) and Woody Plant Medium (WPM) (Lloyd & McCown, 1980) (Appendices B and C). Both basal media were modified as per Owen & Miller (1992) (refer to Appendices B and C). Media were adjusted to pH 5.7 using 1.0 N NaOH, solidified using 0.4% Phytagel™ (Sigma) and autoclaved for 20 minutes at 121°C, 104 kilopascals. Various concentrations (0, 5, 10 and 25 μM) of the auxins 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma D-7299) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Sigma T-5785) were separately tested. One

hundred and eight explants of each explant type were utilized for each treatment (nine explants per petri dish). The 100 x 15 millimeter plastic petri dishes were sealed with Parafilm® (American National Company) and incubated in the dark at 24°C. The explants were inspected for callus growth every week, for four weeks, using a dissecting microscope (NorthWest, Model# 1962711).

2.3.3 Embryogenic Tissue Initiation and Isolation

After four weeks, the explants were transferred to embryogenic tissue (ET) initiation media. The ET initiation media consisted of the same basal medium with either no plant growth regulators (PGRs) or supplemented with 40% the original concentration of auxin. Weekly, for four weeks, presumptive embryogenic tissue (PET) was isolated from the calli/explants and transferred onto fresh medium of the same composition. The tissue was considered PET if it exhibited any of the following traits: mucilaginous, soft, yellow, white or cream in color, and/or nodular in appearance. At the four-week mark, the tissue was observed and photographed using a dissecting microscope equipped with a N2000 Nikon digital camera. The tissue was characterized on morphological features and 12-15 samples from each type of tissue were selected for histological analysis as described below. The remaining isolated PET was then transferred to ET Proliferation medium, which was the same composition as the ET Initiation medium.

2.3.4 Maturation

After four weeks on ET Proliferation medium, the PET was observed for characteristics of being embryogenic. Tissue was considered to be embryogenic if it was

nodular in texture. Embryogenic tissue was selected from the plates exhibiting the traits using a sterilized scalpel. The tissue was placed into 250ml flasks each containing 30ml liquid PGR-free media of the same basal composition. The cultures were shaken for 24 hours at 110 rpm on a rotary shaker (G10 Gyrotory® Shaker, New Brunswick Scientific, N.J., U.S.A). Three 10ml aliquots from each flask were vacuum filtered onto Whatman #1 filter paper and placed onto a MS medium containing 7.57μM ±-cis, trans-Abscisic acid (ABA) (Sigma A-1049). Medium was adjusted to pH 5.7 using 1.0 N NaOH, solidified using 0.4% Phytagel™ (Sigma) and autoclaved for 20 minutes at 121°C, 104 kilopascals.

After eight weeks on maturation medium, the cultures were placed into cold storage (4°C) in the dark for eight weeks. The somatic embryos were then individually transferred onto germination medium consisting of PGR-free MS medium. After eight weeks, the plantlets were transferred to 98 cell trays (2" deep) containing Pro-Mix soil (Halifax Seed Co., Halifax, N.S.). The soil was treated with the fungicide Benomyl® at a concentration of 5ml fungicide/2L of water prior to transplanting. The plantlets were transferred to soil with care being taken not to damage the roots. The plantlets were misted frequently during the transplanting process to avoid desiccation. Each tray was covered with a plastic dome and secured with six cloths pins. The plantlets were misted daily and watered three times a week. Each week, a cloths pin was removed from each tray to gradually acclimatize the plants to the harsher *ex vitro* environment. Once all of the cloths pins were removed, the dome was removed completely. Survival rates were recorded monthly for three months after transplanting.

2.3.5 Histological Procedures

Within a drop of 3% glutaraldehyde in 0.025M potassium phosphate buffer, tissues were cut into pieces approximately one to two millimeters in length. The sectioned samples were fixed in a 3% glutaraldehyde solution in 0.025M potassium phosphate buffer in a glass 25mL vial equipped with a screw lid. The samples were stored at 4°C until histological processing. One drop of erythrosine B was added to each vial and stained for 30 minutes. The samples were centrifuged for approximately five seconds to remove the supernatant and then placed into an agar medium prior to solidification (0.2% agar in 0.025M potassium phosphate buffer, pH 6.8). After the agar medium solidified, it was cut into small blocks using a fresh razor blade. Dehydration was carried out using an ethanol to 95% butanol series, ethanol was replaced with the secondary solvent butanol. Infiltratation was performed by placing the tissue into a 2.0ml 25% paraffin butanol solution for 30 minutes. Every 30 minutes for 1.5 hours, 1.0 ml of 100% paraffin was added to the vials containing the samples. After 1.5 hours, the samples were stored with the lid off overnight at 57°C. The following morning the paraffin was poured off and replaced with 100% paraffin. The samples were embedded in paraffin, sectioned into 7.0 μM sections using a microtome, stained with hematoxylin and mounted in permount. Serial sections of the tissue were studied and photographed using a compound microscope.

2.3.6 Statistical Analysis

All computations were performed using the SYSTAT statistical analysis package. Non-normally distributed data were analyzed at a 5% probability level using Kruskal-

Wallis, a non-parametric analog for analysis of variance. A conservative testing procedure (Bonferoni) was used for the multiple Kruskal-Wallis test. Normally distributed data were analyzed using ANOVA and means were compared using Fisher's least significant difference (LSD) at the 5% probability level.

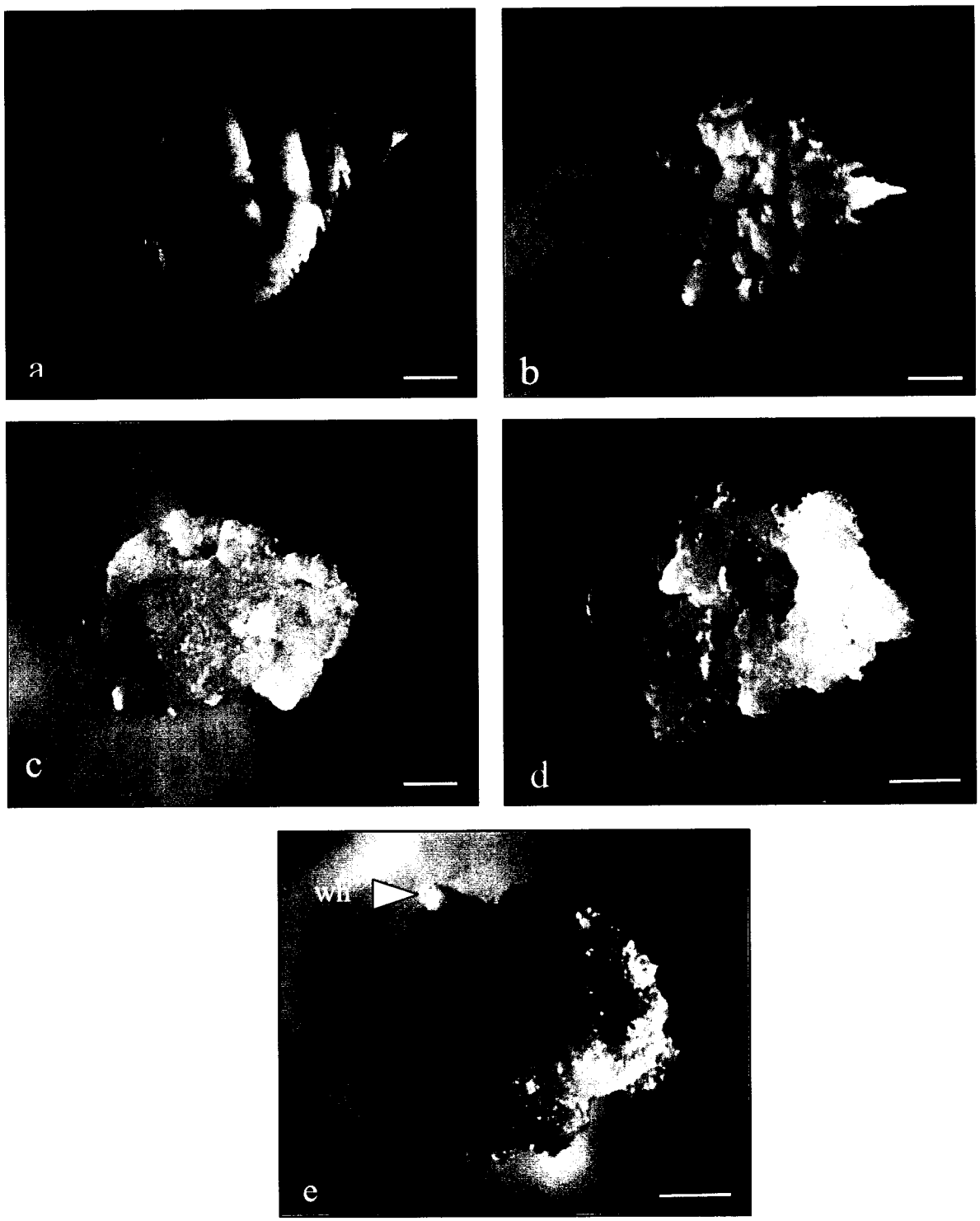
2.4 RESULTS

2.4.1 Callus Induction

Formation of whitish-yellow callus was observed on both leaflet and petiole explants after one week on callus induction media in all treatments except PGR-free (Figure 5a & 6a). After two weeks on callus induction media, calli in all treatments displayed browning, with increased browning throughout weeks three and four (Figure 5b,c & 6b,c). At weeks three and four, small quantities of white mucilaginous calli formed on the brown calli; this was isolated and later produced Presumptive Embryogenic Tissue (PET) (Figure 5d & 6d).

Rhizogenesis, in the form of hair-like projections and aerial root formation was observed on callus by week four (Table 2) (Figure 7). Hair-like projections were observed on callus derived from leaflet and petioles explants in all treatments except leaflet-derived callus grown on WPM supplemented with 2,4-D. Aerial root formation was observed on callus derived from leaflet and petioles explants in all treatments except on callus derived from leaflet or petiole explants cultured on MS medium supplemented with 2,4,5-T. Root formation was especially notable in callus cultures on media supplemented with 2,4-D.

Figure 5. Callus induction from leaflet explants. Leaflets growing on Murashige and Skoog medium supplemented with 10μM 2,4,5-trichlorophenoxyacetic acid a) day one b) after one week c) after two weeks d) after three weeks e) after four weeks, note formation of white mucilaginous callus (wh) on brown callus. Bar 1mm.


a
b
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e

Figure 6. Callus induction from petiole explants growing on Murashige and Skoog medium supplemented with 10μM 2,4,5-trichlorophenoxyacetic acid a) day one b) after one week c) after two weeks d) after four weeks, note formation of white mucilaginous callus (wh) on brown callus. Bar 1mm

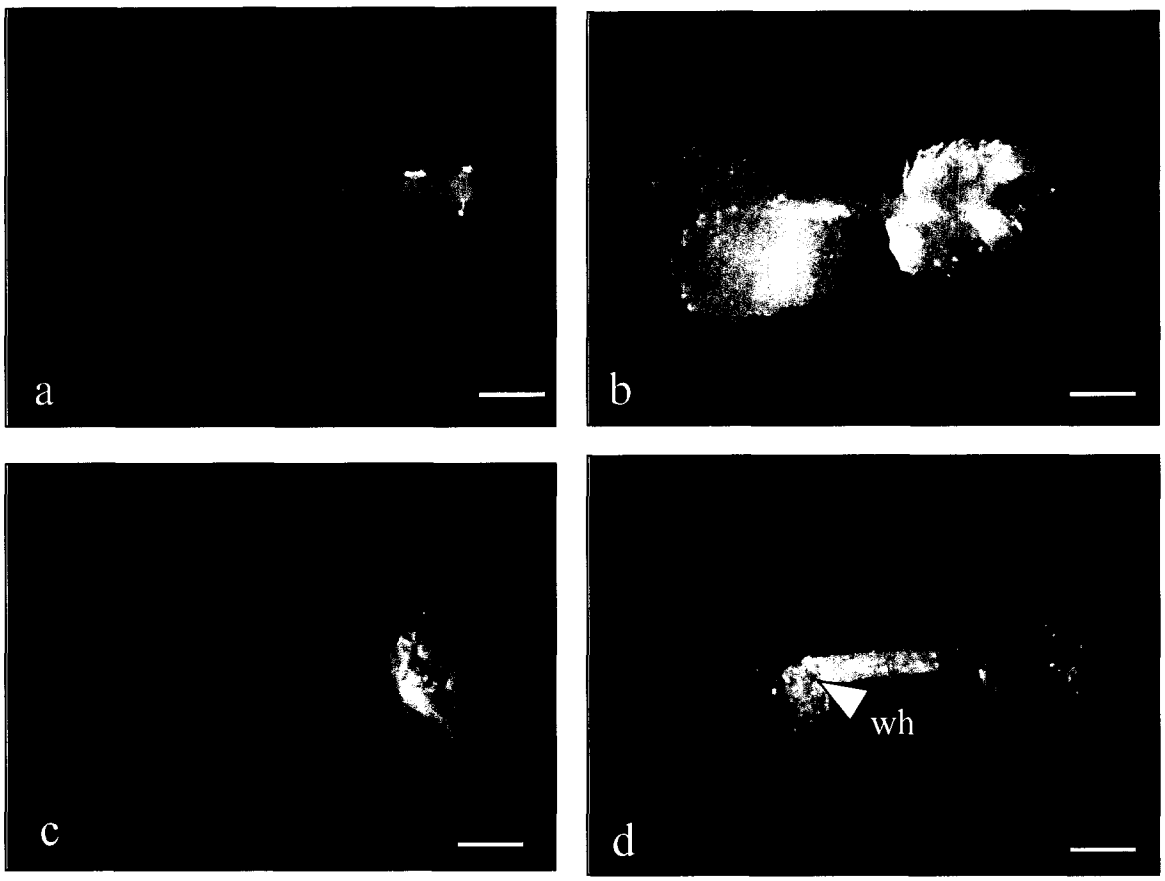
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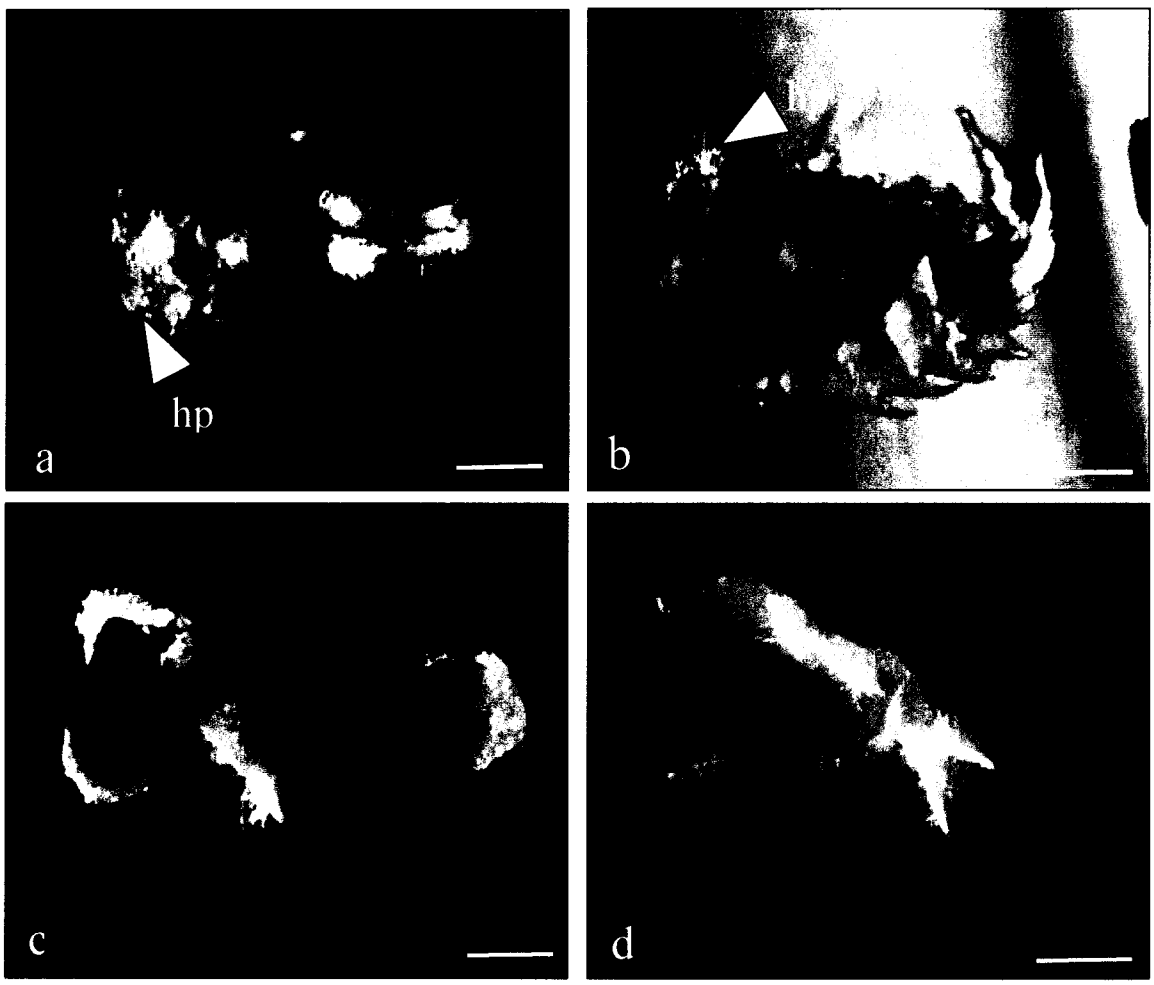
Table 2. Frequency of rhizogenesis occurring on callus cultures after four weeks on callus induction media. (***PGR*** Plant Growth Regulator, ***2,4-D*** 2,4-dichlorophenoxyacetic acid, ***2,4,5-T*** 2,4,5-trichlorophenoxyacetic acid, ***L*** Leaflet, ***P*** Petiole, ***MS*** Murashige and Skoog, ***WPM*** Woody plant medium).

Explant Type	Medium	PGR	Frequency of hair-like projections (%)	Frequency of aerial roots (%)	Mean # aerial roots per explant (± standard deviation)
P	MS	2,4-D	2.5	12.4	1.4 ± 0.3
P	MS	2,4,5-T	4.9	0	0
L	MS	2,4-D	0.6	56.0	3.2 ± 0.6
L	MS	2,4,5-T	5.3	0	0
P	WPM	2,4-D	6.5	6.8	1.4 ± 0.6
P	WPM	2,4,5-T	6.8	0.6	0.3 ± 0.5
L	WPM	2,4-D	0	70.3	4.8 ± 1.2
L	WPM	2,4,5-T	8.2	7.3	0.6 ± 0.7

Figure 7. Rhizogenesis occurring on callus cultures after four weeks growth on callus induction media. a and b) hair-like projections (hp) c and d) aerial root formation. Bar 1mm.


hp
a
b
c
d

2.4.2 Characterization of Presumptive Embryogenic Tissue

After the white mucilaginous callus was isolated, it was grown on ET Proliferation medium consisting of either PGR-free medium or medium containing 40% of the original concentration of auxin. The resulting presumptive embryogenic tissue (PET), after four weeks growth, was characterized into 10 different groups based on the morphological traits of color and texture (Table 3).

Type 1 PET was yellow in color with very distinct nodules (Figures 8 & 9). Type 2 was very similar to Type 1 with the distinguishing white color (Figure 10 & 11). Examination of the anatomy of these two PET types revealed the presence of early stage somatic embryos interspersed with callus cells (Figure 12). The somatic embryos consisted of groups of small densely cytoplasmic cells with very prominent nuclei. There was a very well-defined epidermis surrounding each group of cells. The somatic embryos had darkly stained procambium, indicative of the high degree of organization and differentiation within the embryo. Dispersed among the somatic embryos were groups of callus cells. The callus cells were large in size and highly vacuolated with a large central vacuole occupying the majority of the cells interior.

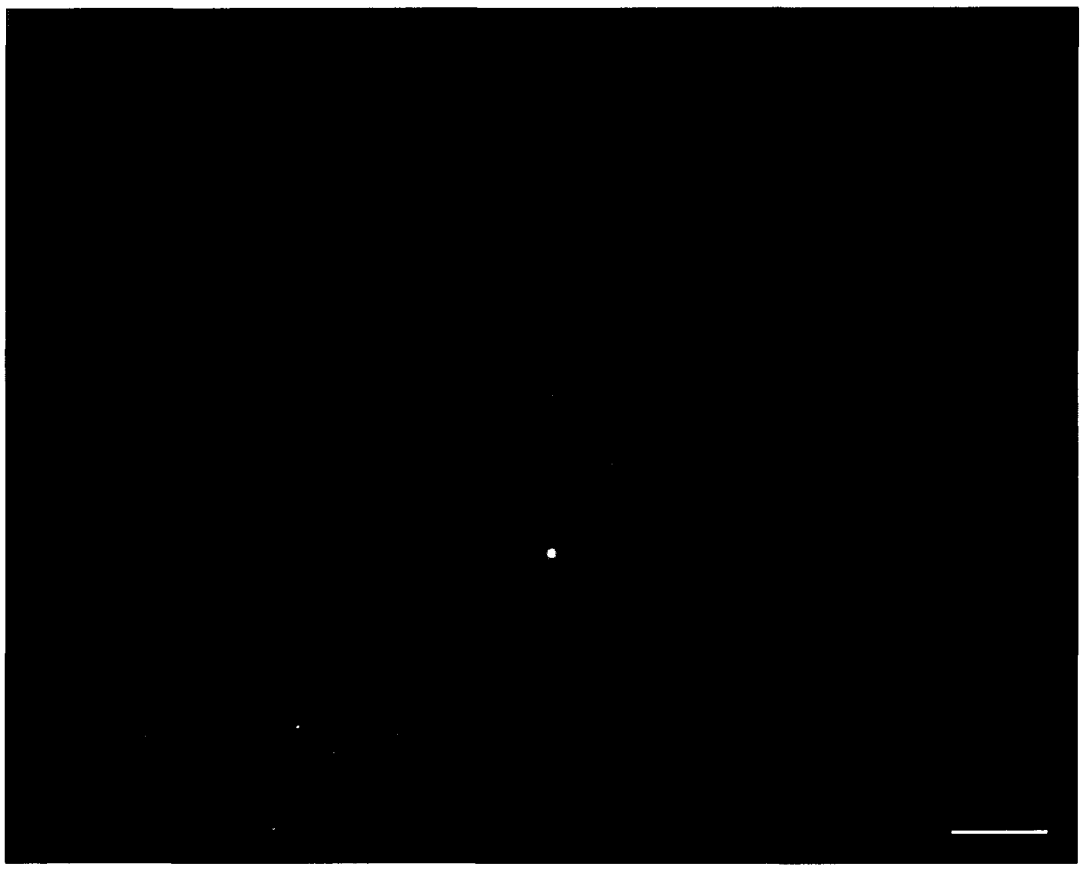
Types 3 and 4 PET were yellow and white in color, respectively (Figures 13 -16). These two tissue types were slightly nodular in appearance, however the nodular structures were not as defined as those present in Types 1 & 2. Histological analysis showed groups of cells with well-defined epidermal layers. These epidermal cells themselves differed from those in Types 1 and 2. The cells in PET Types 3 and 4 were

Table 3. Types of Presumptive Embryogenic Tissue resulting from white mucilaginous callus after four weeks growth on ET initiation media.

Type #	Texture	Color
1	defined nodular	yellow
2	defined nodular	white
3	slightly nodular	yellow
4	slightly nodular	white
5	soft	yellow
6	soft	white
7	crystal-like	yellow – brown
8	large round masses	orange/beige
9	soft	brown
10	hard	brown

Figure 8. Type 1 Presumptive Embryogenic Tissue. Bar 1mm.

Figure 9. Anatomy of Type 1 Presumptive Embryogenic Tissue. Note somatic embryos (se) intermixed with callus (ca).



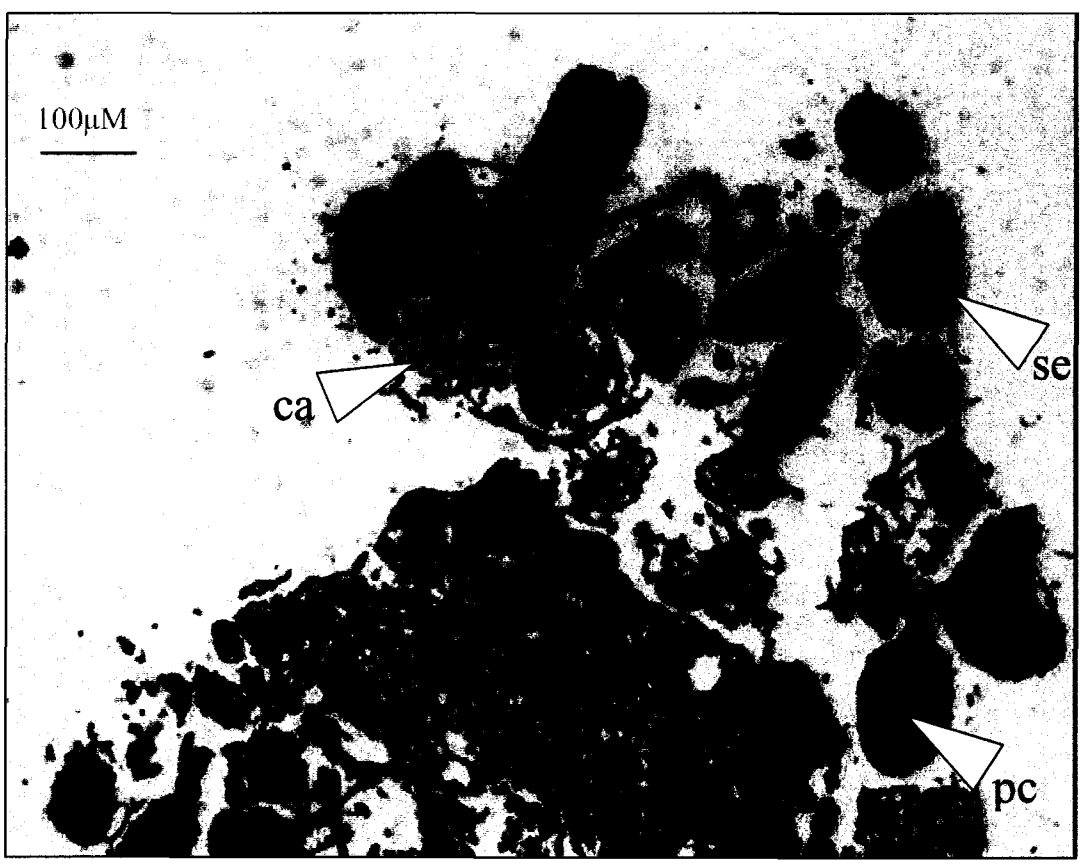
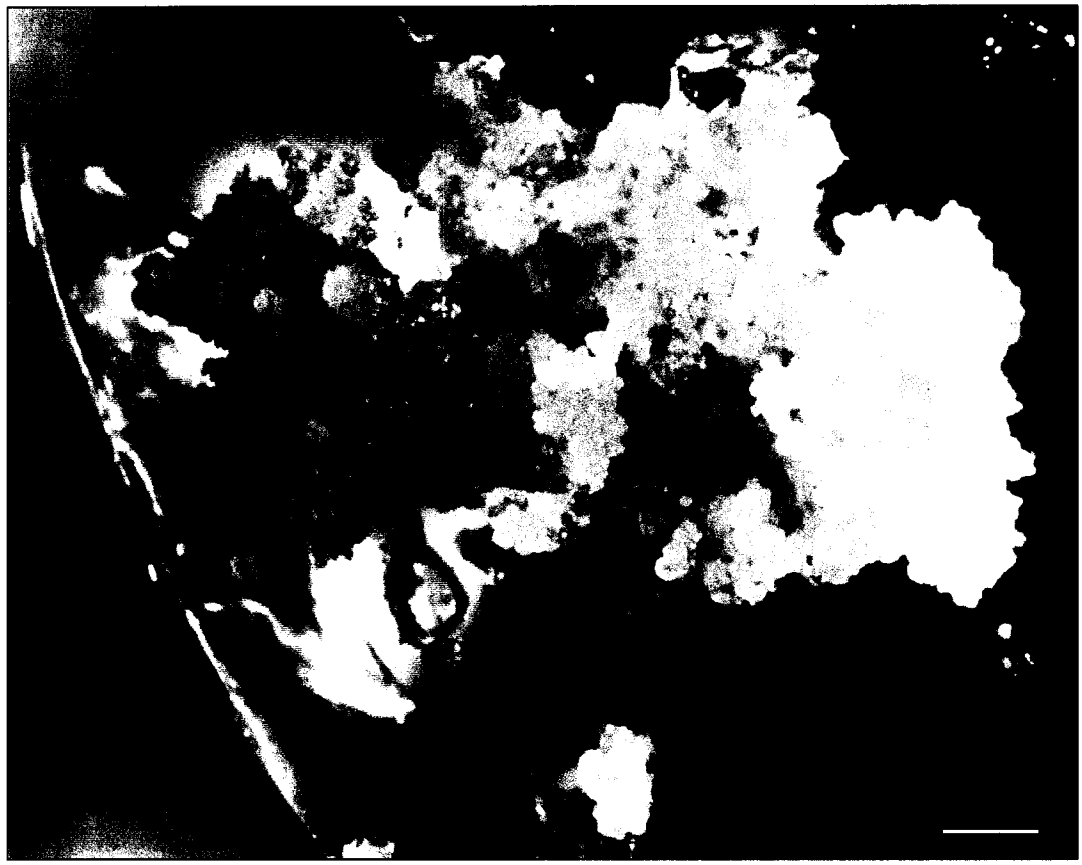

100μM
ca
se
pc

Figure 10. Type 2 Presumptive Embryogenic Tissue. Bar 1mm.

Figure 11. Anatomy of Type 2 Presumptive Embryogenic Tissue. Note somatic embryos (se) intermixed with callus (ca).




se
ca
100 µm

Figure 12. Anatomy of Type 2 somatic embryo.

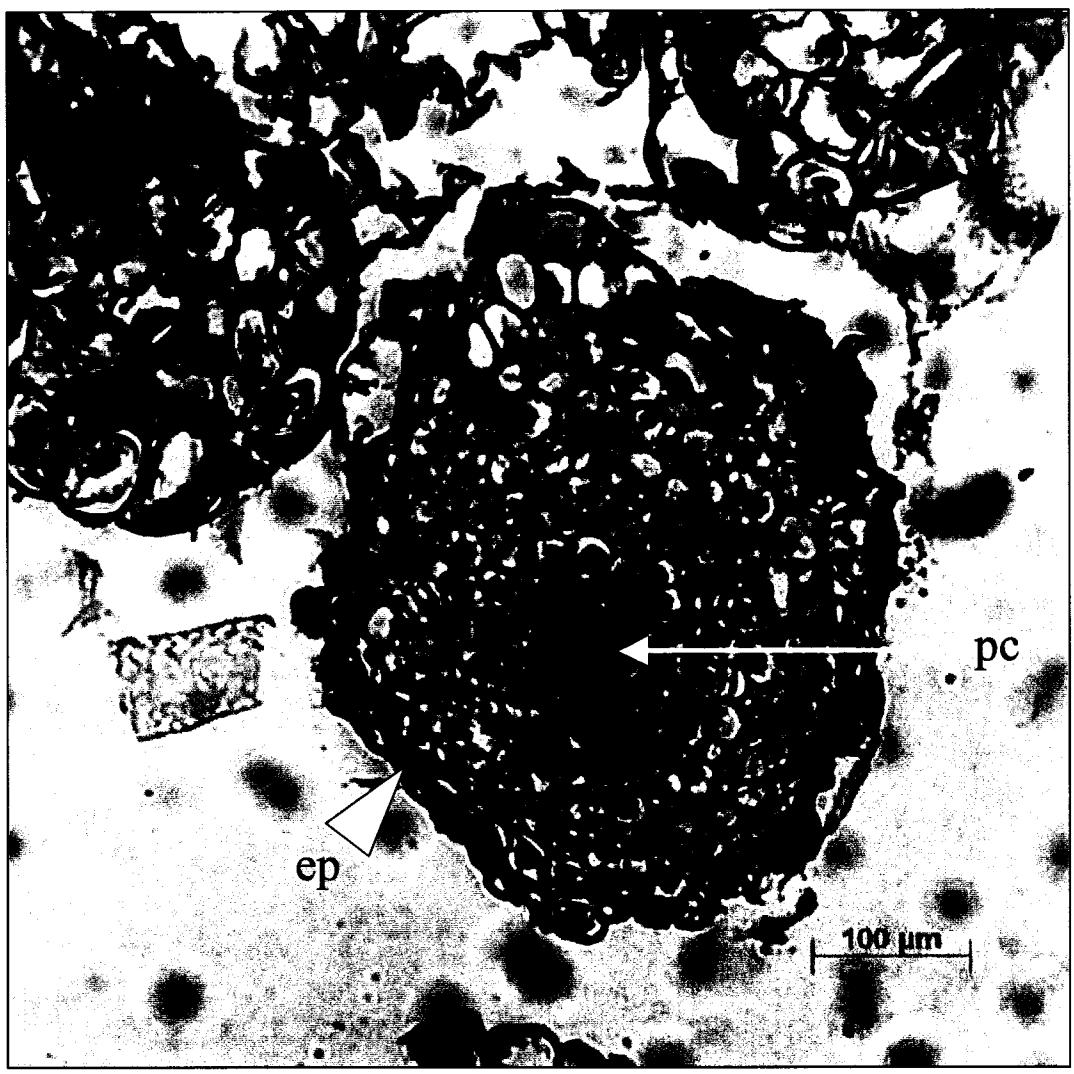
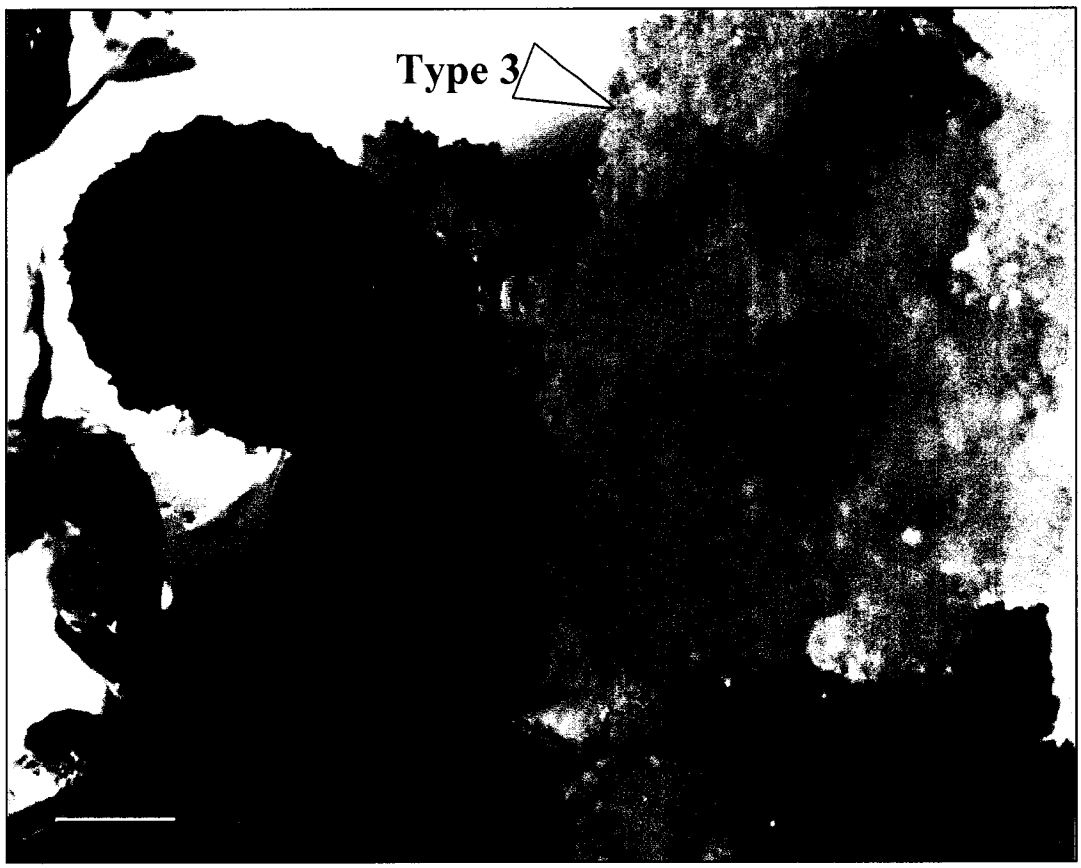

pc
ep
100 µm

Figure 13. Type 3 Presumptive Embryogenic Tissue. Bar 1mm.

Figure 14. Anatomy of Type 3 Presumptive Embryogenic Tissue


Type 3

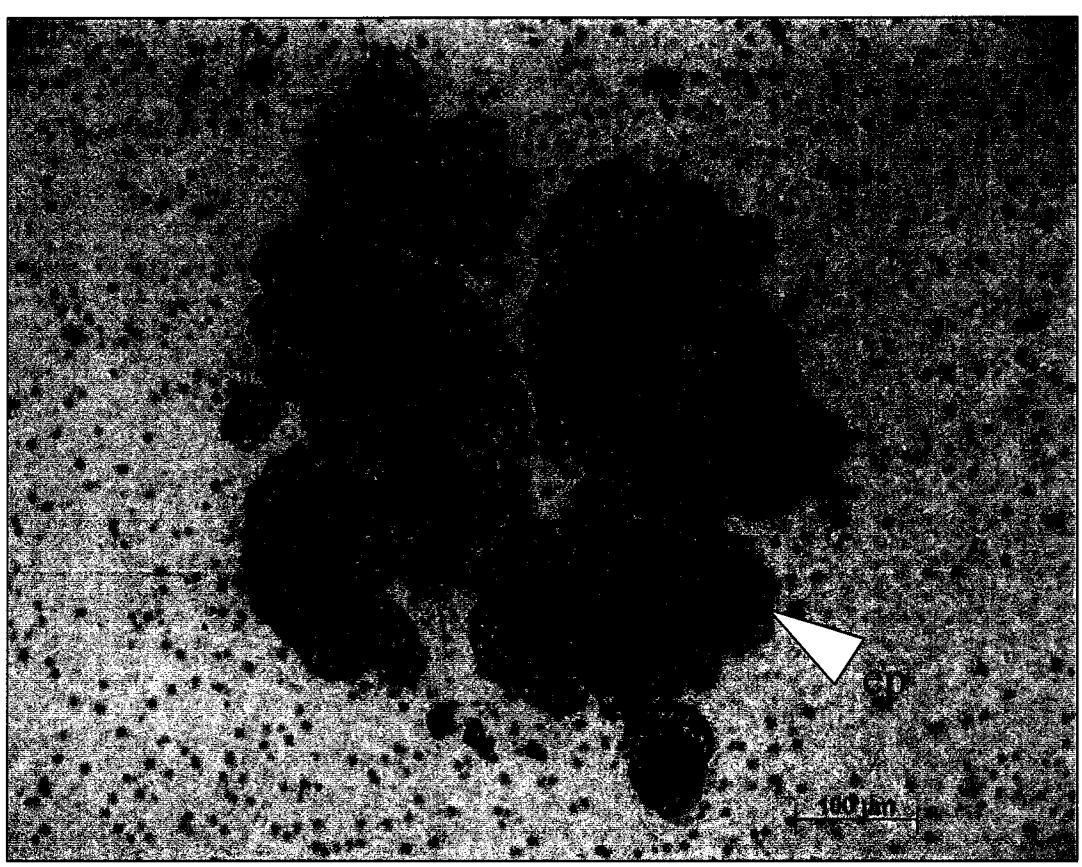
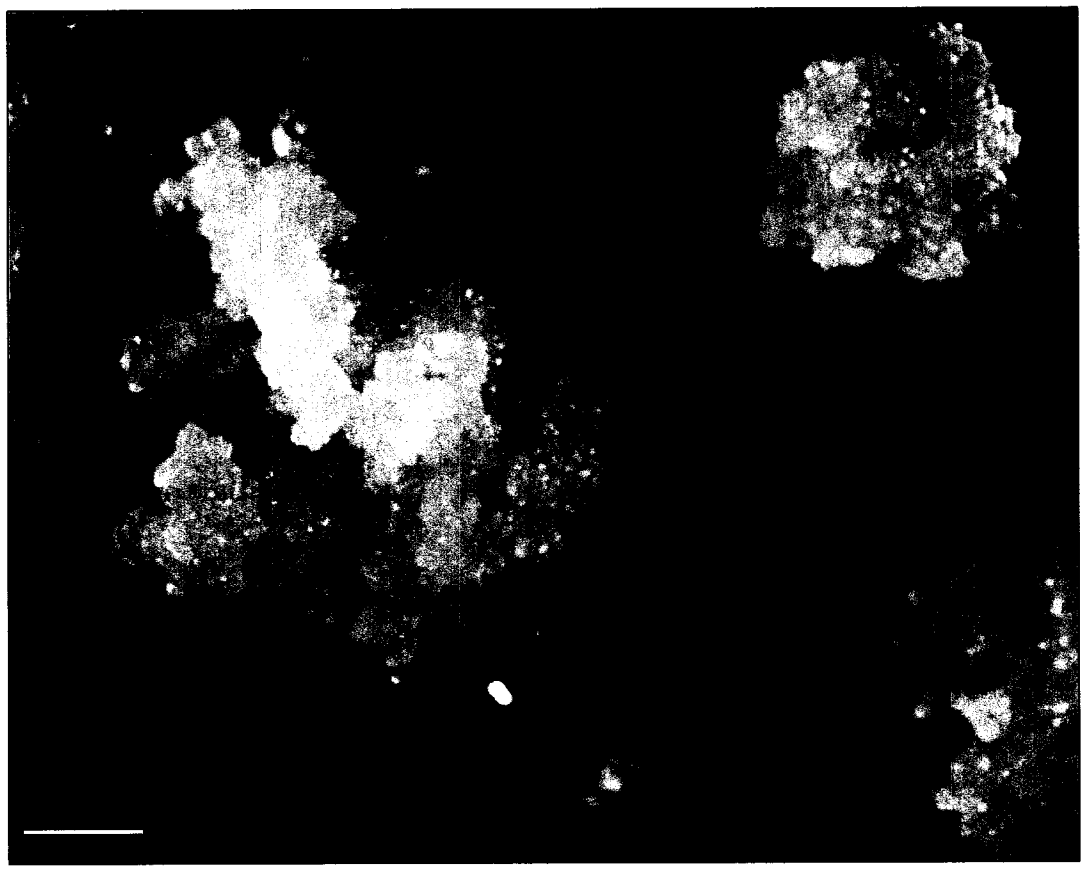
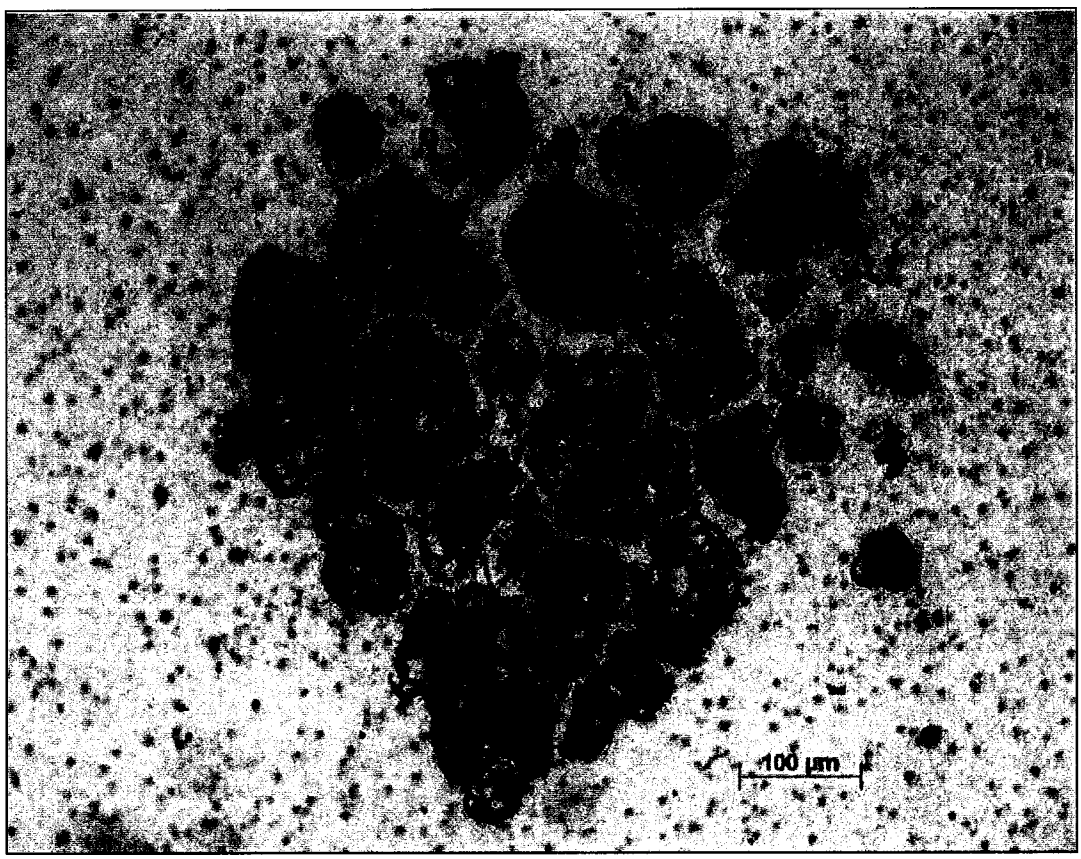

CD
100 μm

Figure 15. Type 4 Presumptive Embryogenic Tissue. Bar 1mm.

Figure 16. Anatomy of Type 4 Presumptive Embryogenic Tissue.




100 µm

highly vacuolated with a large central vacuole occupying the majority of the cells interior. The presence of an epidermis indicates these cells are more highly organized than callus, however they are not somatic embryos. These structures lack the high level of organization seen in somatic embryos, such as the formation of the procambium. The cells seem to have differentiated into parenchyma cells rather than somatic embryos.

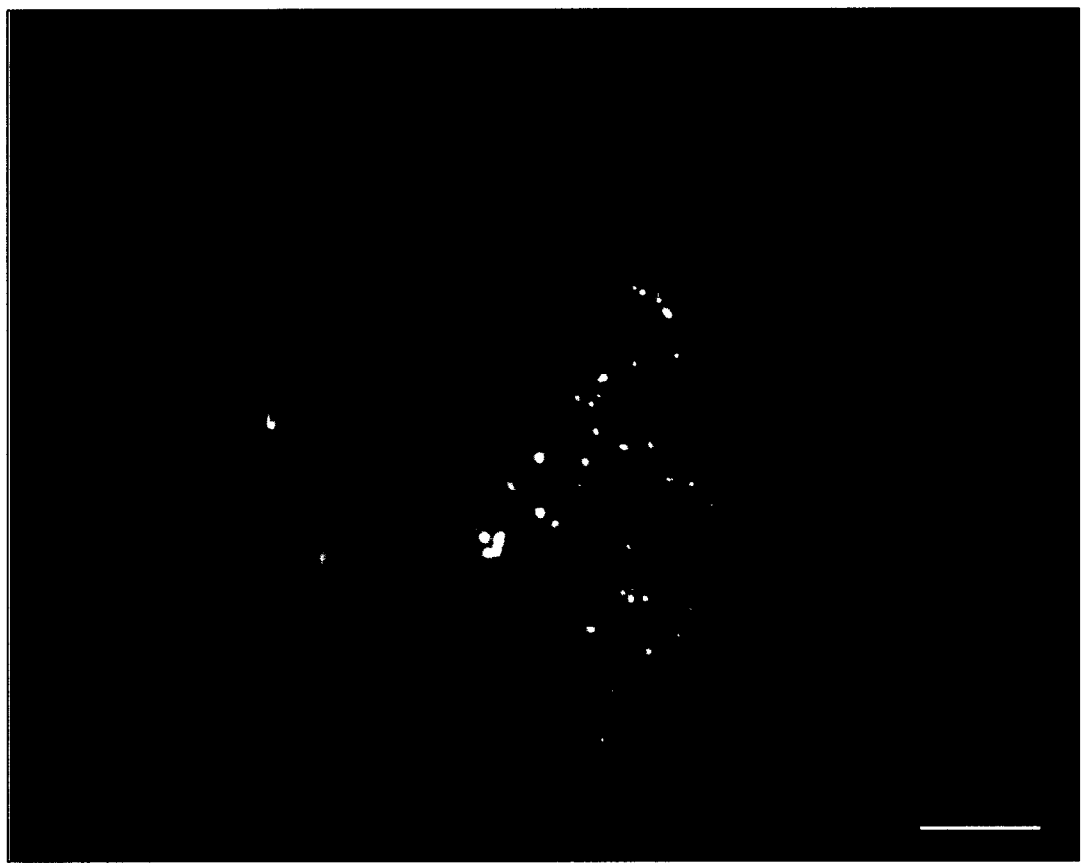
Type 5 and 6 PET were both pliant in nature, differing only in color: Type 5 PET was yellow while Type 6 was white. Type 5 and 6 PET consisted of cells that were soft and easily disrupted as evident by the large number of lysed cells disrupted during the histological process (Figures 17-21). The majority of the intact cells were large, highly vacuolated with thin cell walls (Figure 21). There were smaller, densely cytoplasmic cells with large nucleoli dispersed throughout the middle of the tissue.

Type 7 PET varied in coloration from dark yellow to brown but with a distinct region of whitish tissue, best described as flake-like or crystal-like (Figure 22). Examination of the anatomy of Type 7 PET revealed specialization of cells into vascular tissue as well as epidermal cells (Figure 23). The majority of cells were large and highly vacuolated. These highly vacuolated cells were interspersed with smaller highly cytoplasmic cells, as evident from the dark stain.

The external morphology of Type 8 PET consisted of large round masses of tissue. These large round masses differed vastly from the nodular structures observed in Types 1-4 (Figure 24). Type 8 PET consisted of groups of cells organized in a circular or

Figure 17. Type 5 Presumptive Embryogenic Tissue. Bar 1mm.

Figure 18. Anatomy of Type 5 Presumptive Embryogenic Tissue.



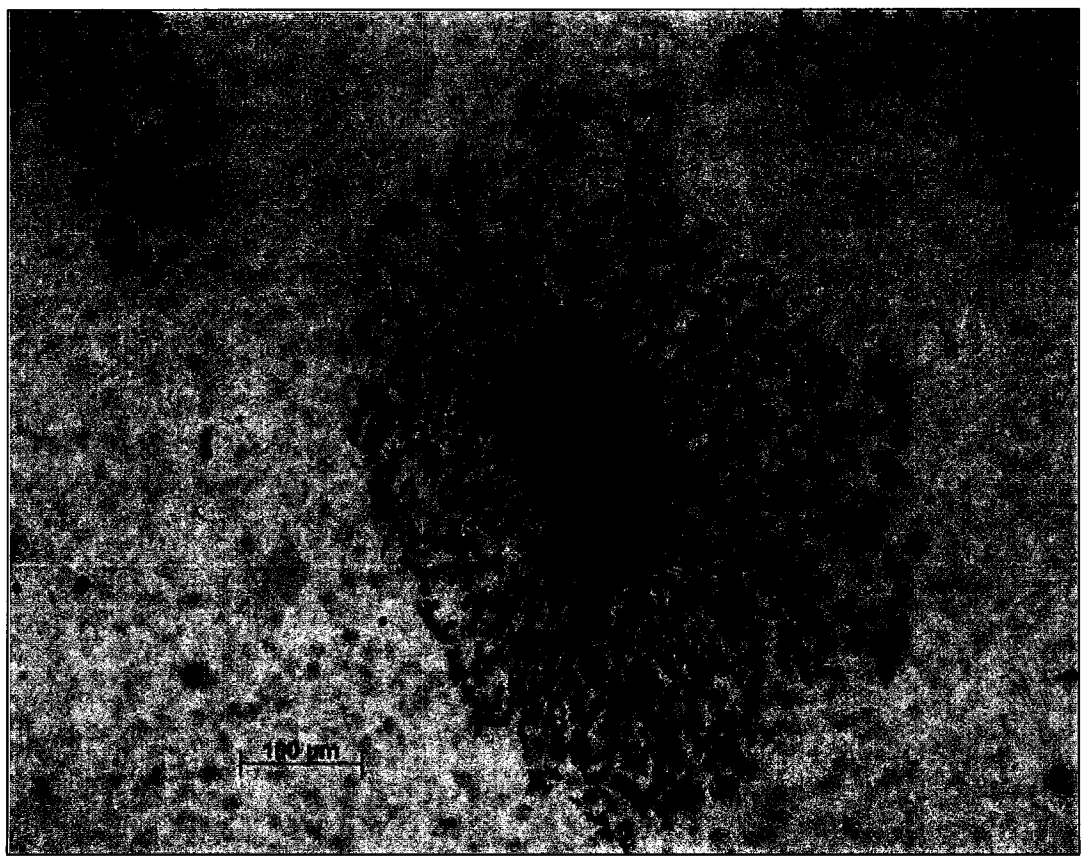

100 nm

Figure 19. Anatomy of Type 5 Presumptive Embryogenic Tissue. Bar 1mm.

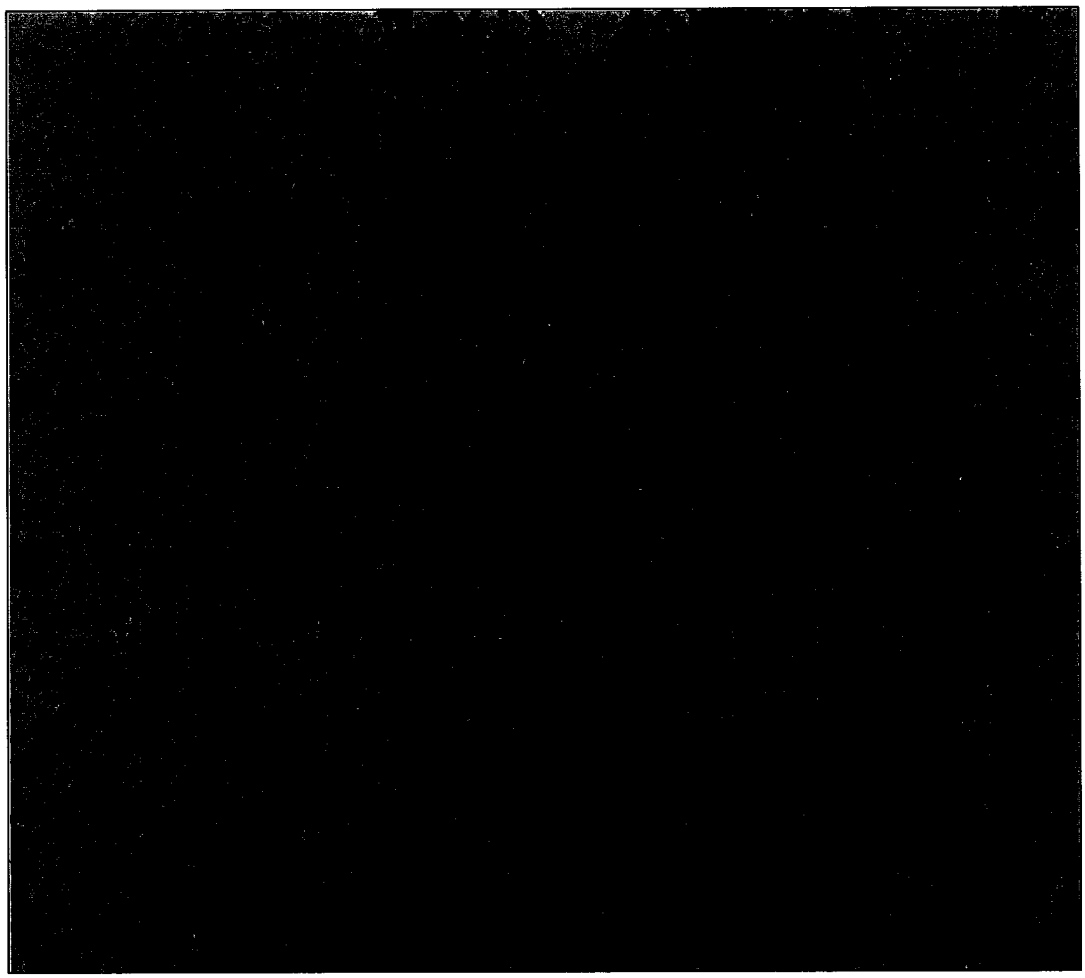
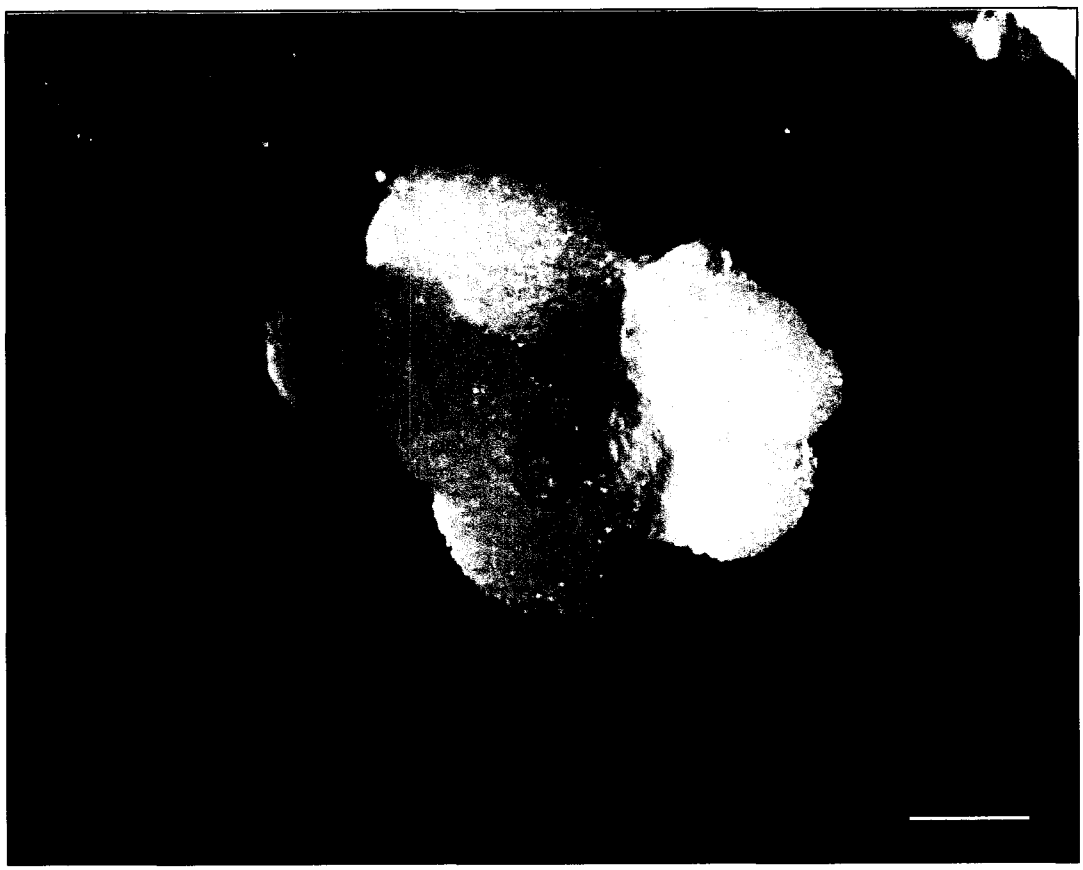


Figure 20. Type 6 Presumptive Embryogenic Tissue. Bar 1mm.

Figure 21. Anatomy of Type 6 Presumptive Embryogenic Tissue.



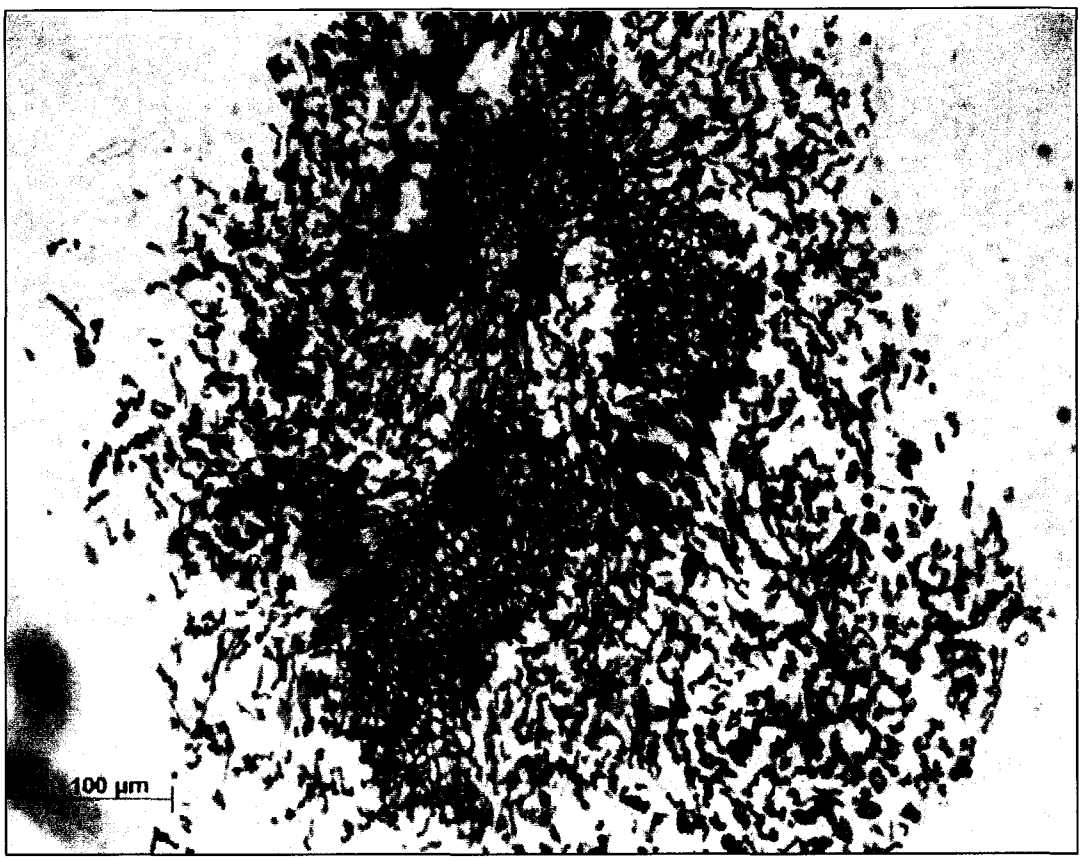
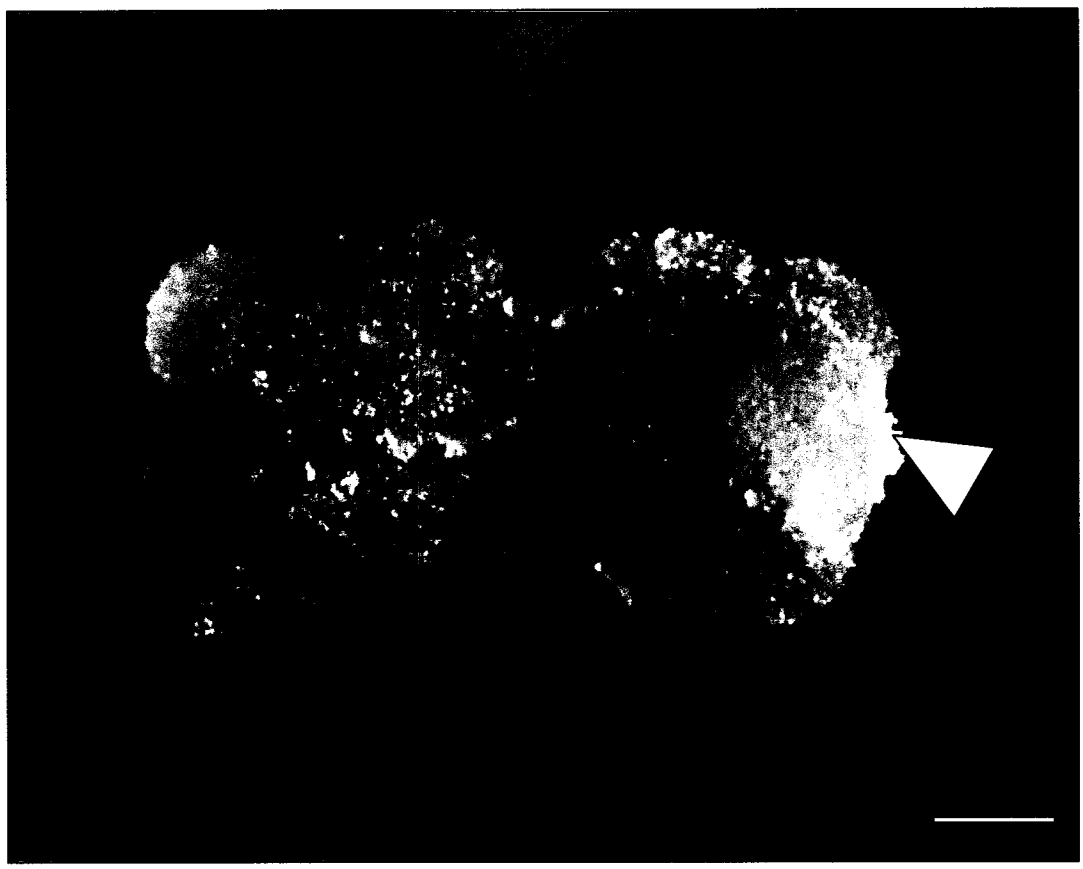
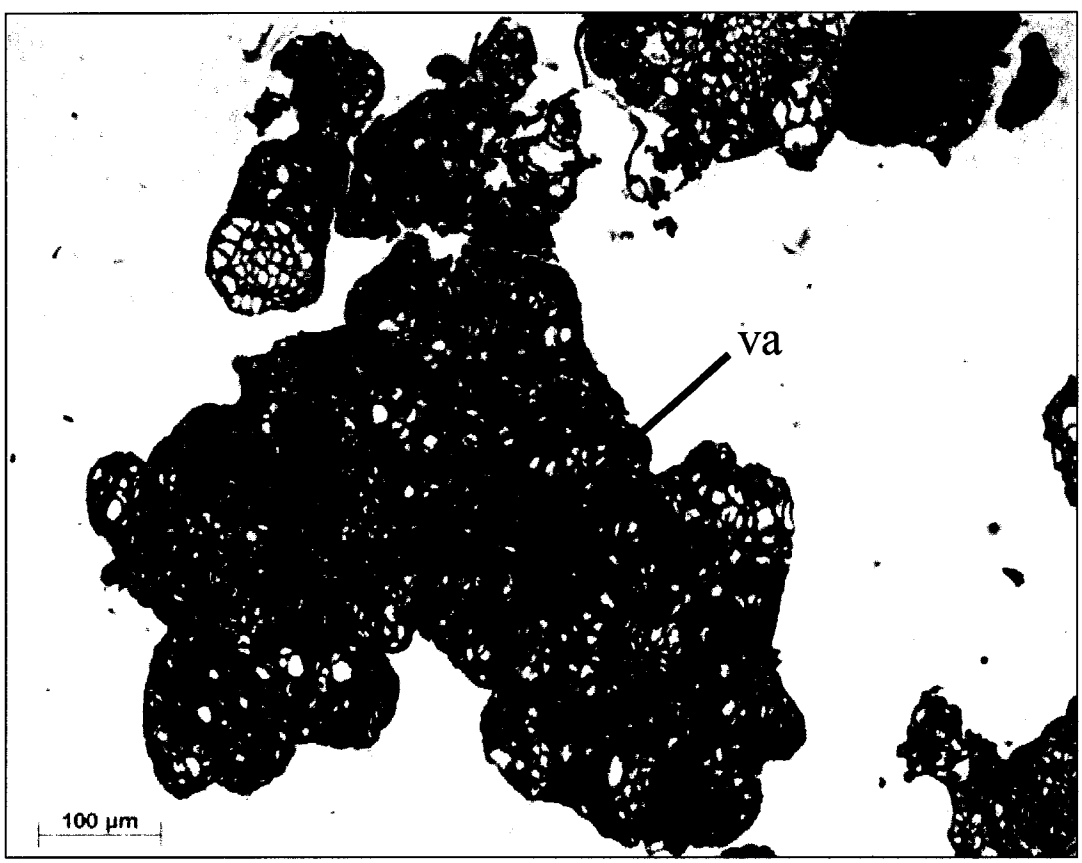

100 µm

Figure 22. Type 7 Presumptive Embryogenic Tissue. Bar 1mm.

Figure 23. Anatomy of Type 7 Presumptive Embryogenic Tissue. Note white, crystal-like texture.




va
100 µm

elliptical shape (Figure 25), as in Types 1 and 2. A closer observation of the cells revealed these were not somatic embryos. The cells were a mix of highly vacuolated cells possessing small nucleoli and highly cytoplasmic cells containing large nucleoli (Figure 26). Although there was a degree of specialization, there was no procambium tissue formation nor was there an organized pattern formation within the cell groups.

Type 9 PET was brown in coloration suggesting necrosis of the tissue was occurring (Figure 27). The tissue did not possess any distinct texture and was soft to the touch. This tissue had many lysed cells, again, likely due to the soft nature of the cells being disrupted during the histological process (Figure 28). Intact cells were largely vacuolated (Figure 29).

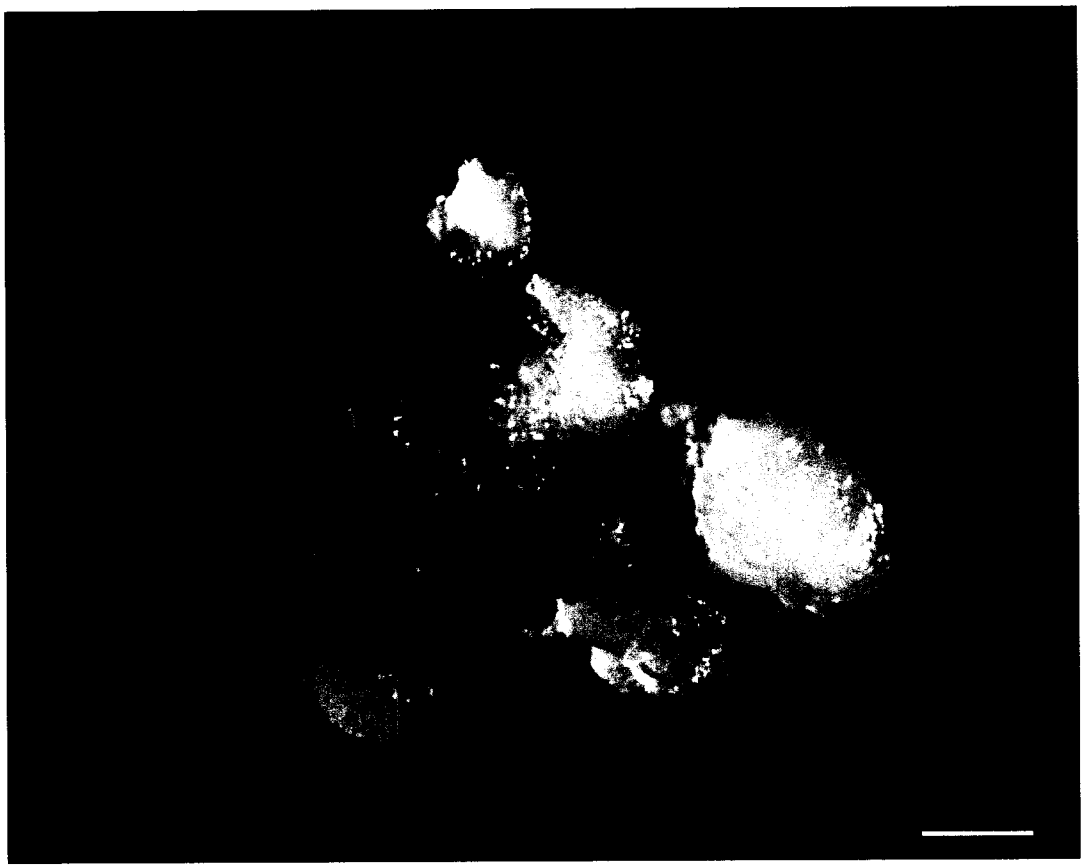
Type 10 PET, like Type 9, was brown in color (Figure 30). Unlike Type 9 however the tissue was not soft to the touch. The cells varied in size and shape (Figure 31). The outer cells were largely vacuolated, while cells in the central area possessed nuclei, some quite prominent in nature (Figure 32). The formation of vascular tissue was observed forming in a circular pattern in the central area of the tissues.

2.4.3 Embryogenic Tissue Initiation

Nodular embryogenic tissue (ET) was produced in both callus induction basal media: MS and WPM. Both media resulted in ET that ranged in color from white, pale yellow to dark yellow. ET was produced in all 2,4,5-T treatments (5, 10 and 25μM) that employ a subsequent transfer to ET initiation media consisting of 40% of the original

Figure 24. Type 8 Presumptive Embryogenic Tissue. Bar 1mm.

Figure 25. Anatomy of Type 8 Presumptive Embryogenic Tissue.



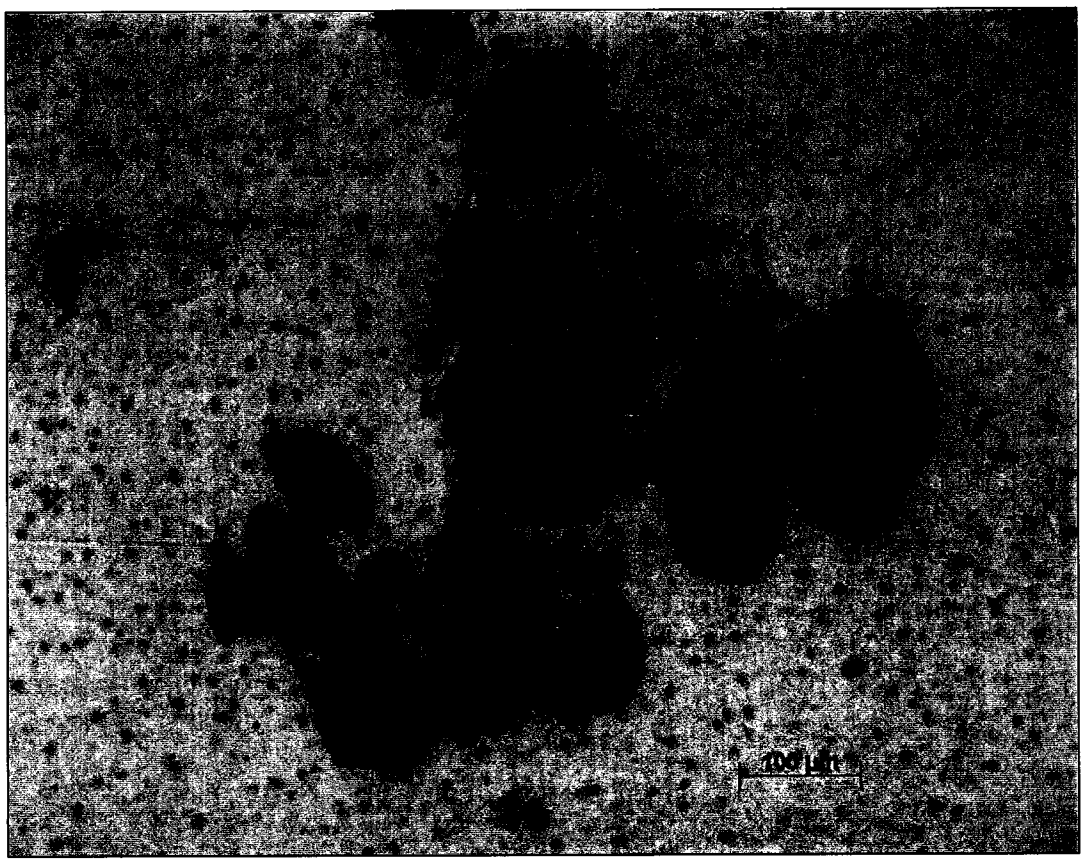


Figure 26. Anatomy of Type 8 Presumptive Embryogenic Tissue.

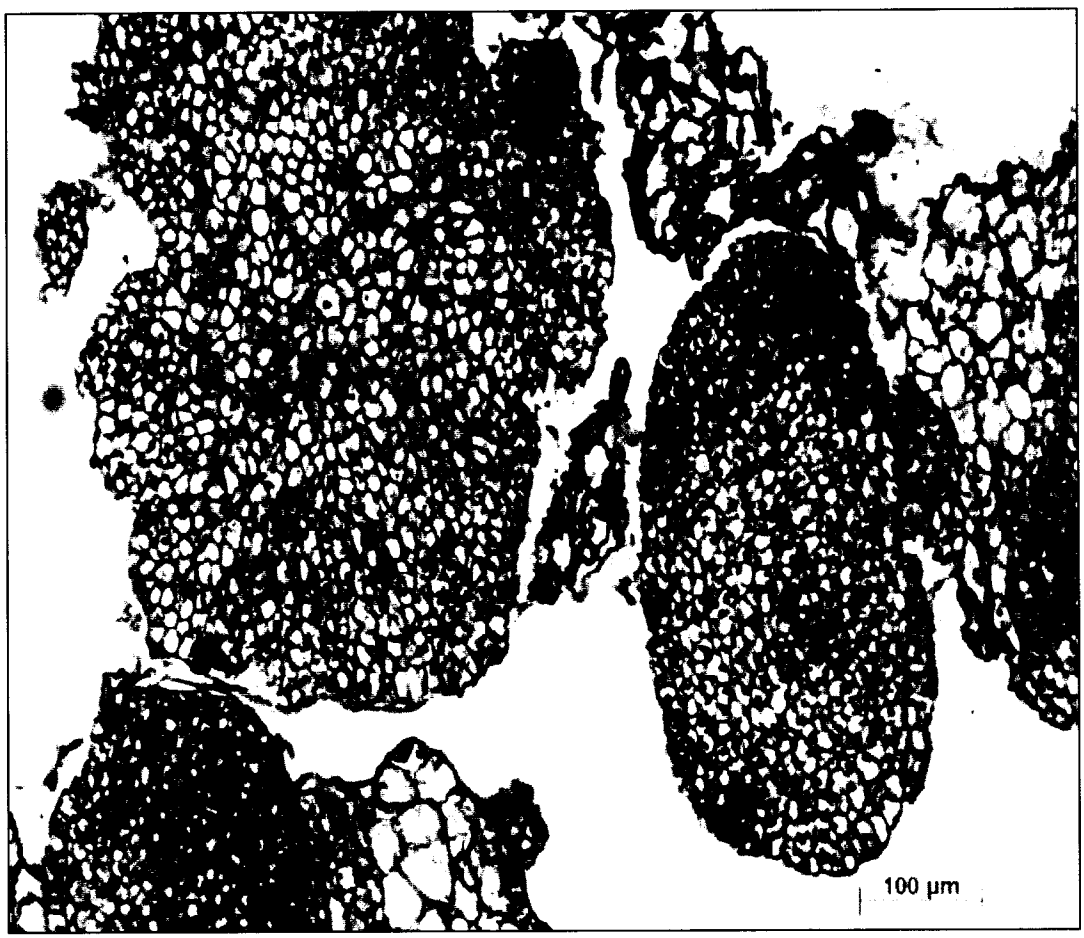
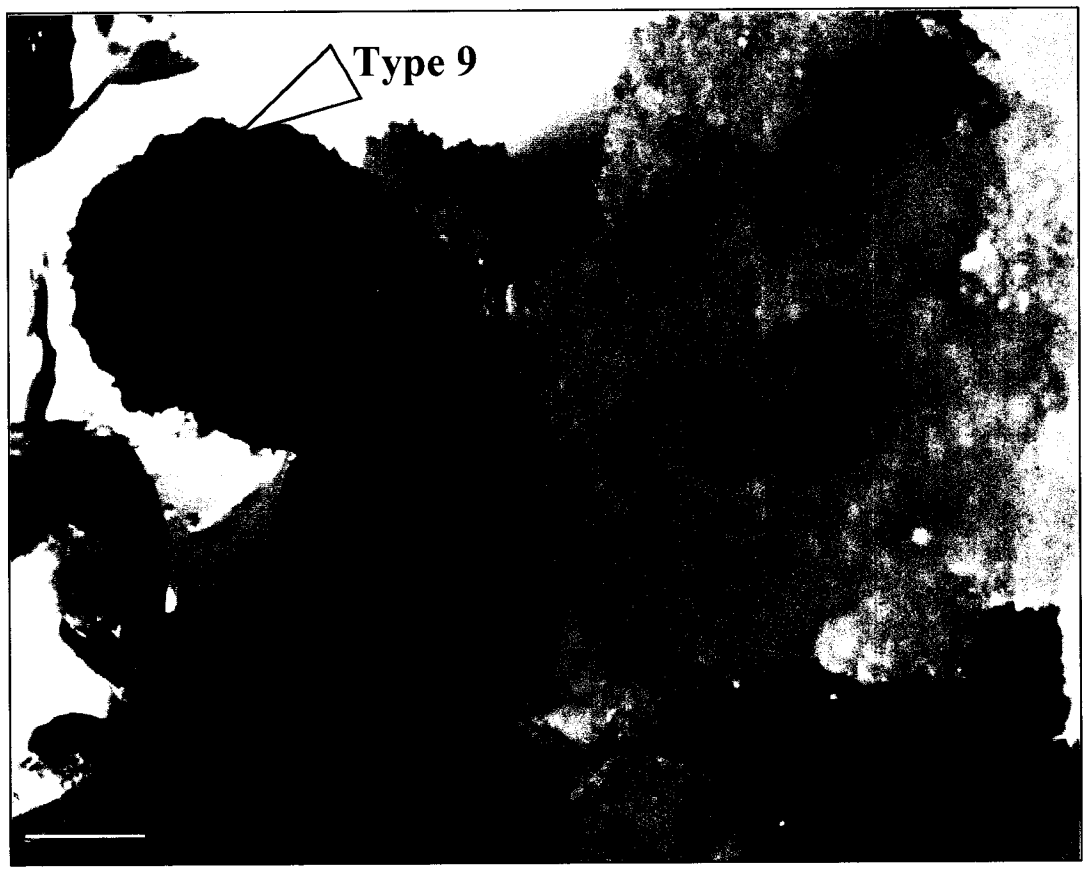

100 µm

Figure 27. Type 9 Presumptive Embryogenic Tissue. Bar 1mm.

Figure 28. Anatomy of Type 9 Presumptive Embryogenic Tissue.


Type 9

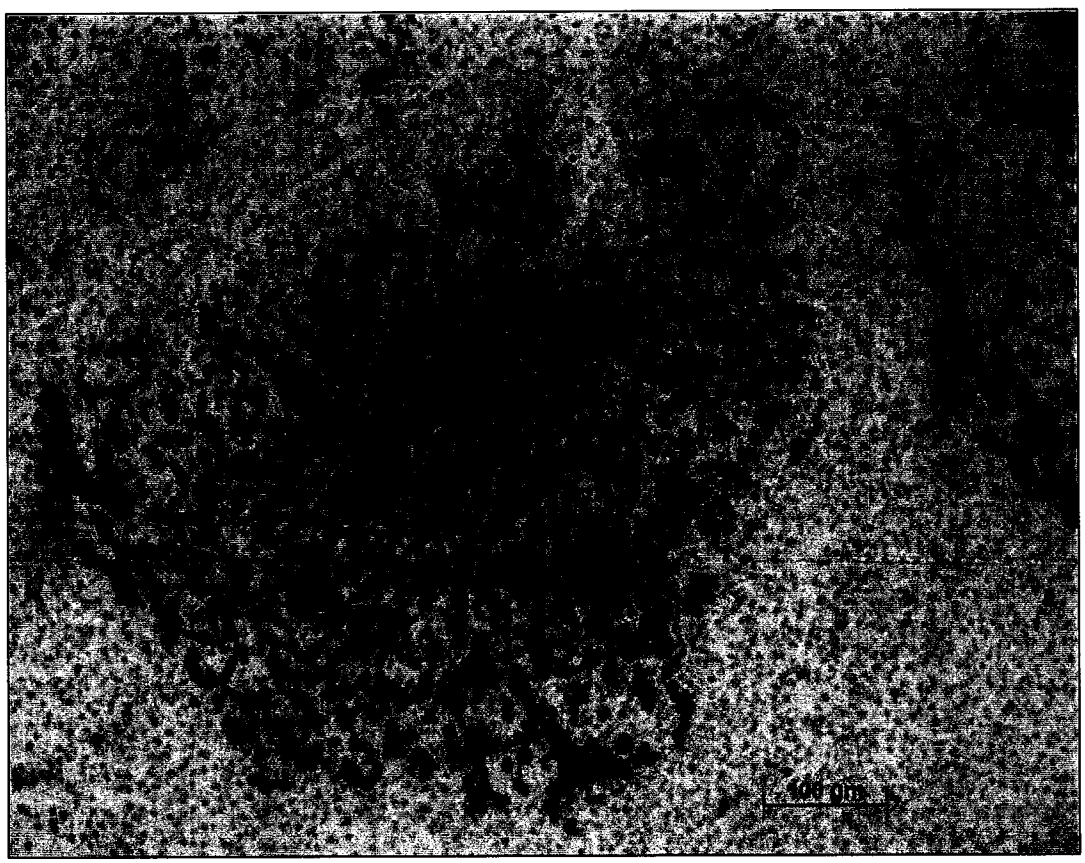


Figure 29. Anatomy of Type 9 Presumptive Embryogenic Tissue

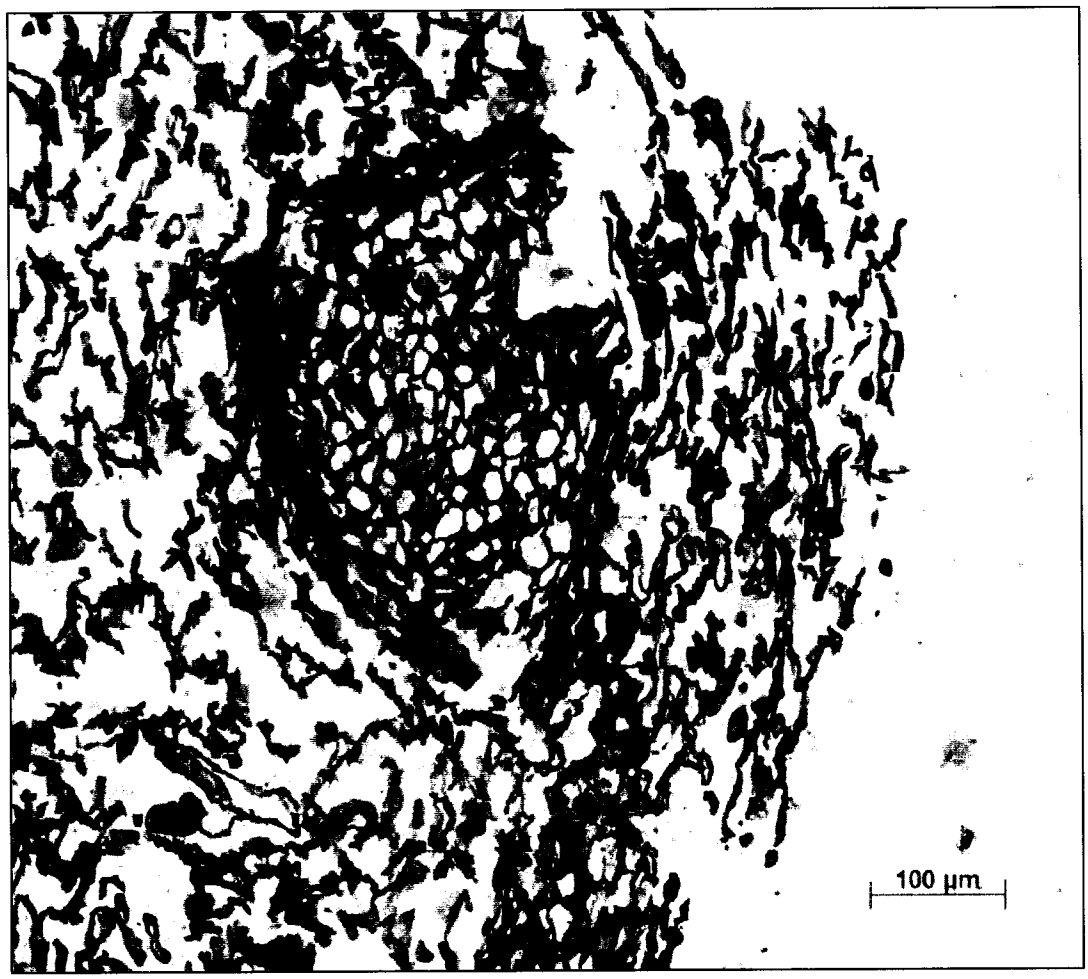

100 µm

Figure 30. Type 10 Presumptive Embryogenic Tissue. Bar 1mm.

Figure 31. Anatomy of Type 10 Presumptive Embryogenic Tissue.

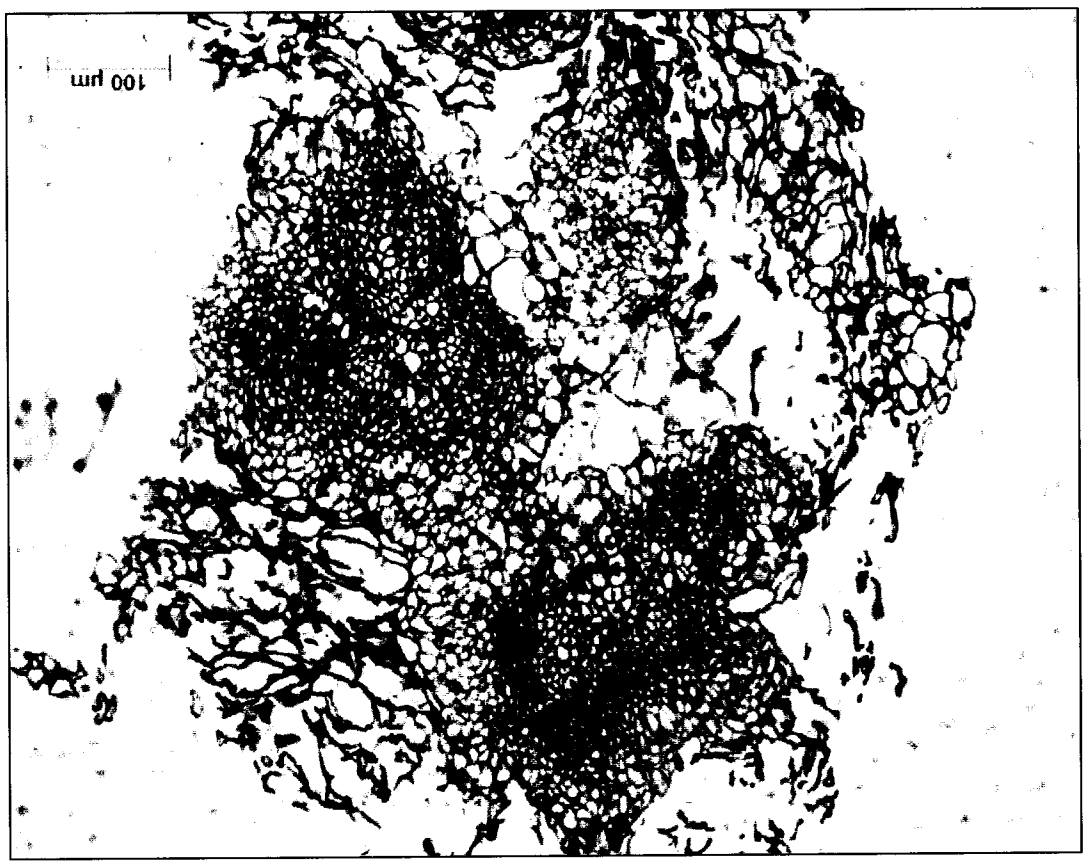
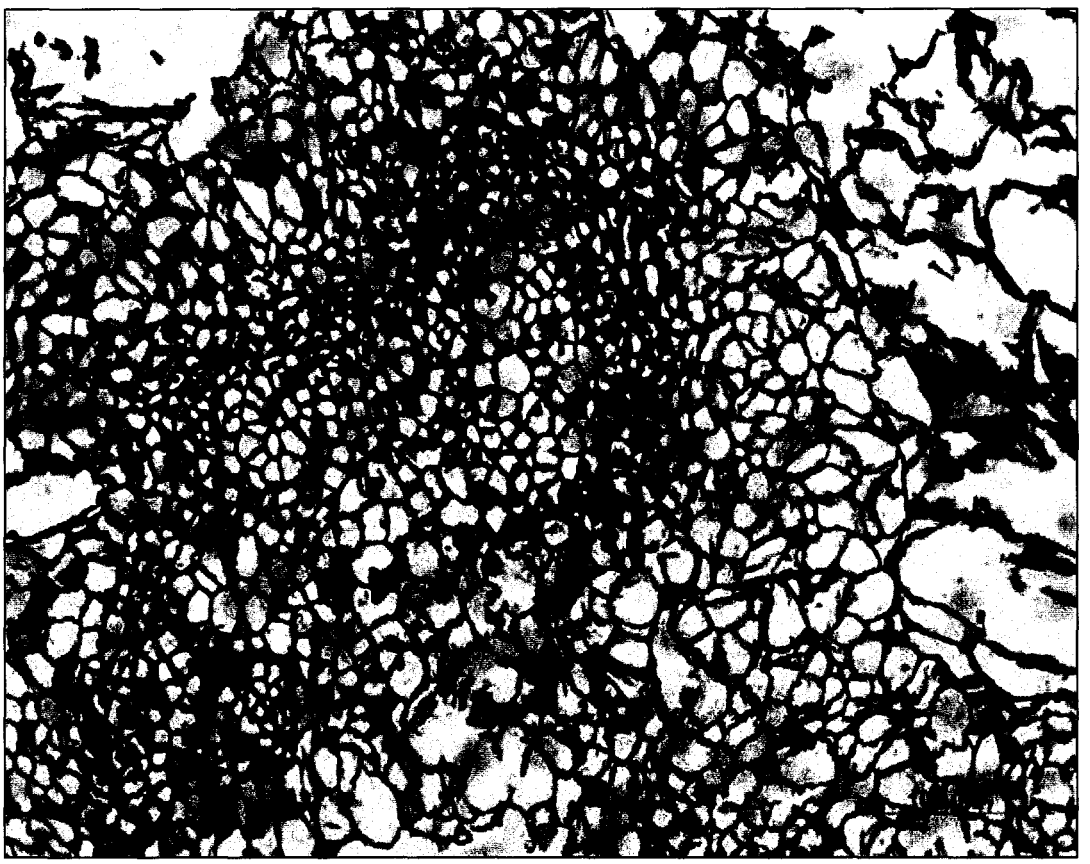

100 µm

Figure 32. Anatomy of Type 10 Presumptive Embryogenic Tissue.



concentration (Table 4). The PGR-free ET initiation media yielded embryogenic tissue only when preceded by the higher concentrations of 2,4,5-T in the callus induction media (10 and 25μM).

Embryogenic tissue production in media supplemented with the various concentrations of 2,4-D was less regular than that of 2,4,5-T. Embryogenic tissue was only produced in the MS callus induction media supplemented with the highest concentration of 2,4-D (25μM) followed by a transfer to 10μM 2,4-D. Callus induction media containing 25μM 2,4-D did not result in ET production when the subsequent ET proliferation medium was PGR-free. In WPM, only the 5μM 2,4-D treatment followed by a transfer to 2μM 2,4-D resulted in embryogenic tissue formation. Embryogenic tissue was produced by both petiole and leaflet explants.

2.4.4 Somatic Embryo Formation

Eight weeks after the embryogenic tissue was placed through the maturation regime, somatic embryos were observed. In all treatments producing embryos, the number of cotyledons varied from one to eight (Figure 33). Maturation was asynchronous with somatic embryos of varying stages of development present (Figure 34). Secondary somatic embryogenesis was also observed occurring at the radicle end of many of the embryos (Figure 35).

MS medium resulted in a significantly greater number of somatic embryos than WPM ($P=0.001$, $H= 413.500$) (Figure 36). 2,4,5-T yielded a significantly higher number

Table 4. Effect of auxin type and concentration, explant type, and basal media on the induction of callus (after 4 weeks) and embryogenic tissue of rose cultivar 'Livin' Easy' (after 12 weeks). The total number of explants for each treatment of callus induction and embryogenic tissue production was 108 and 54, respectively. (***PGR*** Plant Growth Regulator, ***2,4-D*** 2,4-dichlorophenoxy-acetic acid, ***2,4,5-T*** 2,4,5-trichlorophenoxyacetic acid, ***L*** Leaflet, ***P*** Petiole, ***MS*** Murashige and Skoog, ***WPM*** Woody Plant Medium, ***ET*** Embryogenic Tissue).

Auxin Treatment	Auxin (μM)	Explant Type	MS Basal Media			WPM Basal Media		
			Callus Induction (%)	ET Initiation 40% auxin	ET Initiation PGR-free	Callus Induction (%)	ET Initiation 40% auxin	ET Initiation PGR-free
PGR-free	0	L	5.7 ± 2.2	-	-	12.0 ± 6.8	-	-
PGR-free	0	P	1.9 ± 2.6	-	-	14.8 ± 7.9	-	-
2,4-D	5	P	100	-	-	100	+	-
2,4-D	10	P	100	-	-	100	-	-
2,4-D	25	P	100	+	-	100	-	-
2,4-D	5	L	99.1 ± 1.3	-	-	100	-	-
2,4-D	10	L	100	-	-	100	-	-
2,4-D	25	L	100	+	-	100	-	-
2,4,5-T	5	P	100	+	-	100	+	-
2,4,5-T	10	P	100	+	-	100	+	-
2,4,5-T	25	P	99.1 ± 1.3	+	+	100	+	-
2,4,5-T	5	L	100	+	-	100	+	-
2,4,5-T	10	L	100	+	+	100	+	-
2,4,5-T	25	L	100	+	+	100	+	+

Figure 33. Mean frequency rates (and standard deviation) of varying cotyledon numbers observed on somatic embryos produce after six weeks growth on Murashige and Skoog maturation medium supplemented with 7.57μM abscisic acid.

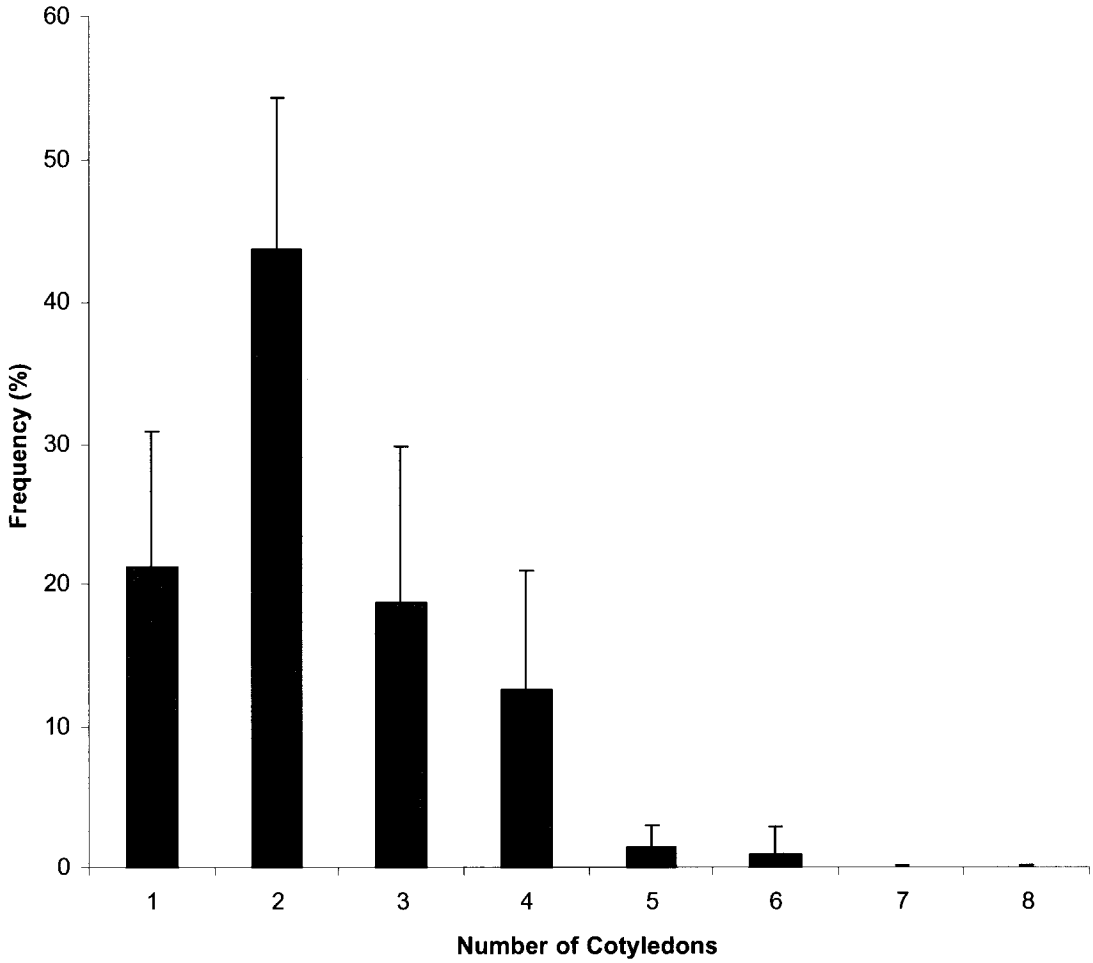
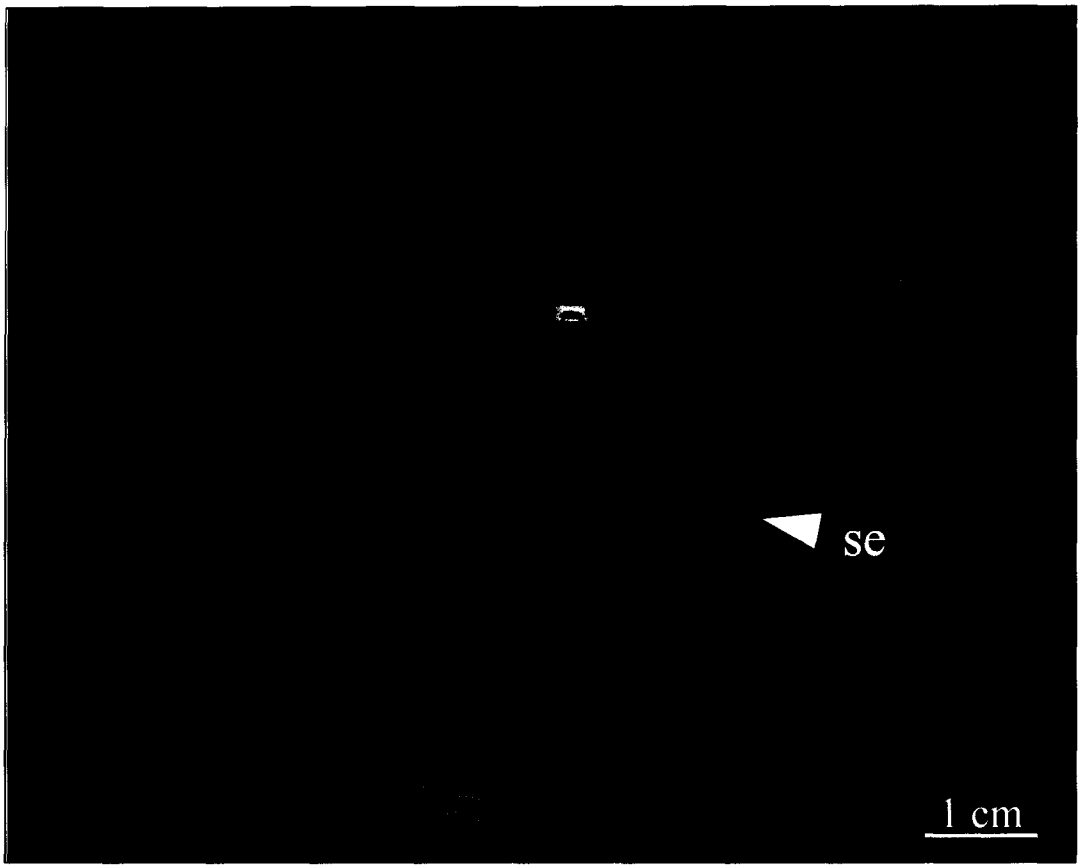

60
50
40
30
20
10
0
Frequency (%)
1
2
3
4
5
6
7
8
Number of Cotyledons

Figure 34. Asynchronous development of somatic embryos growing on Murashige and Skoog maturation medium supplemented with 7.57μM abscisic acid.

Figure 35. Secondary somatic embryogenesis occurring at the radicle ends of somatic embryos. Bar 1mm.


se
1 cm

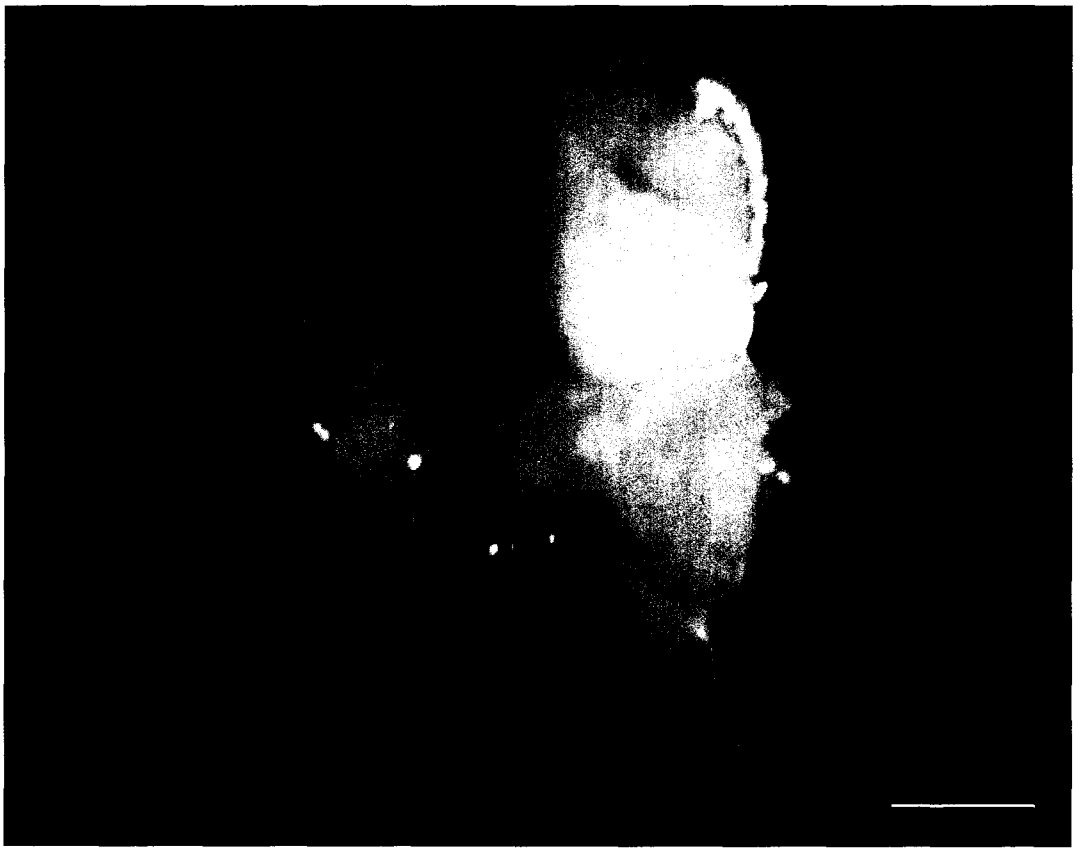
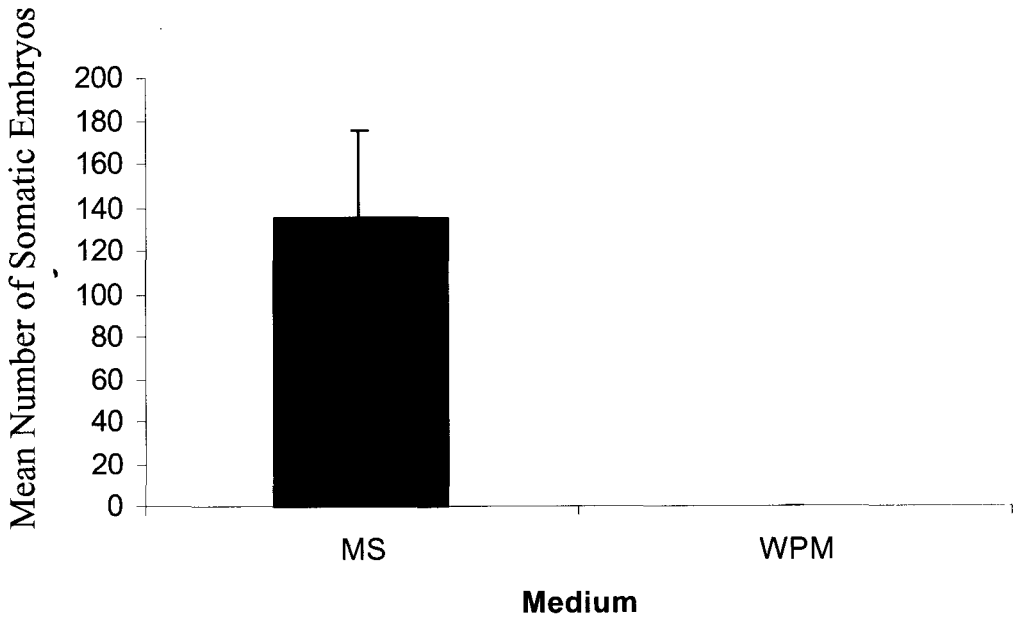
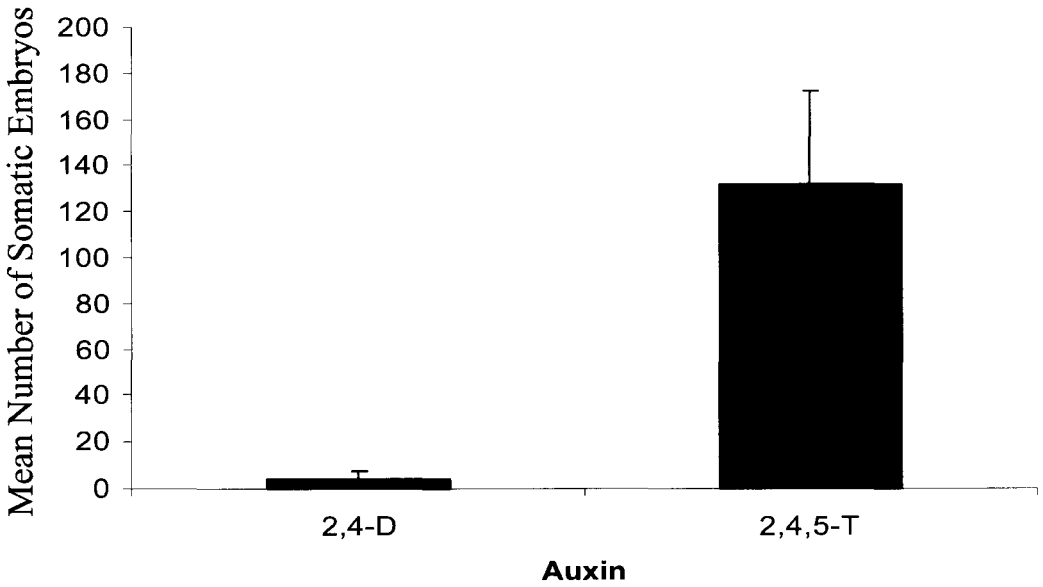


Figure 36. The mean number of somatic embryos (and standard deviation) per treatment in relation to the type of basal medium after eight weeks on maturation medium. The total number of explants for each treatment was 54. (***MS*** Murashige and Skoog, ***WPM*** Woody Plant Medium).

Figure 37. The mean number of somatic embryos (and standard deviation) per treatment in relation to the auxin type after eight weeks on maturation medium. The total number of explants for each treatment was 54. (***2,4-D*** 2,4-dichlorophenoxyacetic acid, ***2,4,5-T*** 2,4,5-trichlorophenoxyacetic acid).


Mean Number of Somatic Embryos
200
180
160
140
120
100
80
60
40
20
0
MS
WPM
Medium


Mean Number of Somatic Embryos
200
180
160
140
120
100
80
60
40
20
0
2,4-D
2,4,5-T
Auxin

of somatic embryos than 2,4-D (P=0.006, H=186) (Figure 37).

A one-way ANOVA determined there was a significant difference among the concentrations of 2,4,5-T employed in MS medium (P=0.035, F=4.991, df = 2, 9) (Figure 38). A Fisher's LSD test determined the control (0μM) was different than 10μM and 25μM (P=0.022 and P=0.001, respectively). The 5μM and 25μM were significantly different (P=0.003). All other pairwise comparisons were not significantly different. There was no significant difference between the number of somatic embryos produced by leaflet and petiole explants (P=0.597, H = 307.500) (Figure 39).

Although there was not a significant difference in the number of somatic embryos produced by transfer of embryogenic tissue to PGR-free medium versus 40% auxin medium (P=0.472, H=261.500), there was a notable difference in terms of embryo quality. 32.3 ± 6.4% of somatic embryos on PGR-free medium appeared abnormal (swollen) (Figure 40).

2.4.5 Germination and Survival Rates

By the end of the first week on germination medium, the somatic embryos had turned green in color and started to elongate. The second and third week consisted of further elongation and the formation of roots. 33.3% to 95.2% of the somatic embryos had germinated after six weeks on MS germination medium (Table 5) (Figure 41). Most (79.2 ± 6.6%) of these germinants had formed roots. Of the somatic embryos that did not germinate, 67.5% had secondary embryogenesis occurring in the form of nodular

Figure 38. Mean number (and standard deviation) of cotyledonary somatic embryos (per 54 explants) produced by varying concentrations of 2,4-dicholorphenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid after eight weeks on maturation medium.

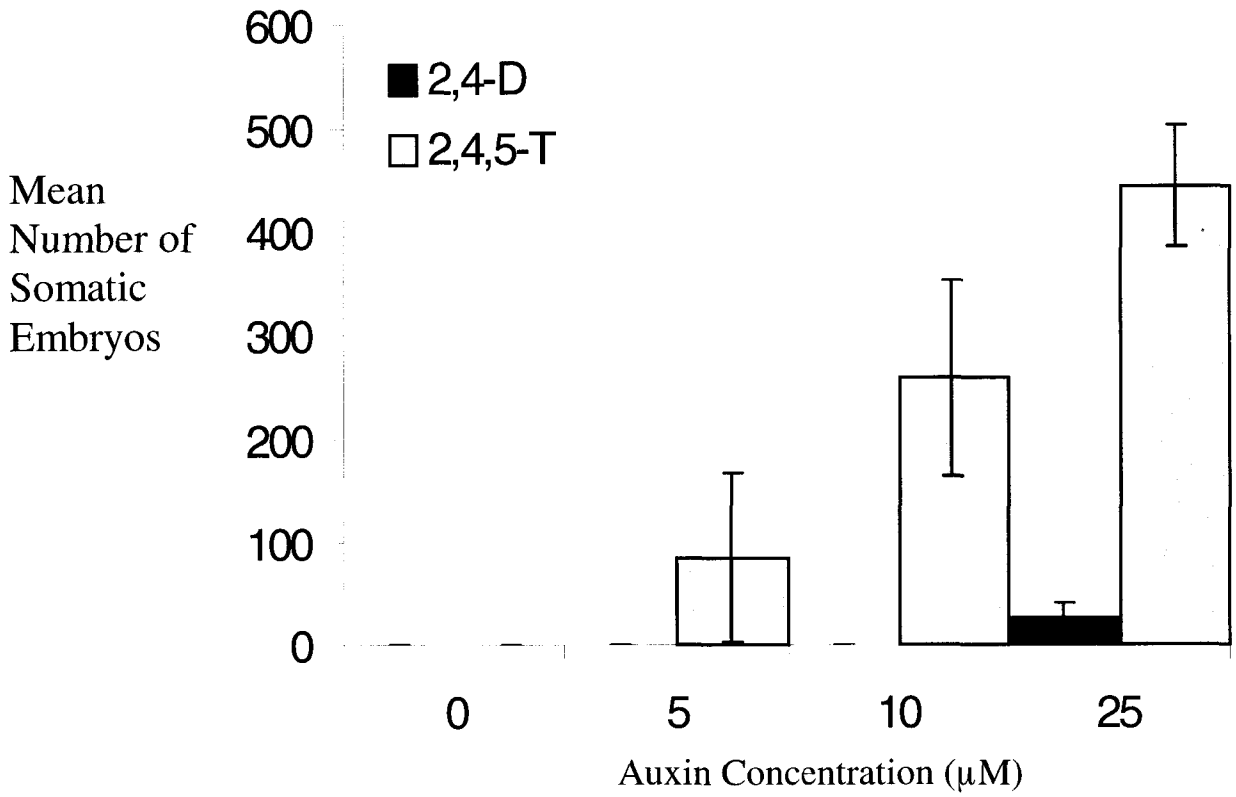

600
500
400
300
200
100
0
2,4-D
2,4,5-T
Mean Number of Somatic Embryos
0
5
10
25
Auxin Concentration (μM)

Figure 39. Effect of explant type on mean number of somatic embryos (and standard deviation) produced after treatment after eight weeks on maturation medium. The total number of explants per treatment was 54.

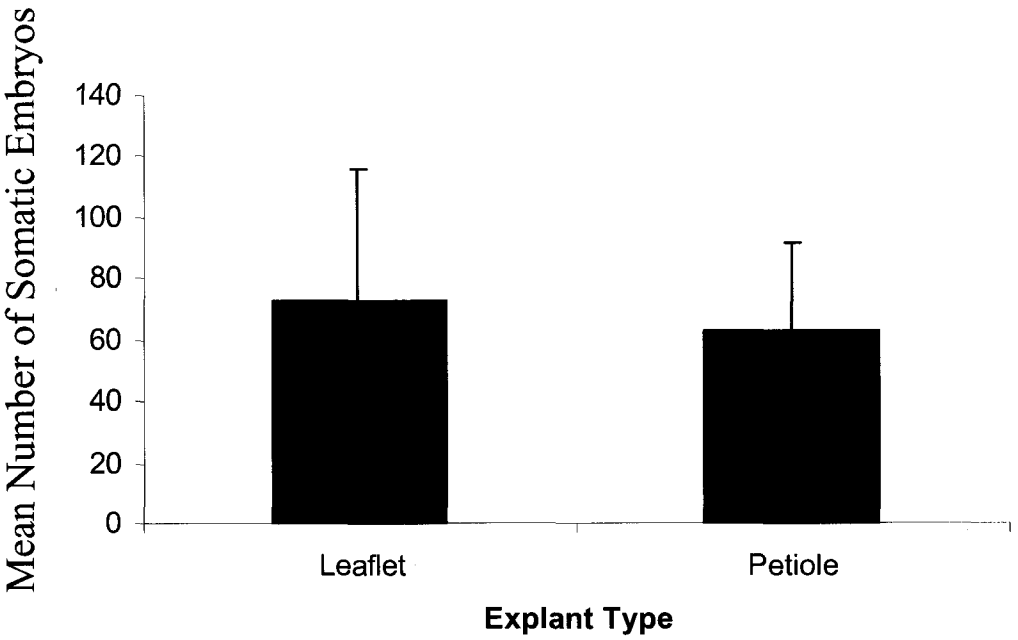

Mean Number of Somatic Embryos
140
120
100
80
60
40
20
0
Leaflet
Petiole
Explant Type

Figure 40. Photograph of abnormal somatic embryo grown on plant growth regulator-free embryogenic tissue proliferation medium.

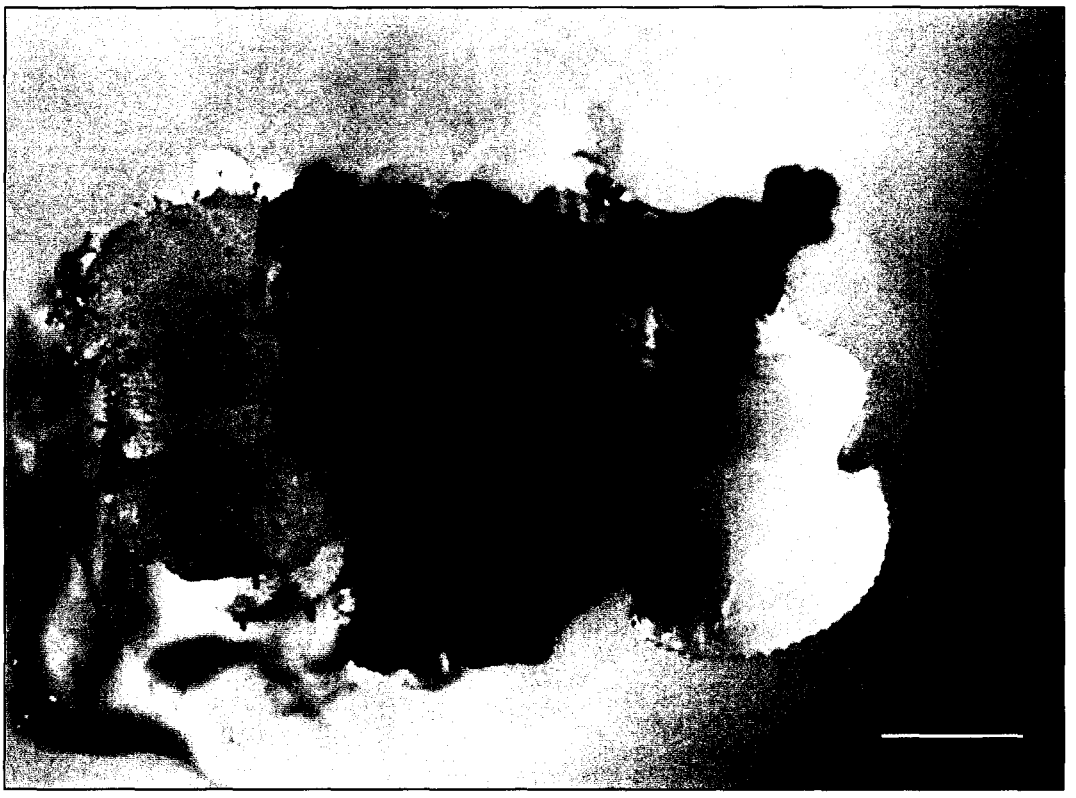
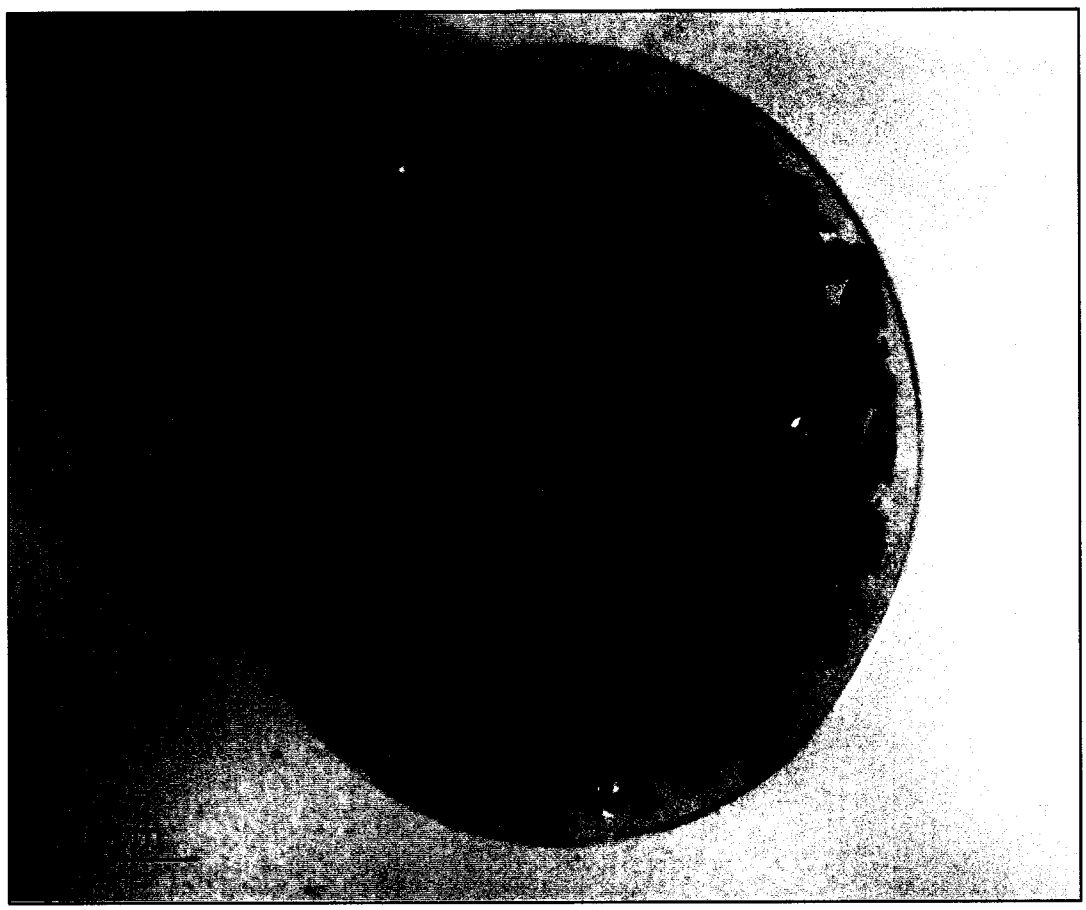


Table 5. Mean germination rate and mean percent of plantlets transplanted to soil after six weeks growth on Murashige and Skoog germination medium. Mean survival rates of transplanted plantlets after three months growth *ex vitro*. (***2,4,5-T*** 2,4,5-trichlorophenoxyacetic acid, ***2,4-D*** 2,4-dichlorophenoxyacetic acid).

Auxin Type	Auxin Concentration (μM)	Proliferation Medium	Explant Type	Mean Germination Rate (%) (± S.D.)	Mean Percent Transplanted (± S.D.)	Mean Survival Rate (%) (± S.D.)
2,4,5-T	5	40%	L	33.3	19.2	28.6
2,4,5-T	5	40%	P	85.5 ± 1.2	15.4	36.4
2,4,5-T	10	40%	L	95.2 ± 4.8	73.0 ± 38.2	83.7 ± 23.1
2,4,5-T	10	40%	P	92.3 ± 4.9	30.5	23.9
2,4,5-T	10	PGR-free	L	78.0	45.1	58.3
2,4,5-T	25	40%	P	94.3 ± 4.1	50.1	50.0
2,4,5-T	25	40%	L	90.4 ± 14.5	54.3 ± 33.9	86.9 ± 3.4
2,4,5-T	25	PGR-free	L	74.7 ± 26.4	33.2 ± 1.2	71.2 ± 27.6
2,4,5-T	25	PGR-free	P	84.5	40.4	61.4
2,4-D	25	40%	L	85.2	45.9	14.0

Figure 41. Plantlets growing on germination medium.



embryogenic tissue formation. Nodular embryogenic tissue was also observed growing at the base of 60.6% of the plantlets, a phenomenon called recurrent somatic embryogenesis (Figure 42). 15.4% to 73.0% of the plantlets were transplanted and acclimatized with survival rates of 14.0% to 86.9% after three months (Table 5) (Figure 43 & 44).

2.5 DISCUSSION

This is the first report of somatic embryogenesis being attempted in *Rosa hybrida* L. cultivar 'Livin' Easy'. The results obtained from the present study will therefore be compared with those obtained with other rose cultivars.

2.5.1 Callus Induction

The high frequency of callus induction when auxin was included in the callus induction medium was expected. It was reported in several previous studies of rose (Marchant *et al.*, 1996; Murali *et el.*, 1996; van der Salm *et al.*, 1996; Rout *et al.*, 1991). The lack of callus formation on explants cultured on PGR-free media was also not surprising as it had previously been reported that 2,4-D is required in media for callus induction from root explants of *Rosa hybrida* L. 'Moneyway' (van der Salm *et al.*, 1996), 'Carefree Beauty' and 'Red Sunblaze' (Li *et al.*, 2002). However, Kunitake *et al.* (1993) reported that both non-embryogenic callus and embryogenic tissue was obtained in *Rosa rugosa* Thunb. cultured on MS medium without PGRs. They used immature seeds and this may explain the difference. Seeds often consist of pre-embryogenic determined cells (PEDCs) that are capable of inducing embryogenesis when cultivated *in vitro* (refer to

Figure 42. Recurrent somatic embryogenesis occurring at the base of plantlets produced from leaflet explants grown on Murashige and Skoog medium supplemented with 10μM 2,4,5- trichlorophenoxyacetic acid. Note embryogenic tissue (ET) and somatic embryos (se) formed at base of rose SE-derived plantlets.

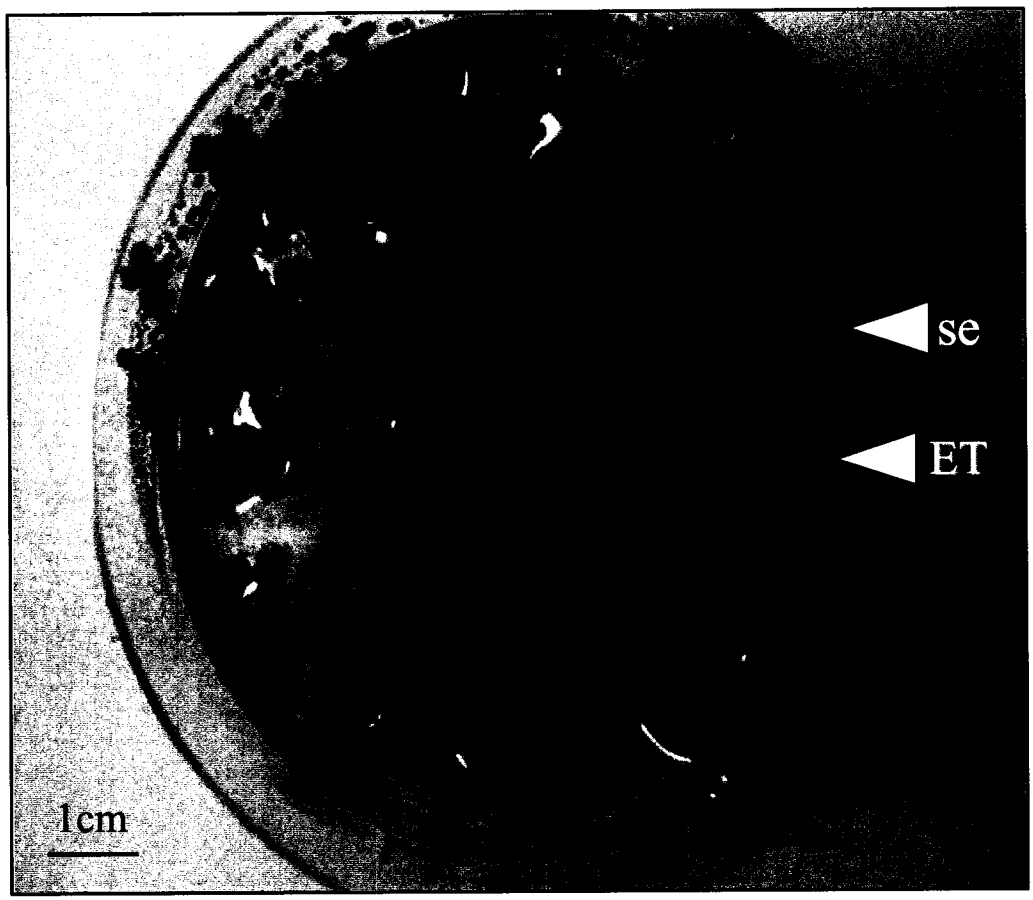
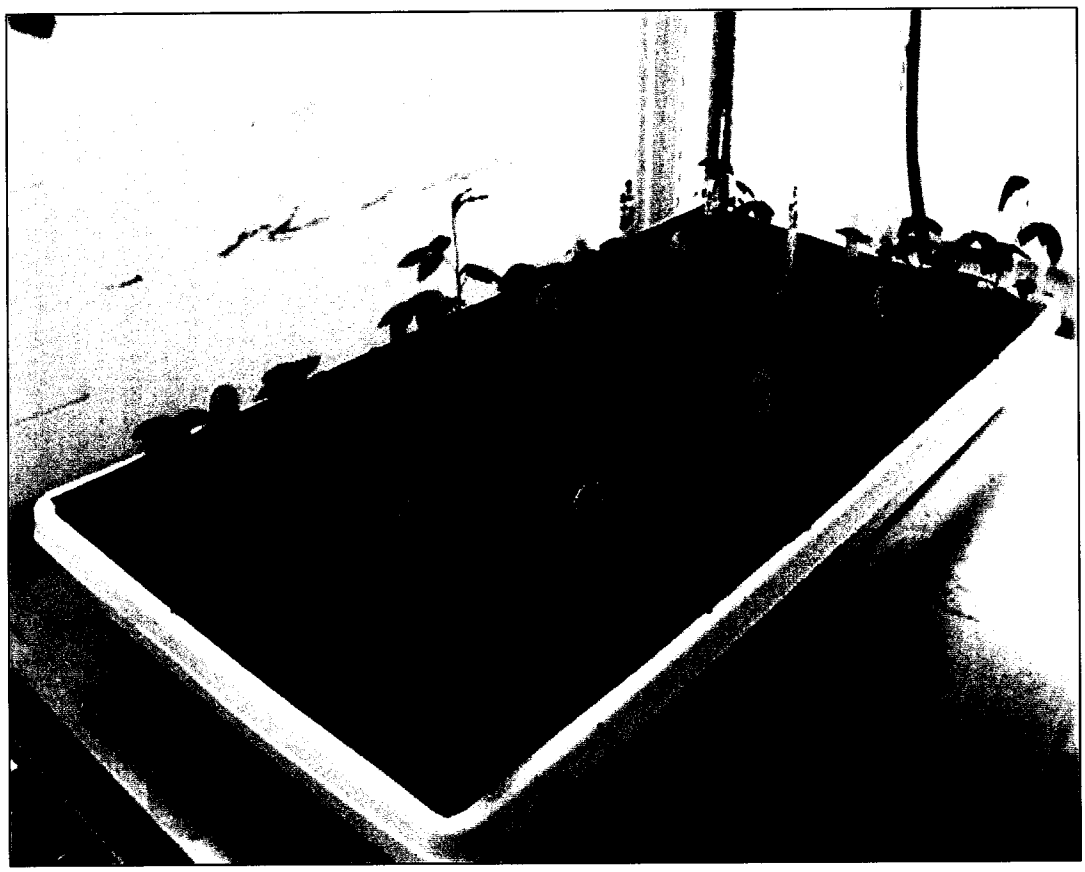

se
ET
1cm

Figure 43. Acclimatized rose plants derived from somatic embryos of *Rosa hybrida* L. cultivar 'Livin' Easy' after two months growing on soil.

Figure 44. Acclimatized rose plants growing *ex vitro* developing flowers and buds after two months growth on soil. Bar 2cm.



chapter one) (Merkle *et al.* 1990).

The browning phenomenon observed in calli on callus induction medium, as the culture time progressed, was also reported by Hameed *et al.* (1993) in callus cultures of *Rosa hybrida* L. cultivars 'Diamond Jubly' and 'Lans France'. Hameed and colleagues used sodium diethyldithiocarbonate (SDC) to prevent browning of the callus as it hampered the establishment of callus cultures. The occurrence of browning in the present study may have been instrumental in initiating embryogenic tissue formation. Somatic embryogenesis can be induced by stresses such as heat shock (Kitamiya *et al.*, 2000) or starvation (Lee *et al.*, 2001) and may therefore be regarded as a survival mechanism by plants in response to unsuitable situations. Such moribund callus may induce the production of competent cells capable of later developing into somatic embryos. Marchant *et al.* (1996) observed that only root explants exhibiting extensive browning produced callus, in rose cultivars 'Trumpeter' and 'Glad Tidings'. Again perhaps as a means of surviving critical circumstances. Puigderrajols *et al.* (2001) in their study of embryogenesis in Cork oak (*Quercus suber* L.) hypocotyls observed a brownish friable callus derived with fragments of necrotic tissue in which isolated, cytoplasm-rich isodiametric cells were present.

Stress induces changes in metabolism and adaptation mechanisms (Lichtenthaler, 1998). Stress causes browning of callus and several variables may have contributed to this moribund state. Wounding is a mechanical stress and is a significant signal for the induction of dedifferentiation (Grosset *et al.*, 1990). Heat shock proteins (hsps) are

expressed during somatic embryo development in response to the stress induced by heat (Coca *et al.*, 1994). Two auxin-responsive genes (*Dchsp-1* and *Dcarg*-1), homologous to hsps, have been isolated in carrot (Kitamiya *et al.*, 2000). *Dchsp-1* is an auxin-induced gene expressed throughout somatic embryo development. *Dcarg*-1 is expressed only during the early phases of embryogenic initiation. The existence of two auxin-induced genes homologous to hsps suggests that a high concentration of auxin may be perceived as a stress condition (Chugh & Khurana, 2002). 2,4-D has been reported to have a dual effect as an auxin and as a stressor (Fehér *et al.*, 2001). 2,4,5-T has been reported to exhibit increased oxidative stress and damage to the thylakoid membrane (Segura-Aguilar *et al.*, 1995).

In addition to the auxins, the basal medium may have contributed to the stress levels in the rose cells. Harsh environments, such as exposing wounded cells or tissues to sub-optimal nutrient supplies generate significant stress effects (Fehér *et al.*, 2003). Some components of MS medium, not present in WPM, have been reported to induce stress in plant cells. The inclusion of cobalt (and lack of nickel) in MS basal medium has been reported to contribute to leaf damage and metabolic stress in tissue culture grown potato plants (Witte *et al.*, 2002). WPM contains neither nickel nor cobalt. MS medium also contains potassium iodide (KI), whereas WPM does not include it. KI has been shown to be toxic to Goldenweed (*Haplopappus gracilis)* cells when cultured under dark conditions (Eriksson, 1965). It is possible that the nutrient formulation of MS basal medium may have exposed the rose cells to more stress resulting in the greater embryogenic tissue induction response.

The occurrence of rhizogenic activity in the form of hair-like projections and root formation is not uncommon in rose. Hsia and Korban (1996) reported the occurrence of highly rhizogenic callus resulting from the application of 2,4-D in the roses *Rosa hybrida* L. 'Carefree Beauty', *Rosa chinensis minima* cultivars 'Red Sunblaze' and 'Baby Katie'. This rhizogenic callus often resulted in subsequent shoot organogenesis and somatic embryogenesis at rates of 3.3% and 6.6%, respectively. Hsia and Korban concluded that a cell's rhizogenic capability might somehow act as a trigger for a cell's competence for organogenesis. It is not known whether or not the rhizogenic activity acts as a trigger, or is merely an *indicator* of a cell's capability for organogenesis. Nevertheless, the presence of rhizogenesis must reflect activation of genes that are not normally expressed in callus. Kim *et al.* (2003b) also observed root formation on callus derived from *in vitro* grown leaf explants of rose grown on MS basal medium supplemented with the auxin naphthalene acetic acid (NAA) and at a less frequent rate with 2,4-D. However, Kim *et al.* (2003b) did not specify if the rhizogenic callus was indicative of somatic embryogenic competence. This phenomenon of auxin root induction is not limited to roses. It has long been known that high levels of auxin, relative to cytokinin levels, stimulate the formation of roots in plant cells (Skoog & Miller, 1965).

2.5.2 Embryogenic Tissue Initiation

Although stress is regarded as a contributing factor in inducing SE, the plant cells must be able to remain viable long enough for survival mechanisms, such as the induction of shoots, roots or embryos, to be activated. Optimal levels of stress induce changes in gene expression and physiology of plant cells whereas stress levels that

exceed cellular tolerance, cause cell death (Lichtenthaler, 1998). Perhaps because of its presence of causative agents or the particular nutrient composition, MS basal was better suited for ET proliferation than the WPM basal medium. The MS basal medium supported the continued growth of the embryogenic tissue, whereas WPM did not.

One of the main differences between MS and WPM basal media is nitrogen concentration. MS medium has a very high nitrogen content (1650 mg/L ammonium nitrate and 1900 mg/L potassium nitrate) compared to WPM (400 mg/L ammonium nitrate and 556 mg/L calcium nitrate). The nitrogen composition of culture medium has been shown to have an effect on embryogenesis and regeneration of plants such as barley (*Hordeum vulgare* L.) (Nuutila *et al.*, 2000) and rice (*Oryza sativa* L.) (Grimes & Hodges, 1990). In addition to the overall higher concentration of nitrogen in MS basal medium than WPM, the quantity of nitrogen in a state readily usable by the plants is also much higher. Plants are most capable of using nitrogen in its reduced form (George, 1993). Reduction refers to the removal of an oxygen molecule from a compound and its replacement with a hydrogen. The ammonium ion (NH_4^+) is a more reduced form of nitrogen compared to the oxidized nitrate ion (NO_3^-). MS basal medium has a much higher concentration of reduced nitrogen in the form of NH_4NO_3 (1650mg/L) than WPM (400mg/L). Morphogenesis is greatly influenced by the total quantity of nitrogen present in the medium but for most plant species, a supply of both reduced nitrogen and nitrate are necessary (George, 1993). It has long been known that the presence of reduced nitrogen is necessary for successful somatic embryogenesis in cell and callus cultures of carrot (*Daucus carota*) (Halperin and Wetheral, 1965; Reinert *et al.*, 1967). Amino acids,

such as proline, casein hydrolysate and glutamine, have been successful in satisfying the reduced nitrogen requirement of media low in nitrogen (Rout *et al.*, 1991). Due to the high cost of amino acid supplements, the use of a high ammonia-containing media such as MS has economic advantages for culture protocols to be used in rose production on a commercial scale.

In addition to basal medium, auxin type also had a significant affect on the induction of somatic embryogenesis in *Rosa hybrida* L. cultivar 'Livin' Easy'. Al-Mazrooei and associates (1997) observed that the use of 2,4,5-T induced embryogenic tissue in a wider range of cultivars of sweet potato [*Ipomoea batatas* (L.) Lam.] than 2,4-D. 2,4,5-T also successfully induced SE in seven cultivars of sweet potato that had responded poorly or not at all to 2,4-D (Al-Mazrooei *et al.*, 1997). 2,4,5-T was also found to be effective in inducing SE in chickpea (*Cicer arietinum* L.) (Sagare *et al.*, 1993) however it was not effective in sesame (*Sesamum indicum* Var. TMV 6) (Jeya Mary & Jayabalan, 1997) or Eucalytptus *(Eucalytptus globules)* (Nugent *et al.*, 2001).

The occurrence of embryogenic tissue formation at high concentrations of 2,4-D (25μM on MS medium) is in contrast to the report by Kunitake *et al.* (1993) who reported that callus formation in *Rosa rugosa* Thunb. was highest on MS medium without growth regulators and no ET was obtained on medium containing a 2,4-D concentration of 22μM. As previously mentioned, perhaps the explant type (seed embryos) may have been a factor in these contrasting results. The high 2,4-D concentration may not have been required for a genetic activation in these PEDCs and instead the 2,4-D may have

induced effects beyond the point of stress, to the level of toxicity.

Embryogenic tissue was produced in the present study by both petiole and leaflet explants. This finding contrasts with that of Marchant *et al.* (1996) who observed embryogenic tissue formation from *in vitro* derived petiole and root explants of the Floribunda rose cultivars 'Trumpeter' and 'Glad Tidings', but not *in vitro* derived leaves. These contrasting results are possibly due to genotype-specific differences among the rose cultivars tested.

2.5.3 The Characterization of Presumptive Embryogenic Tissue

Quite often, the callus induction stage and its subsequent transition to an embryonic state is collectively referred to as early somatic embryogenesis (Kairong *et al.*, 1999; Sato *et al.*, 1995; Momiyama *et al.*, 1995). A greater emphasis on characterizing and defining the stages involved from dedifferentiation through to the transition of callus into an embryogenic state is required in order to elucidate the processes involved in indirect SE. Although characterization of embryogenic tissue may contribute greatly to the understanding of the embryogenic process (Puigderrajols *et al.*, 2001), cellular studies have not been conducted in the *Rosa* sp. The characterization of PET in this study, revealed that several types of tissue are produced during the embryogenic tissue initiation phase of SE. This is the first step towards understanding the processes involved in the transition from callus to embryogenic tissue. Further studies are required to determine if the characteristic tissue types observed in this study are present for other cultivars. In addition, it would be valuable to track the tissue through to maturation to determine the

fate of each tissue type. Markers, such as cell morphology, which indicate the acquisition of embryogenic competence in the induced cells would greatly help elucidate the earliest phases of somatic cell transition (Fehér *et al.*, 2001).

2.5.4 Somatic Embryo Formation

Asynchronous maturation has been reported in many cultivars of rose (Rout *et al.*, 1999; Noriega & Söndahl, 1991). Asynchronous development of embryos has been identified as a problem hindering the scale up of indirect somatic embryogenesis. Methods to overcome this problem have been devised. One such method involves filtering the somatic embryos through a series of filters with varying pore size (Castillón & Kamo, 2002). The larger, more mature somatic embryos are separated from the younger small embryos by size. The occurrence of secondary somatic embryogenesis also contributes to the asynchronous state observed; new embryos being constantly "born" from the older primary embryos. This results in the presence of somatic embryos at varying stages of development.

Secondary somatic embryogenesis has been previously reported in roses (Li *et al.*, 2002; Rout *et al.*, 1991). This phenomenon is considered to be the result of cells escaping integrated group control, permitting them to express their totipotency (Kärkönen, 2000). Secondary SE has potential benefits for the large-scale production of roses in that it creates a constant supply of embryogenic tissue, especially important for cultivars with low SE frequency rates (Li *et al.*, 2002). Secondary SE however also poses problems in the scale up of SE in that it inhibits somatic embryo germination. For

example, in the legume *Medicago truncatula,* secondary SE negatively affects somatic embryo conversion by arresting shoot and root development (das Neves *et al.*, 1999). This appears to be the case in the present study as well, as a high percent of somatic embryos that did not germinate had secondary embryogenesis occurring. It is uncertain if the secondary SE inhibited embryo development or if the secondary SE development was the result of the inhibition. Future studies on this phenomenon could help determine whether secondary SE is a benefit or hindrance.

MS medium resulted in a significantly greater number of somatic embryos than WPM. This concurred with the findings of de Wit and associates (1990) who reported that it was possible to obtain somatic embryos of the rose cultivars 'Domingo' and 'Vickey Brown' on MS medium but not WPM. The observation of abnormal somatic embryos produced on MS medium without growth regulators was also reported by Visessuwan and colleagues in *Rosa hybrida* cv. 'Carl Red' (Visessuwan *et al.*, 1997). The variation in cotyledon number in somatic embryos concurs with many other reports in rose (Li *et al.*, 2002; Marchant *et al.*, 1996; de Wit *et al.*, 1990). Finally, there was no significant difference between the numbers of somatic embryos produced by leaflet versus petiole explants. This is in contrast with previous reports where explant source has been reported to be a contributing factor in rose somatic embryogenesis (de Wit *et al.*, 1990). de Wit and associates found leaflets of *Rosa* sp. 'Domingo' and 'Vickey' formed somatic embryos while petioles did not.

2.5.5 Germination and Survival: Regeneration Rates

The highest germination rate obtained in this study (95.2%) was high in comparison to the highest conversion rates obtained with other rose cultivars in previous studies (Table 6). The rate of conversion of somatic embryos into plantlets in previous reports was often quite low (0-30%) (Rout *et al.*, 1999; Noriega & Söndahl, 1991; Kunitake *et al.*, 1993; Visessuwan *et al.*, 1997; Kim *et al.*, 2003a; Kim *et al.*, 2003b). It is important to remember that germination rates reported in previous studies might reflect differences in embryo selection when transferring embryos to germination medium (Roberts *et al.*, 1995). The present study chose all somatic embryos, and did not select for somatic embryos most closely resembling zygotic embryos. Selecting 'ideal' somatic embryos is a logical procedure but if there is no correlation between 'ideal shape' and ability to germinate, it could give lower germination rates than using all embryos.

Many authors report the enhancement of germination with the addition of various compounds to the germination medium. Sarasan *et al.* (2001) observed an increase in germination rate from 20% in Rosa Heritage x Alister Stella Gray, to 56%, when both 6-benzyladenine (BA) and methyl laurate were added to the germination medium. Murali *et al.* (1996) reported that the 33.3% conversion rate in *Rosa hybrida* L. cultivar 'Arizona' was improved to 93% when phloroglucinal, a root enhancer, was added to the medium.

The capability of somatic embryogenesis to produce bipolar somatic embryos is critical for synthetic seed technology. The formation of both shoots and roots also reduces the time and costs associated with additional steps for rooting. Sarasan *et al.*

Table 6. Highest germination rates obtained in previous studies of SE in *Rosa* sp.

Reference	Cultivar	Explant Type	Germination Rate (%)
Kim *et al.* (2003a)	*R. hybrida* L. cv. 'Sumpath'	protoplasts	30.9
Kim *et al.* (2003b)	*R. hybrida* L. cv. '4th of July'	*in vitro* leaves	11
	R. hybrida L. cv. 'Tournament of Roses'	*in vitro* leaves	8
Castillón & Kamo (2002)	*R. hybrida* L. cv. 'Trumpeter'	not stated	61
	R. hybrida L. cv. 'Dr. Huey'	not stated	39
	R. hybrida L. cv. 'Tineke'	not stated	12
Visessuwan *et al.* (1997)	*R. canina*	*in vitro* leaves	0
	R. hybrida L. cv. 'Carl Red'	*in vitro* leaves	0
Murali *et al.* (1996)	*R. hybrida* L. cv. 'Arizona'	petals	93
Matthews *et al.*, 1991	*R. persica* x *xanthina*	protoplasts	30
Marchant *et al.* (1996)	*R. hybrida* cv. 'Trumpeter'	petiole	17
	R. hybrida cv. 'Glad Tidings'	petiole	9
Kunitake *et al.* (1993)	*Rosa rugosa* Thunb.	immature seeds	3
Noriega & Söndahl (1991)	*R. hybrida* L. cv. 'Royalty'	filaments	12
Rout *et al.* (1991)	*R. hybrida* L. cv. 'Landora'	leaves and stems	12
de Wit *et al.* (1990)	*Rosa* sp. cv. 'Domingo' and 'Vickey Brown'	*in vitro* leaves	60

(2001) reported three types of germination responses in the rose *Rosa* Heritage x Alister Stella Gray; the formation of bipolar germinations possessing both roots and shoots, embryos that formed shoots only (no root formation) and embryos that formed roots (but not shoots).

2.5.6 Recurrent Somatic Embryogenesis

Although there have been several reports of secondary somatic embryogenesis in rose, the present study is the first report of recurrent somatic embryogenesis. (Li *et al.*, 2002; Rout *et al.*, 2001). Although terminology differs among literature (refer to chapter one), a thorough review of previous published studies confirmed that only secondary and repetitive SE somatic embryos has been reported to date in rose. Recurrent SE from regenerated plantlets as observed in the present study has not been described before.

Recurrent somatic embryogenesis is beneficial in bestowing somatic embryogenic systems with a major multiplicative potential for clonal mass propagation (Merkle, 1990). Recurrent systems are especially suited for the continuous production of cells with embryogenic capacity (Fransz and Schel, 1994). Recurrent somatic embryogenesis has been reported for several species including sunflower (*Helianthus maximiliani* Schrader) (Vasic *et al.*, 2001) and barrelclover medick (*Medicago truncatula*) (Nolan *et al.*, 1989). Vasic and associates (2001) utilized leaves of sunflower (*Helianthus maximiliani* Schrader) plants that were obtained via direct somatic embryogenesis as the starting explants for a new cycle of somatic embryogenesis. They recognized the convenience of a cyclic system with a constant supply of starting material. In addition to convenience,

there is an increase in somatic embryogenesis frequency in cycles of repetitive and recurrent embryogenesis compared to primary/indirect embryogenesis (Vasic *et al.*, 2001; Fambrini *et al.*, 1997; Nolan *et al.*, 1989). Further studies demonstrating successful conversion of recurrent ET into plantlets would provide even stronger evidence of the multiplicative potential of recurrent SE in rose.

3. FURTHER OPTIMIZATION OF THE SOMATIC EMBRYOGENESIS SYSTEM

3.1 INTRODUCTION

The preliminary findings in chapter two concluded that Murashige and Skoog (MS) basal medium when supplemented with 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) was successful in inducing somatic embryogenesis in *Rosa hybrida* L. cultivar 'Livin' Easy' leaflet and petiole explants. Several subsequent questions arose related to refining this protocol for the rose cultivar "Livin' Easy' and extending it to other cultivars. This chapter attempts to address these questions.

3.1.1 Efficiency of Somatic Embryogenesis System

One of the areas currently hindering the large-scale propagation of roses via somatic embryogenesis (SE) is the inability to successfully induce SE in some cultivars and low induction rates in others (Rout *et al.*, 1999). The first question arising from chapter two, is at what frequency does 2,4,5-T induce somatic embryogenesis in 'Livin' Easy' and how does this compare to induction rates obtained in previous studies involving *Rosa* sp.?

3.1.2 Tissue Selection for Maturation

As previously stated, embryogenic tissue (ET) is characterized by its nodular texture, soft structure and mucilaginous appearance. The nodular texture is due to the presence of early stage globular somatic embryos. Upon transferring this nodular ET onto maturation medium lacking or with reduced auxin, the embryos continue to develop into cotyledonary embryos. The characterization of presumptive embryogenic tissue

(PET) in chapter two revealed that Type 8 PET was composed of large masses of tissue, rather than the distinct globular structures as observed in Types 1-4. Type 8 PET was, however, soft to the touch and mucilaginous: morphological traits common to embryogenic tissue. Type 8 PET did, in fact, consist of cells with very prominent nuclei, a characteristic of embryogenic cells. This raises the question of whether or not non-nodular PET exhibiting traits characteristic of embryogenic tissue, such as softness and mucilaginous, were capable of forming somatic embryos.

3.1.3 Genotype Specificity

Individual genotypes within a given species vary greatly in terms of embryogenic capability (von Arnold *et al.*, 2002). Although 2,4,5-T was successful in inducing somatic embryogenesis in *Rosa hybrida* L. cultivar 'Livin' Easy', will this compound have the same affect on other cultivars of rose?

3.1.4 The Potential of Recurrent Somatic Embryogenesis

Recurrent somatic embryogenesis has previously been reported in SE systems of several plant species including *Helianthus maximiliani* (sunflower) (Vasic *et al.*, 2001). The embryogenic tissue (ET) observed on the bases of SE-derived plantlets in chapter two indicates a potential usefulness for commercial purposes. The potential commercial benefits of recurrent somatic embryogenesis are only *bona fide* if the tissue produced is in fact capable of regenerating the desired commercial product; plantlets. It is important to determine if the globular embryos within the tissue are capable of further development and germination before prematurely

claiming it to be a tangible benefit of the current SE procedure.

3.2 OBJECTIVES

The objective of the research outlined in this chapter was to investigate further the treatments found optimal in chapter two on somatic embryogenesis of *Rosa hybrida* L. cultivar 'Livin' Easy', to optimize a protocol. The specific objectives were to: 1) Gauge the effectiveness of 2,4,5-T as an inducer of SE in *Rosa hybrida* L. cultivar 'Livin' Easy' by determining the frequencies of callus induction and embryogenic tissue initiation of the optimal treatments 2) Determine the optimal tissue for selection into maturation 3) Gauge the effectiveness of 2,4,5-T in inducing somatic embryogenesis in a variety of cultivars other than 'Livin' Easy' 4) Assess the potential of recurrent somatic embryogenesis.

3.3 MATERIALS AND METHODS

3.3.1 Frequency of SE

Leaflet and petiole explants (5-7 mm in length) were aseptically excised from the previously established *in vitro* shoot cultures of *Rosa hybrida* L. cultivar 'Livin' Easy' as described in chapter two and cultured onto callus induction media. The callus induction media consisted of Murashige and Skoog (MS) basal medium, modified as per Owen & Miller (1992), and supplemented with either 10μM or 25μM 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Sigma T-5785). Media were adjusted to pH 5.7 using 1.0N NaOH, solidified using 0.4% Phytagel™ (Sigma), and autoclaved for 20 minutes at 121°C, 104 kilopascals. Ninety explants of each explant type were utilized for each treatment (nine

explants per petri dish, each petri dish considered a replicate). The 100 x 15 mm plastic petri dishes were sealed with Parafilm® (American National Company) and incubated at 24°C in darkness. After four weeks, the explants were numbered and transferred to ET initiation medium composed of MS basal medium supplemented with 40% of the original 2,4,5-T concentration (4μM or 10μM). PET was isolated as per chapter two, with the exception that the PET was tracked according to the explant it was derived . Maturation, germination, cold storage, and transplanting were carried out as in chapter two. The experiment was conducted thrice. Statistical analyses were performed using the MINITAB statistical analysis package. Normally distributed data were analyzed using ANOVA and means were compared using Fisher's least significant difference (LSD) at the 5% probability level.

3.3.2 Tissue Selection for Maturation: Nodular versus Non-Nodular

Leaflet and petiole explants (5-7 mm in length) were aseptically excised from the previously established *in vitro* shoot cultures of *Rosa hybrida* L. cultivar 'Livin' Easy' as described in chapter two. Two concentrations of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Sigma T-5785) were employed (10 and 25 μM). Murashige and Skoog (MS) basal medium, modified as per Owen & Miller (1992) was utilized as the basal medium. Media were adjusted to pH 5.7 using 1.0N NaOH, solidified using 0.4% Phytagel™ (Sigma), and autoclaved for 20 minutes at 121°C, 104 kilopascals. Four hundred and fifty explants of each explant type were utilized for each treatment (nine explants per petri dish). The 100 x 15 mm plastic petri dishes were sealed with Parafilm® (American National Company) and incubated at 24°C in dark.

For each treatment, 0.25 grams of nodular PET was selected using a sterilized scalpel and placed into each of 250ml flasks containing 30ml liquid MS PGR-free medium. 0.25 grams of soft, mucilaginous, non-nodular PET was also selected from each of the treatments using a sterilized scalpel and placed into each of 250ml flasks containing 30ml liquid MS PGR-free medium. The cultures were shaken for 24 hours at 110 rpm on a rotary shaker (G10 Gyrotory® Shaker, New Brunswick Scientific, N.J., U.S.A). Three 10ml aliquots from each flask were pipetted onto a maturation media containing 7.57 μM ±-cis, trans-Abscisic acid (ABA) (Sigma A-1049). Media were adjusted to pH 5.7 using 1.0N NaOH, solidified using 0.4% Phytagel™ (Sigma) and autoclaved for 20 minutes at 121°C, 104 kilopascals. After eight weeks on maturation medium, the cultures were observed and the number of somatic embryos present was recorded. This study was performed in duplicate. Statistical analyses were conducted using the MINITAB statistical analysis package. Non-normally distributed data were analyzed at a 5% probability level using the non-parametric Mann-Whitney test.

3.3.3 Multi-Cultivar Study

Shoot cultures of the rose cultivars 'Betty Prior', 'Easy Going', 'Graham Thomas', 'John Franklin', 'Morden Sunrise', 'Red Meidiland', 'William Baffin' and *Rosa rugosa* Alba were obtained from *Weeks Roses* (Upland, CA). Rooted cuttings were made from these shoots and grown in a greenhouse at Debert Tree-Breeding Centre, Debert, N.S. The rooted cuttings were collected and stored at 4°C for three days until subjected to the surface sterilization procedure. Shoot cultures were established as described in Chapter two. Leaflet explants (5-7 mm in length) were aseptically excised

from the *in vitro* shoot cultures of each cultivar. Murashige and Skoog (MS) basal medium, modified as per Owen & Miller (1992) was employed as the basal medium. The basal medium was supplemented with either 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Sigma T-5785) or 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma D-7299) at a concentration of 10μM. Media were adjusted to pH 5.7 using 1.0N NaOH, solidified using 0.4% Phytagel™ (Sigma), and autoclaved for 20 minutes at 121°C, 104 kilopascals. Forty five leaflets were utilized for each treatment (nine explants per petri dish, each plate a replicate). The 100 x 15 mm plastic petri dishes were sealed with Parafilm® (American National Company) and incubated at 24°C in the dark. After four weeks, the explants were transferred to MS medium containing 40% the original concentration of auxin. Each week, for two weeks, PET was isolated and placed onto medium of the same composition. The cultures were observed weekly using a dissecting scope (NorthWest, Model# 1962711) for signs of embryogenic response such as the formation of nodular tissue or somatic embryos. The experiment was conducted in duplicate.

3.3.4 Recurrent Somatic Embryogenesis

Embryogenic tissue growing at the base of SE-derived plantlets growing *in vitro* was removed using a sterilized scalpel. 0.25grams of ET was placed into each of five 250ml flasks containing 30ml liquid MS PGR-free medium. The cultures were shaken for 24 hours at 110 rpm on a rotary shaker (G10 Gyrotory® Shaker, New Brunswick Scientific, N.J., U.S.A) and then vacuum filtered onto Whatman #1 filter paper. The filter paper and ET was placed onto a MS maturation medium containing 7.57μM ±-cis, trans-Abscisic acid (ABA) (Sigma A-1049). Medium was adjusted to pH 5.7 using 1.0N

NaOH, solidified using 0.4% Phytagel™ (Sigma) and autoclaved for 20 minutes at 121°C, 104 kilopascals.

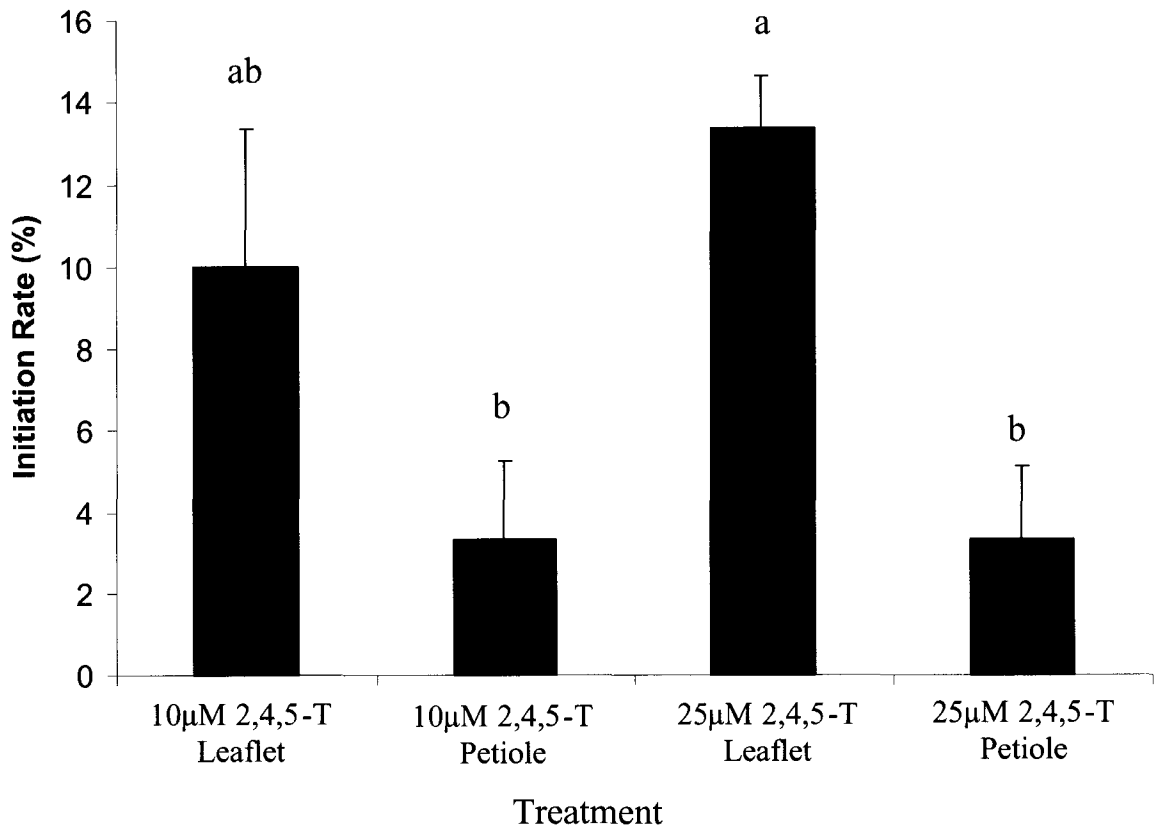
After eight weeks on maturation medium, the cultures were placed into cold storage (4°C) in the dark for eight weeks. The embryos were then individually transferred onto germination medium consisting of PGR-free MS medium. After eight weeks the plantlets were acclimatized and transferred *ex vitro* as described in chapter two.

3.4 RESULTS

3.4.1 Frequency Rates

There was no significant difference among the treatments in regards to callus induction rates after four weeks on callus induction medium (P=0.376, F=1.18). The callus induction rates averaged 98.7 ± 1.5%. Rhizogenic activity, in the form of aerial hairy root formation, was observed on calli in four of the treatments. The rate of root formation varied significantly amongst the four treatments with leaflets cultured on 10μM 2,4,5-T MS medium having the highest rate (24.6 ± 1.6%) (P=0.00, F=73.50). Leaflets grown on 25μM 2,4,5-T MS medium had an aerial root formation rate (17.2 ± 0.8%) significantly greater than that of the treatments involving petiole explants at 10μM and 25μM 2,4,5-T (6.1 ± 2.6% and 4.1 ± 2.4%, respectively). The rate of embryogenic tissue initiation was significantly different amongst the treatments with 25μM 2,4,5-T yielding the highest initiation rate (at 13.3%) (P=0.007, F=8.69) (Figure 45).

Figure 45. Embryogenic tissue initiation rates after four weeks growth on Embryogenic tissue initiation media containing 40% the original concentration of 2,4,5-trichlorophenoxyacetic acid as employed in the callus induction media. Ten replicates per treatment; experiment repeated thrice. Treatments with the same letter denote no significant difference.


16
14
12
10
8
6
4
2
0
Initiation Rate (%)
ab
b
a
b
10μM 2,4,5-T Leaflet
10μM 2,4,5-T Petiole
25μM 2,4,5-T Leaflet
25μM 2,4,5-T Petiole
Treatment

3.4.2 Tissue Selection for Maturation: Nodular versus Non-Nodular

The number of somatic embryos produced from nodular tissue was significantly greater than that from non-nodular tissue per 0.25 grams of embryogenic tissue (P=0.0304, W=26.0) (Figure 46). The mean number of somatic embryos produced per tissue type varied amongst the treatments with nodular tissue ranging from 13.33 to 109. Non-nodular tissue resulted in zero to 1.33 somatic embryos.

3.4.3 Multi-Cultivar Study

By week four on callus induction media, all of the explants had developed callus. The callus induction rates were similar in all treatments and all cultivars averaging 97 ± 4%. The calli produced in 'Graham Thomas', 'Morden Sunrise', 'Easy Going' and 'Red Meidiland' were dark brown on media supplemented with 2,4-D and 2,4,5-T. The calli produced by the cultivar 'William Baffin' was pale yellow on both media. 'Betty Prior' and 'John Franklin' both produced light beige mucilaginous callus. Although the quantity of callus was not measured, there was notably less callus per explant for cultivar 'John Franklin' than the other cultivars. Rhizogenesis, in the form of aerial roots, occurred on the callus of the cultivars 'Betty Prior', 'Easy Going' and 'Morden Sunrise' (Figure 47). Rhizogenesis was only observed in the 2,4-D supplemented media.

Five of the eight cultivars produced embryogenic tissue in response to the 2,4,5-T treatment whereas only two responded to 2,4-D (Table 7). Two cultivars produced both embryogenic tissue and somatic embryos: 'Graham Thomas' on medium containing 2,4,5-T and 'William Baffin' on medium containing 2,4-D (Figure 48). The presumptive

Figure 46. Mean number of somatic embryos produced by 0.25grams of nodular presumptive embryogenic tissue versus non-nodular after eight weeks on maturation medium. Six replicates per treatment; experiment repeated thrice.

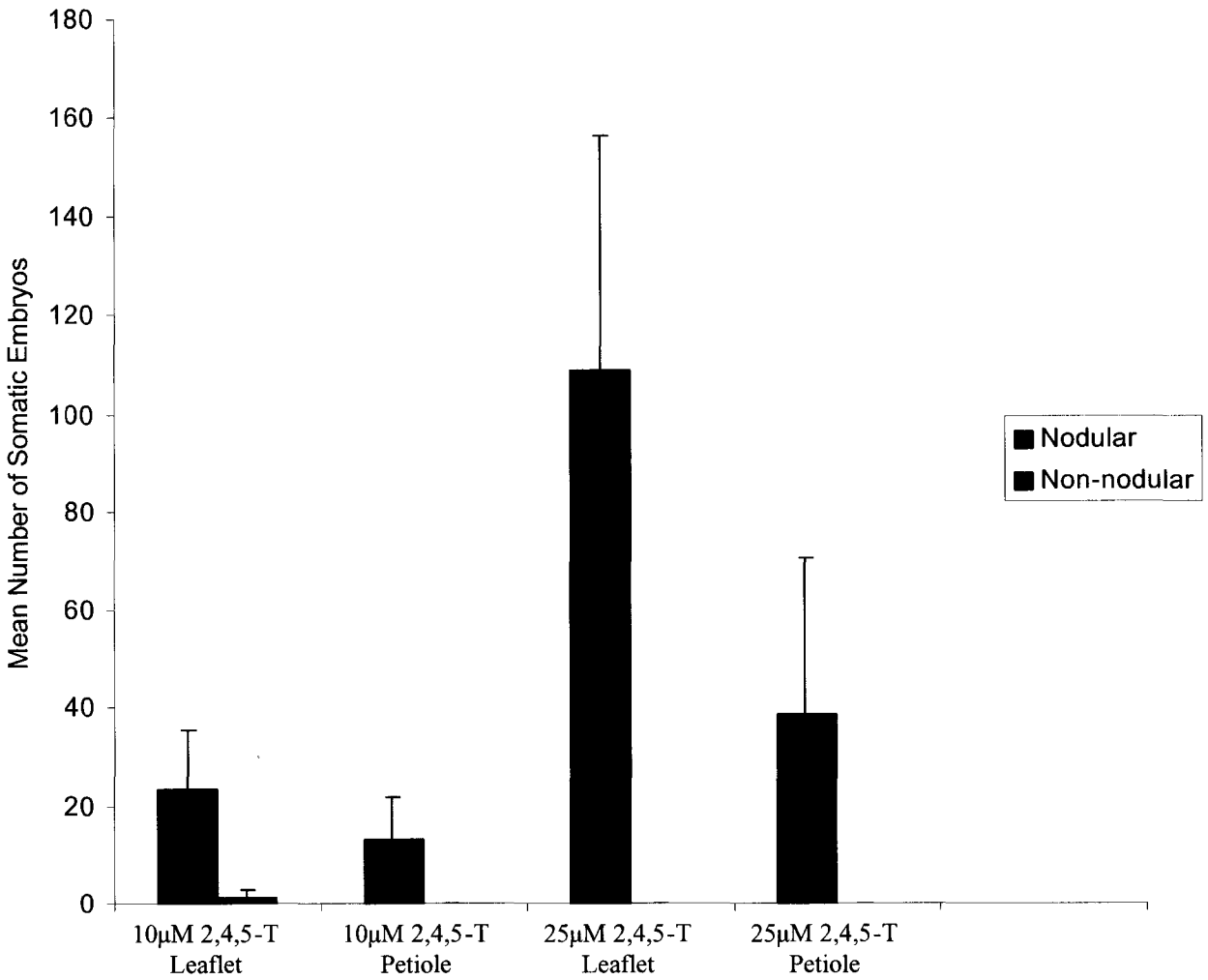

180
160
140
120
100
80
60
40
20
0
Mean Number of Somatic Embryos
Nodular
Non-nodular
10µM 2,4,5-T Leaflet
10µM 2,4,5-T Petiole
25µM 2,4,5-T Leaflet
25µM 2,4,5-T Petiole

Figure 47. Aerial root formation on isolated presumptive embryogenic tissue. Bar 1mm.

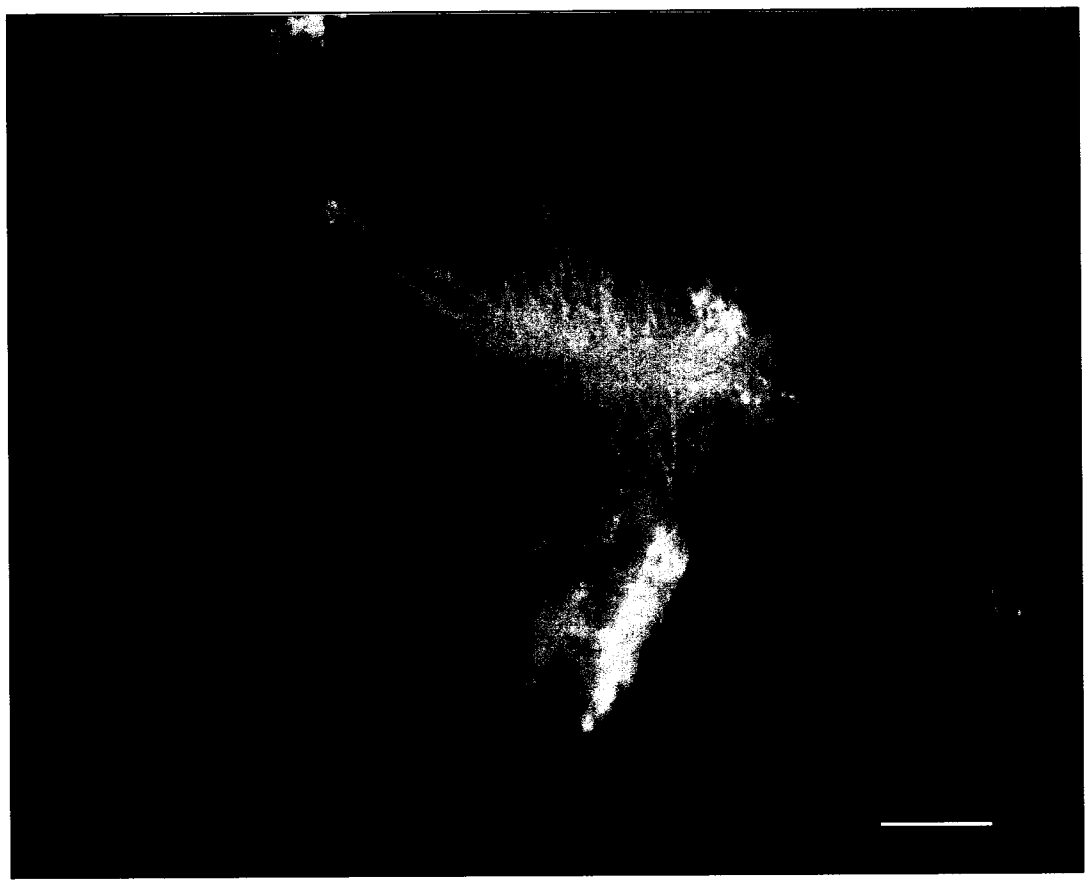


Table 7. Embryogenic responses of various rose cultivars (*Rosa* sp.) to Murashige and Skoog medium containing either 10μM 2,4,5-trichlorophenoxyacetic acid or 10μM 2,4-dichlorophenoxyacetic acid. (***2,4,5-T*** 2,4,5-trichlorophenoxyacetic acid, ***2,4-D*** 2,4-dichlorophenoxyacetic acid, ***ET*** embryogenic tissue, ***se*** somatic embryo).

Cultivar	**ET Initiation Rate (%)**	
	10μM 2,4,5-T	**10μM 2,4-D**
'Betty Prior'	+	+
'Easy Going'	-	-
'Graham Thomas'	+ (se present)	-
'John Franklin'	+	-
'Morden Sunrise'	-	-
'Red Meidiland'	-	-
'*Rosa rugosa* Alba'	+	-
'William Baffin'	+	+ (se present)

Figure 48. Somatic embryos of rose cultivar 'Graham Thomas'. Bar 1mm.

embryogenic tissue (PET) isolated from 'Red Meidiland' was dark brown after four weeks on ET initiation medium. Nodular tissue was not observed in either auxin treatment for this cultivar.

3.4.4 Recurrent Somatic Embryogenesis

In cultivar 'Livin' Easy', a mean of 295.8 ± 91.9 cotyledonary somatic embryos were produced per 0.25g of embryogenic tissue when placed onto maturation medium. The embryos were capable of germinating into plantlets at a rate of 35.9 ± 9.5%. All of the somatic embryos that failed to germinate had secondary SE occurring as nodular tissue.

3.5 DISCUSSION

3.5.1 Frequency Rates of SE Induction in 'Livin' Easy'

The highest rate of embryogenic tissue initiation obtained in this study was in leaflet explants cultured on MS medium supplemented with 25μM 2,4,5-T (13.3%). This rate is about ⅓ of the maximum reported but higher than frequencies reported in many previous studies (Table 8).

3.5.2 Tissue Selection for Maturation

Nodular tissue produced a significantly greater number of somatic embryos than non-nodular tissue. The nodular tissue is more advanced in terms of embryo development and readily identifiable from callus. Although the non-nodular tissue did not produce as many embryos, it did produce some somatic embryos, indicating it was capable of embryogenesis. Further studies to determine if non-nodular tissue is a phase preceding

Table 8. Highest embryogenic tissue initiation rates in previous studies of SE in *Rosa* sp.

Reference	Cultivar	Explant Type	ET Initiation Rate (%)
Kim *et al.*,2003b	*R. hybrida* L. cv. '4th of July'	*in vitro* leaves	24.4
	R. hybrida L. cv. 'Tournament of Roses'	*in vitro* leaves	0
	R. hybrida L. cv. 'Graham Thomas'	*in vitro* leaves	0
	R. hybrida L. cv. 'Sequoia Ruby'	*in vitro* leaves	0
	R. chinensis cv. 'Old Blush'		
Li *et al.*, 2002	*R. hybrida* L. cv. 'Carefree Beauty'	*in vitro* leaves	31.6
	R. hybrida L. cv. 'Grand Gala'	*in vitro* leaves	3.3
	R. chinensis cv. 'Red Sunblaze'	*in vitro* leaves	6.6
Visessuwan *et al.*, 1997	*R. hybrida* L. cv. 'Carl Red'	*in vitro* leaves	13.3
	R. canina	*in vitro* leaves	3.3
Hsia & Korban, 1996	*R. hybrida* L. cv. 'Carefree Beauty'	*in vitro* leaves & stems	25
	R. chinensis cv. 'Red Sunblaze'	*in vitro* leaves & stems	25
	R. chinensis cv. 'Baby Katie'	*in vitro* leaves & stems	10
Murali *et al.*, 1996	*R. hybrida* L. cv. 'Arizona'	petals	3.4
van der Salm *et al.*, 1996	*R. hybrida* L. cv. 'Moneyway'	roots	16
Kunitake *et al.*, 1993	*Rosa rugosa* Thunb.	immature seeds	5.9
Noriega & Söndahl, 1991	*R. hybrida* L. cv, 'Royalty'	filaments	14.2
de Wit *et al.*, 1990	*Rosa* sp. 'Domingo'	*in vitro* leaves	5.8
	Rosa sp. 'Vickey Brown'	*in vitro* leaves	3.0

nodular tissue were formation or a separate entity would provide useful information on the transition from callus to embryogenic tissue in rose SE.

3.5.3 Multi-Cultivar Study

The differences observed in color and texture of callus derived from the various cultivars are similar to previous studies in which variation in callus morphology and color have been reported (review by Roberts *et al.*,1995). Callus morphology in rose also differs with growth regulator treatments (Kim *et al.*, 2003b) and with explant type (Li *et al*, 1995). Although there are initial differences in callus morphology, a transition to pale, nodular, mucilaginous tissue seems to be necessary before somatic embryos are developed (Roberts *et al.*, 1995).

Five out of eight cultivars responded to 2,4,5-T while only two cultivars responded to 2,4-D. These findings support the premise that individual genotypes within a given species vary greatly in terms of the conditions required for embryogenic induction (Kim *et al.* 2003b; von Arnold *et al.*, 2002; Kintzios *et al.*, 1999; Hsia & Korban, 1996; Marchant *et al.*, 1996; de Wit *et al.*, 1990). In a study of 22 different rose cultivars, for their ability to undergo somatic embryogenesis, Murali *et al.* (1996) found that only two responded. Similarly, Kim *et al.*, (2003b) reported only one of the five cultivars successfully underwent somatic embryogenesis even though callus induction ranged from 55-100%.

The findings of the present study demonstrate that 2,4,5-T may be very useful for

inducing SE in cultivars other than 'Livin' Easy'. They also suggest that 2,4,5-T may induce SE in rose cultivars unresponsive to 2,4-D. Although 2,4-D is commonly employed to induce SE in roses, there are many rose cultivars shown recalcitrant to induction by 2,4-D (Li *et al.*, 2002; Murali *et al.*, 1996).

The successful induction of somatic embryogenesis in the cultivar 'Graham Thomas' contrasts a previous study in which SE could not be induced in this cultivar using a combination of 2,4-D and zeatin (Kim *et al.*, 2003b). The lack of embryogenic tissue production in the cultivar 'Easy Going' was somewhat unexpected, as this cultivar is reported to be a sport of 'Livin' Easy'. Due to the genetic similarity between the two cultivars, it was anticipated 'Easy Going' would respond similarly to 'Livin' Easy'. This finding needs further study but demonstrates the stringency of genotype specificity governing rose SE.

3.5.4 Recurrent Somatic Embryogenesis

This is the first report of recurrent somatic embryogenesis in rose and there are no previous studies with which to compare results. The embryogenic tissue produced at the basal ends of regenerated plantlets was capable of producing somatic embryos after being placed through a maturation treatment. The mean number of somatic embryos produced per 0.25grams of tissue was much greater than those obtained from SE of the original explants (refer to chapter two). An increase in embryogenic potential was also reported in recurrent SE of sunflower (*Helianthus maximiliani* Schrader) although reasons for this increase were unknown (Vasic *et al.*, 2001). The somatic embryos produced via

recurrent SE in sunflower were capable of subsequent germination but at a rate much lower than the initial cycle of SE. The high frequency of secondary SE occurring on the somatic embryos may have hindered embryo development. A low conversion rate of somatic embryos from recurrent cycles has been found in previous studies (Raemakers *et al.*, 1995). Although the germination rates in the present study were low (35.8%) compared to initial rates (up to 95.2%), they were high compared to germination rates of embryos derived via indirect SE in previous studies of rose (refer to Table 6 in chapter two). The fact that regeneration of plants occurred from recurrent SE-derived embryos demonstrates a valid potential of recurrent SE to have cost and time advantages in commercial applications.

4. SOMATIC EMBRYO MORPHOLOGY VARIABILITY STUDY

4.1 INTRODUCTION

A critical aspect in the regeneration of roses via somatic embryogenesis is the further development and germination of the somatic embryo (Dohm *et al.*, 2001). Although large numbers of somatic embryos can be obtained rapidly through somatic embryogenesis, the subsequent plants, which are often the desired commercial product, can be difficult to obtain from these embryos. Somatic embryos produced via somatic embryogenesis (SE) may exhibit structural abnormalities that affect their subsequent germination and conversion into plants (Lazerri *et al.*, 1987; Wetzstein and Baker, 1993). Low conversion rates of somatic embryos, in addition to hampering propagation, may also limit their use in synthetic seed technology (Padmanabhan *et al.*, 1998). To achieve high conversion rates, manual or machine sorting of embryos may be necessary to select embryos capable of regenerating plants (Barry-Etienne *et al.*, 2002; Ibaraki, 1999). The sorting and selecting of high quality embryos may also become imperative for the application of automation in somatic embryogenesis (Padmanabhan *et al.*, 1998). One factor currently hindering the application of automation in somatic embryo selection is the uncertainty as to which to embryos have high conversion potential (Ibaraki and Kurata, 2001).

Stuart and colleagues (1985) cited morphological features to be identifiable markers of conversion-competent embryos. Morphological abnormalities in somatic embryos have been reported in roses (Viessuwan *et al.*, 1997; Murali *et al.*, 1996). A normal rose somatic embryo is bipolar with a distinct hypocotyl, radical and two

cotyledons. Abnormal embryos consisting of cotyledons that are fused to form a cup-like structure have been observed (Viessuwan *et al.*, 1997; Murali *et al.*, 1996). These abnormal embryos reportedly fail to germinate. In addition to abnormal cotyledon shape, variation in the cotyledon number in somatic embryos has been reported (Viessuwan *et al.*, 1997; Arene *et al.*, 1993).

4.2 OBJECTIVES

The aim of this study was two-fold: 1) To characterize the morphological heterogeneity of *Rosa hybrida* L. cv. 'Livin Easy' somatic embryos produced using the somatic embryogenic system found optimal from chapter two and 2) To study the impact of the heterogeneity observed in somatic embryos on the conversion into plantlets and subsequent plant development. To characterize the somatic embryos, a morphological and histological comparison was conducted of the various morphological types resulting from the somatic embryogenesis of *Rosa hybrida* L. cultivar 'Livin' Easy'.

4.3 MATERIAL AND METHODS

4.3.1 Production of Somatic Embryos

Leaflet explants (5-7 mm in length) were aseptically excised from the previously established *in vitro* shoot cultures of 'Livin' Easy' (*Rosa hybrida* L.). Four scratches, perpendicular to the midrib, were made on the underside of each leaflet using a sterilized scalpel. The wounded leaflets were placed, underside up, onto MS (Murashige & Skoog, 1962) medium modified as per Owen & Miller (1992). The medium was supplemented

with either 10μM or 25μM 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Sigma T-5785). Medium was adjusted to pH 5.7 using 1.0N NaOH, solidified using 0.4% Phytagel™ (Sigma) and autoclaved for 20 minutes at 121°C, 104 kilopascals.

After four weeks, the explants and resulting callus were transferred to embryogenic tissue (ET) initiation media consisting of MS medium supplemented with 40% the concentration of 2,4,5-T (4 μM or 10μM). Presumptive embryogenic tissue (PET) was isolated from the calli/explants and transferred onto fresh media of the same composition once a week for four weeks. The isolated PET was transferred to fresh medium, of the same composition, after four weeks.

Eight weeks after being isolated, 0.25grams of embryogenic tissue from each treatment was placed into each of three 250ml flasks containing 30ml liquid MS plant growth regulator-free medium. The cultures were shaken for 24 hours at 110 rpm on a rotary shaker (G10 Gyrotory® Shaker, New Brunswick Scientific, N.J., U.S.A). Three 10ml aliquots from each flask were vacuum filtered onto Whatman #1 filter paper and placed onto a MS maturation medium containing 7.57μM ±-cis, trans-Abscisic acid (ABA) (Sigma A-1049). Medium was adjusted to pH 5.7 using 1.0N NaOH, solidified using 0.4% Phytagel™ (Sigma) and autoclaved for 20 minutes at 121°C, 104 kilopascals.

After eight weeks on maturation medium, the cultures were placed into cold storage (4°C) in the dark for eight weeks. After the cold period, the somatic embryos were observed. Twenty somatic embryos from each of the identified embryo types were

randomly selected and fixed for histological analysis (see following section). All other somatic embryos were individually transferred onto germination medium consisting of PGR-free MS medium. The somatic embryos were numbered and observations were made regarding color and cotyledon number. After eight weeks, the plantlets were transplanted and acclimatized as described in chapter two.

4.3.2 Histological Procedures

After eight weeks on maturation medium, 20 somatic embryos from each morphologically different group (where numbers allowed) were fixed in 3% glutaraldehyde in 0.025M potassium phosphate buffer in glass 50mL vials equipped with screw lids. The samples were stored at 4°C until time of histological processing. One drop of erythrosine B was added to each vial and stained for 30 minutes. Dehydration was carried out using an ethanol to 95% butanol series, ethanol was replaced with a secondary solvent, butanol. Infiltration was preformed by placing the tissue into a 25% paraffin butanol solution (1.0 ml butanol and 1.0 ml 50% paraffin in butanol) for 30 minutes. Every 30 minutes afterwards for 1.5 hours, 1.0 ml of 100% paraffin was added to the vials containing the samples. After 1.5 hours, the samples were stored with the lid off overnight at 57°C. The following morning the paraffin was poured off and replaced with 100% paraffin. The samples were embedded in paraffin, sectioned at 7.0 μM using a microtome, stained with hematoxylin and mounted in permount. Serial sections of the tissue were studied and photographed using a compound microscope equipped with a digital camera.

4.3.3 Statistical Analysis

All computations were performed using the MINITAB statistical analysis package. Normally distributed data were analyzed using either ANOVA at the 5% probability level or the Two Sample T-test, as appropriate.

4.4 RESULTS

4.4.1 Somatic Embryo Morphology

Variation in the cotyledon number was observed amongst the somatic embryos (Figure 49). Cotyledon number varied from one to five in the 25μM 2,4,5-T treatment and one to eight in the 10μM 2,4,5-T treatment (Figure 50). Somatic embryos from each cotyledonary group were capable of successful germination (Table 9). Germination rates did not differ significantly amongst the cotyledonary groups (P= 0.513, F= 0.94) nor did they differ between the two 2,4,5-T concentrations (P=0.627, t=0.49). Root formation was observed occurring by week one in both treatments. By week six, 71.8 ± 7.8% of plantlets in the 25μM 2,4,5-T treatment and 40.6 ± 10.4% in the 10μM 2,4,5-T treatment had root formation. The earliest formation of shoots was observed by week three. By week six, shoot formation was observed in 41.0 ± 6.4% of plantlets in the 25μM 2,4,5-T treatment and 52.3 ± 12.6% in the 10μM 2,4,5-T treatment. The shoots did not appear to be 'true epicotyls' in that they did not originate from a primary apical meristem. Shoots seemed to arise from cotyledon axillary meristems located at the junction where the hypocotyl meets the cotyledon (Figure 51). Shoots also formed adventitiously on the hypocotyls (Figure 52). The 10μM 2,4,5-T treatment produced a significantly higher number of adventitious shoots per embryo (5.6 ± 2.5%) than the 25μM 2,4,5-T treatment (1.9 ± 0.9%) (P=0.000,t=-5.93). Adventitious shoot number did not differ significantly

Figure 49. Morphologically different somatic embryos. Bar 1mm.

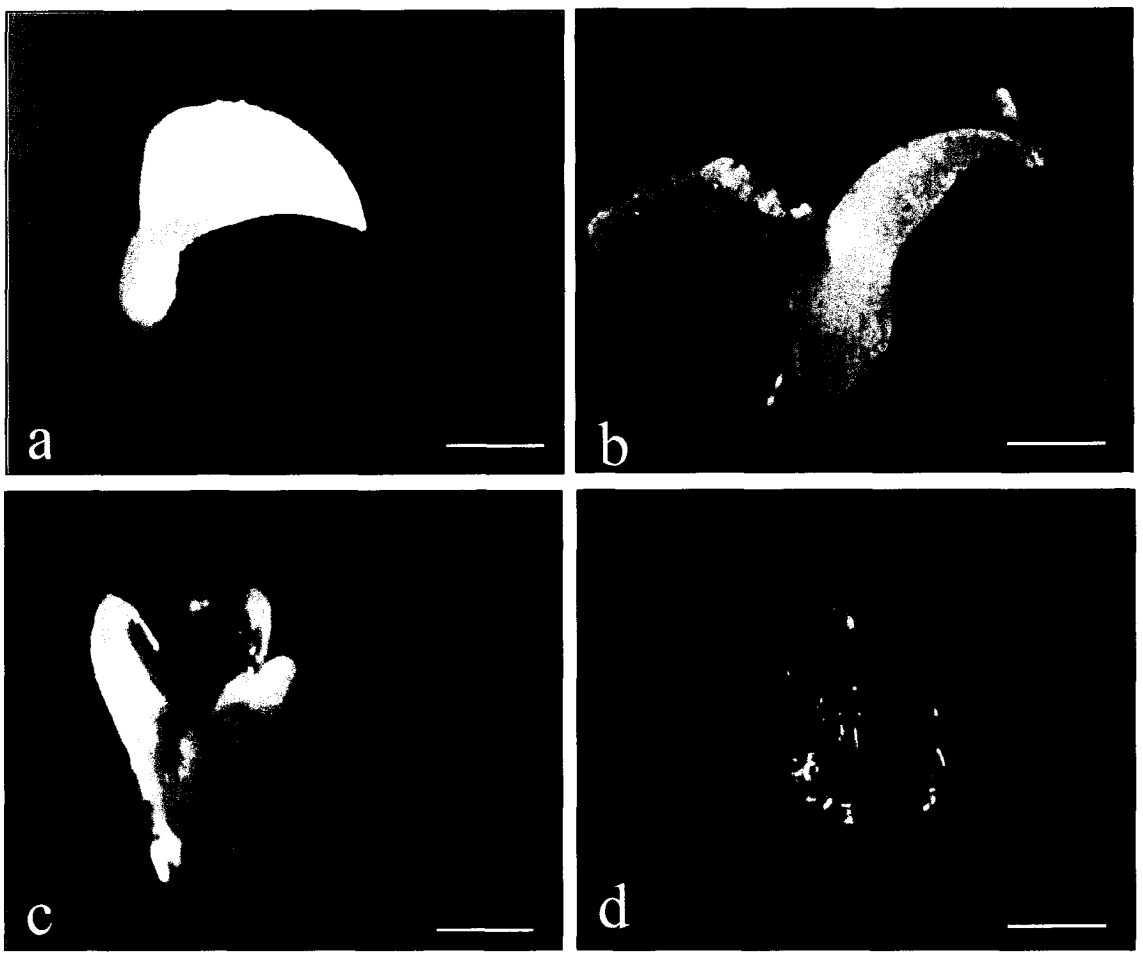

a
b
c
d

Figure 50. Mean frequency rates of somatic embryos possessing varying numbers of cotyledons. Experiment repeated thrice.

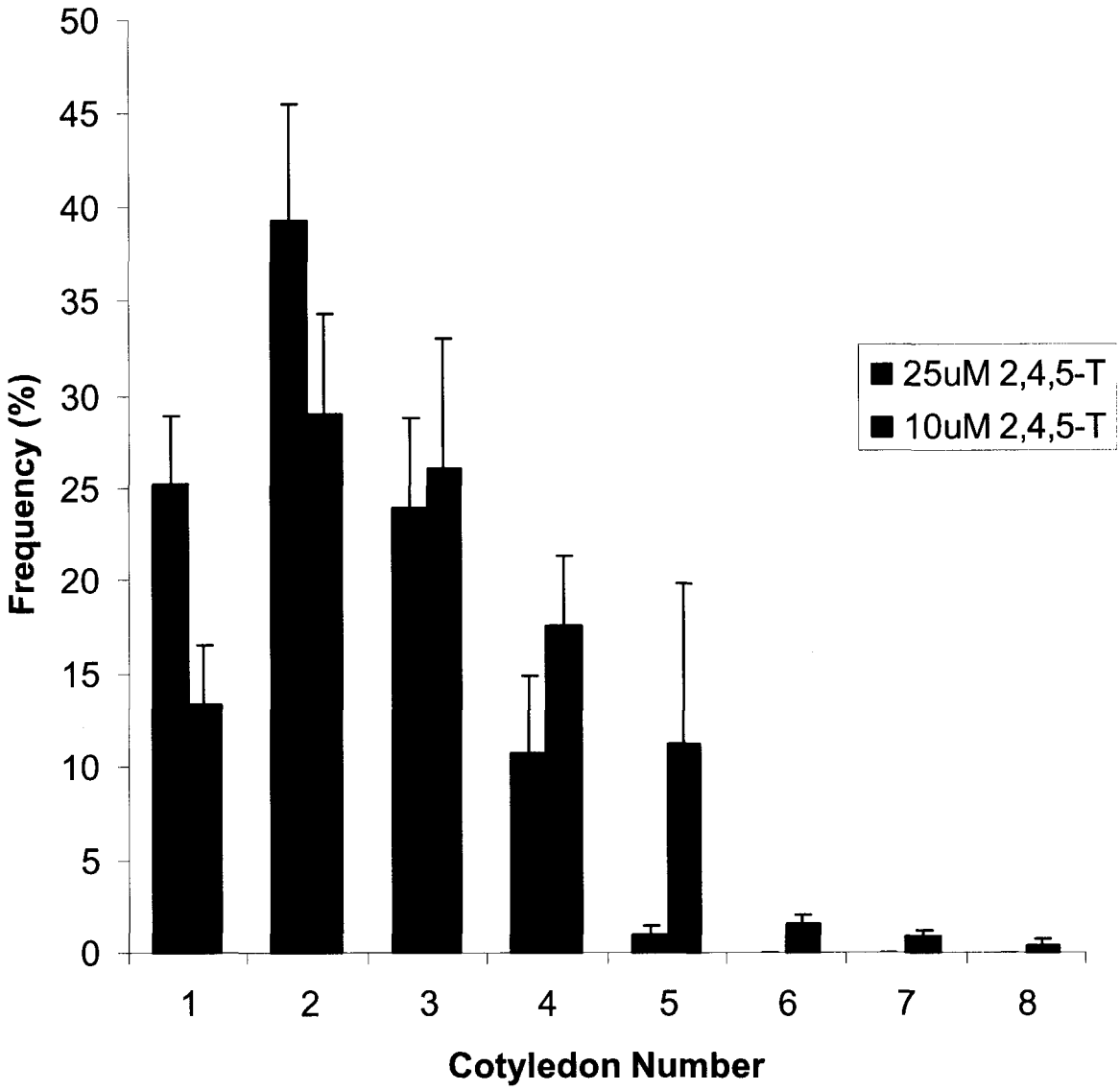
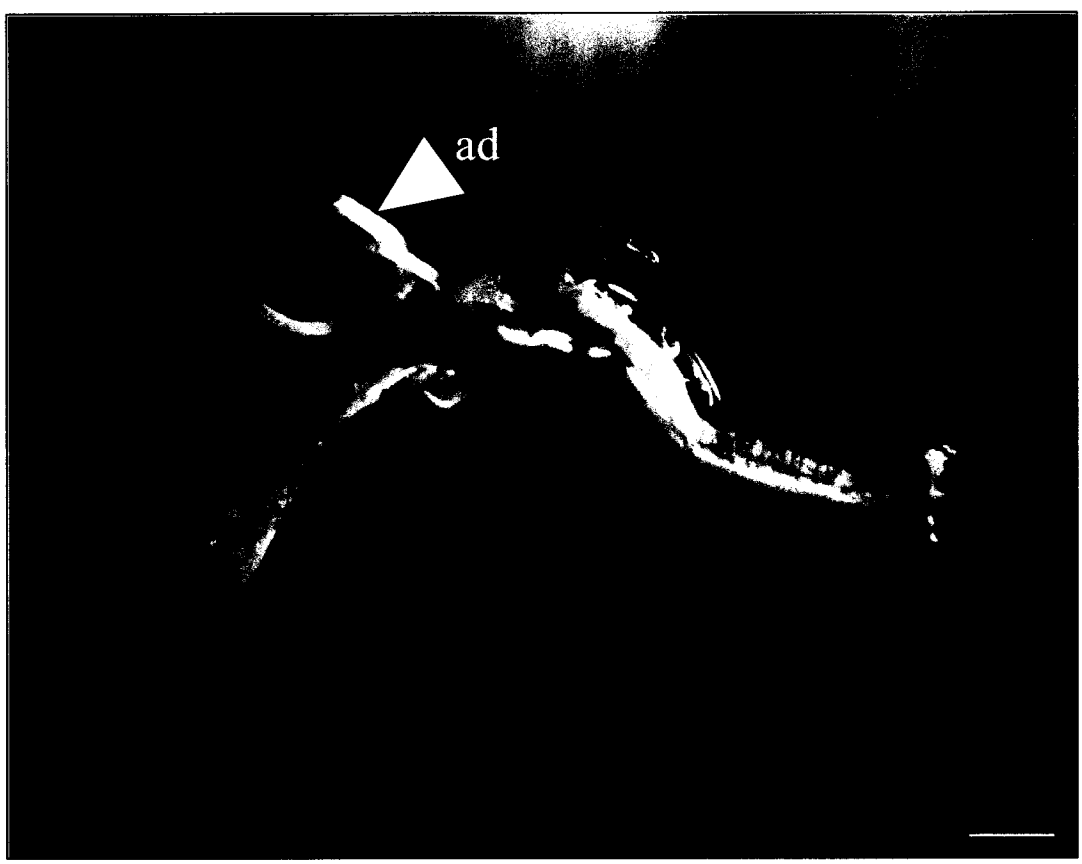

50
45
40
35
30
25
20
15
10
5
0
Frequency (%)
25uM 2,4,5-T
10uM 2,4,5-T
1
2
3
4
5
6
7
8
Cotyledon Number

Table 9. Mean Germination rates and mean percent (and standard deviations) of plantlets transplanted to soil after six weeks growth on Murashige and Skoog germination medium. Mean survival rates of transplanted plantlets after three months growth *ex vitro* (***2,4,5-T*** 2,4,5-trichlorophenoxyacetic acid, ***na*** not applicable).

2,4,5-T (μM)	Cotyledon #	Mean Percent Germination (± S.D.)	Mean Percent Transplanted (± S.D.)	Mean Survival Rate (%) (± S.D.)
25	1	98.0 ± 3.4	44.3 ± 5.2	87.5 ± 11.7
25	2	95 ± 5.0	58.6 ± 14.6	78.1 ± 11.6
25	3	90.2 ± 8.6	63.9 ± 20.2	81.7 ± 8.2
25	4	100	38.9 ± 17.6	81.7 ± 2.4
25	5	100	0	na
10	1	93.5 ± 6.3	40.6 ± 15.4	62.1 ± 13.5
10	2	94.9 ± 8.9	37.3 ± 7.8	69.6 ± 6.4
10	3	95.3 ± 5.0	59.1 ± 15.7	69.2 ± 20.6
10	4	91.7 ± 14.4	36.0 ± 11.4	77.5 ±3.54
10	5	97.8 ± 3.9	69.1 ± 17.0	55.6 ± 10.9
10	6	75 ± 15.4	75 ± 10.3	58.5 ± 12.0
10	7	100	50	54.5 ± 3.7
10	8	100	100	100

Figure 51. Plantlet with shoot formation. Bar 1cm.

Figure 52. Plantlet with adventitious shoot formation (ad). Bar 1cm.


ad

amongst the cotyledonary groups in either treatment (P=0.236, F=1.74 for 25μM 2,4,5-T; P=0.072, F=2.73 for 10μM 2,4,5-T). Abnormal germinants were observed in 9.3 ± 2.1% of the plantlets (Figure 53). The occurrence of abnormal germinants did not differ significantly amongst the cotyledonary groups (P= 0.375, F= 1.09) nor did they differ between the two 2,4,5-T concentrations (P=0.089, t=-1.78).

4.4.2 Anatomy of Somatic Embryos

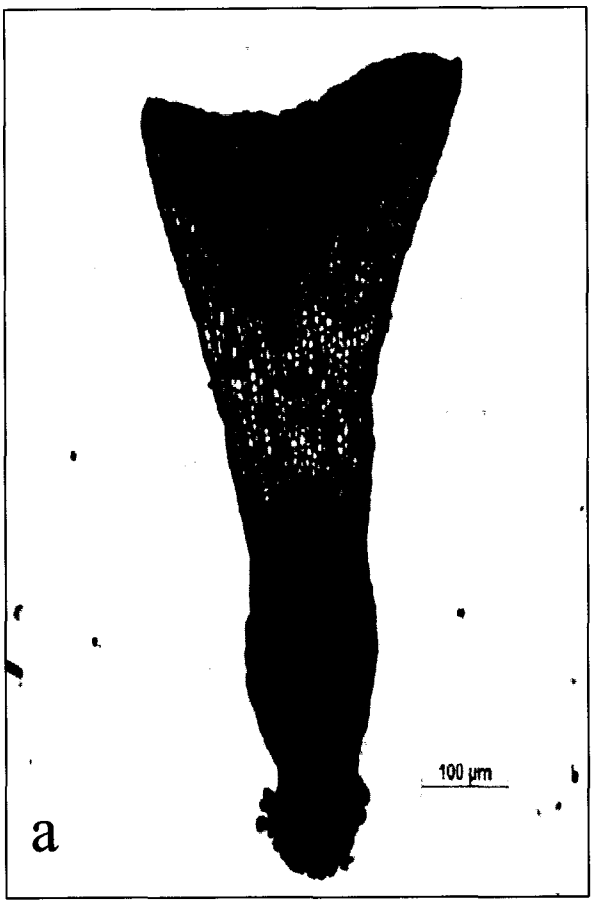
Examination of serial histological sections revealed the somatic embryos had well defined cotyledons, albeit varying in number. Differentiation of the procambium tissue was evident in the central region of many of the somatic embryos (Figures 54-57). The procambium furcated into the cotyledons, even in somatic embryos possessing a number of cotyledons other than the 'normal' two. The radical and hypocotyls were well developed, especially notable in somatic embryos possessing two cotyledons (Figure 55). Observation of the serial histological sections showed a lack of shoot apical meristems in all somatic embryos observed. There were groups of cells located at the base of cotyledons that appeared to remain meristematic activity; evident by their small size, abundant number and unspecialized nature (Figure 54b and c).

4.5 DISCUSSION

It has been reported that the germination frequency of somatic embryos depends largely on morphology (Lee *et al.*, 2001). *Rosa hybrida* L. cultivar 'Arizona' somatic embryos possessing single, concrescent (cup shaped) or two or more fused cotyledons did not germinate (Murali *et al.*,1996). Similarly, *Rosa hybrida* L. and *Rosa canina* embryos

Figure 53. Abnormal germinant. Bar 1cm.

Figure 54. Anatomy of somatic embryos possessing a single cotyledon. Note procambium development (pc), well developed radical and lack of shoot apical meristem.


100 µm
a

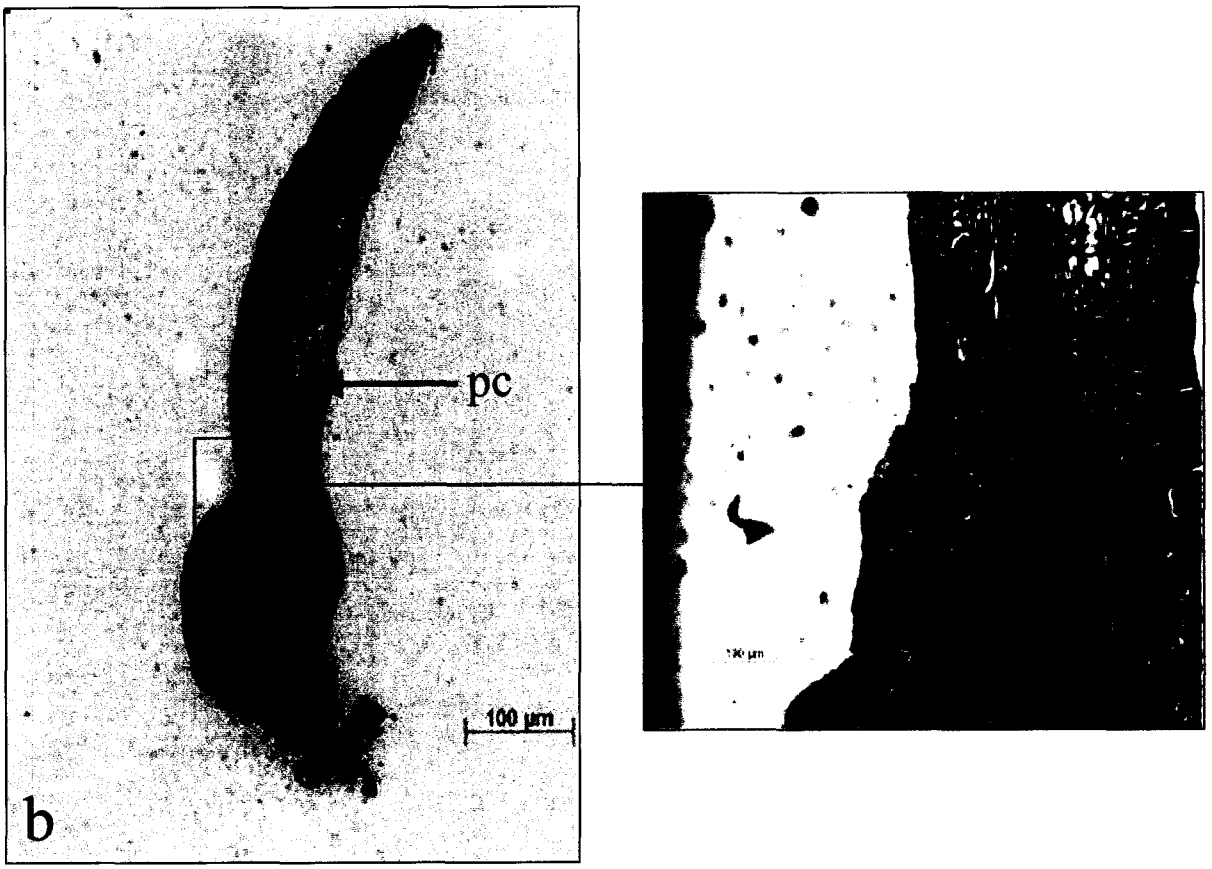

pc
100 µm
b

Figure 55. Anatomy of somatic embryos possessing two cotyledons. Note procambium development (pc), well developed radical and lack of shoot apical meristem.

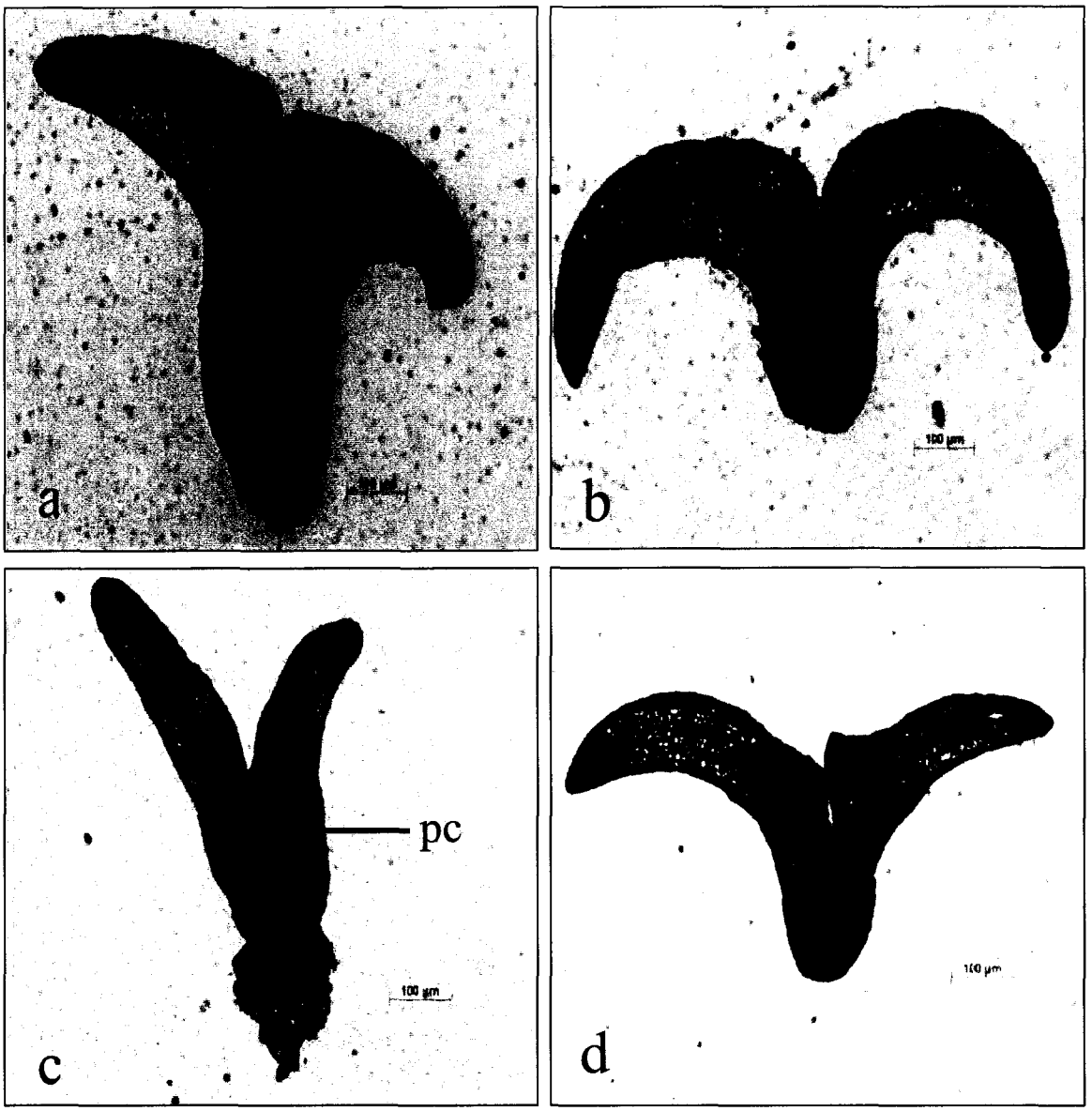

a
b
100 µm
c
pc
100 µm
d
100 µm

Figure 56. Anatomy of somatic embryos possessing three cotyledons. Note procambium development (pc), well developed radical and lack of shoot apical meristem.

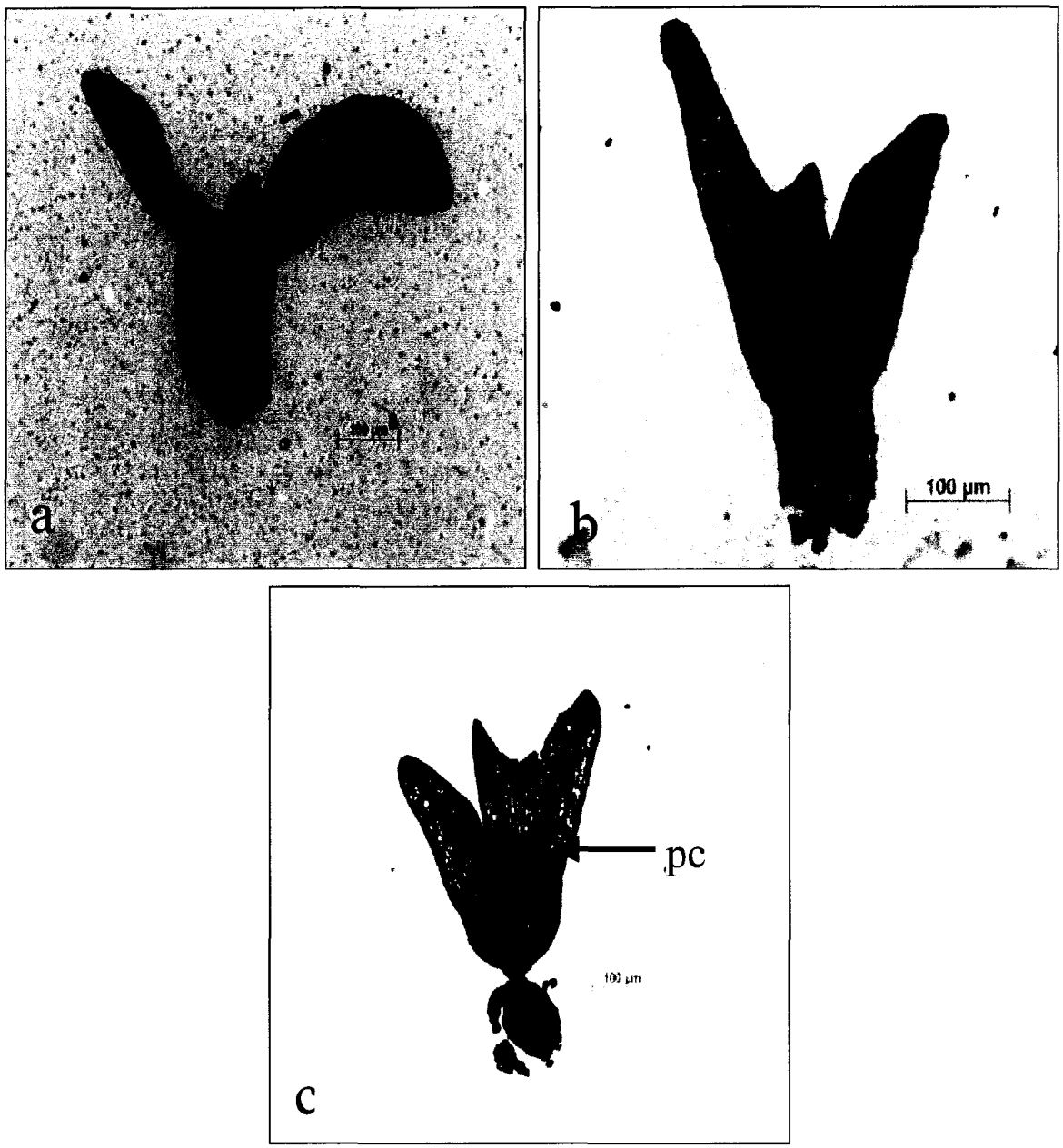
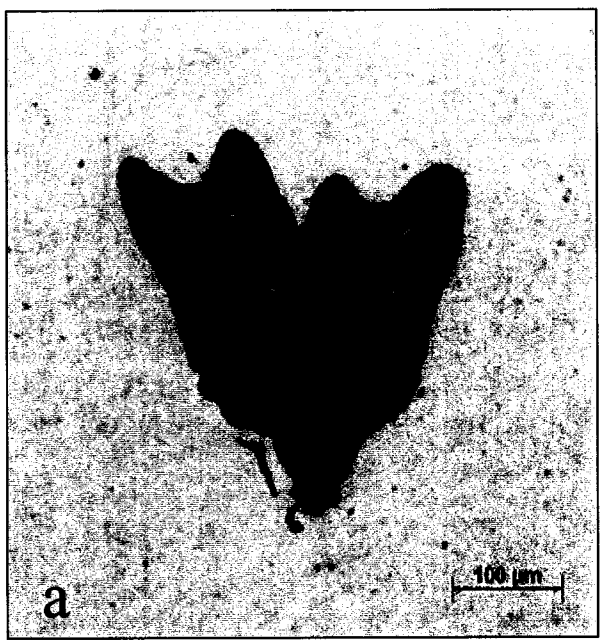
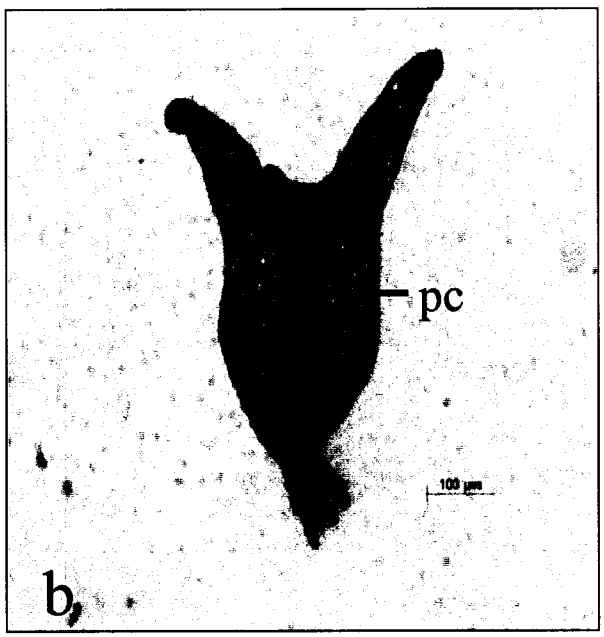
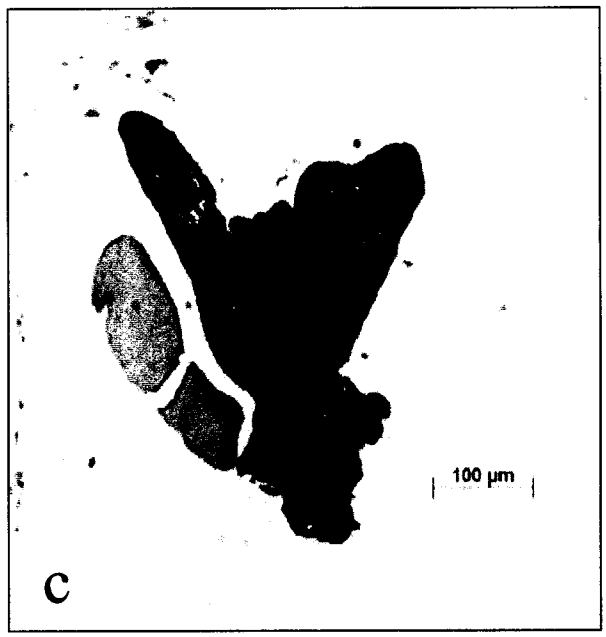

a
b
100 µm
pc
c

Figure 57. Anatomy of somatic embryos possessing various cotyledons a) four cotyledons b) five cotyledons c) six cotyledons. Note procambium tissue differentiation (pc), well developed radical, and lack of shoot apical meristem.


a
100 µm


pc
100 µm
b


100 µm
c

possessing single cotyledons failed to germinate while embryos possessing three and four cotyledons were capable of germination but at very low frequencies (less than 1%) (Viessuwan *et al.*, 1997). Contrary to previous studies, in the present study there was no significant difference in germination rates amongst somatic embryos with varying numbers of cotyledons. Due to this lack of difference amongst germination rates, cotyledon number would not be a useful indication of germination potential as it was in previous studies with carrot (*Daucus carota*) (Lee *et al.*, 2001). Although germination frequency varied with the number of cotyledons in carrot, changing the culture conditions, by starving the somatic embryos, resulted in increased germination rates regardless of embryo morphology (Lee *et al.*, 2001). Lee *et al.* (2001) did not speculate as to how the stress caused by lower levels of nutrients and reduced humidity levels enabled the morphologically diverse somatic embryos to germinate. The findings by Lee *et al.* (2001) provide evidence that even somatic embryos with abnormal cotyledon numbers are capable of germinating at rates similar to normal embryos when specific culture conditions are provided. Similarly, Chengalrayan *et al.* (1997) reported that morphologically abnormal peanut somatic embryos (*Arachis hypogaea*) had high conversion and survival rates in a particular soil type. Although cotyledon number may vary, these findings show the somatic embryos are still physically and genetically equipped to germinate successfully.

Although the somatic embryos in the present study successfully germinated, they did not follow the normal path of zygotic embryogenesis in that shoots arose from axillary meristems. The initial shoot observed upon germination of a zygotic embryo

(called an epicotyl) is formed from the apical meristem; a region located between the cotyledons in which cellular division actively occurs. In the present study, somatic embryos lacked shoot apical meristems. The presence of auxins in medium can lead to poorly developed apical meristems and correspondingly low conversion rates (Merkle *et al.*, 1990). Kunitake and associates (1993) reported difficulty obtaining normal plantlets from somatic embryos of *Rosa rugosa* Thunb. due to a lack of apical meristem formation. It has been reported that without an apical meristem, the embryo is incapable of germinating (Dodeman *et al.*, 1997; Kong & Yeung, 1992). Contradictory to the previous study, Visessuwan *et al.* (1997) reported the germination of rose somatic embryos lacking apical meristems in which normal shoot formation was observed from masses of leaf primordial without apical meristems. These findings are similar to those found in the present study. The observation of shoots originating from the cotyledons and hypocotyls of embryos is also similar to that found in buttercups (*Ranunculus sceleratus)* in which epidermal cells of the hypocotyls failed to differentiate and thus retained meristematic capabilities (Konar *et al.*, 1972).

Root formation occurred after as early as one week while shoot production was delayed. This delay was mostly likely due to the absence of shoot apical meristems (SAMs) and the additional time necessary for adventitious shoots to develop. It is possible that once root development occurred, the roots produced growth regulators (most likely cytokinins). The root-synthesized cytokinins, once transported to the opposite pole, likely enhanced shoot formation at regions where cells had retained meristematic activity. Cytokinins, as discussed in chapter one, are synthesized in plant roots and

translocated to the shoot regions where they stimulate shoot development (Arteca, 1996). The accumulation of root-derived cytokinins are key in shoot initiation and regulating the balance between root and shoot growth (Bangerth *et al.*, 2000; Peres & Kerbauy, 1999). High levels of cytokinins, relative to auxin, are known to result in the formation of shoots (Skoog & Miller, 1965). In addition to stimulating cellular division, cytokinins release plants from apical dominance induced by auxins (Taiz & Zeiger, 1998). In the present study the somatic embryos did not possess SAMs, therefore auxin production could not occur in these regions. In addition, the germination medium was PGR-free, therefore the cytokinin to auxin ratio may have been high since cytokinins were being produced in the roots. Sarasan *et al.* (2001) observed that growth at one pole of rose somatic embryos (*Rosa* Hertiage x Alister Gray) promoted the development at the alternate pole. Sarasan *et al.* (2001) also attributed this growth promotion to the beneficial effect of "hormones" produced at the more advanced end.

This study provides evidence helpful in resolving the controversial debate of whether cotyledons are formed from SAMs or if the SAM and cotyledons arise independently (Bowman & Eshed, 2000). The present findings support the latter view as cotyledon development occurred despite a lack of SAM formation.

Although plants were eventually obtained despite a delay in shoot formation, this may still have disadvantages in terms of commercial applications. A longer culture time necessary for delayed shoot development equates to a higher production cost per plant. In addition, simultaneous bipolar germination is also preferred in artificial seed

technology (Sarasan *et al.*, 2001). Future studies are recommended to address this phenomenon of delay in shoot development. The delay in shoot production may be reduced or eliminated by the addition of cytokinin to the medium, most likely at the ET Initiation stage since it is known that apical meristem formation occurs early in development (von Arnold *et al.*, 2002). During the globular embryo stage, the apical domain of the embryo becomes partitioned into central cells that form the shoot meristem and the lateral cotyledonary primordial (Long *et al.*, 1996). Visessuwan *et al.* (1997) reported the formation of shoots from abnormal somatic embryos was enhanced by the addition of the cytokinin 6-benzylaminopurine to the medium. Sarasan *et al.* (2001) reported the inclusion of methyl laurate and 6-benzyladenine enhanced the bipolar germination of somatic embryos in *Rosa* Heritage x Alister Stella Gray (from less than 20% to 56.5%).

5. GENERAL CONCLUSIONS

This study was the first report of somatic embryogenesis in the commercially valuable rose cultivar 'Livin' Easy' (*Rosa hybrida* L.). A suitable methodology for SE of this rose cultivar was developed. Nodal explants were used to establish *in vitro* shoot cultures of 'Livin' Easy'. Leaflet and petiole explants from these shoot cultures were used as tissue sources for SE induction.

One factor hindering rose SE technology is low induction rates of embryogenic tissue (Rout *et al.*, 1999; Hsia & Korban, 1996). There are also several rose cultivars unresponsive to methods previously employed to induce somatic embryogenesis (Kintzios *et al.*, 1999; Murali *et al.*, 1996). Murashige and Skoog basal medium supported growth and promoted successful induction of embryogenic tissue when supplemented with plant growth regulators, whereas Woody Plant Medium and plant growth regulator-free medium did not. Auxin type and concentration had significant effects on rose SE. The synthetic auxin 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) induced SE over a greater concentration range than the commonly employed 2,4-dichlorophenoxyacetic acid (2,4-D). This is the first report of the utilization of 2,4,5-T for the induction of SE in rose. These findings have obvious benefits and applications for clonal propagation of this rose cultivar. These findings provide an alternative SE inducer for rose, which in turn, may substantially enhance rose SE technology.

Somatic embryo induction occurred within eight weeks when leaflets were placed on embryogenic tissue initiation medium containing 5 - 25μM 2,4,5-T, the optimal

concentration being from 10μM to 25μM. The highest frequency of SE induction (13%) occurred with leaflets cultured on MS medium supplemented with 25μM 2,4,5-T. 2,4-D resulted in successful somatic embryogenesis induction only at a concentration of 25μM.

Somatic embryo formation occurred when leaflet explants were moved to a medium which lacked 2,4,5-T or contained 40% the original concentration. On plant growth regulator-free medium a high percentage of abnormal embryos (32%) developed. Further somatic embryo development occurred after eight weeks on an abscisic acid (ABA)-containing maturation medium followed by an eight week cold period. Tissue that was nodular in structure produced a significantly greater numbers of somatic embryos than non-nodular tissue. Upon transfer to a plant growth regulator-free germination medium, somatic embryos successfully converted into plantlets at high rates up to 95%.

Plantlets growing on germination medium developed nodular embryogenic tissue on 60% of the plantlets, a phenomenon referred to as recurrent somatic embryogenesis. After transfer onto a maturation medium containing ABA, somatic embryo development was observed. Somatic embryos converted into plantlets at a rate of 35% with secondary somatic embryogenesis occurring on embryos that did not germinate to form plantlets. The occurrence of recurrent somatic embryogenesis in this study demonstrates a potential to further enhance SE systems. Recurrent SE creates a cyclic system, in which a constant supply of embryogenic tissue (ET) is produced. A cyclic system greatly reduces the costs

associated with callus and ET induction. This finding holds considerable commercial significance, especially for cultivars that have low frequency rates of induction.

The auxins 2,4,5-T and 2,4-D were also tested in MS medium at a concentration of 10μM, for their effects on leaflet explants of the rose cultivars 'Betty Prior', 'Easy Going', 'Graham Thomas', 'John Franklin', 'Morden Sunrise', 'Red Meidiland', '*Rosa rugosa* Alba' and 'William Baffin'. 2,4,5-T proved successful in inducing embryogenic induction in five out of eight rose cultivars tested. Most notable was 'Graham Thomas', a cultivar in which previous attempts at SE have failed. 2,4-D induced embryogenic induction in two out of the eight cultivars.

Low germination frequency rates are another factor currently preventing the full potential of rose SE from being realized (Sarasan *et al*, 2001; Rout *et al.*, 1999). The variability in morphology of the somatic embryos was investigated to discover what features might predict a high rate of germination. A morphological and histological study was carried out on the somatic embryos resulting from the optimal SE system developed for the cultivar 'Livin' Easy'. The most notable morphological difference among somatic embryos produced from leaflets cultured on MS medium with 10μM or 25μM 2,4,5-T was the variation in cotyledon number; cotyledon number varied from one to eight. Cotyledon number was not a suitable indicator of conversion potential as germination rates did not differ amongst the cotyledonary groups. Observations of the embryos showed that all cotyledonary types were capable of germination at similar rates. The somatic embryos showed differentiation of procambium and well-developed roots. Shoot

formation occurred from axillary meristems or adventitiously from groups of cells on the hypocotyl that retained capacity for cell division. Shoot formation occurred after root formation, presumably enhanced by root-synthesized cytokinins. Histological analysis revealed the somatic embryos, regardless of cotyledon number, lacked shoot apical meristems. The absence of shoot apical meristems explains the delay in shoot production. Despite a delay in shoot production, the plantlets were capable of further development into plants. These findings contribute to the overall knowledge of plantlet production in SE, an area in which information is scarce and in need of further research (Merkle, 1990).

The present study, aside from having many direct commercial benefits, has generated much needed knowledge and information on the various stages involved in rose SE. The overall findings from this study may prove useful in the further advancement of rose SE technology.

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7. APPENDICES

Galley Proof

7.1 Appendix A: Galley Proof

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GENE EXPRESSION DURING INDIRECT SOMATIC EMBRYOGENESIS OF PLANTS

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Somatic embryogenesis is the process by which somatic cells are induced into an embryogenic state, followed by differentiation into embryos. Somatic embryogenesis, in addition to being a method of propagation, can serve as an experimental tool for research into plant embryo development. This is a review of the current literature on *in vitro* plant somatic embryogenesis and the molecular advances made to identify genes expressed during the various stages of this process. Some factors hindering the elucidation of the molecular mechanisms underlying somatic embryogenesis are discussed.

L'embryogenèse somatique est le processus par lequel les cellules somatiques passent à l'état embryogène et se différencient en embryons. En plus de constituer une méthode de propagation, elle peut servir d'outil expérimental de recherche pour développer des embryons de plantes. Le présent document est une revue de la documentation sur l'embryogenèse somatique végétale *in vitro* et sur les progrès réalisés à l'échelle moléculaire pour identifier les gènes exprimés au cours des divers stades du processus. On examine aussi certains facteurs qui rendent difficile l'élucidation des mécanismes moléculaires de l'embryogenèse somatique.

Introduction

Plants are multicellular organisms, composed of highly organized tissues and organs. Plant development, like that of all multicellular organisms, involves the differentiation of cells such that they "acquire distinct metabolic, structural and functional properties" (Taiz & Zeiger 1998). Plant cells differentiate into either sexually reproductive cells or somatic (asexual) cells. Sexually reproductive cells are responsible for the production of a new generation; the fusion of male and female gametes involves the recombination of genetic information through meiosis. This genetic mixing results in offspring that are genetically different from the parent plants. Genetic diversity provides plants with a selective advantage helping to ensure survival of the species during environmental change.

Somatic or asexual cells have two distinct functions: 1) vegetative growth and 2) asexual reproduction. Vegetative growth involves the increase in plant cell size and number. During their lifespan, plants must continuously generate new tissues and organs, for example, to repair the damage caused by herbaceous animals. Asexual reproduction involves the production of plants genetically identical to the single parent plant, a process also known as cloning. Asexual reproduction allows plants to reproduce when the costs of sexual reproduction are too high, environmental conditions are not suitable for sexual reproduction or in cases where individuals may be isolated, in terms of distance, from other individuals. Plants are capable of asexual reproduction and regeneration of lost parts as a result of the totipotent nature of their cells. Many plant cells are totipotent, meaning that under appropriate conditions, they are able to differentiate into "the entire spectrum of cell types" found within the plant (Weigel & Jürgens 2002).

Key words: somatic embryogenesis; totipotency; plant tissue culture; gene expression

For most animal cells, the differentiation process is irreversible while plant cells have the unique ability to *de*differentiate. Upon dedifferentiating, plants can access the genetic information for the entire genome and therefore regain the undifferentiated or embryonic state. Given the sessile state of plants, this ability to "adapt their programme of differentiation and growth" is an important survival mechanism (Roberts et al. 2002). This survival mechanism is evident in a plants' ability to grow back lost parts consumed by animals.

The abilities of plants to reproduce asexually and plant cells to dedifferentiate have been used in plant propagation through plant tissue culture. Plant tissue culture is a broad term referring to the growth of plant cells, tissues or organs under sterile conditions in culture (*in vitro*). One process carried out through plant tissue culture is that of somatic embryogenesis (SE). SE is the development of bipolar embryos from somatic cells and tissues; bipolar meaning they possess both a shoot and a radical end (Sharp et al. 1980). Somatic cells are induced to dedifferentiate and are then reprogrammed to develop into bipolar embryos. The embryos produced via SE, referred to as somatic embryos, are capable of developing into complete plants. The embryos are produced asexually; therefore the resulting plants are genetically identical to the tissue from which the embryos were derived. Although somatic embryogenesis has been reported to occur naturally in species of *Bryophyllum* (Yarbrough 1932) and *Malaxis* (Taylor 1967), it is best known as a pathway used in plant tissue culture to propagate plants. The first report of somatic embryogenesis *in vitro* was in carrot (*Daucus carota* L.) (Steward et al. 1958). The fact that the embryos are bipolar is ideal for propagation in that it allows for simultaneous root and shoot formation. In addition to being a method of propagation, SE is a valuable tool for studying zygotic plant embryo development, which is a difficult task given the intact nature of plant seeds.

Somatic Embryogenesis

General

There are two types of somatic embryogenesis: direct and indirect. Direct SE refers to the process of inducing somatic embryos or embryogenic tissue directly from the differentiated tissue. Zygotic embryos are one of the most successful sources of initiating direct SE (Merkle et al. 1990). Quite often however, the objective of SE is to clone a mature plant after its characteristics and performance have been evaluated and found to be desirable. This objective is especially true of plants whose economic market is driven by consumers' demands for specific floral forms and horticultural traits such as disease resistance (Marchant et al. 1996). Zygotic embryos cannot be used to clone a mature plant since they are produced sexually and therefore are genetically different from the plant that produced them. To clone a mature plant, tissues from the plant itself must be used to induce somatic embryogenesis. Somatic tissues exposed to a high concentration of the plant growth regulator, 2,4-dichlorophenoxyacetic acid (2,4-D) have been reported to undergo direct somatic embryogeneis (Kitamiya et al. 2000). The use of somatic tissues for inducing direct SE is hampered by the fact that not all species are capable of this and that high concentrations of 2,4-D cause genetic mutations that encumber the objective of clonal propagation (Swartz 1991).

Most somatic tissues require a "genetic activation" in order to enter an embryogenic state and be capable of producing embryos. Indirect SE involves an intermediate stage prior to somatic embryo or embryogenic tissue formation. Indirect SE has been induced using somatic tissue in several species, such as African violet (*Saintpaulia ionantha* Wendl.) (Mithila et al. 2003), grape (*Vitis vinifera* L.) (Das et al. 2002), rose (*Rosa* sp.)

(Ibrahim & Debergh 2001, Kintzios et al. 1999, de Wit et al. 1990), sunflower (*Helianthus maximiliani* Schrader) (Vasic et al. 2001), and Siberian ginseng (*Eleutherococcus senticosus*) (Choi et al. 1999). The goal is to induce the source tissue (explant) to dedifferentiate and form callus; a mass of unorganized cells (Figure 1). The callus is not yet committed to differentiate into any organized structure and must be further "genetically activated" to produce embryos. It has been reported that the initiation of embryogenic tissue from differentiated explants often involves "extensive proliferation through an unorganized callus cycle" (Merkle et al. 1990). Factors reportedly used to activate callus growth include growth regulators, such as auxins (Murashige & Tisserat 1977), stress such as starvation of the tissue (Lee et al. 2001), and heat shock. Frequently, exposure to a low concentration of 2,4-D followed by transfer to an even lower concentration of 2,4-D or growth regulator-free medium has been successful in inducing SE in a wide range of plant species, as in Siberian ginseng (*Eleutherococcus senticosus*) (Choi et al. 1999), rose (*Rosa* sp.) (de Wit et al. 1990), oat (*Avena sativa*) (Chen et al. 1994), and carrot (*Daucus carota*) (Toonen et al. 1994). The removal of 2,4-D is not believed to be responsible for initiating SE however it allows the progression of the pre-embryonic cells to the advanced stages of somatic embryo development.

The ultrastructural characteristics of embryogenic cells are typical among many species; consisting of small cells with dense cytoplasmic contents, small vacuoles and large nuclei with very distinct enlarged nucleoli (Williams & Maheswaran 1986). The external morphology of both callus and embryogenic tissue varies greatly amongst and within plant species (Rout et al. 1999). Toonen et al. (1994) reported high morphological variability among single suspension cells of carrot (*Daucus carota*) that were competent to become embryogenic. This variability makes it difficult to distinguish or predict cells with embryogenic competence even within an individual. Positive identification of embryogenic tissue is sometimes only known by the presence of nodular structures. This nodular tissue is often referred to as proembryogenic masses (PEMs) (von Arnold et al. 2002). The nodular structures are actually early globular stage embryos that precede the later stages of dicotymous embryo development, i.e., heart-shaped, torpedo-shaped and cotyledonary embryos (Figure 2).

Once somatic embryos reach the cotyledonary stage, they must go through sequential stages of maturation, a desiccation or cold period, germination, acclimatization and transfer to in the *ex vitro* environment. There is much diversity among and within plant species in terms of the stages and time lines of SE (Das et al. 2002, Ibrahim & Debergh 2001, Vasic et al. 2001, Kintzios et al. 1999, de Wit et al. 1990). Despite this diversity, it is known that the various stages of SE involve the "commitment of specific cells to a sequential pattern of selective gene expression" (Zimmerman 1993, Giroux et al. 1997).

Gene expression during somatic embryogenesis

Each stage of somatic embryogenesis involves the activation and deactivation of genes. Certain plant developmental processes are likely the result of an array of interacting genes, which require the expression of proceeding genes (Torres-Ruiz et al. 1996). The isolation of embryo-specific genes and the characterization of their roles during embryo development are fundamental in the overall understanding of the molecular processes regulating embryogenesis (Magioli et al. 2001). Understanding the genetic control of development is one of the most fundamental questions in biology and remains one of the main research areas of molecular biology today.

The first step in indirect somatic embryogenesis is to dedifferentiate somatic cells to form callus. As previously mentioned, exposure to the plant growth regulator 2,4-D

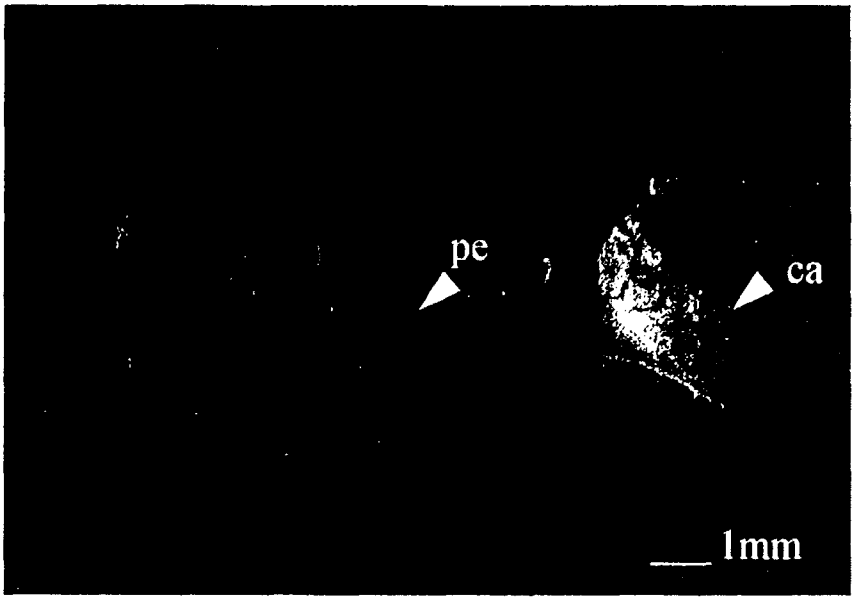

Fig 1 Callus (ca) developed on the excised ends of a rose petiole explant (pe) after two weeks growth on medium containing 5μM 2,4-D.

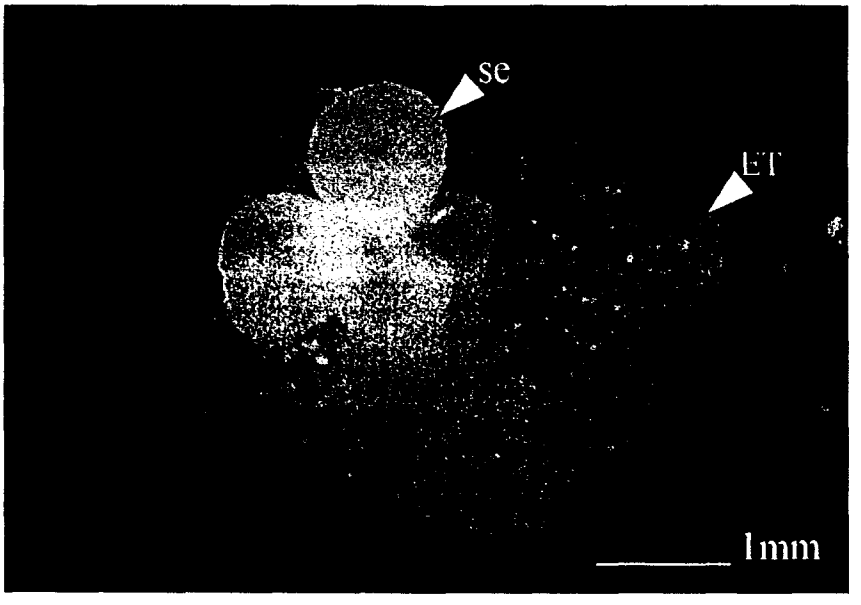

Fig 2 Somatic embryo (se) growing on embryogenic tissue (ET) after transfer of tissue onto growth regulator-free medium.

is one means of achieving this. Reprogrammed gene expression is evident by the synthesis of new messenger ribonucleic acid (mRNA) after exposing cells growing *in vitro* to 2,4-D (Hagen et al. 1984, Theologis 1986, Van der Zaal et al. 1987). The exact stages and mechanisms involved in the transition of callus cells into embryogenic cells are not known. Quite often the callus induction stage and its subsequent transition to an embryonic state is collectively referred to as early somatic embryogenesis (Kairong et al. 1999, Sato et al. 1995, Momiyama et al. 1995).

Recent studies have attempted to identify differences in gene expression between callus and embryogenic tissue. Duncan and associates (2003) found that tissue with high levels of *globulin-1* (*Glb1*) - protein encoded polypeptides is embryogenically competent. In non-embryogenic tissues, very low concentrations of *Glb1* were detected. The *Glb1* protein is known to be synthesized in zygotic embryos shortly after pollination.

In carrot (*Daucus carota*), the Somatic Embryogenesis Recpetor-like Kinase (SERK) gene was found to be a useful marker of single cells possessing competency to form somatic embryos (Schmidt et al. 1997). SERK gene expression was detected in a cohort of slightly elongated and vacuolated cells throughout the developmental stages prior to reaching the globular stage embryos. Somleva and associates (2000) also identified the SERK gene during the induction of "embryogenic cell formation" in single cells of *Dactylis glomerata*.

HBK2, a new gene belonging to class I of the KNOTTED1-like homeobox (KNOX) genes was expressed in Norway spruce (*Picea abies* (L.) Karst.) during proembryogenic masses through to late stage somatic embryo development (Hjortswang et al. 2002). Homeobox genes control cell specification and pattern formation during plant development. The HBK2 gene was expressed only in embryogenic cell lines that resulted in somatic embryo production and not in cell lines that failed to produce embryos. Other related KNOX genes have been reported to be differentially expressed during both zygotic and somatic embryogenesis of maize (*Zea mays* L.) (Zhang et al. 2002).

Kairong and associates (1999) obtained three complementary deoxyribonucleic acids (cDNAs) from early somatic embryogenesis of *Lycium barbarum*, which were not observed in calli. The cDNAs were produced after the transfer of the tissue from 2,4-D to an auxin-free medium. Although the roles of these cDNAs were not discussed, the results of this study supports the notion that callus induction and its transition into an embryogenic state are two distinct processes involving the expression of distinct genes. Likewise, Giroux & Pauls (1997) identified three cDNAs transcripts (*ASET1, ASET2,* and *ASET3)* present in embryogenic tissues of alfalfa (*Medicago sativa* L.) but absent in petioles, mature embryos or non-embryogenic tissue.

Several genes expressed during the various stages of somatic embryo development have been identified. New gene products were reported by Borkind and associates (1988) to be synthesized in plants upon the removal of auxin from the medium. These gene products are required for the transition from globular stage embryos to the heart-shaped stage.

The *CEM6* gene coding for a protein expressed during early embryo development with the highest levels occurring at the early globular stage (Sato et al. 1995) reportedly began to decrease at the later torpedo-shaped stage (Komamine et al. 1999). Magioli and associates (2001) reported the expression of the glycine-rich *Atgrp-5* gene during early embryo development of *Arabidopsis thaliana* and eggplant (*Solanum melongena* L.). *Atgrp-5* was detected in globular and torpedo stage embryos but eventually turned off in later stage cotyledonary embryos.

Several genes expressed during somatic embryo development appear to have characteristics of a class of proteins called Late Embryogenesis Abundant (LEA) proteins (Sunderlíková & Wilhelm 2002, Dure et al. 1981, 1989, Galau et al. 1986). Most of the LEA transcripts increase significantly in somatic embryos at the heart stage (Choi et al. 1987, Wilde et al. 1988, Franz et al.1989, Wurtele et al. 1993). The LEA genes appear to be induced by the application of abscisic acid (ABA) (Hatzopoulos et al. 1990, Goupil et al. 1992) as well as other factors such as water stress (Han et al. 1997). ABA is a growth regulator produced naturally by plants during zygotic embryogenesis and is commonly employed during the maturation stage of SE (von Arnold et al. 2002). Dong & Dunstan (1997) identified five ABA-responsive cDNAs from white spruce (*Picea glauca* L.). Three of the cDNAs (PgEMB12, 14 and 15) were speculated to encode LEA proteins while the other two (PgEMB5 and 23) were not similar to any known DNA or protein sequences. Sunderlíková & Wilhelm (2002) reported the accumulation of mRNAs during the maturation of oak (*Quercus robur* L.) somatic embryos that were similar to that of the *Lea* proteins observed during late cotyledonary embryos development.

Early molecular studies evaluating gene expression in SE focused mainly on the developmental stages of somatic embryos with little attention being paid to the initiation of somatic embryogenesis (Zimmermann 1993). The fact that somatic embryo development is readily divided into distinct stages based on morphological characteristics (globular, heart-shaped, torpedo-shaped and cotyledonary) makes it an easier task to approach than that of the less well-defined stages of early somatic embryogenesis. Although there are both molecular and morphological differences between non-embryogenic and embryogenic tissue, the transition from one stage to the next is still a very grey area. It is difficult to identify differences in gene expression between stages when the stages themselves are not well defined.

Conclusions

Somatic embryogenesis can be induced by several different factors; plant growth regulators, stress, and heat shock. The fact that there is more than one SE inducer suggests that the transition from a somatic cell to an embryogenic cell may have several different molecular routes, further complicating the task of understanding the molecular basis.

A greater emphasis on characterizing and defining the stages involved from dedifferentiation through to the transition of callus into an embryogenic state is required in order to elucidate the processes involved in indirect SE. The understanding of the stages involved in early SE and their underlying molecular mechanisms are linked; advances in one area will facilitate the understanding of the other.

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7. 2 Appendix B: Murashige and Skoog Medium

Murashige & Skoog (MS) Inorganic Salts [1]

Stock	Volume to add to medium (mL/L)	Constituents	Concentration in Stock Solution (g/L)	Final concentration in medium (mg/L)
Major Salts	100	Ammonium nitrate (NH_4NO_3)	16.50	1650
		Calcium chloride ($CaCl \cdot H_2O$)	4.40	440
		Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	3.70	370
		Potassium phosphate ($KH_2 PO_4$)	1.70	170
		Potassium nitrate (KNO_3)	19.00	1900
Minor Salts	10	Boric acid (H_3BO_3)	0.62	6.2
		Cobalt chloride ($CoCl_2 \cdot 6H_2O$)	0.0025	0.025
		Cupric sulfate ($CuSO_4 \cdot 5H_2O$)	0.0025	0.025
		Manganese sulfate ($MnSO_4 \cdot 4H_2O$)	2.23	22.3
		Potassium iodide (KI)	0.083	0.83
		Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$)	0.025	0.25
		Zinc sulfate ($ZnSO_4 \cdot 7H_2O$)[a]	0.86	8.6
Iron	10	Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	2.78	27.8
		$Na_2EDTA \cdot 2H_2O$[b]	3.72	37.2[c]

Organic Additive Stock Solutions

Stock	Volume to add to medium (mL/L)	Constituents	Concentration in Stock Solution (g/L)	Final concentration in medium (mg/L)
Inositol	10	Myo-Inositol	10	100
Vitamins	10	Nicotinic acid	0.5mg/l	0.5
		Pyridoxine HCL	0.5mg/l	0.5
		Thiamine HCL	1.0mg/l	0.1
		Glycine	2.0mg/l	2.0

[a] = Originally printed as $ZnSO_4 \cdot 4H_2O$
[b] = Originally printed as Na_2EDTA
[c] = Originally printed as 37.3 mg/L anhydrous form

[1] Murashige T. and Skoog F. (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiologia Plantarum* **15**: 473-497.

Modifications as per: Owen H.R. and Miller A.R. (1992) An examination and correction of plant tissue culture basal medium formulations. *Plant Cell, Tissue and Organ Culture* **28**: 147-150.

7.3 Appendix C: Woody Plant Medium

7.3 Appendix C: Woody Plant Medium

Woody Plant Medium Inorganic Salts [1]

Stock	Volume to add to medium (mL/L)	Constituents	Concentration in Stock Solution (g/L)	Final concentration in medium (mg/L)
A	20	Ammonium nitrate (NH_4NO_3)	20.0	400
		Calcium nitrate	27.8	556
B	20	Potassium sulfate ($K2SO_4$)	48.5	990
C	5	Calcium chloride ($CaCl_2 \cdot 2H_2O$)	19.2	96
D	5	Potassium phosphate ($KH_2 PO_4$)	34.0	170
		Boric acid (H_3BO_3)	1.24	6.2
		Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$)	0.05	0.25
E	5	Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	74	370
		Manganese sulfate ($MnSO_4 \cdot 4H_2O$)[a]	4.46	22.3
		Zinc sulfate ($ZnSO_4 \cdot 7H_2O$)	1.72	8.6
		Cupric sulfate ($CuSO_4 \cdot 5H_2O$)	0.05	0.25
F	10	Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	5.57	27.8
		$Na_2EDTA \cdot 2H_2O$[b]	7.45	37.2[c]

Organic Additive Stock Solutions

Stock	Volume to add to medium (mL/L)	Constituents	Concentration in Stock Solution (g/L)	Final concentration in medium (mg/L)
Inositol	10	Myo-Inositol	10	100
Vitamins	10	Nicotinic acid	0.5mg/l	0.5
		Pyridoxine HCL	0.5mg/l	0.5
		Thiamine HCL	1.0mg/l	0.1
		Glycine	2.0mg/l	2.0

[a] = Originally printed as $MnSO_4 \cdot H_2O$
[b] = Originally printed as Na_2EDTA
[c] = Originally printed as 37.3 mg/L anhydrous form

[1] Lloyd G. and McCown B. (1980) Combined Proceedings of the International Plant Propagators Society **30**: 421-427.

Modifications as per: Owen H.R. and Miller A.R. (1992) An examination and correction of plant tissue culture basal medium formulations. *Plant Cell, Tissue and Organ Culture* **28**: 147-150.