SOMATIC EMBRYOGENESIS OF PATHOGEN- FREE HORSERADISH (*ARMORACIA RUSTICANA*) PLANTS *IN VITRO*

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Natural Resources and Environmental Sciences
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2009

Urbana, Illinois

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ABSTRACT

Illinois had been the major producer of horseradish in the United States for many years. Horseradish root production in Illinois had recently decreased due to a disease complex that causes root discoloration and rots. The use of pathogen-free (PF) horseradish planting stock was a means for the Illinois horseradish growers to restore root quality and yields. Micropropagation of PF plants through tissue culture could be used to deliver PF planting stock to growers, but it was time-consuming and has high labor costs. In addition, the growers needed millions of plants to supply their planting stock needs. It had been proposed to develop a method to produce whole plants from single cells *via* somatic embryogenesis. To initiate somatic embryogenesis, the Illinois Horseradish 'Doll' cultivar ('Doll') was tested with 2,4 -D (0.25 to 1.0 mg/l) and BA *in vitro*. Somatic embryogenic callus was successfully induced from leaf explants on MS medium supplemented with 2, 4-D. Somatic embryos (SEs) were collected from the callus, germinated, and converted to whole plants. Histological studies of SEs showed that they developed from cells near the vascular bundles. Furthermore, SEs developed through stages that mimicked normal embryo development. SEs germinated to give healthy and vigorous horseradish plants. Since cell suspensions of somatic embryogenic calli yielded plants, it raised the possibility to generate large number plantlets from suspension cultures. In addition, it was predicted that SEs could be encapsulated to make "artificial seeds" that could be used to supply a certified set program.

To my father, Pol. Col Peerapol Wannarat,

my mother, Thapthong Raethong,

my brothers, Phattarasak and Jaturong Wannarat,

my cousins, my uncles

and my aunts

ACKNOWLEDGMENTS

I would like to express my gratitude to Professor emeritus Richard Crang, Associate Professor. Mohammad Babadoost and Associate Professor John B. Masiunas for serving in my committee members and for their suggestions and helps during my research work. I would like to thank Dr. Margaret A. Norton, my unofficial committee members. She provided countless assistances in many ways. My thesis would not be possible without her supports, suggestions, comments and delicious pastries. My thank also passed to Dr. James B. Nardi for his generous assistance during the histological studies. He allowed me to use his laboratory and gave useful suggestion on histological techniques.

My thanks went to my former and present lab members, Dr. Richard M. S. Mulwa, Dr. Asharf Shehatta, Dr. Mark Uchanski, Daniel Weber, JunMyoung Yu and Nathan Woldarchek for their helps and friendship during this work. My sincere gratitude also went to my parents, my brothers, my relatives, all my friends and all my animal companions for their love, support and patience.

I was thankful to Royal Thai Government for giving me an opportunity to study in the United State with a full financial support through five years. I also thanked Mentoring Fellowship from the University of Illinois at Urbana-Champaign, which I received in Fall 2009.

Finally, I wanted to express my high gratitude to my greatest advisor, Prof. Robert M. Skirvin for his encouragement, patience, guidance and support throughout my time of study. He was always supportive and available to his students. I would not have finished the thesis without his guidance and support. I also would like to thank Mrs. Skirvin for her caring and concerns during my study.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
AGPs	Arabinogalactan-proteins
ANOVA	Analysis of variance
BA (BAP)	6-benzylaminopurine
CRD	Completely randomized design
C°	Degrees Celsius
2,4-D	2, 4-dichlorophenoxyacetic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric Acid
GA	Gibberellin
LS	Linsmaier and Skoog (1965)
MS	Murashige and Skoog (1962)
NDM	New Dogashima Medium
NAA	α-naphthaleneacetic acid
PF	Pathogen-free
PGRs	Plant growth regulators
PGR-free MS medium	Plant growth regulator-free Murashige and Skoog medium
SAS	Statistical analysis system
SEs	Somatic embryos
SH	Schenk and Hildebrandt
TC	Tissue culture
TDZ	Thidiazuron
TuMV	*Turnip mosaic virus*

CHAPTER I: RESEARCH BACKGROUND AND THESIS OBJECTIVES

1.1 Introduction

The history, botany, production, breeding methods and utilization of horseradish plants were reviewed by Shehata *et al.* (2009). According to their review, the horseradish (*Armoracia rusticana*) originated in Eastern Europe and Western Asia. It was introduced to cultivation in the United States in the early 1800s by settlers (Hedrick, 1919). It was brought to Illinois in 1856 and now had become an economically important vegetable in the state. The USDA reported that Illinois produced approximately 65% of the total US horseradish grown in the United States (USDA website, 2000)

Since horseradish seed production was rare and the seedlings were not true-to type, seed propagation was mainly used for breeding programs (Walters, 2007; Doll, 2006). Horseradish was propagated asexually from lateral roots (side roots) cut off of the primary roots; when a plant became infected with a pathogen, all subsequent vegetative offspring will also be infected. Thus, conventional propagation resulted in low yielding virus-infected plants.

Pathogen-free (PF) horseradish plants obtained in tissue culture (TC) could be an alternative source of planting stock that could provide productive PF plants (Uchanski, 2007). Horseradish sets were planted at about 7,000/acre; there were about 1,500 acres of horseradish in Illinois creating a theoretical need for 10.5 million plants per year. Thus, the production of PF horseradish plants by TC alone was not feasible due to high production costs and large TC laboratory space requirements. An alternative method to provide PF plants could be by somatic embryogenesis, somatic embryos (SEs) and encapsulation. The goal of this study was to develop a method to provide such planting materials.

1.2 Research problem

1.2.1 Justification

About 65% of the USA's horseradish is cultivated in southwestern Illinois (USDA website, 2000). Over the past several years, the Horseradish Growers of Illinois have lost their horseradish production due to viral and fungal infections (C-FAR, 2000).

Turnip Mosaic Virus (TuMV) caused mottling of horseradish leaves. The disease complexes composed of bacteria and fungus including *Verticillium* sp. were proposed to cause horseradish internal root discoloration. Since late 1999, researchers and extension personnel from the University of Illinois, and the Illinois Natural History Survey had conducted research to develop a certified program to provide PF horseradish plants to growers. To date, they had established and propagated several cultivars of PF horseradish in TC. Most had proved to be relatively stable and shown a low rate of somaclonal variation (Norton *et al.,* 2001; Norton *et al*., 2003; Uchanski, 2007 and Shehata, 2004)

Factors that limit *in vitro* micropropagation via adventitious shoots or auxiliary branching include the intensive labor required for rooting, acclimatization *ex vitro* and the large amounts of space required to accommodate plants until they are ready for planting**.** An alternate, less expensive method to produce large number of plants could be SEs (Redenbaugh, 1993 and Onishi *et al*., 1994). Somatic embryos (SEs) developed directly from single cells and bypass the multiplication (the cutting of plantlets into segments) and rooting steps that would otherwise be required. Somatic embryogenesis was an alternative to conventional TC shooting and rooting procedures. For instance, a 12-liter bioreactor could produce more than one million carrot somatic embryos at a time (Drew, 1980). The process could be used to make synthetic seeds

(Redenbaugh, 1993 and Onishi *et al*., 1994). Because of the minute size of SEs, synthetic seeds would have to make large enough for easy handling and packaging, probably by encapsulation. To study this possibility, it was necessary to establish a protocol to make PF horseradish SEs and then to produce synthetic seed as a delivery system in a certified program.

1.3 Objectives

1. To induce somatic embryogenesis from PF horseradish in vitro.
2. To establish a protocol of somatic embryogenic cell suspension cultures.
3. To study the processes of somatic embryogenesis by histological investigations (paraffin sections).

Long-term goal

1. To develop a protocol to produce synthetic seeds using PF horseradish somatic embryos (SEs)
2. To regenerate horseradish plants from somatic embryos and compare them to conventionally propagated plants.

1.4 The coding system used for Illinois horseradish germplasm

The proper name for accessions taken from the Illinois horseradish germplasm collection involved "ILHR" (Illinois Horseradish) following by its name or code e.g. ILHR 'Doll'. This had been shortened by eliminating of the "ILHR" and using its name or code only, e.g. 'Doll'. Therefore, "ILHR Doll" will be referred to as 'Doll' through the remainder of this dissertation.

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CHAPTER II: LITERATURE REVIEW

2.1 Brassicaceae family and horseradish taxonomy

Horseradish was a member of the Brassicaceae family (Syn. Cruciferae) (USDA website, 2008). Brassicaceae taxonomy had been reviewed by Warwick *et al.* (2006 ab) who compiled an up-to-date list of Brassicaceae family members based on molecular studies and previous reviews. They concluded that the family includes 338 genera and 3,709 species. Their database also included the name, taxon status, synonymy, scientific authority, literature source and verification and the basionym for each species on their checklist.

Al-Shehbaz *et al.* (2007) reported that the United States has the highest number of Brassicaceace species (653 native species in 61 genera); Turkey ranks second with 571 native species in 32 genera.

Horseradish was in the genus *Armoracia* that included three species: *A. lacustris* (A. Gray) Al-Shehbaz & V. M. Bates, *A. sisymbroides* (DC.) Cajander and *A. rusticana* P. Gaertn., B. Mey. & Scherb (the common cultivated horseradish) (Al-Shehbaz *et al*., 2006; Shehata *et al.,* 2009 and Warwick *et al*., 2006b).

2.2 Sexual reproduction in horseradish

Horseradish flowers were perfect, producing both male and female parts on individual flowers, borne on a raceme. Although their flowers were perfect, seed production was rare, making sexual reproduction difficult (Weber, 1949).

Roots were usual propagation material for horseradish. Consequently, the plant did not have to be propagated by seeds. In fact, horseradish had been propagated asexually for so long

that many clones appeared to have lost their ability to set seeds. Luther Burbank once wrote, "The horseradish does, indeed, bloom with the greatest profusion. But the blossoms prove sterile. The plant has entirely, and probably forever, lost the power of producing seed. I have elsewhere [made] a joking offer of one thousand dollars an ounce for horseradish seed. Of course I knew that no horseradish seeds were to be had, yet I would gladly have given then, and I would gladly pay, at the rate of $1,000 an ounce for horseradish seed. But there is not the remotest probability that any one will ever legitimately claim the prize." (Burbank, 1914).

Male sterility and non-functional pollen grains appeared to be the cause of most of the sterility problems in horseradish. For instance, to produce functional pollen grains that can be deposited on a stigma and form pollen tubes, microsporogenesis (pollen grain formation) had to proceed properly. Winiarczyk *et al*. (2007) studied male sterility in horseradish and found that their anther walls that developed properly can shed pollen freely, but at anthesis many anther sacs were observed to be shrunken and dry. Microscopic analysis revealed that microsporogenesis in these anthers were abnormal. Mitosis did not occur in the majority of microspores, so bi- and tri-nuclear pollen grains had formed occasionally. Some microspores failed to vacuolated, and others appeared to have very few starch grains. The authors suggested that these are some of the problems that contribute to male sterility.

Self-incompatibility was the failure of the egg and/or polar nuclei to be fertilized and set seed following self-pollination (Sleper and Poehlman, 2006), and it was common in Brassicaceae (Mable, 2008). The self-incompatibility system in *Brassica* species was a sporophytic system controlled by a single gene at a polymorphic locus designated as the *S* locus (Bateman, 1955). When the pollen had the same *S* phenotype as the stigma, pollen germination and/or pollen tube penetration were disturbed at the stigma surface (Watanabe *et al*., 2003).

Winiarczyk and Bednara (2008) found that self-incompatibility in horseradish was related to the male gametophyte. Three consequences of pollen self-incompatibility were suggested by these authors: 1) horseradish pollen grains were not hydrated when deposited on the stigma, so they did not stick to the stigma surface and fell from the stigma before they began to germinate; 2) pollen tubes failed to reach the ovules; 3) pollen tubes failed to penetrate the ovules to accomplish double fertilization. Even when fertilization occured, embryos often aborted. For instance, histological studies of horseradish embryo sacs suggested that many embryos aborted because they appeared to be unable to utilize the endosperm that accumulates in their embryo sacs. The authors also reported that most of the surviving embryos were deformed.

2.3 Asexual reproduction in horseradish

To ensure generation-to-generation uniformity of clones, horseradish plants were propagated vegetative from harvested roots. In general, the big taproots were harvested for processing, and the side roots were saved as "sets" for the next growing season. The biggest problem with vegetative propagation of horseradish was the buildup of disease. For instance, with each cycle of propagation, sets became more infested with pathogens and the pathogen loaded of the horseradish plant can increase annually. Yield and quality losses were concomitant, and plants exhibiting such yield losses were said to be "running out". Pathogen removal through *in vitro* or thermotherapy methods could restore yields of "run-out" cultivars.

"Running out" was previously misunderstood and was believed to be similar to aging in humans and other animals; annual yield and vigor losses were equated to normal aging. The belief was that plants, like humans and animals, eventually "run out" of energy and vigor and

die. Thus, it was important that new fruit and vegetable cultivars be developed on a regular basis to replace older cultivars that had "run out" (Stadelbacher, 1980).

In order to obtain clean PF horseradish roots several *in vitro* techniques, including tissue culture (TC) and thermotherapy, had been combined with traditional root cutting propagation methods to make PF planting stock (Uchanski, 2007).

2.4 *In vitro* studies in horseradish

2.4.1 *In vitro* horseradish propagation

PF plants were usually obtained by *in vitro* methods. In this section the establishment of horseradish *in vitro* cultures including disinfestations methods, source explants, medium components, and culture conditions will be discussed.

Meyer and Milbrath (1977) were the first to report *in vitro* shoot regeneration from horseradish. They discovered that horseradish leaf explants cultured on a Linsmaier and Skoog (1965) medium (LS) supplemented with α-napthaleneacetic acid (NAA) (1 mgl^{-1}) and kinetin (0.1-0.5 mgl^{-1}) produced adventitious plants that rooted well within about six months.

Gorecka *et al.* (1978) determined that LS medium could be replaced with Murashige and Skoog (1962) medium (MS). They also observed that horseradish buds disinfested in 80% ethanol for 6 minutes, and then in 5%, 7.5% or 10 % chloramines T for 30 or 15 minutes showed lower pathogen contamination rates than other treatments. Goreka (1987) reported that LS medium supplemented with IAA (Indole-3-acetic acid) (0.8 $mg.l^{-1}$) and Kinetin (4.0 $mg.l^{-1}$) induced horseradish leaf explants to form adventitious shoots.

The most common basal medium now used for horseradish culture was MS supplemented with plant growth regulators (PGRs), especially auxins and/or cytokinins. For instance, Kim and

Pank (1988) found that both NAA and 6-benzylaminopurine (BA) were important for horseradish shoot induction. They also compared explant sources and found that leaf segments formed shoots better than petiole, root or pedicel explants. Khadeeva *et al.* (1993b) regenerated plants from horseradish leaf pieces cultured on MS medium supplemented with kinetin (0.25 mgl^{-1}) and NAA (1 mgl^{-1}). Araki *et al*. (1995) discovered that horseradish leaf explants formed callus on MS supplemented with 2,4-D (1 $\mu mol.l^{-1}$) and BA (1 $\mu mol.l^{-1}$). To form adventitious plants the callus had to be subcultured on plant growth regulator-free MS medium (PGR-free MS medium). Kamada *et al*. (1995) compared two explant sources, leaves and roots, under light and darkness on MS supplemented with NAA (0, 0.1 and 1.0 mgl^{-1}) and BA (0, 0.1 and 1.0 mgl^{-1}). They found that horseradish leaf explants grown in the light formed more shoot buds than root explants. Under dark conditions, BA (1.0 mgl^{-1}) and NAA (0.1 mgl^{-1}) promoted shoot bud formation.

Moreover, Sugaya *et al.* (1995) observed that MS medium supplemented with 2, 4-D (0.1-2.0 mgl^{-1}) and BA (0.1 mgl^{-1}) induced horseradish root explants to form callus. Plantlets were regenerated when the callus was grown on PGR-free MS medium. Norton *et al.* (2001) obtained shoot buds from leaf segments cultured on MS medium supplemented with NAA (15 $\mu mol.l^{-1}$). Pawelczak *et al.* (2006) produced that shoot regeneration from horseradish leaf explants on MS medium supplemented with NAA (0.5 mgl^{-1}) and BA (0.1 mgl^{-1}). They demonstrated that BA at 0.2 and 0.4 $mg.l^{-1}$ resulted in leaf deformation and a reduced ability to root.

Horseradish explants could form adventitious shoots directly on PGR-free medium. For example, Uozumi *et al.* (1994) cultured horseradish hairy roots (*Agrobacterium rhizogenes*) and transformed horseradish cells in liquid MS medium without growth regulator supplements in the

dark. They discovered that the hairy root cells formed tumor tissue (neoplastic) that turned green and subsequently formed adventitious shoots when the explants were moved from darkness to light. They also determined that BA promoted shoot primordial enlargement. Mano and Matsuhashi (1995) used modified Gamborg's medium without PGRs to regenerate shoots form *A. rhizogenes*-transformed roots. After two weeks of culture, the explants formed additional roots, and some of these developed shoots, which became whole plants within 38 days.

TC-propagated plants were expected to be identical to the mother plants; however, variation can be found among TC-derived plants (Skirvin, 1978). Larkin and Scowcroft (1981) described genetic and heritable variation among cells, tissues or plantlets as "somaclonal variation." Somaclonal variation could arise either from pre-existing mutant cells associated among parental somatic tissues, or be a mutation induced by the process of TC itself. Some of the factors that might affect somaclonal variation were growth regulator type and concentration, cultivar difference, cultivar age, ploidy level of plants; explant source, length of time *in vitro*, and proliferation rate (Skirvin *et al.,* 2000). Somaclonal variation had been shown to result from a change in karyotype, point mutations, gene amplification, transposable elements, and DNA methylation (Larkin *et al*., 1989; Kaeppler *et al*., 2000, and Skirvin *et al*., 2000).

In horseradish, growers required uniform clones; however, any somaclonal variation that occured among *ex vitro* plants might be of commercial interest and could be stabilized by selection and asexual propagation to yield improved forms of standard cultivars. Many techniques had been used to detect the variations occurred in *in vitro* horseradish. For example, Krsnik-Rasol *et al.* (1994) used a 66-kDa protein to distinguish *A. tumefaciens*-transformed and normal (non-transformed) callus obtained from leaves of crown gall-infected horseradish. Sugaya *et al.* (1995) employed DNA fingerprinting using the Restriction Fragment Length

Polymorphism (RFLP) technique to identify somaclonal variation in horseradish plants derived from root and leaf calli.

2.4.2 Peroxidase production by horseradish *in vitro*

In addition to its culinary uses, horseradish roots had commercial value as a source of peroxidase, a heme-containing enzyme that catalyzes oxidoreduction between hydrogen peroxide and reductants. It was used as a reagent for organic synthesis and biotransformation, as well as in coupled enzyme assays, chemiluminescent assays, immunoassays and the treatment of wastewater (Veitch, 2004). Peroxidase played many physiological roles in plants; some of these were lignifications, suberization, cross-linking of cell wall structural proteins, auxin catabolism, self-defense against pathogens, salt tolerance, and senescence (Elstner and Heupel, 1976; and Hiraga *et al.,* 2001).

Peroxidase was traditionally extracted from field-grown horseradish roots. Because field cultivation of horseradish had decreased due to the disease complex discussed earlier (section 2.4.1: *in vitro* horseradish propagation), the availability of peroxidase had decreased. It may be that TC horseradish cells could become an alternate source of peroxidase; the possibility had been investigated (Shehata, personal communication).

Parkinson *et al*. (1990) established callus from horseradish leaves on modified MS supplemented with 2,4-D (5 mgl^{-1}) and kinetin (0.1 mgl^{-1}) and used them to make cell suspensions from which they planned to harvest peroxidase. They also transformed horseradish petioles with *A. rhizogenes* to make hairy root suspension culture. They compared peroxidase activity between normal callus and transgenic suspension systems and found that the quantity of peroxidase obtained from normal cells was two times higher than that from hairy root cultures.

Saitou *et al*. (1991) studied peroxidase activity in horseradish plants regenerated from *A. rhizogenes*-transformed leaves and petioles. There were three types of regenerated plants: normal type (with normal leaf and stem morphology), wrinkled type (with either wrinkled or small leaves and short or normal-sized stem) and a rooty type (with short shoots and long adventitious roots). The rooty-type plants showed the highest peroxidase activity. Khadeeva *et al.* (1993a) established callus from horseradish leaves on MS medium modified as in Meyer and Milbrath (1977). They found that the peroxidase content in cell suspensions was equal to or higher than in field-grown roots.

Sudhir and Mukundan (2002) generated adventitious roots from horseradish leaves that had been cultured on MS medium supplemented with indole-3-butyric acid (IBA, 1.0 mgl^{-1}). The peroxidase activity of the adventitious roots was higher than that expressed by either leaf- and root-derived adventitious shoots or transgenic hairy root cultures. Soudek *et al*. (2005) compared the peroxidase activity among transformed hairy roots, callus cultures and natural horseradish roots, and found that callus produced the most peroxidase. They also reported that cadmium, cobalt, nickel, and lead ion supplements in MS culture appeared to increase peroxidase concentrations.

2.5 Somatic embryogenesis

Embryos were normally produced *in vivo* following the union of female and male gametes resulting in a bipolar structure that included both a shoot and a root meristem apex (Dodeman *et al*., 1997). However, not all embryos developed sexually. Tissues such as the nucellus and synergid cells could develop into structures that were similar to zygotic embryos; however, these structures developed asexually and were thus clones of the parent plant

developing through a process called 'apomixis' (e.g. *Citrus, Mangifera,* and *Eugenia, etc*.) (Raghavan, 1976). Somatic embryogenesis was first reported in 1958 (Reinert, 1958 and Steward, 1958); since then information on somatic embryogenesis has accumulated greatly.

Somatic embryogenesis was a process whereby somatic cells go through a series of developmental stages that mimiced the zygotic development pathway to result in the formation of an embryo-like (somatic) structure that was said to be bipolar because it had both a shoot and root axis (Dodeman *et al*., 1997). In the modern literature, somatic embryos (SEs) were defined as non-zygotic embryos that had no vascular connection to the maternal tissues (Haccius, 1978). Yeung (1995) discussed three events that can lead to somatic embryo formation: competence, induction and determination. Competence was the capacity to respond to specific signals. Induction occured when a signal produced a unique developmental response from competent tissue. Determination was a process by which the developmental fate of a cell or group of cells becomes fixed and thus limited to a particular developmental pathway.

SEs developed in two ways: direct somatic embryogenesis and indirect somatic embryogenesis. Indirect somatic embryogenesis, SEs arose directly from tissues without callus formation. Some tissues that express direct SE formation were pre-embryogenic determined cells (PEDCs). PEDCs were cells that associate with immature tissues including seedling parts, and inflorescence parts that were presumed to have a pre-determined embryogenic capacity (induced-embryogenic determined cells). PEDCs required only growth regulators or favorable conditions to induce cell division and expression of embryogenesis. For indirect somatic embryogenesis, a callus phase was required to facilitate embryogenesis. Presumably callus formation helped the tissue revert to a relatively undifferentiated stage that was more conducive to regeneration (Sharp *et al*., 1982).

Merkle *et al.* (1990) suggested that distinctions between direct and indirect embryogenesis depended on the timing of dedifferentiation and the acquisition of competence. In direct somatic embryogenesis, the cells responded to an inductive signal and became to determine in a short time without callus formation. On the other hand, indirect somatic embryogenesis required a longer time because the cells had to go through a cell proliferation (callus formation) stage to acquire competence.

Not all determined cells of inductive tissues were able to divide and gave rise to SEs. Regardless of the pattern of somatic embryogenesis, SEs arising from either direct or indirect somatic embryogenesis could have originated from either a single cell (unicellular origin) or a group of cells (multicellular origin) (Quiroz-Figueroa *et al.*, 2006; Williams and Maheswaran, 1986 and Yeung, 1995).

For the unicellular origin, only a single cell of the epidermal layer of explant tissue actively divided to form a basal part that becomes a suspensor-like structure that connects the SE to the maternal tissue (Williams and Maheswaran, 1986 and Quiroz-Figueroa *et al.*, 2006). The histological section series of carrot somatic embryogenic callus done by Haccius (1978) showed that single cells divided to form polyembryonal cell masses. Salaj *et al.* (2008) found similar results using different methods. They used the expression of the *Arabidopsis* Somatic Embryogenesis Receptor-like Kinase 1 (*AtSERK*1) gene that functions during somatic embryogenesis to locate competent *A. thaliana* cells that will develop into SEs. The assays showed that *A. thaliana* SEs developed from competent single cells and competent small cell clusters from an epidermal cell layer of embryogenic callus.

When SEs had a multicellular origin, their basal region might be fused directly to their maternal tissue without forming a suspensor-like structure. For example, Gui *et al.* (1991)

studied the morphological changes during somatic embryogenesis in a Chinese medicinal plant (*Acanthopanax senticosus)*. Their histological study demonstrated that the first sign of somatic embryogenesis was active cell division in a group of epidermal cells at the callus surface. Later, these active cells developed tiny cylindrical protrusions that became globular-shaped embryos that later developed into cotyledon shaped embryos. Histological studies of direct somatic embryogenesis induced from moth orchid (*Phalaenopsis amabilis*) leaf explants also showed that somatic embryos were formed from small cells with dense cytoplasm that originated from the epidermal cell layer. These active cells proliferated to become nodular masses, and SEs developed from the outer cell layers of these structures (Chen and Chang, 2006).

2.6 Identification of somatic embryogenic cells

In some cases somatic embryogenic cells could be distinguished from non-embryogenic cells by callus morphology. Quiroz-Figueroa *et al.* (2006) suggested that, in general, an embryogenic callus was nodular with a smooth surface. In contrast, non-embryogenic callus was rough, friable and translucent. However, they recommended the use of histological procedures to confirm whether the calli has become embryogenic.

In general, embryogenic cells were reported to be small and highly cytoplasmic. Nomura and Komamine (1999) discovered that carrot SEs were most likely to regenerate from homogenous small, round and cytoplasm-rich single suspension-cultured cells. Based on many histological studies, primordial somatic embryogenic cells in general were dense in nucleoplasm with a cytoplasm rich in protein, and had a prominent central nucleus (Atmane *et al*., 2000; Namasivayam *et al*., 2006 and Sa′enz *et al.,* 2006). In contrast, Oka *et al*. (1995) reported that non-embryogenic cells of barley (*Hordeum vulgare* L.) were elongated and vacuolated.

Another sign of somatic embryogenesis was an accumulation of starch in somatic cell plastids. Starch grains were found in plastids of somatic embryogenic cells of camellia (*Camellia japonica*, Barciela and Vieitez, 1993) and a wild sunflower (*Helianthus smithii*, Laparra *et al.,* 1997). Atmane *et al*. (2000) also reported that embryogenic cells of marsh clubmoss (*Lycopodiella inundata* L.) were relatively non-vacuolated but had starch grains. Pescador *et al.* (2008) suggested that starch accumulation is important for SE development and conversion.

In addition, callose could be used to indicate cells that were entering the somatic embryogenesis pathway. For example, *Eleutheroccus senticosus* zygotic embryos were plasmolyzed with sucrose or mannitol (1 mol) to form SEs. After 12 hrs of plasmolyzing pretreatment, it was found that the callose was deposited between the plasma membrane and the cell wall of hypocotyls of the zygotic embryos (You *et al.,* 2006). The surface cells of camellia (*Camellia japonica)* globular SEs appeared to be covered with callose and cutin (Pedroso and Pais, 1995).

The next marker employed to detect somatic embryogenic cells is plasmodesma. Very early-stage SEs that contain actively dividing meristematic cells seemed to have functional plasmodesmata. To illustrate, Anzidei *et al.* (2000) demonstrated that embryogenic callus of fennel (*Foeniculum vulgare* Miller) contained actively dividing meristematic cells forming numerous plasmodesmata that could become non-functional at a later stage in the callus development. Ehlers *et al.,* (1999) reported that longitudinal sections of globular embryo-like structures of *Molinia caeruleu* showed plugged and nonfunctional plasmodesmata. Similarly, older cell walls of somatic embryogenic callus of *Gentiana punctata* L. were markedly

thickened, multi-layered, folded, and without plasmodesmata, while the walls of newly divided cells contained plasmodesmata (Mikula *et al.,* 2004).

Arabinogalactan proteins (AGPs) were another indicator used to identify cells that were likely to undergo somatic embryogenesis. AGPs were located on the outer side of the plasma membrane (Rumyantseva, 2005). To position AGPs in the cell wall, AGPs were bound to monoclonal antibodies (MAbs) at their cell wall epitopes (Knox *et al.,* 1990; Pennell and Roberts, 1990; Pennell *et al*., 1991). Pennell *et al.* (1992), as well as McCabe *et al*. (1997), used MAb JIM8 to detect the expression of the AGP epitope among carrot cells from suspension culture; they found that the JIM8 epitope was recognized only in the cell walls of carrot cells that would develop into SEs.

Genes expressed during somatic embryogenesis were also used as molecular markers to tag embryogenic cells in carrot, including *Late Embryogenesis Abundant* (*LEA*) genes (Zimmerman, 1993); *Somatic Embryogenesis Receptor-like Kinase (SERK)* genes (Schmidt *et al*., 1997) and the *Carrot Early Somatic Embryogenesis 1 (C-ESE1)* gene (Takahata *et al.,* 2004) as well as the maize genes (*knotted1 (knl)* gene and *ZmLEC1* gene, Zhang *et al*. 2002).

2.7 Initiation of *in vitro* somatic embryogenesis

To initiate *in vitro* somatic embryogenesis, three major steps were involved: 1) induction of somatic embryos; 2) development and maturation of somatic embryos, and 3) conversion of somatic embryos.

2.8 Induction of somatic embryos

The factors involved in SE initiation were discussed in general in this section. The specific methods used to initiate SEs in the *Brassica* family will be discussed in section 2.8.5.

2.8.1 Explant types

Somatic embryos were induced from many plant parts: axillary buds (Oggema *et al*., 2007); zygotic embryos (Canhoto *et al*., 1996; Choi *et al*., 1999; Choi and Jeong, 2002; Mikula *et al*., 2004; Bassuner *et al*., 2007; Moura *et al*., 2008); leaves (Sharma and Kumar, 1994; Ke *et al.,* 1995; Fki *et al*., 2003; Ipekci and Gozukirmizi, 2003; Chithra *et al*., 2005; D'Onofrio *et al*., 2006), roots (Wang, *et al*., 2006), cotyledons (Zhou *et al*., 2006; Wu *et al.,* 2007), hypocotyls (Michalczuk *et al.,* 1992; Martin and Mohanty, 2002), shoot tips (Ganapathi *et al.,* 2001; Tokuhara and Mii, 2001), internodal sections (Craig *et al*., 1997; Chithra *et al*., 2005), young inflorescences (Gupta and Conger, 1999; Jain *et al.,* 2005) as well as ovules and anther filaments (Choi *et al.,* 1998).

2.8.2 Culture media

The methods used to initiate SEs depended on the species (Kantharajah and Golegaonkar, 2004), but the most common medium used to induce somatic embryogenesis was the Murashige and Skoog (MS) medium (1962). However, some plants required other media. For instance, Talwar and Rashid (1990) demonstrated that N_6 medium (Chu *et al.,* 1975) favored somatic embryo formation from millet (*Pennisetum typhoides*). Modified B-5 (Gamborg *et al*., 1968) was used for SE induction in 'Chardonnay' grapevine (*Vitis vinifera* L., Jayasankar *et al*., 2001). New Dagashima Medium was used to initiate embryogenic callus of *Phalaenopsi*s orchid

(Tokuhara and Mii, 2001). Basal medium was used to induce SEs from macaw palm (*Acrocomia aculeate* (Jacq) Lodd. Ex Martius, Moura *et al.,* 2008).

2.8.3 Plant growth regulators (PGRs)

PGRs affected somatic embryogenesis (Feher *et al.*, 2003; Arnold *et al*., 2002); in general, both auxins and cytokinins played major roles as direct inducers of somatic embryogenesis (Arnold *et al*., 2002; Feher *et al*., 2003; Gaj, 2004). Yeung (1995) proposed that the induction of embryogenic callus depended on a 'competent' explant being stimulated by a particular inducer. When competent explants were stimulated with the correct inducer, the explant dedifferentiated and the cells were reprogrammed, leading to somatic embryogenesis.

2.8.3.1 Auxin

Natural auxin played an important role in embryogenesis. The most common naturally occurring form of auxin was IAA (Tanaka *et al.,* 2006). The relationship between IAA and embryogenesis was discussed by Firml *et al.* (2003). They studied the pattern of endogenous IAA distribution in transformed *Arabidopsis* zygotic embryos and found that the auxin localizing area with the highest concentration of IAA (IAA maxima) helped establish the apical-basal axis of embryos. As a sexual embryo develops, IAA was redistributed from surrounding cells to increase IAA concentration gradients, which in turn triggers cell differentiation (Appendix A).

2,4-D, a weed killer that was a synthetic analog of the natural auxin, was believed to be the most important PGR for SE induction (Feher *et al*., 2003; Quiroz-Figueroa *et al*., 2006). For example, 2,4-D was used to induce SEs from pineapple guava (*Feijoa sellowiana* Berg, Canhoto *et al*., 1996) and date plam (*Phoenix dactylifera* L, Fki *et al*., 2003) callus. Various hypotheses

to explain how 2,4-D induces somatic embryogenesis initiation had been proposed, but the process remained unclear.

First, Dudit *et al*. (1991) suggested that 2,4-D induced DNA synthesis and cell division, important events for the induction of early stages of carrot somatic embryogenesis. Similarly, Fujima and Komamine (1980) reported that 2,4-D-treated carrot embryogenic cell clusters underwent a rapid cell division to form globular embryos.

Endogenous IAA levels might be affected by 2,4-D. Michalczuk *et al.* (1992) found that 2,4-D induced carrot embryogenic callus had a higher level of endogenous IAA (about 1000 ng/g of fresh weight) than callus grown on 2,4-D-free medium (100 ng/g of fresh weight). In contrast, the endogenous IAA level of non-embryogenic callus remained stable with and without 2,4-D treatment. The authors hypothesized that a high endogenous auxin environment was required for embryogenic cells to divide rapidly to form globular embryos. Similar observations were reported by many researchers including Cook *et al*. (1993); Liu *et al.* (1993); and Jimenez (2001 and 2005).

Next, using 2,4-D might stress explants to form SEs because it is an auxin herbicide (Feher *at el*., 2003). 2,4-D mimicked the action of IAA but it was stable and not degradable by the plant as rapidly as endogenous IAA. At low concentrations IAA stimulated cell division but high concentrations (auxin overdose) IAA could cause plants to grow to death. Similarily, a high concentration of 2,4-D, depending on tissue sensitivity, could disturb plant growth (Grossman, 2000).

Binding sites of IAA might be affected by 2,4-D. Deshpande and Hall (2000) studied the physiology of auxin receptors in the plasma membrane of auxin herbicide-resistant wild mustard

(*Sinapsis arvensis*) protoplasts. The authors suggested that the plant changed the conformation of the auxin-binding sites on the membrane to being resistant to the auxinic herbicides.

In addition to 2,4-D, other auxins had been used to initiate SEs. For instance, α-naphthaleneacetic acid was used to induce SEs from North American ginseng (*Panax quinquefolius L.*, Zhou and Brown, 2006). Dicamba was used in wheat (Russian spring variety 'Tajoznaja', Filippov *et al*., 2006) and betal nut (*Areca catechu*, Wang *et al*., 2006).

2.8.3.2 Other PGRs

Cytokinins such as thidiazuron (TDZ) had been used to induce SEs in *Paulownia elongata* (Ipeki and Gozukirmizi, 2003). For some plants combinations of both auxins and cytokinins were important for SE induction. Mikula *et al.* (2004) induced somatic embryos from zygotic embryos of *Gentiana punctata* L. cultured on MS medium supplemented with dicamba (4.5 $\mu mol.l^{-1}$), NAA (0.54 $\mu mol.l^{-1}$), 6-benzylaminopurine (BAP, 8.88 $\mu mol.l^{-1}$) and adenine hemisulfate (0.43 $mmol.l^{-1}$).

Abscisic acid (ABA) had been found to be necessary for the SE induction for some plants including orchardgrass (*Dactylis glomerata*, Bell *et al.,* 1993), carrot (*Daucus carota* L., Nishiwaki *et al.,* 2000) and hybrid Bermuda grass (*Cynodon dactylon* × *Cynodon transvaalensis* cv. 'Tifgreen', Li and Qu, 2002). Kikuchi showed that carrot (*Daucus carota* L. cv. US-Harumakigosun) apical tip segments formed no SEs on MS medium without growth regulators (control) or on medium with fluridone (an ABA inhibitor). However, when ABA was added to the fluridone medium, SEs formed, demonstrating the importance of ABA for shoot formation in carrot.

Gibberellin (GA) had been reported to impede SE formation in carrot (*Daucus carota*, Fujimura and Komamine, 1975), citrus (*Citrus sinensis* [L]. Osbeck *cv.* Shamouti, Kochba *et al.,* 1978); geranium (*Pelargonium x hortorum* Bailey, Hutchinson *et al.,* 1997), chickpea (*Cicer arietinum*, Hita *et al.,* 1997) and *Medicago sativa* L. cv. Rangelander (Rudus *et al*., 2002).

However, PGRs were not always required to induce SE formation. Wu *et al. (*2007) reported that cotyledon tissues of king protea (*Protea cynaroides*) formed SEs directly on MS without growth regulators, while MS medium supplemented with auxin and/or cytokinin suppressed the formation of SEs. Nair and Gupta (2006) stated that germinating black pepper micropylar tissues from (*Piper nigrum* L.) seeds formed SEs on PGR-free Schenk and Hildebrandt (SH) medium (1972).

2.8.4 Other SE inducers

Other compounds that affected SE induction were polyamines and AGPs. The effect of nitric oxide (NO) on auxin regulation during embryogenesis was reported by Otvos *et al.* (2005). They suggested that NO promoted auxin-mediated activation of cell division and embryogenic cell formation in alfafa cell cultures. Silveira *et al.* (2006) informed that *Araucaria angustifolia* (Bert.) O. Ktze. embryogenic cells supplemented with polyamines, especially putrescine accumulated more NO than the suspensor cells; they suggested that NO synthesis might be related to the maintenance of embryo-suspensor polarity. Pereira-Netto *et al*. (2007) found that AGPs extracted from cashew-nut tree exudate gums stimulated somatic embryogenesis of carrot cells (*Daucas carota* L. *cv*. Barsillia) cultured in MS liquid medium supplemented with 2,4-D (1 $\mu mol.l^{-1}$).

Matthys-Rochon (2005) suggested that peptides such as phytosulfokinines (PSKs) and pathogen-related proteins (PR proteins) might be involved in embryo formation. There were other minor inducers that had been used to induce carrot somatic embryogenesis including high sucrose concentration (Kamada *et al.,* 1993), heavy metal ions (Kiyosue *et al.,* 1990) and high temperature (Kamada *et al*., 1989). Additionally, a high concentration of sucrose could induce somatic embryogenesis in carrot (Kikuchi *et al*., 2006). Similar to Ikeda-Iwai *et al*. (2003), shoot apical tips of *Arabidopsis thaliana* (ecotypes Col, L*er* and Ws) formed SEs on B5 medium containing mannitol (0.7 phosephatase), after which the explants were transferred to B5 medium supplemented with 2,4-D (4.5μ $mol.l^{-1}$).

2.8.5 Induction of SEs in the Brassicaceae family

Most Brassicaceae plants formed the SEs somatic tissues (somatic embryogenesis), but some *Brassica* asexually-derived embryos could be developed directly from the microspores through androgenesis, a process that differs from somatic embryogenesis. Thus, somatic embryogeneis and androgenesis in the Brassicaceae will be discussed in this section.

2.8.5.1 Somatic embryogenesis in the Brassicaceae family

Somatic embryogenesis had been studied in many crops in the genus *Brassica* including *Brassica napus* (Koh and Loh, 2000), *B*. *juncea* (Singh and Sachan, 1999) and *B*. *oleracea* (Donato *et al*., 2000). Members of the Brassicaceae family have been induced to form SEs from various tissues and organs including hypocotyls (Leroy *et al.*, 2000; Martin and Mohanty, 2002; Majd *et al*., 2006), leaves (Deane *et al*., 1997; Donato *et al.,* 1999), cotyledons (Choi *et al*.,

1996; Turgut *et al.,* 1998; Koh and Loh*,* 2000) as well as zygotic embryos and ovules (Choi *et al.,* 1998).

The medium most commonly used to induce SEs in the Brassicaceae family is MS supplemented with auxins and cytokinins. For example, MS medium supplemented with 2,4-D (1.0 mgl^{-1}) and kinetin (0.1 mgl^{-1}) induced somatic embryogenesis in *B. oleracea* and *B. napus* (Martin and Mohanty, 2002). SEs were induced from hypocotyls of *B. napus* on MS medium containing 2,4-D (1.0 mgl^{-1}), NAA (2.0 mgl^{-1}) and BAP (2.0 mgl^{-1}) (Majd *et al*. 2006). *B. oleracea* formed SEs on MS with IAA (1.0 mgl^{-1}) and kinetin (0.5 mgl^{-1}) (Pareek and Chandra, 1978). Additionally, Gupta *et al.* (1990) showed that a gibberellic acid (GA_3) supplement placed on MS medium containing kinetin (1.0 mgl^{-1}) and 2,4-D (1.0 mgl^{-1}) enhanced embryogenic callus of *B. nigra*.

2.8.5.2 Androgenesis in the Brassicaceae family

Brassica microspores could develop SEs directly though a process called "androgenesis" or "microspore embryogenesis" (Maraschin *et al*., 2005). Microspore embryogenesis occured by reprogramming the inducible immature pollen grains or their microspores to become SEs directly via embryogenesis, diverting them from their original developmental pathway towards becoming pollen grains (Segui-Simarro and Nuez, 2008).

Briefly, mechanically isolated uninucleate microspores, excised inflorescences, shed pollen or anthers cultured on either the chemically defined N6 or MS media were treated with stresses (cold, heat, carbon starvation, or colchicine) to induce androgenesis (Datta, 2005 and Shariatpanahi *et al.,* 2006). Maraschin *et al.* (2005) suggested that there were three main phases of embryogenic development during androgenesis: Phase I involved an acquisition of

embryogenic potential. During this phase stress stimulated microspores to dedifferentiate from microspores to embryogenic status. Phase II was the initiation of the microspore cell divisions to form multicellular embryo-like structures contained within the exine wall. Phase III was a "pattern formation" where embryo-like structures were released from their exine wall to develop as bipolar as opposed to generative and vegetative cells.

Touraev *et al.* (1997); Smykal (2000); and Pechan and Smykal (2001) all suggested that uninucleate haploid microspores that underwent the first mitosis division symmetrically would develop into embryos. For example, Zaki and Dickinson (1991) showed that *B. napus* microspores at the stage prior to the first pollen mitosis (PM I) cultured on colchicine (25 mgl^{-1}) for 12 hours formed 5,000 to 22,000 embryos containing cotyledons per 200,000 microspores plated. Their cytological study showed that colchicine-treated microspores underwent PM I symmetrically to form multicellular embryos.

Rapeseed (*B. napus*) (Cegielska-Taras and Szaa, 1997; Iwanowska *et al.,* 1997 and Segui-Simarro and Nuez, 2008) and several *Brassica* species have yielded SEs from microspore cultures: *B. oleracea* (Vyvadilova *et al.,* 1998; Dias, 1999); *B. carinata* (Barro and Martin, 1999); *B. juncea* (Prem *et al.,* 2008) and *B. pekinesis* (Chokyu *et al.,* 1995). The major cellular events that microspores normally underwent during pollen development and the androgenesis were discussed further Appendix B.

2.9 Development, maturation and conversion of somatic embryos

After somatic embryogenesis had been induced, SEs emerge either directly from the explant surface (direct somatic embryogenesis) or they were formed indirectly from callus (indirect somatic embryogenesis) (Sharp *et al*., 1982). In this section some of the methods used

to encourage SE development and maturation as well as the conversion of the SEs to plantlets will be discusssed. The terminologies of germination and conversion were defined in Chapter 3 section 3.1.2.

2.9.1 Indirect somatic embryogenesis

Indirect somatic embryogenic callus had been reported to be a compact nodular callus that includes globular embryo-like structures, as in the case of purple mistress (*Moricandia arvensis,* Craig *et al.,* 1997) and finger root (*Boesenbergia rotunda*, Tan *et al*., 2005). The embryogenic callus could also become a friable complex. For example, Ganapathi *et al.,* (2001) reported that somatic embryogenic callus of banana cv. Rasthali (*Musa* spp. AAB group) was whitish and friable; two other plants that had formed friable embryogenic callus were date palm (*Phoenix dactylifera* L, Fki *et al*., 2003) and a woody medicinal plant (*Rutula aquatica*, Chithra *et al*., 2005). Somatic embryogenic callus was also found to be associated with non-embryogenic callus (Talwar and Rashid, 1990 and Nhut *et al*., 2006). Somatic embryogenic calli had to be carefully selected from non-embryogenic callus after several cycles of subculturing (Wang *et al*., 2006).

After the somatic embryogenic callus became relatively homogenous, it was transferred to maturation media to encourage further development of its SEs. However, Sharma and Kumar (1994) demonstarted that luckynut (*Thevetia peruviana* L.) somatic embryogenic callus did not have an indefinite life span; the amount of callus decreased as the number of subcultured cycles increased on the induction medium.

To mature SEs from the globular-like state to a cotyledon-like stage, the maturation medium was required. The cotyledon-like structures developed further after being moved to

germination and conversion medium where they germinated and produced roots and shoots to become whole plants. For example, SEs of American ginseng (*Panax quinquefolius* L.) developed to a cotyledonary stage on maturation medium with 0.5% activated charcoal without PGRs. However, their shoots and roots developed no further until they were moved from maturation medium to conversion medium supplemented with GA_3 (10 to 20 mgl^{-1}). Then 85% of them converted to plantlets (Zhou and Brown, 2006).

For many species maturation medium did not need to contain PGRs. Examples include the woody perennial trees (*Kalopanax pictus*, Moon *et al.,* 2005) and blue gum (*Eucalyptus globulus*, Pinto *et al.,* 2008). Maltose was reported to encourage maturation of SEs of a five-needle pine (*Pinus armandi* var. *amamiana*, Maruyama *et al*., 2007) and European chestnut (*Castanea sativa*, Corredoira *et al.,* 2008).

Germination and conversion media often included GA_3. Plants that respond to GA_3 include a woody perennial tree (*Kalopanac pictus*, Moon *et al.,* 2005), alfafa (*Medicago sativa* L., Kepczynska and Zielinska, 2006) and okra (*Abelmoschus esculentus* L., Ganesan *et al*., 2007). Ascorbic acid was reported to improve germination and conversion rates in white spruce (*Picea glauca*) SEs (Stasolla *et al*., 2006). ABA (2 mgl^{-1}) was used to improve SE germination in Persian walnut (*Juglans regia* L., Vahdati *et al*., 2008).

2.9.2 Direct somatic embryogenesis

When SEs were subcultured on induction medium, the number of SEs resulting from direct somatic embryogenesis could be increased. For example, Chen and Chang (2006) showed that the SEs of *Phalenopsis amabilis* var *formosa* that had formed directly from leaf explants

could be multiplied as secondary SEs on half-strength MS supplemented with TDZ, a very strong compound with cytokinin-like activity.

PGR-free medium could promote both SE maturation and development in globular-stage *Paulownia elongata* SEs (Ipekci and Gozukirmizi, 2003) and sweet potato (*Ipomoea batatas* L. Lam, Oggema *et al*., 2007).

SEs in some plants required PGR supplements for them to mature and convert into plantlets. For example, Mandal and Datta (2005) found that in the SEs formed from ray florets of *Chrysanthemum morifolium* on MS medium supplemented with 2,4-D and BA germinated on the same medium after subculturing. Liu *et al*., (2007) discovered that directly-formed SEs of sea-buckthorn (*Hippophae rhamnoides* L.) were both induced and germinated on SH medium supplemented with kinetin (1.0 mgl^{-1}) and IAA (0.2 and 0.5 mgl^{-1}). Khan *et al*. (2005) stated that cayenne pepper (*Capsicum annuum* L.) stem segments and shoot tips cultured on MS medium supplemented with TDZ formed SEs directly; the SEs were matured on the same medium. The SEs developed shoots and roots on MS conversion medium supplemented with IBA.

Gibberellin had been shown to affect germination of directly formed SEs of some plants. Wu *et al.* (2007) found that SEs from king protea (*Protea cynaroides)* germinated only on media containing either no growth regulators or media with GA_3 (0.3 $\mu mol.l^{-1}$ or 1 $\mu mol.l^{-1}$). At GA_3 concentrations (>1.0 mM) all germinating embryos were malformed. Most (60%) of the SEs of habanero pepper (*Capsicum chinense* Jacq.) induced directly from hypocotyls on MS with 2,4-D (9.05 $\mu mol.l^{-1}$) formed roots and shoots when they were cultured with ABA (1.89 $\mu mol.l^{-1}$) and GA_3 (1.1 $\mu mol.l^{-1}$) (Lopez-Puc *et al.,* 2006).

Carbon source, cold treatment and activated charcoal could also promote SE germination. Corredoira *et al.* (2008) reported that 3% maltose and cold treatment (at 4 °C, two months) improved the SE maturation and conversion rate of European chestnut (*Castanea sativa* Mill). Activated charcoal had been observed to promote germination of some SEs. Vikrant Rashid (2003) revealed that SEs differentiated directly from the rachis of immature inflorescences of kodomillet (*Paspalum scrobiculatum* L. cv. PSC 1) on MS or N6 medium supplemented with various concentrations of 2,4-D (4.5–22.5 μmol.l^{-1}). The *Paspalum* SEs germinated best if they were precultured on an induction medium supplemented with activated charcoal before being transferred to germination medium without charcoal.

2.10 Applications of somatic embryogenesis

The importance of somatic embryogenesis had been discussed in many review papers (Arnold *et al*., 2002; Gupta and Timmis, 2005; Ibaraki and Kurata, 2001; Paek *et at*., 2005; Raemaker *et al.*, 1995). Some potential uses for somatic embryogenesis will be discussed in this section. First, since SEs underwent developmental stages similar to those occurring in zygotic embryogenesis (Dodeman *et al.*, 1997), SEs could be used as a model system for embryological studies where early-stage zygotic embryos are difficult to obtain. The studies of the molecular basis of gene regulation in somatic embryogenesis had been conducted primarily on *Arabidopsis thaliana*. Transformed *A. thaliana* SEs had been developed to find the signaling pathways and the genes that were required to regulate somatic embryogenesis (Gaj, 2004 and Hecht *et al*., 2001).

Another application for SEs was for plant propagation. Somatic embryogenesis was an attractive alternative to cuttings or other asexual methods that might not provide enough

propagules. Some examples of where somatic embryogenesis had proved useful include: 1) *Cyclamen prison* Mill is propagated from F1-hybrid seeds which were expensive and not sufficiently uniform (Lyngved *et al.*, 2008); 2) transgenic Siberian ginseng (*Eleutherococcus senticosus*) used to produce an immunity protein to make edible vaccine, was difficult to propagate from seeds. The Siberian ginseng seeds typically required an 18 month-stratification chilling treatment to induce their germination (Kang *et al.* 2006); 3) liquid cultures of hybrid banana 'FHIA-18' (AAAB) SEs regenerated from somatic embryogenesis were scaled up in a bioreactor. When the plants regenerated from SEs were tested for somaclonal variation in a field, 0.13% exhibited somaclonal variation compared to 5% for plants propagated from shoot tip culture and suckers (Wong *et al*., 2006).

Another benefit of SEs for plant propagation was that they could be used to scale-up plant quantities by using bioreactors to yield virtually unlimited numbers of SEs that could be germinated and converted to whole plants (Arnold *et al.*, 2002). Thus, bioreactors could reduce labor costs and yield a high percentage of uniform embryos (Ibaraki and Kurata, 2001). For example, Shohael *et al.* (2005) inoculated 10 g of ginseng (*Eleutherococcus sessiliflorus*) embryogenic cells (fresh weight) into a 3 1iter balloon-type bubble bioreactor and obtained 121.8 g mature SEs at different developmental stages. Gupta and Timmis (2005) reported that they were able to establish somatic embryonal-suspensor mass cultures of conifers in a bioreactor.

Another advantage of SEs was that they produced root and shoot meristems in a single step (Gupta and Timmis, 2005). This benefit cuts labor costs related to root induction on a separate medium. Furthermore, many SEs, including carrot, alfafa, celery, lettuce and spruce were encapsulated in aginate gels to make artifical seeds that can be directly planted into the field (Redenbaugh, 1992). In addition to their use for propagation, SEs could be used for plant

transformation, haploid breeding, and metabolite production in which the zygotic embryos yield commercially important metabolites (Raemarker *et al.*, 1995). Somatic embryogenic culture could be also cryopreserved to establish gene banks (Arnold *et al*., 2002).

2.11 References

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CHAPTER III: ESTABLISHMENT OF A SOMATIC EMBRYOGENESIS REGENERATION PROTOCOL FOR HORSERADISH

3.1 Introduction

Somatic embryogenesis had been studied in many crops (Chapter II: section 2.5). The objective of this study was to induce somatic embryogenesis and somatic embryos (SEs) from PF horseradish explants, and a protocol used to establish somatic embryogenic callus from 'Doll' horseradish will be discussed in this chapter.

In general, somatic embryogenic callus was induced with auxins, either alone or in combination with cytokinins. The best explants for somatic embryogenesis were inflorescences and vegetative explants, depending on the species (discussed earlier in Chapter II: section 2.7).

For example, Tokuhara and Mii, (2001) obtained pale yellow granular calli from excised *Phalaenopsis* shoot tip explants on New Dogashima Medium (NDM) supplemented with NAA (0.5 μmol.l^{-1}), BA (4.4 μmol.l^{-1}) and sucrose (29.2 mmol.l^{-1}M). Fki *et al.* (2003) successfully induced the immature inflorescences of date palm (*Phoenix dactyliferea* L. cv. Deglet Nour) to produce a small white nodular friable embryogenic callus on MS medium supplemented with 2,4-D (10 mg.l^{-1}). Chithra *et al.* (2005) found the MS medium supplemented with 2, 4-D at 0.45 μmol.l^{-1} was the most effective medium to generate a friable embryogenic callus from leaf and internod explants of a rare rhoeophytic woody medicinal plant (*Rotula aquatic* Lour.*)*. Jain *et al*. (2005) informed that young immature inflorescences of hybrid bermudagrass [*Cynodon dactylon* (L.) Pers × C. *transvaalensis* Burtt Davy] cv. Tifton 85) were more likely to form embryogenic callus than either apical shoots or nodal segments on MS medium supplemented with 2, 4-D (4 mg.l^{-1}) and casein hydrolysate (0.01 and 200 mg.l^{-1}). Gaj (2004) reviewed 124 articles published

on somatic embryogenesis in many plant species and found that one-fifth of the researchers used immature zygotic embryos to generate their SEs. The reason zygotic embryos are so useful may relate to a report made by Ni *et al*. (2001) who found that *Arabidopsis* zygotic embryos contained high levels of endogenous IAA.

3.1.1 Somatic embryogenesis in horseradish

Somatic embryogenesis had been investigated in horseradish by Shigeta and Sato (1994) who obtained somatic embryogenic callus and somatic embryos (SEs) from horseradish leaf segments grown with 2, 4-D (0.1 to 2.0 $mg.l^{-1}$). To increase the size of the embryogenic callus, they suggested adding BA (0.1 or 0.5 $mg.l^{-1}$) to MS medium supplemented with 2, 4-D (0.1 to 2.0 $mg.l^{-1}$). Unfortunately, the cultivar they used was not specified in their publications.

Additionally, Shehata (2004) reported that 'Doll' petioles, induced with 2,4-D (0.2 $mg.l^{-1}$), produced friable calli that were useful for establishing liquid suspension cultures. When transferred to a solid medium without growth regulators, these calli produced shoots, but none of them were of obvious somatic embryogenic origin.

To investigate the possibility of producing somatic embryogenic callus and SEs from 'Doll' TC, various explants and 2, 4-D concentrations were tested either alone or in combination with BA for their usefulness in this process.

3.1.2 Germination and conversion of SEs

Somatic embryogenesis could be an efficient way to produce horseradish plants commercially. SEs had shoot and root apical meristems similar to zygotic embryos, but they have no seed coats (testa) (Kysely and Jacobsen, 1990). To deliver SE-derived plants to

customers, it was important that SEs be able to undergo germination and conversion to become whole plants with both roots and shoots, resembling seedlings derived from sexual embryos. These studies were also initiated to investigate the processes of SE germination and conversion in comparison to sexually derived seedlings.

SE germination was a process of initial elongation of the embryogenic roots; and the conversion was survival and growth of the plant *ex vitro* (Bhojwani and Soh, 2001). Seed germination occured in two steps: imbibition (seeds take up water or imbibe), and radicle emergence and epicotyl elongation. There were the appearance of true leaves and photosynthesis. The penetration of the embryo's radicle through seed structures such as testa and endosperm is called 'visible germination'. Later stages involved mobilizing storage food (Bewley, 1997).

Information regarding the germination process in horseradish was not well documented. To better understand seed germination in horseradish, germination among our few horseradish seeds was compared to some members (*Arabidopsis thaliana* and *Lepidium sativum*) of the Brassicaceae that does exhibit good seed set and germination. To illustrate, Muller *et al.* (2006) investigated the anatomy of *A. thaliana* and *L. sativum* (garden cress) seeds and their germination physiology. Because the morphology of these two seeds appeared to be similar to horseradish seeds and all were Brassicaceae, it was assumed that horseradish seed should have similar germination steps. The mature seeds of *A. thaliana* and *L. sativum* seeds were reported to be endospermless; the single-celled aleurone layer was diploid maternal tissue and was part of the inner testa, so embryos depended on their cotyledons for food when they germinated (a feature common to most *Brassica* species). Within their seed coat, the embryos consisted of two cotyledons with a radicle surrounded with a thin layer of endosperm. The embryos had

micropylar endosperm which was seen as a cap-like structure covering their radicle tips. The germination of *A. thaliana* and *L. sativum* seeds proceeded on PGR-free medium as follows: the testa ruptured, endosperm weakened, the micropylar endosperm broke down, and radicle emerged. It was also found that ABA delays the onset and decreased the rate of the endosperm weakening process, and GA stimulated endosperm weakening.

3.2 Materials and methods

3.2.1 Media preparation and culture conditions

The basic medium used in all *in vitro* studies was Murashige and Skoog (MS, 1962) high mineral salts medium supplemented with agar (7 g/l), sucrose (30 $g.l^{-1}$) and myo-inositol (100 $mg.l^{-1}$) at pH 5.6, adjusted prior to autoclaving. All *in vitro* treatments were maintained in a culture room with cool white fluorescent lights (35 $\mu mol.m^{-2}.s^{-1}$ photosynthetically active radiation [PAR]) to provide a 16 h photoperiod at 21±1°C.

3.2.2 Plant Materials

3.2.2.1 Establishment of pathogen-free (PF) horseradish plants *in vitro*

Some of the horseradish cultivars discussed in this thesis had been established in TC prior to the initiation of these studies. Briefly, the cultivars had been collected either from the University of Illinois horseradish germplasm collection (UIUC, Urbana) or from cooperators in the Collinsville, IL area. All cultivars had been established *in vitro* by Norton *et al*. (2001) as discussed earlier (Chapter II: section 2.4.1.). TC plants used in the present studies had been made pathogen-free (PF) by Uchanski (2007). Briefly, apical domes (300-500 cells in size and *ca.* 0.1 mm in length) were excised from *in vitro* shoots under a dissecting microscope and

individually cultured in wells of a 24-well polystyrene, cell culture cluster (Costar® 3524). Each well contained *ca.* 2 ml of PGR-free MS. The cell cluster vessels were placed in the culture room under the growing conditions described earlier for *in vitro* culture. To assess the virus status of surviving meristems, samples of each plant were assayed for turnip mosaic virus (*TuMV*) by enzyme-linked immunosorbent assay (ELISA) and reverse-transcriptase polymerase chain reaction (RT-PCR). Those that tested negative for *TuMV* were presumed also to be free of most other viruses. There were no obvious signs of bacterial or fungal infestation on the TC medium, hence, they were designated pathogen-free (PF) (Uchanski, 2007).

Some of the PF plants made by Uchanski (2007) were increased though many cycles of axillary bud multiplication *in vitro* (Norton *et al*., 2001). To ensure constant supplies of PF plants, a portion of the PF horseradish plants were stored as stock clones in an incubator at 4 °C. These were the clones that were used for the experiments discussed in the remainder of this study.

3.2.2.2 Micropropagation system for PF horseradish cultivars

To prepare the *in vitro* stocks of PF horseradish clones for this research, PF cultivars were taken from the chilled incubator, where they had been stored in jars (described earlier) at 4 °C in PGR-free MS for six to twelve months.

To reinitiate growth, the jars of plants were first placed in a laminar flow hood overnight (Bellco glass, Inc® New Jersey) to acclimate them to room temperature. The next day clumps of shoots were cut into single microcuttings and transferred to MS medium supplemented with BA (2 $\mu mol.l^{-1}$) (Norton *et al.,* 2001).

Within 14 days many new axillary buds appeared. Some of these buds were separated and transferred to PGR-free MS to develop shoots and roots. Two- to three-week-old leaves

were harvested from these plantlets and used to develop methods for induction of embryogenic callus.

3.2.3 Preliminary studies of explant sources and 2,4-D for SE induction

3.2.3.1 '22C' and '1590' horseradish cultivars

To investigate somatic embryogenic callus induction in horseradish, five PF horseradish cultivars ('22C', '1069', '1590', '7586' and 'Doll') were chosen for preliminary study. The PF horseradish cultivars used in this study were selected using a genetic relationship dendrogram. The selected clones represented a range of genetic diversity among the 101 horseradish cultivars evaluated by Hamblin *et al*. (2002).

In spring 2006, following *in vitro* establishment, two of the five cultivars ('22C' and '1590') were found to be contaminated with a vigorously growing yellow contamination that appeared to come from internal regions of the explants themselves. These two cultivars were discarded; the remaining three cultivars ('Doll', '1069', and '7586') were used for the preliminary studies.

3.2.3.2 'Doll', '1069' and '7586' horseradish cultivars

In the summer of 2006, PF TC 'Doll' was investigated for its ability to initiate somatic embryogenesis. Leaves and petioles were used for these studies. To begin the preliminary study, two- to three-week-old TC horseradish leaves and petioles were cut from proliferating cultures (Norton *et al*., 2001) and explanted on MS medium supplemented with 2,4-D at 0.5 and 1.0 $mg.l^{-1}$ (recommended by Shigeta and Sato, 1994) and a control without 2,4-D. Fifteen to twenty-five explants were used for each treatment; the leaf and petiole explants were evaluated

after four weeks in culture. The experiments were repeated three times. Without 2,4-D explants formed only shoots; with 2,4-D callus formed was randomly picked from both 2,4-D (0.5 and 1.0 $mg.l^{-1}$) treatments and photographed under a stereomicroscope with a digital camera (16 calli per treatment); the pictures were printed, and the number of SEs per callus was assessed by counting. The results were reported in section 3.3.1.

To investigate the possible role of 2,4-D on horseradish somatic embryogenic callus induction, PF horseradish leaf and petiole explants of '1069' and '7586' were placed on MS media with two levels of 2,4-D (0.5, and 1.0 $mg.l^{-1}$). Fifteen to twenty-five explants were used for each treatment; the leaf and petiole explants were evaluated after four weeks in culture. The experiments were repeated two times. Callus of '1069' and '7586' yielded slow growing compact calli without obvious globular structures. Since 'Doll' had formed SE and '1069' and '7586' did not, PF 'Doll' was chosen for all subsequent *in vitro* experiments.

3.2.4 Effect of 2,4-D on induction of somatic embryogenesis on leaf and petiole explants

To further investigate the role of 2,4-D and the explant source on SE induction, a wider range of 2,4-D concentrations was tested with leaves and petioles harvested from two- to three-week-old TC PF 'Doll'. Leaves were cut into squares (0.5 × 0.5 cm) that included a mid-vein. Petioles were cut from the leaf blades *ca.* 0.5 cm^2. The explants were cultured in Petri dishes with MS medium supplemented with four levels of 2,4-D (0.5, 1.0, 1.5, and 2.0 $mg.l^{-1}$). PGR-free MS medium was used as control. Explants were assigned to treatments in completely randomized design (CRD). Each treatment included five Petri dishes with five explants on each dish. They were incubated under light and culture room conditions described earlier (section 3.2.1). Each treatment was replicated four times. After four to six weeks of culture, the calli

were determined their types, color and diameter. To rate the embryogenic potential of callus, an overall score for embryo production was established by estimating the number SEs formed. The scores were divided into three categories: 1) "3" for the embryogenic callus with 26 or more SEs per callus; 2) "2" for the embryogenic callus with 10 to 25 SEs per callus; and 3) "1" for the embryogenic callus with 0 to 10 SEs. All calli were rated in the experiment. The diameters of calli were analyzed by using ANOVA of SAS institute, Cary, NC (2007).

3.2.5 Role of BA and 2,4-D on SE induction on leaf explants

In the previous experiment (section 3.3.2), it was shown that 2,4-D at either 0.5 or 1.0 $mg.l^{-1}$can initiate somatic embryogenic callus from leaf explants *in vitro*. Since cytokinins were traditionally used to encourage adventitious shoot initiation and growth, it was hypothesized that calli grown on a media with both BA and 2,4-D would produce more embryogenic tissue than medium supplemented with 2,4-D alone. To test this possibility, various concentrations of 2,4-D and a single level of BA were tested. The concentrations of 2,4-D used in this experiment were 0.25, 0.5. 0.75 and 1.0 $mg.l^{-1}$. BA was used at one concentration, 0.25 $mg.l^{-1}$. The overall embryogenic callus formation was scored by using the "1 to 3" scale discussed in section 3.3.2.

To investigate the effect of 6-Benzylaminopurine (BA), a plant growth regulator, on somatic embryogenic callus induction, leaves and petioles were harvested from two-to three-week-old TC PF 'Doll' (section 3.2.2). The leaf explants were placed on MS media containing one of four concentrations of 2,4-D (0.25, 0.5, 0.75 and 1.0 $mg.l^{-1}$) with or without a single level of BA (0.25 $mg.l^{-1}$). PGR- free MS was used as a control group. The cultures were grown in the culture room under the conditions described above. Each treatment included five Petri dishes; each contained five explants. The treatments were repeated four times, and experiments were

conducted as a CRD. Once embryogenic callus formed, the type, color and diameter of callus were recorded. ANOVA of SAS institute, Cary, NC (2007) were used for diameter analysis.

3.2.6 Somatic embryogenic callus maintenance

Once somatic embryogenic callus was induced, it was usually maintained and subcultured on the same medium, *i.e.* the somatic embryogenic callus medium. In the previous experiment (3.2.5) all leaves grown on medium containing only 2,4-D formed embryogenic callus. The callus grown on 2,4-D (0.25 $mg.l^{-1}$) seemed to be the most friable; it was chosen for this experiment (discussed in section 3.3.3). To test the suitability of using 2,4-D (0.25 $mg.l^{-1}$) MS induction medium as a maintenance medium for 'Doll' embryogenic calli, 60 to 72 clumps of pea size (6.75 mm in diameter) embryogenic tissue were harvested from MS induction medium. These embryogenic calli were transferred to and cultured on fresh induction medium for four weeks. After four weeks in culture, the callus diameters were measured. The experiment was repeated two times.

3.2.7 Germination and conversion of SEs

Twenty-five two- to three-week-old horseradish leaf explants were grown on MS medium supplemented with 2,4-D (1.0 $mg.l^{-1}$) using five explants per petri dish. After four weeks in culture, embryogenic calli with SEs formed. Somatic embryos at the globular and cotyledonary stages were pricked from the embryogenic calli and transferred individually to culture tubes containing PGR-free MS medium (10 ml/tube). A total of 336 SEs were collected; five replications contained 50 SEs each; one replicate contained 86 SEs. Somatic embryo

development was observed weekly using a stereo microscope. The number of germinating SEs and the number of germinating SEs that converted to plantlets was recorded.

Somatic embryos that had healthy roots (one to three centimeters in length) and at least two true leaves were considered to have completed conversion and to be ready to move to soil (Figure 1a). Converted SEs were lifted from their tubes and their roots freed of clinging agar by swirling them in a tub of warm water. They were then transferred to soil in nine-celled packs (5 × 5 × 2.25 inch) filled with Sunshine® soil mix (Sungo Horticulture, Canada Ltd, Appendix C). The plants were acclimatized in a plastic tub (16 × 9 × 5 inch) that was covered with plastic wrap and grown under cool white florescent light in the laboratory (Figure 1b). After three to four days of acclimatization, the plantlets were transferred to benches under a shade cloth in the UIUC greenhouse (Figure 1c). After one month in the greenhouse, the survival rate was recorded.

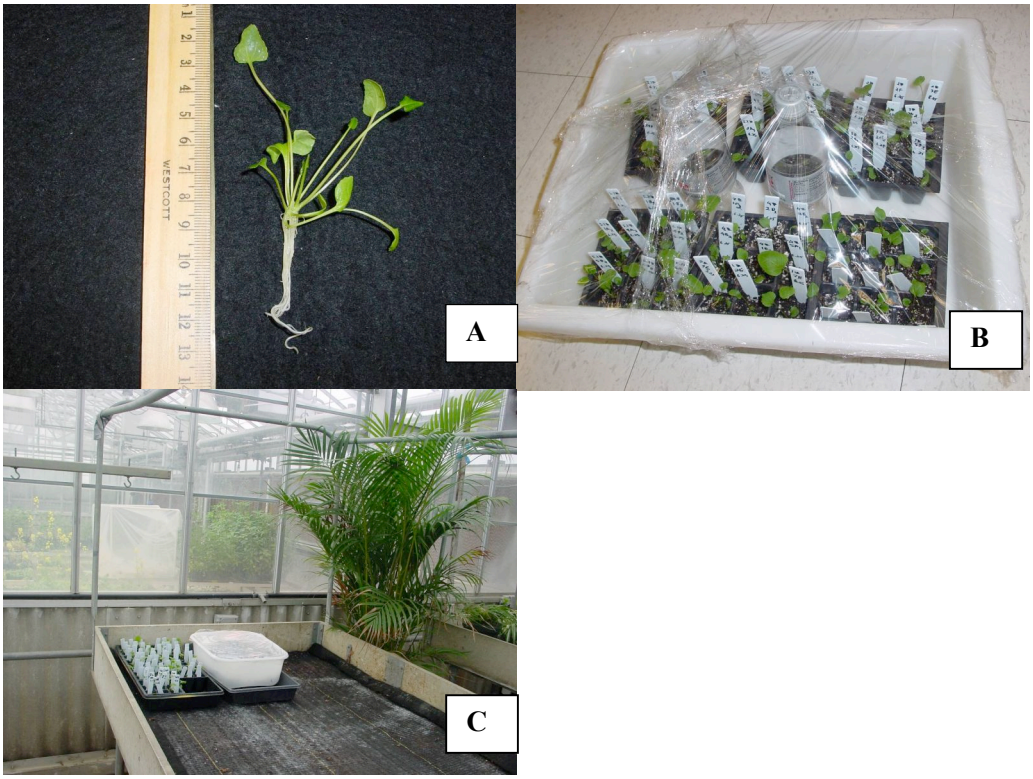

Figure 1. ‘Doll’ horseradish somatic embryos have undergone conversion to yield a healthy plant with a strong root and shoot system.
A, the converted horseradish plantlet was ready to be moved to soil *ex vitro*.
B, the horseradish plantlets derived from ‘Doll’ somatic embryos were acclimatized either in our laboratory.
C, they later moved into a greenhouse under mist.

3.2.8 Horseradish seed germination

Another objective of this thesis was to compare SE development to *in vitro* germination of sexual embryos. Because horseradish seeds are rare and difficult to obtain, and no vernalized ‘Doll’ horseradish plants flowered *ex vitro* (data not presented), open pollinated horseradish seeds were gathered from the UIUC horseradish germplasm collection for these studies.

3.2.8.1 Germination of '807A', '1053A' and '1069' horseradish seeds

3.2.8.1.1 Seed collection

In late May, 2008, various cultivars in the UIUC horseradish germplasm collection were observed to be in flower; among these were the cultivars '807A', '1069' and '1053A' (Figure 2a and b). In late July, 2008, flowers and seeds were harvested from germplasm-selected plants, identified with a tag, wrapped in a plastic bag to prevent seed loss or mixing and returned to our lab. Seeds were removed from the dried silique by shaking the flower clusters over white paper. Seed lots of each cultivar were stored in glass Petri dishes at room temperature until needed.

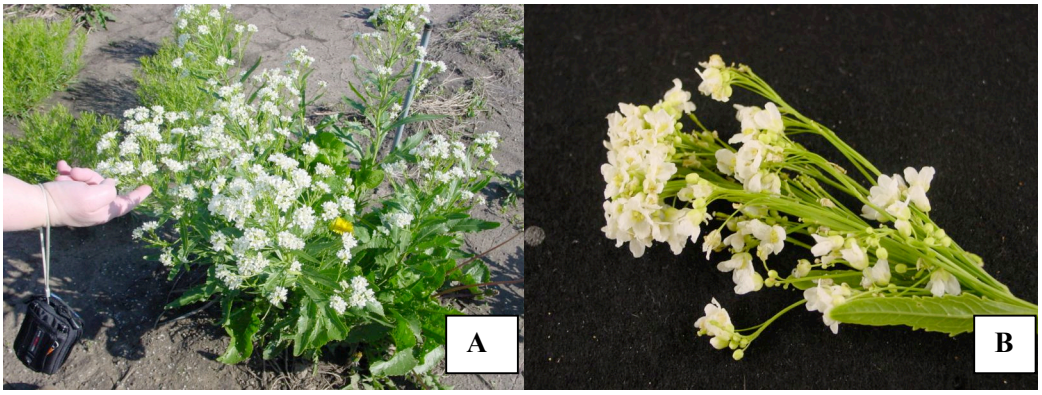

Figure 2. Horseradish plants in the UIUC horseradish germplasm collection.
A, flowering horseradish plants
B, an individual horseradish flower clusters.

3.2.8.1.2 Seed disinfestations and seed germination

Since horseradish seeds were very small (average diameter of seeds = *ca*. 1.5 mm), disinfestation was difficult. Seeds were disinfested by wrapping them in nylon mesh bags, closed with paper clips and placed into bottles containing 15 ml of a 0.6% sodium hypochlorite solution (10% v/v commercial bleach) supplemented with a surfactant reagent (0.1% triton X-100). The bottles were shaken on a rotary shaker (100 rpm) for five to ten minutes. The seed bags were rinsed with sterile water two times, five minutes per rinse. The seeds were transferred

individually with sterile forceps to culture tubes (25 × 150 mm) containing PGR-free MS medium. Six to eleven seeds were available for each replicate, one seed per tube. There were three replicates. Their germination progression was monitored weekly.

3.3 Results

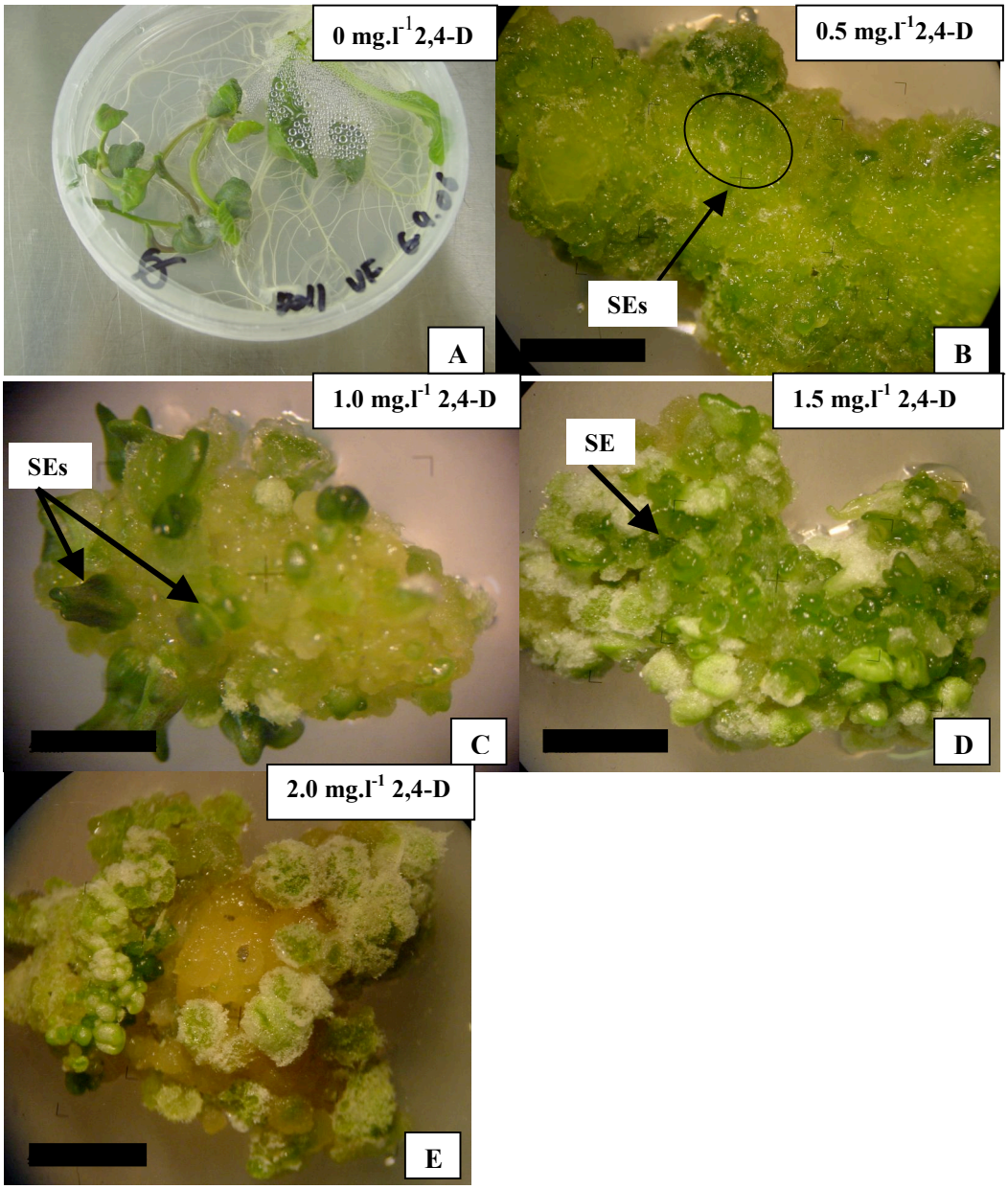

Figure 3. The induction of somatic embryogenic callus on 'Doll' horseradish leaf explants on MS medium with various 2,4-D concentrations.
A, 0 mg.l^{-1}: explants formed roots and adventious shoots (bar = 5mm).
B, 0.5 mg.l^{-1}: explant formed embryogenic callus with minuscule globular embryos (SEs) (arrow) (bar = 5 mm).
C, 1.0 mg.l^{-1}: explants MS formed embryogenic callus and visible SEs; with some had cotyledon (arrow) (bar = 5mm).
D, 1.5 mg.l^{-1}: explants formed embryogenic callus, globular SEs that failed to develop further and non-embryogenic structures (arrow) (bar = 5mm).
E, 2.0 mg.l^{-1}:explants formed non-embryogenic callus and SE-like structures that failed to develop further (bar = 5mm).

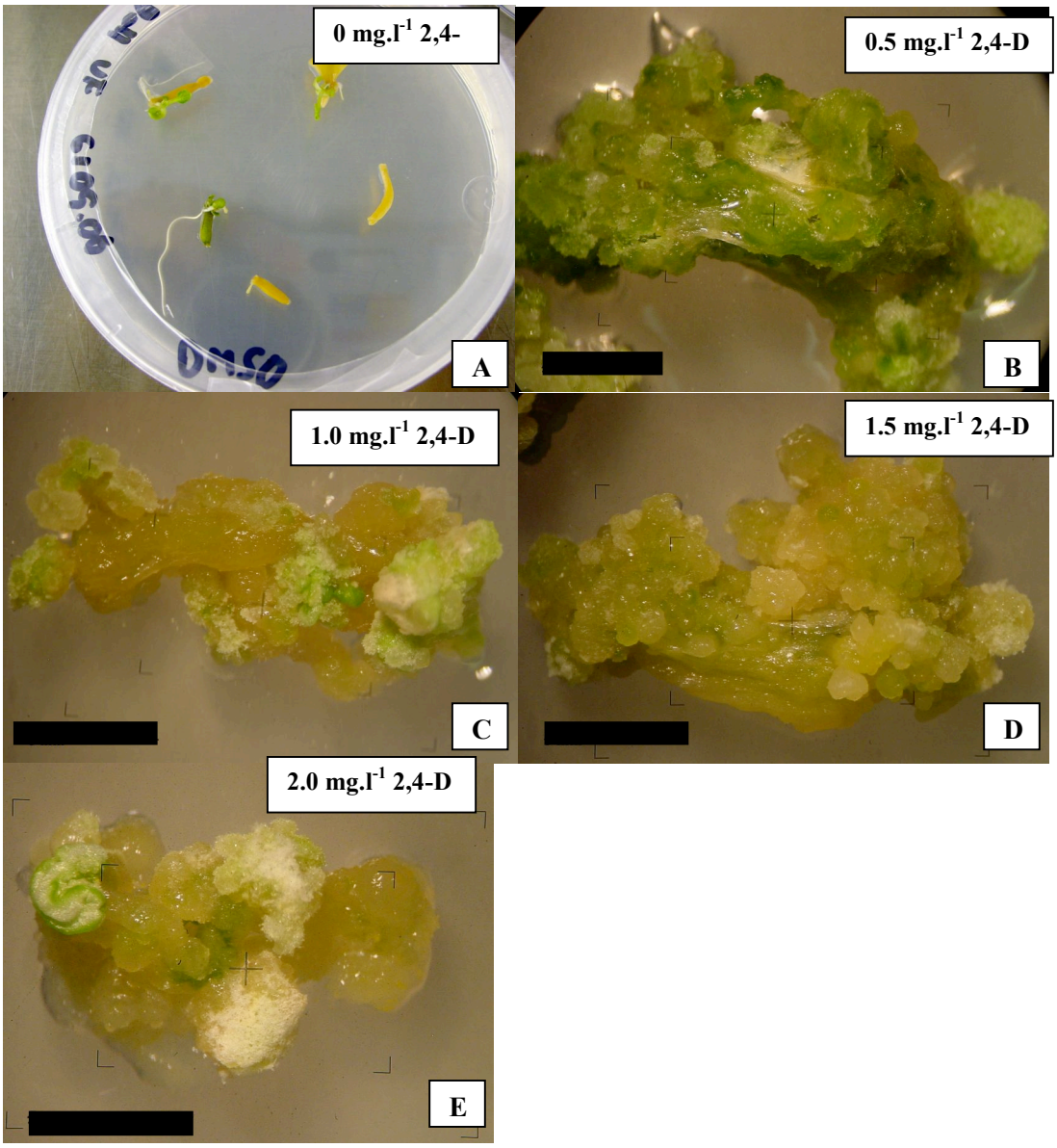

Figure 4. The induction of somatic embryogenic callus on 'Doll' horseradish petiole explants MS medium with various 2,4-D concentrations.
A, 0 mg.l^{-1}: explants died (bar = 5mm).
B, 0.5 mg.l^{-1}: explant formed lightly green non-embryogenic callus (bar = 5mm).
C, 1.0 mg.l^{-1}: explants formed white+green+yellow non-embryogenic callus (bar = 5mm).
D, 1.5 mg.l^{-1}: explants formed white+green+yellow non-embryogenic callus (bar = 5mm).
E, 2.0 mg.l^{-1}:explants formed white+green+yellow non-embryogenic callus (bar = 5mm).

3.3.1 Preliminary studies of explant sources and 2,4-D for SE induction.

Leaf explants grown on PGR-free MS formed adventitious plants from their cut edges (Figure 3a). Leaf explants cultured on MS medium with 2,4-D (0.5 $mg.l^{-1}$) formed light green loose granular calli with globular green SE-like structures (Table 1 and Figure 3b). The calli induced on 1.0 $mg.l^{-1}$ 2,4-D were light green to yellowish with structures that resembled globular-stage SEs, as well as SEs with cotyledon-like structures (Table 1 and Figure 3c). The calli produced on medium with 1.0 $mg.l^{-1}$ 2,4-D were less friable than those produced by the 0.5 $mg.l^{-1}$ 2,4-D treatment.

The relative numbers of SEs formed by each 2,4-D treatment (0.5 and 1.0 $mg.l^{-1}$2,4-D) were quantified by counting. The absolute number of SEs produced ranged from numerous (> 60 SEs) but too small to count (minuscule SEs, Figure 3b) to countable (visible SE, Figure 3c). The number of SEs from 1.0 $mg.l^{-1}$ 2,4-D treatment ranged from 0 to 113. However, the average numbers of SE-like structures found from both treatments were not significantly different. The average number of SEs on calli treated with 0.5 $mg.l^{-1}$ 2,4-D was 26. The average number of SEs induced by 1.0 $mg.l^{-1}$ 2,4-D was 33 (Table 1). Therefore, SEs on embryogenic calli treated with 2,4-D at 0.5 to 1.0 $mg.l^{-1}$ were either visible SEs (25 to 30/ side) on each side of each callus, or they contained countless diminutive SEs per callus.

Within four weeks of culture, petiole explants cultured on PGR-free MS medium either died or produced one to two adventitious plantlets along their cut edges. Petiole explants grown on 2,4-D (0.5 and 1.0 $mg.l^{-1}$) formed non-embryogenic were yellow to green compact calli; no SE-like structures were observed.

Based on these results, it was concluded that 2,4-D was required to induce induction of leaf and petiole tissue to produce somatic embryogenic callus. The embryogenic calli from leaf

explants appeared to be light yellow to green granular with obvious SE-like structures. Leaf explants regenerated SEs at both 0.5 and 1.0 mg.l^{-1}2,4-D. However, petioles did not form embryogenic calli on the same induction medium. It was hypothesized that petioles may require a higher level of 2,4-D to become embryogenic. In order to verify this, another study of the ability of leaf and petiole segments to regenerate somatic embryogenic callus was initiated. Leaf and petiole segments were grown on TC medium with 0, 0.5, 1.0, 1.5 and 2.0 (mg.l^{-1}) 2,4-D. The results will be discussed in section 3.3.2 (below). Later some of the somatic embryogenic calli of these experiments were used for histological investigations and somatic embryogenic suspension culture studies.

3.3.2 Effect of 2,4-D on induction of somatic embryogenesis on leaf and petiole explants

To overcome the contamination issue, carbenicillin (50 µg.ml^{-1}), a strong antibiotic, was added to MS medium (the details are discussed in Appendix E). Briefly, calli grown with carbenicillin (50 µg.ml^{-1}) supplements remained relatively contamination-free for the first six weeks in culture but contaminants were present after six weeks in culture. Although contaminated, cultures growing with bacteria appeared to grow vigorously and remain healthy.

It was noticed that all levels of 2,4-D (0.5, 1.0, 1.5 and 2.0 mg.l^{-1}) induced leaf explants to form calli (Table 2). However, the calli grown on medium with 0.5 and 1.0 (mg.l^{-1}) 2,4-D had the highest score for embryo production; the size of the calli produced by these treatments was not significantly different. The leaf explants treated with 1.5 and 2.0 (mg.l^{-1}) 2,4-D received the lowest embryogenic scoring because they formed the least amount of embryogenic tissue; in addition, their callus was dry, friable, yellow to white calli and was assumed to be non-embryogenic calli (Figure 3). Petiole explants at 1.5 and 2.0 mg.l^{-1} 2,4-D formed no obvious

embryogenic calli. Instead they produced friable yellow granular calli without SE-like structures (Table 2 and Figure 4). Based on these experiments it was concluded that MS media with either 0.5 or 1.0 ($mg.l^{-1}$) 2,4-D were best for initiating somatic embryogenic callus from PF 'Doll' leaf horseradish. Petioles formed no somatic embryogenic calli, so only leaf explants were used for the following study (3.3.3).

3.3.3 The role of BA and 2,4-D on SE induction on leaf explants.

After six weeks in culture control leaf explants grown on PGR-free medium had rooted and formed adventitious shoots (Table 3). All leaf explants treated with both BA and 2,4-D formed dry white to green solid calli without SE-like structures. All leaf explants cultured on medium supplemented with 2,4-D alone produced visible globular and cotyledonary SE-like structures borne on green granular calli (Table 3). All 2,4-D-only treatments received the same embryogenic callus formation scores of '3' (having 26 SEs or more per callus). The calli induced by 0.25 $mg.l^{-1}$2,4-D appeared more friable than the other 2,4-D-only treatments; their overall embryogenic callus formation score was as high as that produced by 1.0 $mg.l^{-1}$2,4-D. At 1.0 $mg.l^{-1}$2,4-D some of the leaf explants formed embryogenic callus that produced irregular SE-like structures (discussed in section 4.4.1, Figure 13). Thus, MS with 2,4-D (1.0 $mg.l^{-1}$) might be not a good medium for long-term use. Based on the results of this experiment. The induction of embryogenic calli from 'Doll' leaf explants was best achieved with 2,4-D at 0.25 $mg.l^{-1}$. To minimize the risk of mutation in embryogenic calli due to excessive plant growth regulators, the 2,4-D used to induce SE *in vitro* should be lowered to 0.25 $mg.l^{-1}$. From the results, it can be concluded that the addition of BA to MS medium with 2,4-D retards SE formation. However, 2,4-D alone has a strong effect on SE regeneration from leaf explants (Figure 5).

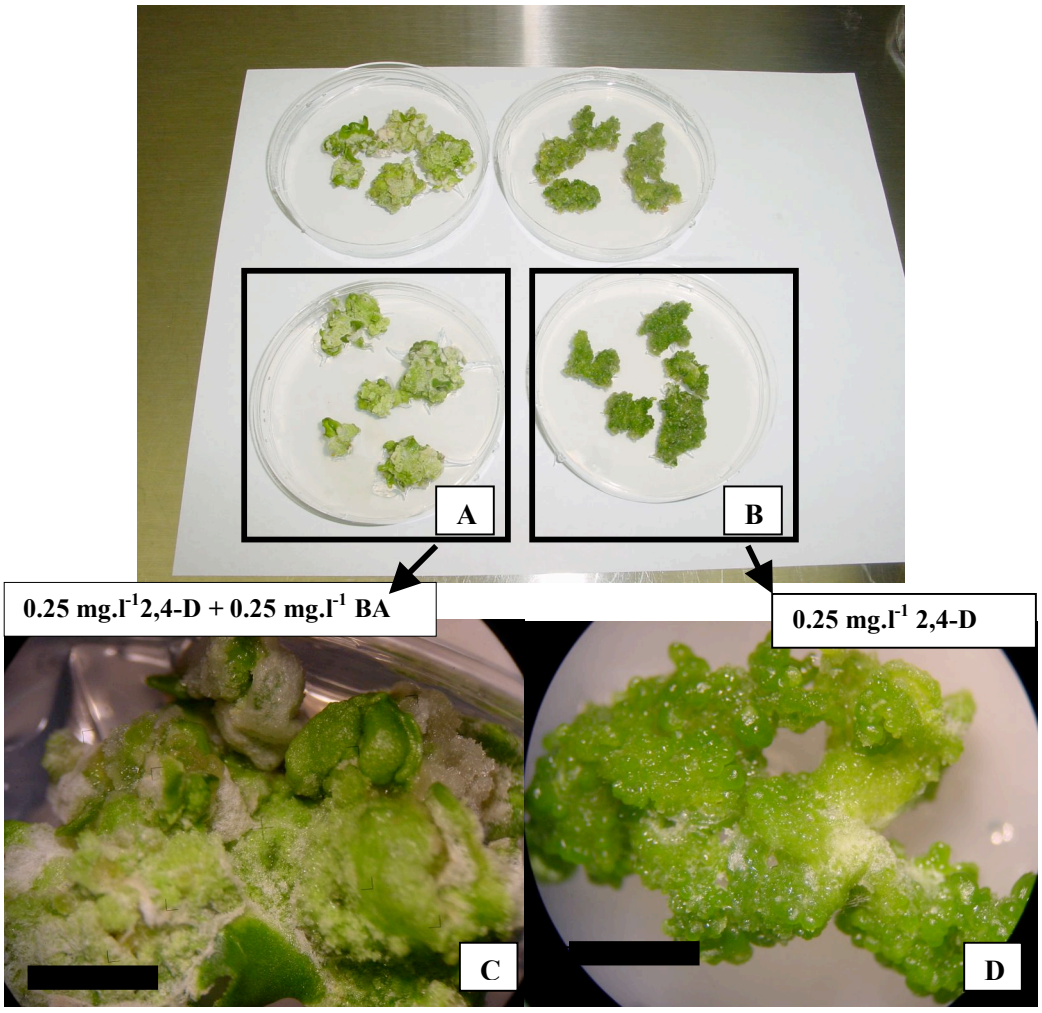

Figure 5. 'Doll' horseradish leaf explants treated with 2,4-D with and without BA.
A, C, the leaf explants treated with (0.25 mg.l^{-1}) and BA (0.25 mg.l^{-1}) formed non-embryogenic calli (bar for C = 5 mm).
B, D, those leaf explants treated with 2,4-D alone (0.25 mg.l^{-1}) formed embryogenic calli (bar for d = 5 mm).

3.3.4 Somatic embryogenic callus maintenance

After four weeks in culture, callus diameters were assessed, and the mean diameter of the calli were analyzed by SAS (2006); the callus diameter ranged from 6.75 to 12.24 $\pm$ 0.21 mm (S.E.= standard error of the mean). Since callus diameter increased about two times the original

size, and the ability to form SEs was retained. 2,4-D at 0.25 $mg.l^{-1}$ was a useful medium for both embryogenic tissue callus initiation medium and maintenance.

3.3.5 Germination and conversion rates of Doll' Somatic embryos (SEs)

'Doll' somatic embryos began to germinate after one week in culture; germination was evidenced by radicle elongation, and true leaves developed later. After three weeks in culture, the germinating SEs had formed healthy roots that were at least one to two centimeters in length and had produced at least two healthy leaves. After four to five weeks in culture, the SEs had completely converted to plantlets with well-formed roots and leaves. The germination rate of 'Doll' SEs was quite high: among 336 SEs cultured on germination medium, 65% of them germinated (215 out of 336). Half (50%) of the germinating SEs converted to plantlets (121 out of 215).

All of the plantlets transferred to soil developed new young leaves and vigorous and healthy roots after three weeks in the greenhouse. After six weeks in the greenhouse, they had become healthy horseradish plants with fully expanded leaves and healthy roots.

3.3.6 Germination of '807A', '1053A' and '1069' horseradish seeds

Field-collected horseradish seeds were very dirty, and their *in vitro* contamination rate was as high as 38% (28 contaminated seeds out of 75 seeds). Contamination percentage decreased slightly when the period of disinfestation in bleach solution was increased from five to ten minutes (data not presented). After one to three weeks *in vitro*, the radicles began to elongate, and hypocotyls and epicotyls appeared later. The rate of germination was slow and sporadic. After four to five weeks in culture, only 18% of the seeds showed signs of germination; among the 75 seeds that germinated, only three seedlings developed fully to become whole plants (Figures 7 and 8).

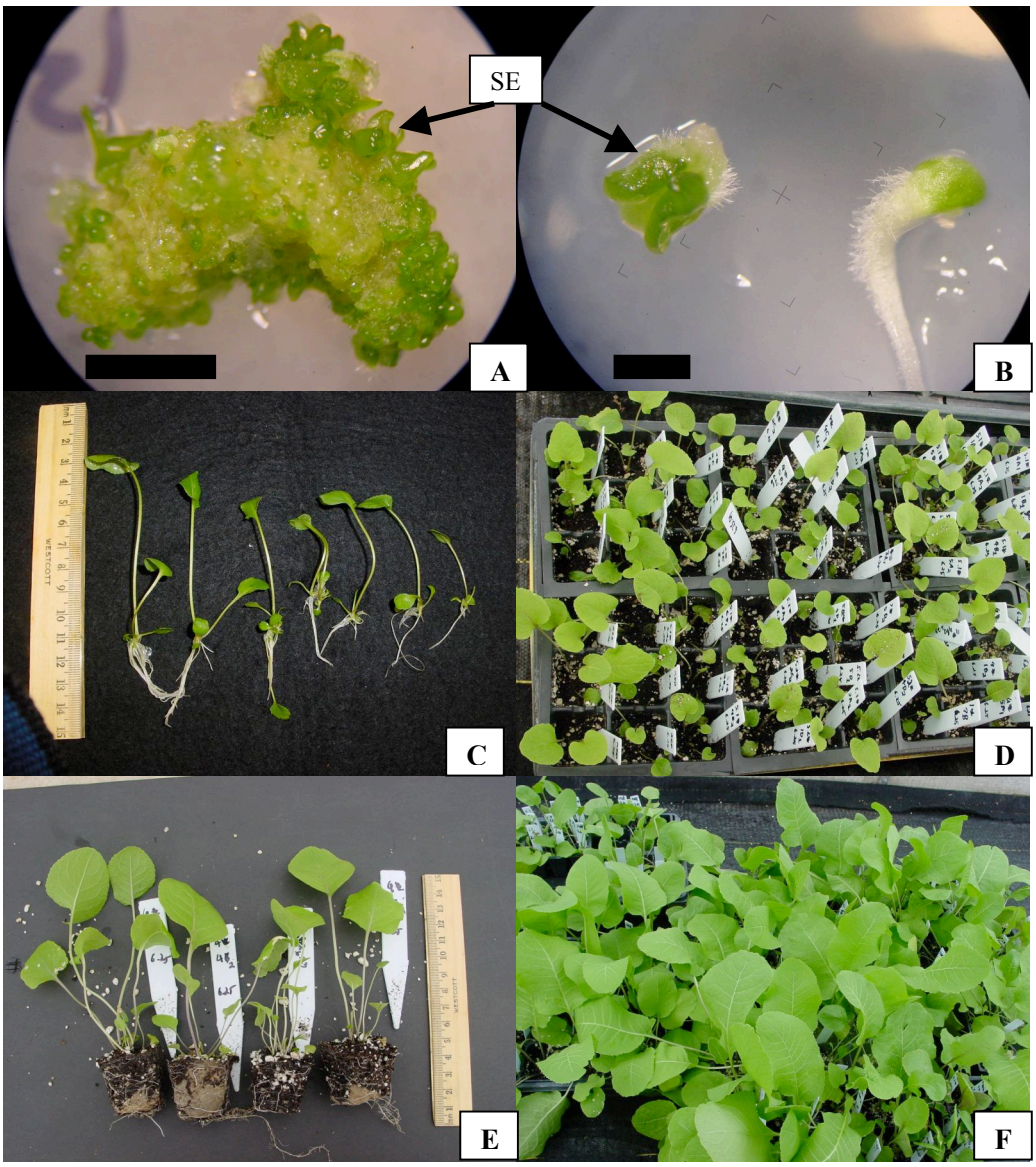

Figure 6. Germination and conversion of horseradish somatic embryos.
A, Somatic embryos were pricked from embryogenic 'Doll' horseradish callus (bar = 5mm).
B, after one week on PGR-free MS, they germinated (bar = 1 mm).
C, after three weeks in culture, they have undergone conversion and developed intact root and shoot systems and they are ready for acclimatization to *ex vitro* conditions under periodic mist.
D, E, F, respectively, acclimatized horseradish plants after one week; three weeks and six weeks in a greenhouse.

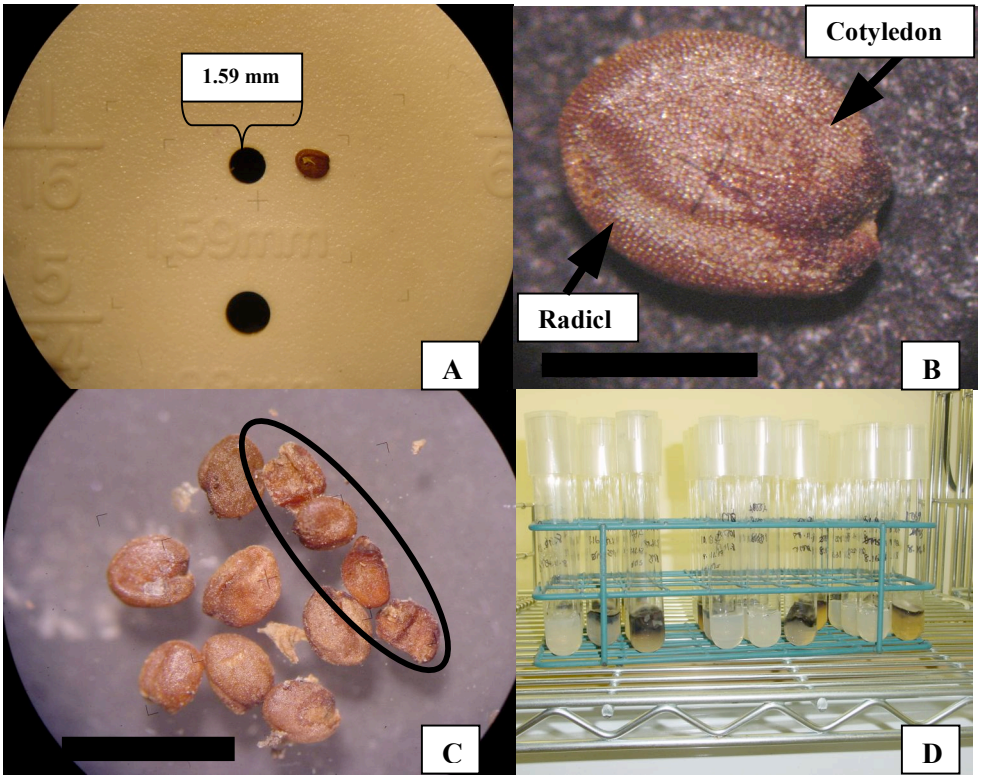

Figure 7. Horseradish seeds.
A, they are very small, *ca* 1.5 mm in diameter.
B, it includes both a "radicle" and "cotyledon" (bar = 1mm).
C, some seeds are shrunken and presumed to be dead (circle) (bar = 5mm).
D, the tubes contain field harvested horseradish seeds that germinated on plant growth regulator free [PGR-free] MS medium. The high rate of contamination is obvious by the darkened medium.

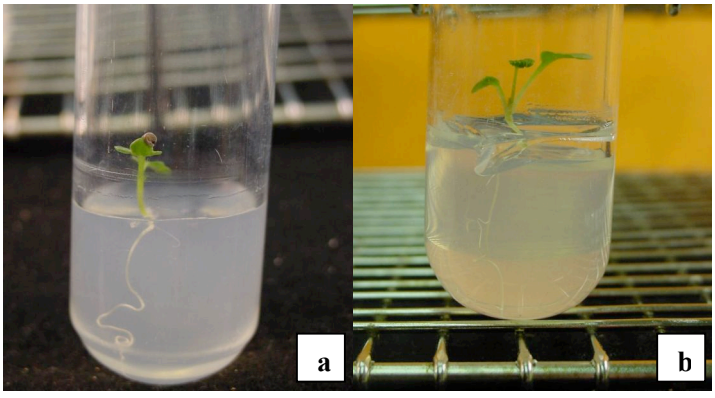

Figure 8. Germinating horseradish seeds. A, '807A'. B,'1069'.

3.4 Discussion

3.4.1 Explant source and SE formation

The ability to form SEs depended on explant source. According to the literature (Chapter II, section 2.8.1), somatic embryogenic calli have been induced from a number of plant parts including leaves, stems, seeds, and floral parts. In horseradish, vegetative tissues were the explant sources used for my studies with many reasons. First, among the vegetative organs available from horseradish plants, roots would be the logical explant of choice, but root tissue has exhibited such a high rate of contamination (personal observation, data not presented), that they were not used. Next, horseradish does not normally produce flowers or set seeds, so its sexual parts were not useful as explants for the present studies (personal observation, data not presented, Stokes, 1955 and Weber, 1949). Thus, these studies were conducted using leaf and petioles explants only.

After comparing leaf and petiole explants for their ability to produce somatic embryogenic callus, leaf explants were found to be better than petiole explants. The reason for this may be related to leaf and petiole anatomy. The histological studies discussed in Chapter V showed that somatic embryogenic cell masses developed from vascular tissue in the veins (Figure 16 c,d). Horseradish petioles possed a single bundle of vascular tissue (Figure 16a); leaf blades were flattened and their veins branch to make the typical net-like venation of dicotyledonous leaves. Because of the branched net venation of flattened leaves, leaf explants had more exposed veins (and potential sites for regeneration) than petioles. In addition, the flattened leaf blades had more surface contact with the TC medium than rounded petiole explants. Thus, the flattened leaf explants made better contact with 2,4-D medium than petioles, facilitating more 2,4-D interaction. In future experiments, it might be useful to cut petioles in

half transversely and plant them cut surface down on the TC medium to increase surface contact and see if regeneration percentages improve.

In the present study, when 100 leaf explants were grown on MS medium with various 2,4-D concentrations, leaf explants on PGR-free MS medium all quickly rooted, formed adventitious shoots and developed to whole plants. Those on 2,4-D media formed SEs. Among 100 petiole explants on the same media, 75% of those on PGR-free MS medium died; the other 25% rooted, shooted and developed weak plants (Table 2). This could also be the situation for horseradish leaves and petioles that endogenous hormone concentration in the horseradish plants may vary among regions of the plants since leaf explants appeared to complement the PGRs encountered *in vitro* to yield better regeneration percentages than petiole explants.

3.4.2 PGRs and SE induction

The ability of PGRs to induce 'Doll' horseradish leaf and petiole explants to form embryogenic callus was tested. Results from these present studies confirmed that 2,4-D can induce somatic embryogenesis in horseradish. At 0.25 to 1.0 $mg.l^{-1}$, 2,4-D leaf explants were stimulated to form green nodular embryogenic callus with SEs. When BA was added to 2,4-D media, leaf explants formed only non-embryogenic calli. These results differed from the findings of Shigeta and Sato (1994), who reported that horseradish leaf explants developed embryogenic callus on 2,4-D (0.1 $mg.l^{-1}$) combined with BA (0.1 $mg.l^{-1}$). Similarly, Mevy *et al.* (1997) found 2,4-D (0.2 $mg.l^{-1}$) and kinetin (0.2 $mg.l^{-1}$) promoted horseradish embryogenic calli. Parkinson *et al.* (1990) used 2,4-D (5 $mg.l^{-1}$) and kinetin (0.1 $mg.l^{-1}$) to establish friable callus. The fact that 'Doll' horseradish leaf explants did not respond to BA may be genotype specific. Genotype specific somatic embryogenesis has been reported in other crops including alfalfa

(Chen *et al.,* 1987) sweet potato, (Triqui *et al.,* 2008), *Gentiana kuroo* (Agniezka and Rybczunski, 2008) and eggplant (Mir *et al*., 2008).

3.4.3 Germination and conversion of 'Doll' SEs

The germination and conversion efficiency of 'Doll' SEs in comparison to sexually-derived seeds and seedlings of 'Doll' were investigated. 'Doll' plants were vernalized for either 6 or 8 weeks. Unfortunately, 'Doll' plants did not produce flowers, so no sexually-derived seeds were available. Seeds collected from other horseradish cultivars germinated poorly. Many authors have made similar observations. For example, Stokes (1955) reported that some of their sexually-derived horseradish embryos differentiated *in vivo* to produce cotyledons and a radicle by 18 days post pollination. However, most of the seeds subsequently either aborted or failed to complete germination. Seed failure was often due to endosperm-maternal tissue incompatibility.

In this study only 14 of 75 (18.7%) sexually-derived horseradish seeds germinated on PGR-free MS medium as evidenced by testa rupture, radicle penetrate, cotyledon appearance; of these, only 3 of 75 (4%) became seedlings. Most of field-harvested seeds were lost due to contamination during the first and second transferring on *in vitro* germination medium.

The ability of SEs to germinate and convert to plantlets depended on the species. Some require no PGRs; for example, *Cedrela fissilis* germinated on PGR-free MS medium (Villa *et al*., 2009). In contrast, some species did not germinate and/or undergo conversion unless they were pretreated with specific PGRs or receive specific drying treatments (Bhojwani and Soh, 2001). Persian walnut SEs showed better germination and conversion rate when they were matured on maturation medium with ABA (2.0 $mg.l^{-1}$) (Vahdati *et al.,* 2007) than the ABA-free treatment group. However, in this study it was found that half (50%) of the 'Doll' horseradish SEs were

able to germinate directly on PGR-free MS medium. Sexually-derived horseradish seeds germinated much less efficiently than SEs (*ca*. 4% vs 50%, respectively).

By comparing the development of horseradish embryos to published germination studies in some other *Brassica* embryos (Muller *et al.,* 2006), it was possible to better interpret the germination and conversion pattern of the SEs in this study. It appeared that 'Doll' SEs germinated in the same way as sexual embryos of *A. thaliana* and *L. sativum*. On PGR-free MS medium, the radicle of SEs elongated as the first sign of visible germination; then they formed leaf and root systems to become plantlets. 'Doll' SEs did not appear to require dormancy-breaking treatments to germinate and undergo conversion. A future study could be to investigate the usefulness of GA and ABA for improving the germination rate in 'Doll' SEs, as was the case for *A. thaliana* and *L. sativum.*

Thus, SE germination and conversion mimic the natural germination pathway of the Brassicacae. Successful horseradish as artificial seeds would be expected to germinate efficiently and yield whole plants identical to the mother plant. This system could be used to produce the hundreds of thousands of cloned PF horseradish plants that are needed annually to support the local horseradish industry. Furthermore, it is proposed that germinating SEs could be a good way to deliver pathogen-free plants to the commercial grower.

Table 1. The induction of somatic embryogenic callus and somatic embryos (SEs) on pathogen-free 'Doll' horseradish leaf explants grown on MS medium containing 2,4-D after four weeks *in vitro* (preliminary studies).

Explant	2,4-D ($mg\ l^{-1}$)	No. of explants (n)	No. of Embryogenic calli with both visible globular and cotelydonary-like structures (%)	No of SEs per callus (Avg. $\pm$ std error [Y])
Leaf	0	63	0	0
	0.5	55 [Z]	30 (55.5%)	$(26 \pm 6.9)^{a}$ [X]
	1.0	63	56 (88.8%)	$(33 \pm 8.5)^{a}$ [X]
Petiole	0	63	0	0
	0.5	60 [Z]	0	0
	1.0	63	0	0

[Z] some of the explants lost due to contamination.

[Y] std error = standard error of mean from numbers of SEs found on 16 embryogenic calli

[X] means followed by the same letter are not significantly different at $P<0.05$ according to T-Test

Table 2. The induction of somatic embryogenic callus and somatic embryo (SEs) on pathogen-free horseradish 'Doll' leaf and petiole explants on MS medium containing 2,4-D after six weeks *in vitro* (n=100).

Explant	2,4-D (mg l^{-1})	No. of explants with embryogenic callus	No of SEs /explant (estimated)	Avg. calli diameter (cm)
Leaf	0	0	0 [Z]	0
	0.5	100	> 30	1.99[a X]
	1.0	100	> 30	1.93[ab X]
	1.5	100	10-30	1.90[ab X]
	2.0	100	<10	1.82[b X]
Petiole	0	0	0 [Z]	0
	0.5	0	0 [Y]	0
	1.0	0	0 [Y]	0
	1.5	0	0 [Y]	0
	2.0	0	0 [Y]	0

[Z] several adventitious shoots formed but there was no obvious callus or somatic embryos

[Y] petiole explants developed non-embryogenic callus.

[X] means followed by the same letter are not significantly different at $P<0.05$ according to Tukey's Studentized Range Test

Table 3. The induction of somatic embryogenesis and somatic embryos (SEs) on pathogen−free 'Doll' horseradish leaf explants grown on MS medium containing 2,4-D and/or BA after six weeks *in vitro.*

2,4-D ($mg\ l^{-1}$)	BA ($mg\ l^{-1}$)	No of explants	No. of explants with embryogenic callus	No of SEs/explant (estimated)	Callus diameter (cm)
0	0	95	0 [Z]	0 [Z]	0
0.25	0	100	95	>30	2.01[a X]
0.5	0	100	100	>30	1.91[ab X]
0.75	0	100	100	>30	1.85[bc X]
1.0	0	100	100	>30	1.76[c X]
0.25	0.25	95	0 [Y]	0 [Y]	0
0.5	0.25	100	0 [Y]	0 [Y]	0
0.75	0.25	100	0 [Y]	0 [Y]	0
1.0	0.25	100	0 [Y]	0 [Y]	0

[Z] several adventitious shoots formed but there was no obvious callus or somatic embryos.

[Y] with BA (0.25 $mg.l^{-1}$), leaf explants developed non-embryogenic callus.

[X] means followed by the same letter are not significantly different at $P<0.05$ according to Tukey's Studentized Range Test

Table 4**.** *In vitro* germination of open pollinated horseradish (HR) seeds.

HR cultivars	No. of seeds (n)	Germination n (%)	Surviving seedling n (%)
807A	27	7	0
1069	27	4	1
1053A	21	2	2
Total	75	14 (18.7%)	3 (4%)

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CHAPTER IV: SOMATIC EMBRYOGENIC CELL SUSPENSION CULTURE PROTOCOLS FOR 'DOLL' HORSERADISH

4.1 Introduction

The use of somatic embryogenesis could reduce the labor costs and time required to introduce new or PF horseradish clones. This would be accomplished by eliminating the field-based hand labor normally required for set propagation. In chapter III, we demonstrated that 'Doll' horseradish leaf explants can produce SEs *in vitro*. To study SE development for producing synthetic seeds, it was important to first develop 'Doll' horseradish somatic embryogenic cell suspension culture protocols. Thus, in this chapter the establishment of somatic embryogenic suspension cultures in general (section 4.1.1), and the methods used to initiate 'Doll' somatic embryogenic suspension culture (section 4.1.2, and 4.2) that suitable for use as synthetic seeds will be discussed.

4.1.1 Somatic embryogenic suspension culture

The general process of embryogenic suspension culture involved the initiation and subculture of cell suspensions, synchronization of cell divisions to the same stage of somatic embryogenic cell development, and maturation of somatic embryos (Ibaraki and Kurata, 2001). Suspension cultures had been used to make SEs in many species including gladiolus (*Gladiolus* x *grandiflorus* cv. Peter Pears, Remotti, 1995), cowpea (*Vigna unguiculata* L., Ramakrishnan *et al*., 2005), banana (*Musa* AAA cv. Grande naine, Wong *et al.*, 2006) and bringal (*Solanum melongena* L., Hossain *et al*., 2007).

To initiate somatic embryogenic cell suspensions of any species, friable embryogenic calli were collected from agar-based induction medium transferred to the same medium but without agar (liquid medium). When agitated on a rotary shaker (approx. 90 to 130 rpm), free single embryogenic cells and small clusters were released. To maintain viable embryogenic cell suspension cultures, the suspension culture should be subcultured when it contained the highest number of viable embryogenic cells. The subculture time depended on the species, but it was usually at two-week intervals (Kim *et al.,* 2004; and Li and Kurata, 2005). For example, Vargas *et al*. (2005) demonstrated that embryogenic cell suspensions of potato *(Solanum tuberosum*) growing in MS media with 2,4-D (0.5 $mg.l^{-1}$) and kinetin (0.5 $mg.l^{-1}$) increased their cell numbers from 2.2×10^4 viable cells/ml to 6.87×10^4 viable cells/ml in up to the first thirteen days in culture. After the thirteenth day the number of viable embryogenic potato cells decreased because the cells began to die due to nutrient depletion.

The relative turbidity of a suspension culture was a simple way to estimate when the cell suspensions reached their exponential growth phase, and it was time to sub-culture the cell suspension. However, to measure the growth rate of plant cells in suspension cultures precisely, cell volume was established by one or more of these procedures: settled cell volume, packed cell volume, fresh cell weight, dry cell weight, or cell counting (Godoy-Hernandez and Vazquez-Flota, 2005).

Embryogenic cell suspension cultures at the initiation stage often had embryogenic cells mixed with another cell types (Kim *et al.*, 2004). For example, banana (*Musa* AAA cv. Grande naine) suspensions contained five types of cell aggregates: Type I consisted of isolated cells or small cell aggregates composed of non-joined cells. Type II included opaque compact embryogenic cells. Type III was comparable to those of Type II but contained a peripheral

proliferation zone of embryogenic cells. Type IV cells were compact proembryos. Type V was compact and composed of a central zone with meristemic cells and a peripheral zone with starchy cells. All types eventually formed SEs except Type V aggregates (Georget *et al.*, 2000). Similarly, Remotti (1995) reported that embryogenic *Gladiolus* suspension cultures at the exponential stage, they contained a large number of single cells as well as smaller cell aggregates that could grow into larger aggregates.

A common way to separate single embryogenic cells from non-embryogenic cells and to synchronize embryogenic single cell suspension is to filter the cell suspension through mesh of various diameters (Giuliano *et al.,* 1983). For instance, carrot (*Daucus carota*) single cell embryogenic suspensions developed best from the fractions that were smaller than 22 μm in diameter (Toonen *et al*., 1994). Horsegram (*Macrotyloma uniflorum* Lam.) embryogenic cell suspensions were obtained after filtering their suspension cell cultures through 125 μm stainless steel sieves (Mohamed *et al*., 2004).

Other parameters that affect embryogenic cell suspension includes carefully dissociating embryogenic calli from non-embryogenic calli (Kim *et al.*, 2004) and gradually increasing the volume of primary culture medium (Mikula *et al.,* 2005).

4.1.2 Horseradish suspension cultures

Various methods used to initiate horseradish suspension cultures have been reported. For example, Parkinson *et al*. (1990) first induced friable callus by growing leaf explants on MS with 3% sucrose, 2,4-D (5 $mg.l^{-1}$) and kinetin (0.1 $mg.l^{-1}$). The calli were transferred to MS liquid callus induction medium. The suspension culture was subcultured weekly by culturing one part suspension in three parts fresh medium. Once the suspension culture began its late exponential

phase, 25g/l fresh weight of cell aggregates were collected and transferred into 50 ml of fresh medium..

Shigeta and Sato (1994) cultured horseradish embryogenic calli in MS liquid media supplemented with 2, 4-D (0.1 mg.l^{-1}) and BA (0.1 mg.l^{-1}) and reported that within four to six weeks various sized cell clusters formed in the suspension culture. They separated the various cluster sizes (< 100 µm, 100 µm to 1 mm and > 1mm[1]) by filtering them through stainless steel filters. After four weeks in culture, SEs developed spontaneously from cell aggregates between 100 µm to 1 mm in diameter in PGR-free MS liquid medium. The clumps that were greater than1 mm in diameter were resuspended in embryogenic induction liquid medium to initiate a new somatic embryogenic suspension.

Mevy *et al*. (1997) initiated horseradish suspension cultures by transferring three grams of 28-day-old embryogenic calli to 100 ml of MS liquid media with kinetin (0.2 mg.l^{-1}) and 2,4-D (0.2 mg.l^{-1}) contained in a 250 ml-flask. The suspensions were subcultured at 21 day intervals for six months until they became fine (single cells and small cell clusters) suspension cultures.

4.2 Materials and methods

4.2.1 Preliminary studies to initiate somatic embryogenic cell suspensions of 'Doll' horseradish - Part I

The objective of the first preliminary study was to initiate somatic embryogenic cell suspension cultures. Five-week-old friable embryogenic 'Doll' calli grown on MS agar media with 2,4-D (0.25 mg.l^{-1}) were used for this study. One gram of embryogenic callus was weighed and transferred to a 125-ml Erlenmeyer flask containing 20 ml of MS liquid media with 2,4-D

[1]= the specific pore mesh sizes used in the experiments were not specified by the authors

(0.25 $mg.l^{-1}$). Four flasks were prepared and shaken on a rotary shaker (100 RPM) for eight weeks with intervals three to four week subcultures. All were grown under the controlled temperature and lighting conditions previously described.

After three weeks in suspension, calli were separated from the supernatant by removing the culture from the shaker, allowing the cells and aggregates to settle to the bottom of the flasks. The supernatant from each flask, which contained cells and small aggregates, was then subcultured by transferring it into a new 50 ml graduated centrifuge tubes. Half of the supernatant (10 mls) was discarded. Fresh 0.25 ($mg.l^{-1}$) 2,4-D MS liquid media was added to each tube and its supernatant until the total of volume reached 20 ml. All (20 mls media + 1 gm of calli) were then transferred from the centrifuge tubes to clean 125 ml Erlenmeyer flasks. The flasks were placed on the shaker for four to five weeks under conditions previously described.

After a total of eight weeks in culture, the suspension cultures were filtered through various sized nylon mesh in order to separate the cell clumps from the suspension medium. The pore sizes of the various meshes were 0.1, 0.5, 1.0 and 1.32 mm. The first flask (F1) was filtered through the 1.32 mm pore size mesh. The second, third and fourth flasks (F2, F3 and F4) were filtered through the 1.0, 0.5 and 0.1 mm meshes, respectively. 10 ml of the filtered cell suspensions were collected and transferred into 10 ml MS liquid media without 2,4-D. The cell aggregates that were too large to pass through the mesh were moved to PGR-free MS agar media. All growth was assessed four weeks later.

4.2.2 Preliminary studies to initiate somatic embryogenic cell suspensions of 'Doll' horseradish - Part II

In a second attempt to establish somatic embryogenic cell suspensions, the previous experiment was repeated with some modifications based on the findings made in the first preliminary study (4.2.1). The amount of calli used to initiate the suspension culture was increased, and the growth rate of suspensions was estimated.

The suspensions in this study were established from friable embryogenic calli that included some SE-like structures. The SEs had been induced on MS agar media with 2,4-D (0.25 $mg.l^{-1}$) and then subcultured on the same fresh induction media. In this study, 125 ml Erlenmeyer flasks were used as culture vessels.

The initiatial suspension was made by increasing the ratio of callus to medium volume to two grams per 20 ml of MS liquid media supplemented with 2,4-D (0.25 $mg.l^{-1}$). Four flasks (specified as F1, F2, F3 and F4) were made. All flasks were shaken on the rotary shaker for eight weeks under the controlled light and temperature condition described earlier (4.2.1).

The suspensions were subcultured at four and eight weeks. At each subculture, all calli were removed from each suspension flask, and the quantity of free cells and cell aggregates were determined by using the packed cell volume method (Godoy-Hernandez and Vazquez-Flota, 2005). Briefly, supernatants were poured into 50 ml graduated centrifuge tubes, centrifuged (at 3,000×g for 5 minutes) and cell sediment volumes measured. Next, 50% of the clear supernatant was drawn from the tubes. Fresh medium was added until the volume of the cell aggregates and medium reached 25 ml. The suspension cultures were returned to the shaker under the conditions described earlier. The volumes of cell sediments from all four flasks were again measured after five and eight weeks in culture by packed cell volume as described earlier.

After eight weeks in culture (the second subculture), the suspension cultures were again monitored for their packed cell volume. The suspension cultures in each tube were then swirled and filtered through a 1.3 mm nylon mesh. The four to six millimeter diameter clumps were discarded. Aggregates smaller than four millimeter in diameter were picked off the mesh, returned to the same suspension culture and then filtered through a 1.0 mm pore size nylon mesh. The cell aggregates left on the nylon mesh were rinsed with PGR-free MS liquid media and suspended in 125 ml flask containing 15 ml of PGR-free MS liquid media for four weeks. The supernatant was discarded.

4.3 Results and discussions

4.3.1. Preliminary studies to initiate somatic embryogenic cell suspensions of 'Doll' horseradish - Part I

After three weeks in culture, it was observed that the suspension cultures became milky and cloudy, suggesting a large number of rapidly dividing cells. These cells were subcultured to fresh liquid medium and placed on the shaker for another five weeks in culture. After another five weeks in culture, various sized yellow-green clumps formed in all four flasks (the approximate diameter of each clump was about 6 mm). The clusters were filtered through various nylon mesh sizes and cultured for another four weeks in PGR-free MS liquid media.

After four weeks on PGR-free MS liquid media, the suspensions established from cell clusters that had passed through the 1.32 mm pore size differentiated fibrous root-like structures (Figure 9a). When these fibrous structures were transferred to fresh PGR-free MS liquid media, they rooted and formed shoots within four weeks (Figure 9b); they developed into a whole plants

after another four weeks on the medium (Figure 9c). None of the suspension fractions filtered through 0.1, 0.5 and 1.0 mm pore sized mesh formed either plantlets or fibrous structures.

After another four weeks in culture the cell aggregates that had been too large to pass through the meshes (aggregates greater than 0.5 mm pore sized mesh) rooted and produced adventitious shoots (Figure 9d). Cell clusters smaller than 0.5 mm diameter did not form plants.

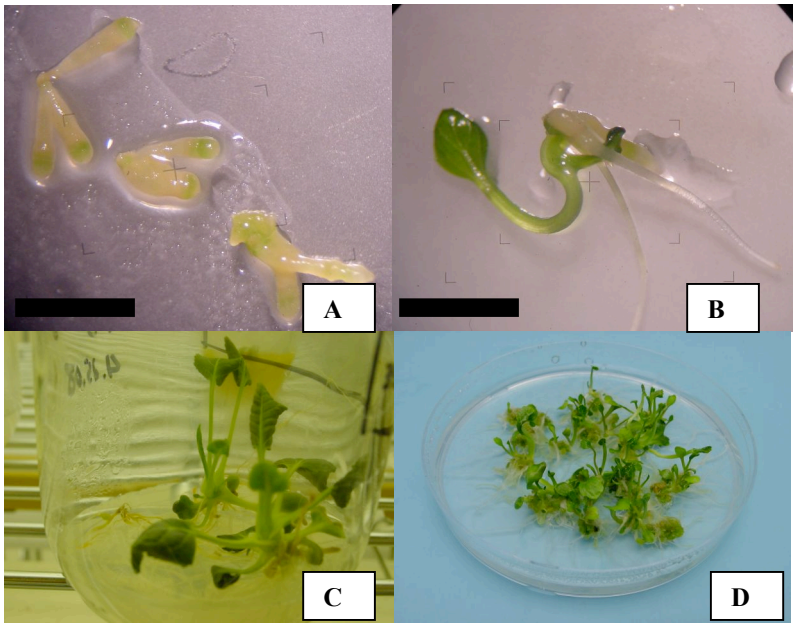

Figure 9. 'Doll' horseradish embryogenic calli grown on solid MS with 2,4-D (0.25 mg.l^{-1}) and moved to MS liquid medium with the same 2,4-D concentration for four weeks formed clumps of cells that were filtered through 1.32 mm pore sized mesh and cultured in PGR-free MS liquid media for eight weeks.
A, after another four weeks in culture, they formed root-like structures. (bar = 5mm)
B, the root-like structures were transferred to fresh PGR-free MS liquid medium for another four weeks where they formed adventitious shoots. (bar = 5mm)
C, the adventitious shoots were moved to PGR-free MS solid media; they developed into plantlets after another four weeks in culture.
D, the cell aggregates larger than 1.32 mm were collected and grown on PGR-free MS solid medium. They formed roots and shoots after four weeks in culture.

In summary, four-week-old suspension cultures grown on solid medium with 2, 4-D (0.25 $mg.l^{-1}$) yielded various-sized cell aggregates. Cell aggregates greater than 1.3 mm in diameter developed plantlets on PGR-free MS media within another four weeks. SEs derived from callus clumps from 0.5 to 1.3 mm in diameter required another 12 weeks in PGR-free MS media to germinate and convert to plants. Although 2, 4-D was essential for stimulating somatic embryogenesis, it did not appear to be important for the maturation of SEs. Based on the SE results and the plantlets produced using these methods, it may be possible to use suspension cultures to produce large numbers of horseradish SEs. Furthermore, it is thought these could be induced to germinate into whole plants.

4.3.2 Preliminary studies to initiate somatic embryogenic cell suspensions of 'Doll' horseradish - Part II

Suspension cultures became turbid after four weeks. In this study, the packed cell volume of the turbid suspension cultures was 5 ml after four to five weeks in culture. After another four weeks, various-sized cell aggregates formed in the suspension cultures; the aggregate clumps were yellowish green and differed in size from approximately 1 to 6 mm in diameter (Figure 10). Regardless of aggregate size, the average number of cell aggregates that formed was 45 aggregates per culture vessel. Eight-week-old packed cells that were not uniform and included large size clumps as well as small size clump increased to 7.5 ml (Figure 11).

Eight-week-old suspension cultures were filtered through 1.3 mm and 1.0 mm pore sizes, respectively. After the second filtering, the suspension filtrate was discarded and the cell aggregates left on the nylon mesh (1.0 mm pore size) were collected, rinsed and re-suspended in 15 ml of PGR-free MS liquid media in 125 ml flasks; the cultures were allowed to grow for

another four weeks. After another four weeks, the cell aggregates that had previously adhered to the nylon mesh had developed three types of cell structures: large green clusters (0.5 -1 cm in length) with shoots (Figure 12a), root-like structures (Figure 12.b) and cell clusters with somatic embryo-like structures (arrow) (Figure 12c).

When the green clusters with shoots and root-like structures were transferred to PGR-free MS liquid media plantlets produced roots within four weeks. From this study it was found that SEs differentiated from specific-sized cell aggregates ranging from 1 to 3 mm in diameter. In addition, the smaller-sized aggregates became larger; they were more likely to differentiate. Their diameter could be increased by leaving them in suspension cultures with 2,4-D (0.25 $mg.l^{-1}$) for four weeks.

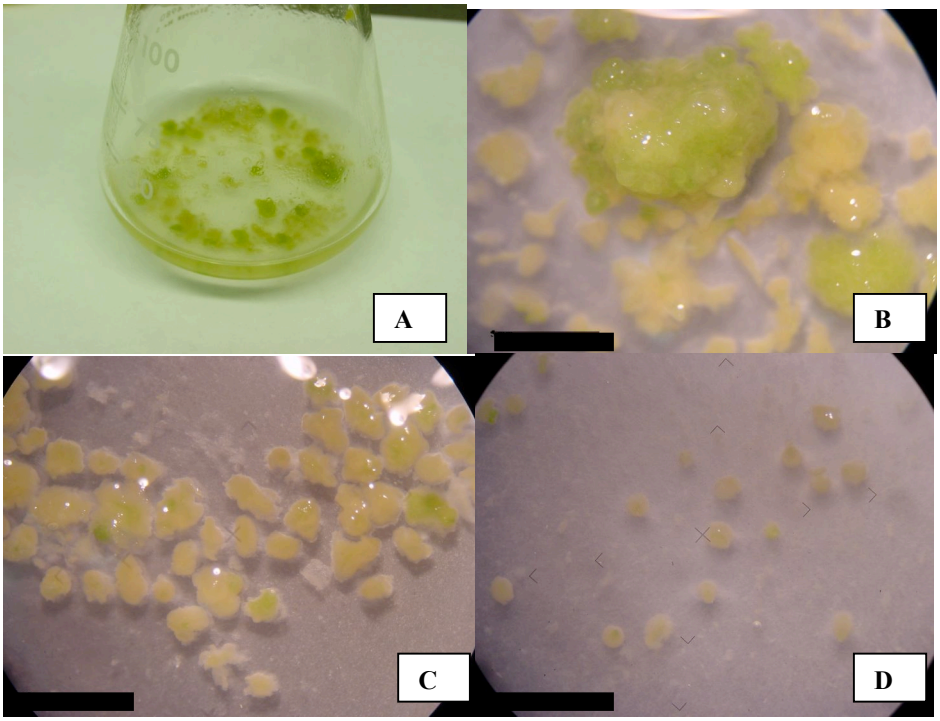

Figure 10. 'Doll' horseradish embryogenic suspension culture in MS liquid medium with 2,4-D (0.25 $mg.l^{-1}$).
A, cell clumps formed after four weeks in culture. The clumps were of various in sizes.
B, 4-6 mm in diameter clumps (bar = 5 mm).
C, 2-4 mm in diameter clumps (bar = 5mm).
D, 1-2 mm in diameter clumps (bar = 5mm).

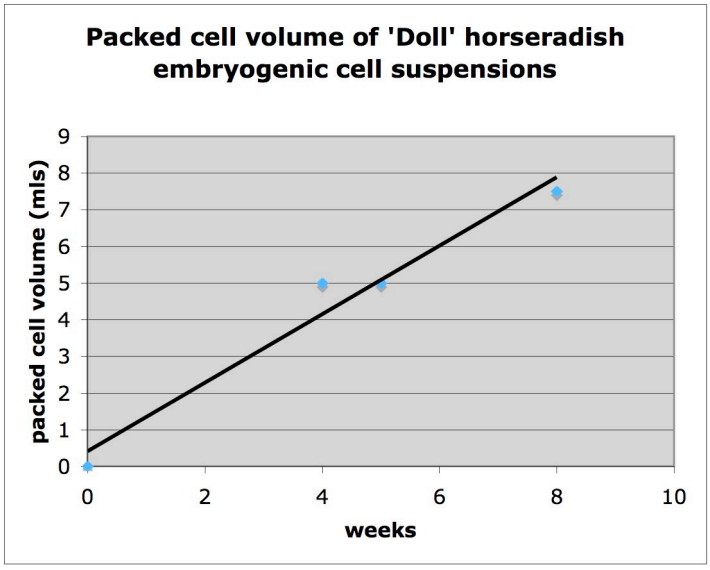

Figure 11. Growth (packed cell volume) of 'Doll' horseradish embryogenic cell suspensions during eight weeks in culture, n=4.

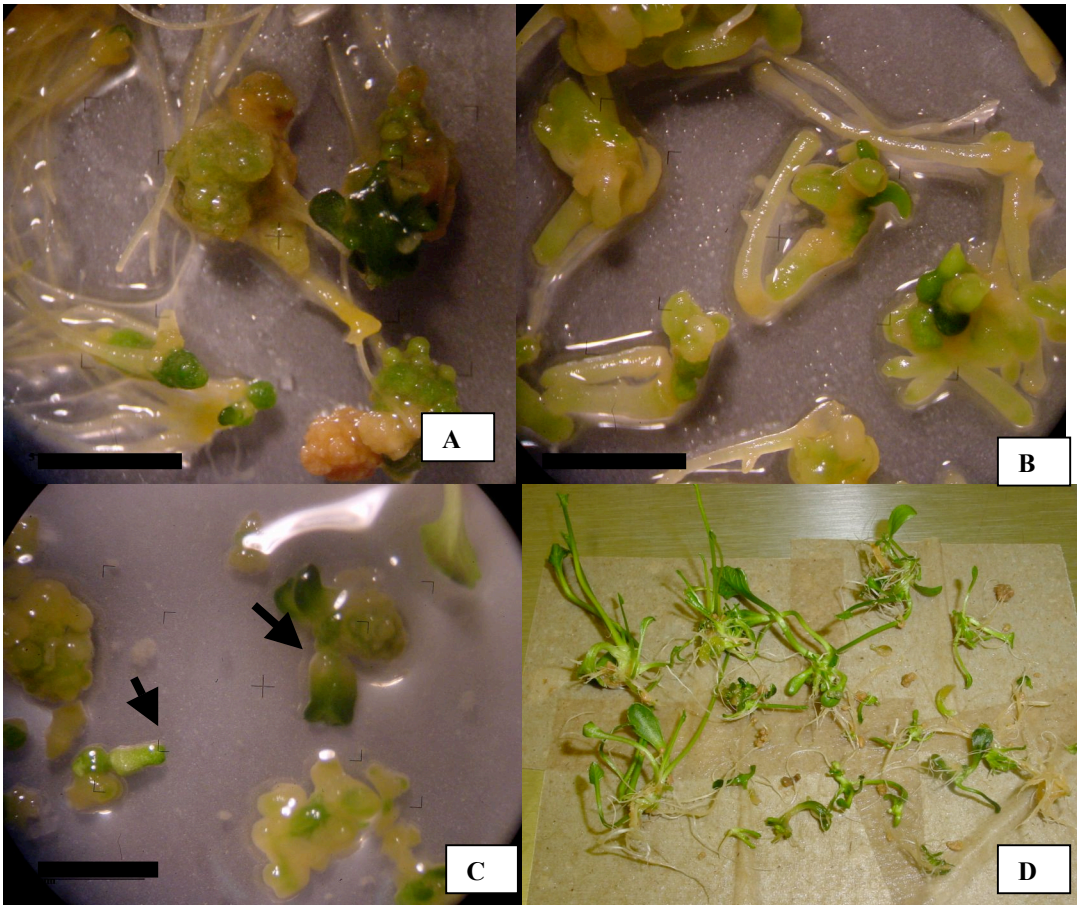

Figure 12. After filtering 'Doll' embryogenic suspensions through various nylon meshes. Cell aggregates were cultured in PGR-free MS liquid medium for four weeks. After four weeks, they formed three types of structures;
A, large green clusters (0.5 -1 cm in length) with shoots (bar = 5mm).
B, root-like structures (bar = 5mm).
C, cell cluster with somatic embryos-like structures (arrows) (bar = 5 mm).
D, the green cluster and root-like structures later developed to plantlets.

In conclusion, the first two preliminary suspension culture studies (section 4.3.1 and 4.3.2) highlighted several difficulties that must be overcome in SE production. First, after eight weeks in culture the suspension cultures formed cell aggregates that regenerated to plantlets only after being transferred to PGR-free MS liquid medium. Second, the establishment of embryogenic suspension cultures is a long process. The primary suspension cultures cannot be

used for SE production because they contain both somatic embryogenic and non-embryogenic cells. They must be filtered to separate the mixed cell types. Subsequently, the filtered cells must be subcultured for several cycles to increase the numbers of somatic embryogenic cells in the suspension cultures. Because these studies showed that 'Doll' horseradish embryogenic cell suspension cultures took four weeks to form cell aggregates, the filtered suspension cultures should be subcultured weekly. Shigeta and Sato (1994) did not mentioned on the subculture sequences in their publication. According to Mevy *et al*. (1997), they subcultured their horseradish embryogenic suspension cultures regularly for seven months before they were to obtain fine embroygenic suspension. However, the future studies should concern at shortening the length of establishment of horseradish suspension culture.

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CHAPTER V: HISTOLOGICAL STUDIES OF 'DOLL' HORSERADISH SOMATIC EMBRYOGENESIS

5.1 Introduction

Somatic embryogenesis was a pathway that yields SEs that were identical to the source plants and will germinate similarly to zygotic embryos. There were two ways that adventitious shoots could be achieved in TC--somatic embryogenesis and organogenesis. Somatic embryos could be distinguished from shoots of organogenic origin because they contained both shoot and root apical meristems and had no vascular connection with their mother tissue. In contrast, adventitious shoots formed by via an organogenic pathway had only shoot apical meristems and exhibited vascular connections to the mother tissue (Haccius, 1978 and Bassuner *et al.,* 2007). To confirm that horseradish SEs developed through somatic embryogenesis and not *via* organogenesis, the ontogeny of regenerating embryogenic callus had to be explored. In this section the importance of histological studies in somatic embryogenesis and histological procedures used to study the situation will be discussed. Then these observations will be used to describe the observed process of SE formation and development in horseradish.

5.1.1 The importance of histology for the study of somatic embryogenesis

In order to study the ontogeny of various tissues and organs including somatic embryogenesis, histological serial sectioning was commonly used. Serial sectioning referred to a series of sections from a single specimen cut in sequence. A light microscope was ideal for classical histological studies with serial sections being used to illustrate the 3-D relationship and to confirm structural organization of somatic embryos from cell induction to differentiation. In

the present study, somatic embryogenic calli were investigated anatomically for signs of SE development (the details of SE cells and their characteristics were discussed in chapter II; section 2.6).

To begin the investigation of somatic embryogenesis, tissues at various stages of development through somatic embryogenesis were chosen. For these studies, the histological methods described by Jensen (1962) were used. Briefly, all horseradish tissues were killed and fixed in formalin-aceto-alcohol (FAA) fixative. After fixing, the tissues were processed through a graded ethanol series to remove water from the cells, then moved to butyl alcohol. Next, they were infiltrated with paraffin (Paraplast®) and were embedded in molds. The infiltrated and embedded tissues were sectioned on a rotary microtome (American Optical Scientific®). Tissue ribbons were generated and mounted on glass slides, and the paraffin was removed by dissolving it in xylene. Finally, the tissues were stained with Safranin O and fast green and permanently mounted on glass slides with cover glasses.

The histological literature of SE induction suggested that the somatic embryogenic pathway began with embryogenic callus that actively divided and then developed embryo-like structures that advanced through globular, heart, torpedo and cotyledonary developmental stages (Gui *et al.*, 1991; Alemanno *et al.*, 1996; Quiroz-Figueroa *et al*., 2002; Azpeitia *et al*., 2003; Mandal and Gupta, 2003 and Sharma and Millam, 2004).

Microscopic studies using serial sections were used to trace the origin of somatic embryogenic primordial cells and showed that they could originate from many locations within callus tissues. For instance, embryogenic callus of cacao (*Theobroma cacao*, Alemanno *et al*., 1996), myrtle (*Myrtus communis* L., Canhoto *et al.,* 1996) and winter oilseed rape (*Brassica napus*, Namasivayam *et al*., 2006) formed somatic embryogenic primordial cells in their

epidermal and subepidermal regions. Embryogenic callus of chicory (*Cichorium intybus,* Dubois *et al.,* 1991) and peach palm (*Bactris gasipaes,* Steinmacher *et al*., 2007) formed their multicellular proembryoids near their vascular bundles.

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) had been used to study the ultrastructure of competent embryogenic cells. Dubois *et al.* (1991), for example, studied SEMs of *Cichorium intybus* proembryos that had just emerged through the epidermis of the explant. Scanning electron microscopy showed that the proembryo cell surfaces were linked by a fibrous network that was not found on the cell surfaces of proembryos cultured in suspension. The authors suggested that the fibrous network might help isolate embryogenic cells from the influence of surrounding cells while they developed toward somatic embryogenesis. Namasivayam *et al*. (2006) observed the surfaces of *Brassica napus* embryogenic tissue in comparison with embryogenic tissue and non-embryogenic tissue. A fibrous material was found to form a net covering the surfaces of the proembryo, but no sign of this fibrillar material was observed on non-embryogenic surfaces. The fibrillar material disappeared from the surface of SEs once they had protruded out of the epidermal cell layer to develop into mature SEs. Transmission electron microscopy studies showed that this fibril material that was present outside the outer epidermal cell wall of the pre-embryo was an amorphous layer filled with numerous small osmiophilic granules (a vesicle produced by a cell wall that can be visualized when stained with osmium).

Histology was used in the present study in order to investigate whether the 'Doll' horseradish leaf explants cultured on MS medium supplemented with 1.0 $mg.l^{-1}$ 2,4-D could form somatic embryogenic callus. Callus at various stages of SE development were histologically examined. These included samples from the youngest tissue stage that showed no

obvious sign of SE formation, to the oldest tissue stage that had clear signs of globular or cotyledonary SE-like structures.

5.2 Materials and methods

5.2.1 Preliminary studies of 'Doll' horseradish leaf segments cultured on induction medium with 2, 4-D (0, 0.25 and 1.0 mg.l^{-1}).

The objective of this study was to monitor how horseradish leaf explants responded to 2,4-D with regard to somatic embryogenic calli formation. Based on information gathered during this study, later experiments were planned when samples would be harvested for histological studies (5.2.2).

Leaves from two- to three-week-old plants from PF 'Doll' TC horseradish plantlets that had been maintained on PGR-free MS medium (Figure 13) were harvested and cultured on MS supplemented with 2,4-D (0, 0.25 and 1.0 mg.l^{-1}). Five explants were placed on each Petri dish. Five Petri dishes were assigned for one treatment. Ten to fifteen explants from each treatment were photographed after 2, 4, 10, 13, 19 and 29 days of culture. The number of SEs that developed on embryogenic calli in each treatment were counted on the photograph using a stereo microscope.

Figure 13. 'Doll' horseradish TC plants in PGR-free MS medium. These were used as stock plants.

5.2.2 Histological studies of 'Doll' horseradish leaf segments cultured on MS supplemented with 2,4-D.

5.2.2.1 Tissue preparation and plant materials

PF 'Doll' TC horseradish plants were multiplied on MS medium supplemented with BA (2 $\mu mol.l^{-1}$) and transferred to PGR-free MS medium for further growth. Two- to three-week-old TC horseradish leaves were cut into square explants (0.5 × 0.5 cm) and cultured on MS medium supplemented with 2,4-D (1.0 mgl^{-1}).

5.2.2.2 Killing and fixation

Tissue specimens were collected from callus that had grown on initiation medium (2,4-D 1.0 $mg.l^{-1}$ MS medium) after 0, 7, 13, 19, 25 or 40 days. They were killed and fixed in FAA (Appendix D). Specimen bottles containing the horseradish samples were placed in a desiccator jar under vacuum to help the fixatives penetrate throughout the tissues. The completely infiltrated tissues that sank to the bottom of the specimen bottle under the vacuum were processed through an ethanol dehydration series (Sass, 1958).

5.2.2.3 Dehydration

To dehydrate the fixed tissues, they were washed briefly in 50% ethyl alcohol. Then tissues were run through a tertiary butyl alcohol (TBA) series (Appendix D). The tissues were left in each TBA solution for two hours except for the final solution of 100% TBA solution where tissues were left for a period of eight hours to overnight; this step was repeated three times (Jensen, 1962).

5.2.2.4 Paraffin infiltration and embedding

Dehydrated tissues were placed in small bottles half filled with solid paraffin; 100% TBA was poured into the bottle to cover the tissues. Then the bottles were capped and kept in an oven at 61°C (5°C higher than the melting point (56 °C) of the paraffin). Four hours later the paraffin bottles were uncapped and the mixture of TBA and paraffin was poured off. Fresh melted paraffin was added two more times at six-hour intervals to facilitate paraffin infiltration. Next, the infiltrated tissues were embedded in Peel-A-Way® embedded plastic molds that were half loaded with molten warm paraffin. A warm needle was used to orientate the tissues vertically or horizontally in the mold under a stereo microscope. The molds with their fixed infiltrated paraffin tissues were allowed to cool to room temperature and then mounted on wooden blocks for support during sectioning (Jensen, 1962).

5.2.2.5 Sectioning and affixing sections to slides

The paraffin around the tissue was trimmed to a rectangular cube with a sharp single edged razor blade. If the paraffin block was too soft because of a warm room temperature, it was hardened by placing it in an ice cube tray in a refrigerator at about 4 °C prior to trimming and sectioning. The trimmed paraffin block was mounted parallel to the knife-edge of a rotary microtome (AO Scientific®) that had been set at a specific angle (approx. 30-45°). A long paraffin ribbon consisting of segments with a thickness of 5 to 10 μm was produced. Then the ribbon was cut and placed on new clean slides that had been pre-washed in 100% acidified ethyl alcohol. The ribbon segments were floated on water, captured on the slide surface, and the slides were then laid on a slide warmer (45 °C). The heat caused the ribbon tissue to extend and flatten (Sass, 1958).

5.2.2.6 Staining and mounting slides

The paraffin was removed from the slides in a single xylene rinse (two to five minutes) and then processed through a progressive staining series that included safranin and fast green using a procedure adapted from Sass's protocol (1958) (Appendix E). Safranin stains lignified cell walls and chromosomes; fast green stains cellulose cell walls and cytoplasm. After staining, cover slips were placed on the slides and the slides were allowed to dry completely before the slides were examined under a compound light microscope (Nikon Eclipse E600) connected to a digital camera.

5.3 Results and discussion

5.3.1. Preliminary studies of 'Doll' horseradish leaf segments cultured on induction medium with 2, 4-D (0, 0.25 and 1.0 mg.l^{-1}).

During the studies the time required for the horseradish explants to regenerate callus and develop SEs through specific stages on induction medium were observed and recorded. Horseradish leaf squares (0.5 × 0.5 cm) cultured on MS supplemented with either 0.25 or 1.0 mg.l^{-1} 2,4-D formed embryogenic callus (Figure 14, g –r); leaf segments grown on the control medium (Figure 14, a-f) did not.

By the second day of culturing, all explants grown with 2,4-D showed vein discoloration from green to pale yellow. By the fourth day of culturing, the explants cultured on 2,4-D medium (both 0.25 and 1.0 mg.l^{-1}) had formed yellow calli. Globular SEs formed on these calli around the tenth day of culture. There were more globular SEs on calli treated with 1.0 mg.l^{-1}2,4-D than calli treated with 0.25 (mg.l^{-1}) 2,4-D. All explants treated with either level of 2,4-D developed cotyledonary-shaped SEs within 21 to 30 days in culture (Figure 14 l, r). It was also found that

SEs which remained on the induction medium for at least two months without subculture, failed to convert to plantlets unless they were picked out and placed on PGR-free MS medium for germination. Leaf explants grown on control medium (PGR-free MS medium) enlarged within one week of culture (Figure 14b) and rooted by their tenth day in culture (Figure 14c). Roots emerged from the explant's cut surface (Figure 14d). By the 19^{th} day in culture, shoots had formed from the root (Figure 14e). Whole plantlets were observed by the 30^{th} day in culture (Figure 14 f).

It was observed that in addition to forming SEs, somatic embryogenic callus on both 2,4-D (0.25 $mg.l^{-1}$and 1.0 $mg.l^{-1}$) media formed swollen SE-like structures (Figure 15). These malformed structures were white and roundish. Some appeared to have cotyledons (Figure 15 a,b) but some of them were round (Figure 15 c,d). The malformed structures appeared to be SE-like structures that had formed abnormally, probably as the result of 2,4-D interactions (Skirvin, 1978; Ziv, 1999). The situation was not explored further. In order to reduce the risk of selecting off-type, it was decided to use the embryogenic calli induced at the lowest 2,4-D level (0.25 $mg.l^{-1}$) for the remainder of the suspension studies. Future investigations should consider making histological studies of these malformed structures in order to determine their nature.

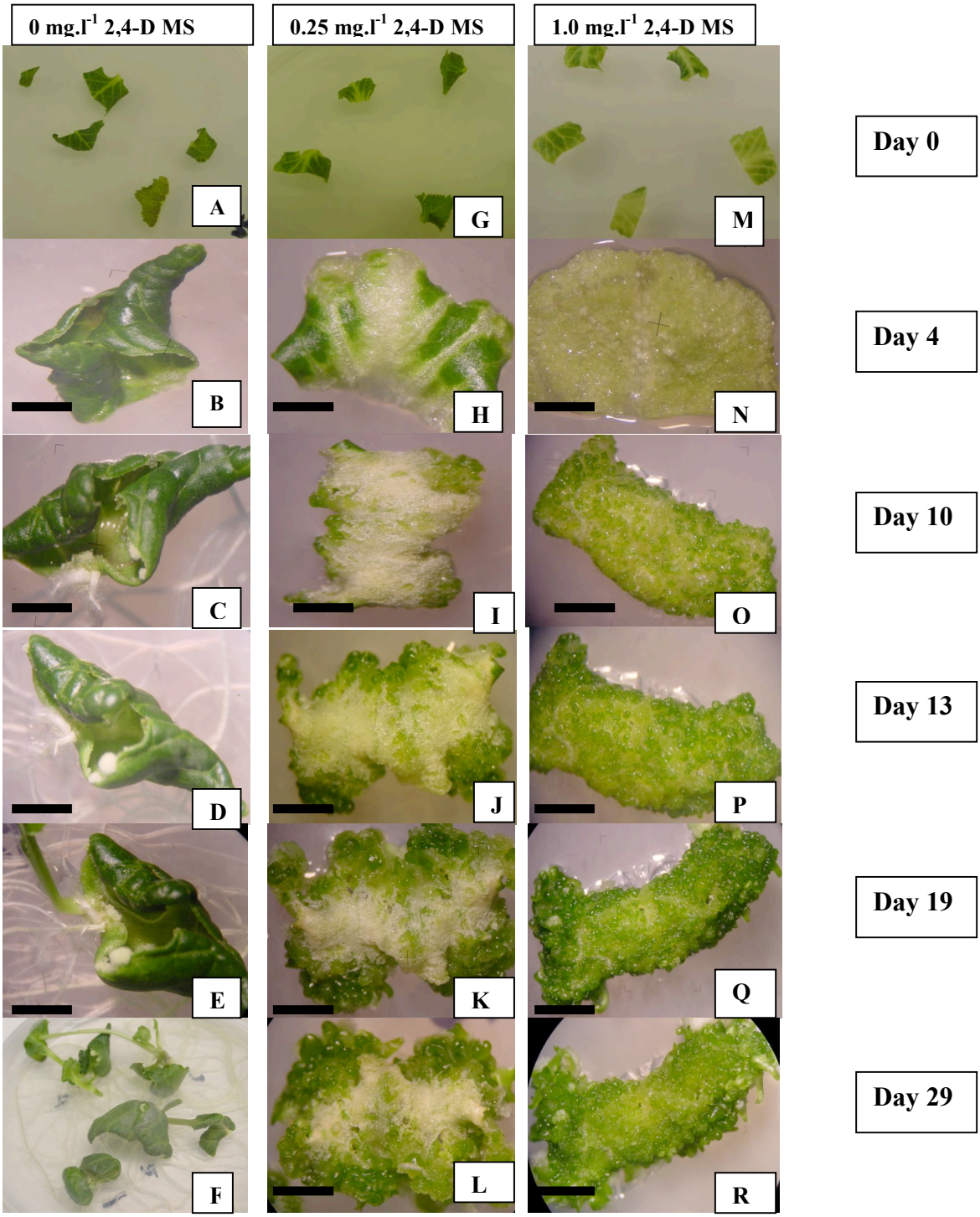

Figure 14. 'Doll' horseradish leaf explants on MS medium with 0, 0.25 or 1.0 mg.l^{-1}2,4-D. A-F, on control medium (0 mg.l^{-1} 2,4-D) leaf explants developed roots and adventitious buds that grew to plants (bar = 5mm). G-E and M-R, respectively, leaf explants on MS with 0.25 or 1.0 mg.l^{-1}2,4-D formed embryogenic callus with somatic embryos (bar = 5mm).

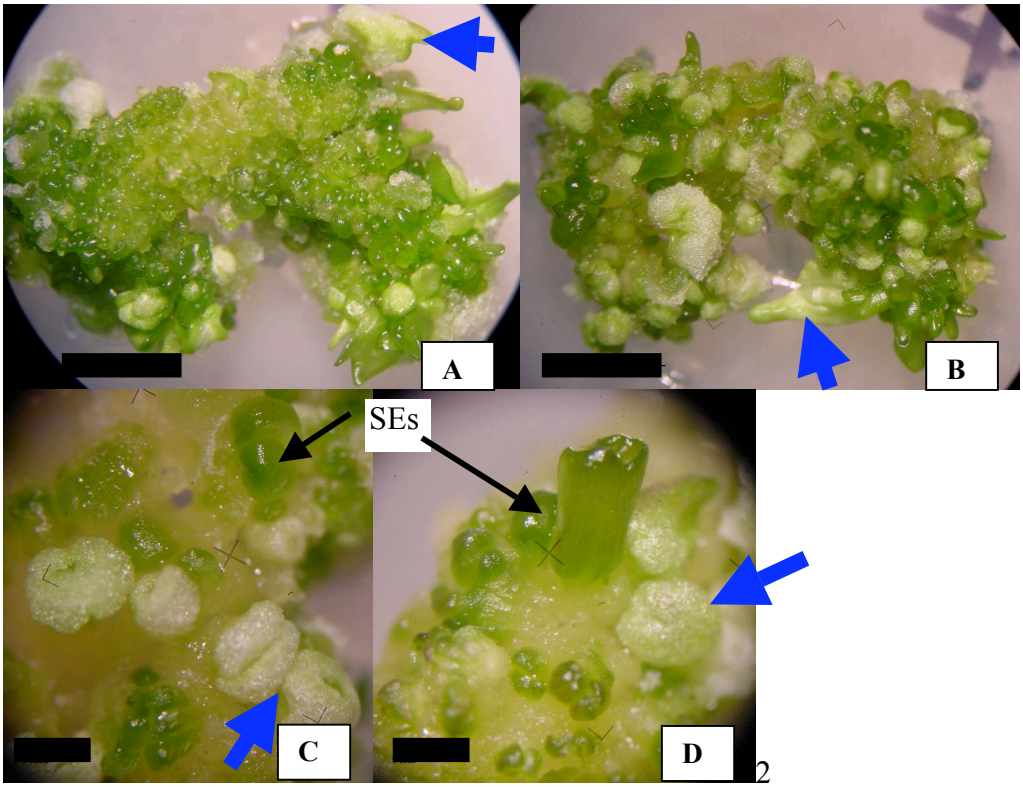

Figure 15. 'Doll' horseradish leaf explants on MS medium with 2,4-D (1.0 mg.l^{-1}) formed green somatic embryo-like structures as well as swollen white somatic like structures.
A-D, swollen white SE-like structures were indicated by blue arrows (SE= green somatic embryo-like structure, bar for a,b = 5mm; c,d = 1mm).

5.3.2 Histological studies of 'Doll' horseradish leaf segments cultured on MS with 2,4-D.

Leaf explants cultured on MS with 1.0 mg.l^{-1} 2,4-D formed a higher number of cotyledonary SE-like structures than those grown on a lower level of 2,4-D (0.25 mg.l^{-1}) (43 vs. 9, respectively; Table 5). Thus, embryogenic calli grown on the 2,4-D (1.0 mg.l^{-1}) MS medium were chosen for the histological studies.

Samples at various stages of SE development were collected, fixed, and paraffin embedded using the procedures discussed in section 5.2.2. Cross-sections of fresh leaf blades at day 0 (Figure 16b) showed that a leaf midvein was composed of an epidermal layer (outermost),

mesophyll cells (spongy parenchyma) and vascular bundles (innermost). The vascular bundles were surrounded by compact, spongy parenchymal cells without obvious intercellular spaces or palisade parenchymal cells (Figure 16b). The adaxial and abaxial epidermises were not obvious in the cross-sections; it was assumed that they had been removed or lost during histological processing.

Leaf blade cross-sections suggested that within seven days of culture there were many layers of actively dividing cells (Figure 16c); arising from the area of the vascular bundles outward toward the mesophyll layer. Regions of active division were indicated by safranin-stained red nuclei. The cross-sections of the explants indicated that periclinal cell divisions had occurred among cells in the vicinity of the tissues surrounding the spiral thickenings of the xylem cells (Figure 16d).

Cross-sections of thirteen-day-old calli showed densely packed cells that appeared to arise from the area of the vascular bundle layer. Globular embryo-like structures were present at this time (Figure 16e). After nineteen days of culture, the globular embryos became more obvious in longitudinal section (Figure 16f). These calli contained many dome-shaped cell masses that resembled globular SEs and were observed all along the vascular bundle axes.

Longitudinal sections of single cotyledonary SE-like structures individually picked from somatic embryogenic callus after forty-five days in culture, (Figure 16 b,c) had no obvious vascular connections between the SE-like structure and the parental tissue. This suggested that the young plants arose by somatic embryogenesis–not organogenesis. This observation was similar to the report by Haccius (1978), who found that carrot SEs had no vascular connection between their tissues and the mother tissues. The cotyledon SE-like structures of the present study had no obvious apical meristem, so it was assumed they were not bipolar structures. In

fact, the cotyledonary SE-like structures of ‘Doll’ appeared to contain only a root tip meristem. However, 50% of germinating ‘Doll’ SEs converted to whole plants (section 3.3.5) with roots and shoots. It appeared that the apical meristem may form later than the root meristem, possibly during germination. Similarly, microscope studies of sweet potato (*Ipomoea batatas* L.) SEs showed that torpedo-stage SEs contained limited or no apical meristems, but they still converted to plantlets (Padmanabhan *et al.,* 1998).

It was concluded that ‘Doll’ leaf explants treated with 2,4-D can produce embryogenic calli that originated from parenchyma cells in vascular tissue area. Furthermore, the SE-like structures that develop on embryogenic callus were the result of somatic embryogenesis, not organogenesis, since there appeared to be no vascular connection to the mother plant.

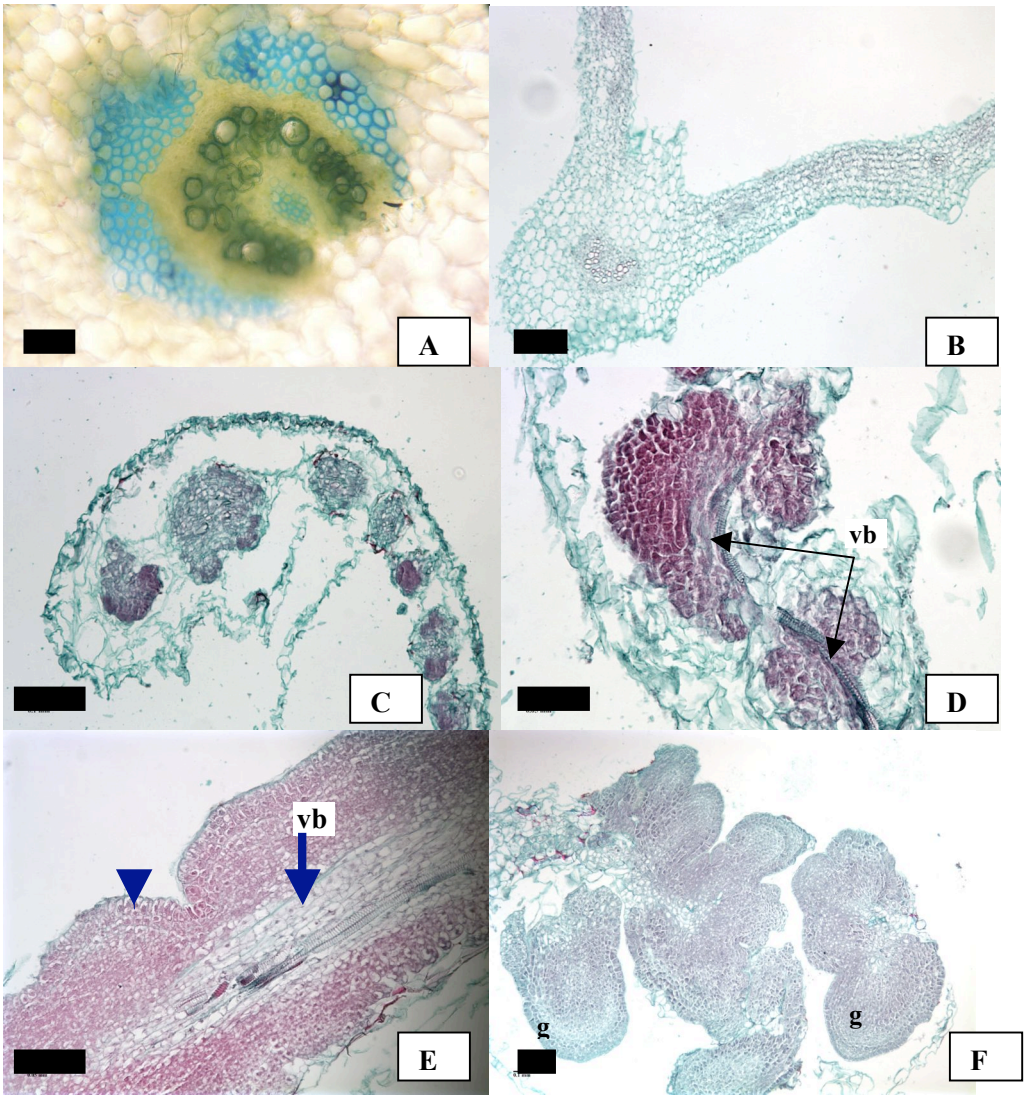

Figure 16. Histological sections of 'Doll' horseradish petiole and leaf explants. Leaf explants grown on MS medium with 2,4-D (1.0 mg.l^{-1}) formed embryogenic callus within four weeks in culture.
A, a free hand cross-section of a petiole explant at day 0 in culture (bar = 0.05 mm)
B, a cross-section of the leaf blade explant at day 0 in culture. The mid-rib of a leaf blade was composed of a vascular bundle (**vb**) surrounding by mesophyll cells (bar = 0.1 mm).
C, D, after 7 days in culture cross sections of the leaf explants showed cell division to be arising near the vascular bundle tissue layers (bar for c = 0.1 mm, bar for d = 0.05mm).
E, longitudinal sections of embryogenic callus formed after 13 days in culture. The new active meristematic cell layers (thick arrow) were found to be dividing outward from the vascular bundles (bar = 0.05 mm).
F, longitudinal section of embryogenic callus after 19 days in culture shows many globular SE like structures (**g**) adhering together on the calli (bar = 0.1mm).

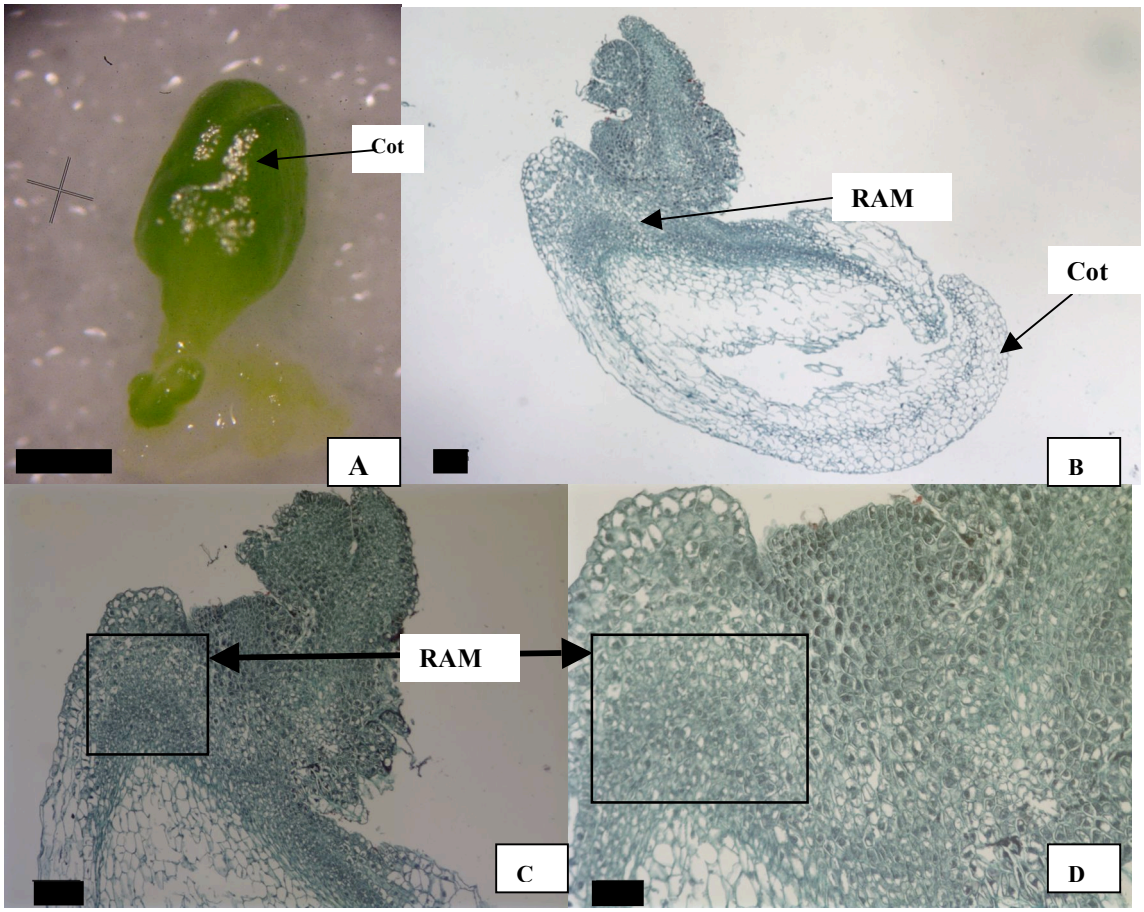

Figure 17. Histological sections of 40-day-old 'Doll' somatic embryo (SE)-like structures.
A, Somatic embryo–like structure with cotyledons was picked from 40-day-old 'Doll' embryogenic callus and photographed before being processed through paraffin sectioning (bar = 1 mm).
B, C, D, longitudinal sections of the SE-like structure showed that they had no vascular connection to vascular tissues between their root apical meristem and the mother tissue. (Cot=cotyledon, RAM= root apical meristerm, bar b,c = 0.1mm; d = 0.05mm).

Table 5. The formation of green cotyledonary SE-like structures and swollen white SE-like structures on MS supplemented with 2,4-D, n=1.

2,4-D concentration ($mg.l^{-1}$)	Average no. of cotelydonary SE-like structures	Average. no. of malformed SE-structures
0	0	0
0.25	9	1
1.0	43	4

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SUMMARY AND CONCLUSION

Illinois was the leading producer of horseradish in the United States; harvesting more than 2,000 acres of horseradish roots each year (USDA, 2000). However, during the past few years growers had encountered reduced horseradish yields due to a disease complex that infected horseradish roots, leading to internal root discoloration and rots. To help Illinois growers overcome this problem, our laboratory proposed to develop a certified horseradish planting stock program to supply pathogen-free (PF) horseradish plants root sets to the growers. During the past seven years, members of our laboratory had investigated the feasibility of such a system. Shehata (2004) established Illinois horseradish plants in TC and tested their performance under field conditions. Uchanski (2007) developed a tissue culture-based system to evaluate the pathogen status of horseradish clones and then proceeded to make PF horseradish plants that he tested in commercial growers' fields. Illinois growers were anxious to commercialize certified PF plants, but did not have the laboratory or greenhouse space to provide the thounsands of plants that would be required to supply their annual needs.

Somatic embryogenesis was a process whereby single cells become embryo-like, developed through stages that mimicked those that sexually-derived embryos underwent, and yielded somatic embryos (SEs) with cotyledons and a root that could germinate and grew like a normal seed. The first objective of this research was to initiate somatic embryogenesis from *in vitro* PF horseradish vegetative tissues. To induce somatic embryogenic callus, 'Doll' horseradish leaf and petiole explants were treated with 2,4-D (0.25 to 1.0 mg/l) on Murashige and Skoog (MS) medium (1962). Leaf explants formed green nodular compact embryogenic calli with SEs; petioles formed only non-

embryogenic callus. At 0.25 to 1.0 mg/l 2,4-D, leaf explants formed somatic embryogenic calli with globular and cotyledonary SEs. The ability of BA (6-benzylaminopurine, 0.25 mg/l) to enhance the formation of somatic embryogenic callus was tested on MS with of 2,4-D (0.25, 0.5, 0.75 and 1.0 mg/l) induction medium. Leaf explants grown with BA formed no embryogenic callus regardless of 2,4-D concentration. To test the ability of SEs to develop into whole plants, SEs were germinated on PGR-free MS medium; 65% of them germinated and 50% of the germinating SEs converted to whole horseradish plants. These studies demonstrated that MS supplemented with 2,4-D at 0.25 to 1.0 mg/l is efficient for the induction of somatic embryogenic calli from 'Doll' leaf explants.

The next objective of the study was to establish a somatic embryogenic cell suspension culture offerred a continuous source of SEs for propagation. Embryogenic callus induced by 2,4-D (0.25 mg/l) on MS agar medium were cultured on the same induction medium in an agar-free (liquid) form. After eight weeks in culture, somatic embryogenic suspension cultures were established; however, most tended to form cell clumps. Some of the clumps developed into whole plants when 2,4-D was removed from the culture medium.

Histological sections of somatic embryogenic callus and SEs at various stages of development showed that somatic embryogenic callus formed from vascular bundle tissues. Sections of individual SEs showed that they had no vascular connection to their mother plant tissue, suggesting that at least some of them had arisen by somatic embryogenesis and not solely by organogenesis. Although some of the SEs did not have obvious apical meristems, all had root meristems. Regardless of the apical meristem

status, most of the SEs tested germinated to yield whole plantlets. The ability of leaf tissue to produce SEs that will germinate and yield plants suggests that somatic embryogenesis could be used to make large numbers of plants.

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APPENDIX A: A PROPOSED MODEL OF AUXIN DISTRIBUTION DURING EMBRYOGENESIS OF TRANSFORMED *ARABIDOPSIS*

A.1 Auxin distribution model

Brown and Floyd (2008) reported that one-celled transformed *Arabidopsis* zygotes divided into two daughter cells; the apical cell developed into the embryo proper, and the basal cell became the suspensor cell. It was found that auxin accumulated in the apical cell of pre-globular (8-cell stage) embryos. IAA maxima reversed to the basal part once the embryo became globular (32-cell stage). Later, IAA moved from the quiescent center of the root meristem to the collumela precursors giving a biopolar orientation to the embryo.

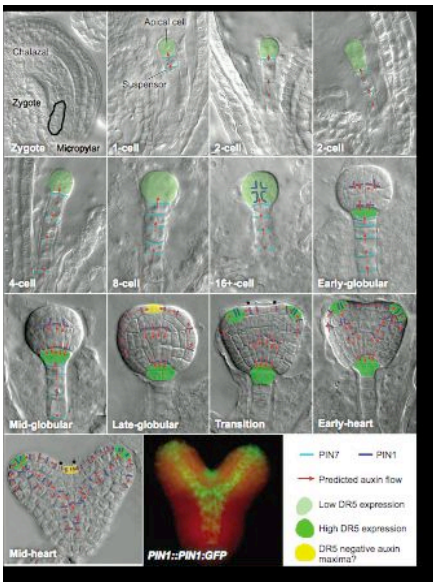

Figure 18. Auxin distribution in transformed *Arabidopsis* embryos --green area indicated high auxin accumulation (Adapted from Bowman and Floyd, 2008).

Auxin flows in plants two ways. The first way is a rapid long distance transportation system: auxin synthesized in the shoots (source) is loaded passively into mature phloem that transport an auxin throughout the whole plant and is unloaded the at sink tissues such as the root.

The second way is short distance transportation, the 'auxin polar transport'. When auxin is in the apoplast, it becomes protonated and lipophilic and can diffuse across cell membranes. Once auxin is in the cytoplasm, it becomes anionic and can no longer diffuse across the plasma membrane on its own, but can only exit the cell with the help of an active transporter protein. IAA translocation from cell to cell requires the aid of specific transporters.

The polar auxin transport proteins are divided into three families: AUXIN RESISTANT 1 LIKE AUX 1(AUX1/LAX) uptake symporters, PIND FORMED (PIN) efflux carriers and P-GLYCOPROTEIN (MDR/PGP/ABCB) efflux/conditional transporters (Titapiwatanakun and Murphy, 2009). PIN genes are expressed during embryogenesis in *Arabidopsis* (Friml *et al.,* 2003). The PIN protein is involved in auxin efflux (polar IAA transport out of the cell) (Tanaka *et al*., 2006).

1-N-naphthylphthalamic acid (NPA), an auxin inhibitor, is used to prevent the efflux of auxin by blocking polar auxin transport as well as causing abnormal accumulations of endogenous IAA. The mechanism that NPA uses to control auxin efflux is unclear (Grossman, 2003). NPA has also been used to study auxin-dependent development during embryogenesis, and also in SE development. For example, Larsson *et al.* (2007) found that the NPA treated Norway spruce SEs developed abnormally. The Norway spruce proembryogenic masses (PEMs) were induced on 2,4-D culture medium. When PEMs were grown on 2,4-D-free medium (maturation medium), SEs with eight separate cotyledons and a distinct shoot meristerm were produced. SEs on maturation medium supplemented with NPA showed abnormalities such as partially fused cotyledons, abnormal apical shoot meristems, fused cotyledons and no distinct shoot apical meristem, aborted cotyledon development, or abnormal shoot and root apical meristems. NPA-treated SEs also had a high endogenous IAA content (*ca*.16 pg mg / fresh

weight) compared to SEs from the non-NPA treatment (*ca.*10 pg mg/fresh weight). The authors suggested that endogenous IAA levels increased because auxin transport was blocked by NPA. Changes in endogenous IAA might affect cell fate as well as cell division of SEs during their development.

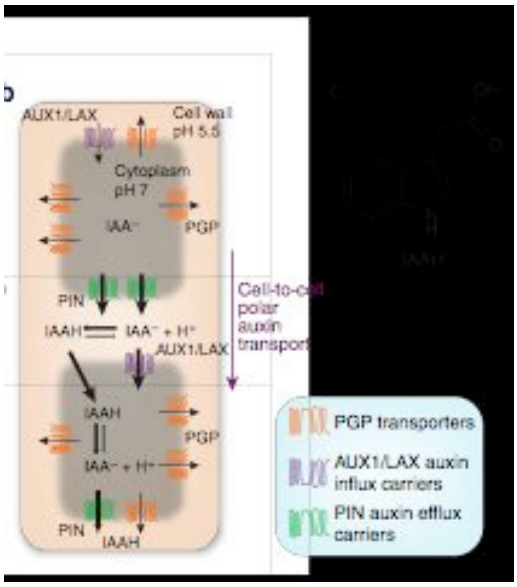

Figure 19. The chemiosmotic model of polar auxin transport in *Arabidopsis* cells including three auxin transporter families (Adapted from Robert and Friml, 2009).

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APPENDIX B: POLLEN DEVELOPMENT AND PATHWAYS OF ANDROGENESIS

B.1 Pollen development

During normal pollen development, microspore mother cells go through meiosis to form a tetrad of four haploid microspores each with its own callose envelope; the four haploid microspores are still contained by the original tetrad's callose wall. Next, the tetrad's cell wall is digested by a mechanism called 'callase dissolution' to release four haploid microspores. Soon after meiosis is complete, each microspore forms a new pollen wall with an outer layer (exine) and innermost layer (intine); pollen wall formation ceases soon after the free microspores enter their first pollen mitosis division (Boavida *et al.*, 2005). Next, each free microspore undergoes a single asymmetric mitotic cell division (the first pollen mitosis: PM I) to give two unequal-sized daughter cells: a large vegetative cell and a relatively small generative cell contained within the vegetative cell. The vegetative cell contains the bulk of the pollen's cytoplasm including mitochondria and stored metabolites that will be used for pollen tube germination. The generative cell is completely enclosed by the vegetative cell cytoplasm that includes a few organelles and stored metabolites. There are now two cells enclosed within the pollen grain's cell wall. Following another round of callase dissolution at the generative cell's wall, the pollen becomes one cell with two haploid nuclei (bicellular pollen grain). Later, depending on the species, as the pollen grain germinates, the generative cell can undergo a further symmetrical mitotic division to form the two sperm cells that are responsible for fertilization (Twell and Howden, 1998).

B.2 Androgenesis pathway

Microspore-derived embryos were formed by many pathways. Reynolds (1997), Touraev *et al.* (1997), Smykal (2000) and Touraev *et al*. (2001) suggested that microspore-derived embryos can develop from either repeated division of the generative cell or repeated division of the vegetative cell or division of both the vegetative and generative cell. Sunderland *et al*. (1974) and Sunderland and Evan (1980) reported that microspores from thorn-apple (*Datura innoxia*) anthers and barley (*Hordeum vulgare*) anthers formed microspore-derived embryos by three pathways as follows:

1) Pathway A: after the first microspore mitosis, the vegetative nucleus divided to form a multicellular embryo-like structure that was still enclosed in the pollen grain's wall. The generative nucleus subsequently became either fragmented, disappeared or turned pycnotic and degenerated without dividing.

2) Pathway B: some microspores entered the first mitosis symmetrically and divided to give two equal vegetative-like nuclei that further divided to form multicelluar embryo-like structures.

3) Pathway C: the generative nucleus doubled its DNA content during two cycles of DNA synthesis without mitosis to become a diploid (2n) nucleus; the vegetative nucleus then underwent one mitosis cycle to yield two haploid (n) nuclei.

Another way to form a microspore-derived embryo is by the repeated division of the generative cell. For instance, Raghavan, (1976) reported that henbane (*Hyoscyamus niger*) uninucleate microspores became androgenic after the first mitosis, when the generative cell divided to form a group of cells that gave rise to multicellular embryos. The vegetative cell either did not divide or divided only a few times to form a multicellular suspensor-like structure.

There are some cellular markers that can be used to indicate which microspores will become embryogenic. One of these is a centralized nucleus surrounded by 'stair-like' cytoplasmic strands; another is a symmetrical mitotic division (Shariatpanahi *et al.*, 2006).

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APPENDIX C: SUNSHINE ® SOIL MIX

C.1 Soil mix components

Sunshine soil mix LC1*(Sungo Horticulture, Canada Ltd.) composes of peat and perlite.

(Comment: general soil less, also used as a rooting medium. A substitute for pro-mix BX. Good for Arabidopsis).

C.2 References

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APPENDIX D: HISTOLOGICAL TECHNIQUES

The study of cells and tissues at the microscopic level is called "histology" and involves two major processes: tissue preparation and microscopic examination. Tissue preparation, a process of making thin (10 μm or less) tissue sections that are mounted on glass slides for microscopic examination, has five major steps: killing/fixation, dehydration, embedding, staining, and sectioning. Depending on the goals and detail to be studied, various microscopes can be used to examine cells and tissues: *e.g*. a light optical microscope, a transmission electron microscope (TEM), a scanning electron microscope (SEM) or some combination of these.

D.1 Fixative and tissue dehydration (Jensen, 1962)

Killing is a process used to terminate the life of the protoplasm. Fixation is designed to preserve cell structures and their organelles. The FAA mixture is the standard preservative used for plant histological studies. The fixative used in these histological studies was the standard FAA mixtures (90 cc. of 50% ethyl alcohol +5 cc. of glacial acetic acid+ 5 cc. of 40% formaldehyde). The tertiary butyl alcohol (TBA) series were used to dehydrate embryogenic tissue samples.

Table 6. TBA series

Series	H_20 (ml)	95% alcohol (ml)	100%TBA (ml)	Dehydration time (hrs)
50	50	40	10	2
70	30	50	20	2
85	30	50	35	2
95	0	45	55	2
100	0	25	75	2
TBA	0	0	100	8-overnight ***

*** repeat three times

D.2 Tissue staining (Sass, 1962)

Tissues were stained with the progressive staining series of safranin and fast green:

1. Xylene, 2-5 min
2. Absolute ethyl alcohol, 2-5 min
3. 95% ethyl alcohol, 2-5 min
4. 70% ethyl alcohol, 2-5 min
5. 50% ethyl alcohol, 2-5 min
6. 30% ethyl alcohol, 2-5 min
7. Distilled water, 1-2 min
8. Safranin solution, 1-2 hours
9. Water, change until colorless
10. 30% ethyl alcohol, 2-5 min
11. 50% ethyl alcohol, 2-5 min
12. 70% ethyl alcohol, 2-5 min
13. 95% ethyl alcohol, 2-5 min
14. Fast green in 95% alcohol, 5-30 second
15. Absolute ethyl alcohol (1^{st} rinse), 2-5 min
16. Absolute ethyl alcohol (2^{nd} rinse), 2-5 min
17. Xylene and mounting slides with Permount ®sigma and covering with cover glasses

D.3 References

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APPENDIX E: STUDIES OF *IN VITRO* CONTAMINATION IN 'DOLL' HORSERADISH SOMATIC EMBRYOGENIC CALLUS CULTURES

E.1 Introduction

Near the end of the thesis research, contamination of cultures with bacteria became a serious problem. For almost two years micropropagated PF 'Doll' horseradish shoots had been subcultured to fresh TC medium regularly. During this time the TC plants were subcultured monthly; no obvious contamination was observed on either horseradish shoot proliferation medium or somatic embryogenic callus induction medium. However, late in the second year of these studies, yellow contaminants were found growing among calli originating from leaves harvested from "clean" plants. The yellow contaminants were not obvious until callus had been on embryogenic induction medium at least five to six weeks. Consequently, the liquid culture studies were temporarily ended because clean embryogenic calli were not available to initiate more aseptic suspension cultures.

Thus, *in vitro* contamination in horseradish somatic embryogenic calli had to be eliminated before the original research plan was continued. In most cases, the best way to solve contamination problems is to discard all contaminated cultures and start again. Such a radical step would require that 'Doll' horseradish plants be re-meristemed to establish a new set of PF horseradish plants. This was not feasible within the time allocated for this project. For this reason, using antimicrobial agent to eradicate the yellow contaminants from the 'Doll' horseradish embryogenic calli was investigated in these studies.

E.2 Review literature

E.2.1 *In vitro* contamination

In vitro contamination is absolutely unacceptable for long-term maintenance of tissue culture and international exchange of germplasm. As a general practice, contaminated plant tissue and cell cultures are discarded as soon as the contamination is found. Large scale commercial TC enterprises are only a little concerned by small numbers of contaminated and discarded cultures because cultures are plentiful, but contamination can become a serious problem when it occurs on rare, valuable or irreplaceable cultures (Leifert and Cassells, 2001). For example, Houwe *et al.* (1998) of INIBAP (International Network for Improvement of Banana and Plantain *Musa* germplasm transit center) reported that some of their banana germplasm TCs occasionally had latent bacteria that appear around the base of their shoot tip cultures, even after repeated subculturing. Similarly, Reed *et al*. (1995) reported an incident of bacterial contamination among TCs initiated from mint (*Mentha* sp.) accessions conserved as potted plants in a greenhouse at the USDA-ARS National Clonal Repository in Corvallis, Oregon.

Bacteria, yeast and filamentous fungi are the most common contaminants in plant tissue and cell cultures (Leifert and Cassells, 2001). There are three major ways that contaminants are introduced to plant tissue and cell cultures:

1. Contaminants are on or within the explants themselves. These contaminants are either pathogens of the crop, or they can be common environmental microorganisms associated with the crop. For instance, Knauss and Miller (1978) found that *Erwinia carotovora*, a pathogen of ornamental tropical foliage plants, was a common contaminant in Bosten fern (*Nephrolepsis exaltata* "Bostoniensis" (L.) Schott) and strawberry begonia (*Saxifraga sarmentosa* L.) TC

cultures. Although the contaminants should have been killed during traditional disinfestations procedures, some organisms survive because they hide in biofilms or within the explants themselves as endophytes. Skirvin *et al.* (1999) suggested that leaf scales and other projections including plant hairs could hide contaminant spores or other reproductive structures. Plant hairs cause the entrapment of air bubbles that decreases the contact area between disinfesting agents and explant surfaces. Likewise, particular anatomic structures can harbor contaminants. Ali *et al.* (2007) showed that guava seeds have a bristly "seed cap" that appears to harbor contaminants and is responsible for up to 100% of the contamination they encountered *in vitro*.

2. Contaminants can enter TC accidentally due to poor aseptic techniques during subculturing and pouring of media (Cassells, 2001 and Leifert and Cassells, 2001). Leifert *et al.* (1991) reported that half of the bacteria isolated from 12 different TC plant species belonged to bacteria groups found on human skin, other human tissues or other mammals (*Staphylococcus, Micrococcus,* and *Lacbacillus*).

3. Mites and/or thrips sometimes survive disinfestations and can be a source of contamination. Fungal mites carry bacteria and fungi on their bodies and actively inoculate TC medium with them (Blake, 1988; and Bhagwat and Lane, 2003). For instance, *Sideroptes graminis* mites carry *Fusarium poe* (Leifert and Woodward, 1998). Mites and thrips can be either plant inhabitants or plant pests *in vivo*. They are easily transported by dust, insects and by human activity, and they enter TCs through loose culture vessel lids (Blake, 1988). It has been observed in our lab that even Parafilm®-sealed vessel lids do not completely prevent the invasion of all mites and thrips.

The presence of contamination is either expressed quickly, usually within a few days, or is not obvious until weeks or months later. Regardless of the source, contaminants that persist

through subculturing become serious problems because the organisms can remain inactive (latent) when TC environments are not suitable for their growth; contamination is not always immediately obvious. For example, bacteria usually grow best when incubated at temperatures of 35 °C or greater; the average temperatures used for plant tissue cultures are near 25 °C.

Another way that microbial contamination can be suppressed *in vitro* is by acidification of culture media and by exudates from the plant tissues themselves (Cassells, 2001). Plant TC media are usually adjusted to a pH near 5.7 at which point the growth of many bacteria are suppressed. Bacteria culture media, on the other hand, are usually adjusted to a pH near neutral (7.0), even though some bacteria have specific pH requirements above and below neutral. Changes in the TC environment such as temperature, moisture, pH and media components also can stimulate or repress rapid growth of contaminants *in vitro* (Cassells, 2001 and Leifert and Cassells, 2001).

To overcome such contaminants, Leifert and Woodward (1998) reported that it was important to know the nature of the contaminants (*e.g.* bacteria or fungus), and in some cases, their genus and species. The authors suggested that identification of bacteria at the genus level could help pinpoint the source of bacterial contamination. Each of the above authors reported that the collection of this information facilitated eradication of their contaminants and the establishment of new clean cultures to replace the contaminated ones (Buckley *et al*., 1995; Reed *et al*., 1995; Tanprasert and Reed, 1997).

Many techniques have been used to identify bacterial TC contamination. To differentiate commonly isolated genera, the contaminants must be isolated on diagnostic media to obtain pure cultures. Then the isolated bacterial contaminants are assessed by the Gram reaction and other standard bacteriological tests such as the anaerobic growth, the oxidase test, and the urease test

(Schaad, 1980). Some researchers have reported using specific kits or assays to aid in their process. An Analytical Profile Index (API) test kit was used to identify bacteria to their species (Leifert *et al*., 1989). Additionally, fatty acid profile analysis was used to identify bacteria to a group, either a genus or a species (Stead *et al.,* 1992); Buckley *et al.,* 1995); Cassells and Tahmatsidou, 1996 and Thomas, 2007). Biolog® Gram-negative and Gram-positive microplates can be used to identify bacteria to the genus and species level (Tanprasert and Reed, 1997).

Molecular techniques can be utilized to detect plant pathogenic bacteria and viruses (Lopez *et al*., 2008). For instance, a highly conserved 16S ribosomal DNA gene sequence is commonly used to identify bacterial species (Clarridge, 2004) in TC: *Bacillus pumilus* in potato (*Solanum turberosum*, Isenegger *et al*., 2003) and grape TC (*Vitis vinifera* L., Thomas, 2004); *Stenotrophomonas maltophilia* and *Achromobacter* sp. in holly cultures (*Ilex dumosa* var. *dumosa* R., Luna *et al.,* 2008); *Pseudomonas reactans* in enset (*Ensete ventricosum*, Birmeta *et al.,* 2004) and *Herbaspirillum huttiense*s in eucalyptus (*Eucalyptus grandis* vs. E. *smithii*, Picoli *et al.,* 2005).

E.2.2 *Antimicrobial* agents

Antimicrobial agents are useful to eliminate contaminants from TC cultures. However, regular use of antimicrobial agents in TC medium can result in selection of microorganisms with resistance to the agent (Falkiner, 1998).

Antibiotics have been used to eliminate bacterial contaminations. Barret and Cassells (1994) stated that cefotaxime (500 $mg.l^{-1}$) eliminated bacteria (*Xanthomonas campestris* pv. *Pelargonii*) from *Pelargonium × domesticum* cv. 'Grand Slam' explants in TC. Buckley *et al.* (1995) identified bacteria including *Pseudomonas fluorescens, Agrobacterium, Xanthomonas*,

Micrococcus, *Cornyebacterium* and *Curtobaterium* that caused contamination in mints (*Mentha* spp.) TC. Reed *et al.* (1995) used streptomycin (1000 μg/ml) to eradicate the bacterial contamination in their TC mints. Fellner *et al*. (1996) found that contaminated callus of two garlic species (*Allium sativum* L. *and A. longicuspis* Regel) grown on culture medium supplemented with erythromycin (10 $mg.l^{-1}$), gentamycin (10 $mg.l^{-1}$) and imipenem (5 $mg.l^{-1}$) showed no further growth of three bacteria (*Bacillus circulans, Staphylococcus xylosus* and *Staphylococcus warneri)*. According to Tanprasert and Reed (1997), bacteria-contaminated strawberry runners (*Fragaria X ananassa* Duch *cv.* Jucunda) treated with a combination of timentin, streptomycin sulfate and gentamycin successfully removed *Pseudomonas corugata* from their cultures. Houwe *et al.* (1998) observed that within one month of treating contaminated banana (*Musa* spp.) shoot tips with rifampicin (100 $mg.l^{-1}$), 100% of their plants were contaminant-free with no obvious phytotoxicity. Luna *et al.,* (2008) reported that cefotaxime (0.5 $mg.ml^{-1}$) helped to remove two bacteria (*Stenotrophomonas maltophilia* and *Achromobacter* sp.) from contaminated holly (*Ilex dumosa* var. *dumosa* R.) TCs.

Picoli *et al*. (2005) reported that one bacterium (*Herbaspillium huttiense)* and one yeast (unidentified species) caused the contamination in their *Eucalyptus* TCs. They compared sulfadiazine, streptomycin, kanamycin and penicillin at minimal inhibitory concentrations for their ability to inhibit the growth of yeast and bacteria. Their studies showed that only sulfadiazine, streptomycin and kanamycin at 256 and 512 $mg.l^{-1}$ were effective, as evidenced by inhibition halos on the growth medium.

E.2.2.1 Carbenicillin

Carbenicillin, a derivative of pencillin, is often used in media designed to select transformed plant tissue and eliminate *Agrobacterium* from transgenic plant tissues containing

transgenes (Ogawa and Mil, 2007). Carbenicillin is reported to provide a broad spectrum of antibiotic activity and can kill both gram negative and gram positive bacteria by inhibiting peptidoglycan cross-linking during bacterial cell wall synthesis (Pollock *et al.,* 1983)

Antibiotics have been used to control bacterial growth, but they also can affect the growth of *in vitro* plant tissue. Carbenicillin can inhibit the growth of plant tissues. For example, Pollock *et al.* (1983) studied the phytoxicity of 21 antibiotics to protoplast-derived tobacco cells (*Nicotiana plumbaginifolia)*. Filter-sterilized antibiotic solutions were added to the protoplast-derived cells plated on Petri dishes. The relative plating efficiency showed that cells treated with ampicillin, carbenicillin, cefoxitin and cefotaxime showed the least toxicity levels at ranges from 0 to 100 µg/ml. Tang *et al.* (2000) evaluate the production of secondary SEs by transformed walnut (*Juglans regia*) treated with four antibiotics: ampicillin, cefotaxime, carbenicillin and timentin. Among the four antibiotics, carbenicillin at 100 to 1000 $mg.l^{-1}$ minimally reduced the rate of SE production. Mihaljevic *et al.* (2001) reported that carbenicillin at 500 $mg.l^{-1}$ reduced the growth of SE embryogenic callus of *Picea omorika* (Panc. Purk) as compared to the control group without antibiotics.

Carbenicillin has been reported to stimulate growth *in vitro* for some plant species. Bhau and Wakhlu (2001) suggested that shoot formation by elephant tusk cactus (*Coryphantha elephantidens*) was increased when cultured on MS medium supplemented with kinetin (2 $mg.l^{-1}$), 2,4-D (0.5 $mg.l^{-1}$) and carbenicillin (600 $mg.l^{-1}$). Suzuki and Nakano (2003) found that carbencillin at 200 to 500 $mg.l^{-1}$ promoted both callus growth and SE formation in transformed grape hyacinth (*Muscari armeniacum*). Rahman *et al.* (2004) showed that carbenicillin promoted root branching of *Beta vulgaris, Capsicum annum* and *Glehnia littoralis* roots. Dai and Castillo (2007) stated that the addition of carbenicillin at 250 to 500 $mg.l^{-1}$ to

Woody Plant medium (WPM) supplemented with BA (5 µmol) and IBA (5 µmol) promoted shoot regeneration from leaf explants of two *Buddleia* cultivars.

In some cases, carbenicillin appears to be able to provide both auxin and bactericidal effects. It is composed of a ß-lactan and an aromatic ring that is related to the auxin structure. For example, 2,4-D and NAA contain either a phenol or benzyl group connecting to an acetic acid side chain (Rahman *et al*., 2004). Holford and Newbury (1992) used HPLC, GC and GC-MS to compare samples of two compounds: Phenylacetic acid (PAA), a natural auxin, and a carbencillin breakdown product. The analyses showed that carbenicillin could break down to give physiologically active levels of PAA. Lin *et al. (*1995) cultured transformed tobacco leaves on MS supplemented with BA and carbenicillin (0, 250, 500, 1,000 and 2,000 $mg.l^{-1}$) and found that as carbenicillin concentration was increased from 250 to 2,000 $mg.l^{-1}$, callus formation also increased, but shoot formation decreased. The study confirmed the observation that carbenicillin can provide growth regulator-like effects for transformed tobacco leaf explants.

E.3 Materials and methods

E.3.1 Preliminary test of using carbenicillin in embryogenic induction medium

Since the contaminant appeared to be bacterial, antibiotic was selected as a likely candidate for their control. To study the effects of carbenicillin on horseradish leaf segments, two-week-old leaves were harvested from PF TC 'Doll' horseradish and cut into a square (*ca.* 0.5 × 0.5 cm). Five explants were grown on each Petri (15×100 mm) dishes containing about 30 mls of embryogenic induction medium supplemented with 2,4-D (0.25 $mg.l^{-1}$) with or without carbenicillin (50 $\mu g.ml^{-1}$). Each treatment included twenty-five to fifty explants and each treatment was repeated two times. After six weeks in culture all calli were screened under a stereomicroscope; "clean" calli had no obvious yellow colonies and "contaminated" calli had yellow colonies that had formed on agar surfaces where the calli touched it. The numbers of contaminated and non-contaminated calli were counted. To assess whether the carbenicillin killed the yellow bacteria or just slowed its growth, seventy-two small portions (*ca.* = 6.75 mm diameter) of "clean" embryogenic calli were collected and placed on fresh carbenicillin-free MS media with 2,4-D (0.25 $mg.l^{-1}$). The numbers of contaminated calli were assessed after four weeks in culture.

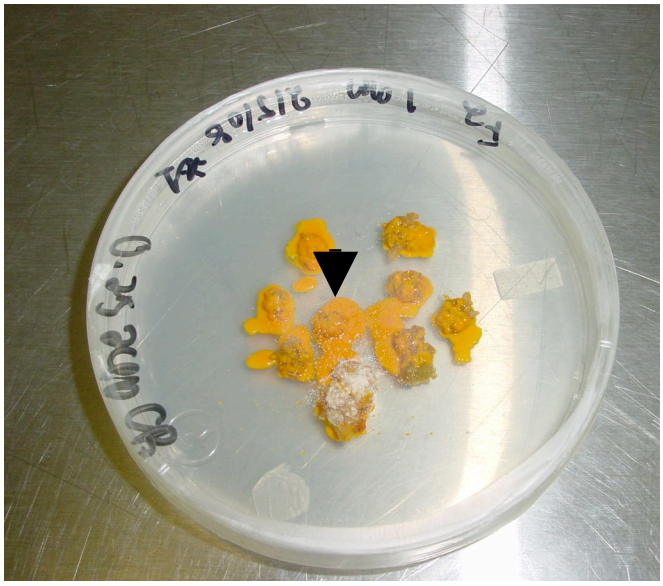

Figure 20. Nine-month-old embryogenic 'Doll' horseradish calli heavily contaminated with an unknown yellow contaminant (arrow).

E.3.2 Carbenicillin phytotoxicity to 'Doll' somatic embryogenic calli

Based on the results, the reputed "clean" somatic embryogenic callus cultures were often found to harbor yellow bacteria that grew to become colonies after four to five weeks in culture. Carbenicillin at 50 $\mu g.ml^{-1}$ apparently was not strong enough to kill all the colonies of yellow bacteria, but merely suppressed their cell division. Thus, when carbenicillin was removed from the embryogenic induction medium, yellow bacteria no longer suppressed and they began to divide again. To study the situation the phytoxicity of carbenicillin to PF horseradish plant tissues had to be investigated.

The basic media used in this study was Murashige and Skoong (MS, 1962) medium supplemented with 2,4-D (0.25 $mg.l^{-1}$) with four levels of carbenicillin (0, 200, 500 and 2,000 $\mu g.ml^{-1}$). Two- to three-week-old PF TC horseradish leaves were cut from proliferating cultures and placed onto treatment media. There were five Petri dishes per treatments; five explants per Petri dish. The experiments were arranged in a complete randomized design and repeated three

times. The contamination rate of each treatment was assessed at the end of five weeks. The number of SEs that formed from calli was estimated.

E.3.3 Carbenicillin and liquid media

In the previous experiment, carbenicillin was included in agar-based somatic embryogenic induction medium. Since carbenicillin had failed to kill all bacteria in previous experiments (E.3.2), further investigation was needed. We hypothesized that since leaf explants on solid medium contact the medium only on one side of the explant surface, carbenicillin killed only the yellow bacteria that inhabited the side of the leaf that touched the agar. Bacteria on the other side of the leaf that did not touch the agar remained unaffected and may have been responsible for the continuing contamination problem. If true, all parts of leaf explants liquid growing in media with carbenicillin would come in contact with the antibiotic and thus more effective control the yellow bacteria.

The explants used to establish the liquid cultures were leaf squares (0.5 × 0.5 cm) of two- to three-week-old *in vitro* leaves. MS medium with 2, 4-D (0.25 $mg.l^{-1}$) was used as an embryogenic induction medium. The explants were grown on three media:

1) Induction agar medium without carbenicilllin for four weeks (control)

2) Induction agar medium with carbenicillin (50 $\mu g.ml^{-1}$) for four weeks

3) Induction liquid medium with carbenicillin (50 $\mu g.ml^{-1}$) for ten days, followed by transfer of explants to induction agar media supplemented with carbenicillin (50 $\mu g.ml^{-1}$) for three more weeks.

Contamination incidence was recorded after four weeks in culture. After that, all calli from every treatment were transferred to induction agar medium without carbenicillin for another

four weeks. The numbers of contaminated and non-contaminated calli were evaluated. The experiments were repeated three times**.**

E.3.4 The combination of carbenicillin and spectinomycin

In the previous experiments (sections E.3.1, E.3.2 and E.3.3) carbenicillin had been added to MS medium in an attempt to control the yellow bacterial contaminants. Although carbenicillin was found to stop bacterial growth temporarily, within a few weeks the organism(s) again became visible and grew vigorously. Apparently carbenicillin was only inhibiting, not killing, the yellow bacteria. Supena *et al.* (2006) found that the addition of two antibiotics, timentin at 200 $mg.l^{-1}$ and rifampicin at 10 $mg.l^{-1}$, could prevent bacterial contamination in shed-microspore culture of Indonesian hot pepper (*Capsicum annum* L.) cultivars. It was hypothesized that a second antibiotic might help solve our bacterial contamination problem.

Spectinomycin was selected for use in the present study for several reasons. First, Poulsen (1996) reported that spectinomycin has been used to select transformed *Brassica* species. Second, spectinomycin kills bacteria in a different way than carbenicillin. Carbenicillin's mode of action involves the interruption of bacterial cell wall synthesis (Pollock *et al*., 1983). Spectinomycin's mode of action relates to its ability to inhibit protein synthesis in bacteria. Third when protoplast-derived tobacco cells (*Nicotiana plumbaginifolia*) were treated with spectinomycin at 0.1 to 100 ($\mu g.ml^{-1}$), it was found to be nontoxic.

To investigate the effectiveness of using two antibiotics for contaminated horseradish cultures, spectinomycin was added to the carbenicillin medium described earlier. It was assumed that the combined effect of carbenicillin's ability to inhibit bacterial cell wall synthesis and spectinomycin's ability to inhibit protein synthesis should kill the yellow bacteria contaminants.

To evaluate their effectiveness, the growth of yellow bacterial contaminants and the morphology of embryogenic callus were studied in this experiment.

Two- to three-week-old TC horseradish leaves, five leaves per Petri dish, were used in this experiment. The leaves were induced to produce embryogenic calli on 2,4-D (0.25 $mg.l^{-1}$) MS medium. There were three treatments: 1) Combination of carbenicillin and spectinomycin: twenty-five leaf explants were cut and grown on the embryogenic induction medium supplemented with 50 $\mu g.ml^{-1}$ carbenicillin for two weeks. After two weeks in culture, these twenty-five leaf explants were transferred to grow on fresh embryogenic induction medium supplemented with spectinomycin (50 $\mu g.ml^{-1}$) for another two weeks. Filter sterilized spectinomycin stock solution had been added to the culture medium after autoclaving. 2) Carbenicillin: twenty-five leaf explants were cultured on MS medium carbenicillin (50 $\mu g.ml^{-1}$) for four weeks and 3) Control: twenty-five leaf explants were cultured on MS medium with neither carbenicillin nor spectinomycin for four weeks.

After four weeks in culture, the morphology of these calli was evaluated and the number of cultures with the yellow contaminants counted. To investigate the contamination status of the cells grown on the two antibiotic media, all embryogenic calli from both treatments, as well as control calli, were transferred to induction medium without antibiotics for four weeks. The rate of yellow contamination was reassessed after another four weeks in culture. The experiment was repeated four times.

E.4 Results and discussion

E.4.1 Preliminary test using carbenicillin in embryogenic induction medium

After six weeks in culture on 2,4-D medium, leaves formed embryogenic calli on all media, both with and without carbenicillin treatments. Leaf explants from both treatments produced green granular calli that include SE-like structures. Contamination on media with carbenicillin was as low as 14.3% (10 out of 70) and 85.7% (60 out of 70) appeared to be clean. In contrast, the control without the carbenicillin supplement was 100% contaminated (Figure 21, Table 7). Next, 72 samples of "clean" embryogenic calli were subcultured on carbenicillin-free MS induction medium for another five weeks. After another five weeks, 11 out of 72 (15.2%) were free from the yellow contaminants but 61 out of 72 (84.8%) appeared to have yellow contamination. Based on these results, it was hypothesized that in most cases the carbenicillin at 50 $\mu g.ml^{-1}$ had merely suppressed the growth of the yellow bacteria, not killed it. However, 15% of embryogenic calli were freed of contamination. To test this possibility, in the next experiment the phytotoxicity of carbenicillin at concentration levels greater than 50 $\mu g.ml^{-1}$ was tested in 'Doll' horseradish embryogenic callus.

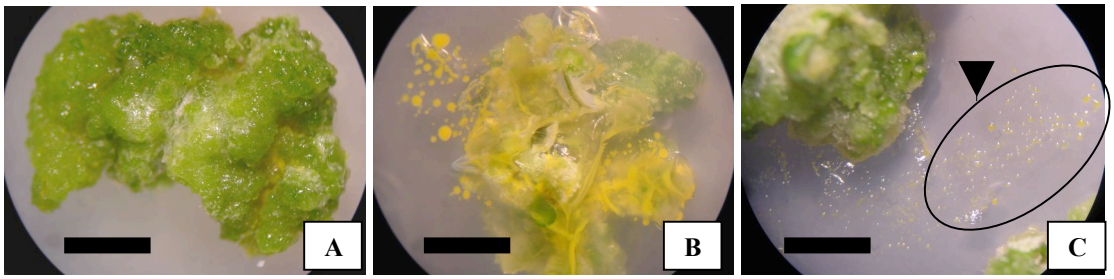

Figure 21. 'Doll' horseradish embryogenic calli grown on MS induction medium with carbenicillin after 45 days in culture.
A, a top view of the embryogenic callus (bar = 5mm).
B, a bottom view of the embryogenic calli; it showed that the yellow contaminants forming small colonies on induction medium (bar = 5mm).
C, the yellow contaminants were not always obvious to the naked eye and had to be observed under a stereomicroscope (bar = 5mm).

F.4.2 Phytotoxicity of carbenicillin to PF 'Doll' somatic embryogenic calli

After four weeks in culture, all leaf explants in every treatment group had formed embryogenic callus with no obvious yellow bacterial contamination. The embryogenic callus treated by carbenicillin at 0 (control), 200, 500 and 2,000 µg.ml^{-1} formed green nodular compact calli with globular SE like structures. Embryogenic calli treated with carbenicillin at 500µg.ml^{-1} or lower produced SEs at an average of 30 or greater (figure 22a) per callus. At the highest dose of carbenicillin (2,000 µg.ml^{-1}) some leaf explants died early in the experiments, while survivors formed green nodular compact callus with fewer prominent globular SEs (figure 22b).

When the culture plates were assessed eight weeks later, it was found that 96.45% of the embryogenic calli treated with 2000 µg.ml^{-1} carbenicillin were clean, while other treatment groups (0, 200, and 500 µg.ml^{-1} carbenicillin) were 52 to 95% contaminated (Table 8).

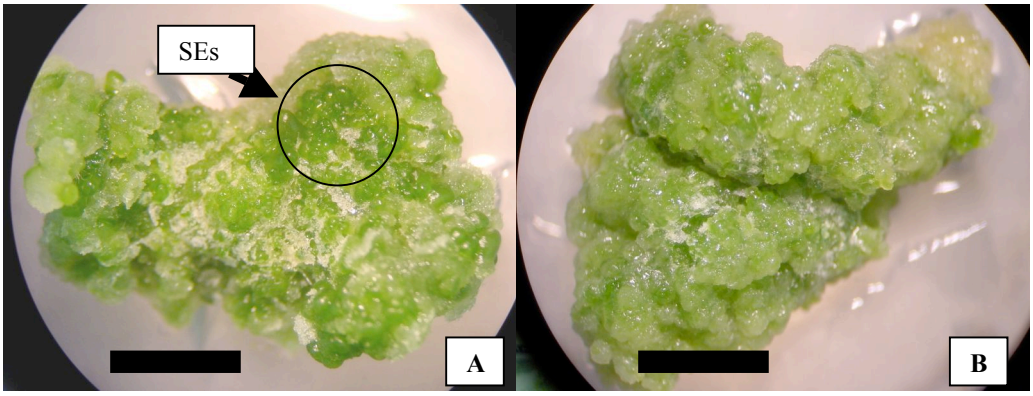

Figure 22. Phytotoxicity of carbenicillin on 'Doll' horseradish embryogenic callus formation.
A, 'Doll' horseradish embryogenic calli grown on MS with 2,4-D 0(.25 mg.l^{-1}) without carbenicillin formed a great number of globular SE-like structures (bar = 5mm)
B, Those embryogenic calli grown on induction medium with carbenicillin (2000 µg.ml^{-1}) formed few number of globular SE-like structures (bar = 5mm).

E.4.3 Antibiotics for controlling contamination

E.4.3.1 Carbenicillin and liquid media

After ten days in culture, leaf explants cultured in MS liquid medium with 2,4-D (0.25 $mg.l^{-1}$) and carbenicillin (50 $\mu g.ml^{-1}$) became enlarged in length and width (Figure 23a). The growing leaf tissue became light yellow in color and the tissues appeared to be soft and fragile (Figure 23b). Leaf explants grown on MS solid induction medium with or without carbenicillin formed green nodular embryogenic calli after four weeks in culture. After three weeks in culture, the leaf explants moved from the carbenicillin liquid treatment to the MS solid induction medium with carbenicillin formed green nodular compact embryogenic calli with globular SE-like structures. The leaves that began in liquid medium seemed to have a higher number of estimated SEs (*ca.* 50 SEs or greater per one side of callus) than that formed on on solid MS induction medium (Figure 23 c,d; Table 9). Contamination was not observed until the fourth week for the control and 70 to 90% of all treatments were contaminated after eight weeks in culture.

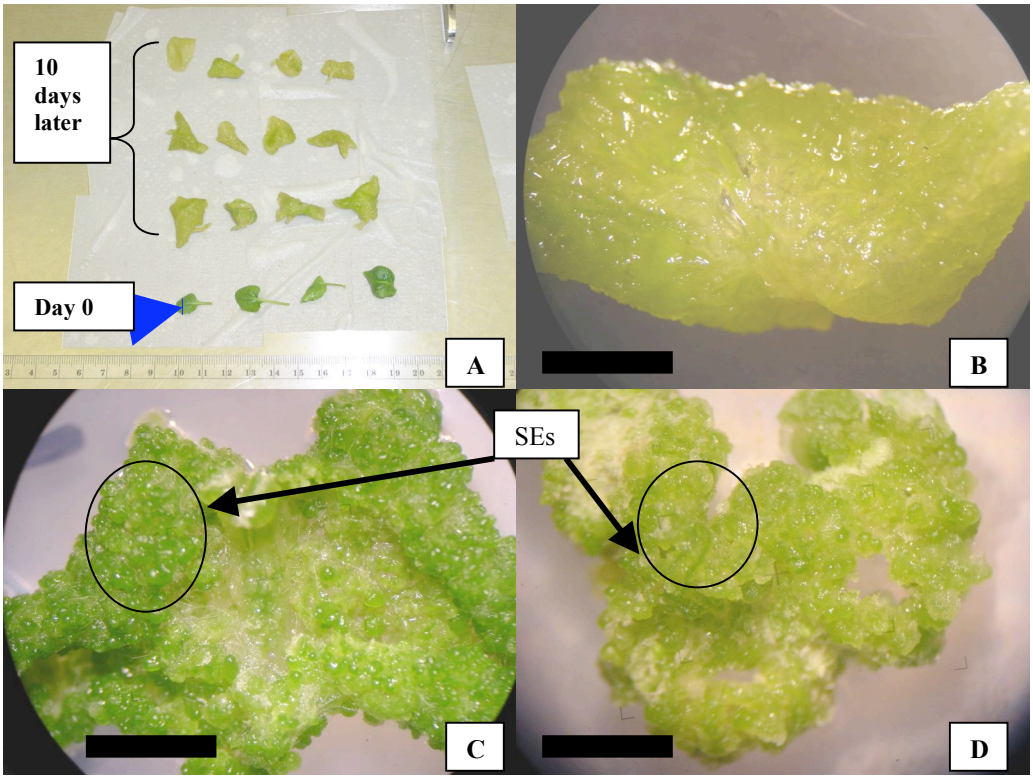

Figure 23. 'Doll' leaf horseradish explants treated with agar or liquid MS with carbenicillin.
A, after ten days in culture, 'Doll' horseradish leaf explants on liquid medium with 2,4-D (0.25 $mg.l^{-1}$) and carbenicillin (50µg.ml^{-1}) increased in size compared to fresh picked *in vitro* leaves (blue arrow).
B, In general, leaf tissues were soft and yellowish (bar = 5mm).
C, after the leaf tissues were moved to MS agar induction medium with carbenicillin for another three weeks, green nodular compact embryogenic calli with SEs formed (bar = 5mm).
D, those leaf explants grown the MS agar induction medium with carbenicillin formed embryogenic calli but fewer somatic embryos (bar = 5mm).

E.4.3.2 The combination of carbenicillin and spectinomycin

For the first four weeks in culture, the yellow contamination was not observed on any of the embryogenic calli grown on MS induction medium with carbenicillin alone or supplemented with spectinomycin. However, after eight weeks, the yellow bacterial contaminants were observed on about half of the embryogenic calli from both antibiotic treatment groups. Most (92%) of control calli that received no antibiotic treatment were contaminated. While the antibiotic treated groups were 49.4% to 57% contaminated (Table 10). Also, it appeared that the

spectinomycin had a negative effect on embryogenic tissue since the leaf explants grown on spectinomycin formed light yellow nodular calli reduced SE production (Figure 24 a,b)

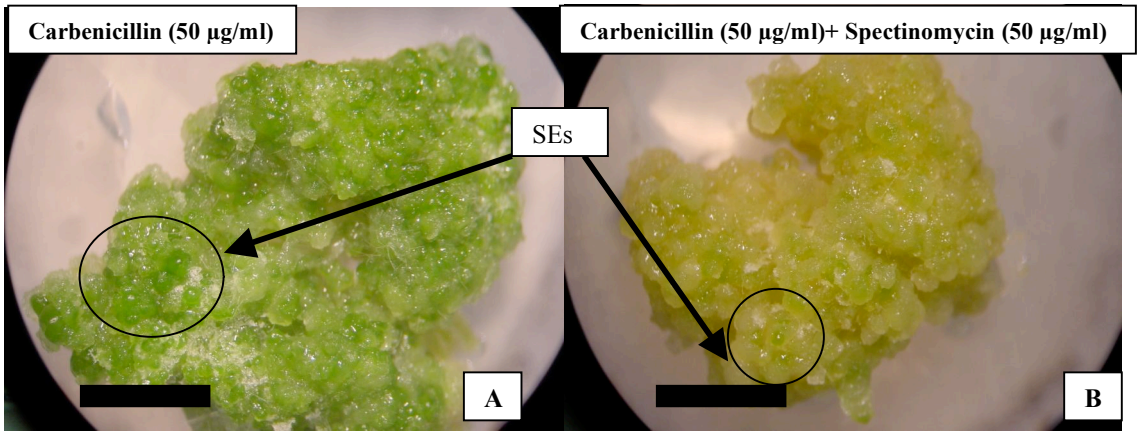

Figure 24. 'Doll' horseradish leaf explants treated with carbenicillin or with a combination of carbenicillin and spectinomycin.
A, leaf explants of 'Doll' horseradish grown with carbenicillin formed a large number of somatic embryos (bar 5 mm).
B, leaf explants grown with both spectinomycin and carbenicillin formed yellow nodular callus with few somatic embryos (bar = 5mm).

In summary, the yellow contaminants was hypothesized that the yellow contaminants might be latent bacteria since the leaf explants used in these studies had been harvested from clean mother plants, and the bacteria grew slowly to develop obvious colonies within five to six weeks. The presence of the yellow bacteria may confound the embryogenic callus formation in 'Doll' horseradish. However, there is no proof that having bacteria would affect the callus response.

Regardless of concentration (50 to 500 $\mu g.ml^{-1}$), carbenicillin did not appear to kill contaminants, but it suppressed bacterial growth for 45 days. Beyond 4 to 8 weeks in culture, yellow bacteria became more reactive and aggressively produced more colonies. The high level of carbenicillin at 2,000 $\mu g.ml^{-1}$ inhibited bacteria growth but it also reduced the number of globular SE-like structures formed on leaf-derived embryogenic calli. When leaf explants were

grown in liquid carbenicillin medium with 2,4-D at 0.25 $mg.l^{-1}$, the growth of 'Doll' leaves and the formation of somatic embryogenic callus was promoted. Carbenicillin has been reported to have a stimulatory effect on SE formation in some plants.

The presence of a second antibiotic (spectinomycin at 50 $\mu g.ml^{-1}$) in addition to carbenicillin (50 $\mu g.ml^{-1}$) did not kill yellow bacteria. When the antibiotics were removed, yellow colonies formed. Moreover, spectinomycin seemed to have an inhibitory effect on 'Doll' horseradish embryogenic calli formation since the leaf explants grown with spectinomycin medium formed only complex yellow nodular callus and few SEs (10 SEs or fewer). Based on these studies, it can conclude that neither carbenicillin nor spectinomycin can eliminate yellow bacterial contaminants. In future studies other antibiotics should be considered.

Table 7. The growth of yellow contaminants in 'Doll' horseradish embryogenic callus on embryogenic induction agar MS containing 2,4-D (0.25 $mg.l^{-1}$) and carbenicillin after six weeks *in vitro*.

Carbenicillin ($\mu g.ml^{-1}$)	Explant (n)	Contaminant status	
		No. with bacterial contaminant (n) (%)	No. without bacterial contaminants (n) (%)
0	50	50 (100%)	0 (0%)
50	70	10 (14.3%)	60 (85.7%)

Table 8. The growth of yellow contaminants in 'Doll' horseradish embryogenic callus on embryogenic induction agar MS containing 2,4-D (0.25 $mg.l^{-1}$) and carbenicillin at various concentrations after eight weeks *in vitro* (n=75).

Carbenicillin ($\mu g.ml^{-1}$)	No. of explants with embryogenic callus	No. embryogenic calli contaminated with yellow bacteria (n) (%)	No. of clean embryogenic calli (n) (%)	No. of SEs on embryogenic callus /explant (estimated)
0	73	70 (95.9%)	3 (4.1%)	>30
200	74	39 (52.7%)	35 (57.3%)	>30
500	74	39 (52.7%)	35 (57.3%)	>30
2,000	68	3 (4.4%)	65 (96.4%)	<10

Table 9. The growth of yellow contaminants in 'Doll' horseradish embryogenic callus on embryogenic induction agar MS containing 2,4-D (0.25 $mg.l^{-1}$) and carbenicillin (50$\mu g.ml^{-1}$) after eight weeks *in vitro* (n=75).

Treatments	No. contaminated embryogenic calli with yellow bacteria (n) (%)	No. of clean embryogenic calli (n) (%)	No. of SEs on embryogenic callus /explant (estimated)
Without carbenicillin + Agar (control)	73 (97.3%)	2 (2.7%)	>30
With carbenicillin +Agar	57 (76%)	18 (24%)	>30
With carbenicillin –Agar (liquid)	61 (81.3%)	14(19.7%)	>30

Table 10. The growth of yellow contaminants in 'Doll' horseradish embryogenic callus on embryogenic induction agar MS containing 2,4-D (0.25 $mg.l^{-1}$) with or without carbenecillin (CB) and spectinomycin (ST) after eight weeks *in vitro*.

CB ($\mu g.ml^{-1}$)	ST ($\mu g.ml^{-1}$)	No. of Explant (n)	No. contaminated embryogenic calli (n) (%)	No. of clean embryogenic calli (n) (%)	No. of SEs on embryogenic callus /explant (estimated)
0	0	95	88 (92.6%)	7 (7.4%)	>30
50	0	95	46 (49.4%)	49 (51.6%)	>30
50	50	100	57 (57%)	43 (43%)	>10

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Miss Wannasiri Wannarat was born in Pattanee, Thailand April 2nd, 1978. She grew up with her two caring younger brothers and with the greatest love from her father, Police Colonel Peerapol Wannarat, and mother, Mrs. Thapthong Raethong. When she was eighteen, she dreamed to be a scientist. During her undergraduate studies she received scholarships from Chulalongkorn University in 1996-1997 and from the Institute for the Promotion of Teaching Science and Technology (IPST) in 1998-1999. In 1999 she earned her BS in biology from Chulalongkorn University. She won another scholarship from IPST to pursue her MS in an animal physiology program at the same university and graduated in 2002. After feeling a lot of guilt for killing several hundred hamsters while completing her master's thesis, she chose to do Ph.D. involving plants instead. In 2003, she was awarded a scholarship by the Royal Thai Government to study in a Ph.D. program related to plant cell and tissue culture. She came to University of Illinois at Urbana-Champaign in 2004 and became a Ph.D. student under supervision of the greatest advisor, Dr. Robert M. Skirvin in 2006. Most of her research involved in somatic embryogenesis of horseradish. After her PhD. completion, she will return to Thailand. She will work as a researcher in a unit of plant biotechnology at Kasersart Agricultural and Agro-Industrial Product Improvement (KAPI), Kasetsart University, Bangkok. I wished to thank the Royal Thai Government for giving me this opportunity.