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Analysis of Tomato Synthetic Seeds for the Development of an Optimized
Encapsulation System

John E. Porter

Thesis submitted to the
Davis College of Agriculture, Forestry and Consumer Sciences

in partial fulfillment of the requirements for
the degree of

Master of Science
in
Horticulture

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2008

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ABSTRACT

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John E. Porter

In vitro propagules of tomato (*Solanum lycopersicum* L.) were used to evaluate the effects of alginate encapsulation and subsequent storage for synthetic seed (synseed) production. Nodal segments were encapsulated in hollow alginate beads with and without supplemental nutrients, auxin (0, 2.5 and 5 μ M IBA) and fungicide (0, 50 and 100 μ M Daconil 2787). Synseeds were stored for 0, 30 and 90 days at $4\pm 1^\circ\text{C}$ in darkness and were given a light treatment (none vs. one week at $25\pm 2^\circ\text{C}$ with a 16 hour photoperiod) before sowing in a greenhouse hydroponic system. There was no survival across all treatments of *ex vitro* sown synseeds. Gene expression profiling was conducted on nodal segments of 'Big Beef' in stored (0, 30 and 90 days) hollow alginate beads using microarrays. Lack of hybridization was attributed to RNA degradation during storage. Apical meristems of 'Big Beef' were encapsulated in hollow or solid alginate beads, with or without supplemental nutrients, and stored for 0, 15 and 30 days at $4\pm 1^\circ\text{C}$. Synseeds were sown *in vitro* and incubated at $25\pm 2^\circ\text{C}$. Hollow bead synseeds had a higher survival rate than solid bead synseeds. Supplemental nutrients also improved survival and conversion of synseeds. Storage of synseeds for 15 and 30 days resulted in significantly lower survival and conversion rates when compared to 0 days of storage.

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INTRODUCTION

Garden tomato (*Solanum lycopersicum* L.) is a member of the Solanaceae family. It is second only to potato (*Solanum tuberosum* L.) in worldwide vegetable crop production, with 1.3 billion metric tons produced on 4.6 million hectares in 2006 (FAO Statistical Database, 2008). Tomato contains many different important compounds for human nutrition such as vitamins A and C and carotenoids, including lycopene (Bhatia et al., 2004; Fei et al., 2004). Extensive research on tomato has contributed to its use as a model plant species for genetic studies, fruit development, disease interactions and physiological investigations (Fei et al., 2004) because it represents a nutritionally and commercially important crop family (Fei et al., 2006). At the genetic level, a sequenced nuclear tomato genome is near completion (Fei et al., 2004), which would allow for further, more focused studies in expressional and physiological responses.

Genetically stable populations must be preserved to maintain genetic resources of tomato and other important plants in germplasm storage facilities to ensure conservation of cultivars, breeding lines and genotypes for breeding and research purposes. Moreover, stocks of germplasm must be easily maintained and free of disease to provide an affordable and reliable source of plants for research (Danso and Ford-Lloyd, 2003). Many current genetic storage techniques use sexually produced seeds, which produce genetically variable populations and have the potential to carry seed-borne pathogens (Hartmann et al., 2002). Another method of germplasm storage is the use of tissue culture, with the negative component being maintenance regimes are labor intensive (Hartmann et al., 2002).

Storage and transport of clonal populations *in vitro* can be made possible via production of synthetic seeds. Synthetic seeds (synseeds) are composed of propagules, such as nodes, shoot tips, callus or somatic embryos, which are derived from step two of micropropagation (Standardi and Piccioni, 1998). These propagules are then encapsulated in a gelatinous matrix, such as sodium alginate (Standardi and Piccioni, 1998; Murashige, 1978). Efforts have focused on species such as potato (Nyende et al., 2003; Patel et al., 2000; Sarkar et al., 1998), apple (Piccioni 1997; Brishia et al., 2002; Micheli et al., 2002) banana (Hassanein et al., 2005; Priya and Shakila, 2003) and orchid (Divakaran et al., 2006; Saiprasad and Polisetty, 2003). Expansion of this technique to include other important plant species and to study changes in encapsulated and stored propagules will improve the potential to produce viable synseed technology.

Principles of Plant Tissue Culture

Tissue of culture has become a widely used method for axenic plant propagation (culturing one organism that is entirely free of all other contaminating organisms) plant propagation. The process involves growing explanted cells, tissues or organs in an aseptic environment. The most common application of tissue culture is to produce clonal populations of plants with fixed desirable traits. Clones are, by definition, genetically identical and are offspring from a single parent genotype (Hartmann et al., 2002). Multiple plants are produced in the absence of seeds, thus species lacking proper pollinators or those with very low germination rates can be propagated successfully (Saiprasad, 2003). Aseptic culture techniques can 1) diminish the probability of spreading diseases, pathogens and pests to the progeny of the cultured

plants, and 2) eliminate systemic pathogens from plants in culture, which is especially useful in plants with high infection rates, such as potato (Nyende et al., 2003).

Micropropagation

Micropropagation has the potential to produce numerous plants per year, while traditional propagation techniques may have limited production numbers in the same period (Hartmann et al., 2002). This benefit is especially important for plants that are sterile, slow to propagate, or have low fecundity, such as bulbous crops. Introduction of new cultivars to the consumer market also is promoted, as traditional propagation methods would take many years to produce enough plants for introduction (Hartmann et al., 2002). The high commercial value and potential for genetic manipulation of tomato has lead to a multitude of *in vitro* and micropropagation studies (Bhatia et al., 2004). Tissue culture methods have been applied to making improvements in tomato through selection for resistance to biotic and abiotic stresses, development of haploids, production of somatic hybrids and mass propagation (Bhatia et al., 2004).

Micropropagation is carried out in a series of four steps. The first step is establishment of explants (propagule), which is that portion of the parent plant excised and introduced into culture (Hartmann et al., 2002). This step involves acclimation of the explant to the culture environment. The explant is first surface disinfested using solutions of sodium hypochlorite (household bleach) and/or ethanol. Debris is removed from the tissue and associated microorganisms are killed. The disinfested explant is placed on a culture medium that contains all of the essential elements for plant growth, including macro- and microelements, vitamins and nutrients. Since plants in culture have low photosynthetic rates, organic sources of carbon, such as sucrose or maltose,

must be included in the medium (El-Bakry, 2002). Generally, the medium is supplemented with plant growth regulators, such as auxins and cytokinins, in ratios that promote shoot or root development (Hartmann et al., 2002). A high ratio of cytokinin to auxin results in growth of shoots, while a higher concentration of auxin results in root growth (Hartmann et al., 2002). Equal concentrations of each result in the growth in undifferentiated callus tissue (Hartmann et al., 2002).

The second step of micropropagation involves directing the multiplication of plant tissues and organs along one of three developmental pathways; axillary shoot formation, adventitious shoot formation or somatic embryogenesis (Hartmann et al., 2002). Axillary shoot proliferation involves the culture of shoot tips and axillary nodes. These segments usually contain only the apical or axillary meristem and a few undeveloped leaf scales (Hartmann et al., 2002). Exposure of these tissues to cytokinins, such as 6-benzyladenine (BA, BAP), results in axillary shoot proliferation. These shoots, which resemble miniature stem cuttings, then are separated and established for planting or are reintroduced to culture for further multiplication. When axis tips are cultured after stock plants are exposed to warm temperatures, plants are free of viruses (Hartmann et al., 2002; Quak, 1977). This method has been effective in producing disease free lines of economically important plants (Murashige et al., 1974). It also increases the probability of genotype retention, in that plants derived via axillary shoot cultures are less likely to develop mutations *in vitro* than tissues produced adventitiously (Hartmann et al., 2002).

Growth of shoots from non-nodal explants such as stems, leaves, or roots results in adventitious shoot formation. This method is similar to axillary shoot proliferation, the

major difference being the use of non-nodal explants for establishment of the culture (Hartmann et al., 2002). Adventitious growth can produce higher rates of multiplication than axillary shoot cultures (Brischia et al., 2002). Formation of shoots from a relatively small number of cells, however, can result in a high number of aberrant plants from mutations (somaclonal variation) (Hartmann et al., 2002).

Somatic embryogenesis, the growth of embryo-like structures from somatic tissue, is the most complicated micropropagation pathway (von Arnold et al., 2002). Species specific ratios of plant growth regulators in tissue culture media promotes adventitious growth of somatic embryos on explant tissues (von Arnold et al., 2002; Standardi and Piccioni 1998). Somatic embryogenesis produces propagules that have a bipolar structure, containing both shoot and root apical meristems (Standardi and Piccioni, 1998). These propagules are ideal for encapsulation for synseed production, since both shoots and roots are already present on the explant. Somatic embryos are adventitiously derived and are therefore susceptible to somaclonal variation (Standardi and Piccioni, 1998). The potential for somaclonal variation to occur in adventitious or somatic embryo derived plants can be problematic when the goal is germplasm storage (Isabel et al., 1996).

The third step usually involves rooting of the multiplied explants in a medium containing higher concentrations of auxin, such as 3-indole butyric acid (IBA), to encourage rooting (Hartmann et al., 2002). Supplied carbon source is reduced, humidity is decreased and light is increased to acclimatize the plant to *ex vitro* conditions (Hartmann et al., 2002).

The fourth step involves transferring and acclimatizing plantlets to a greenhouse potting medium and growth environment (Hartmann et al., 2002). To accomplish this, humidity levels are decreased and light intensities are increased.

Principles of Synseed Technology

Synseeds were first proposed by Murashige (1978) and have since become an emergent field in plant cell and tissue culture research (Standardi and Piccioni, 1998). Initially, synseeds consisted of propagules produced only from alginate encapsulated somatic embryos (adventitious embryogenic structures from somatic tissue) originating from rapidly multiplying vegetative cell cultures (Standardi and Piccioni, 1998; Murashige 1978). However, low production rates and quality, as well as the potential for somaclonal variation constrained production to only a few species (Standardi and Piccioni, 1998). As a result, the technology has expanded to include propagules such as shoots, cell aggregates, nodes and any other totipotent (potential to develop into any cell or tissue type or whole organism) tissue (Standardi and Piccioni, 1998). The use of *in vitro*-derived axillary buds and shoot tips provides inexpensive, easily obtained propagules for storage (Danson and Ford-Lloyd, 2003). Nodal segments of eggplant (Huda et al., 2007) and potato (Sarkar et al., 1998), both relatives of tomato, have been successfully used to produce synseeds.

Synseed technology has many applications. Difficult-to-propagate species, such as certain orchids, can be mass produced (Saiprasad and Polisetty, 2003). Disease susceptible species, such as potato, may be grown free of viruses and other pathogens utilizing micropropagation and synseed technology (Nyende, et al., 2003). The technology provides a possible method for germplasm storage, transport and utilization

of clonal plant populations (Ozden-Tokatli et al., 2008; Naik and Chand, 2006; Singh et al., 2006; Danso and Ford-Lloyd, 2003). While great strides have been taken in the development of this technology, its potential has yet to be fully recognized and an analog to natural seed has yet to be realized (Kumar et al., 2005).

Synseeds composed of alginate encapsulated somatic embryos resemble natural seed in that the propagule has a bipolar structure (Standardi and Piccioni, 1998). Production of synseeds using somatic embryos is limited to species for which somatic embryogenesis protocols exist, such as carrot (Patel et al., 2000) and alfalfa (Senaratna et al., 1990). In contrast, synseeds have been produced from somatic tissues such as shoot tips, axillary nodes and callus in a broader range of species, including rice (Kumar et al., 2005), potato (Nyende et al., 2003), the genera *Malus* and *Rubus* (Piccioni and Standardi, 1995) and other woody species (Gardi et al., 1999).

The production of green, phenotypically normal plants from synseeds containing either unipolar (nodes or shoots) or bipolar propagules (somatic embryos) is called conversion (Redenbaugh et al., 1986). Conversion of synseeds to plantlets is analogous to the germination of seedlings from seeds (Standardi and Piccioni, 1998), and the rate of conversion is often used as a marker of synseed viability.

Two issues are raised concerning the quality of synseeds made from each type of explant. First, somaclonal variation in somatic embryos may adversely affect propagule quality (Larkin and Scowcroft, 1981). However, it has been suggested that the developmental pathway for somatic embryogenesis selects against most mutations (von Arnold et al., 2002). Second, non-embryonic propagules do not undergo a natural

dormancy phase (Gardi et al., 1999). Regeneration quality could decrease over time as a result of nutrient depletion over time within the synseed (West et al., 2006).

Synseed encapsulation provides multiple benefits that can affect the quality and storability of the propagules. Techniques do exist where somatic embryos are desiccated and stored cryogenically sans encapsulation (Kim et al., 2006). However, there is no protection from damage from the environment or from handling practices, either of which could cause propagule damage or mortality (Fabre and Dereuddre, 1990).

Two common encapsulation methods are currently utilized, each with its own benefits and drawbacks. The first method involves solid bead encapsulation, and it has remained largely unchanged since its introduction by Murashige (1976). Vegetative propagules are suspended in a viscous sodium alginate solution containing basic micro and macronutrients (Standardi and Piccioni, 1998; Murashige, 1976). The suspended propagules then are deposited in a solution of calcium chloride to harden (Standardi and Piccioni, 1998; Murashige, 1976). The exchange of the calcium ions for sodium ions results in cross linkage of the alginate cations and produces a solid gel matrix (Patel et al., 2000).

While solid bead encapsulation is a relatively simple procedure, propagules can be damaged during production and storage (Patel et al., 2000). Protrusion of the propagule from the surface of the gel matrix due to incomplete coverage of the propagule by the sodium alginate is the most common problem (West et al., 2006; Patel et al., 2000). Detrimental effects include desiccation, susceptibility to shearing forces, early emergence and necrosis (Patel et al., 2000).

Patel et al. (2000) proposed hollow bead encapsulation as an alternative method to protect propagules from any damage related to protrusion or early emergence. This method utilizes a process that produces a semi-liquid center surrounded by solid gel. A propagule is suspended in a semi-liquid containing a thickening agent and calcium chloride and then dropped into a solution of 0.8% sodium alginate (Patel et al., 2000). At the surface of the drop, a layer of calcium alginate forms the outer gel coating. After 20 minutes, the beads are rinsed and then stirred in a 1% calcium chloride solution for hardening (Patel et al., 2000). Carrot embryogenic callus demonstrated increased growth in hollow synthetic seeds when compared to solid synthetic seeds (Patel et al., 2000).

Basics of Gene Expression Profiling

Further development of synseed technology depends on understanding the underlying physiological processes occurring in the explants during encapsulated storage. To improve the technology in terms of explant longevity and quality, questions regarding physiological processes and reactions that decrease both vigor and viability must be answered (Gardi et al., 1999).

Physiological processes that explants undergo during encapsulated storage have yet to be studied, especially as they are impacted by temperature and metabolic stresses. Within the self-contained environment of a synseed, the metabolic rate of the explant could be a factor in sustaining biological activity, since nutrient availability is limited to that present in the encapsulation media (Gardi et al., 1999). Plant tolerance to storage temperature and conditions also is an important consideration for survival and conversion ability of stored propagules (West et al., 2006; Nyende et al., 2003).

Gene expressional analysis is a standard research tool used to examine differential responses in plant physiology. Recent tools of analysis include RNA gel-blot analysis, differential display, cDNA library sequencing and serial analysis of gene expression (SAGE) (Alba et al., 2004). Output of these tools is constrained by lack of genome-wide comparisons (RNA gel-blot, lack of quantitative results (differential display), and requirement of extensive sequence information, high labor intensity and low sensitivity (cDNA library sequencing and SAGE) (Alba et al., 2004).

Expression Profiling via Microarray Analysis

Microarrays are composed of either cDNA or oligonucleotide probes printed microscopically immobilized on a glass slide or other solid surface a high-fidelity robotic system (Shalon et al., 1996). Fluorescently-labeled cDNA samples from transcripts expressed in different treatments are hybridized to the slide, creating a unique array of hybridization signal spots (Shalon et al., 1996). These signals identify which genes are being differentially or uniformly expressed between or across each sample (Shalon et al., 1996). This technique characterizes many mRNA transcripts concurrently, is semi-quantitative, and is sensitive to low-abundance transcripts (Alba et al., 2004). However, microarrays are constrained by sequence information and size of EST collections (Alba et al., 2004). This is not a problem where complete genome information exists, but many investigators have no choice but to focus their microarray analyses on targeted subsets of the genome (Alba et al., 2004).

Illumination of gene expression profiles for complex processes involved in physiological, developmental and response pathways has lead to a vast collection of shared knowledge in public databases (Alba et al., 2004). Such databases include the

Arabidopsis Information Resource (TAIR; <http://www.arabidopsis.org/home.html>), the Solanaceae Genomics Network (SGN; <http://sgn.cornell.edu>) that includes tomato, potato and eggplant, and GRAMENE (<http://www.gramene.org>), which encompasses rice, maize, wheat and barley.

Microarray Analysis of Tomato - the TOM1 cDNA Microarray

The most commonly used microarray system for tomato and other closely related species is the TOM1 microarray produced by Boyce Thompson Institute for Plant Research at Cornell University (<http://bti.cornell.edu/www.libproxy.wvu.edu/CGEP/CGEP.html>). This microarray is composed of 12,899 EST clones, representing 8500 independent tomato genes (Alba et al., 2004). These slides have been utilized in studies investigating tomato fruit ripening (Fei et al., 2004, Alba et al., 2005); oxidase and wound responses (Sagi et al., 2004); tomato ethylene signal transduction (Adams-Phillips et al., 2003); and pathogen responses (Gibly et al., 2004, Bonshtien et al., 2005). TOM1 data, along with all other tomato microarray research findings, are brought together in a suite of three integrated software component, the Tomato Expression Database (TED) (Fei et al., 2006).

Research Objectives

The overall objective of this study was to identify factors promoting the development of an optimized synseed encapsulation protocol for tomato. Specifically: 1) compatibility of tomato synseeds with *ex vitro* planting environments was examined using hollow bead synseeds planted in a greenhouse hydroponic system, 2) physiological responses of encapsulated tomato synseeds to the encapsulation environment was examined with gene expression profiling, and 3) evaluation of the

effects of hollow and solid bead encapsulation methods, nutrient inclusion and storage time on survival and conversion of tomato synseeds after planting *in vitro*.

MATERIALS AND METHODS

Source of Explants for Alginate Encapsulation

Two F1 hybrid cultivars of *Solanum lycopersicum* L. were chosen for use in this study on the basis of maturity time of early (<60 days) or late (>70 days)) and flowering type (indeterminate and determinate). These cultivars were 'Big Beef' (late/indeterminate) and 'Sweet Olive' (early/determinate).

Explants were excised from *in vitro* grown seedlings of seeds obtained from Park Seed Company¹, unless otherwise noted. Seeds of each cultivar were surface disinfested by a 10 minute incubation in sodium hypochlorite and polyoxyethylene (20) sorbitan monolaurate (Tween 20) surfactant followed by three rinses with sterile deionized distilled water. Seeds were sown on tomato growth media composed of Murashige-Skoog (MS) media supplemented with Gamborg B5 vitamins and 3% maltose according to El Bakry (2000) in sealed 1.06 liter glass vessels (standard canning jars) (Murashige and Skoog, 1962; Gamborg et al., 1976). The seeds were incubated at 25±2°C with a 16-hour photoperiod provided by cool white fluorescent lights that provided a photon flux of approximately 40 $\mu\text{mol m}^{-2}\text{s}^{-1}$ (*in vitro* growth conditions). New seeds were sown for each experiment to provide a source of uniform propagules.

Alginate Encapsulation and Cold Storage of Tomato Synseeds

Nodes were excised from tomato seedlings two to four weeks after sowing. Segments were prepared by excising three to five millimeter segments of tomato stem surrounding a node. Hollow bead synseeds were produced by suspension of

¹ Park Seed Company, Greenwood, SC

propagules in a semi-liquid 1.5% carboxymethylcellulose² solution containing 1% calcium chloride (Patel et al., 2000). Propagules coated with the solution were then dropped via a 10 ml volume pipette with tips cut to have an opening diameter of one cm into 50 ml 0.8% sodium alginate solution. Solid bead synseeds were produced by suspension of propagules in 1.5% sodium alginate solution and dropped via a large 10 ml pipette with tips cut to have an opening diameter of one cm into 50 ml 1% calcium chloride. After a 10-minute gellation period, both solid and hollow bead synseeds were washed with sterile distilled deionized water and hardened for 20 minutes in 50 ml 1% calcium chloride. Synseeds were washed again in sterile distilled deionized water and then placed in 100 x 15 mm plastic Petri dishes³ sealed with Parafilm⁴. The sealed Petri dishes containing synseeds were stored at 4±1°C in darkness (storage conditions) for 0, 15, 30 or 90 days depending upon experimental use. For *in vitro* studies, synseeds were reintroduced to *in vitro* growth conditions (previously described in *Source of Explants for Alginate Encapsulation*)

Direct Planting of Synseeds in a Hydroponic System

Hollow bead tomato synseeds were tested in a hydroponic system to evaluate direct planting in an *ex vitro* growth environment. Synseeds were sown directly into a greenhouse environment in an ebb-and-flow hydroponic system. The ebb-and-flow system consisted of a 105 x 185 x 5 cm black plastic tray with a corrugated base for drainage. The hydroponic solution containing normal strength (1x) Peters Professional[®]

² Sigma-Aldrich, St. Louis, MO

³ Fisherbrand Disposable Sterilized, Manufactured by Fisher Scientific

⁴ Manufactured by Pechiney Plastic Packaging, Inc., Chicago, IL

Hydro-Sol^{®5} water soluble fertilizer (5-11-26) was introduced via an electric pump from a 150 liter plastic storage container reservoir through a tube fitting inserted into the base of the tray and drained through a similar fitting at the opposite end. Synseeds were sown on impressions (Figure 1a) in inert medium-grade vermiculite made using a small wooden dowel in 35 x 66 cm Speedling^{®6} foam trays with 128 cells in a randomized block design (Figure 1b). The system was covered with 30% opacity shade cloth to reduce light intensity. Three treatments were tested in a 3x3x3x2x2 factorial combination of auxin concentration (0, 2.5 and 5 μ M IBA) fungicide concentration (0, 50 and 100 μ M Daconil 2787⁷), storage time (0, 30 and 90 days), cultivar ('Big Beef' and 'Sun Olive') and light treatment (direct planting from storage (previously described) or one week in *in vitro* growth conditions (previously described)) to determine effects on survival and conversion rates of directly sown synseeds in a greenhouse hydroponic system. Data were collected on survival and conversion of each propagule 15 days after introduction to culture.

Expression Profiling of Tomato Synseeds

Total RNA Isolation from Propagules

This experiment was conducted concurrently with the *ex vitro* planting experiment. Hollow-bead encapsulated tomato synseeds of 'Big Beef' were stored (previously described in *Alginate Encapsulation and Cold Storage of Tomato Synseeds*) for one, 30 and 90 days at 4°C, and gene expression was measured at each time. Total

⁵ The Scotts Company, Marysville, Ohio

⁶ Speedling Incorporated, Sun City, FL

⁷ GardenTech, Lexington, Ky

Figure 1 Ex vitro planting of tomato synseeds: a) synseed placement in the planting cell b) arrangement of treatments in the hydroponic system.

RNA was extracted by the guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). One gram samples of each treatment was ground in liquid nitrogen in a mortar and pestle, followed by addition of 1ml of RNA extraction buffer (commercially available as TRIzol Reagent⁸). Samples were ground until macerated and the extraction buffer was thawed, at which point they were decanted into microcentrifuge tubes (Portillo et al., 2006). Samples were incubated at room temperature for 5 minutes, homogenized via vortexing in two 15 second intervals and centrifuged at 12,000xg at 4°C for 10 minutes. The supernatant containing total RNA was transferred to a new tube and the pellet discarded. The addition of 300 µl of chloroform to each tube was followed by a 15 second vortexing and 3 minute room temperature incubation. The tubes then were centrifuged at 10,000xg at 4°C for 15 minutes.

The aqueous phase was pipetted from each tube and placed in new screw-cap tube and the lower phase and interphase discarded. Isopropanol at half the volume of the aqueous phase was added, along with a half-volume of 0.8M sodium citrate/1.2M NaCl buffer solution (Portillo et al., 2006). Samples were mixed by gentle inversion and incubated for 10 minutes at room temperature to allow for RNA precipitation. Samples then were centrifuged at 10,000xg at 4°C for 10 minutes. The supernatant was discarded and the pellet washed with 700 µl of 75% ethanol by brief vortexing. The sample was centrifuged again at 10,000xg and the ethanol discarded. The pellet was dried under vacuum centrifugation for 10 minutes and re-suspended in 10-40 µl of Milli-Q⁹ water. Total RNA yield was assessed using the GeneQuant nucleic acid

⁸ Cat. No 15596-026, Invitrogen Corporation, Carlsbad, CA

⁹ Millipore Corporation, Billerica, MA

spectrometer. Total RNA quality was assessed by 1.2% formaldehyde denaturing agarose gel electrophoresis and visualized using 0.1 µg/ml ethidium bromide staining and a UV-transilluminator.

Fluorescent Labeling of Total RNA

Samples of total RNA were amplified and labeled with Cy3 and Cy5 fluorescent dyes from the Amersham CyeDye Post-Labeling Reactive Dye Pack¹⁰ via the protocol provided with the SuperScript Indirect cDNA Labeling Kit¹¹. Enough labeled cDNA was prepared for three biological replicates at each of the three time points. Following amplification and labeling, samples were stored at -80°C until hybridization to the TOM1 array. Samples of cDNA for the 1-month and 3-month stored propagules were labeled with Cy3 and samples of the propagules stored for 1-day were labeled with Cy5. Labeling efficiency for each sample was assessed using a Nano-Drop 1000¹² photometer.

Hybridizing Cy-Labeled cDNA Targets to the TOM1 Microarray

Expression profiling was performed on the samples using TOM1 microarray slides from the Center for Gene Expression Profiling at the Boyce Thompson Institute for Plant Research at Cornell University (<http://bti.cornell.edu.CGEP/CGEP.html>). Arrays were incubated in a pre-hybridization solution (5X SSC, 0.1% SDS and 1% BSA) at 43°C for 45 minutes. Following incubation, arrays were rinsed by dipping 10 times in Milli-Q[®] H₂O, another 10 dips in isopropyl alcohol, and finally 10 dips in Milli-Q[®] ultra-purified water. Slides were dried via centrifugation in Falcon[™] tubes at 1500 rpm for 30

¹⁰ RPN5661, GE Healthcare Biosciences AB, Uppsala, Sweden

¹¹ Cat. Number L1014-01, Invitrogen Corporation, Carlsbad, CA

¹² Thermo Fisher Scientific, Wilmington, DE

seconds. LifterSlips[™]¹³ cover slips also were rinsed by dipping in Milli-Q[®] ultra-purified water.

To prepare Cy-labeled cDNA targets for hybridization to the array, the Cy-cDNA labeling reactions were assessed first via the procedure outlined in TIGR's SOP #M004 (http://pga.tigr.org/sop/M004_1a.pdf) and total pmol of synthesized cDNA and incorporated CyDye calculated. The labeling protocol indicated optimal reactions produce concentrations of >6000 pmols of cDNA and >200 pmol CyDye per 60 µl with a nucleotide/dye ratio of <30. Volumes of labeled cDNA equal to 14 pmol of CyDye were combined in a microcentrifuge tube and dried using a roto-evaporator. Samples were prepared by mixing labeled cDNA from the one day time point with each of the samples of the 30 and 90 day storage points.

The combined cDNA targets were re-suspended in 75 µl of Livesey's hybridization solution (50 µl de-ionized formamide, 25 µl 20X SSC, 10 µl 50 Denhard's¹⁴ solution, 5 µl 10% SDS, 2.5 µl 0.2M monobasic K-phosphate and 7.5 µl sterile Milli-Q[®] water. The re-suspended targets were incubated at 95°C for 5 minutes and then centrifuged at maximum speed for one minute. "Blocked" microarrays were placed in Corning hybridization chambers¹⁵ and 63 µl of re-suspended cDNA targets were pipette onto the array surface. The loaded array was covered with a clean, dry LifterSlip[™] and then incubated in a 43°C water bath for 16 hours in the dark.

The arrays were dipped in a foil-covered Coplin¹⁶ staining jar containing a solution of 1X SSC and 0.2% SDS pre-heated to 43°C so that the LifterSlip[™] to allow

¹³ Cat. No 22X50I-2-4711, Erie Scientific Company, Portsmouth, NH

¹⁴ Cat. No 100512-266, VWR, West Chester, PA

¹⁵ Cat #2551, Corning Costar, Lowell, MA

¹⁶ Cat. No 25457-200, WVR, West Chester, PA

removal of the cover slip. The array slide was moved to another covered jar filled with the same pre-heated solution and incubated for 10 minutes and then washed in a covered jar of 0.1X SSC and 0.2% SDS at room temperature and with gentle agitation. The arrays were washed a final time in 0.1X SSC buffer and dried via centrifugation in a slide centrifuge.

Scanning and Interpreting Arrays

Arrays were scanned immediately after drying using a ScanArray Lite¹⁷ confocal microarray scanner and the associated ScanArray Express software. Following laser focusing and balancing the two channels, 10 µm scans were conducted with laser power at 90% of the maximum and photomultiplier tube settings at 60-85% of maximum. The Cy3 and Cy5 fluors were excited at 543/570 nm and 633/670 nm respectively, and emissions were captured in a digital image. Images were analyzed using ScanArray¹⁸.

Planting of Synseeds In Vitro

Shoot apical meristems were excised from seedlings sown on tomato growth media (previously described in *Source of Explants for Alginate Encapsulation*) and grown in *in vitro* conditions (previously described in *Source of Explants for Alginate Encapsulation*) for two weeks. Synseeds were produced using the hollow bead method introduced by Patel et al. (2000) or by the solid bead method outlined by West et al. (2006). Synseeds were stored for 0, 15 and 30 days (previously described in *Alginate Encapsulation and Cold Storage of Tomato Synseeds*). Following storage, synseeds

¹⁷ PerkinElmer, Wellesley, MA

¹⁸ MB Eisen, <http://rana.lbl.gov/EisenSoftware.htm>

were sown on tomato growth media (previously described) and incubated in *in vitro* growth conditions (previously described in *Source of Explants for Alginate Encapsulation*). The treatments were assessed in a 2x2x3 factorial design of encapsulation method, incorporation of nutrients, and storage time to determine the effects on reintroduction to *in vitro* culture conditions. Data was collected on survival and conversion of each propagule 15 days after introduction to culture.

Data Collection and Statistical Analysis

All growth experiments were arranged as completely random designs and conducted twice. A minimum of 5 replications were used in each run of every experiment. All data were analyzed using the General Linear Model (GLM) of SAS¹⁹. Survival was based on the lack of discoloration and necrosis in explants and conversion was based on the visible elongation of the axillary bud.

¹⁹ SAS Institute, Inc

RESULTS AND DISCUSSION

Direct Planting of Synseeds in a Hydroponic System

Synseeds removed from cold storage appeared undamaged and viable directly after removal. Synseeds receiving a one-week light pre-treatment exhibited a dark discoloration and a hyperhydric state at the end of the treatment (Figure 2), while those planted directly from cold storage appeared viable at planting. There was no survival of tomato explants across all synseed treatments two weeks post-planting *ex vitro*. Inspection of the synseeds two weeks post planting revealed: 1) no contamination on any of the synseeds, including those not treated with fungicide, 2) reduced volume of synseeds, despite incubation in a hydroponic environment and 3) necrosis of all propagules within all synseeds (Figure 3). The complete mortality of all synseed treatments suggested that tomato was incompatible with the encapsulation environment.

Multiple factors are possible contributors to the failure of the synseeds to germinate. The inability of tomato to survive in encapsulated, stored form is the most significant constraint. There are no reports of vegetative tomato propagules in synseed production, though Garret (1991) demonstrated encapsulation of tomato seeds. While other species in *Solanum* have been successfully encapsulated (Huda et al., 2007; Nyende et al., 2003; Patel et al., 2000), tomato appears to be unsuitable for synseed production. Adriani et al. (2000) found encapsulation to be detrimental to propagules of kiwifruit and reported low survival of encapsulated microcuttings. However, cold treatment of the explant source prior to excision improved survival and vigor (Adriani et

Figure 2 Tomato synseeds after 0, 30 and 90 days of storage following a one week light treatment.

Figure 3 Tomato propagules from synseeds after 2 weeks in a greenhouse hydroponic system.

al., 2000). Implementation of a cold-treatment prior to excision of tomato propagules for encapsulation may similarly improve survival and vigor.

The capability of synseeds to be planted *ex vitro* has received limited attention. Utomo et al. (2008) proposed the use of solid synseeds as a possible tool for introduction of smooth cordgrass along coastlines for erosion, wave and sediment protection, but evidence for the use of synseeds in a direct-to-soil planting test was not reported. Nyende et al. (2003) also promoted the use of solid synseeds for direct introduction of potato to soil. The synseeds successfully germinated, but synseeds were cultured *in vitro* on growth media for two weeks before planting. In contrast, Preece and West (2006) were successfully in sowed *Hibiscus moscheutos* directly into a greenhouse hydroponic system, both with and without light pretreatment.

Expression Profiling of Tomato Synseed

Extraction of total RNA from the synseeds of 'Big Beef' stored for 0, 30 and 90 days resulted in samples of total RNA ranging from 1.5 to 6.54 µg (Table 1). Contamination from DNA or protein was absent, so the lower purity rates of some samples was attributed to residual sodium salts in the isopropanol precipitation step. Sodium salts reduce polysaccharides in the sample (Portillo et al., 2006) and can be difficult to remove, despite an extra ethanol wash step. Denaturing gel electrophoresis of the total RNA samples showed characteristic smears in samples A and B from the 0 day storage period and sample B from the 30 day storage (Figure 4).

Dye incorporation of cDNA samples was low in samples where RNA was absent on the electrophoresis gel (Table 2). Both samples A and B from the 0 day samples exhibited incorporation above 40 pmol of dye and sample B from the 30 day time point

Table 1 Total RNA yield and purity from tomato synseeds

Sample		RNA (μ g)	Purity (%)
0 days	A	6.54	93
	B	3.72	60
	C	2.20	93
30 days	A	4.35	90
	B	3.00	70
	C	4.65	87
90 days	A	4.80	74
	B	1.50	57
	C	4.20	87

Figure 4 RNA gel electrophoresis of propagules from tomato synseeds

Table 2 cDNA yield and CyDye incorporation of tomato synthetic seed samples.

Sample		Dye	Total cDNA (pmol)	Total Dye (pmol)	cDNA/dye
0 Days	A	Cy5	1146.6	53.9	21.3
	B	Cy5	280.8	58.8	4.8
	C	Cy5	180.8	0	-
30 days	A	Cy3	226.4	19.6	11.6
	B	Cy3	588.5	34.3	17.2
	C	Cy3	180.8	0	-
90 days	A	Cy3	166.1	4.9	33.9
	B	Cy3	709.52	4.9	144.8
	C	Cy3	196.294	14.7	13.35

had incorporated dye above 30 pmol. Samples exhibiting suboptimal labeling were relabeled using the Cy3 and Cy5 dyes, but no additional dye incorporation occurred.

Two samples from each storage period (A and B from 0 and 30 days; B and C from 90 days) were used in the TOM1 hybridization experiment. Pairings were made with 0 day samples (Cy5 labeled) and one of the other storage period samples (Cy3 labeled) (Table 3). The arrays were scanned immediately following hybridization (Figure 5). Only two slides, three and six (Figure 6), exhibited hybridized cDNA. In both cases, the cDNA was from the Cy5 labeled samples from the 0 day time point. The absence of hybridization of any of the 30 and 90 day samples suggested that degradation of RNA had occurred during encapsulated storage, showing reduced biological activity after 30 days in storage and suggesting that encapsulated nodal segments of tomato are not compatible with encapsulated cold storage techniques. Low yields of RNA were likely attributed to a combination of labile molecules and tomato tissue necrosis.

Planting of Synseeds In Vitro

Synseeds were sown 0, 15 and 30 days after encapsulation because synseeds stored longer than 30 days in the expression profiling experiment exhibited low biological activity. Encapsulation method, nutrient inclusion and storage period significantly affected synseed survival after 15 days of reintroduction into *in vitro* culture (Appendix Table 1). The interactions between nutrient inclusion/storage time and encapsulation method/storage time also significantly affected synseed survival (Appendix Table 1). Nutrient inclusion and storage period also significantly affected synseed conversion (Appendix Table 2).

Table 3 Microarray slide assignment of tomato synseed samples

Slide	Cy3 sample	Cy5 sample
1	90d C	0d A
2	90d B	0d B
3	30d B	0d A
4	90d B	0d A
5	30d A	0d B
6	30d B	0d B

Figure 5 Microarray slides of tomato synseed CyDye labeled total RNA.

Figure 6 Cy5 labeled tomato synseed cDNA from 0 days of storage hybridized to the TOM1 array.

Hollow bead synseeds had a significantly higher mean survival rate (75.0%) as compared to solid bead synseeds (61.7%). Patel et al. (2000) reports improved callus development of carrot in hollow beads as compared to solid beads and an 81 percent conversion rate in shoot tips of potato. Patel et al. (2000) suggest a possible toxic effect of the alginate matrix from high levels of calcium and sodium ions. The effect would be more pronounced when the propagule is in direct contact with the matrix, such as in solid bead encapsulation. The semi-liquid core of hollow bead synseeds reduces contact with the matrix and would reduce the effect of the toxicity of the matrix. Alginate beads demonstrate impermeability to gas exchange (Sakamoto et al., 1992). Inclusion of the semi-liquid core produces a thinner layer of alginate (Patel et al., 2000) and therefore allows a greater exchange of gasses between the interior of the synseed and the environment, which may increase survival in solid beads.

Synseeds with supplemental nutrients had a significantly higher mean survival rate (86.7%) as compared to synseeds without supplemental nutrients (48.3%). Synseeds with supplemental nutrients also had a significantly higher mean conversion rate (50.0%) as compared to synseeds without supplemental nutrients (31.7%). Seedlings of germinated synseeds over all storage treatments were less chlorotic with supplemental nutrients than without. Ozden-Tokatli et al. (2008), Huda et al., (2007), Saiprasad and Polisetty (2003), and Gardi et al. (1999) collectively found that solid synseeds containing an “artificial endosperm” of species specific inorganic and organic nutrients, including carbon source, have higher survival and conversion rates than synseeds without. Encapsulated explants require supplemental nutrients because of continued respiration and reduced photosynthesis during storage (Gardi et al, 1999),

therefore supplementation increases survival and vigor of plants during storage. Incorporation of MS salts in the encapsulation material of solid beads improves conversion time of synseeds by as much as 6.2 days to formation of the first leaf and 10.2 days to the formation of the first roots in orchid pseudobulbs (Saiprasad and Polisetty, 2003). Prior to this study, inclusion of nutrients has not been utilized in hollow bead encapsulation evaluations, however, this method is still in the early stages of development (Patel et al., 2000).

Survival of synseeds stored for 0, 15 and 30 days declined perceptively between 0 and 15 days, but did not change significantly from 15 to 30 days storage (Table 4). A similar trend was observed for conversion rates, with more than a seven-fold difference between 0 and fifteen days storage. Poor conversion rates for tomato synseeds stored for 15 and 30 days suggested that tomato propagules were unable to tolerate applied encapsulation and storage regimens. Singh et al. (2006) and Saiprasad and Polisetty (2003), report a reduction of survival rates as storage time increases, suggesting that conditions during storage reduce viability and vigor of encapsulated propagules.

One possible explanation of the incompatibility of tomato with synseed storage is the high cold sensitivity of tomato plants (Zhang et al., 2004). Compensation for cold sensitivity through development of cold-tolerant tomatoes (Zhang et al., 2004) of tomato could further development of tomato synseed technology. Development of synseed techniques that do not rely on cold storage of encapsulated propagules, such as dehydration and cryopreservation (Kim et al., 2006; Fabre and Dereuddre, 1990), may also provide a viable synseed protocol for tomato. The toxic effect of the alginate matrix proposed by Patel et al., (2000) and the gas impermeability proposed by Sakamoto

Table 4 Effect of storage time on survival and conversion of tomato synseeds.

Storage Time	n	Mean Survival (%)²	Mean Conversion(%)²
0 days	40	92.5 a	92.5 a
15 days	40	57.5 b	12.5 b
30 days	40	55.0 b	15.0 b
Significance ¹		**	**

¹ ** Significant at the 1% level according to the F-test

² T-test for paired comparisons.

et al. (1992) also could be potential sources of incompatibility to encapsulated storage, since encapsulation method is a significant factor in survival. Implementation of new materials in synseed production may improve overall survival and vigor of propagules and improve the range of compatible species and applications.

CONCLUSIONS AND SUGGESTIONS

Worldwide, nearly 600 articles document the practice of storage of non-embryogenic seeds for purposes of research and germplasm conservation. However, scientists lack consensus on the basics of encapsulation. Synseed structure and composition must be evaluated to establish guidelines for optimal encapsulation. This study, while narrow in scope, showed that synseed bead nutrient composition and solid or hollow structure were important factors in the survival and growth of encapsulated tomato propagules. Survival of synseeds produced using the solid bead method was 29% lower than that of synseeds produced using the hollow bead method. While the growth of the surviving solid synseeds was high enough to offset this mortality rate, storage conditions must be considered suboptimal

This study confirms that incorporation of inorganic and organic nutrient sources in synseeds is necessary to aid in the survival and vigor of stored propagules. Survival and conversion of unstored synseeds was improved with nutrient supplements. Storage of encapsulated tomato propagules failed to produce viable plants. High mortality and low conversion of tomato synseeds stored for 15 and 30 days demonstrated a strong incompatibility with these environments. Similarly, the low survivability of conversion *ex vitro* of the tomato hollow bead synseeds also indicated negative sensitivity to encapsulation.

An attempt was made to measure genes expressed in stored and unstored synseed propagules by microarray analysis, but hybridization failed to occur using probes from stored samples. Lack of signal was attributed to RNA degradation from tissue deterioration and reduction of biological activity during storage.

This study makes four original contributions to the field of hollow bead synseed research. First, tomato vegetative propagules were introduced into hollow and solid synseed environments. Second, use of vegetative propagules of tomato for synseed storage is unsuitable with current protocols. Third, hollow bead encapsulation was shown to be a superior encapsulation method compared to solid bead encapsulation for survival of vegetative propagules. Last, gene expression profiling provided direct evidence that synseeds lost viability after 30 days in storage.

Further research and evaluation needs to be done to study the physiological effects of encapsulated storage on synseeds. Further research should concentrate on:

- 1) Evaluation of the effects of temperature on stored tomato synseeds by analyzing survival and conversion after storage at multiple temperatures, and
- 2) Gene expressional analysis of tomato synseeds stored for brief periods (< 2 weeks) to evaluate specific expressional responses to storage

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APPENDIX

Appendix Table 1 Analysis of variance for the effects of encapsulation method (solid or hollow bead), nutrient inclusion and storage time (0, 15 and 30 days) on survival of tomato synthetic seeds two weeks after in vitro planting.

Source of Variation	df	Mean Square	F Value
Run	1	0.03333333	0.26
Rep	4	0.17916667	1.41
Encapsulation (Encap)	1	0.53333333	4.21 *
Nutrients (Nutr)	1	4.03333333	31.83 **
Storage Time (ST)	2	1.75833333	13.88 **
Encap*Nutr	1	0.13333333	1.05
Encap*ST	2	0.90833333	7.17 **
Nutr*ST	2	0.80833333	6.38 **
Encap*Nutr*ST	2	0.25833333	2.04
Error	103	0.12669903	

¹ *, ** Significant at the 5% and 1% levels, respectively, according to the F-test

Appendix Table 2 Analysis of variance for the effects of encapsulation type (hollow or solid bead), nutrient inclusion and storage time (0, 15 or 30 days) on conversion of tomato synseeds two weeks after in vitro planting.

Source of Variation	df	Mean Square	F Value
Run	1	0.30000000	3.04
Rep	4	0.03333333	0.34
Encapsulation (Encap)	1	0.13333333	1.35
Nutrients (Nutr)	1	0.83333333	8.44 **
Storage Time (ST)	2	8.27500000	83.84 **
Encap*Nutr	1	0.03333333	0.34
Encap*ST	2	0.10833333	1.10
Nutr*ST	2	0.15833333	1.60
Encap*Nutr*ST	2	0.05833333	0.59
Error	103	0.09870550	

¹ ** - Significant at the 1% level, according to the F-test

VITA

John E. Porter

Education

May 2004 *Bachelors of Science Degree*
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August 2008 *Masters of Science Degree*
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Work Experience

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