A MORPHOLOGICAL AND HISTOLOGICAL STUDY OF CALLUS INITIATION AND SOMATIC EMBRYOGENESIS IN *ROSA HYBRIDA*

BY

KERRY ANN NICHOL DUST

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Botany
University of Manitoba
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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Abstract

2,4-D and a wounding treatment, alone and in combination, were used for callus initiation from micropropagated leaflets of *Rosa hybrida* L. cv John Davis. The only initiation treatment that resulted in callus formation was the combination of 2,4-D (5 mg/ml) and wounding treatment. Histological examination of the leaflets from the callus initiation treatments revealed potentially embryogenic cells were present in week-old calli. Examination of calli clusters and embryogenic tissue allowed the identification and characterization of non-embryogenic and embryogenic cells. Somatic embryos were initiated from the embryogenic tissue and were fixed and examined using light and fluorescence microscopy. The somatic embryos followed similar stages of development as have been described in the literature, e.g. globular, heart, torpedo and cotyledonary stages. The protoderm was the first tissue to differentiate. It was observed in the globular stage. The beginnings of the procambium, ground tissue and cotyledons were observed in the late globular/ early heart-shaped stage. The root apical meristem (RAM) was developed by the cotyledonary stage. Shoot apical meristem (SAM) development was rarely observed. The lowest level of Abscisic Acid (ABA) tested, (0.1 mg/L), combined with highest level of sucrose tested, (40 g/L), resulted in the most mature somatic embryos. Maturation treatments including PEG1500 (polyethylene glycol 1500) yielded lower numbers of mature somatic embryos. Histological examination of mature rose zygotic embryos showed that the tissues of zygotic embryos are more regular, yet similar to somatic embryos.

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List of Abbreviations

ABA	Abscisic acid
AGP	Arabinogalactan protein
cot	Cotyledon
cw	Cell wall
CZ	Central zone
et	Embryogenic tissue
gt	Ground tissue
IAA	Indole acetic acid
lc	leaf callus
mr	mid rib
N	Nuclei
NAA	Naphthalene acetic acid
nec	Non-embryogenic callus
pb	Protein body
PAS	Periodic acid- Schiffs reaction
pc	Procambium
pd	Protoderm
PEG	Polyethylene glycol
pin-1	*pin-formed 1* mutant
PB	protein body
PZ	Peripheral zone
RAM	Root apical meristem
RZ	Rib zone
S	Scratch
SAM	Shoot apical meristem
sc	Seed coat
sg	Starch grain
ss	Secondary structure
stm	*shoot meristemless* mutant
STM	Shoot meristemless gene
trp	Tryptophan
UV	Ultraviolet
V	Vacuole
2,4-D	2,4-dichlorophenoxyacetic acid

Introduction

Roses *(Rosa hybrida* L.) have been cultivated for thousands of years and are a valuable part of the horticultural trade. In Canada, more than 38 million roses are produced for cut flowers every year and over 700,000 are sold as potted plants (Hole and Fallis 1997). The rose's beautiful flowers are also coveted for their fragrant essential oils by the perfume industry.

Propagation of roses is commonly achieved by grafting, budding or conventional cutting techniques (Roberts et al. 1995). Grafting and budding are quicker than conventional cuttings, but all three methods are labor intensive, limited by available plant material and season. Tissue culture methods such as micropropagation have been developed to alleviate limitations imposed by shortages of plant material and seasonal constraints, however these methods are also labor intensive. Protocols for another tissue culture method, somatic embryogenesis, have been developed which have the potential to produce thousands of propagules *in vitro* with minimal labor demands (reviewed by Roberts 1995).

Somatic embryogenesis is the process of producing embryos from tissues not normally associated with sexual reproduction. This process occurs naturally in some species of plants and can be induced *in vitro* in many. *In vitro*, somatic embryos can either occur directly from cells of organized tissues or indirectly from a callus (Williams and Maheswaran 1986).

Somatic embryos are organized bipolar structures that do not have vascular connections with the maternal tissue (Williams and Maheswaren 1986). While both somatic and zygotic embryos often undergo similar morphological stages, e.g. globular, heart-shaped, torpedo and cotyledonary stages, organization of somatic and zygotic embryos often differs at the histological level. For example, somatic embryos often lack a well developed shoot apical meristem (SAM) (Nickle and Yeung 1993). The lack of a well developed SAM has been cited for the poor germination rates often associated with somatic

embryos (Ramsean-Fortner and Yeung 2000). A deformed or absent SAM or RAM could be responsible for the low germination rates of somatic embryos. The SAM will give rise to the above ground portion of the plant and the RAM will give rise to the below ground portion of the plant after germination. For somatic embryos to be commercially viable the maturation protocols must be improved so that germination rates will rise.

Somatic embryogenesis has been reported in roses by several authors (Noriega and Sondahl 1991, Van der Salm et al. 1996 and Marchant et al. 1996), however very little information describing histological aspects of somatic embryogenesis has been published (Rout et al. 1998).

Microscopy is a useful and informative means to describe and document somatic embryogenesis. Rout et al. (1998) have done some preliminary histological work on rose somatic embryogenesis. They have described that callus cells become thick walled prior to embryo initiation but they did not describe nor characterize the different types of callus, nor did they indicate if rose somatic embryos follow similar morphological patterns as typical rose zygotic embryos. No information was provided on the quality of the meristems or the germination rates of the somatic embryos in the study by Rout et al. (1998).

The purpose of this study was to use light and fluorescence microscopy to examine callus initiation and somatic embryogenesis from micropropagated rose leaflets. This involved assessing different callus initiation treatments and characterizing cell and tissue types in callus. Discernible changes that may indicate the onset of embryogenesis were be sought and comparisons between somatic and zygotic embryos will be made. Finally, maturation protocols using Abscisic acid (ABA) and osmotic agents were used in an attempt to improve maturation and increase germination rates.

Literature Review

Somatic Embryogenesis

Somatic embryogenesis is the process whereby embryos are regenerated from somatic cells or tissues *in vitro* through the manipulation of growth media components, particularly plant growth regulators (Ammirato 1983). The resulting propagules are clones of the parent tissue, as the process of genetic recombination associated with sexual reproduction does not occur. Somatic embryos were first produced from carrot (*Daucus carota)* tissues by Steward et al.(1958) and Reinhert (1959). Since then, somatic embryogenesis has been reported in hundreds of species including both angiosperms and gymnosperms (Williams and Maheswaren 1986).

The protocol for inducing somatic embryogenesis has been well documented for many species including roses (Ammirato 1983; Marchant et al. 1996). There are two main methods of somatic embryogenesis: direct and indirect. The direct method involves producing embryos directly from single cells in the explant. No callus initiation stage is involved. The success of the direct method depends on the type explant used, e.g., the explant must have cells that are capable of immediately giving rise to an embryo. The direct method has been successfully used to produce somatic embryos from *Cichorium* (chicory) roots (Dubois et al. 1990). Direct somatic embryogenesis is the less common of the two methods, has never been reported as a successful somatic embryogenesis method for roses and therefore is not considered in this study.

The indirect method of somatic embryogenesis is the one suited to roses (Marchant et al. 1996). The protocol involves four main steps. First the cells of an explanted tissue are treated with plant growth regulators, usually an auxin, to induce a mass of undifferentiated cells known as a callus. Then the dedifferentiated cells of the callus are induced to become embryogenic by incubating the callus on a plant growth regulator-free medium for a month or longer. Once an embryogenic tissue is observed, it is cultured on a medium that

will allow maturation. This media may contain abscisic acid (ABA) and/or osmotic agents. Somatic embryos proceed through four morphological stages: globular, heart shaped, torpedo and cotyledonary stages. Finally, a cold treatment or desiccation step may be required prior to germination of the mature somatic embryos.

A successful somatic embryo is one that has a root apical meristem (RAM) and a shoot apical meristem (SAM) and is capable of germination, e.g. characteristics that a zygotic embryo of the same species would possess. Although the process in general seems straight forward, the details of the protocols may vary from species to species or even cultivar to cultivar because factors such as explant sources and media components (especially plant growth regulators) can affect the success of somatic embryogenesis (Williams and Maheswaren 1986).

Factors Affecting Somatic Embryogenesis

Explant Source

The explant source chosen for somatic embryogenesis will influence the success of callus and /or somatic embryo initiation. Immature tissues such as zygotic embryos, micropropagated tissues and seedling hypocotyls are ideal explants since they possess cells that readily dedifferentiate and divide, e.g. meristematic and/ or totipotent cells (Ammirato 1983). When choosing an explant the developmental stage and tissue type should be considered.

Meristematic cells are undifferentiated cells from which all other cells are derived. Because of their ability to differentiate, it is not surprising that meristematic cells are one of the most effective types of cells for callus and somatic embryo initiation. Regions of meristematic cells are found in many types of tissues especially areas of tissue growth, e.g. shoot apical meristems, lateral buds, vascular cambium and root apical meristems.

The productiveness of an explanted tissue may depend on the amount of meristematic cells present in the tissue. As a tissue matures, most of its cells become differentiated to function in specific roles, e.g. tracheids for water transport. As a result, the older regions of the plant will contain fewer meristematic cells than the younger growing points, e.g. shoot meristems. Thus, the maturity of the explanted tissue may affect the amount of callus or somatic embryos it will produce. For example Levi and Sink (1991) found that explanted lateral buds and *in vitro* crowns (shoot apices) of asparagus (*Asparagus officinalis* L.) produced 9 and somatic embryos per gram of callus respectively, while explanted spear cross sections only produced 4. They attributed the difference in somatic embryo production to the greater abundance of meristematic cells present in lateral buds and *in vitro* crowns (which are relatively immature, actively growing regions of tissue) than in cross sections of spears (more mature, differentiated tissue). Unfortunately, they did not mention if the quantity of callus produced was affected by explant type. If all three explant types in Levi and Sink's study produced a similar volume of callus, that might indicate that non-meristematic cells were also involved in callus production, but only the callus cells derived from the meristematic cells were capable of somatic embryo formation. Their report insinuates that only meristematic cells were involved, when quite possibly differentiated cells such as epidermal cells could have contributed to callus production. It is not known if all callus cells are metabolically similar until differentiation, or if their metabolism is determined by their mother cell.

Many plant cells are totipotent, e.g. differentiated somatic cells with the potential to dedifferentiate into an embryogenic cell that can give rise to a multicellular embryo and eventually a new plant. This was first demonstrated by Steward et al.(1958) who used carrot root phloem tissue to produce a callus. He observed that some callus cells separated from the rest of the callus and, when media conditions were altered (e.g. plant growth regulator levels reduced) some of these cells became embryogenic. (A similar study was done at the same time

by Reinhert (1959).) However phloem tissue contains many types of cells, possibly including meristematic cells of the vascular cambium, yet Steward et al.(1958) did not identify which cell types were responsible for callus formation. One can only assume that the cells that gave rise to the first somatic embryos, were differentiated phloem cells and not meristematic cells. Or possibly, more than one cell type in an explant will become callogenic. It is not known if the callus derived from different cells differs in its ability to yield somatic embryos. However it is known that all cells in a callus do not become embryogenic, thus, one cannot say with complete certainty that all cells are totipotent.

Several types of explants have been used successfully for rose somatic embryogenesis. However, the cells that are stimulated to produce a callus have not been identified. Souq (1987) was the first to report the production of rose somatic embryos. He obtained calli from explanted leaves, filaments and stamens. There have been subsequent reports of rose somatic embryos derived from leaflet calli (DeWit et al. 1990; Rout et al. 1991; Hsia and Korban 1996), petiole calli (Marchant et al. 1996), filament calli (Noriega and Sondahl 1991; Firoozabady et al. 1994), immature seeds (Kunitake et al. 1993), root calli (Van der Salm et al. 1996; Marchant et al. 1996) and petal calli (Murali et al. 1996). It would seem that in the case of roses, most somatic tissues are capable of giving rise to somatic embryos.

Auxins

The first stage in most protocols for somatic embryogenesis is a callus induction stage. This stage involves explanting a tissue onto a medium that contains high levels of auxins. For example, a range of 1 to 10 mg/L of 2,4-D (2,4-dichlorophenoxyacetic acid) has been used in the callus induction media for rose somatic embryogenesis (Rout et al. 1991; Noriega and Sondahl 1991; Marchant et al. 1996). The specific physiological effects of auxin on explanted tissues are unknown, but including auxins in a medium is known to interrupt the explanted cells' normal functions, resulting in an undifferentiated callus.

Interestingly, Ribnicky et al. (2002) have described how the auxin levels in unfertilized carrot ovules spike up 80 fold upon fertilization. This seems to indicate that zygotic embryos need to be exposed to a high auxin environment for the early stages of embryogenesis to proceed.

There are several types of auxins. IAA (indole acetic acid) is the most common type of auxin naturally found in plant cells. Synthetic auxins such as 2,4-D and naphthalene acetic acid (NAA) have similar physiological effects as the natural auxins but must be supplied in the culture medium. 2,4-D is often used in tissue culture because it is more stable and less prone to conjugation and catabolism than natural auxins (Ribnicky et al. 1996).

The biologically active form of an auxin is known as "free auxin". Free auxins are able to stimulate cell elongation, cell division and dedifferentiation, e.g. produce a callus. The cellular levels of free auxins can be modulated by biosynthesis, conjugation to sugars, alcohols or amino acids and catabolism. Auxin biosynthesis and metabolism is thoroughly reviewed in Normanly et al. (1995).

Auxins are known to stimulate callus proliferation and somatic embryogenesis in cultures, however the mechanism by which this occurs is not clear. One theory suggests that exogenous auxins act indirectly on explanted cells by affecting their endogenous IAA levels which in turn causes a callus and embryogenic response. For example, Michalczuk et al. (1992) observed high levels of endogenous IAA (548 ng of total IAA/g fresh weight) were present in explanted carrot cells cultured on a medium containing 1 mg/L of 2,4-D. When these cells were transferred to a medium that was free from exogenous 2,4-D, the levels of endogenous IAA decreased to 30 ng total IAA/g fresh weight and somatic embryos formed (Michalczuk et al. 1992). They attributed the embryogenic response to the level of free auxin in the cells. These results are also supported by the fact that 2,4-D has been shown to increase the level of auxin binding proteins in the membranes of carrot cells (Loschiavo et al. 1991).

Other studies suggest that exogenous auxins stimulate the explant cells directly, causing callus proliferation and embryogenesis via auxin effects on the cells. Ribnicky et al. (1996) measured endogenous IAA concentrations in carrot hypocotyls that were cultured on media that contained 2,4-D, or NAA or IAA (at 10 mM). Their results did not indicate that endogenous IAA levels were changed by exogenously applied auxins, thus they suggested that the exogenous auxins were directly affecting the explanted cells and causing a callogenic and embryogenic response. To determine this, Ribnicky et al. (1996) observed the conjugation levels of three exogenously applied auxins after one week and four weeks in culture. They found that NAA was 90% conjugated and IAA was 99% conjugated after one week on culture and 2,4-D was 35% conjugated after 4 weeks. These conjugation levels were related to the auxin responses observed in the carrot cell cultures. For example, the cultures supplied with 2,4-D had the lowest amount of conjugation and therefore had the highest amount of free auxins. 2,4-D caused a callus to proliferate that was capable of forming somatic embryos when the 2,4-D was removed. NAA was highly conjugated, and was only able to cause a callus response. However, this callus was not capable of forming somatic embryos. Exogenously applied IAA was almost completely conjugated and could not cause callus to proliferate. Not surprisingly, the tissues with high levels of free auxins showed the strongest auxin responses.

The differences in conjugation levels observed by Ribnicky et al. (1996) were probably due to structural differences between the auxin molecules. IAA is the natural form of auxin and thus will be susceptible to enzymes that are present in the cell, e.g., IAA-Glc synthase, an enzyme that causes conjugation of IAA with glucose. NAA has a structure that is only slightly different from IAA and thus it may be susceptible to similar enzymes. The structure of 2,4-D however is quite different from the other two auxins in this study, and not as readily affected by naturally occurring enzymes (Figure LR-1).

Exogenously applied IAA was found to affect endogenous IAA metabolism (Ribnicky et al. 1996). It activated the IAA conjugation system and had a

negative effect on de novo IAA biosynthesis, which lowered the level of free IAA (the active form of IAA). The lowered levels of free IAA may explain why exogenously applied IAA was not capable of sustaining a callus like the other auxin treatments. Ribnicky et al.(1996) concluded that a threshold level of free auxin or free IAA is required to initiate embryogenic competence, but for the next stage, embryo development, to proceed, the level of free auxin must be lowered.

Endogenous auxins play an important role in embryo development. Auxin transport and distribution have a very important role in ensuring the coordinated growth of organs and maintaining polarity during plant development. The role of auxin in embryo development has been studied using Arabidopsis (Mouse cress) *pin-formed 1* (*pin-1)* mutant (Okada et al. 1991). This mutant has a severely reduced ability for auxin transport, resulting in an abnormal inflorescence axis, flowers and leaves; and the embryos of the *pin-1* mutant have a fused collar-like arrangement of their cotyledons as opposed to the normal bilateral arrangement. These phenotypes can be reproduced in wild type embryos and seedlings in the presence of auxin transport inhibitors. The application of polar auxin transport inhibitors to immature zygotic embryos of Canola (*Brassica napus* L.) has resulted in embryos with a similar fused cotyledon phenotype (Liu et al. 1993). Although exogenous auxins are reduced or removed for somatic embryo development, this mutant study suggests that polar auxin transport is actively involved in determining the transition from radial to bilateral symmetry in the globular dicotyledonous embryo.

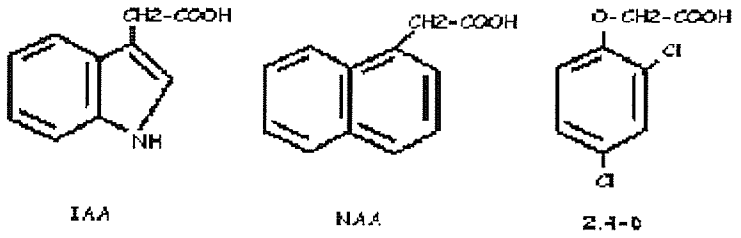

Figure LR1 The chemical structures of three types of auxins: Indole Acetic acid (IAA), Napthalene Acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D)

Abscisic Acid

Abscisic acid (ABA) is a naturally occurring plant growth regulator. Although the chemical structure of ABA has been known for some time, its biosynthetic pathway has yet to be determined. Two pathways have been suggested for ABA synthesis: a direct pathway involving a 15 carbon precursor derived from farnosyl pyrophosphate and an indirect pathway where ABA is derived by the degradation of a carotenoid. Regardless of the pathway, mevalonic acid is known to be the ultimate precursor of ABA synthesis. For a complete review of ABA biosynthesis and physiology see Zeevaart and Creelman (1988).

Although well known for its role in stress responses, ABA has also been shown to be involved in many aspects of seed and embryo development. ABA levels have been observed to increase during the development of the zygotic embryo of many plant species, stimulating the production of seed storage proteins, promoting dormancy and preventing precocious germination (Kamada and Harada 1981). For example, Roberts et al. (1991) found that when they included 40 to 50 mg/L of ABA in the media, interior spruce (*Picea glauca* (Monench) Voss subspecies *engelmanni* (Parry x Engelmann)) somatic embryos

did not germinate precociously and were structurally similar to their zygotic counterparts. Roberts et al. (1991) also observed that including ABA in the media allowed seed storage proteins to develop. They observed that 34.8% +/- 5.4 of the proteins in somatic embryos cultured on media containing 40 mg/L of ABA were storage proteins compared to 19.4%+/-1.9 of proteins in somatic embryos cultured on 20 mg/L ABA. Thus it appears that ABA is a useful component in many maturation media used for somatic embryogenesis.

ABA has been shown to reduce morphological abnormalities which are often present in cultures of somatic embryos, e.g. too few or too many cotyledons, or malformed embryos. Ammirato (1987) reported that growing Caraway (*Carum carvi* L.) somatic embryos, in the presence of low levels of ABA (0.1 to 1.0 mg/L), increased the number of somatic embryos with normal morphologies (they appeared similar to zygotic embryos) and earlier exposure to ABA resulted in higher numbers of normal embryos.

Similarly, ABA has been demonstrated to improve the development of the shoot apical meristem (SAM) in somatic embryos of both angiosperms and gymnosperms. Carrot somatic embryos treated with 15 mg/L ABA had more densely staining meristematic type cells in the apical notch than those that were not treated with ABA (Nickle and Yeung 1993). This apical notch characteristic was shared by somatic embryos capable of normal germination. Thus, Nickle and Yeung (1993) concluded that ABA may be an induction agent for meristematic organization, or it may extend the competence of cells to become meristematic. Similar results were obtained by Kong and Yeung (1992, 1994) using white spruce (*Picea glauca* Moensch.) somatic embryos.

ABA has been used in somatic embryogenesis protocols for roses. For example Noriega and Sondahl (1991) observed that early stages of somatic embryos differentiated when embryogenic tissue was transferred to a medium containing ABA, but further embryogenic tissue proliferation was inhibited in the presence of ABA. However ABA has not been linked with controlling precocious germination and promoting normal development of rose somatic embryos.

Carbon sources and Osmoticum

The carbon source used in the tissue culture medium has two important roles, as a source of energy and as an osmoticum (Jeannin et al. 1995). Tissues *in vitro* rely on carbon from the medium for energy, as the light levels (<120 μ mol/m^2 s) to which they are exposed are usually too low for photosynthesis to occur (Caspellades et al. 1991).

Sucrose is the most commonly used energy source for somatic embryogenesis, however, sugars other than sucrose can be used to promote somatic embryo development. For example, fructose was observed to stimulate the formation of asparagus somatic embryos (Levi and Sink 1991).

Sucrose, fructose and other carbon sources that are supplied to the medium as a source of energy also affect the osmotic potential of the culture. As sugars are metabolized, the osmotic potential of the culture medium changes.

Similar to ABA effects on maturation, a low osmotic potential in the maturation medium has been found to prevent premature germination and promote the accumulation of storage reserves in somatic embryos. For example, in the presence of high levels of sucrose, alfalfa (*Medicago sativa* L.) and spruce somatic embryos were observed to synthesize storage proteins (Xu et al. 1990; Roberts et al. 1991). Osmotic stress has been shown to increase endogenous ABA levels in angiosperms (Loveys et al. 1975; Robinson and Barrit 1990) and this may be why osmotic agents are effective in somatic embryo maturation protocols.

Hexitols such as inositol, mannitol and sorbitol have been used as osmotic agents in somatic embryo cultures. Unlike sucrose, these sugar alcohols are not easily metabolized by the plant tissue and therefore may be effective for regulation of the osmotic potential. For example, Roberts et al. (1991) found that mannitol was the most effective carbon source for the maturation of spruce somatic embryos. However, Tremblay and Tremblay (1991) found that hexitols as the sole carbon source could not support somatic

embryogenesis in spruce, indicating that an additional carbohydrate source is required for nutrition.

The osmotic agent polyethylene glycol (PEG) is a neutral polymer, highly soluble in water and is commercially available in a variety of molecular weights, e.g. PEG 1500 (the number indicates the molecular size). PEG 1500 to PEG 6000 have very high molecular weights and are typically used as osmotic agents in tissue culture protocols. Due its large size, PEG is not able to penetrate cell walls, and therefore may not be as easily metabolized by the cell (Rains 1989). Since PEG is not normally metabolized the osmotic potential of the culture remains constant (Attree et al. 1992).

PEG has been used as an osmotic agent for embryo maturation in many conifer species (Xin et al. 1997; Attree et al. 1992). PEG has also been used in microspore cultures of *Brassica napus* (Argentine canola) (Ilic-Grubor et al. 1998) to induce osmotic stress. However PEG has not been included in any rose somatic embryo protocols to date.

Cell to Cell Communication

There is little known about the cell to cell communication processes involved in cell differentiation and somatic embryogenesis (McCabe et al. 1997). Cells in a plant differentiate according to their positions and use cell to cell communication to assess their positions. For example, suspensor cells are able to develop into embryos if the original embryo is aborted (Yeung and Meinke 1993). The mechanism, which controls this, is not well understood, although there is evidence that soluble signal molecules are involved. For example, Siegel and Verbeke (1989) observed that when certain carpel epidermal cells of periwinkle flowers contact each other, they redifferentiate into parenchyma cells and fuse. However, if agarose inlays are inserted into the contact site between the carpels, the signal molecule that mediates this change is captured in the agarose. Then if the agarose is placed on some epidermal cells that wouldn't

normally fuse, the signal trapped in the agarose will cause these epidermal cells to redifferentiate into parenchyma and fuse. This evidence suggests that plant cells communicate with each other using soluble signals.

It has been established that, in indirect somatic embryogenesis, cells must redifferentiate and give rise to somatic embryos. The cell to cell interactions, which control this, are only beginning to be understood. It has been observed that cells cultured at low densities in suspension cultures are unable to form somatic embryos. However, cells cultured at high densities or in a medium preconditioned by a high density culture that allows the accumulation of certain factors in the medium can produce somatic embryos (Smith and Sung 1985; Devries et al. 1988). Some of these factors in conditioned growth medium have been identified as arabinogalactan proteins.

Arabinogalactan proteins (AGPs) are 90-100kDa glycoproteins with high levels of arabinosyl and galactosyl residues as well as high levels of alanine, threonine, and hydroxyproline residues. AGPs have been associated with many biological processes such as plant development, cell to cell adhesion, pollen stigma recognition and disease resistance (Clarke et al. 1979; Fincher et al. 1983; Pennell and Roberts, 1990). These soluble signals are secreted into the spaces between cell walls, are present on plasma membranes and are also localized in cytoplasmic organelles (Anderson et al. 1977; Moody et al. 1988). Since AGPs are secreted into cell wall spaces, these substances can be found in the culture medium of suspension cultured plants. For example, certain purified AGPs isolated from the culture medium of *Daucus* embryos were able to promote the formation of pro-embryogenic masses in previously non-embryogenic lines when added to the culture medium in nanomolar concentrations. On the other hand, AGPs isolated from the culture of non-embryogenic lines acted negatively on the formation of pro-embryogenic masses. This suggests that specific AGPs are involved in somatic embryogenesis (Kreuger and VanHolst 1993). AGPs have been isolated, purified and characterized from the culture medium and plasma membranes of

cells in a rose suspension culture, however the authors did not state whether this culture was embryogenic (Komalavilas et al. 1991).

Pennell et al. (1992) used a monoclonal antibody (JIM8) to identify cells with embryogenic potential. The monoclonal antibody (JIM8) recognizes a carbohydrate epitope present in AGPs (Pennell et al. 1992). It was originally suggested that cells with embryogenic potential could be identified by the presence of this epitope on their cell wall, as the epitope was absent on the cell walls of cells with low embryogenic potential. It was suggested that these cells displaying the AGP cell wall epitope recognized by the JIM8 monoclonal antibody were in a transition state between competent and embryogenic states (Pennell et al. 1992). However, cell tracking of JIM8 labeled cells failed to show that these cells became embryogenic (Toonen at el., 1996). Thus they suggested that it is possible that the labeled cells do not form embryos themselves but instead produce AGPs that promote somatic embryogenesis (Toonen at el., 1996). A study by McCabe et al. (1997) supports this (Toonen et al. 1996) theory. They found that the cells, which labeled with JIM8 underwent an asymmetrical division, yielding cells which later developed into somatic embryos, but did not label with JIM8 (called state C cells). Notably, the state C cells could not become embryogenic if the JIM8 cells were removed from the culture. Therefore the removal of a population of cells carrying the JIM8 epitope resulted in a decrease in the production of somatic embryos in the remaining cell culture. These results lead McCabe et al. (1997), to conclude that soluble signals released by JIM8 labeled cells are necessary for the development of state C cells into somatic embryos. Interestingly, in *Brassica* zygotic embryos a JIM8 epitope has been identified on suspensor cells and on hypopheseal cells but not on direct descendants of the apical daughter cell (Pennell et al. 1992).

Somatic versus Zygotic Embryogenesis

For many species, the development of somatic embryos has been reported to follow a similar morphological pattern to that of developing zygotic embryos of the same species, e.g. globular, heart-shaped, torpedo-shaped and cotyledonary stages (Yeung et al. 1996;Ammirato 1983) (Figure LR-2).

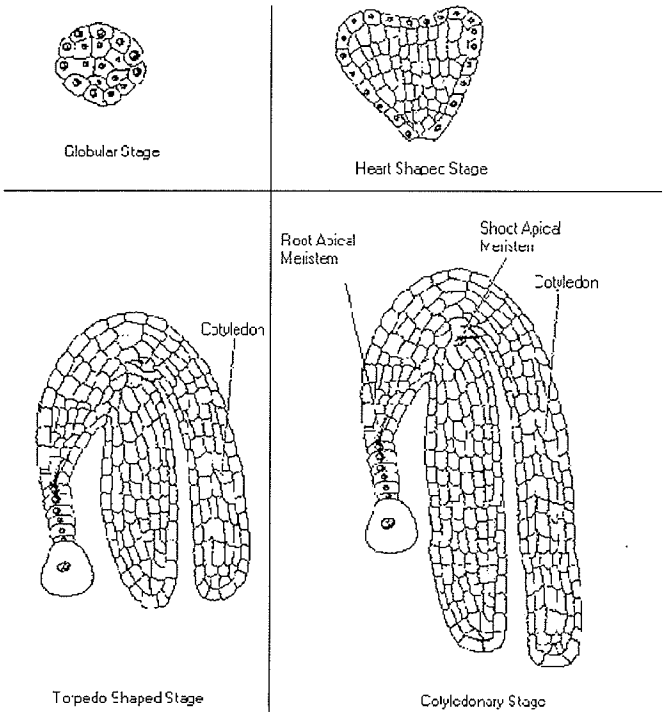

Figure LR2. Four Morphologies of Somatic and Zygotic embryogenesis.
(Adapted from Gilbert and Raunio 1997)

Protoderm

The first meristem to differentiate in zygotic embryogenesis is the protoderm. Generally it is differentiated by the 16 cell stage, but may differentiate as early as the octant stage of zygotic embryogenesis in some species (Krishnamurthy 1994). Periclinal divisions in the early globular stages of canola zygotic embryogenesis result in the outer layer of cells becoming the protoderm (Yeung et al. 1996) and once differentiated, the protoderm cells only divide anticlinally (perpendicularly to the surface).

Somatic embryos often do not form a protoderm at the corresponding stage of development to that of the zygotic embryo and the protoderm may be incomplete (Halperin and Wetherell 1964; McWilliam et al. 1974). However the formation of the protoderm may be essential for embryogenesis to be completed. For example, the carrot *temperature sensitive* somatic embryo mutant lacks a properly differentiated protoderm, which may relate to the failure of these embryos to undergo normal development (van Engelen and de Vries 1992; de Jong et al. 1993). *In situ* hybridization techniques have localized a lipid transfer protein gene that is expressed only in the protoderm cells of carrot somatic embryos and the protoderm and suspensor cells of carrot zygotic embryos (Sterk et al. 1991; Meijer et al. 1993). The failure of carrot somatic embryos to produce a protoderm and the lipid transfer protein resulted in the failure of further embryo development (Sterk et al. 1991). Telmer et al. (1995) suggested that the delimitation of the protoderm may be a structural marker for the commitment to embryogenesis. However the formation of secondary embryos from protoderm cells in somatic embryos, suggests that the protoderm cells of somatic embryos are not as fully determined as those of zygotic embryos (Yeung et al. 1996; Rout et al. 1998).

Lackie and Yeung (1996) observed positive staining by Nile red of the cuticular material of the protoderm during zygotic embryogenesis of carrot. They suggested that the presence of cuticular material may be an early marker for protoderm differentiation. However they did not determine the role of this cuticular substance, but suggested that it may be an inherent property of protoderm cells and that the cuticular substance may have a role in the architecture of the cell walls which is necessary for subsequent morphological stages of embryogenesis. Sterk et al. (1991) suggested that cuticular substances may protect embryos from osmotic changes in the environment.

Ground Meristem and Procambium

The central mass of cells enclosed by the protoderm gives rise to the

ground meristem and the procambium during late globular to early heart-shaped stages in zygotic embryogenesis. The ground meristem will give rise to the cortex and the procambium cells will yield the vascular tissues in the seedling. The cells which are to become the ground meristem have an isodiametric shape, they are enlarged, vacuolated cells, which do not stain darkly, while the cells which are to make up the procambium are elongated, less vacuolated and have a denser cytoplasm (Krishnamurthy 1994). In the late globular stage, canola zygotic embryos developed elongated cells with densely staining cytoplasm that formed the procambium and at the same time the ground meristem was initiated adjacent to the procambial cells (Yeung et al. 1996).

Similar to zygotic embryos, somatic embryos generally develop a procambium and ground meristem during the late globular stage. In rubber plant (*Hevea brasiliensis)* and Geranium (*Pelargonium domesticum* Bailey 'Madame Loyal') somatic embryos, the procambium and ground meristem form by the early heart stage (Michaux-Ferriere et al. 1992; Wilson et al. 1994).

Shoot Apical Meristem (SAM)

The SAM forms during embryogenesis and gives rise to the bulk of the above ground portion of the plant. Cytohistochemical studies of SAMs from various angiosperms indicate that they share many structural characteristics (Steeves and Sussex 1989).

The tunica corpus pattern of cell division is a characteristic feature of the angiosperm SAM (Figure LR-3). The tunica makes up the outermost layer (or layers) of cells. The cells of the tunica only divide anticlinally and this pattern of cell division results in a layered appearance of cells. The corpus is found under the tunica layer(s) and the cells of the corpus divide both anticlinally and periclinally.

The SAM has been described as having three zones, based on the arrangement of staining patterns and cell divisions (Steeves and Sussex 1989). The central zone is made up of large, vacuolated cells at the top of the SAM.

Cells divide less frequently in this zone compared to the other zones and the cells of the central zone serve to maintain a supply of undifferentiated cells to be incorporated into either leaf or stem tissues. The peripheral zone is found on the periphery of the central zone, on the outer edge of the SAM. The cells in the peripheral zone are small, have tiny vacuoles and divide rapidly. The peripheral zone cells serve as initials for new organ primordia. The rib zone is found under the central zone. The cells of the rib zone divide and expand rapidly, and give rise to stem structures.

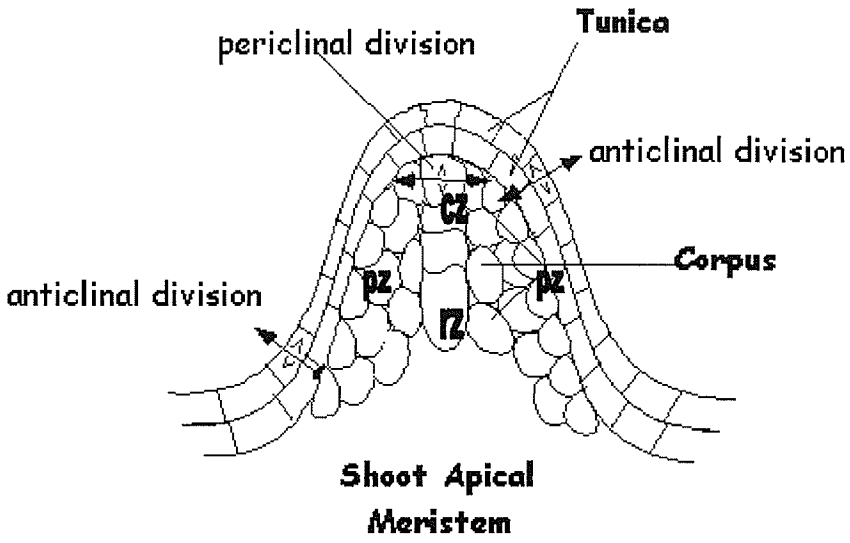

Figure LR3. Shoot apical meristem with tunica corpus organization.

According to Krishnamurthy (1994), the initiation of cells for the future shoot apical meristem (epiphysis) occurs at approximately the same time as the protoderm differentiates in the zygotic embryo. Pritchard (1964) described the cells of the epiphysis as being larger than the adjacent cells, more vacuolated, and mitotically quiescent. These cells also have large nuclei and low RNA and protein content. In zygotic embryos of *Nerium oleander* (oleander), the appearance of partially differentiated meristematic cells in the globular embryo

was the earliest evidence of the SAM initiation (Mahlberg 1960). These cells are believed to be direct descendants from the protoderm.

The STM (shoot meristemless) gene is involved in shoot meristem organization throughout plant development. Barton and Poethig (1993) found that the STM gene is expressed in the cells of the tunica and corpus (located between the cotyledon primordia), suggesting that these cells are SAM initials. Poethig (1996) also used clonal analysis to determine that the cells located between the cotyledon primordia are destined to become the SAM.

The *shoot meristemless* mutants (*stm*) have also been used to study SAM development. The *stm* mutant in *Arabidopsis* lacks a histologically defined embryogenic SAM, but has cotyledons, a hypocotyl and a RAM (Barton and Poethig 1993). Barton and Poethig (1993) also observed the first detectable difference between the wild type embryo and the *stm* embryo at or just prior to the torpedo stage of embryogenesis. They observed that the wild type embryo had a *tunica corpus* pattern of cell division at the torpedo stage, but the *stm* embryo lacked such a structure, indicating that SAM formation is initiated early in embryogenesis, prior to the torpedo stage.

There is also a weak *stm* allele. Mutants carrying the weak *stm* allele also lack a fully functional SAM, but unlike the strong *stm* allele, the weak *stm* embryos have a small number of cells with meristematic features where the SAM should be. In seedlings of weak stm mutants, leaves are formed as early as a week after germination, but growth is limited to two or three leaves. From the axils of these leaves, more leaves arise, until there are no more meristematic cells available. Analyses of these weak *stm* mutants indicate that STM is required to specify SAM cells of the embryo and maintain undifferentiated cells in the central zone.

In somatic embryogenesis, SAM development is very sporadic. In many species, several anomalous patterns of SAM development have been observed, e.g. failure to develop a functional meristem (Nickle and Yeung 1993), extended cell division in the shoot apex (Ammirato 1983) and precocious vacuolation in

the shoot cortex (Fujii et al. 1990). Within one species, sweet potato (*Ipomoea batatas* (L.) Lam.), four patterns of SAM development were observed that resulted in failed conversion: a lack of an organized SAM, a lack of developing cells in the SAM region, a flattened apical meristem, and low meristematic activity throughout the entire embryo (Padmanabhan et al. 1998). They cited auxin as the likely reason for these strange patterns but doesn't explain (or test) what auxin's role in pattern formation might be. Instead he refers to the study done by Michalczuk et al. (1992) (discussed previously). Similarly Vasil and Vasil (1982) stated the presence of 2,4-D in the culture medium may be responsible for the dissimilarities between somatic and zygotic embryos.

Root Apical Meristem (RAM)

The root apical meristem (RAM) consists of one to three tiers of initials (depending on the species), surrounding a quiescent center, which is made up of cells that are mitotically inactive. The proximal initials, above the quiescent center, give rise to concentric layers of root tissues, e.g. the lateral root cap, epidermis, the cortex, the endodermis, pericycle and vascular tissue. The distal initials, below the quiescent center, add cell tiers to the central root cap. According to Scheres et al. (1994) the architecture of the RAM is organized in the heart-shaped embryo, when the embryonic root axis begins to extend.

Root apical meristem (RAM) formation in some species, such as *Stellaria media* (Chick weed) and canola, can be traced to the formation of hypophyseal cells (Krishnamurthy 1994; Yeung et al. 1996). The RAM initials develop late in somatic embryogenesis (Krishnamurthy 1994). The RAM may develop abnormally in somatic embryos but RAM abnormalities are less common than SAM abnormalities. Padmanabhan et al. (1998) observed that most embryos had a well developed RAM regardless of the quality of SAM present in the sweet potato somatic embryos.

The absence of a SAM or RAM may be responsible for the low germination rates observed for somatic embryos. (Recall that normal

germination is achieved if a root, a shoot and a hypocotyl emerge and elongate.) If a SAM has not developed, a shoot will not emerge during germination. Similarly if a RAM is absent a root will not emerge at germination.

Rose Zygotic Embryogenesis

In the 1920's Soueges and Schnarf devised a system to describe and classify early embryo development. They described five classes of dicot embryos and named them: Cruciferen, Chenopodiaceen, Astereen, Solanaceen and Caryophyllaceen. These names were later changed to: Crucifer, Chenopodiad, Asterad, Solanad, and Caryophyllad (Maheshwari 1950). Johanson (1950) described a sixth class of embryo, Piperad, and he replaced the name of the Cruciferad with Onagrad.

The zygotic embryo ontogenesis exhibited by members of the Rosaceae family is referred to as the Asterad type (Johanson 1950). This type of embryogenesis is distinguished by the longitudinal partitioning of the terminal cell and the blending of the terminal and basal cells to form the embryo.

Variations in the sequence and timing of cell divisions and the orientation of cell wall formation can vary between angiosperm taxa, and even within the same species (Natesh and Rau 1984). *Geum urbanum* (*Rosaceae*, subfamily *Rosoideae*) is the only known representative of the Rosoideae subfamily (which includes *R. hybrida*) for which zygotic embryo development has been described (Johanson 1950) (Figure LR4).

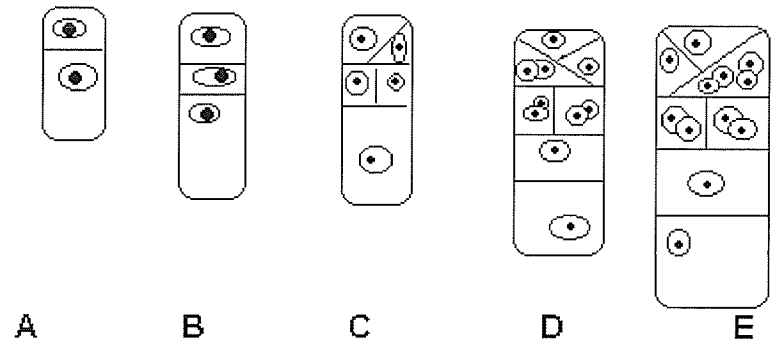

Figure LR-4. *Geum urbanum* early embryogenesis modified from Johanson (1950). Stages A through E represent longitudinal sections of *G. urbanum* early embryogenesis.

The zygote of *Geum* first divides transversely to form a small distal cell and a large basal cell (Fig LR-4 A). The basal cell divides transversely and the distal cell divides obliquely for a total of four cells (Fig LR-4 B). The two upper cells give rise to the cotyledonary portion, the middle cell will result in the hypocotyledonary region and the basal cell develops the initial of the root apex and the suspensor (Fig LR-4 C). The two apical cells undergo further divisions and a triangular cell results at the apex of the proembryo, the subsequent divisions of this triangular cell give rise to the epiphysis (Fig LR-4 D). The epiphysis divides slowly. It is divided into two juxtaposed cells that are subsequently divided and form four circumaxial (surrounding the axis) cells. These four cells are separated by tangential horizontal walls yielding two groups of four cells, (one above the other). The upper group will become the epidermis at the shoot apex (tunica), the lower group will give rise to the corpus. Once the cotyledons begin to develop the limits of the epiphysis cannot be recognized with any certainty. According to Johanson (1950), the first elements of the protoderm are defined by the eight celled proembryo, and the procambium and ground tissue initials are developed shortly after the protoderm has been initiated. The hypophysis (RAM inital) is also initiated after the protoderm has

been established. The description of *Geum* zygotic embryo development by Johanson (1950) does not continue past the globular embryo stage.

The morphological development of zygotic embryos of the Rosaceae, beyond the globular stage, has been recorded by Burger et al. (1990). They investigated the effect of embryo age on the ability of embryos to respond to culture media and documented four stages of rose embryo development at 7, 28, 56, and 75 days post pollination. The 7day stage lacked any organ differentiation. At 28 days post pollination the embryo had reached a second stage where the protoderm, procambium and ground tissues were present, as was a clearly defined embryo axis. At 28 days post pollination, the embryo appeared to be in the late heart-shaped stage and constituted the best explant source for organogenesis perhaps suggesting that the cells of the heart shaped embryos are less differentiated than the later stages. The third stage observed at 56 days post pollination had well defined cotyledons with vascular traces similar to embryos in the torpedo stage of embryogenesis. The final stage was a fully mature zygotic embryo at 75 days post pollination with well defined meristems.

Conclusions

The development of somatic embryos has been well documented for many species including roses. Most somatic embryogenesis protocols follow four main steps: callus initiation, embryo initiation, maturation and germination.

First the cells of an explanted tissue are treated with plant growth regulators, usually an auxin, to induce a mass of undifferentiated cells known as a callus. Generally, explants that are comprised of immature tissues or those that possess meristematic regions have been shown to be the most prolific (Vasil 1987). The type of auxin used in this step can also affect the success of the culture. There are several types of auxins, each with different capabilities for

callus proliferation. 2,4-D has been found to be one of the most effective (Evans et al.1981).

In the next step, the dedifferentiated cells of the callus are induced to become embryogenic by incubating the callus on a medium free of plant growth regulators for a month or longer in 24 hour darkness.

Once embryogenic tissue forms, the embryos are isolated and grown on a maturation medium that may contain abscisic acid and/or osmotic agents. ABA can affect the accumulation of protein storage bodies and prevent precocious germination in somatic embryos (Yeung et al. 1996). ABA has also been associated with improving SAM development in somatic embryos (Ammirato 1987). Osmotic agents have been shown to have similar effects as ABA, and have been used in maturation protocols for conifer somatic embryos (Attree et al. 1992).

Germination is the final step in the somatic embryogenesis protocol. Germination is considered to be a success if a root, a shoot and a hypocotyl emerge and elongate. This step is where most of the protocols fail. Often the shoot, the root or both fail to emerge and as a result, the germination rates for somatic embryos tend to be low.

Although the protocol seems straightforward, it may not be successful for all species or even all cultivars. Developing somatic embryos often resemble their zygotic counterparts in terms of morphology. A globular embryo precedes a heart-shaped form. A torpedo-shaped embryo forms next and finally a cotyledonary stage embryo results.

The development of the primary meristems has been documented for somatic embryo development in a few species. Generally a protoderm is the first tissue to form in both the somatic and zygotic globular embryo. The ground tissue and procambium are the next tissues to develop. The tissues in the zygotic embryos are much more organized than those of the somatic embryos. A successful somatic embryo is one that has a RAM and a SAM and is capable of germination. However unlike zygotic embryos, somatic embryos often lack a

well formed SAM and /or RAM. The lack of the SAM or RAM is likely the reason for the low germination rates.

The main reason that somatic embryos are not being produced commercially is that they have very low germination and conversion rates. The development of somatic embryos must be improved before they will be an efficient clonal propagation alternative. However improvement is contingent on increasing our understanding of somatic embryo development.

Materials and Methods

Establishment of Explants

Vegetative shoots of *Rosa hybrida* cv John Davis were excised from greenhouse grown plants and their leaves were removed. The shoots were disinfested under running tap water for 15 minutes, followed by 10 minutes in 10% Javex bleach solution with 2 drops of Tween 20 emulsifier. The shoots were rinsed in sterilized deionized water and then washed in hydrogen peroxide (3%) for 15 minutes. Two more rinses in deionized water followed. In a laminar flow hood the shoots were cut into pieces approximately 1 cm long, and contained one lateral axillary bud. The basal ends of these pieces were placed vertically in the rose propagation medium.

Plant Pathways Inc.'s standard propagation medium was used for micropropagation. It consisted of modified Murashige and Skoog basal salts (Murashige and Skoog 1964) (Appendix A). The pH was adjusted to 5.7 before autoclaving at 121 °C for 20 minutes.

The cultures were grown in 150mL glass jars, capped with plastic lids (Sigma Magenta B Caps) and were maintained at 23 +/- 1 °C under low intensity illumination (34W Sylvania Supersaver cool white fluorescent lamps) for 16 hours daily. The lateral buds produced multiple shoots that were divided into individual shoots and transferred to fresh media every 4 weeks. The excised leaflets from these shoots were used for explants for somatic embryogenesis.

Callus Initiation

Leaflets, selected randomly from micropropagated 'John Davis' rose propagules, were shallowly scratched with a scalpel across the midrib (abaxial side) to encourage callus formation and placed on the callus initiation media with the abaxial side up. The callus initiation media was chosen based on a

preliminary experiment looking at different callus initiation media. The composition of the callus initiation medium chosen for this study is detailed in appendix B. The cultures were grown in petri dishes sealed with parafilm in 24 hour darkness at 23+/-1 °C for 6 weeks.

To determine the location of callus initiation in the explanted leaflets and to determine if wounding and/ or auxins influence callus initiation, 3 leaflet treatments were devised. In the first treatment leaflets were scratched (as described above) and placed on a callus initiation medium which contains 5 mg/L 2,4-D. In the second treatment the leaflets were placed on the callus initiation medium which contained 5 mg/L 2,4-D. In the third treatment the leaflets were scratched (as described above) and placed on a callus initiation medium without 2,4-D. The leaflets were placed in 24 hour darkness at 23+/-1 °C for 10 days until calli were observed. The entire leaflets were fixed, infiltrated, embedded and sectioned as described below.

Embryo Initiation

Soft, wet calli were removed from the callus initiation cultures and placed on embryo initiation medium (Appendix C). The embryo initiation medium was the same as the callus initiation medium except that it did not contain any 2,4-D. The cultures were grown in petri dishes sealed with Parafilm. These cultures were grown in 24 hour darkness at 23+/- 1° C for 6 to 10 weeks or until embryogenic tissue appeared.

Embryo Maturation

Yellowish white, friable, embryogenic tissue was selected from the cultures and suspended in an Erlenmeyer flask containing liquid embryo initiation medium for 15 minutes. The embryogenic tissue was separated into smaller pieces by vigorously shaking the flask. The suspension culture was

poured onto a #1- 70mm Whatman filter paper lining a Buchner funnel and the excess liquid was vacuumed away. Then the filter paper was placed on fresh, solid, embryo initiation media (Appendix C) and cultured in 24 hour darkness at 23+/- 1° C for 6 weeks. After 6 weeks embryos at all stages of development were present. The embryos were selected based on their morphology and were fixed whole.

Somatic Embryo Maturation Treatments

Embryogenic tissue was incubated on 10 types of maturation media to compare the effects of ABA levels and osmoticum on the development and germination of somatic embryos. 0.1 g of embryogenic tissue was suspended in 100 mL embryo initiation media for 15 minutes before being transferred to filter paper as described above. The filter paper was placed on to 10 different maturation media as follows:

1. 20 g/L sucrose, PGR free
2. 40 g/L sucrose, PGR free
3. 40 g/L sucrose and 0.1 mg/L ABA
4. 40 g/L Sucrose and 1 mg/L ABA
5. 20 g/L Sucrose and 0.1 mg/L ABA
6. 20 g/L Sucrose and 1 mg/L ABA
7. 20 g/L Sucrose and 0.1 mg/L ABA and 1 mg/L PEG
8. 20 g/L Sucrose and 1.0 mg/L ABA and 1 mg/L PEG
9. 20 g/L Sucrose and 1 mg/L PEG
10. 40 g/L Sucrose and 1 mg/L PEG

The rest of the media components are outlined in Appendix C. There were 10 plates per treatment. For 6 weeks the cultures were kept in 24 hour darkness at 23+/-1° C. After 6 weeks cotyledonary stage embryos were counted and placed on germination medium (See Appendix D) and placed in the fridge at 4°C

for 12 weeks.

After the 12 week cold treatment the embryos were placed in the growing room at 23 +/- 1° C under low intensity Illumination (34W Sylvania Supersaver cool white fluorescent lamps) for 16 hours daily for 2 weeks. The percentage of embryos which germinated with a root, hypocotyl and epicotyl was calculated for each treatment.

Zygotic Embryogenesis

To obtain all stages of zygotic embryos, unopened 'John Davis' rose buds were emasculated and pollinated at Plant Pathways Inc in 1998. Zygotic embryos were collected every week starting after one week and continuing to 12 weeks. Two hips were collected at each collection date.

A second collection of zygotic embryos was taken from randomly pollinated roses in the fall of 1999 at Plant Pathways Inc. The rose hips in this collection were mature and contained mature embryos. A sharp knife was used to remove the stony seed coats before fixation.

A third collection was taken in the summer of 2000. The roses were emasculated and pollinated at Agriculture and Agrifood Canada Research Station at Morden, MB. The hips were collected weekly beginning at 21 days post pollination until 75 days post pollination. At the first and second collection dates, 20 to 50 carpels were collected from each hip. By the fourth collection date (42 days post pollination), there were less than a dozen embryos per hip. The embryos were removed from the hips by shallowly slicing the perimeter of the seed with a scalpel. For the younger stages when a gentle pressure was applied to the seed the immature embryo would slide out. The embryos were fixed as described below.

Histology

Fixation

Karnovsky's fixative (4% paraformaldehyde and 8% glutaraldehyde) was used to fix tissue samples. Samples were fixed for 2 hours at room temperature (22+/-1 °C) and 24 hours at 4+/- 1° C. After fixation and prior to dehydration the samples were washed three times for 20 minutes with 0.025 M phosphate buffer. As the embryos matured, a vacuum was used to ensure penetration of fixative.

The samples were dehydrated in a graded alcohol series of 30, 50, 70, 90 and 95% ethanol for 20 minutes in each concentration of alcohol. Followed by three 10 minute dehydrations in 100% ethanol.

The samples were infiltrated in a one to one ratio of L.R.White resin to 100% ethanol and placed on a rotator for 2 hours, then in 3:1 L.R.White:100% ethanol for a further 2 hours. Finally the samples were twice infiltrated in 100% L.R White and placed on a rotator for 24 hours.

The samples were arranged in fresh L.R.White in small Fisher brand nested aluminum pans and baked overnight at 45+/-1 °C for the L.R.White to harden. The embedded samples were removed using a jeweller's saw and glued to plastic Beem capsules with Le Page's 5 minute Epoxy. After drying at least 24 hours, the mounted samples were trimmed into a trapezoid shape using razor blades.

Sectioning

The samples were sectioned on a Sorvall Porter Blum JB-4 rotary microtome, using glass knives with water troughs. The 0.75 – 1μm sections were floated onto the water and removed with a fine glass rod. The sections were placed on a drop of water on 0.5 % gelatin coated slides. The slides were dried on a slide warmer (75 °C) prior to staining.

Staining

Toluidine blue O

Toluidine blue O (c. index # 52040 British Drug Houses Limited) was used as a general stain. It is a metachromatic stain which can be used to indicate DNA (blue), RNA (purple), polycarboxylic acids (red), and ploy phenols or lignin (green) (O'Brien and McCully 1981). A drop of 1% Toluidine blue was added to the sections and the slide was placed on a 75°C slide warmer for 1 to 2 minutes. The stain was then rinsed off the slide under running tap water until all the excess stain was removed. The sections were mounted in 70% sucrose and overlaid with a cover slip. Brightfield microscopy was used to observe these sections.

Periodic Acid Schiff (PAS)

PAS staining was used to stain compounds containing vicinal glycol groups such as starch, pectin, and hemicellulose (O'Brien and McCully 1981). The sections were placed in a 1% Periodic acid solution (G Frederick Smith Chemical Company) for 20 minutes to oxidise vicinal glycol groups to aldehydes. The sections were rinsed under running tap water for 5 minutes, then placed in Schiffs reagent (Fisher Scientific) for 30 minutes to allow the reagent to complex with the newly formed aldehydes. The sections were next rinsed in 0.5% $Na_2S_2O_5$ and finally in running water for 5 minutes. The sections were mounted in glycerol under a cover slip. Brightfield and fluorescence microscopy were used to observe these sections. For fluorescence microscopy green light excitation (Nikon G excitation filter cassette; excitation filter G 535-550 nm, dichroic mirror DM 575 nm and eyepiece side absorption filter slide 580 W.)

Aniline Blue Black (ABB)

Aniline Blue Black was used to indicate proteins. This stain was added after PAS staining. The stain was added to the slides for 30 seconds, then

rinsed off under running water. Brightfield microscopy was used to observe these sections.

Aniline Blue/Calcofluor stain

Aniline blue was used to indicate callose, a β,1-3 glucan, in the cell walls (Smith and McCully 1978). Calcofluor white M2R (Polysciences) was used to indicate β, 1-4 glucans such as cellulose and hemicellulose (Hughes and McCully 1975). The sections were stained by mounting them in a 0.05% solution of aniline blue and 0.1% Calcofluor solution for 2 minutes. The slides were then briefly rinsed under tap water and dried. A drop of glycerin was placed on the cover slip and the sections were examined by fluorescence microscopy using ultraviolet light excitation (Nikon UV excitation filter cassette; excitation filter U 330-380 nm, dichroic mirror DM 400 and eyepiece side absorption filter slide 420 W).

Sudan Black

Sudan black was used to indicate the presence of lipids. Saturated solution of Sudan black was mixed with equal parts of ethanol and placed on the sections for 5 minutes. The stain was rinsed off with tap water. A cover slip was mounted in 70% sucrose. The sections were observed using light microscopy.

Light and Fluorescence Microscopy and Photomicroscopy

A Nikon Optiphot brightfield microscope equipped with EF fluorescence and AFX photomicrographic attachments was used for examination and microphotography of sections. A high pressure 50W mercury lamp was used as a UV source. Glycerin immersion objective lenses (Nikon CF UV-F) were used for fluorescence microscopy and Nikon CF Plan-Achromat dry objective lenses were used for light microscopy. Both light and fluorescence micrographs were recorded using the Nikon Microflex AFX control box and the photomicrographic

attachment and camera back. Kodak ektachrome 64 slide film and T-max black and white film were used. Color slide film was developed at Don's Photo (Winnipeg).

The Kodak T-max Black and white film was developed following the standard procedures.

Results

The anatomical changes and characteristics associated with the different developmental stages of somatic embryogenesis have been summarized in table 1.

Table 1. Anatomical characteristics of the developmental stages of somatic embryos.

	Calli	Pro-embryogenic Masses	Globular stage embryo	Heart shaped stage embryo	Torpedo shaped stage embryo	Mature/ Cotyledonary Somatic Embryo	Mature Zygotic Embryo
Vacuolated Cells	•		•				
Meristematic cells		•					
Starch grains		•		•	•	•	•
Protein bodies		•		•	•	•	•
Callose		•					
Protoderm			•	•	•	•	•
Ground Tissue				•	•	•	•
Procambium				•	•	•	•
RAM					•	•	•
SAM						•*	•

* indicates characterictic often absent or deformed.

Callus initiation

After ten days in culture, soft, yellow calli formed along the wounded midrib and petiole of explanted leaflets in treatment A (5 mg/L 2,4-D, wounded) and on the petiole of leaflets in treatment B (5 mg/L 2,4-D, not-wounded). However, calli were not observed on explants in treatment C (2,4-D free, wounded). Figure 9 shows calli forming on an explanted leaflet after two weeks on treatment A. After four to six weeks the entire blade of the leaflet was covered with a callus derived from vascular cells of the midrib. The petiole and midrib calli formed a single mass therefore they were treated as a single piece of

callus.

A transverse section of a leaflet from treatment A (Figure 1) shows disrupted adaxial and abaxial epidermis, disrupted mesophyll and a mass of callus where the vascular tissue should be. An enlarged view of a callus mass (Figure 2) reveals a pattern of outwardly radiating cells. Also visible are areas of smaller, darkly staining cells with prominent nuclei in these callus masses (Figure 3). These cells had similar physical characteristics to meristmeatic cells, such as darkly staining cytoplasm, and smaller, isodiametric shaped cells. These meristematic type cells were not observed in either of the other two treatments.

Leaflets in treatment B had some protrusions on the leaf blade, but callus was only present on the petiole where it had been cut during explanting. A transverse section of a leaflet cultured on treatment B revealed an intact adaxial epidermis (Figure 4). However some cells of the abaxial epidermis were elongated resulting in protrusions and large intercellular spaces as shown in Figure 6. The midrib and vascular tissues were well organized and undisturbed (Figure 5).

Leaflets in treatment C did not have any callus after ten days in culture. A transverse section of a leaflet in treatment C revealed an intact epidermis, relatively small intercellular spaces among the parenchyma and a well organized midrib and vascular tissue (Figures 7 and 8).

Embryo initiation

After 4 to 6 weeks on the callus initiation medium, soft yellow calli were transferred to a hormone-free medium for embryo initiation. After four weeks on this later medium, most of the non-embryogenic callus became brown. Finally, light yellow embryogenic tissue was observed on the top and sides of the brown callus after eight to ten weeks on embryo initiation medium. Figures 10 and 11 show light colored embryogenic tissue growing on the top of brown non-

embryogenic callus. The embryogenic tissue was soft, friable (easily teased apart) and contained small spherical structures termed pro-embryogenic masses. Pro-embryogenic masses had the potential to develop into somatic embryos when transferred to a maturation medium. Both early and late pro-embryogenic masses were usually present in a single culture. Late pro-embryogenic masses had a distinctly rounded border while the early pro-embryogenic masses did not (Figure 12).

Upon microscopic observation, non-embryogenic callus was observed to be made up of elongated and irregularly shaped cells which did not stain darkly because they were highly vacuolated (Figure 13). The cells of pro-embryogenic masses were observed to be much different from non-embryogenic callus cells, as they were smaller, isodiametric cells which formed clusters of closely associated cells (Figure 14). Cells of pro-embryogenic masses also had smaller vacuoles, more prominent nucleoli and stained more intensely with cationic stains, such as Toluidine blue, compared to non embryogenic callus cells.

When stained with PAS, amylosplasts were observed in most of the pro-embryogenic masses but not in the non-embryogenic callus cells (Figures 13 and 14). Similarly, when aniline blue black stain for proteins was used as a counter stain to PAS, many proteins were observed in the pro-embryogenic masses, but not in the non-embryogenic callus cells.

Forming a border around the pro-embryogenic masses, were cells that appeared to be collapsed, as well as cells that resembled the empty cells observed in callus. This boundary of cells was termed the transition zone (Figure 14).

PAS stained cells were observed using epi-fluorescence combined with a green filter. The carbohydrates inside of the pro-embryogenic masses fluoresced orange while the non-embryogenic callus cells had an orange cell wall but appeared empty inside. The transition zone appeared thicker and more prominent when the PAS stain and epi-fluorescence techniques were used (Figure 15).

The pro-embryogenic masses were found to be positive for aniline blue when observed with epi-fluorescence microscopy. A callose was present on the cell walls of the pro-embryogenic masses (Figure 16), but not on the cell walls of non-embryogenic cells.

After embryogenic tissue was cultured on a hormone-free maturation medium for six to eight weeks, somatic embryos at globular and heart stages appeared. By twelve weeks all stages of somatic embryos were observed. New somatic embryos were forming as others matured and as a result, all stages were often present in the same culture (Figure 17).

Somatic Embryogenesis

Globular shaped embryos

Globular shaped embryos appeared spherical when viewed from the top however a longitudinal view showed that they were rounded on top and then tapered to a point (Figure 18). Generally, embryos at the globular shaped stage were less than 1 mm wide at their widest point and less than 1 mm long at their longest axis.

As shown in Figure 19, the first and only primary tissue to form at this stage was the protoderm. The protoderm was often irregular and incomplete. Also the cells of the protoderm were slightly smaller than the cells which they enclosed. As in the pro-embryogenic masses there were very few intercellular spaces between the cells of the globular embryo.

However, unlike cells of pro-embryogenic masses, the cells of the globular embryo did not stain darkly and appeared empty e.g. when PAS stain was used, the cell walls stained pink while the interior of the cell remained colorless (Figure 19). This indicates that the cells of the globular embryo may have a different metabolic program than the cells of the pro-embryogenic masses. Unlike those of the pro-embryogenic masses, cells of the globular stage embryos stained with PAS/ABB contained neither amyloplasts nor

proteins. Also a callose layer was not observed when the fluorochrome Aniline blue was used to stain globular embryos.

At the tapered point of the embryo, a tissue by which the embryo was attached to the embryogenic tissue was observed; it was named the proximal pole attachment tissue. This tissue was present in most stages of somatic embryos, although it became reduced in the later stages.

Heart shaped embryos

Towards the end of the globular embryo stage, cotyledons began to form, leading the embryo into the next stage of development. The developing cotyledons were responsible for the heart-shaped appearance of this stage of somatic embryos. The heart shaped embryos were approximately 1mm long and, like the globular stage embryos, they are not completely opaque (Fig. 20).

The procambium and ground tissue developed during the heart shaped stage. In Figures 21 and 22, a longitudinal section of a heart shaped stage somatic embryo, the procambium and ground tissue can be observed. The cells of the ground tissue were isodiametric and larger than the cells of the developing procambium. The cells of the ground tissue were highly vacuolated and when stained with PAS and ABB, proteins and amyloplasts were observed. They were also separated by intercellular spaces. There were not any intercellular spaces associated with the smaller, elongated cells of the procambium. Procambial cells also contained some proteins.

Torpedo stage embryos

As the cotyledons elongated and the embryos became opaque and white they were considered to have reached the torpedo stage. Although smaller at approximately <2mm wide and <2mm long, the torpedo stage somatic embryo began to resemble a mature somatic embryo (Figure 23).

The procambium extended into the elongating cotyledons and the ground tissue continued to accumulate storage proteins and starches. There was not

any evidence of shoot apical meristem development at this stage, but the root apical meristem was developing (Figure 24). Figure 25 shows protein bodies present in the ground tissue of a torpedo stage somatic embryo.

Cotyledonary stage embryos

The cotyledons continued to elongate and often began to curve away from the main axis of the embryo. This was the beginning of the cotyledonary stage. Cotyledonary stage embryos were approximately 4 mm wide (at their widest point) and 3 mm long (Figure 26).

Somatic embryos with one, two and three cotyledons were observed (Figures 26-28). Zygotic embryos of roses normally only have two cotyledons therefore only somatic embryos with two cotyledons were used for these anatomical studies.

In most mature somatic embryos, the protoderm, procambium, ground tissue and the root apical meristem (RAM) were all present by this stage (Figure 29). Although a few SAMs were observed this was a rare event (Figures 29 and 30).

Figure 32 provides a more detailed look at the organization of the RAM. The cells of the RAM were small and well organized.

A poorly organized SAM is demonstrated in Figures 31. For comparison, a relatively organized SAM is shown in Figure 30. The cells of the SAM are medium to large, and various shapes. The cells in the organized SAM were in a tunica corpus pattern. In the poorly organized SAMs there was no tunica corpus pattern, instead there were intercellular spaces, and isodiametric cells. Nuclei were observed in many of the vacuolated SAM cells.

PAS and ABB stains revealed the presence of more carbohydrates and proteins than in the preceding stages. Figure 34 shows the large protein bodies that were present in the cotyledon and ground tissue of the cotyledonary stage somatic embryo.

Secondary structures growing out of the protoderm along the hypocotyl

region and cotyledons were observed (Figure 33). The cells of the secondary structures resemble the cells that make up the protoderm and the ground tissue of the cotyledonary stage somatic embryo.

Zygotic embryogenesis

The zygotic embryos of roses were excised from their stony seed coats (Figure 35) to reveal an embryo that shared some similarities with somatic embryos (Figures 36). At an average of 4 mm long and 2 mm wide mature rose zygotic embryos were similar to cotyledonary stage somatic embryos in size. Also similar was the creamy white color of both the zygotic and somatic embryos. However the zygotic embryos were more compact and oval shaped while the somatic embryos are more "Y" shaped (when they have two cotyledons). The zygotic embryos had larger cotyledons and shorter hypocotyls compared to the somatic embryos. Unlike somatic embryos, all of the zygotic embryos in this study had two cotyledons.

The zygotic embryos had well defined regions of tissues (Figure 37). The protoderm was made up of uniform cells and was complete, the ground tissue and procambium were also well defined and made up of uniform cells. The RAM and SAM were both present, and well developed in zygotic embryos (Figures 37-39). Similar to the somatic embryos, secondary growths were observed on the protoderm of some of the zygotic embryos.

The PAS and ABB stains showed that there are large protein bodies and carbohydrates present in the corresponding stages of somatic and zygotic embryos (Figures 40-42). Staining with Sudan black B did not indicate lipids were present in either zygotic or somatic embryos.

Immature Zygotic Embryos

The sections of early stages of zygotic carpels revealed an unexpected

lack of embryos. At the earliest stages (21 days post pollination), over 30 potential embryos were collected and fixed per hip. At later stages (42 days post pollination and older) there were less than a dozen embryos present per hip. Unlike the older embryos, all of the immature zygotic embryos did not contain any embryos (Figures 44-47).

Maturation and germination

The average number of cotyledonary embryos produced per plate for 10 maturation treatments was counted and summarized in Table 2a. The maturation treatment which produced the highest number of somatic embryos per plate was #3 (high sucrose (40 g/L) and low ABA (0.1 mg/L). It produced an average of 13.3 +/- 4.8 embryos per plate. The treatment which produced the next highest number of cotyledonary embryos was #5 which contained low sucrose (20 g/L) and low ABA levels (0.1 mg/L). On average 7.9 +/- 4.6 cotyledonary stage embryos were produced per plate in #5. The maturation treatment which produced the lowest number of cotyledonary stage embryos, an average of 1.8 +/- 2.3 embryo per plate, was #10 (high sucrose (40 g/L) and PEG (1.0 mg/L). Treatment #6 (with low sucrose (20 g/L) and high ABA (1.0 mg/L)) had the second lowest number of cotyledonary embryos per plate at 2.6 +/- 1.5.

Statistical analysis found the effects of ABA, sucrose and ABA by sucrose interactions to significantly affect the number of embryos produced (Table 2b).

The percentage of embryos that had germinated completely (root, epicotyl and hypocotyl elongation) after 3 weeks in the growing room was calculated (Table 3). The maturation treatment that had the highest germination rate was #7 (low sucrose (20 g/L) low ABA (0.1 mg/L) and PEG (1.0 mg/L)) at 18 %. The maturation treatments #1-5 and #9 had very similar germination rates of 14 to 16%. The lowest germination rate was for treatment #6 with no germination, followed by #8 with 9% and #10 with 10% germination.

Table 2a. Average number of cotyledonary stage somatic embryos per plate after 6 weeks on maturation treatments.

Maturation Treatment	Average # of embryos/plate
1 (20 g/L sucrose)	7.0 +/- 3.2
2 (40 g/L sucrose)	6.9 +/- 3.6
3 (40 g/L sucrose, 0.1 mg/L ABA)	13.3 +/- 4.8
4 (40 g/L sucrose, 1.0 mg/L ABA)	4.7 +/- 3.0
5 (20 g/L sucrose, 0.1 mg/L ABA)	7.9 +/- 4.6
6 (20 g/L sucrose, 1.0 mg/L ABA)	2.6 +/- 1.5
7 (20 g/L sucrose, 0.1 mg/L ABA, 1.0 mg/L PEG)	4.8 +/- 3.16
8 (20 g/L sucrose, 1.0 mg/L ABA, 1.0 mg/L PEG)	2.6 +/- 2.6
9 (20 g/L sucrose, 1.0 mg/L PEG)	5.3 +/- 4.1
10 (40 g/L sucrose, 1.0 mg/L PEG	1.8 +/- 2.3

Table 2b. Anova table: Significance of ABA, Sucrose and ABA x Sucrose effects on the # of embryos produced.

Source	DF	Sum of Squares	F ratio	Prob>F
Model	5	590.0824	8.2	<0.0001
ABA	2	401.9042	13.9625	<0.0001
Sucrose	1	81.7713	5.6816	0.0192
ABA x Sucrose	2	193.6062	6.7260	0.0019
Error	94	1352.8776		

Table 3. Germination rate of embryos from ten maturation treatments after 12 weeks in cold storage, followed by 3 weeks under growing room conditions (22+/- 1 ° C and 16 h light). Germination requires the presence of a root, a hypocotyl and an epicotyl.

Maturation Treatment	% germination
1	15
2	14
3	16
4	14
5	14
6	0
7	18
8	9
9	14
10	10

Figure Descriptions

Figure 1 Light micrograph of a transverse section of a micropropagated rose leaflet, which was subjected to callus initiation treatment A (wounding and 2,4-D), showing the disrupted epidermis and mesophyll cells due to the formation of callus near the vascular tissues (arrows). PAS/ABB stain. Bar=390μm.

Figure 2 Light micrograph of a transverse section of a micropropagated rose leaflet, which was subjected to callus initiation treatment A (wounding and 2,4-D), showing the callus radiating outward from the vascular tissue (arrows). PAS/ABB stain. Bar=275μm.

Figure 3 Light micrograph of a transverse section of a micropropagated rose leaflet, which was subjected to callus initiation treatment A, showing a cluster of potentially embryogenic cells among the callus (arrows). These cells have large nuclei and darkly staining cytoplasms that are characteristic of embryogenic cells. PAS/ABB stain. Bar=20μm.

Figure 4 Light micrograph of a transverse section of a micropropagated rose leaflet, which was subjected to callus initiation treatment B (2,4-D alone), showing the disrupted epidermis (e) (arrow) and large intercellular spaces (is) among the colenchyma cells (col). The vascular tissue (vt) is intact and organized. Also present are bundle sheath cells (bs) and stomata (st). PAS/ABB stain. Bar=85μm.

Figure 5 Light micrograph of a transverse section of a micropropagated rose leaflet, which was subjected to callus initiation treatment B (2,4-D alone), showing the vascular tissue is intact and organized. PAS/ABB stain. Bar=40μm.

Figure 6 Light micrograph of a transverse section of a micropropagated rose leaflet, which was subjected to callus initiation treatment B (2,4-D alone), showing the disrupted epidermis (arrow) and large intercellular spaces among the parenchyma cells. PAS/ABB stain. Bar=40μm.

Figure 7 Light micrograph of a transverse section of a micropropagated rose leaflet, which was subjected to callus initiation treatment C (wounding alone), showing the epidermis (e) and vascular tissues (vt) are intact. There are some intercellular spaces (is) among the colenchyma (col) and parenchyma cells (par). PAS/ABB stain. Bar=85μm.

Figure 8 Light micrograph of a transverse section of a micropropagated rose leaflet, which was subjected to callus initiation treatment C, showing organized, intact vascular tissues (vt) with xylem (x) and phloem (p) surrounded by intercellular spaces (is), parenchyma (par) and colenchyma. PAS/ABB stain. Bar=50μm.

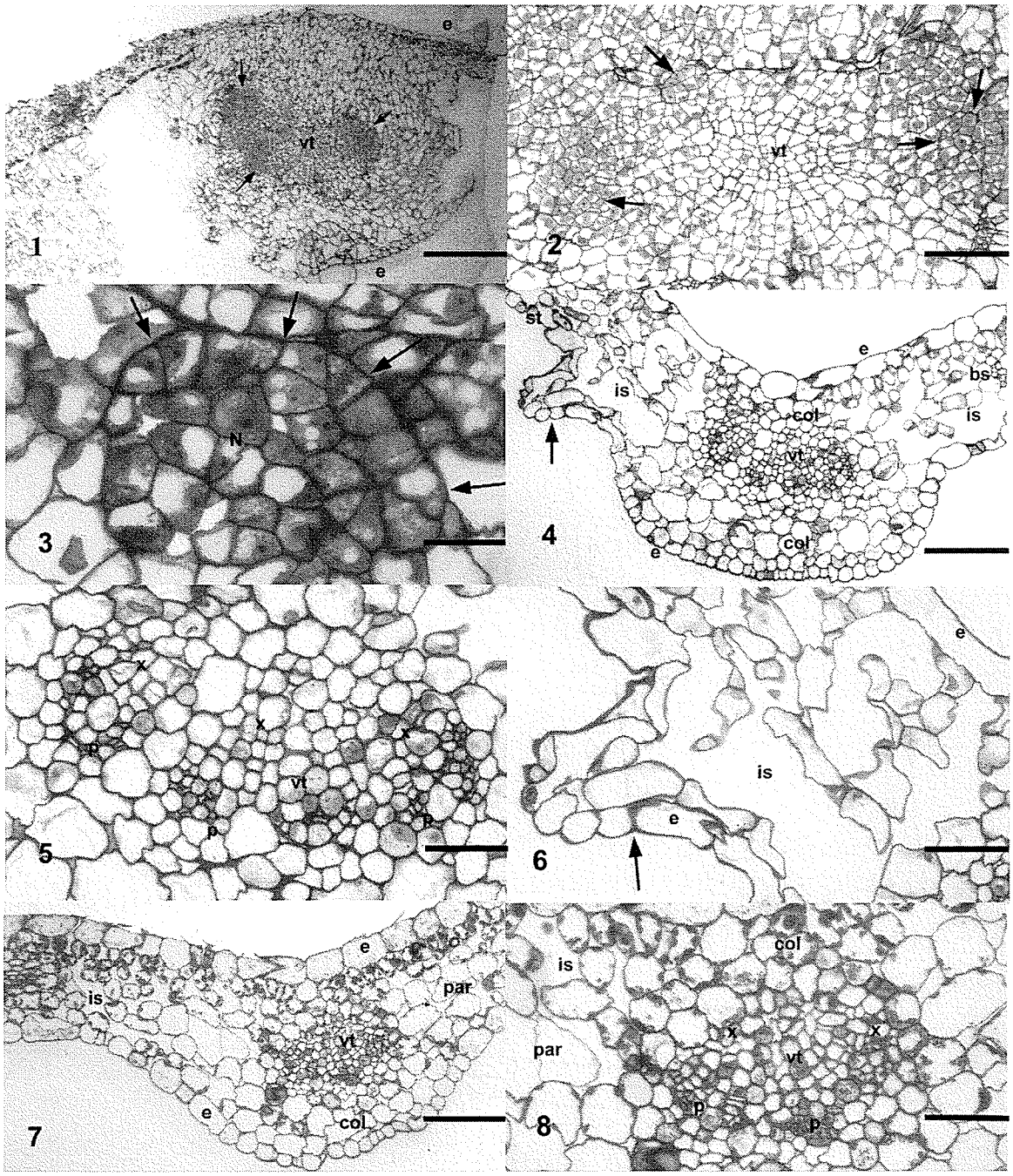
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Figure 9 Light macrograph of a micropropagated rose leaflet from callus initiation treatment A (wounding and 2,4-D) showing the formation of leaflet callus (lc) and phloem callus (pc) along the midrib (mr) of the abaxial surface of the leaflet where it was scratched (s). Ruler= 1 mm units.

Figure 10 Light macrograph of a rose leaflet which has been subjected to callus initiation treatment A for 10 weeks and then transferred to embryo initiation medium (without auxins) for 8 weeks. The cells of the leaflet have been transformed into clusters of brown non-embryogenic calli (nec) and lighter colored embryogenic tissue (et). PAS stain. Bar=150μm.

Figure 11 Light macrograph, showing the pro-embryogenic masses (pem) (arrows) among the embryogenic tissue (et) from figure 10. PAS stain. Bar=25μm.

Figure 12 Light macrograph of a mass of embryogenic tissue (et) that has been teased apart with a scalpel to better display the pro-embryogenic masses (pem), immature embryogenic tissues and non-embryogenic tissues (arrow). PAS stain. Bar=500μm.

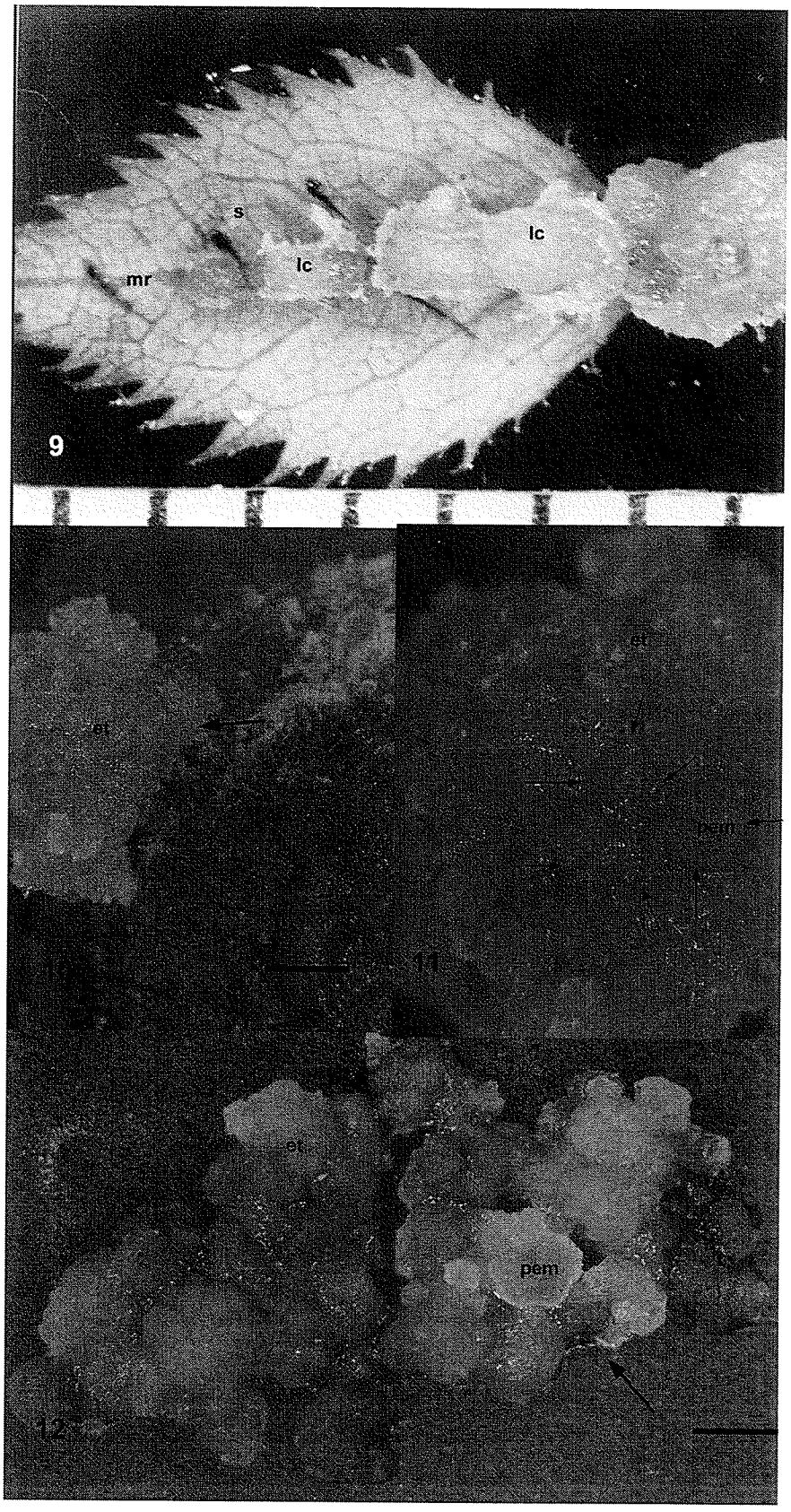
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Figure 13 Light micrograph of a section of a piece of callus derived from a rose leaflet cultured on callus initiation treatment A (wounding and 2,4-D). Non embryogenic calli, typically consist of highly vacuolated non-embryogenic cells. PAS stain. Bar=40μm.

Figure 14 Light micrograph of a section of a piece of embryogenic tissue derived from a rose leaflet which was on callus initiation treatment A for 10 weeks and then transferred to a embryo initiation medium (without auxins) for 6 weeks. Visible are typical, embryogenic cells and pro-embryogenic masses (pem). Note the presence of starch grains (sg) in the embryogenic cells. The transition zone is indicated by arrows. There are a few non-embryogenic cells present (nec). PAS stain. Bar=40μm.

Figure 15 Fluorescence micrograph of a section of embryogenic tissue (et) from a rose leaflet, showing the abundance of PAS positive material in the cell walls and the starch grains (arrow). Some non-embryogenic tissues are also visible (nec). PAS stain. Bar=80μm.

Figure 16 Fluorescence micrograph of a section of embryogenic tissue from a rose leaflet, showing the abundance of AB positive material (callose) (arrows) on the cell walls of pro-embryogenic masses (pem). Calcofluor and AB stain. Bar=80μm.

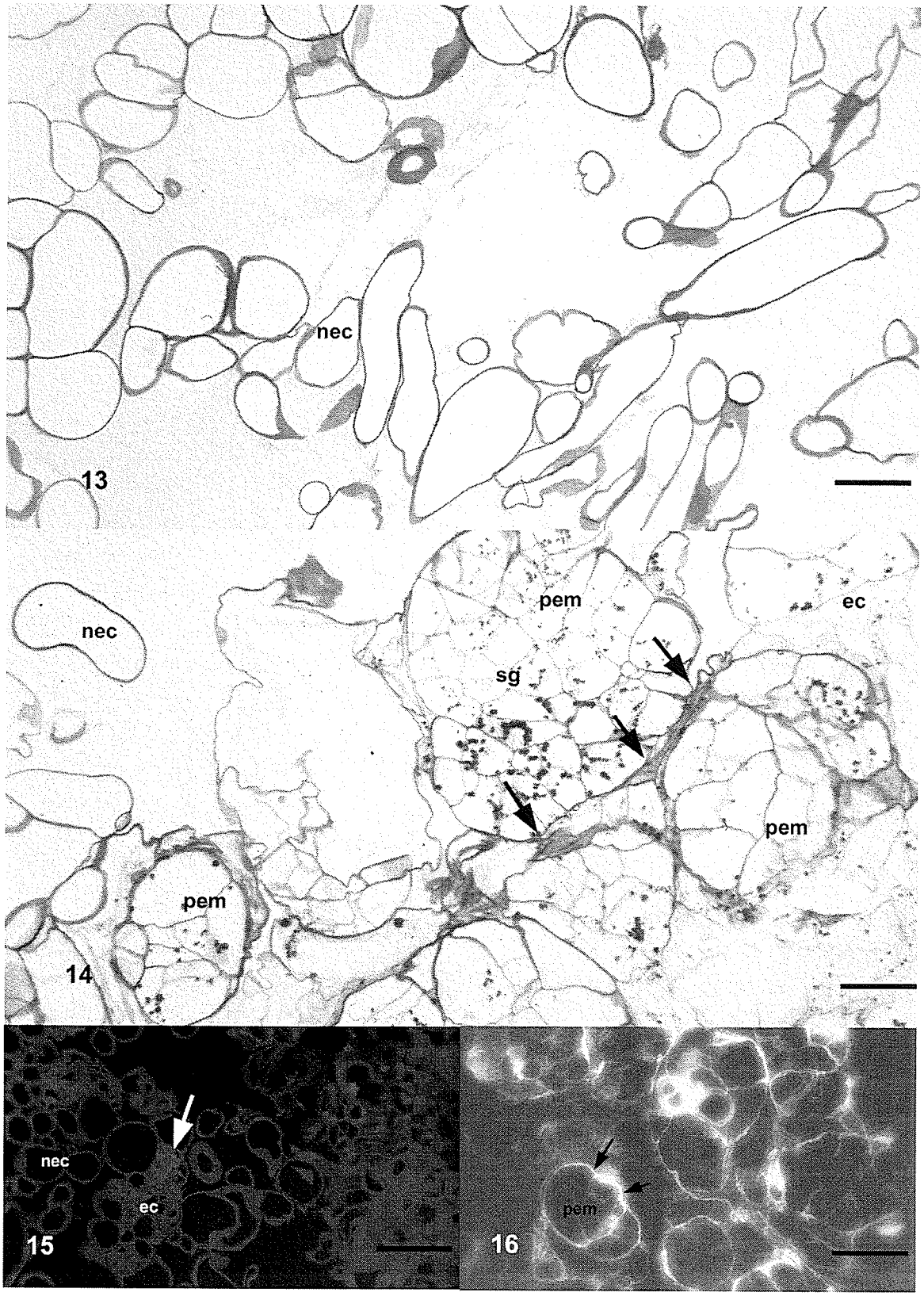
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Figure 17 Light macrograph of a typical culture of rose somatic embryos. Many somatic embryos of different morphological stages can be present simultaneously. Shown here are globular (ge) heart (he),and cotyledonary stages (cot). Bar=500μm.

Figure 18 Light macrograph of a mass of globular stage rose somatic embryos (ge) showing the different poles. Shown here are the proximal pole (pp) where the embryo attaches to the culture medium, medial distal pole (mdp) site of the future SAM; and lateral distal pole (ldp) site of the future cotyledons. Bar=900μm.

Figure 19 Light microgaph of a longitudinal section of a globular stage rose somatic embryo showing the protoderm (pd) (the first primary meristem to develop) and the vacuolated parenchyma cells. Also shown here are the proximal pole (pp) where the embryo attaches to the culture medium, medial distal pole (mdp) site of the future SAM; and lateral distal pole (ldp) site of the future cotyledons. PAS stained. Bar=95μm.

Figure 20 Light macrograph of heart shaped stage rose somatic embryo. At this stage the cotyledons are beginning to develop and an apical notch is present. The embryo appears to be yellowish and semi-transparent. Ruler= 1mm

Figure 21 Light micrograph of a longitudinal section of a heart shaped rose somatic embryo (he) showing the procambium (pc) and ground tissue (gt), (the next primary meristems to develop after the protoderm), as well as the cotyledons (cot) and apical notch (arrow). PAS/ABB stain. Bar=250μm.

Figure 22 Light micrograph of a longitudinal section of a heart shaped rose somatic embryo (he) showing the three primary meristems present at this stage; the protoderm (pd) the ground tissue (gt) and the procambium (pc). PAS/ABB stain. Bar=1054μm.

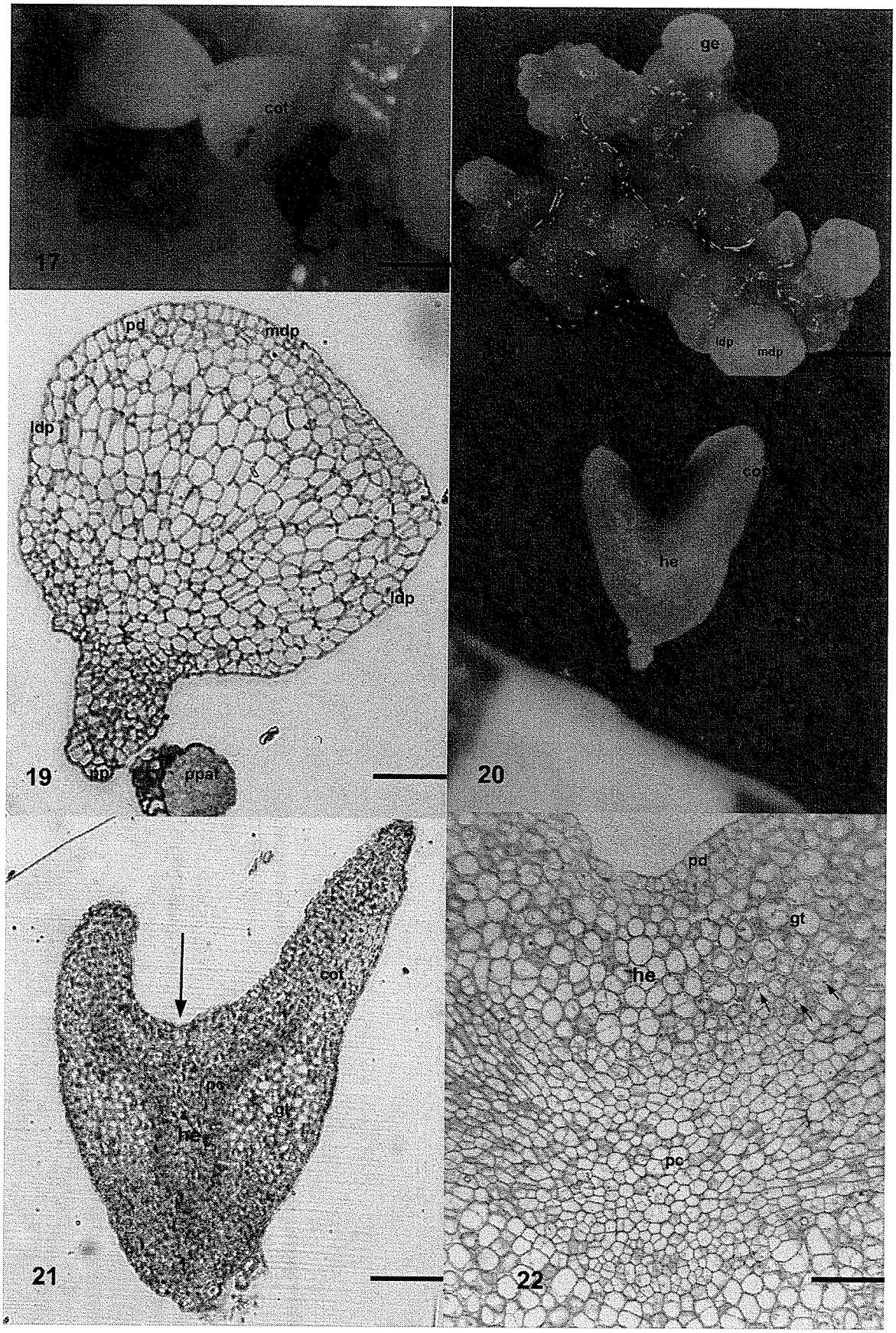
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Figure 23 Light macrograph of torpedo-shaped rose somatic embryo (te). The cotyledons (cot) continue to elongate. Arrow indicates the apical notch where the SAM should form. The embryo has become opaque and creamy white in color. Ruler= 1mm

Figure 24 Light micrograph of a longitudinal section of a torpedo shaped stage rose somatic embryo showing the procambium (pc), ground tissue (gt) and cotyledons (cot), however there is no evidence of a SAM at the shoot pole (sp). The RAM is present at the root pole (rp). PAS/ABB stain. Bar=250μm.

Figure 25 Light micrograph of protein storage bodies (arrows) in the torpedo stage rose somatic embryo. Bar=25μm.

Figure 26 Light macrograph of cotyledonary stage rose somatic embryo showing shoot pole (sp), root pole (rp) and cotyledons (cot). The cotyledons have begun to curve away from the main axis of the somatic embryo. Morphologically, the embryo is complete at this stage but storage bodies may continue to develop. Ruler= 1mm

Figure 27 Light macrograph of rose somatic embryos with three cotyledons (cot). Ruler=1mm.

Figure 28 Light macrograph of rose somatic embryos with single cotyledons (cot). Also visible is the proximal pole attachment tissue (ppat) at the root pole (rp). Ruler=1mm

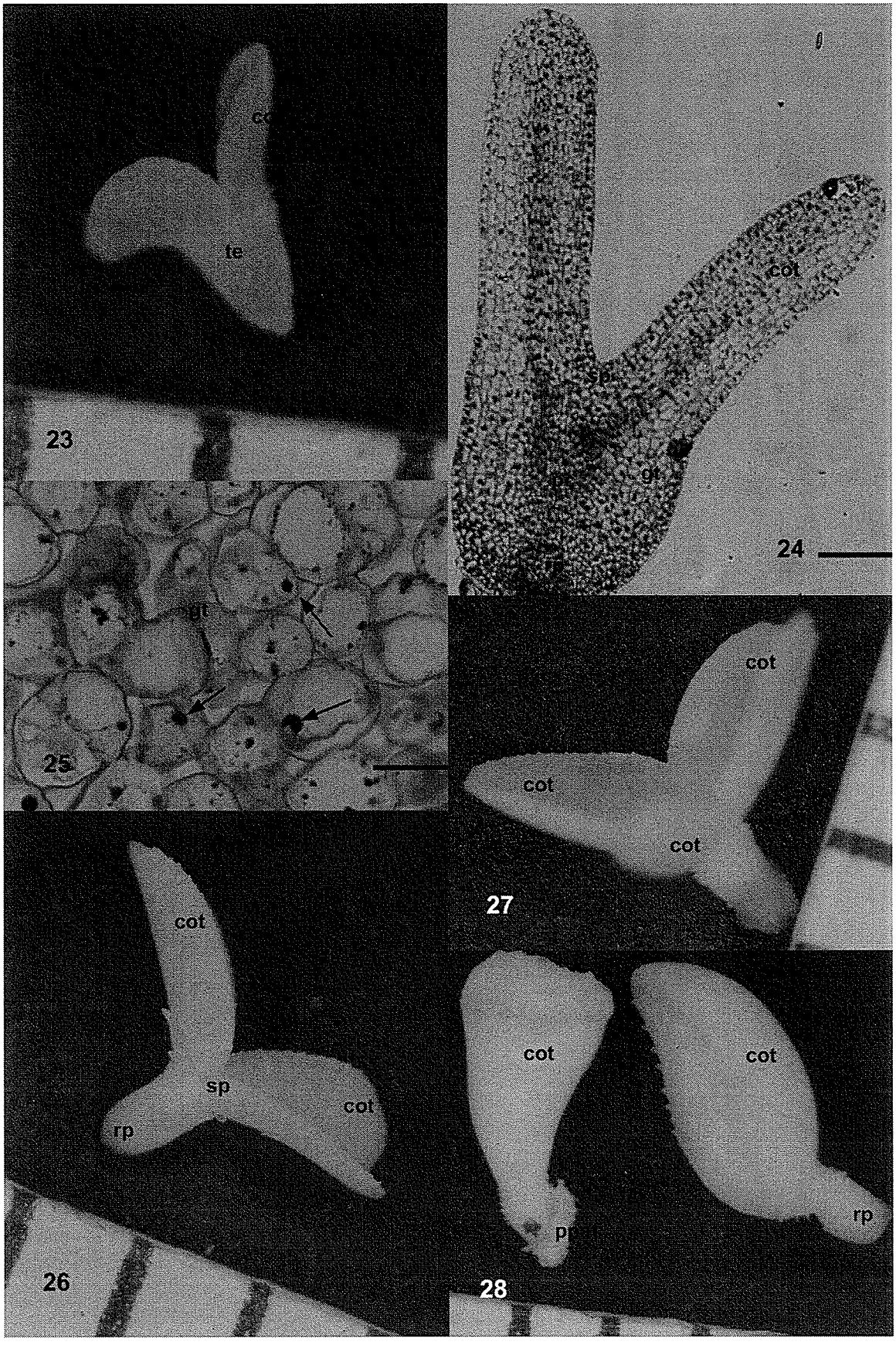
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Figure 29 Light micrograph of a longitudinal section of a cotyledonary stage rose somatic embryo showing a well developed SAM (arrow), ground tissue, procambium, protoderm and root pole. PAS/ABB stain. Bar=300μm.

Figure 30 Light micrograph of a longitudinal section of a cotyledonary stage rose somatic embryo showing a well developed SAM, with a tunica (t) and corpus (c) structure. Also visible is protoderm (pd), ground tissue (gt), procambium (pc) and part of the cotyledon (cot). PAS/ABB stain. Bar=125μm.

Figure 31 Light micrograph of a longitudinal section of a cotyledonary stage rose somatic embryo showing a poorly developed SAM (arrow), with large intercellular spaces (is) and parenchyma cells where the tunica and corpus structure should be. Also visible is protoderm (pd), ground tissue (gt), and procambium (pc). PAS/ABB stain. Bar=60μm.

Figure 32 Light micrograph of a longitudinal section of a cotyledonary stage rose somatic embryo showing a typical RAM. Also visible is protoderm (pd), ground tissue (gt), and procambium (pc). PAS stain. Bar=60μm.

Figure 33 Light micrograph of a cotyledonary stage rose somatic embryo showing some secondary structures (ss) arising from the protoderm (pd) adjacent to ground tissue (gt). PAS stain. Bar=60μm.

Figure 34 Light micrograph of a cotyledonary stage rose somatic embryo showing some protein bodies (arrows) in the ground tissues. PAS/ABB stain. Bar=50μm.

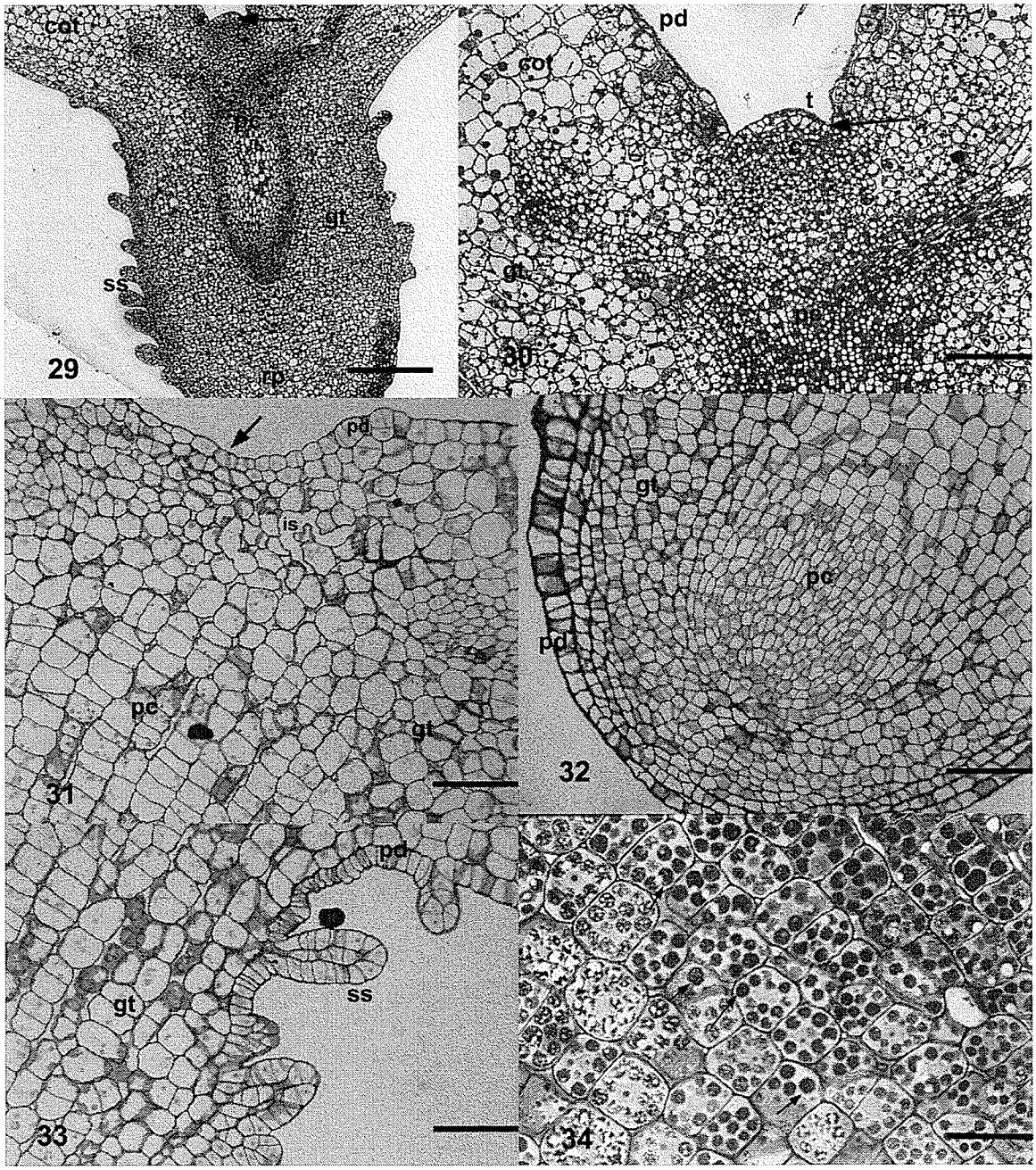
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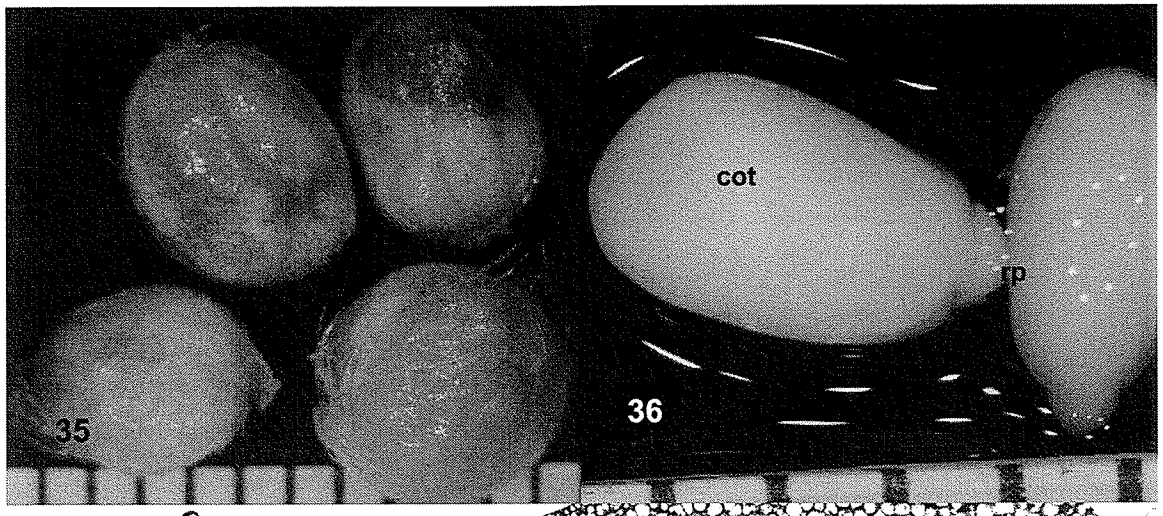
Figure 35 Light macrograph of mature rose seeds, showing the hard, brown seed coat. Ruler=1mm.

Figure 36 Light macrograph of mature rose zygotic embryos after their seed coats were removed. The cotyledon (cot) and root pole (rp) are labeled. Ruler=1mm.

Figure 37 Light micrograph of a longitudinal section of a mature rose zygotic embryo showing a typical organization of all of the primary meristems: protoderm (pd), ground tissue (gt), procambium (pc). Also labeled are the root pole and the cotyledons (cot). The shoot pole is indicated by the arrow. PAS/ABB stain. Bar=200μm.

Figure 38 Light micrograph of a transverse section of a mature rose zygotic embryo showing a typical organization of the RAM. There are also secondary structures (ss) protruding from the protoderm (pd), similar to those in the mature somatic embryo. Ground tissue (gt) and procambium (pc) are also visible. PAS stain. Bar=20μm.

Figure 39 Light micrograph of a longitudinal section of a mature rose zygotic embryo showing a typical organization of the SAM. Notice the tunica (t) corpus (c) pattern. Arrows indicate protein bodies.PAS/ABB stain. Bar=60μm.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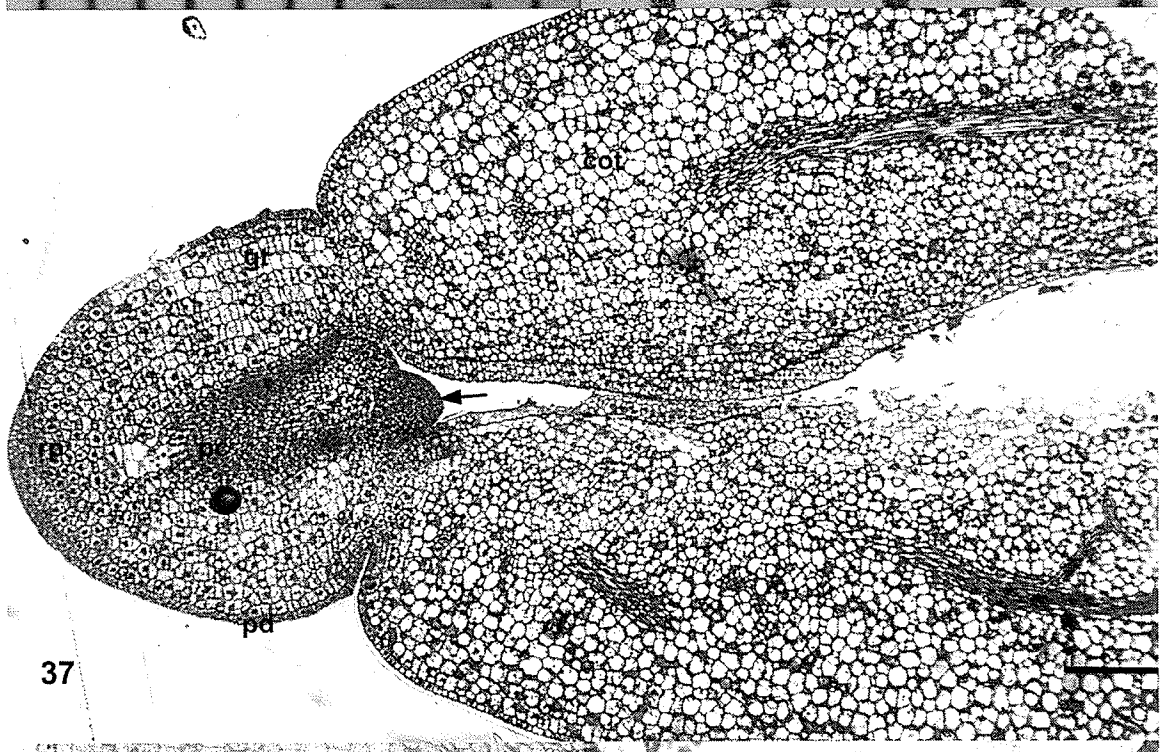
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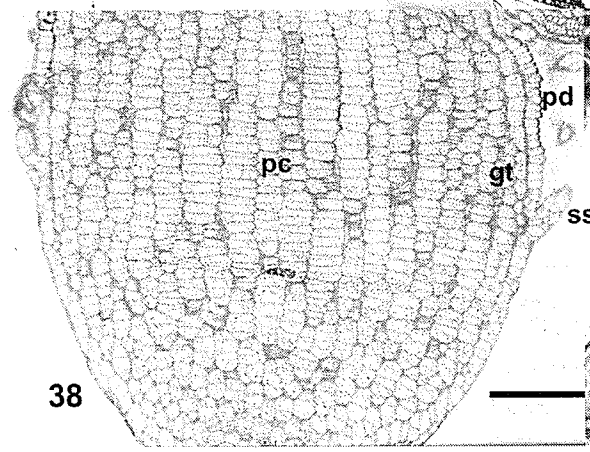
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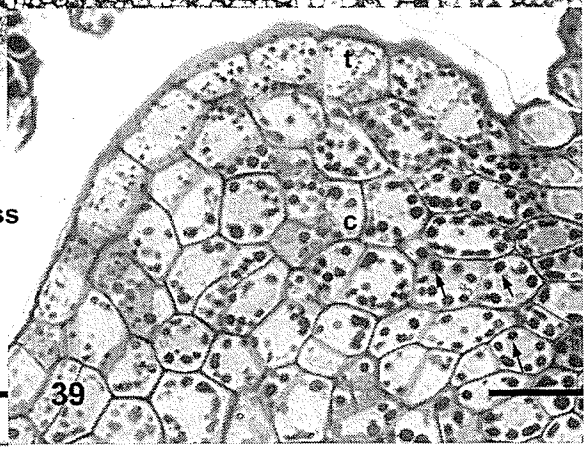
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Figure 40 Light micrograph of a transverse section of a mature rose zygotic embryo showing the accumulation of dark blue stained protein bodies in the cotyledons. PAS/ABB stain. Bar =30μm.

Figure 41 Light micrograph of a transverse section of a mature rose zygotic embryo showing the accumulation of dark blue stained protein bodies in the ground tissue of the hypocotyl. PAS/ABB stain. Bar =30μm.

Figure 42 Light micrograph of a transverse section of a mature rose zygotic embryo showing the accumulation of dark blue stained protein bodies in the protoderm. PAS/ABB stain. Bar =30μm.

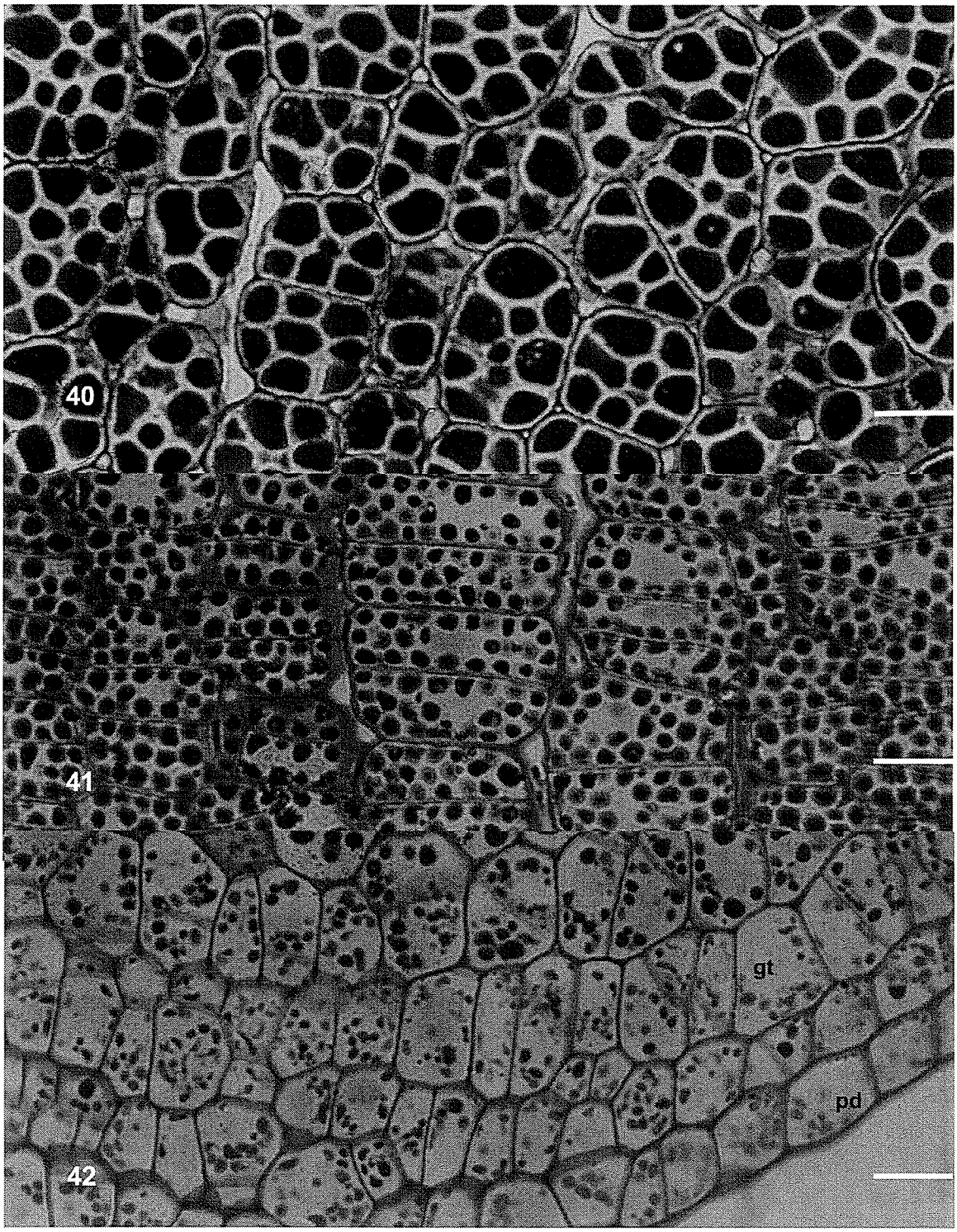
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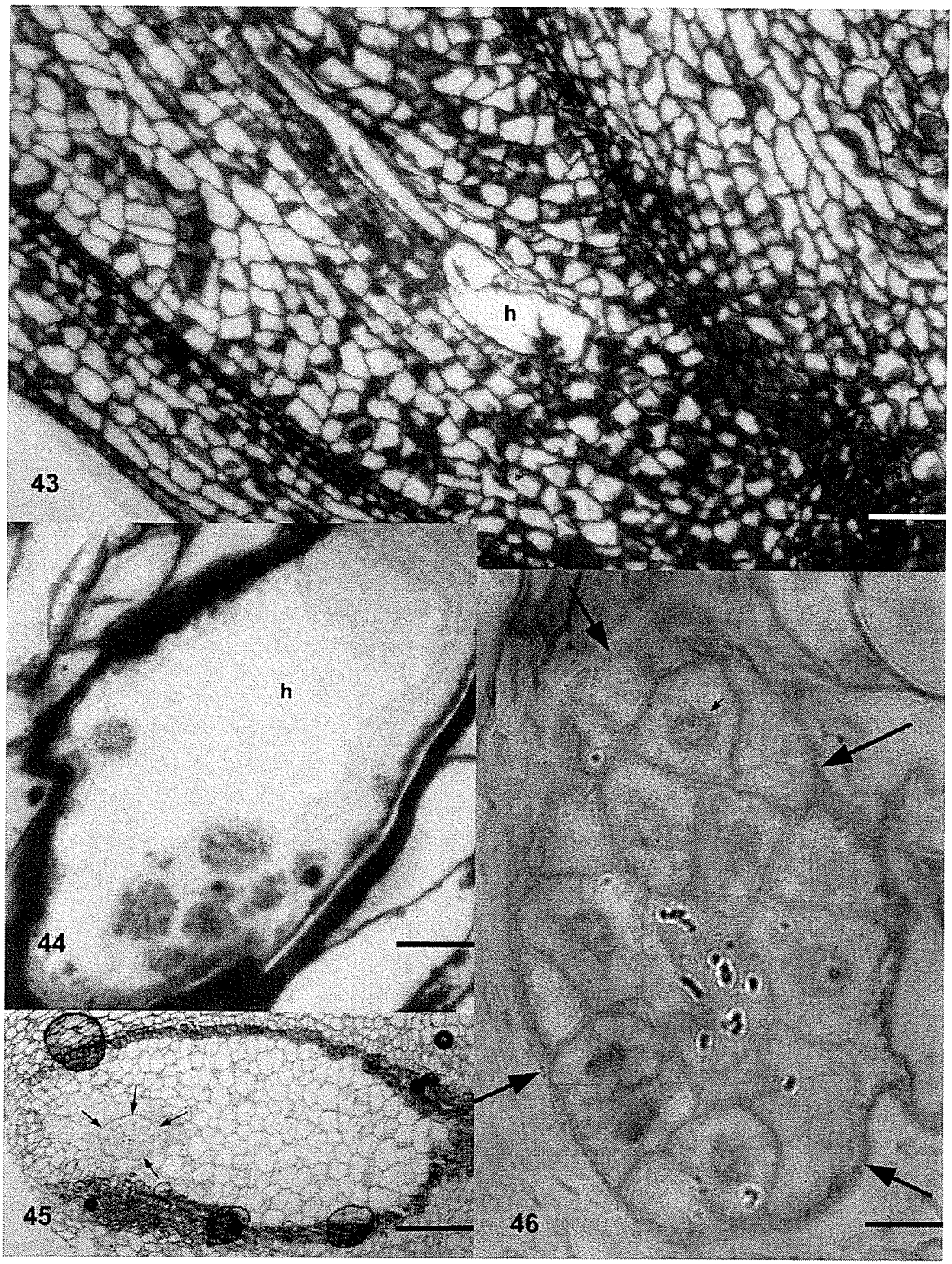
Figure 43 Light micrograph of a longitudinal section of an immature rose zygotic embryo showing a hole (h) where the embryo should be. PAS/ABB stain. Bar= 400μm.

Figure 44 Light micrograph of a longitudinal section an immature rose zygotic embryo showing a closer view of the seed coat, a hole (h) and some undetermined cells where the embryo should be. PAS/ABB stain. Bar=200μm.

Figure 45 Light micrograph of a longitudinal section an immature rose zygotic embryo showing some undetermined cells that are possibly embryogenic (arrows). Toluidine blue stain. Bar=400μm.

Figure 46 Light micrograph of a longitudinal section an immature rose zygotic embryo showing a closer view of the undetermined cells with their large nuclei (arrows). Toluidine blue stain. Bar=200μm.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Discussion

Callus Initiation

Callus initiation is the first stage in indirect somatic embryogenesis protocols (Ammirato 1983; Williams and Maheswaren 1986). Callus is believed to be a necessary precursor in indirect somatic embryogenesis protocols. Recall that there are two ways to achieve somatic embryogenesis, a direct method (somatic embryos derived directly from a single cell) and an indirect method (somatic embryos resulting from the dedifferentiation and re-differentiation of several cells). The most successful method for John Davis roses is the indirect method and therefore that is the method used in this study.

After five to 10 days, wounded, micropropagated rose leaflets explanted onto a callus initiation medium containing 5 mg/mL 2,4-D, will form a callus. However, if either the wounding treatment or the auxin was omitted, a callus did not form. This indicates that wounding and auxin treatments may have a synergistic effect on callus initiation.

Auxins are known to stimulate cell division and callus initiation in cells. 2,4-D has been used successfully in many callus initiation protocols for somatic embryogenesis (Evans et al. 1981). 2,4-D enters plant cells, by carrier mediated transport, involving influx carriers. However, it is not excreted as efficiently by the efflux carriers, leading to a build up of 2,4-D inside the cells (Imhoff et al. 2000). When this happens, 2,4-D acts like a typical auxin, causing cell elongation, division, changes in the plasma membrane's electrical properties, increasing the H+-ATPase activity, acidification of the apoplast and cell wall loosening (Macdonald 1997).

Cells differ in their response to exogenously applied auxins. The maturity and level of differentiation of a cell have been suggested as factors affecting callogenic response (Levi and Sink 1991). The structure of the micropropagated leaflet (Johansson et al. 1992) shows that there are immature, undifferentiated

cells associated with the vascular bundle that would normally differentiate into phloem or xylem tissues. Immature vascular elements respond readily to treatment with auxins. For example, young xylem and phloem elements of carrot seedling hypocotyls are the first to cells to begin dividing a few days after explanting on to a callus initiation media containing auxins (Guzzo et al. 1994). Similarly, wounded John Davis rose leaflets explanted onto a callus initiation medium for seven days will yield a callus along the midrib and vascular bundles. Although it is difficult to trace the origin of the callus back to individual cells, it appears that the immature cells of the vascular bundle are the primary contributors to the observed calli.

Epidermal cells of the John Davis micropropagated rose leaflets are not able to dedifferentiate and form a callus when placed in direct contact with media containing 5 mg/L 2,4-D for 10 days. The epidermal cells of micropropagated rose leaflets do not have a cuticle or waxy layer that could act as a protective barrier (Johansson et al. 1992), thus it doesn't seem that this would explain the lack of response. It is possible that the epidermal cells will eventually form a callus, as some of the epidermal cells appear to be abnormally elongated. (Cell elongation is known to be one type of auxin effect.) However, the epidermal cells may act as a barrier preventing or retarding the auxins from reaching the parenchyma cells they enclose. Therefore, one way wounding may contribute to the callus response is by disturbing the epidermal cells. When the epidermis is disturbed via wounding, 2,4-D is able to penetrate past the epidermis and cause a callus response in the parenchyma cells.

Another way wounding may contribute to a callus response is by inducing a wound response. When a plant tissue is wounded a variety of physiological and anatomical changes are triggered (Leon et al. 2001). The purpose of a wound response is to prevent desiccation, provide protection from pathogens and herbivores and facilitate healing. The type of response elucidated depends on the species and the type of wound incurred. For example, Rittinger et al. (1987) studied the wound responses of twelve different species (including

spruce, cedar, ferns, barley, cucumber, apple and sour cherry). The wound responses of many of these species included a deposit of lignin and suberin. Interestingly, the apple foliage did not show any lignification at the wound site. However, the apple leaves were wounded as a result of a fungal infection, while all of the other species were wounded by cutting the tissues with a razor blade. This suggests that the type of wound can affect the plant's response.

In herbaceous and woody dicots, cell proliferation is a common wound response. Rittinger et al. (1987) observed that cell division was induced in the cells next to the wounded cells of the sour cherry foliage (that was cut with a razor blade). Similarly cell division as a result of the scalpel scratches may have been stimulated in the John Davis rose leaflets. Possibly the exogenously supplied auxins were able to maintain and increase the cell division signals initiated by the wounded leaflet in response to the wound. However the control leaflets in treatment C (wounding without auxins), did not reveal any layers of cells near the wounds. This may be because the leaflets were observed at 7 days after wounding, and had not divided yet. In the Rittinger et al. (1987) study the new cell layers in the sour cherry leaves were observed fourteen days after wounding.

Within a few minutes to a few hours after wounding, specific signals for the activation of wound response genes are generated (Leon et al. 2001). The proteins encoded by the defense genes may have a role in tissue repair, producing substances to inhibit herbivory, participate in wound signaling pathways, or adjust the plant's metabolism to the imposed nutritional demands (Leon et al. 2001).

Plant growth regulators that have been reported to accumulate in wounded tissues include jasmonic acid, ABA and ethylene. It is interesting to note that Gaspar (1996) reports that jasmonic acid, has an inhibitory effect on callus production. In fact, jasmonic acid, ethylene and ABA are all known to inhibit cell division and cell growth. Therefore if these three hormones were produced by the wounded John Davis rose leaflets, one would not expect a

callus to form. The high levels of exogenously supplied auxins may drown out any ABA or jasmonic acid effects. On the other hand, the high levels of auxins in the culture would be expected to increase ethylene synthesis from the tissue.

Auxins may have a negative effect on the expression of wound inducible genes. The endogenous levels of IAA were found to decline in wounded tobacco tissues (Thornburg et al. 1991). So the high levels of exogenous auxins in the culture medium may have overridden the jasmonic acid and ABA induced genes that prevent cell division, growth and callus production. Therefore a callus formed.

Callus Characteristics

In carrot cell suspension cultures derived from carrot phloem tissue, two types of cells with embryogenic potential have been identified: namely, spherical, vacuolate cells that are ~12μM in diameter, and spherical, cytoplasmically rich cells that are also ~12 μM in diameter (Nomura and Komamine 1985). On the other hand, elongated vacuolate cells were found to give rise to very few somatic embryos (Nomura and Komamine 1985). Similarly, when a piece of callus derived from John Davis rose leaflets is viewed in cross section, two types of cells can be observed: elongated, vacuolated non-embryogenic cells; and spherical, cytoplasmically rich meristematic cells (Figure 14). The John Davis rose leaflet callus is initiated near the vascular bundles and radiates outwardly. The majority of the cells in the callus are elongated vacuolate, non-embryogenic, e.g. these cells are not meristematic and do not produce somatic embryos. Towards the center of the callus, surrounded by non-embryogenic cells, are meristematic cells. These cells are isodiametric, with darkly staining cytoplasm, possibly capable of differentiating into callus cells. In contrast, the older callus clusters appear to be composed entirely of non-embryogenic cells. It could be that the meristematic cells were just missed in the sections due to their relative scarcity. Or perhaps the embryogenic cells cease to exist in the older clusters.

The incubation of John Davis rose leaflet calli on a medium free from auxins results in the emergence of clumps of embryogenic tissue (containing pro-embryos). First, the yellowish calli turns brown after a few days on this new medium. This browning could be due to the death of the outer calli cells due to the lack of auxins, or oxidation as a result in changes in the gases in the culture vessel. With the transfer to the new media, the environment of the culture changes. The tissues are relieved from the build up of gasses, such as ethylene (recall it is also a plant growth regulator associated with wounding), and waste products present in the old culture. The new environment offers a fully replenished complement of nutrients. However, if a callus is transferred to a fresh medium containing high levels of auxins, browning is less severe but somatic embryos do not develop. It seems that new resources combined with the absence of auxins may provide a better environment for embryogenic tissue to proliferate, while hampering the survival and proliferation of callus cells. Once embryogenic tissue has been established and isolated the amount of tissue can be increased using low levels of auxins (<1-2mg/mL).

After 6-10 weeks on this new medium a cream colored mass of embryogenic tissue will appear on the top or sides of the calli. The embryogenic tissue exists only on the surface of the callus and cannot be traced below the surface. The embryogenic cells are closely associated into masses called pro-embryogenic masses, first described and characterized in *Daucus* cultures by Halperin (1966). These masses are the precursors to globular stage embryos, but organized tissues such as a protoderm cannot be identified at this stage. Pro-embryogenic masses are often isolated from the non-embryogenic tissues and each other by one or several mechanisms discussed later, e.g. a transition zone.

PAS and ABB staining reveal that in John Davis rose cultures, the cells of pro-embryogenic masses contain starch grains and protein bodies. Protein bodies and starches were absent in non-embryogenic callus cells, indicating a different metabolic program is operating in these cells.

Isolation/ Transition Zones

The isolation of pre-globular embryos by a thickened cell wall, a callose layer and the disappearance of plasmodesmata has been observed in embryogenic cultures of several species such as *Daucus* (carrot), *Vitis* (grapevine), *Cocos* (coconut) and *Cichorium* (chicory) (Wetherell 1984; Faure 1996; Verdiel et al. 2001; Dubois et al. 1990). Some callose deposits were present on the outer cell walls of John Davis rose pro-embryogenic masses. Also, thickened cells walls and zones of non-embryogenic and collapsed cells, termed transition zones, surround the pro-embryogenic masses. Calcofluor staining of the rose leaflet calli did not reveal the presence of plasmodesmata connecting the pro-embryogenic masses to other tissues. Thus it seems that isolation of pre-globular embryos is a common characteristic of somatic embryos of many species including John Davis roses.

Williams and Maheswaran (1986) have suggested that embryogenic tissue must be isolated from non-embryogenic parent tissue for embryogenesis to occur because they have different metabolic programs and needs (e.g. embryogenic tissues accumulate storage bodies while non-embryogenic tissues do not). The disrupted cells observed in the transition zones around the John Davis rose somatic embryos may be an artifact of preparation for microscopy or they could be breaking down in response to the changes in the culture. In *Camellia* cultures the thick walled proembryos were separated from the nonembryogenic tissue as the inner layers of parent tissue degraded (Barciela and Vietez 1993). Franz and Schel (1991) also observed degradation of tissues surrounding the somatic embryos and suggested that the degrading tissues serve as a source of nutrition for the developing embryogenic cells. The observation of starch accumulations in the degrading *Camellia* cells supports this hypothesis, as starch metabolism would provide a source of energy for the developing embryos (Franz and Schel 1991). In John Davis rose cultures

however, starch grains were only observed in the pro-embryogenic masses, never in the non-embryogenic callus cells. Therefore the breakdown of non embryogenic cells in the transition zone does not provide the embryogenic cells with a source of nutrition from starch.

The transition zone is also responsible for the extremely friable nature of embryogenic tissue. The transition zones of non-embryogenic cells between the pro-embryogenic masses serve as the fault line.

Polarity

Polarity is established early on in zygotic embryogenesis (Jurgens 2001). In the zygote, soon after fertilization, there is a reorganization of organelles followed by an asymmetrical division resulting in a basal cell (future suspensor) and a cell that will give rise to the embryo. It has been suggested that an asymmetrical cell division is also necessary for the initiation of somatic embryogenesis (McCabe et al. 1997). An initial, asymmetrical division has been demonstrated during somatic embryogenesis of both Vitis and Daucus (Faure et al. 1996; Halperin 1966). Faure et al. (1996) demonstrated that somatic embryogenesis in Vitis begins with an asymmetrical division yielding two cells of different structure. A larger basal cell, which would yield the suspensor and a smaller apical cell, which would give rise to the embryo. However, in the present study, the first evidence of polarity in somatic embryos was observed at the globular stage. Distinct poles are visible at this stage (e.g. the point of attachment to the callus is the proximal pole and the future site of the SAM and cotyledons are the median and lateral distal poles respectively).

Unlike Vitis somatic embryos, a suspensor was not observed to be associated with rose somatic embryos. However a suspensor may have been present, but was obscured by the proximal pole attachment tissue. This tissue is present in all stages of somatic embryos, although it is reduced in the later stages. This tissue occurs at the proximal pole and anchors the embryo to the

rest of the embryogenic tissue and/or to the culture medium. It is possible that nutrients may be transferred to the developing embryo via the proximal pole attachment tissue, yet this tissue is a mass of cells and does not resemble a suspensor.

It is not known how polarity is influenced. Some reports suggest that polarity is controlled maternally. For example, seedlings derived from sin1 mutant mother plants show defects in the apical basal axis regardless of the paternal gene input (Ray et al. 1996). This suggests maternal control over polarity. Conversely, maize zygotes formed from isolated egg and sperm cells in vitro acquired polarity before their first asymmetrical division indicating that maternal influence may not control polarity (Breton et al. 1995). Similarly somatic embryos of Daucus and Vitis demonstrate polarity from the first division.

Embryogenesis

Both the zygotic and the somatic embryo go through four main morphological stages of development: the globular, heart shaped, torpedo shaped and cotyledonary stages (Krishnamurthy 1994).

It is very difficult to obtain and fix rose zygotic embryos. Mature rose hips of John Davis crosses, contained 5-10 embryos enclosed in a very hard seed coat, during removal from which, the embryo is often damaged. In the immature rose hips there were fifty or more carpels that may or may not be enclosing embryos. However maybe only 5 to 10 carpels contained viable embryos, as that is how many were present in mature hips. Perhaps the others were not fertilized, or were aborted. At the earliest stages they are all morphologically identical. It is also possible that the failure to obtain the earlier zygotic stages is due to poor fixation. Trying to improve fixation by increasing its duration, applying a vacuum (for fixation, dehydration and infiltration), and removing the seed coat, still may not improve the success of obtaining the earliest stages of

rose zygotic embryos. The young seeds often had huge holes in them (where the embryo should have been) when they are sectioned.

Burger et al. (1990) had success fixing rose zygotic embryos using formalin-propanoic acid alcohol and infiltrating them in paraffin. They have described and illustrated zygotic embryogenesis in four stages of rose zygotic embryogenesis: 7, 28, 56 and 75 days post pollination. This translates into the pre-globular, late heart, torpedo and cotyledonary stages. From their study, the morphology and development of rose zygotic embryos was observed to be similar to that of somatic embryos.

Primary Meristems

In general all of the tissues in the zygotic embryo are well organized compared to those of the somatic embryo, likely because the environmental conditions encountered by the zygotic embryos are much different than those the somatic embryos are subjected to. For example the protoderm of the zygotic embryo is made up of uniform cells, while the protoderm in the somatic embryo is usually made up of cells of irregular sizes. Likewise, the organization of the SAM in zygotic and somatic embryos can vary greatly. The SAM in zygotic embryos is arranged in a tunica corpus pattern with fairly uniform cells, but in somatic embryos a SAM may not even develop if the culture conditions are not ideal. If a SAM is present, it may be composed of irregularly sized and shaped cells, and the tunica corpus pattern may be incomplete.

The protoderm is the primary tissue from which the epidermis will arise. The protoderm is the first of the primary tissues to develop, and is first observed in the globular stage of somatic embryos. In somatic embryos, especially in the earliest stages, it is not uncommon for the cells of the protoderm to exhibit irregular shapes and sizes (Krishnamurthy 1994). This holds true for rose somatic embryogenesis. Although not observed at the pre-globular and globular stages of zygotic embryogenesis, the protoderm in later stages appears to be

well organized, e.g., it is made up of isodiametric cells, which only divide periclinally (Burger et al. 1990).

The ground tissue and procambium give rise to the tissues responsible for storage and for transport, respectively. The ground tissue and procambium begin to develop around the same time in both zygotic and somatic embryos, e.g. the late globular to early heart shaped stages. Unlike the protoderm these tissues are fairly well organized in somatic embryos, e.g., they are made up of uniform cells.

RAM and SAM

The root apical meristem (RAM) is the region of the embryo that will produce the root upon germination. The beginnings of the RAM can sometimes be observed in the torpedo stage rose somatic embryo, and by the cotyledonary stage, the RAM is fully developed. In *Arabidopsis* zygotic embryos the origin of the RAM can be traced back to the octant stage (Jurgens 2001).

In *Rosa* the RAM is of the open variety, with continuous files of cells that span the cortex and the root cap. It is structurally similar in both zygotic and somatic embryos.

In zygotic embryos of *Brassica napus*, the patterns of cell division are so regular that a distinct lineage can be identified for each tissue (Yeung et al. 1996). Yeung et al. (1996) were able to identify the cells in the apical portion of the early *Brassica napus* globular embryo that are the precursors of the SAM. These are called the epiphyseal cells. The patterns of cell division in somatic embryos are irregular, thus defining cell lineages is not an easy task. The epiphyseal cells were not identified in any rose embryos in this study.

The SAM is often deformed or absent in somatic embryos, leading to one of the most common problems: a low conversion rate (germination failure) Nickle and Yeung 1993). The presence of a SAM is not commonly observed in John Davis rose somatic embryos (e.g. only 1 was found out of approximately 100

cotyledonary stage somatic embryos sectioned). In most cotyledonary stage John Davis rose somatic embryos there are intercellular spaces, and an irregular protoderm where the SAM should be. In the exceptional case where a SAM is observed, it has a *tunica corpus* pattern, similar to the zygotic embryo. On the other hand, all of the cotyledonary stage zygotic embryos had a well developed SAM.

Both structural (Yeung et al. 1996) and molecular genetics (Mayer et al. 1998) studies indicate that the SAM is initiated prior to the globular stage of development. Ramsean-Fortner and Yeung (2000) have found that SAM development in pre-globular and globular embryos is extremely vulnerable to unfavorable environments. To test this, they applied TIBA (an auxin transport inhibitor) to both globular and heart shaped stage *Brassica* somatic embryos and found that abnormal SAM development (lack of *tunica corpus* type of SAM) occurred only in the globular stage embryos. Thus they concluded that during this period, (stages prior to and including the globular stage), culture conditions need to be conducive to meristem formation (e.g. auxin transport should not be interrupted). They also concluded that after the heart stage is reached the SAM is established and its organization is not disturbed by exogenous influences. Therefore it is likely that the culture conditions for the preglobular to gobular John Davis rose embryos must be ideal for SAM development to proceed.

There are a few hypotheses regarding the role of polar auxin transport and SAM formation. It has been suggested that auxin synthesis occurs in the apical half of the embryo. The auxin is then transported to the basal pole (towards the RAM). Liu et al. (1993) suggests that this transport system could lead to a pocket in the apical notch where the concentration of auxin is lower than in the surrounding cells. They showed that when a polar auxin inhibitor such as TIBA is used, auxin does not get transported to the basal part of the embryo. As a result a ring of cotyledonary tissue develops instead of two separate cotyledons and SAM formation is disrupted (Liu et al. 1993). This indicates that high levels of auxin are not conducive to SAM formation.

It is also possible that the epiphyseal cells (SAM precursors) respond differently to polar auxin transport and can adopt a different fate from the cells around them. They may be able to react differently to auxins. Ramsean-Fortner and Yeung (2000), suggest that perhaps the unique genetic expressions of epiphyseal cells are able to modify the auxin levels via conjugation or degradation or maybe they can alter their sensitivity to auxin by changing the number of auxin receptors available.

Storage Bodies

Using a PAS/ABB stain, starch grains and protein bodies are observed in the embryogenic tissue and in the ground tissue of the heart shaped somatic embryos and beyond. Not surprisingly, the amount of storage protein increases with the age of the embryos. It seems that as the embryo matures, the stream of nutrients is channeled from growth to accumulation of reserves. The cotyledonary stage zygotic embryos have more storage proteins than their somatic counterparts.

Plant growth regulator levels can affect the development of starch grains. The development of starch grains in cultured tobacco cells is stimulated by a reduction of auxins in the medium, while an increase in cytokinin levels stimulates their development (Miyazawa et al. 1999). Rose embryogenic tissue is matured on an auxin free medium and this may explain the presence of starch grains in the pro-embryogenic masses but would not explain the disappearance of starch grains in the globular embryos. Perhaps all of the storage reserves in the embryogenic tissue are used up when the metabolic programs of the pro-embryogenic masses cells switch to support the many divisions needed to form the globular embryo.

Although most of the proteins in seeds have structural or metabolic roles, all seeds contain at least one group of proteins that serves as a source of amino acids during germination (Shewry et al. 1995). The development of storage

proteins may be regulated by nutrition as protein bodies often act as a sink for excess nitrogen. Storage proteins are produced at high levels in specific tissues and at certain stages of development (Shewry et al. 1995). In the case of rose somatic embryos, storage proteins were observed in all stages except the globular stage. The amount of protein increases with maturity and as the amount of storage materials increases the appearance of the somatic embryo changes from semi-transparent to opaque.

Lipids were expected to be present in the mature embryos. However, when sudan black stain was used to detect lipids in somatic and zygotic embryos, none were observed. It is likely that the lipids were unintentionally removed during sample preparation. Glutaraldehyde has been found to destroy lipids in samples during the fixation process (O'Brien and McCully 1981).

Secondary Structures

An interesting characteristic of both the somatic and zygotic embryos is the secondary structures protruding from the protoderm in the hypocotyl region. These structures are made up of a peninsula of parenchyma cells surrounded by the epidermis. They are more abundant on somatic embryos (5-10 structures per embryo) than on zygotic embryos (1-3 structures per embryo)

There are two hypotheses regarding their function. These structures may be the beginnings of prickles. Prickles (often mistakenly called thorns) are modifications of the epidermal cells. A cross section of a mature rose prickle by Sengbusch (2001) shows that epidermal cells, underlying bark tissue and some parenchyma cells make up the prickle, but no vascular tissues. Similarly, the structures observed on the rose somatic embryos do not have any vascular tissues and are only composed of epidermal and parenchyma cells.

The other hypothesis is that these are the beginning of secondary embryos. Secondary embryos are very common in somatic embryogenesis cultures however they usually form at the base of the embryo (near the RAM) or

from the proximal pole attachment tissue. Therefore, it is doubtful that these are secondary embryos.

Odd number of cotyledons

Somatic embryos with one to three cotyledons are common. Somatic embryos that also had a single, fused ring of cotyledonary tissue instead of two cotyledons, were described by Liu et al. (1993). They attributed this fused structure to high levels of auxins in the apical portion of the embryo during SAM development. It would seem logical that the odd cotyledon morphologies observed in rose somatic embryos, were also due to abnormal levels of auxins. Polar transport inhibitors might be used in an attempt to determine if that is what was happening.

Maturation and Germination

In an attempt to replicate the natural conditions of maturation, certain components, such as ABA and osmoticum are often added to the culture medium.

ABA is a plant growth regulator that has been linked to dormancy and found to improve the development of somatic embryos (Nickle and Yeung 1993). For example, histological comparisons were made between larch somatic embryos matured on media with and with out ABA. After four weeks, the somatic embryos cultured on media containing ABA developed normally compared to the somatic embryos cultured on the ABA free media, e.g. the somatic embryos matured on the ABA free media had large vacuolated cells (Gutmann et al. 1996). Similarly, carrot somatic embryos, grown in the presence of ABA have normally developed SAMs, e.g. they have uniform cells without large vacuoles or intercellular spaces (Nickle and Yeung 1994). In the present study, lower levels of ABA (0.1 mg/ml) were associated with higher numbers of mature rose somatic

embryos than the treatments that included higher levels (1.0 mg/ml). The rose somatic embryos subjected to the maturation treatments in the present study were not examined histologically.

In cultures of John Davis rose somatic embryos, the combination of ABA (0.1 mg/ml) and sucrose (40g/L) in the maturation medium produce the highest number cotyledonary stage (mature) embryos. Despite the fact that the combination of ABA and polyethylene glycol, has been found to improve somatic embryo maturation in other species, especially conifers (Attree et al. 1993), the treatments that included polyethylene glycol, in this study, resulted in the lowest number of mature rose somatic embryos.

Polyethylene glycol is a non-plasmolysing osmotic agent, e.g. it will not enter the cell. This type of osmoticum restricts water uptake by cells and provides a "natural drought stress" (Attree et al. 1993). It is available in different molecular weights, with a range of PEG (1500) to PEG (4000) being commonly used as in somatic embryogenesis cultures (Attree et al. 1993). Attree et al. (1991) were the first to report the use of polyethylene glycol as a promoter of somatic embryo maturation in *Picea glauca* cultures. They found that adding 5 to 7.5 % PEG (4000) to the maturation media increased maturation frequency by two to three times and increased desiccation tolerance.

Sucrose has two roles in somatic embryogenesis. It plays a nutritional role, acting as a source of carbohydrates and acts as an osmotic agent. However because sucrose is metabolized by the culture, its osmotic effects decrease over time. Sucrose appears to be very important in the maturation of rose somatic embryos as the treatments which yielded the highest number of mature somatic embryos were those treatments which included 20-40 g/L of sucrose.

Like ABA, osmotic agents such as polyethylene glycol and sucrose, prevent precocious germination and are also linked to increased protein synthesis and increased germination rates (Xu et al. 1990). Osmotic agents are capable of causing osmotic stress, which is known to increase endogenous

levels of ABA. This is likely why osmotic agents and ABA have similar effects on embryogenesis (promote dormancy/ prevent precocious germination) (Finklestein and Crouch 1986).

Although PEG can speed up the maturation of somatic embryos in some species, a histological study by Find (1997) reported that Norway spruce somatic embryos cultured on media containing PEG were smaller and had large intercellular spaces in the SAM. Therefore the inclusion of ABA, sucrose, and/or PEG in the maturation media does not necessarily result in higher germination rates. In fact the average germination rate for rose somatic embryos, regardless of maturation treatment, was only 12.4%.

Another reason for the poor germination rate may be because of elevated levels of ABA remaining in the somatic embryos, preventing germination. Treatment with fluridone or gibberellins may be required to break the dormancy. Nickle and Yeung (1994) found that application of fluridone to cultures reduced endogenous ABA effects on somatic embryos.

Fluridone is a herbicide that is known to disrupt the production of endogenous ABA (Srinivasan and Vasil 1986) and has been shown to promote precocious germination as discussed earlier (Grappin et al. 2000). However other studies have shown that somatic embryos that were germinated with the aid of fluridone had poor quality, albino shoots (Srinivasan and Vasil 1986; Zaghmout and Torello 1990). So fluridone may not be the best solution to overcoming dormancy in somatic embryos.

The transition from embryogenesis to germination is regulated by a balance between gibberellins and ABA (Debeaujon and Koornneef 2000). There are a few theories to explain the role of exogenous gibberellins in the control of germination. First, it is believed that gibberellins are able to induce the expression of hydrolyzing enzymes which are able to breakdown the endosperm or other tissues that provide mechanical resistance to radical protrusion (Groot and Karssen 1987). A recent study by Debeaujon and Koornneef (2000) supports this theory. They found that *Arabidopsis ga1* (gibberellin deficient)

mutants will not germinate without exogenously applied gibberellins, however, if the testa on seeds is removed or sufficiently weakened, germination will occur without exogenously applied gibberellins. Thus it seems that gibberellins play a role in loosening or breaking down the testa in some species.

It was proposed that the role for gibberellins in dormancy release/ germination was simply to be present in sufficient levels to promote germination as soon as ABA synthesis was inhibited (Le Page- Devgivry et al. (1996). However, the results of a more recent study by Grappin et al. (2000) indicate that GA_3 is more involved in dormancy release than originally thought. For example, they found that exogenously supplied GA_3 (100uM), antagonized ABA accumulation in dormant, imbibed *Nicotiana* seeds, resulting in dormancy release (Grappin et al. 2000). Thus they proposed that gibberellins play an active role in controlling this process. However, it has not been determined whether the effect GA_3 has on ABA levels is mediated by inhibiting ABA synthesis or by stimulating degradation of ABA. In the Grappin et al. (2000) study, fluridone (an inhibitor of ABA synthesis) was found to be almost as effective as GA_3 in decreasing ABA accumulation. On the other hand, it has been reported that when GA_3 is applied to lettuce seeds a decrease in endogenous ABA occurs (Toyomasu et al. 1994). Further studies will need to be done to test whether gibberellins affect dormancy release by interrupting ABA synthesis or causing degradation of ABA or possibly both synthesis and degradation occur to varying degrees.

After the maturation treatments a cold period was used to prepare all of the somatic embryos for germination. It is believed that a cold treatment can promote germination by inducing gibberellin biosynthesis (Yamaguchi et al. 1998) or by increasing sensitivity to gibberellins (Karsen and Lacka 1986). Even with the cold treatment, the average germination rate was only 12.4%.

Future

To improve germination of somatic embryos, SAM development is key.

This study has found that the occurrence of a fully developed SAM (e.g. has a tunic corpus pattern) is rare in John Davis rose somatic embryos. Although the earliest evidence of a SAM in rose somatic embryos was at the cotyledon stage, work must be done at the earlier stages e.g. globular stage to ensure the SAM develops properly. The study by Ramsean-Fortner and Yeung (2000) indicates that auxin levels are critical for proper SAM formation in *Brassica* somatic embryos. More evidence needs be gathered on plant growth regulators and their effects on rose somatic embryogenesis.

Another area of interest for future research may be to investigate whether somalclonal variation is a factor in this John Davis rose somatic embryogenesis system. If the purpose of utilizing somatic embryogenesis is for clonal propagation this information would be quite important. Many plants species propagated by somatic embryogenesis, including roses have been found to exhibit some genotypic and phenotypic variation (Arene et al. 1993).

Conclusions

A combination of 2,4-D and wounding caused the most efficient callus initiation. Omission of either factor precluded the formation of a callus. Thus it seems that auxin and wounding act synergistically on callus inititiation. The wounding was likely effective because it allowed the auxins to penetrate past the protective epidermis and contact the cells that were more sensitive to the auxin.

The cells near the vascular bundles appeared to be the most affected by the callus initiation treatments. A large callus was present along the midrib of the leaflet within a week. Immature parenchyma cells are associated with the vascular bundles. These cells are known to differentiate into vascular tissues if the need arises and so it makes sense that they would have some meristematic characteristics.

There were two types of tissues present in the seven day old callus. Non-embryogenic callus, made up of elongated, vacuolated, non-embryogenic/ non-meristematic types of cells, and meristmatic tissues. The meristematic tissues were made up of small, isodiametric cells that were closely associated with one another. They had darkly staining cytoplasm and large nuclei.

The calli clusters had to be removed from the auxin rich callus initiation medium and placed on auxin free medium for the initiation of embryos. It was not determined if the meristmatic tissue observed at seven days of culture persisted and gave rise to the embryogenic tissue which formed on the periphery of the calli clusters after approximately 6 weeks on embryo initiation medium. Morphologically embryogenic tissue is lighter in color and more friable than the non-embryogenic callus on which it forms.

The somatic embryos followed the expected morphological patterns of development. They proceeded through a globular stage, heart shaped stage, torpedo shaped stage and finally a cotyledonary stage. The primary meristems were similar in appearance to those of the zygotic embryos although they were a

bit less organized in the somatic embryos. The protoderm was the first tissue to be observed, followed by the ground tissue and the procambium, then finally a RAM and occasionally a SAM. The SAM did not form in most of the somatic embryos. Usually there were large parenchyma and intercellular spaces where the SAM should be. When a SAM was present, it had a tunica corpus pattern of cells and appeared similar to the SAM of a zygotic embryo. Further studies on SAM development are needed. The mature stage somatic embryos had fewer storage proteins than the mature zygotic embryos.

Maturation seemed to be improved by the addition of sucrose and low levels of ABA to the medium. Treatments including high levels of ABA (1.0 mg/mL) or PEG did not produce many mature stage somatic embryos. Regardless of the maturation method, the germination rate was similar for all treatments (12.4% on average). It seems that there might be high levels of endogenous ABA in the somatic embryos. Perhaps a treatment with gibberellins or fluridone or red light may overcome this.

Appendix A

Micropropagation media

Media components	Amount
Ammonium nitrate (NH_4NO_3)	1856 mg/L
Potassium nitrate (KNO_3)	1425 mg/L
Calcium chloride ($CaCl_2$)	300 mg/L
Magnesium chloride ($MgSO_4$)	278 mg/L
Potassium phosphate (KH_2PO_4)	128 mg/L
Manganese sulfate ($MnSO_4$)	33.8 mg/L
Zinc sulfate ($ZnSO_4$)	21.2 mg/L
Boric acid (H_3BO_3)	6.2 mg/L
Potassium iodide (KI)	0.83 mg/L
Sodium molybdate ($NaMoO_4$)	0.25 mg/L
Copper sulfate ($CuSO_4$)	0.025 mg/L
Cobalt chloride ($CoCl_2$)	0.025 mg/L
Ferrous sulfate ($FeSO_4$)	27.8 mg/L
Na_2EDTA	37.2 mg/L
Inositol	100 mg/L
Thiamine	0.1 mg/L
Niacin	0.5 mg/L
Pyridoxine	0.5 mg/L
Benzyladenine	0.5 mg/L
Sucrose	20 g/L
Phytagar	6 g/L

Appendix B

Callus initiation media

Media components	Amount
Ammonium nitrate (NH_4NO_3)	1856 mg/L
Potassium nitrate (KNO_3)	1425 mg/L
Calcium chloride ($CaCl_2$)	300 mg/L
Magnesium chloride ($MgSO_4$)	278 mg/L
Potassium phosphate (KH_2PO_4)	128 mg/L
Manganese sulfate ($MnSO_4$)	33.8 mg/L
Zinc sulfate ($ZnSO_4$)	21.2 mg/L
Boric acid (H_3BO_3)	6.2 mg/L
Potassium iodide (KI)	0.83 mg/L
Sodium molybdate ($NaMoO_4$)	0.25 mg/L
Copper sulfate ($CuSO_4$)	0.025 mg/L
Cobalt chloride ($CoCl_2$)	0.025 mg/L
Ferrous sulfate ($FeSO_4$)	27.8 mg/L
Na_2EDTA	37.2 mg/L
Inositol	100 mg/L
Thiamine	0.1 mg/L
Niacin	0.5 mg/L
Pyridoxine	0.5 mg/L
2,4-D	5 mg/L
Sucrose	20 g/L
Phytagel	4 g/L
Casein hydrolysate	1 g/L

Appendix C

Embryo Initiation/ Maturation media

Media components	Amount
Ammonium nitrate (NH_4NO_3)	1856 mg/L
Potassium nitrate (KNO_3)	1425 mg/L
Calcium chloride ($CaCl_2$)	300 mg/L
Magnesium chloride ($MgSO_4$)	278 mg/L
Potassium phosphate (KH_2PO_4)	128 mg/L
Manganese sulfate ($MnSO_4$)	33.8 mg/L
Zinc sulfate ($ZnSO_4$)	21.2 mg/L
Boric acid (H_3BO_3)	6.2 mg/L
Potassium iodide (KI)	0.83 mg/L
Sodium molybdate ($NaMoO_4$)	0.25 mg/L
Copper sulfate ($CuSO_4$)	0.025 mg/L
Cobalt chloride ($CoCl_2$)	0.025 mg/L
Ferrous sulfate ($FeSO_4$)	27.8 mg/L
Na_2EDTA	37.2 mg/L
Inositol	100 mg/L
Thiamine	0.1 mg/L
Niacin	0.5 mg/L
Pyridoxine	0.5 mg/L
2,4-D	0
Sucrose	20 g/L
Phytagel	4 g/L

Appendix D

Germination media

Media components	Amount
Ammonium nitrate (NH_4NO_3)	928 mg/L
Potassium nitrate (KNO_3)	712.5 mg/L
Calcium chloride ($CaCl_2$)	150 mg/L
Magnesium chloride ($MgSO_4$)	139 mg/L
Potassium phosphate (KH_2PO_4)	64 mg/L
Manganese sulfate ($MnSO_4$)	33.8 mg/L
Zinc sulfate ($ZnSO_4$)	21.2 mg/L
Boric acid (H_3BO_3)	6.2 mg/L
Potassium iodide (KI)	0.83 mg/L
Sodium molybdate ($NaMoO_4$)	0.25 mg/L
Copper sulfate ($CuSO_4$)	0.025 mg/L
Cobalt chloride ($CoCl_2$)	0.025 mg/L
Ferrous sulfate ($FeSO_4$)	27.8 mg/L
Na_2EDTA	37.2 mg/L
Inositol	100 mg/L
Thiamine	0.1 mg/L
Niacin	0.5 mg/L
Pyridoxine	0.5 mg/L
Sucrose	20 g/L
Phytagel	3 g/L

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