SYMMETRIC PROTOPLAST FUSION IN INTERSERIAL *SYRINGA* (OLEACEAE) HYBRIDIZATION

A Thesis
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

Nathan Allen Maren

In Partial Fulfillment of the Requirements
for the Degree of
MASTER OF SCIENCE

Major Program:
Horticulture and Forestry

June 2016

Fargo, North Dakota

North Dakota State University
Graduate School

Title

Symmetric Protoplast Fusion in Interserial *Syringa* (Oleaceae) Hybridization

By

Nathan Allen Maren

The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

SUPERVISORY COMMITTEE:

Dr. Todd P. West
Chair

Dr. Wenhao Dai

Dr. Phillip McClean

Dr. Tao Wang

Approved:

6/20/16
Date

Harlene Hatterman-Valenti
Department Chair

ABSTRACT

Few other woody plants embody the preeminence of temperate woody plants in garden cultivation like the lilacs. In spite of their relationship, the trees lack the diversity of cultivated floral forms observed within the shrub lineages. Typical selection and cross-pollination schemes within the tree lilacs or between trees and shrubs have failed to yield the diversity of colors and fragrances on a tree form. With somatic fusion in *Citrus* spp. as a guideline for *Syringa* spp. protoplast isolation and culture, experiments were designed to optimize the conditions through somatic fusion. Protoplast isolation experiments revealed yield increases with increased exposure to cell wall degrading enzymes as well as losses in viability with increased exposure. Electrofusion experiments yielded somatic hybrids, yet further investigation is necessary to optimize the fusion electroporation settings and beyond.

ACKNOWLEDGEMENTS

The body of this work could not have been done without the help of multitudes of people. The genesis of this project is owed to the inspiration of Vernon Black former Vice President of Production at Bailey Inc. Vern's personal interest in the breeding projects encouraged my interest and fueled my desire to learn and be a better plantsman. Dr. Todd West's willingness to champion the project and mentorship in scientific principles were great examples of his dedication to the horticultural industry and the development of independent thinking. Todd always encouraged examination of what is known and where the gap in knowledge lies. Learning about the intricacies of tissue culture were made possible thanks to the guidance of Juan Ramon Franco Coronado. Dr. Deborah McCown and Dr. Brent McCown provided the means to conduct all of the supporting micropropagation work beyond the basic principles. The body of work would have been entirely unmanageable without the support from Victoria Magnussen, Rian Lee and Justin Hegstad. Be it words of encouragement or advice on the mundane tasks of plant tissue culture, Vickie was a bastion in the conduct of in vitro and biotechnological aspects of the projects. Rian was incredibly supportive in all aspects of the genetics and molecular marker work; going the extra mile to help me understand the processes and concepts underlying the work. Justin and Rian would regularly support my greater understanding of the world of science and prompt me to reach beyond the immediate community of plant science. The assistance of NDSUs Plant Sciences Department and the training provided by Dr. Jude Grosser and the staff of the Citrus Research and Extension Center (CREC) in protoplast isolation and somatic fusion permitted the technical skill development required by such a project. Joseph Rothluetner from the Morton Arboretum provided the knowledge base necessitated by the application of flow cytometric analysis.

DEDICATION

This work is dedicated to the people who push me to be better than I was, am or will be. I would like to pay special tribute to my grandfather Ronald Wynn Coy. He is the rock of our family, a pillar of his community, and the most loving man. In his honor I push myself to live right and make choices that I won't regret. In all aspects of my life I endeavor to conduct myself with the utmost integrity as he had. By his ethic I devote myself to the betterment of my family and the communities that I can call home.

My dear friend and mentor Dr. David Zlesak has devoted his life to plant science and gardening. With David's guidance and mentoring each stride to the next level of academic rigor was met with appreciation for the learning process and gratitude for the foundational work that permit continued advancement. The pursuits of mankind are accomplished because we are the benefactors of our ancestor's achievements. I strive to make worthy contributions that will add to the scientific consensus and furthermore dedicate myself such that my descendants can do the same.

The people that push me the most are too small to understand their impact. My daughters make me strive to be a better person in every way and parenthood has been the litmus test of my foundations. Each day I have a new opportunity to reflect, recapitulate or construct in them the constitution of being a good person. As a child of a single mother, I am grateful to have an amazing partner to help navigate those situations. Kelsey brings out the best in our family and I am a lucky man to be able to share this life with her.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AC	Activated Charcoal.
BA	6-Benzylaminopurine
MS	Nutritive medium composition as described by Murashige and Skoog 1962
NAA	Naphthalene acetic acid
PPM™	Plant Preservative Mixture is a proprietary preservative/biocide affecting key enzymes in the Krebs cycle and the electron transport chain of prokaryotic organisms and some forms of fungal contamination (Plant Cell Technology, 1823 Jefferson Place NW, Washington D.C., 20036, USA)
SWPM	A woody plant media (WPM) based basal salt formulation modified for the micropropagation of *Syringa spp*. (Appendix Table A-1).
TDZ	Thidiazuron.
WPM	Woody Plant Medium as described by Lloyd and McCown (1981)
ZEA	Trans-zeatin

LIST OF DEFINITIONS

Channel .....the final composition of a parameter that represent the amplified signal information collected on an event (Flow cytometry)

Events .....a unit of data about which measures of the fluorescence signal intensity or light scatter for each channel are measured. (Flow cytometry)

Competence.....the ability of an explant to respond to changes in the cultural conditions such as hormone signaling, for the directed formation of roots, shoots, or embryos

Totipotency .....the ability of a single cell to develop into the differentiated tissues of a typical functional organ or organism

Parameter .....the measures of fluorescent light intensity and/or scatter generated when an object passes through a laser

LIST OF APPENDIX TABLES

CHAPTER 1. INTRODUCTION

The genus of lilacs (*Syringa*) is comprised of many temperate garden plants made popular over the long history of gardening. Their diversity in flower form, color and fragrance make them a sight to behold in the early spring through mid-summer. The botanical name *Syringa* comes from the Greek word syrinx meaning hallow stem. The most common name for *Syringa*, however, likely derives its common name from the Persian word *lilak* or *lilaf*, which means blue. However, the tree form lilacs lack a blue floral color in any cultivated variety. Nevertheless, the tree lilacs have been an economically important ornamental tree since the description of Peking lilac (*Syringa pekinensis* Rupr.) in 1742 by Pierre d' Incarville and represents the first recorded lilac collection for garden use in Europe. The floral characteristics of the tree lilacs have placed them morphologically close to the privets (*Ligustrum* spp.) and have such led to differences in opinion regarding their taxonomic position. The similarity of the floral morphology between *Ligustrum spp*. and the *Syringa* series *Ligustrina* explains the taxonomic series naming of the *Ligustrina*. The dehiscent scimitar shaped capsules of the *Ligustrina* distinguish the *Syringa* and as such place them outside of the *Ligustrum* genera, which has a non-dehiscent berry-like drupe (Dirr, 1998). The *Ligustrina* are categorized as trees, but depending on the species may be found to grow with habit characteristics of a large shrub. *Syringa pekinensis* represents the smaller of the two species reaching a mature height ranging 2-5(-10) m, while the range spans 4-10(-15) m with *S. reticulata* (Blume) H. Hara (Fiala and Vrgutman, 2008). In addition to the two to three-week mid-summer floral display the more attractive features of the tree lilacs include a beautiful cherry-like, sometimes, exfoliating bark. The bark can range in color from an orange to red brown or brown and mature from a smooth to peeling texture developing into scaly brown to gray (Dirr, 1998; Fiala, 1998).

Tree Lilac Origins and Cultivation

Originating from the south central parts of Manchuria (southwestern Inner Mongolia) to the Japanese island of Hokkaido the tree lilacs have evolved under a myriad of conditions, which make them particularly well suited for their cultivation in a wide array of conditions. Their adaptability and beauty in floral display have made them an appealing source of mid-summer blooming ornamental trees. Tree lilacs thrive when the environment has cooler summers and freezing winters (Dirr, 1998). The ability to reliably set abundant flowers and fruit is consistent with an evolutionary history in temperate climates, requiring a vernalization period (Fiala and Vrgutman, 2008). The tree lilacs have few insect problems that plague their cultivation. Lilac borers and scale insects are the most common insect problems, while diseases such as bacterial blight, *Phytophthora* and powdery mildew plague landscape level trees under stressful conditions (Dirr, 1998). Their adaptability to a wide variety of landscape level sites make them suitable for mass plantings, specimen trees and are even tolerant of saline conditions, which make them well suited as a boulevard tree.

The taxonomic placement of the tree lilacs within the genus *Syringa* has been debated since western botanists first encountered them in the orient (Fiala and Vrgutman, 1998). Today the placement of the tree lilacs is recognized as paraphyletic to the genus and according to Li et al. (2012) represent one of six sub-classifications, called series, within genus *Syringa*. Parsimonious tree construction from maximum parsimony and Bayesian inference analysis place the series: *Pinnatifoliae, Ligustrae*, *Ligustrina*, *Villosae,* and *Pubescentes* divergent from within series *Syringa* 17.67 million years ago (mya) (Li et al., 2012). The taxonomic relationship of the tree lilacs places the series *Ligustrina* divergent from *Ligustrae* (*Ligustrum spp*.), approximately 11.39 mya, and series *Villosae* thereafter at 9.65 mya during the warming in the middle Miocene

(Li et al., 2012). The divergence of these groups meant the adaptive capacity for the shrubs to produce beautiful pinks, lilac, and violet to blue colored flowers or the loss of that ability in the trees. The more recent evolution and dissemination of the *Ligustrina* throughout the world, are owed to the movement of seeds from several individual collection trips throughout China from 1742 to present (Fiala and Vrgutman, 2008). Today seed propagation is still one of the most common forms of plant proliferation for the species of tree lilacs.

Commercialization and Production

The widespread popularity of the genus has a market demand that supports several nurseries throughout the world that devote a majority of their crop production capacity to the group. At some nurseries in the US, tree lilacs are the single most important production crops as compared to any other product category in cultivation. Cultivated varieties, hereafter cultivar, of the trees lilacs are often a challenging species to produce clonally from vegetative or hardwood shoot cuttings. Most frequently the trees are propagated by grafting or in vitro micropropagation. These successful cultivation of tree lilac through these techniques are very skill dependent and labor intensive. The skilled practitioner in the nursery trade must seed propagate trees in orders of magnitude in excess of the actual annual needs to yield a continual source of useful seedling rootstocks. The rootstock onto which a scion is grafted, or as often the case with *Syringa reticulata* budded, needs to have the vigor and stem diameter that will best suit the nurseryman's needs. The care and culture of landscape ready trees of three plus feet can take between three and five years to cultivate. Often the number one limiting factor to the economical production of tree lilacs is the cost of propagation. Alternative propagation strategies, such as in vitro propagation have also been challenging for producers and researchers alike (G. Suttle, personal communication). To date, the publications on the in vitro clonal propagation of lilac do not

include any form of tree lilac. The publications that do work with the tree lilacs often source their explant material from seeds or seedlings. The nurseries who produce them are typically unwilling to share their experience with public entities.

Improvement of Ligustrina

Lilacs, particularly the shrubs, have been cultivated for their fragrant and showy flowers, but in spite of their relationship, the trees do not share the diversity of cultivated forms. Selection and hybridization schemes in the tree lilacs have successfully introduced cultivars with variations of foliage color and variegation, flower panicle size or floret density, bark color or textural difference, as well as changes in mature habit. However, these strategies have not been able to provide the marketplace with cultivars of diverse flower color or fragrance. Though often described as having a creamy white to yellow flower color, the floral display may confound the appearance of color as anthers are formed on elongated filaments that extend well beyond the corolla. As of yet a solitary variation in flower color has been identified in a single plant of undescribed origin out of Beijing Botanical Gardens known in the commercial trade as *Syringa pekinensis* ‘Zhang Zhiming’ Beijing Gold™ (Chicagoland Grows®, Inc., 2012). Any improvement in floral color or fragrance characteristics in the tree lilacs would diversify the color palette of mid-summer blooming ornamental trees.

In the development of improved plant characteristics, traditional plant breeding approaches with controlled crossing and recurrent selection can be challenging with long lived perennial species. Juvenility period, the maturity period from the seedling stage to mature flowering stage of development, can be long in woody perennials. The juvenile period can vary from three months with some rosaceous species and hybrids or up to several decades as with some magnolias. The juvenile period of *S. reticulata* may take anywhere from three to six years

depending on several factors including but not limited to the growing conditions and provenance of the original propagule. These challenges are compounded by the heterozygosity within landrace selections and the lack of trait fixation found in commercially available cultivars. These challenges make the plant improvement process of trees and other woody ornamentals protracted in outcome and in terms of the human lifespan may require intergenerational efforts to complete a particular goal. As such scouting landraces and naturalized stands for standing variation is the more common approach to tree improvement.

In efforts to diversify the floral habits of the tree lilacs, attempts to hybridize the various taxonomic series has led to a long history of failures (Kim & Jansen, 1998; Fiala and Vrugtman, 2008; J. Alexander III, personal communication). The true nature of the sexual incompatibilities between lilac series are not entirely known. As described above, the tree lilacs are paraphyletic within subgenus *Syringa* and having evolved most recently from the series of *Villosae* 9.65 million years ago (Li, 2012). This evolutionary history has formed the approach to look at more recent divergence for probable compatibility in the development of interseries hybrids. Conversely, early divergence of these different series may be appealing to increase the germplasm background for future developments.

Previous attempts at interserial hybridization between tree and shrub lilacs by Nathan Maren and Dr. David Zlesak of the University of Wisconsin-River Falls resulted in the appearance of a fertilized zygote. Failure to completely develop a functional endosperm suggest delayed incompatibility resulting in the abortion of seed before complete maturation. An approach commonly applied to vegetatively propagated crops, would be to induce auto-tetraploidy in both species of the hybrid cross and attempt further crossing. The mating of two autopolyploids can yield plants that maintain typical bivalent pairing at meiosis, where gametes

form double the typical haploid chromosome number and result in the production of fertile offspring. This phenomenon is often exploited in the production of sterile plants such as bananas, watermelon, and citrus. However, this is a long-term process as the recovery of true autopolyploids and rearing those plants to a flowering stage will likely take years before either parents flower.

In vitro techniques in plant improvement may be a great approach to forging interspecific, or interserial, hybrids. Somatic hybridization is one such in vitro technique in plant improvement that has demonstrated the ability to overcome the barriers of sexual incompatibility across species and familial divides. Somatic hybridization or protoplast fusion is a procedure in hybridization whereby somatic cells of one genotype isolated from their cell walls (protoplasts) are fused with protoplasts of another genotype. The resulting hybrids offer the opportunity to introduce characteristics of the cytoplasm from both parents that may otherwise be excluded in typical sexual fertilization. In spite of somatic hybridization being a valuable tool in bringing together the heritable characteristics of distantly related species, the technique has yet to reach its full potential in the plant sciences (Grosser et al. 2005). Supporting the use of this technique in a hereto unexplored genus provides an opportunity to study the mechanisms and conditions that affect the culturing of plant protoplasts. This research will provide benchmark studies into the research and eventual introduction of a series tree form lilac with a diverse color and aroma palate.

References

Chicagoland Grows®, Inc. 2012. Plant Introduction Program - Plant Release Bulletin #34: Zhang Zhiming Peking Lilac – Beijing Gold™. Retrieved January 17, 2015, from http://www.chicagolandgrows.org/downloads/beijing_gold.pdf

Dirr, M.A. 1998. Manual of woody landscape plants. 5th ed. Stipes Publ., Champaign, IL.

Fiala, J.L. and F. Vrugtman. 2008. Lilacs: A gardener's encyclopedia. 2nd ed. Timber Press, Portland, OR.

Grosser, J.W. and F.G. Gmitter, Jr. 2005. 2004 SIVB proceedings: Thinking outside the cell: Applications of somatic hybridization and cybridization in crop improvement, with Citrus as a model. In Vitro Cell. Dev. Biol. Plant 41:220–225.

Kim, K.J. and R.K. Jansen. 1998. A chloroplast DNA phylogeny of lilacs (Syringa, Oleaceae): Plastome groups show a strong correlation with crossing groups. Am. J. Bot. 85(9):1338-1351.

Li, J., B. Goldman-Huertas, J. DeYoung, and J. Alexander III. 2012. Phylogenetics and diversification of *Syringa* inferred from nuclear and plastid DNA sequences. Castanea 77(1):82-88.

CHAPTER 2. IN VITRO CULTURE AND REGENERATION

Abstract

The in vitro establishment of lilac (*Syringa* spp.) plant tissues for micropropagation or biotechnological purposes can be challenging. Several sources of lilac (*Syringa* spp.) explant tissues were used to establish an in vitro grown stock for experimentation. Experiments to regenerate whole plants directly from clonal somatic tissues via adventitious organogenesis directed the formulation of treatments in proceeding trials, but did not generate whole lilac plants. Concomitant replication of previous studies in somatic embryogenesis from zygotic embryos yielded somatic embryos with reproducible, albeit low, yields of regenerated tissues. Environmental manipulations such as temperature, hormone formulation, cytokinin: auxin ratio and hormone concentration, were targeted for the directed manipulation of somatic tissues to a regeneration competent condition. Internodal stem segments or leaf mesophyll tissues from in vitro grown plants were applied to treatment mediums with a modified woody plant medium basal salt formulation with factor level variation in thidiazuron (TDZ) (0, 1.4, and 4.0 μM) and naphthalene acetic acid (NAA) (0, 5, 10, 20 μM) for a competence inductive period of two weeks. Tissues were subsequently cultured onto a common somatic embryo inductive media. Somatic embryos did not form on any treatments following subculture.

Introduction

In vitro propagation of plant tissues and the establishment of aseptic cultures has a dramatic impact on plant physiology. The establishment phase of micropropagation is characterized by an acclimatization period that results in drastic shifts in morpho-physiological arrangements including how plant mineral nutrition is acquired and translocated as well as a shift from autotrophy to heterotrophy (George et al., 2008). This process is generally genotype and

physiological age dependent and may affect the duration of stage one establishment and stabilization. Stabilization of in vitro culture is marked by the serially similar response of sub-cultured explants on exposure to new media. The stabilization phase may be short or may require multiple strategies to overcome the natural barriers and stress responses (McCown, 2000).

Common challenging responses to the initiation of cultures includes the formation of tannins, phenolic acids, plant defensive compounds and other wound responsive cellular developments. These responses may be classified in the hypersensitive responses associated with biotic stressors (Ikeuchi et al., 2013). Lilacs (*Syringa* spp.) in particular produce high volumes of secondary metabolites during the early stages of in vitro culture. The common lilacs (*S. vulgaris* L.) predominantly produce hydroxyphenylethanol glycosides whereas the tree lilacs (*S. reticulata* (Blume) H.Hara and *S. pekinensis* Rupr.) produce secoirioid glucosides (Ellis et al., 1983; Bi et al., 2011). While some of these products have been used in medicinal tinctures for their expectorant, anti-asthmatic and antioxidant properties, these responses can have a drastic negative impact on the establishment and stabilization of micropropagation in vitro.

The challenges of introducing woody tissues into the in vitro environment can be compounded by plant anatomical features such as bud scales, bark exfoliation and pubescence that harbor insect and microorganism contaminants. Researchers apply several different techniques to eliminate these contaminants via tissue exposure to sodium hypochlorite solutions, long duration water rinses and even exposure to solutions containing heavy metals like a silver nitrate that also serve to reduce the ethylene mediated defensive response (Janick, 1986). Alternatively, control of explant contaminant load can be accomplished through close monitoring and pest management of the vegetative shoots during development. Vegetative shoot forcing is one method in nursery culture that has addressed production volume short-falls. Dormant woody

stems are encouraged to develop vegetative shoots when cut stems are exposed to carbohydrate containing solutions. Vegetative growth may benefit from the inclusion of plant growth regulating substances such as cytokinins and gibberellins (Preece, 2008). As found in the post-harvest preservation of cut floral stems the inclusion of citric acid or acidification of the forcing solution helps to increase vascular system conductivity and mitigate problems of microorganism contamination (Durkin, 1981).

Some responses to the acclimatization of in vitro culturing can be detrimental to the establishment of cultures while other responses offer exploitable opportunities. The phenomenon of callus formation, and totipotency or ability of a single cell to form a whole plant, is essential for the regeneration of whole plants for many biotechnological applications. In the context of tissue and organ specialization, called differentiation, original explant source has a significant effect on the ability to respond to hormonal signaling, or competence, for the formation of de novo shoots or somatic embryos (George et al., 2008; Liu et al., 2003; Sugiyama, 1999).

Iwase et al. (2011) reported that wound induced calluses in *Arabidopsis thaliana* (L.) Heynh. have expression patterns more consistent with the development of new shoots as compared to calluses induced on auxin rich media. Whereas calluses formed on auxin rich substrates appeared as a mass of tissue with an organizational structure and gene expression patterns similar to that of root initials (Atta et al., 2009; Sugimoto et al., 2010; Iwase et al., 2011). The dedifferentiation of plant cells from terminally differentiated tissues requires cells to undergo a cascade of changes within the cell. These modifications permit the continued growth and development of signal responsive competent cells (Iwase et al., 2011). Some of the morphological feature changes are measurable and have been correlated to embryogenic

regeneration capacitance in Pea (*Pisum sativum* L.), grass pea (*Lathyrus sativus* L.) as well as the model species *Medicago truncatula* Gaertn. and *Arabidopsis thaliana* (Ochatt et al., 2010).

The pinnacle of differentiation in woody plants results in the physiological ability to form flowers and reproduce. Since maturity or physiological age in explants is believed to be one of a few most important determinants in the successful yield of whole plants from protoplasts most experimenters choose to utilize embryos, seedlings, embryogenic calluses or suspension cells (Liu et al., 2003; Ochatt et al., 1995; Dos Santos et al., 1980). In plant gametogenesis there is an increasing amount of information to suggest a whole genome epigenetic change in the forming of microspores and megaspores (She et al., 2013).

The thermal and photoperiodic impact on flowering and crop productivity have been understood by farmers long before DNA was understood to be the unit of inheritance. The ability of the entire plant to successfully mature and reproduce requires whole organism communication and regulation to mitigate aberrant changes in chromatin and histone marks from environmental influences (King, 2015). As found in field plant reproductive biology, phase changes in morphology can be encouraged by the directed applications of environmental stressors like osmotic, temperature or hormonal stress (Poethig, 1990; Von Aderkas and Bonga, 2000). Plant regeneration from somatic cells in vitro is marked by changes similar changes in methylation, demethylation, and chromatin remodeling to those found in situ (Xu & Huang, 2014; De-la Peña et al., 2015). These changes provide the necessary access for active transcription and foster changes in the regulation of micro-RNA's and other transcription factors (Gordon et al., 2007; Mirouze and Paszkowski, 2011; Pulianmackal et al., 2014). Biotin, a metabolism coenzyme associated with mitochondrial and cytoplasmic carboxylases, plays a significant role in cell signaling and has been more recently implicated with a direct role in directing the recruitment of

histone modifying transferases in animals (Hassan and Zempleni, 2006; Nikolau et al., 2003). In plants the role of biotin, thiamine, riboflavin and several of the B vitamins have played important roles in the development of somatic embryos, adventitious organogenesis, and the rearing of rescued embryos from wide hybrid crosses (Al-Khayri, 2001; Von Arnold, 2008). These considerations follow in the experiments that were executed in the attempt to culture and rear whole plants in vitro.

Materials and Methods

Introduction of Explants in situ Sources

Dormant woody stems were collected after an approximate minimum accumulation of 2000 chilling hours on the campus of North Dakota State University (NDSU; Fargo, ND, USA). Stems were trimmed roughly 2 cm on either side of a dormant bud and placed in a sealable glass vessel. A sufficient volume of a 10% household bleach (8.25%–NaOCl) solution (0.825% v/v solution of sodium hypochlorite with additional 0.1% polysorbate) was added to immerse the tissues. Vessels were sealed and vigorously shaken for 5-20 minutes before they were placed in a laminar flow cabinet. The solution was decanted and replaced by a minimum of three exchanges of distilled deionized water (ddH_2O) (18.2 MΩ) or until bubbles from the polysorbate containing solution were eliminated. Tissues were then trimmed at the bicipital end to expose green healthy vascular tissue and placed into prepared sterile test tubes containing nutritive media as described in the initiation of cultures section below.

Vegetative Shoot Production from Dormant Hardwood Explants

Separate solutions were prepared in advance of explant exposure or transfer to new solutions. Solution A contained a 4% (w/v) sucrose with 0.2% activated charcoal (AC) in ddH2O adjusted to 3.5±0.1 pH with 1 N HCl. Solution B contained 20 μM 6-Benzyladenine (BA) and

2% (v/v) Plant Preservation Mixture (PPM™, a proprietary biocide affecting key enzymes in the Krebs cycle and the electron transport chain; Plant Cell Technology, 1823 Jefferson Place NW, Washington D.C., 20036, USA) in ddH2O water adjusted as necessary to 3.5±0.1 pH with 1 N HCl or 1 N KOH . The two solutions were autoclaved at 121 °C at 15 PSI for 20 min. then stored in the dark at 4 °C before use. Preceding initial or subsequent culture approximately 10mL of each solution were mixed in sterilized 25 X 100 mm test tubes.

Dormant woody stems were collected after an approximate minimum accumulation of 2000 chilling hours on the campus of NDSU. Explants were trimmed to three or four node explants of 5-8 mm in diameter. A length of internode at the bicipital end was left for continual trimming of the explants during shoot elongation. Buds that would be immersed in shoot forcing solution were removed to prevent rapid contamination of the solution. A clean blunt cut was made to the distal end roughly 5-10 mm away from the closest vegetative bud to prevent desiccation of the vasculature. A long angled cut was made at the bicipital end of the explant preceding their introduction to solutions. Explants were transferred on a 2 to 3-day cycle into 15-20 mL solutions containing equal volumes of solutions A and B. When mucilaginous masses accumulated at the bicipital end explants were lightly brushed in a 70% ethanol solution. All explants were re-trimmed with a long angled fresh cut and transferred to new solutions. Explants were grown in an incubator (Model 818, Precision Scientific, 170 Marcel Drive, Winchester, VA 22602, USA) with 16/8-hour light (36-40 μmol s-1)/dark cycling with concomitant ambient temperature fluctuation of 23/20 °C.

Initiation of Cultures

Potted plants of Nathan Maren's personal collection were stored in underground storage space. The underground storage space consisted of a plastic covered earthen floor crawl space

under the individual's residence in a dark condition around 5-10 °C. Plants were periodically watered to maintain humidity as moisture was drawn from the pot or tissue. Etiolated vegetation was trimmed from *S.* x*prestoniae* 'Dancing Druid', *S.* x*prestoniae* 'Miss Canada', *S.* x*prestoniae* 'Donald Wynman', *S.* x*prestoniae* 'James Macfarlane', and *S.* x*prestoniae* 'Minuet' and placed in sealable plastic bags during transport to the facilities of NDSU. Aseptic introduction of tissues followed the same procedure outlined in the introduction of explants from in situ sources section. Explants were introduced into vessels containing prepared medium formulations as described by Murashige and Skoog (1962) (MS), Driver and Kuniyuki (1984) (DKW), or Lloyd and McCown (1980) (WPM). Unless otherwise noted, the following and all remaining procedures within this section were conducted in a horizontal laminar flow hood. Test tubes (25 X100 mm) containing each medium formulation containing 3% sucrose, either 2 µM thidiazuron (TDZ) or 6-benzylaminopurine (BA), and were adjusted to a final 5.8 pH. Agar (0.7% w/v) was melted into solution and poured into before autoclaving at 121 °C at 15 PSI for 20 minutes. When phenolic acid residues accumulated in new cultures, tissues were trimmed upon transfer to new culture vessels as necessitated by population size.

Standard Micropropagation

Plants were sub-cultured on a four-week growing cycle on WPM based media. Syringa Woody Plant Medium (SWPM) (Appendix Table A-1.) in addition to the basic WPM media formulation contained 50% of carbohydrates as D-Maltose (M588; PhytoTechnology Laboratories®, P.O. Box 12205, Shawnee Mission, KS 66282, USA), 5 µM trans-Zeatin (Z007; Caisson Laboratories, 836 South 100, East Smithfield, UT 84335, USA), 1.45 µM Calcium D-gluconate monohydrate (G4625; Sigma-Aldrich® Co., 3050 Spruce Street, St. Louis, MO 53103, USA), 277 µM additional Myo-Inositol, and increased nitrogen ion contents. Ammoniacal

nitrogen and potassium nitrate were added to match ammonium to nitrate ratios similar to those described in MS through the addition of 18.7 mM ammonium nitrate and 16.3 mM potassium nitrate. Mediums were adjusted in accordance with a post autoclaving pH of 5.4 as this varied on the size of the pre-autoclave batch size. Mediums were solidified with a combination of 0.4% w/v agar and 0.14% w/v Gelrite® (CP Kelco U.S., Inc., Cumberland Center II, 3100 Cumberland Boulevard Suite 600, Atlanta, Georgia 30339, USA) The final *Syringa* spp. micropropagation media manipulations included a 50% exchange of sucrose for maltose.

Regeneration of Whole Plants from Differentiated Somatic Tissues

In vitro grown tissues at the end of a four week growing cycle were harvested for tissues (leaves or internodal stem segments) in the conduct of regeneration experiments. Internodal stem segments from *S.* x*chinensis* or leaves from *S.* x*prestoniae* 'Dancing Druid' were trimmed from nodes and placed in a petri dish of sterilized ddH20 while accumulating sufficient explant volume to fill a couple of treatments. Explants were applied to SWPM (Appendix Table A-1) mediums containing an additional 1 g•L^{-1} malt extract (218630, Becton, Dickinson and Company, 1 Becton Drive Franklin Lakes, New Jersey 07417, USA), 1 g•L^{-1} Amicase® (Kerry Group Services Ltd., Prince's Street, Tralee, Co. Kerry, V92 EH11, Ireland), 0.01 mg•L^{-1} D-biotin (B140, PhytoTechnology Laboratories®), 400 mg•L^{-1} L-Glutamine (G229, PhytoTechnology Laboratories®), 3.5% w/v sucrose, 1.5% w/v maltose and a factorial arrangement treatment of TDZ (0, 1.4, 4.0 μM) and NAA (0, 5, 10, 20 μM). Treatments were cultured in an incubator with a diurnal cycle with eight hours in the dark at 23 °C and varying day to day temperatures between 25-36 °C for 16 hours at 36-40 μmol s^{-1} of light for the competence induction phase (Fig. 2-1). After two weeks of culture on competence inductive treatments two to three whole explants were sub-cultured to test tubes or 60 mm petri dishes.

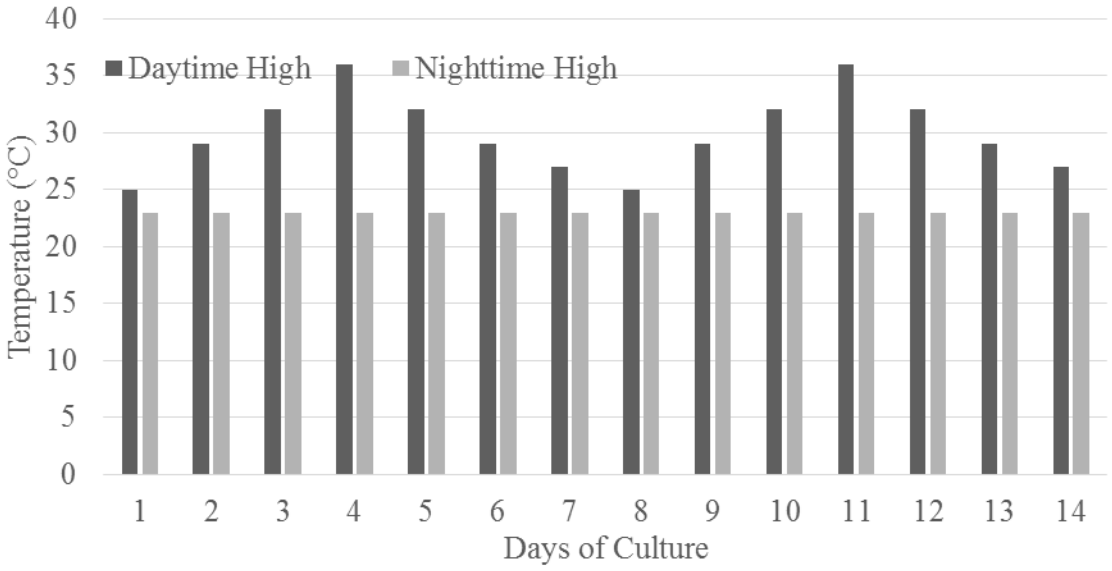

Fig. 2-1. Environmental control regime for the competence induction phase of regeneration experiments.

Somatic embryogenesis mediums contained the same basic SWPM media composition outlined in the competence induction treatments with 50% of the nitrogen content, 1 mg•L^{-1} riboflavin (R9504, Sigma-Aldrich®, Co), 5µM 2-(N-morpholino)ethanesulfonic acid (MES) (M825, PhytoTechnology Laboratories®).

Results and Discussion

Introduction from Dormant Woody Tissues.

Many attempts were made to introduce tissues from the landscape to initiate cultures of several species and cultivars. As found in *Rosa* spp., the best tissues were often derived from the second and third sub-terminal nodes of the previous growing seasons most vigorous shoots Hsia and Korban (1996). Smaller diameter woody explants were significantly wounded during the disinfestation procedure with the standard 10% v/v sodium hypochlorite solution. Disinfestation treatments of lower exposure time would result in loss of cultures due to contamination. Larger diameter explants would precociously exude phenolic acids into the media, which would

eventually cause the vasculature to become necrotic and most likely prevented continued flow of nutrition to growing shoots.

Plants etiolated at 5-10 °C in underground storage in February of 2014 were the best explant source for initiation of cultures. Many of these tissues were aseptically introduced into culture without incident. Promotion of vegetative shoot growth from dormant landscape tissues followed these successes. Constriction of the vasculature predominated the early attempts with this technique. Bacterial streaming and the rapid accumulation of phenolic acids appeared to be the most common causes of cambial discoloration and/or necrosis. To maximize shoot growth a fresh cut was made to the bicipital end and placed in fresh sterile solutions on a 2-3 day cycle. The addition of 0.1% AC (w/v) and PPM™ (1% v/v) was particularly effective at reducing the rate of wound closure between subcultures. To prevent the potential neutralizing activity of AC on plant hormones before explant exposure the forcing solution was split into two separate solutions. PPM™ could be and was substituted with more effective antibiotic(s) with some aseptic culture of *Ulmus americana*, *Prunus glandulosa*, and *Magnolia* x*loebneri* being introduced. Initiation of cultures from shoot forcing treatments were more variable in their acclimatization to culture than were etiolated shoots from whole plants. Shoot forcing treatments that included gibberellic acid (GA) during elongation fared poorly in the subsequent culturing of shoots. Yang and Reed (1991) found that bud break on shoots forced in solutions containing GA were developmentally hastened with insufficient chilling hours whereas some treatments that had accumulated sufficient chilling hours were deleterious.

Many cultures of lilac were initiated on MS media containing 2 μM TDZ. New shoots initiated on WPM media containing 2.2 μM BA had a reduced number of vitreous shoots produced and reduced the amount of callus growth at the bicipital end of new explants. This is

likely more related to the choice of cytokinin than the media formulation, though the incidence of vitreous shoot development was never eliminated. During the acclimatization of cultures, the effect of TDZ had a lasting effect on the proliferation of calluses and shoot growth in subsequent cultures. Plants grown on BA containing media were variable in response when subcultured onto common media. Whereas explants derived from shoots grown on TDZ containing media would often grow several shoots when subcultured onto new mediums. Many of TDZ grown shoots were vitreous, or encourage lateral bud to develop new shoots before the end of a subculture. The progression and decline of cultures beseeched an alternative plant growth medium formulation. Plants of several varieties would show nutritional deficiency symptoms such as speckling of the leaf blade, followed by collapse of the petiole, loss of the upper leaves and finally girdling the apices. Dr. Deborah McCown of Knight Hollow Nursery Inc. (7911 Forsythia Ct, Middleton, WI 53562, USA), a wholesale producer of micropropagated ornamentals specializing in *Syringa* spp., recommended an increase in the nitrogen content of the WPM basic formulation, the addition of calcium gluconic acid, exchange of BA for ZEA, use of a combinatorial gel matrix with agar and gelrite and greater attention to post-autoclave pH. Proportional species manipulation of nitrogen to MS levels, the addition of 1.44 mM calcium gluconate and exchange of 2.0 μM BA for 5 μM ZEA promoted the development of a regular and predictable cyclic growth for most cultivars. The final *Syringa* spp. micropropagation media manipulations included a 50% exchange of sucrose for maltose based on the significant increase in growth rates observed in suspension cultures of *S. pekinensis* ‘Zhang Zhaming’.

In attempts to regenerate whole plants from terminally differentiated tissues, a series of experiments were pursued. Testing different basic formulations of tissue culture media at the outset of micropropagation and adventive regeneration resulted in little variation between the MS

and WPM. Experiments tested dark inductive periods to light treatments. All treatments contained combinations of a full factor level treatment varying a single auxin (2,4-Dichlorophenoxyacetic acid (2,4-D), Naphthalene Acetic Acid (NAA)) and single cytokinin (Thidiazuron (TDZ), 6-Benzyladenine (BA), and Trans-Zeatin (ZEA)). Early unrepeated experiments revealed no significant differences between the media formulations during attempts at direct organogenesis. Media formulation was abandoned as a treatment factor by the fifth run of experiments and all subsequent runs of the experiment were conducted in an incubation chamber per the conditions outlined in the materials and methods.

As found in *Azadirachta indica* A. Juss, low concentrations of TDZ tended to promote nodular green callus (Murthy and Saxena, 1998). These findings are consistent with the outcomes of *Syringa* spp. treatments as shown in Fig. 2-2. A sample of tissues at the subculture from the competence inductive period were dissected and examined microscopically. Internodal stem segments had developed callus with patterns consistent with the formation of an embryonic suspensor. The suspensor elements were localized to the vascular tissues, and most prominent with treatments of low or no TDZ and mid to high rates of NAA. Furthermore, these tissues developed a polarity-based difference in callus color and derivation. Later examinations of undisturbed tissues left in contact with the competence inductive treatments revealed a large population of cells with an angular shape and abundant green flecking consistent with the overdevelopment of tracheid cells of the vascular xylem.

Thidiazuron as a plant growth regulator treatment for in vitro regeneration has been commonly tested and successfully applied within the *Oleaceae* (Hammatt, 1994; Karami et al., 2009). The caveat, as reported by Murthy et al. (1998) is the accumulation of TDZ and the

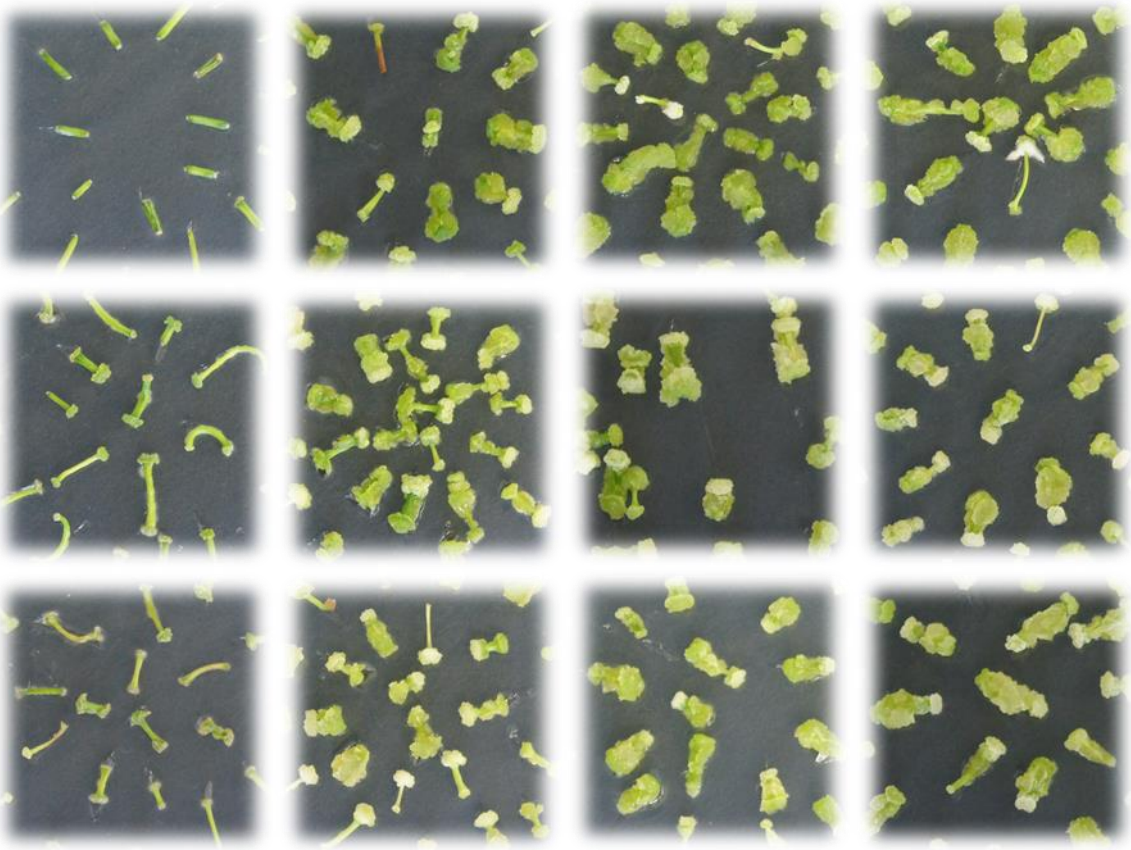

Fig. 2-2. Callus growth response from competence induction treatments.
Left to right: Naphthalene acetic acid (NAA) 0, 5, 10, 20 μM; Top to bottom: Thidiazuron (TDZ) 0, 1.4, 4 μM

residual effect on subsequent culture of shoots for in vitro use or ex vitro establishment. *Syringa* micropropagation cultures frequently responded during early cultivation. The appearance of rich green calluses on embryogenesis inductive treatments suggest a correlation between the outcome and TDZ. Per the observations on cell developments from competence induction treatments, it would be worth testing directed morphogenesis in *Syringa* with greater attention on the effect of duration of exposure. Pulse treatments, short term exposure to hormones or other ques, of high levels of auxins or cytokinins is consistent with the reports in the literature. In *Arabidopsis thaliana* explants, Gordon et al. (2007) reported changes in fluorescently labeled reporters of auxin responsive *DR5* expression inside of the first five days of culture, which subsequently

diminished thereafter. The change in abundance of suspected suspensor cells to those more consistent in appearance with vascular tracheids suggest the proportion population of different cell types proliferated differently over time. Preliminary experiments were conducted to test if tissue severance from the original explant would promote the development of the cells of interest only, but all tissues failed within seven days.

The changes that plant cells undergo in the development of de novo organs is a complex process with phases of development that vary between species (Ochatt et al. 2010). In broader fields of biology and medicine, new horizons in the development of therapies for cancer and tissue loss are evolving with continued stem cell research. Studies focusing on the promotion of tissue specific somatic cells to dedifferentiate and develop a pluripotent condition has become routine in stem cell research (L. Reynolds, personal communication). The integration of reprogramming signals such as the transcription factors: *c-Myc*, *Oct3/4*, *Sox2*, and *Klf4* have been demonstrated to induce pluripotency in stem cells of human, monkey, pig, rat, mouse, dog and rabbit (Takahashi and Yamanaka, 2006; Liao et al., 2009; Li et al., 2009; Liu et al., 2008; Esteban et al., 2009; Shimada et al., 2009; Honda et al., 2010). In plant systems, plant growth regulating substances and nutritional composition are often manipulated to direct differentiation. However, the link between hormone signaling and its effects on tissue developmental fate is an indirect pathway complexed by many of the aforementioned pre-conditions. The application transcription factors via direct delivery of purified proteins or plasmids may someday provide a convenient means to direct regeneration.

References

Al-Khayri, J., 2001. Optimization of biotin and thiamine requirements for somatic embryogenesis of date palm (*Phoenix dactylifera* L.). In Vitro Cell. Dev. Biol.-Plant. 37:453-456

Atta. R., L. Laurens, E. Boucheron-Dubuisson, A. Guivarc'h, E. Carnero, V. Giraudat-Pautot, P. Rech, and D. Chriqui. 2009. Pluripotency of Arabidopsis xylem pericycle underlies shoot regeneration from root and hypocotyl explants grown in vitro. Plant J. 57: 626–644.

Bi, X., W. Li, T. Sasaki, Q. Li, N. Mitsuhata, Y. Asada, Q. Zhang, and K. Koike. 2011. Secoiridoid glucosides and related compounds from *Syringa reticulata* and their antioxidant activities. Bioorg. Med. Chem. Let. 21:6426-6429.

De-la Peña, C., G.I. Nic-Can, R.M. Galaz-Ávalos, R. Avilez-Montalvo, and V.M. Loyola-Vargas. 2015. The role of chromatin modifications in somatic embryogenesis in plants. Frontiers Plant Sci. doi:10.3389/fpls.2015.00635.

Durkin, D.J. 1981. Factors effecting hydration of cut flowers. Acta Hortic. 113, 109-118. doi: 10.17660/ActaHortic.1981.113.15.

Dos Santos, A.V.P., D.E. Outka, E.C. Cocking, and M.R. Davey. 1980. Organogenesis and somatic embryogenesis in tissues derived from leaf protoplasts and leaf explants of Medicago sativa. Z. Pflanz. 99(3):261-270.

Driver, J.A. and A.H. Kuniyuki.1984. In vitro propagation of paradox walnut rootstock. HortSci. 19(4):507-509.

Ellis, B.E. 1983. Production of hydroxyphenylethanol glycosides in suspension cultures of *Syringa vulgaris.* PhytoChem. 22(9):1941-1943.

Esteban, M. A., J. Xu, J. Yang, M. Peng, D. Qin, W. Li, Z. Jiang, J. Chen, K. Deng, M. Zhong, J. Cai, L. Lai, and D. Pei. 2009. Generation of induced pluripotent stem cell lines from Tibetan miniature pig. J. Biol. Chem. 284(26):17634–17640 doi:10.1074/jbc.M109.008938.

George, E.F., M.A. Hall, and G.J. De Klerk. 2008. Plant propagation by tissue culture: Vol 1 The background. 3rd ed. Springer, Dordecht, the Netherlands.

Gordon, S.P., M.G. Heisler, G. Venugopala Reddy, C. Ohno, P. Das, and E.M. Meyerowitz. 2007. Pattern formation during de novo assembly of the Arabidopsis shoot meristem. Development 134:3539-3548 doi:10.1242/dev.010298.

Hammatt, N. 1994. Shoot initiation in the leaflet axils of compound leaves from micropropagated shoots of juvenile and mature common ash (*Fraxinus excelsior* L.). J. Exp. Bot. 45(275):871-875.

Hassan. Y.I. and J. Zempleni. 2006. Epigenetic regulatioin of chromatin structure and gene function by biotin. J. Nutr. 136(7):1763-1765.

Honda, A., M. Hirose, M. Hatori, S. Matoba, H. Miyoshi, K. Inoue, and A. Ogura. 2010. Generation of induced pluripotent stem cells in rabbits: potential experimental models for

human regenerative medicine. J. Biol. Chem. 285(41), 31362–31369 doi:10.1074/jbc.M110.150540.

Hsia, C.N. and S.S. Korban. 1996. Factors affecting in vitro establishment and shoot proliferation of *Rosa hybrida* L. and *Rosa chinensis minima*. In Vitro Cell. Dev. Biol.–Plant 32:217–222.

Ikeuchi M, K. Sugimoto, and A. Iwase. 2013. Plant callus: Mechanisms of induction and repression. The Plant Cell 25:3159-3173.

Iwase, A., N. Mitsuda, T. Koyama, K. Hiratsu, M. Kojima, T. Arai, Y. Inoue, M. Seki, H. Sakakibara, K. Sugimoto, and M. Ohme-Takagi. 2011. The AP2/ERF transcription factor WIND1 controls cell dedifferentiation in Arabidopsis. Curr. Biol. 21:508-514.

Janick, J. 1986. Horticulture science. 4th ed. W.H. Freeman, New York, USA.

Karami, O., A. Deljou, and K. Piri. 2009. Adventitious shoot formation from leaf explants of oleaster (*Eleagnus angustifolia* L.). Prop. Orn. Plants. 9(1):50-52.

King, G. 2015. Crop epigenetics and the molecular hardware of genotype x environment interactions. Front. Plant Sci. doi: 10.3389/fpls.2015.00968.

Li, W., W. Wei, S. Zhu, J. Zhu, Y. Shi, T. Lin, E. Hao, A. Hayek, H. Deng, and S. Ding. 2009.Generation of rat and human induced pluripotent stem cells by combining genetic reprogramming and chemical inhibitors. Cell Stem Cell 4(1):16–19 doi:10.1016/j.stem.2008.11.014.

Liao, J., C. Cui, S. Chen, J. Ren, J. Chen, Y. Gao, H. Li, N. Jia, L. Cheng, H. Xiao, and L. Xiao. 2009. Generation of induced pluripotent stem cell lines from adult rat cells. Cell Stem Cell 4(1):11–15 doi:10.1016/j.stem.2008.11.013.

Liu, J., X. Xu, and X. Deng. 2003. Protoplast isolation, culture and application to genetic improvement of woody plants. J of Food Agri & Envir 1(3-4):112-120.

Liu, H., F. Zhu, J. Yong, P. Zhang, P. Hou, H. Li, W. Jiang, J. Cai, M. Liu, K. Cui, X. Qu, T. Xiang, D. Lu, X. Chi, G. Gao, W. Ji, M. Ding, and H. Deng. 2008. Generation of induced pluripotent stem cells from adult rhesus monkey fibroblasts. Cell Stem Cell 3(6):587–590 doi:10.1016/j.stem.2008.10.014.

Lloyd, G. and B. McCown. 1980. Commercially feasible micropropagation of mountain laurel, Kalmia latifolia, by use of shoot tip culture

McCown, B.H. 2000. Recalcitrance of woody and herbaceous perennial plants: Dealing with genetic predeterminism. In Vitro Cell. Dev. Biol. - Plant 36(3):149-154.

Mirouze, M. and J. Paszkowski. 2011. Epigenetic contribution to stress adaptation in plants. Curr. Opin. Plant Biol. 14:267-274.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Murthy, B.N.S. and P.K. Saxena. 1998. Somatic embryogenesis and plant regeneration of neem (*Azadirachta indica* A. Juss.). Plant Cell Rpt. 17:469-475.

Murthy, B.N.S., S.J. Murch, P.K. Saxena. 1998. Thidiazuron: A potent regulator of in vitro plant morphogenesis. In Vitro Cell. Dev. Biol. Plant. 34: 267-275.

Nikolau, B.J., J.B Ohlrogge, and E.S. Wurtele. 2003 Plant biotin-containing carboxylases. Arch. Biochem. Biophys. 414:211-222.

Ochatt, S.J., F.M. Atif, E.M. Patat-Ochatt, L. Jacas, and Conreux. 2010. Competence versus recalcitrance for in vitro regeneration. Not. Bot. Hort. Agrobot. Cluj. 38(2):102-108.

Ochatt, S.J. and E.M. Patat-Ochatt. 1995. Protoplast technology for the breeding of top-fruit trees (*Prunus*, *Pyrus*, *Malus*, *Rubus*) and woody ornamentals. Euphytica 85:287-294.

Poethig, R. 1990. Phase change and the regulation of shoot morphogenesis in plants. Science, 250(4983):923-930. Retrieved from http://www.jstor.org/stable/2878230.

Preece, J. 2008. Stock plant physiological factors affecting growth and morphogenesis. p. 403-422. In: Plant propagation by tissue culture: Vol 1 The background. 3rd ed. Springer, Dordecht, the Netherlands.

Pulianmackal, A.J., A.V.K. Kareem, K. Durgaprasad, Z.B. Trivedi, and K. Prasad. 2014. Competence and regulatory interactions during regeneration in plants. Front. Plant Sci.| http://dx.doi.org/10.3389/fpls.2014.00142.

She, W., D. Grimanelli, K. Rutowicz, M.W.J. Whitehead, M. Puzio, M. Kotliński, A. Jerzmanowski, and C. Baroux. 2013. Chromatin reprogramming during the somatic-to-reproductive cell fate transition in plants. Development. 140:4008-4019. doi:10.1242/dev.095034

Shimada, H., A. Nakada, Y. Hashimoto, K. Shigeno, Y. Shionoya, and T. Nakamura. 2009. Generation of canine induced pluripotent stem cells by retroviral transduction and chemical inhibitors. Mol. Reprod. Dev.77(1):2 doi:10.1002/mrd.21117.

Sugimoto, K., Y. Jiao, and E.M. Meyerowitz. 2010. Arabidopsis regeneration from multiple tissues occurs via a root development pathway. Dev. Cell 18:463–471.

Sugiyama, M. 1999. Organogenesis in vitro. Curr. Opin. Plant Biol. 2:61–64 http://biomednet.com/elecref/1369526600200061.

Takahashi, K., and S. Yamanaka. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 126:663–676 doi:10.1016/j.cell.2006.07.024.

Von Aderkas, P. and J.M. Bonga. 2000, Influencing micropropagation and somatic embryogenesis in mature trees by manipulation of phase change, stress and culture environment. Tree Phys. 20:921-928.

Von Arnold, S. 2008. Somatic embryogenesis. p. 335-354. In: Plant propagation by tissue culture: Vol 1 The background. 3rd ed. Springer, Dordecht, the Netherlands.

Xu, L. and H. Huang. 2014. Genetic and epigenetic controls of plant regeneration. Curr. Top. Dev. Biol. 108, 1–33. doi: 10.1016/B978-0-12-391498-9.00009-7

Yang, G. and P.E. Reed. 1991. Influence of plant growth regulators and season on growth responses of forced woody stems. Acta Hort. 300:173-176.

CHAPTER 3. PROTOPLAST ISOLATION AND FUSION

Abstract

Few other woody plants embody the preeminence of temperate woody plants in garden cultivation like the lilacs. In spite of their relationship, the trees lack the diversity of cultivated floral forms observed within the shrub lineages. Typical selection and cross-pollination schemes within the tree lilacs have revealed few inherent variations and interspecies crosses have repeatedly failed between the two groups. Somatic hybridization is an in vitro technique in plant improvement that has demonstrated the ability to overcome the barriers of sexual incompatibility across species and familial divides. Protoplast isolation experiments examined various enzyme concentrations, formulations, and durations of exposure on protoplast liberation and viability. Leaf tissues from in vitro grown plants representing the taxonomic series *Syringa* (*Syringa* x*chinensis*) and series *Villosae* (*S.* x*prestoniae* 'Dancing Druid') were used as source material for those experiments. Protoplast quantity and quality were significantly affected by the interaction of enzyme treatments and the duration of exposure ($P < 0.05$). Equal volumes of leaf derived protoplasts of *S.* x*chinensis* or *S.* x*prestoniae* 'Dancing Druid' were mixed with fluorescein diacetate (FITC) labeled protoplasts derived from zygotic embryos of *S. reticulata* 'Ivory Silk' for electrofusion experiments. The alignment field strength and duration were kept constant to test permeation voltage strength and application duration for differences in hybrid yield. Flow cytometric analysis revealed putative hybrid signals in all treatment conditions. Differences in field strength or application duration treatments were not revealed to have a significant impact on hybrid yield.

Introduction

The first studies in protoplast isolation were done by the work of JAF Klercker in 1892 (Klercker, 1892). Klercker (1982) used leaves of the water plant *Stratiotes aloides* L. dissected to release the cellular contents into a hypertonic solution for further examination. The work was forgotten until the isolation and commercial productions of pectinase and cellulase enzymes from bacteria (Davey et. al 2005a). These cell wall digesting enzymes were pivotal in bringing reproducible results to the study of "naked" cells of cell wall bound organisms. Today the isolation of protoplasts involves a composite of enzymes that break down the glycosidic linkages in large and small chain branched polysaccharides that make up the cell wall and intercellular pectin. The enzyme cocktail with other media components are empirically designed to liberate high volumes of protoplasts of similar condition. Protoplast isolation and culture can be utilized for a number of different purposes from transient gene expression assays in plant and fungal physiology studies to applications like somatic fusion.

Regeneration of whole plants from differentiated tissues can be elusive without compounding the organismal need to restore a functional cell wall, metabolism and cytokinesis. As expected with other tissue culture systems, genotype is a significant contributor to the success of protoplast isolation and culture (Liu et al., 2003). Though tissues may be isolated from any number of plant parts, explant source and physiological age have been shown to have a significant impact on the regeneration capacity of isolated protoplasts (Davey et al., 2005a; Davey et al., 2005b; Eeckhaut, 2013; Liu et al. 2003). In applied plant breeding efforts, at least one parent in the hybridization scheme is a regeneration competent tissue source such as an embryogenic callus, embryo tissues or leaves of juvenile plants (Grosser. J. personal communication).

The waxy cuticle and epidermis of plant tissues are evolutionary adaptations to regulate interactions with the environment. As such these protective layers can inhibit the access of protoplast digestive solutions and limit the liberation of protoplasts from their cell walls. When isolating protoplasts, manipulation of the osmotic environment is essential to the liberation of protoplasts from their cell walls and subsequent maintenance of cell turgor (Klercker, 1892). The manipulation of solution osmolarity is usually accomplished by varying sugar or sugar alcohol concentrations in the mediums used for various applications during protoplast preparation and use. The treatment of tissues in a hypertonic solution before enzyme exposure is called plasmolysis. Plasmolysis conditioning reduces vacuole water content and plasma membrane contact with the cell wall thus providing greater contact of digestive solutions with the cell wall. Plasmolysis was essential for Ochatt (1994) who in the early 1990's isolated *Forsythia* x*intermedia* 'Spring Glory' and other lilac species.

The components of the digestion media are fairly conserved between experiments and experimenters for studies in protoplast research (Liu et al., 2003). The digestion or isolation medium components are similar in composition to the plasmolysis media with tissue source specific osmoticum adjustments and the addition of enzymes. Plasmolysis and isolation media often contain calcium, magnesium, potassium and phosphates to assist in the maintenance of plasma membrane stability (Liu et al., 2003). The most common basic protoplast preparation solution, Cell Protoplast Wash (CPW), is similar in composition to the commonly used phosphate buffered saline solutions used in other biological systems research (Banks and Evans, 1976).

Protoplast isolation treatments are often conducted in the dark to prevent media photooxidation and free radical interactions with liberated protoplasts. A protocol of best fit for

each variable component is empirically derived. The most common variables in a protoplast isolation experiment vary duration of exposure to the quality, quantity, and proportions of different enzyme combinations. Additional constituents such as pH buffers (2-(N-morpholino) ethanesulfolic acid (MES), monosodium or monopotassium phosphates) and phenolic acid binding agents such as polyvinylpyrrolidone (PVP) may be added for the mitigation of cellular or organelle damage during the digestion process.

Once protoplasts have been liberated from their cell walls, observations on cell size will indicate how media osmolarity is affecting the cellular solution equilibrium. The consideration of the size variance bear effects on the incidence of spontaneous fusion, recovery of the cell and the restoration of mitosis. Plasmolysis is not only a favorable practice for liberating mesophyll derived protoplasts, but has furthermore been found to reduce spontaneous cell fusion incidence (Davey et al., 2005b). The importance of monitoring the osmolar equilibrium of medium and protoplast cannot be overstated as protoplast liberation is rarely the end goal. The successful application of somatic fusion or treatments involved in DNA uptake require some level of plasma membrane disruption.

Once protoplasts are liberated from their cell walls, protoplasts are purified to eliminate enzyme solutions and unusable cellular debris. A strained and rinsed suspension of the protoplast digest is then purified via equilibrium (isopycnic) centrifugation on a solution density gradient. A low density solution is carefully layered over a higher density solution utilizing varying sugars, sugar alcohols, or poly-sucrose (i.e. Ficoll®). Purification of the digested material in a Percoll® (GE Healthcare Bio-Sciences, P.O. Box 643065 Pittsburgh, PA 15264, USA) density gradient (colloidal suspension of PVP coated silica) may help to precipitate phenolic acid contaminants if necessary. The principle of the technique permits the suspension of the digest solution in either

the high or low density solution as long as the difference between the solution concentration favors suspending the viable protoplasts.

Quantification of protoplasts can be made with most standard hemocytometers with a slide and cover slip depth and adjustments to protoplast solution density can be made to a typical 0.5-10 X 10^6 cells•mL^{-1} depending on the needs of the system. Validation and quantification of protoplast viability is conducted via fluorescein diacetate (FDA) staining of protoplasts. Fluorescein diacetate is readily mobile across the plasma membrane and once met with biologically active esterase cleaves the molecule to liberate fluorescein that accumulates within the cytosol. The assay itself is a particularly convenient tool for routine examinations and the evaluation of treatment conditions on of protoplast colonies.

De Filippis et al. (2000) reported an almost two-fold advantage in electrofusion byproducts over chemically induced fusion. Their report cited several advantages, including an increase in the potential for the regeneration of hybrid plants over that of simply isolated products alone. Among those observations in electrically stimulated hybrids were the increased oxygen consumption profiles, as compared to chemically fused hybrids, as well as greater membrane and organelle integrity (De Filippis et al., 2000).

The principles of protoplast culture, particularly osmoticum, play an important role in electrofusion with additional caveats. The electrofusion solution ionic conductivity will change the effective field strength applied during the alignment and fusion phases. For fusion to occur a neighboring cell, must be in close contact when the plasma membranes disassociate. Cell contact in electrofusion is brought about by the application of a dielectrophoretic field where an alternating current passes through the cells as the result of greater intracellular electrical conductivity than that of the fusion solution. During diploinduction the anode and cathode circuit

closes as neighboring cells come in greater cell-to-cell contact in what has been referred to as a pearl chain. Estimations on protoplast yield and mean cell size impact the choice of the biparental solution density and field strength necessary for reversible electrical breakdown in plasma membrane integrity (Zimmerman et al., 1974). The resulting contact between cells and the simultaneous application of a direct field current permit the transient plasma membrane disruption in those cells in contact with one another. The minimum applied field strength voltage necessary for membrane potential breakdown between two adjoining cells is two volts (Zimmerman et al., 1974). The critical field strength necessary for membrane breakdown is related to contact and which changes with cell size and so critical field strength is estimated as two volts divided by 75% of the mean cell diameter (cm) of the two parental populations in solution (Zimmerman et al., 1974). Many permutations on the number of pulses and voltages applied have been evaluated but empirical evaluations and the best parameter settings are unique to each system. Aggregation of protoplasts of common origin may complicate the process and the occurrence for autopolyploidy is common. The (pre)treatments that results in the greatest yield of true hybrids is thereby the determinant of the system success.

Protoplast isolation, and fusion where applicable, are stressful procedures and their subsequent culturing is paramount to the regeneration of whole plants. As mentioned above, low voltage electrical stimulation has been demonstrated to aid in the restoration of the cell cyclic growth and development, but several other factors determine the ability to restore the cell wall and subsequent mitotic division (De Filippis et al., 2000; Davey et al., 2005a&b). The ability to restore the cell growth and development cycle stems from the capacity of those cell lines to respire and their competence for hormonal signal perception. Cell wall formation may occur within an hour after isolation of protoplasts; however, as was the case for *Forsythia* x*intermedia*

and other woody plants cell wall development may lag for several days (Ochatt et al. 1994, Ochatt et al. 1995). For forsythia and lilacs, the microcallus to callus phase can take up to a month before final plating (Ochatt, S. personal communication). The culturing phase requires cells to be nurtured in a progressively reduced osmolarity toward the final culture osmoticum while directing the signals necessary for typical metabolism and cytokinesis.

Materials and Methods

Leaf explant tissues for the conduct of all protoplast isolation and fusion experiments were derived from in vitro grown plants sub-cultured on a four-week growing cycle on modified (WPM) Lloyd and McCown (1980) based media termed Syringa Woody Plant Medium (SWPM) (Appendix Table A-1.). In addition to the basic WPM media formulation contained 50% of carbohydrates as D-Maltose (M588; PhytoTechnology Laboratories®, P.O. Box 12205, Shawnee Mission, KS 66282, USA), 5 µM trans-Zeatin (Z007; Caisson Laboratories, 836 South 100, East Smithfield, UT 84335, USA), 1.45 µM Calcium D-gluconate monohydrate (G4625; Sigma-Aldrich® Co., 3050 Spruce Street, St. Louis, MO 53103, USA), 277 µM additional Myo-Inositol, and increased nitrogen ion contents. Ammoniacal nitrogen and potassium nitrate were added to match ammonium to nitrate ratios similar to those described in MS through the addition of 18.7 mM ammonium nitrate and 16.3 mM potassium nitrate. Mediums were adjusted in accordance with a post autoclaving pH of 5.4 as this varied on the size of the pre-autoclave batch size. Mediums contained 10 $g \cdot L^{-1}$ sucrose and10 $g \cdot L^{-1}$ maltose and were solidified with a combination of 0.4% w/v agar and 0.14% w/v Gelrite® (CP Kelco U.S., Inc., Cumberland Center II, 3100 Cumberland Boulevard Suite 600, Atlanta, Georgia 30339, USA)

Protoplast Isolation

Plants at the end of a four-week subculture cycle were used for experiments involving leaf tissues for protoplast isolation. Protoplast culture medium (designated medium 81) was modeled after the *Citrus spp*. protoplast culture medium (Grosser et al., 2010). *Citrus spp*. protoplast culture medium is principally designed from 8P medium (Kao and Michayluk, 1975) to contain at least the levels of the macro- and micronutrient basic salts, as well vitamins utilized in Murashige and Tucker (1969). Medium 81 is a protoplast culture medium designed principally on 8P, containing tissue culture media components with the macro- and micronutrient basic salts, as well as vitamins utilized in SWPM. In addition, 150 mg•L^{-1} additional Amicase® (A2427; Sigma-Aldrich®, Co) casein acid hydrosylate (Liu et al., 2013), 5mM 2-(N-morpholino)ethanesulfonic acid (MES) and 1% polyvinylpyrolidone (PVP10; Sigma-Aldrich®, Co) were added to the culture medium per the recommendations of Dr. Sergio Ochatt (personal communication). Mediums were adjusted to a pH of 5.5 as necessary with 1 NHCL or 1 NKOH and then filter sterilized.

Leaf tissues were collected into 100 mm sterile petri dishes containing 8 mL of 0.6M 81 protoplast culture medium until approximately 2-3g of leaf tissue had been collected. Leaves were finely cut using a double edged razor blade and set aside for a brief plasmolysis period of 15-25 minutes (Ochatt, 1994). After the plasmolysis period, enzyme solutions (Table 3) containing varying concentrations of cellulase or pectinase solutions were diluted into the 0.6M 81 medium and gently distributed before 15 minutes of vacuum infiltration at 50 kPa. Treatments were blocked by replicate since collecting sufficient tissue volumes and procedures involved in processing were time consuming. All plates were sealed with Parafilm® M (Bemis®, P.O. Box 2968, Oshkosh, WI 54903, USA) and placed on an incubating shaker at ~30 rpm, 28°C in the

dark. Digestion solutions were sampled at designated timeframes of 16, 24, and 32 ±2 hours of digestion for analysis. Samples were collected by placing a 100 µm cell strainer into each treatment and drawing 1 mL of the digestion slurry. Samples were placed in 1.5 mL microcentrifuge tubes and spun at 900 rpm for 9 minutes. 850 µL of the supernatant was removed and the pellet was suspended in the same volume of a CPW medium containing 25% (w/v) sucrose (CPW25S; Banks and Evans, 1976).

Table 3-1. Enzyme digestion solution concentrations on dilution

	Enzyme Category I	Proportion (w/v)	Enzyme Category II	Proportion (w/v)	Enzyme Category III	Proportion (w/v)
1	Onozuka RS[1]	1.0%	Hemicellulase[2]	0.75%		
2	Onozuka RS[1]	1.0%	Hemicellulase[2]	1.00%		
3	Onozuka RS[1]	1.0%	Hemicellulase[2]	0.75%	Driselase®[3]	0.10%
4	Onozuka RS[1]	1.0%	Macerozyme R-10[1]	0.75%		
5	Onozuka RS[1]	1.0%	Macerozyme R-10[1]	1.00%		
6	Onozuka RS[1]	1.0%	Macerozyme R-10[1]	0.75%	Driselase®[3]	0.10%

1. Yakult Pharmaceutical Industry Co., Ltd., 16-21 Ginza 7-Chome, Chuo-Ku,Tokyo 104-0061 Japan.
2. No. H2125, Sigma-Aldrich, Co., 3050 Spruce Street St. Louis, MO 53103, USA.
3. No. D9515, Sigma-Aldrich, Co., 3050 Spruce Street St. Louis, MO 53103, USA. (Driselase is a registered trademark of ASKA Animal Health Co. Ltd.)

Microscopy analysis

Protoplasts were stained with fluorescein diacetate according to Power et al. (1989). Stained protoplast solutions were added to a 0.1 mm Neubauer improved hemocytometer and examined under x10 magnification on the Zeiss Axio Imager M2 platform. Standard brightfield images and fluorescence images captured on the DAPI channel were captured on the Zeiss

AxioCam HRc Rev3. Manual counts of all images were conducted from image acquisitions. Only those data within the focal plane that could readily be distinguished as plant cells were counted into total counts. Minimum distinguishing features included a rounded (not angular) appearance and the presence of a surrounding cell boundary (plasma membrane). Viability counts were made on the basis of presence of fluorescence in 365-nm channel images and the presence of a veritable cell count in bright field images as shown in Fig. 3-1.

Flow cytometry

The remaining sample volume from replicates were spun at 900 rpm for 9 minutes. 850 µL of the supernatant was removed and the pellet was suspended in the same volume of a LB01 solution (Dolezal et al., 1989). Tubes were placed on ice in the absence of light for a period of 5-20 min. Data was collected on minimum of 100,000 events on a polygonal gate set to on the biparametric contour plot of the FL2-A (585/40nm) versus FL3-A (≥675nm) fluorescence emission channels as described by D.W. Galbraith (2009) or a maximum of 10 min of data collection. Figure 3-2 is a graphical representation of the parameters used in the evaluation of the cytometry data.

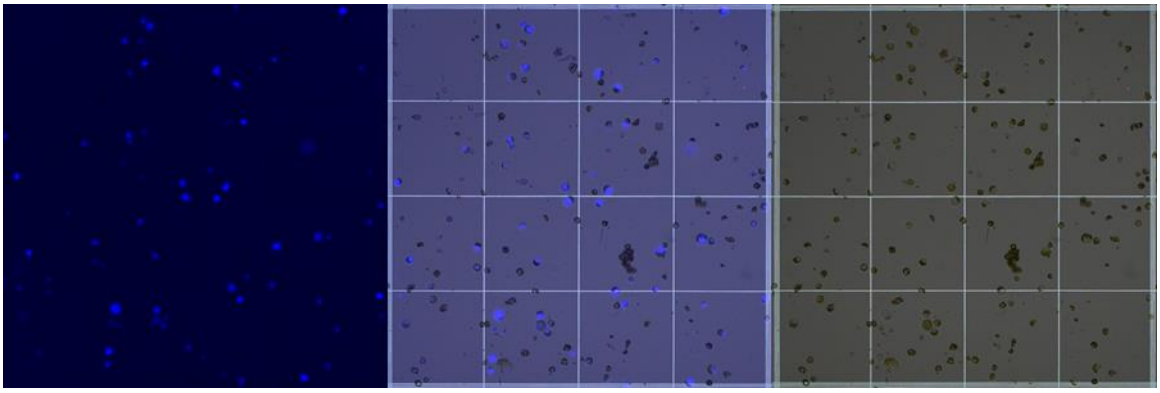

Fig. 3-1. Microscope slide example used in the evaluation of protoplast counts and viability. Left. Fluorescent image capture of fluorescein diacetate stained (viable) protoplasts captured on the DAPI band pass filter. Middle. Overlay of the fluorescent image and bright field image used in the determination of viable protoplast count. Right. Standard bright field image.

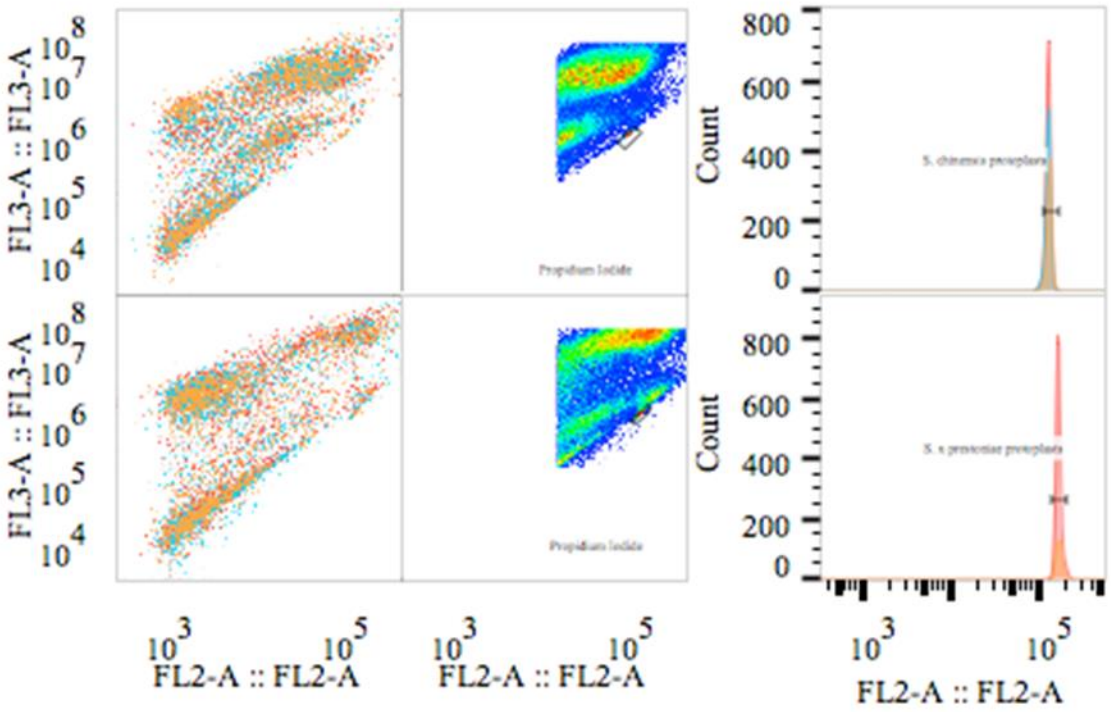

Fig. 3-2. Flow cytometric analysis of protoplast isolation treatments.
Top. Left. Biparametric contour plot of the FL2-A (585/40nm) versus FL3-A (≥675nm) fluorescence emission from a consensus plot of a typical protoplast isolation treatment with *Syringa* x*chinensis*. Top. Middle. Biparametric contour of the FL3-A versus FL2-A plot on which final cell counts were gated. Top. Right. Uniparametric histogram of the consensus peaks for a typical protoplast isolation treatment. Bottom. Left. Biparametric contour plot of the FL2-A versus FL3-A fluorescence emission from a consensus plot of a typical protoplast isolation treatment with *S.* x*prestoniae* 'Dancing Druid' Bottom. Middle. Biparametric contour of the FL3-A versus FL2-A plot on which final cell counts were gated. Bottom. Right. Uniparametric histogram of the consensus peaks for a typical protoplast isolation treatment.

Somatic Fusion

Protoplasts were digested from in vitro grown leaves of both *S.* x*chinensis* and *S.* x*prestoniae* 'Dancing Druid' as outlined in the protoplast isolation section for parent A in fusion experiments. Protoplast digest solutions were washed and purified according to procedure outline in protocol 10.3 in Grosser et al. (2010). Parent B was derived from excised zygotic embryos of

S. reticulata 'Ivory Silk'. Mature seeds were first acid scarified in 96% H_2SO_4 for approximately three min and then continuously rinsed in distilled water until embryo excision. Once excised seeds were held in a 3% sucrose solution (3.5 pH) with additional 0.006% Carbenicillin and Cefotaxamine until 1-2 min disinfestation with a 1% sodium dichloroisocyanurate dihydrate (NaDICC; D253, PhytoTechnology Laboratories®, P.O. Box 12205, Shawnee Mission, KS 66282, USA) solution. Seeds were rinsed thoroughly in a 13% D-mannitol CPW (CPW13M) solution before placement in 60 mm petri dishes. Rinse solutions were removed with a Pasteur pipette and 4 mL of CPW13M containing 30 μM of fluorescein 5-isothiocyanate (FITC; F7250, Sigma-Aldrich®, Co) Embryos were coarsely chopped with a flame sterilized double edged razor blade followed by the addition of 1.5 mL of enzyme solution 6 (see Table 1). Dishes were sealed in Parafilm®, wrapped in aluminum foil and placed on an incubating rotary shaker at 28° C and 30 rpm for 16-24 hours. Protoplast purification proceeded as described for leaf protoplasts, modified where 13% D-mannitol containing CPW (CPW13M) solution was used in place of CPW25S and a 9% D-mannitol containing CPW (CPW9M) solution was overlain for creation of the density gradient.

Electrofusion solutions and the preparation of protoplast from each parent followed protocol 10.6 as described in Grosser et al. (2010). Protoplast quantitation was evaluated under standard bright field microscopy and adjustments to protoplast density were made to 0.5×10^6 cells•mL^{-1}. Following the preliminary procedural evaluations outlined in Nissing (2007) square wave pulse number was evaluated on the 200μm microfusion chamber slide. Final fusion treatments were conducted in the Eppendorf Helix Fusion chamber. 125 μL of each population were homogeneously mixed in the Helix Fusion chamber cuvette. Electroporation setting evaluations commonly applied a pre- and post-fusion alignment setting of 1.6 V for 95 s and 3

bursts of all varying combinations of the square wave pulse voltage settings of 1000, 2000, and 4000 V•cm^{-1} and either 40 or 80 μs durations. Ten minutes were allotted for protoplast hybrids to regain their conformation before the cuvette was disturbed. The fusion core was rinsed with 1 mL of protoplast culture media and subsequently transferred to a microcentrifuge tube and placed in a low light condition while the remaining treatment replicates were accumulated.

Flow Cytometric Analysis of Hybrid Protoplasts

As the ability to distinguish flow cytometry output relies heavily on relative measures and differences in fluorescent signal intensity, a number of positive and negative controls were examined. Preliminary and unreplicated controls were used to determine plausible hybrid locations within the biparametric contour and uniparamentric histogram plots. Chlorophyll autofluorescence was used as the alternative fluorescent signature to identify or distinguish hybrids. Leaf mesophyll derived protoplasts were stained with FITC to represent the hybrid population. Pure unbiased protoplast solutions of each parental type were run with each experiment as shown in Fig.3-3. Homogenous mixes of the pure parental lines suspended in fusion media were mixed immediately before analysis to prevent possible spontaneous fusion. Flow cytometric analysis of protoplast fusion hybridization treatments were used to establish a hybrid count as the gating procedure is shown in Fig. 3-4. Uniparametric histograms of ungated FL1-A channel output were rough gated and concurrently gated with FL3-A channel histograms. The final gating was adopted to fit the maximum value within each heterogeneous hybrid population. As such a different between population and as such was unique to cultivar by cultivar combination as best shown in Fig. 3-4 D vs. H.

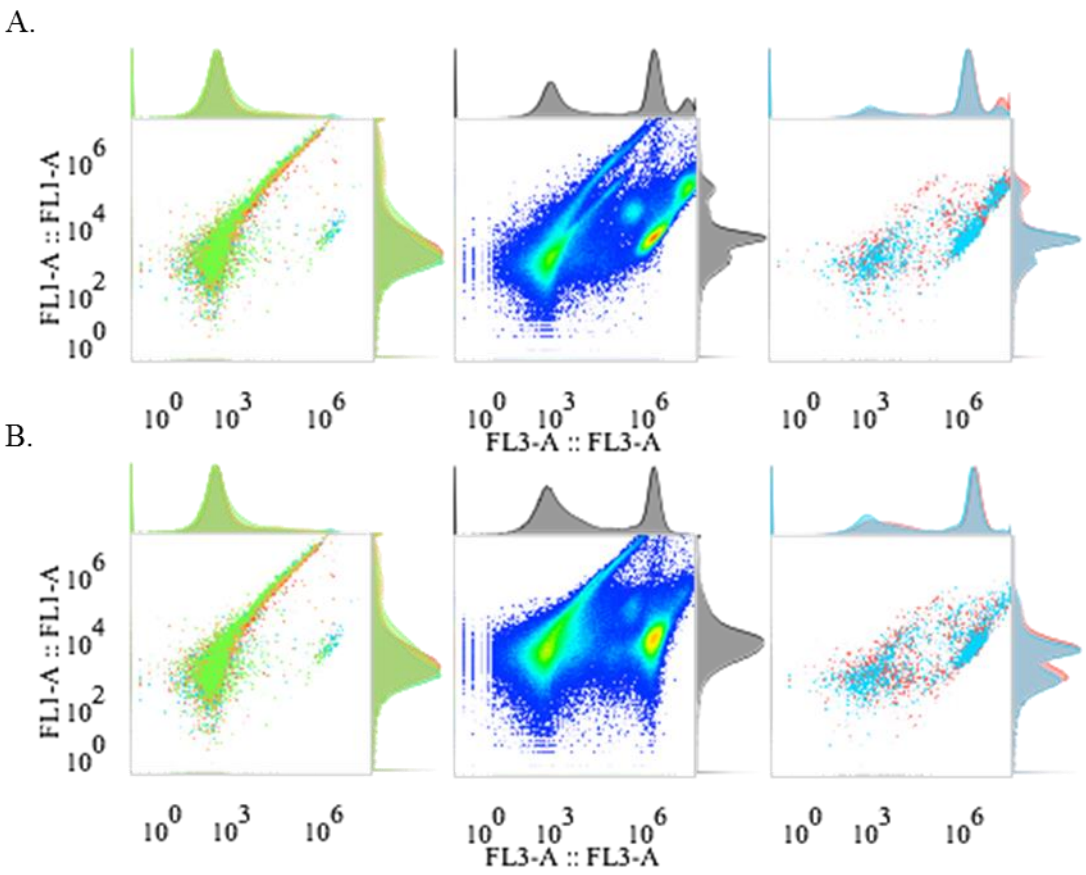

Fig. 3-3. Biparametric plots of control samples used in the evaluation of somatic fusion hybrid populations.
All plots are the biparametric representations of the FL3-A (≥675nm) versus FL1-A (530/30nm) fluorescence emission. Contours on parallel axes represent uniparametric histograms and relative signal strength contributing to the biparametric plots. From left to right: A-1. Consensus plot from *Syringa reticulata* 'Ivory Silk' protoplasts stained with Fluorescein 5-isothiocyanate (FITC). A-2. Homogenous mix of protoplasts from FITC stained *S. reticulata* 'Ivory Silk' and *S.* x*chinensis*. A-3. Consensus plot from *S.* x*chinensis* protoplasts. B-1. Consensus plot from *S. reticulata* 'Ivory Silk' protoplasts stained with FITC. B-2. Homogenous mix of protoplasts from FITC stained *S. reticulata* 'Ivory Silk' and *S.* x*prestoniae* 'Dancing Druid'. B-3. Consensus plot from *S.* x*prestoniae* 'Dancing Druid' protoplasts.

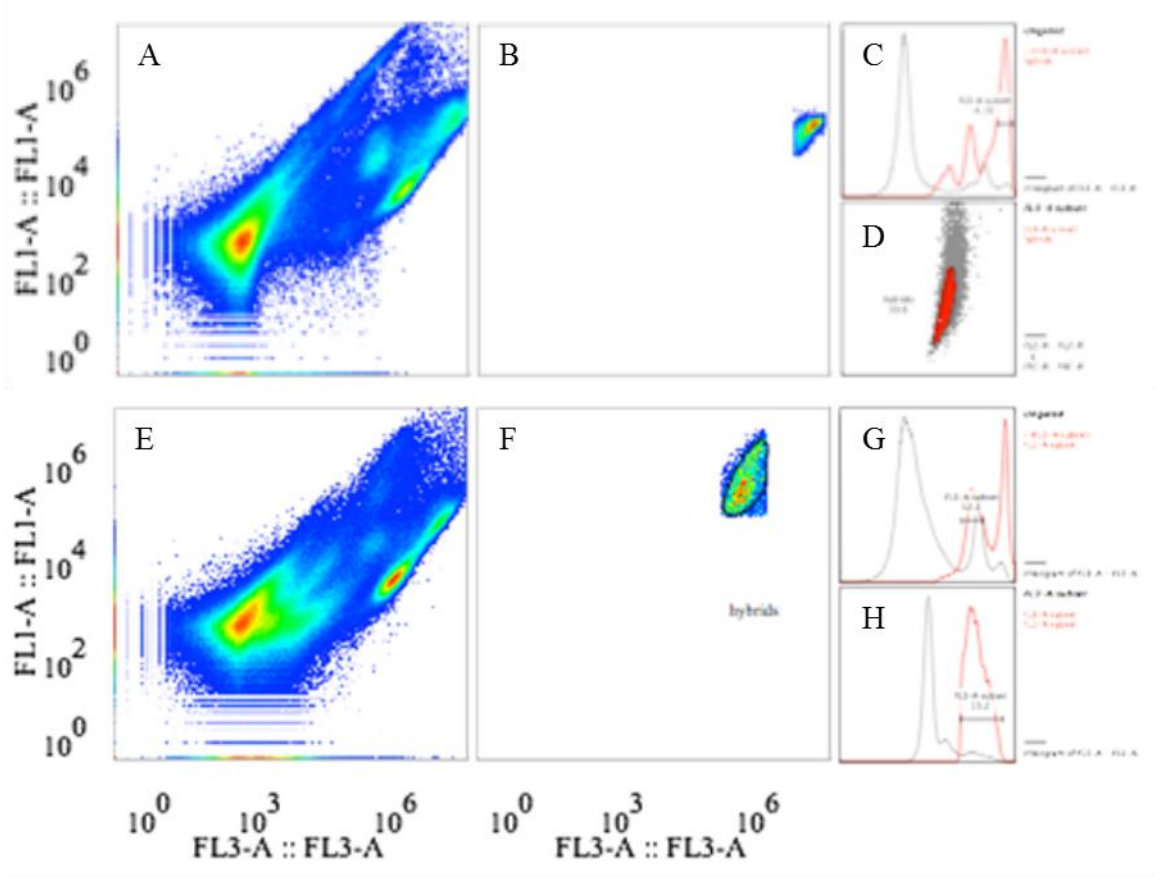

Fig. 3-4. Flow cytometric analysis of protoplast fusion hybridization.
A. Biparametric contour plot of the FL3-A (≥675nm) versus FL1-A (530/30nm) fluorescence emission from a single plot of a typical protoplast fusion treatment between *Syringa* x*prestoniae* 'Dancing Druid' and *Syringa reticulata* 'Ivory Silk'. B. Biparametric contour of the FL3-A versus FL1-A plot on which final hybrid counts were determined. These were preceded by gating on the FL2-A versus FSC-A region of panel D. C. Uniparametric histogram of the FL3-A emission with ungated FL1-A (grey) compared to gated FL1-A (red) D. Biparametric gating procedure applied to on the FL2-A versus FSC-A region of panel E. Biparametric contour plot of the FL3-A versus FL1-A fluorescence emission from a single plot of a typical protoplast fusion treatment between *S.* x*chinensis* and *S. reticulata* 'Ivory Silk'. F. Biparametric contour of the FL3-A versus FL1-A plot on which final hybrid counts were determined. These were preceded by gating on the FL1-A (red) uniparamentric histogram region of panel H. G. Uniparametric histogram of the FL1-A emission with ungated FL3-A (grey) compared to gated FL3-A (red) D. Uniparametric histogram of the FL1-A emission resulting from the FL3-A subpopulation gates (G) with ungated FL1-A (grey) compared to gated FL1-A (red)

Experimental Design and Statistical Analysis

Experiments in protoplast isolation were conducted as a repeated measures experiment of a randomized complete block design with three replications. Replicate blocks were randomized during the filling of experimental treatments and all treatments were treated as complete at the washing of digest solutions with CPW25S. Images of treatments were taken in random order as were the flow cytometric analyses. Genotypes were run in separate experiments and all experiments were repeated once. Data was analyzed with the mixed procedure in SAS (version 9.3, SAS Institute, Cary, NC) and time was grouped to define the heterogeneity of the covariance R matrix. An unstructured covariance matrix estimation was specified for the covariance structure of G. Random statements: run*trt run*duration run*trt*duration were used to for the F test was applied in the estimation the least square means and differences in least square means.

Experiments in protoplast fusion were conducted as a randomized complete block design with three replications. Treatments were randomized within blocks during the filling of experimental treatments and all treatments were treated as complete on the addition of protoplast culture medium. Flow cytometric analysis followed the treatment order. Genotypes were run in separate experiments and all experiments were repeated once. Data was analyzed with the mixed procedure in SAS (version 9.3, SAS Institute, Cary, NC). The random statements: run rep(run) run*volts run*durat run*volts*durat were set to ensure the correct F test was applied in the estimation the least square means and differences in least square means.

Results and Discussion

According to Liu et al. (2003), longer durations of exposure of the explant tissue to digestive solutions increases protoplast yield. In the isolation of lilac protoplasts longer digestion exposures did yield higher volumes of lilac protoplasts in several treatments ($P < 0.05$).

However, wide variations in yield preclude the detection of smaller differences in treatments and mandate refinement of technique. The most probable causes for variation are related to experimental design errors and the variation that arises when up to six hours lapse between replicates of the same treatments. Treatments containing hemicellulases from *Aspergillus niger* were significantly different ($P < 0.05$) from several other treatments with the exception of enzyme treatment combinations of similar composition as shown in Fig. 3-5. The value of hemicellulase and the knowledge gained from continued inclusion in future tests was evident in the first experiments and did not change with time. The manufacturing label list a large range of the effective unit concentration (0.3-3.0 units/mg) and narrow glycolytic activity. By comparison the macerating enzymes in either Macerozyme R-10 or Driselase® readily produce sufficient volumes of protoplasts for further work. Treatments containing Driselase®, in particular, were

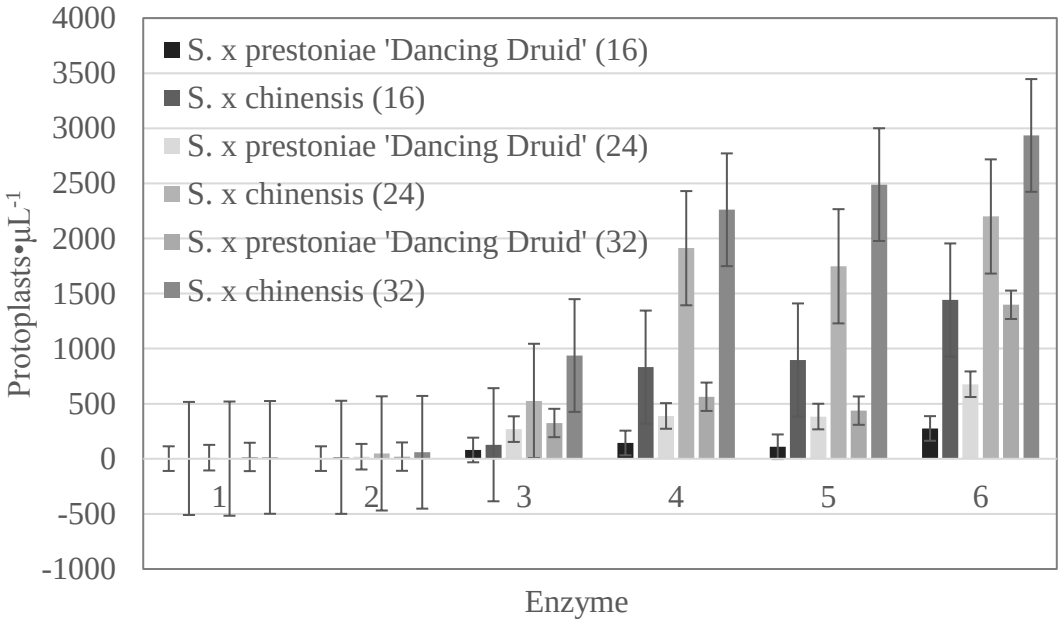

Fig. 3-5. Mean comparisons (±SE) of total protoplast per microliter yields as determined by microscopy data.

very effective at the low concentrations used. Treatments containing Driselase® at higher exposure times liberated indistinguishable protoplast yields from those with many of the Macerozyme containing treatments. The diversity of glycolytic activity, as reported by the vendor, in Driselase® provide a reasonable explanation for the insignificant differences between treatments three, four and five (see Table 3-1.) at 32 hours of digestion. This may also indicate a plausible explanation as to why the flow cytometry analysis yielded such disparate results from microscopic analysis.

Many of the Macerozyme + Driselase® replicates in either genotype contained a solution rich in tissues and organelles. The tissue diversity and quantity would rarely perturb the microscopy analysis, but would occasionally congest the sampling tube on the flow cytometer and add to the total event count for a given sample. The upper limit for event sampling was frequently met within a very small fraction of the sample and the impact of sample size is the most probable explanation for a wide variance.

The optimization of the digestion and early release of protoplasts from their cell walls yield poor environmental conditions under increased exposure times leading to losses in cell viability (Ortin-Parraga and Burgos, 2003). The Flowjo v10.0 cytometer data analysis suite helped to evaluate the fcs flow cytometry data to look for patterns in cell size and complexity. Measurements on mean cell size with FSC-A and various comparisons of other parameters were attempted, but determined a size calibration standard was needed to make such estimates. Total events were compared to the relative debris content were evaluated with several of the plot parameter comparisons available with the four channel BD Accuri C6 output.

Since removal of the epidermis is not a viable option for all plants it becomes apparent that preplasmolysis, vacuum infiltration, and the ability to finely dissect leaves with minimal

damage become increasingly important. While it may or may not be necessary to assess and optimize all of these options in future research a clear understanding of their roles will be important. Specifically, the procedure must attempt to mitigate cell damage and subsequent release of plant phenolic residues into the digestion solution. As a practitioner becomes familiar with the protoplast isolation system the timeframes for preparation and practical use of protoplasts place an idealized timeframe for digestion incubation at around 12-16 hours.

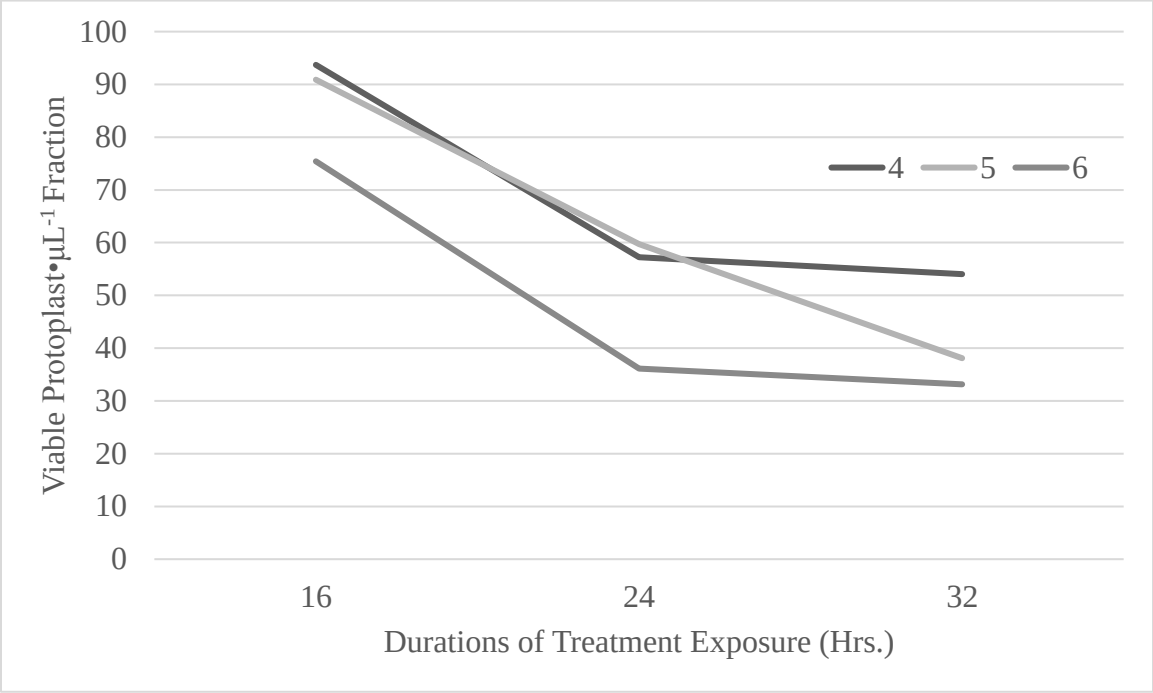

Fig. 3-6. Representation of significant ($P < 0.05$) least square means from viable and total protoplast yields of *Syringa* x*chinensis*.

Optimizing the procedures preceding further protoplast manipulations must focus on maximum protoplast liberation, to within a 3-12 hour timespan. The pattern demonstrated in Fig. 3-6 is representative of the loss in protoplast viability over time, but may or may not capture the peak viable yield only representative of one side of the distribution and furthermore not evident in viable count histograms (Fig. 3-7). Most digestion solutions, regardless of formulation, are

utilized around one to two percent (Liu et al., 2003). The best case scenario with dicotyledonous leaf tissues would be where the epidermis can be removed mechanically or chemically with little tissue damage to the underlying cells. In transient expression assays with *Arabidopsis thaliana* protoplasts, leaves have been physically pulled apart by adhering tape to either side of a leaf and pulling them apart (Wu et al., 2009). While this technique appears at the outset to improve short-term protoplast yield, the challenges in the maintenance of sterile cultures would need to be addressed. Removal of the epidermis alone would likely improve protoplast yields, but the waxy cuticle alone has been inhibitory in preliminary screenings utilizing surfactants and highly acidic or basic solutions.

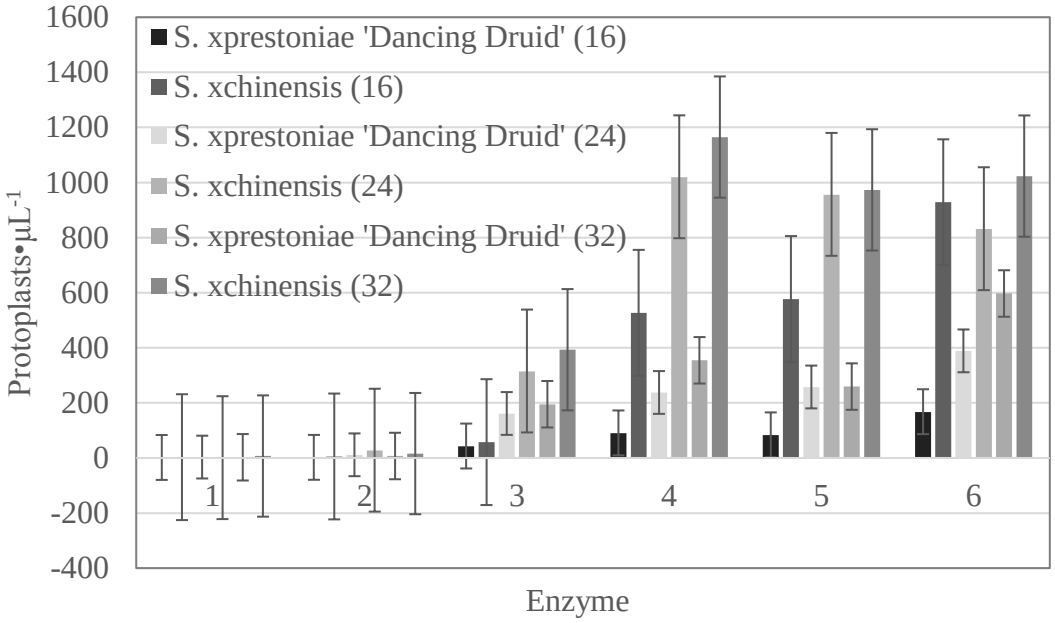

Fig. 3-7. Mean comparisons (±SE) of viable protoplast per microliter yield.

Hand-counting protoplasts is advantageous for being able to distinguish patterns. The pattern that seems to arise in free protoplasts is that cells with varying chloroplast content appear to have differential fluorescence emissions when stained with the cell viability indicator FDA. In

order to determine how these cell types might arise in the byproducts of the protoplast digestion slurry, it was simply a matter of looking at the transverse sections of a typical leaf blade. In the transverse sections of a dicotyledonous plant leaf blade it is possible to distinguish a few elements that are common: the cuticle, epidermis, palisade layer, spongy mesophyll, the vascular bundle(s), and supporting tissues. The distinction worth noting is where the cells with the most abundant chloroplasts reside (chlorenchyma) and where cells with lower chloroplast abundance localize (collenchyma) as seen with the transverse section of *Syringa vulgaris* (Fig. 3-8.). It is in this perspective that one can establish that these differences may have a relationship associated with another phenomenon observed in other plant in vitro applications: regeneration.

The parenchymatous cells of the leaf blade are thought to be the most plastic and make them well suited to handle the in vivo stresses of wound responses or the reinforcement of tissues as a result of tropisms such as light (phototropism), gravity (gravitropism), or touch

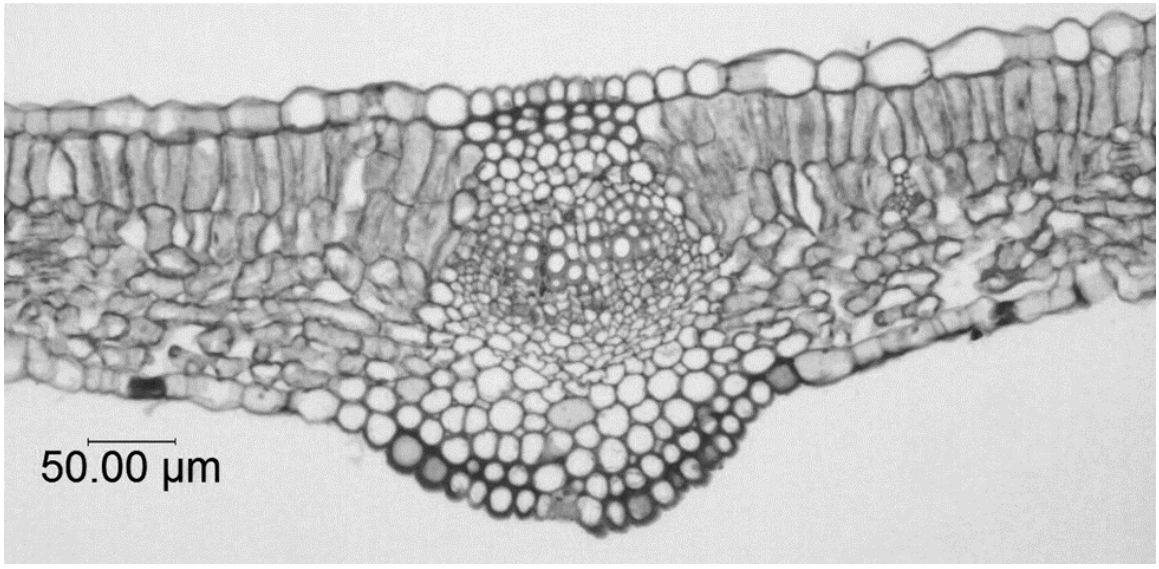

Fig. 3-8. Syringa vulgaris transverse leaf blade section.

(thigmotropism). Moreover, the plasticity of these tissues make them an excellent tissue source for studies in plant biology, biotechnological applications and genetics studies. Often the treatments that are applied in a regeneration procedure include the wounding of the leaf tissue or the placement of the leaf tissue in an abaxial or adaxial orientation on treatment media. Such was the case for Estruch et al. (1991) who found that cytokinin overexpression led to adventive bud formation on the adaxial surface of the leaf in close proximity to the vascular system. The histological patterns that arise in regeneration from leaf tissue appears to be a phenomenon of varied study in the recent past, but has been documented as early as 1959 with *Begonia* spp. leaves by Schraudolf and Reinhardt (1959). According to Fahn (1994), attention to the vasculature commonly involves description of the active bundle sheath cells as well as the supporting tissues that surround them. Attfield and Evans (1991) went on to support Torrey's 1986 description of the adventive organogenesis in tobacco (*Nicotiana tabacum*) as having a similar organizational position as the adventive tissues found in the root pericycle. These root pericycle tissues are the source of tissue.

In personal communication with biologist Mihai Costea, a biologist at Wilfred Laurier University with histological experience in *Syringa* and the *Oleaceae*, the abundance of these cells within the leaf tissue not only localize to the venation on many dicotyledonous plants, but are furthermore most abundant at the base of leaf blades. Their appearance under a typical compound microscope is also reminiscent of another kind of cell: a meristem. According to Fahn (1990), meristems are usually thin-walled more isodiametric in shape, and rich in protoplasm. When meristematic cells are liberated from their cell walls they can furthermore be characterized by their lack of pigmented plastids, which are often in the proplastid (undifferentiated) stage of development (Fahn, 1990).

The aforementioned distinguishing features of leaf blade also have an impact on the consideration to utilize native chlorophyll as a means to distinguish the hybrid populations. While chloroplasts emit a strong fluorescent signal, variation in the signal strength may yield reduced hybrid detection. Regardless, the hybrid populations derived from the various electroporation settings revealed no significant differences in treatments. Treatments applied regularly produced somatic fusion hybrids (Fig 3-9), yet the yields of these different groups was small. The individual runs of hybridization treatments had significant voltage treatment effects in the *S*. xprestoniae 'Dancing Druid' x S. reticulata 'Ivory Silk' populations, yet the outcome in the second run did not confirm the same results.

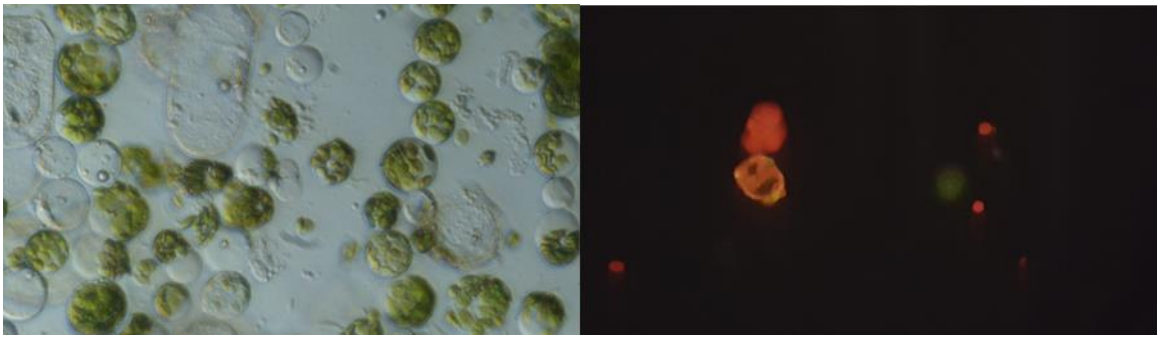

Fig. 3-9. Protoplast fusion hybridization and heterogeneous mix of plant protoplasts. Left is a representation of pearl chain alignment under dielectrophoretic field conditions following fusion (x40). Right is a fluorescent image of FITC labeled protoplasts (green) chlorophyll emission (red) and characteristic oblong shape associated with suspected hybrids (orange) (x40).

The challenge in identifying hybrids in the number of plots that can be generated is no small task. The challenge of purifying channel data on a two dimensional plot are two-fold. Once the population resulting from a subset of the population of other plots (gating) are plotted in the same or different channel distribution plots, a shift in the intensity (as shown in Fig. 3-4. E vs. F) can result and parent or grandparent gates may need to be adjusted accordingly. When multiple

gating parameters need to be used to find the sub: sub-populations it is fastest to set the gating parameters concurrently. There is no substitute for experience, and each plot may require additional fine resolution adjustment to set the gates in accordance with the data as it was plotted. The last and perhaps most important issue is that hybrids present data from both populations in the entire plot. The unique signature of hybrids may not be evident in any of the typical uniparamentric histograms or biparametric contour plots. Three dimensional representations of biparametric contour plots (terrain plots) presented the data in a way that clearly identified features unique to hybrid populations. Unfortunately, software malfunctions prevented their presentation here. Redenbaugh et al. (1982) utilized a single channel versus a representation of two channels (proportional) in terrain plots to identify the fluorescent signal composition of hybrids. The means to distinguish different populations based on the fluorescent signals is a prerequisite to sort the heterokaryon population from the heterogeneous fusion mix.

References

Attfield, E.M. and P.K. Evans. 1991. Developmental pattern of root and shoot organogenesis in cultured leaf explants of Nicotianum tabacum cv. Xanthi nc. J. Exp. Bot. 42(234): 51-57.

Banks, M.S. and P.K. Evans. 1976. A comparison of the isolation and culture of mesophyll protoplasts from several Nicotiana species and their hybrids. Plant Sci. Lett. 7:409-416.

Galbraith, D.W. 2009. Simultaneous flow cytometric quantification of plant nuclear DNA contents over the full range of described angiosperm 2C values. Cytometry Part A. 75A:692-698.

Davey M.R., P. Anthony, J.B. Power, and K.C. Lowe. 2005a. Plant protoplast technology: Current status. Acta Phys Plant 27(1):117-129.

Davey M.R., P. Anthony, J.B. Power, and K.C. Lowe. 2005b. Plant protoplasts: Status and biotechnological perspectives. Biotech. Adv. 23:131-171.

De Filippis, L.F., R. Hampp, and H. Ziegler. 2000. Membrane permeability changes and ultrastructural abnormalities observed during protoplast fusion. J. Plant Phys. 156:628-634.

Doležal, J., P. Binarová, and S. Lucretti. 1989. Analysis of nuclear DNA content in plant cells by flow cytometry. Biologia Plantarum (Praha). 31(2):113-120.

Eeckhaut, T., P.S. Lakshmanan, D. Deryckere, E. Van Bockstaele, and J. Van Huylenbroeck. 1991. Progress in plant protoplast research. Planta. 238:991-1003.

Fahn, A. 1990. Plant anatomy. 4th ed. Pergamon Press. Oxford, New York.

Grosser, J.W., M. Ćalović, and E.S. Louzada. 2010. Protoplast fusion technology – Somatic hybridization and cybridization, p. 175–198. In: M.R. Davey and P. Anthony. Plant cell culture: Essential methods. Wiley-Blackwell. The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK.

Kao, K.N. and M.R. Michayluk. 1975. Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. Planta. 126:105-110.

Klercker J.A.F. 1892. Eine methode zur isolierung lebender protoplasten. Ofvers. Vetensk. Akad. Forh. Stokh. 9: 463-475.

Liu, J., X. Xu, X. Deng. 2003. Protoplast isolation, culture and application to genetic improvement of woody plants. J. Food Agri. & Envir. 1(3-4):112-120.

Liu, C.P., L. Yang, H.L. Shen, and J.M. Cong. 2013. Somatic embryogenesis and plant regeneration from immature embryo cotyledons of *Syringa reticulata* Blume var. *Mandshurica* Hara. Prop. Orn. Plants. 13(2):65-72.

Lloyd, G. and B. McCown. 1980. Commercially feasible micro-propagation of Mountain Laurel (*Kalmia latifolia*), by use of shoot-tip culture. Proc. Intern. Plant Prop. Soc. 30:421-427.

Murashige, T., D.P.H. Tucker. 1969. Growth factor requirements of *Citrus* tissue culture. Proc. First Intl. Citrus Symp. 3:1155–1161.

Nickrent, D.L., Costea, M., Barcelona, J.F., Pelser, P.B. & Nixon, K. 2006 onwards) PhytoImages. Available from: http://www.phytoimages.siu.edu.

Nissing, E., A. Müller, and N. Weiß. 2007. Determinations of suitable electrofusion parameters for the somatic hybridization of mint protoplasts with the Eppendorf Multiporator®. Applications. Note142:1-5.

Ochatt, S.J. 1994. Plant regeneration from protoplasts of *Forsythia* X *intermedia* cv. Spring Glory. Abstracts IAPTC VIII Int. Congr. Plant Tiss Cult. Florence, Italy: 27.

Ochatt, S.J., and E.M. Patat-Ochatt. 1995. Protoplast technology for the breeding of top-fruit trees (*Prunus, Pyrus, Malus, Rubus*) and woody ornamentals. Euphytica 85:287-294 Kluwer Academic Publishers, Netherlands.

Power, J.B., M.R. Davey, M.S. McLellan, and D. Wilson. 1989. Laboratory manual: Plant tissue culture. Plant Genetic Manipulation Group. Dept. of Botany. Univ. of Nottingham.

Schraudolf, H. and J. Reinert. 1959. Nature. 184:465-466. In: Plant Propagation by tissue Culture.(George EF & Sherrington PD.eds.) 1984. Eastern Press, England.

Torrey, J.G. 1986. Endogenous and exogenous influences of the regulation of lateral root formation. p. 31-66. In: M.B. Jackson. Developments in plant and soil sciences. Vol. 20. New root formation in plants and cuttings. Martinus Nijhoff Publishers. Dordrecht.

Wu, F.H., S.C. Shen, L.Y. Lee, S.H. Lee, M.T. Chan and C.S. Lin. 2009. Tape-Arabidopsis sandwich - a simpler Arabidopsis protoplast isolation method. Plant Meth. 5:16 doi: 10.1186/1746-4811-5-16.

Zimmerman, U., G. Pilwat, and F. Rieman. 1974. Dielectric breakdown of cell membranes. Biophys. J. 14:881-899.

CHAPTER 4. MOLECULAR MARKER ESTABLISHMENT FOR THE IDENTIFICATION OF HETEROKARYONS

Abstract

The application of marker assisted selection is essential in the development of somatic fusion hybrids. Auto-polyploidization is inevitable when contact between heterogeneous cells is left to chance. While simple karyotype and phenotypic assessments can be made on cell colonies and eventually on regenerated plants, subsequent culturing of cell colonies of unknown hybrid origin is costly. A molecular marker based assay was developed in preparation of assays used to differentiate heterokaryons from allopolyploids. Introns within low-copy nuclear genes were targeted to identify polymorphic markers for marker assisted selection. Since low-copy nuclear coding sequences have an increased selective pressure for sequence integrity these regions would be unlikely to give rise to an informative polymorphism. Insertion or deletion (indel) mutations within non-coding introns, may give rise to informative and cost effective molecular markers. In the development of a suite of informative markers we targeted four genes within the flavanol and anthocyanin biosynthetic pathways. Intron spanning degenerate primers were designed based on the BLAST consensus sequence alignments from members of the core eudicots. Preliminary screenings revealed the highest consistency and information content when exonic primers spanning intron 2 of a putative dihydroflavonol 4-reductase (DFR) gene was applied to the 13 *Syringa* species, S. hybrids and additional genera tested from within the *Oleaceae*. Amplicon sequencing revealed consistent 5-13 base pair differences unique to each taxonomic series of *Syringa* tested. As a codominant marker system these kinds of markers can be a very effective means to identify and distinguish heterokaryons and allopolyploids in a diverse population of similar hybrids.

Introduction

Since the 1970's, molecular marker assisted selection (MAS) has been a tool that has allowed breeders the opportunity to move from phenotypic scoring novel germplasm to a genotypic selection system. The utility of MAS is vast and can be applied to marker trait associations for characteristics of interest, taxonomic ordering and phylogenetic studies. The applications of molecular markers are increasingly important for scientists and breeders to understand and apply. The applications of the marker system, and the resources at a breeders disposal are important considerations in the integration of MAS to the program. To date information on molecular marker systems suited for applied breeding purposes in lilac (*Syringa* spp.) improvement are not common. Microsatellites have been described in the species conservation efforts of *S. josikaea* (Lendvay et al., 2013) and germplasm collection integrity has been successfully maintained with the application of RAPD's (Kochieva, et al. 2004).

The correct identification of hybrids in the in vitro breeding of hybrids, somatic fusion, makes the successful application of molecular markers tantamount to success. When a heterogeneous population of protoplasts are stimulated with short electrical bursts, thousands of individual hybridization events occur simultaneously. These electrical impulses stimulate the transient cell membrane disruption and pore formation that permit spontaneous fusion of two or more cells. The inevitability for fusing multiple cells of the same species makes the process of culturing colonies of cells of unknown origin a daunting task to sort through. Verification of hybridity permits sorting through colonies to find a population of cells worthy of continued culture, and the effort involved in regeneration. One particular method to determine true heterokaryons, or cells with nuclear material derived from multiple origins, would be similar to the techniques applied in phylogenetic or diversity studies.

Low copy highly conserved genetic sequences have a low threshold tolerance for mutations and as such, few mutations have occurred throughout evolution. Mutations of these types of genes have a high opportunity cost since loss of function changes are potentially lethal. As the coding sequences would have an increased pressure for the maintenance of sequence integrity these regions would be highly unlikely to give rise to an informative marker. However, the variations giving rise to an informative insertion or deletions (indels) may be found in the less conserved intron regions of the sequence (Sang, 2002; McClean et al., 2004 & 2007). Since non-coding intron regions have a reduced impact on the plant physiology, mutations within the intron may give rise to informative molecular markers. The indels could be readily identified through a simple polymerase chain reaction (PCR) based assay where the primers are based on the forward 5' end of the exon sequence and the reverse of the 3' end of the downstream coding sequence that reside on either side of an intron of interest. Different alleles could readily be scored through differential sorting of amplicons based on the length of fragment sequences in an electrophoretic gel (McClean et al., 2004).

Sequences like that of dihydroflavonol 4-reductase (DFR), chalcone isomerase (CHI), flavanone 3-hydroxylase (FHT), and flavonol synthase (FLS) are examples of highly conserved low copy genetic sequences that are involved in the flavonol and anthocyanin biosynthetic pathway. The nucleotide sequence has been determined in a number of species and as such can give a predictive genetic primer design template. The 20 amino acids that make up proteins are coded by 64 possible codon combinations. Therefore, multiple codon sequences can give rise to the same translated amino acid; called degeneracy. Multiple species with alignment of nucleotide sequences of the same gene can also give rise to the same translated protein sequence yielding differences in areas where a primer may be chosen; called redundancy. Alignment of sequenced

DFR genes from multiple genera with a shared evolutionary history can elucidate introns flanked with exons of satisfactory shared homology for primer design.

Materials and Methods

Plant Material

Leaf tissues were sampled for the analysis of sequence diversity. Plants of different taxonomic series were originally selected on phylogenetic similarities between the different groups with greater, moderate and divergent evolutionary histories (Kim and Jansen, 1998; Li et al., 2012). The cultivars included in this study included representatives from four taxonomic groups of the *Syringa* series *Ligustrina*: *S. pekinensis* 'Zhang Zhiming' (01), *S. pekinensis* 'Morton' (12), series *Syringa*: *S.* x*chinensis* (03), series *Pubescentes*: *S.* 'Penda' (24), and series *Villosae*: *S.* x*prestoniae* 'Miss Canada' (06), *S.* x*prestoniae* 'James Mcfarlane' (05), *S.* x*prestoniae* 'Donald Wynman' (07), *S.* x*prestoniae* 'Minuet' (13), *S.* x*prestoniae* 'Dancing Druid' (14).

DNA Isolation, PCR Amplification and Sequence Analysis

Leaf tissues were excised from tissue culture grown plants and landscape plantings on the campus of North Dakota State University (NDSU; Fargo, ND, USA). Approximately 500-1000 mg of tissue was collected from each sample species, frozen in liquid nitrogen and stored at -80 °C until all tested varieties had been accumulated. Under liquid nitrogen the tissue was crushed and approximately 50-100 mg of coarse ground tissue was collected for extraction using the IBI Scientific Genomic Plant DNA Mini Kit (IB47231, IBI Scientific 9861 Kapp Court Peosta, IA 52068, USA). DNA concentration was quantified on the NanoDrop™ 2000 spectrophotometer (ND-2000, Thermo Fisher Scientific, 81 Wyman Street, Waltham, MA 02451, USA). DNA concentration was adjusted to 15-20 ng/µL by adding MilliQ water or concentrated on a Savant

DNA120 vacuum concentrator. Tissue samples were preliminary screened with a degenerate primer set as described in McClean et al. (2004). The DFR nucleotide sequences from *Forsythia* x*intermedia* (Y09127.1), *Capsicum annuum* (JN885196.1), *Camellia sinensis* (AY648027.1), *Fragaria vesca* (KC894053.1), *Solenostemon scutellarioides* (EF522155.1 & EF522156.1), *Torenia hybrida* (AB012924.1), *Mimulus aurantiacus* (EU305679.1 & EU305680.1), *Angelonia angustifolia* (KF285561.1 & KJ817183.1), *Erythranthe lewisii* (KJ011136.1), and *Scutellaria viscidula* (FJ605512.1) were aligned with Multalin software (http://multalin.toulouse.inra.fr/multalin/ multalin.html) to assess intron and exon sequence diversity for plausible intron spanning primer sites (Corpet, 1988). Primers were designed based on sequence data from *Forsythia* x *intermedia* (Y09127.1) spanning the second intron as forward 5'-GTG TTC ACT TCC TCT GCT GGA ACT GT-3' (FiDFR-i2) and reverse 5'-GGG AGT TAA CAT CCG AAC TAG TGA AGT A-3' (FiDFR-e2r). Fifteen nanograms of DNA were used for each cultivar and amplified using the reagent concentrations described in Brady et al. (1998) and amplification conditions of 94 °C for 3 min; 45 cycles of 94 °C for 20 seconds, 55 °C for 30 seconds, and 72 °C for 1 min; 1 cycle of 72 °C for 10 min. Amplified fragments were then purified on a 2% agarose containing Tris-borate-EDTA (TBE) gel. Fragments were cut from the gel and further purified with the Wizard® SV Gel and PCR Clean-up System (A9281, Promega Corporation 2800 Woods Hollow Road, Madison, WI 53711, USA). Amplified fragments were sequenced on the ABI 3730xl DNA Sequencers by Eton Biosciences (Eton Bioscience, Inc. 5820 Oberlin Drive, Suite 108, San Diego, CA 92121, USA). Sequence data were aligned with Multalin (Corpet, 1988).

Results and Discussion

Preliminary screenings with degenerate primers specific to anthocyanin and flavonol genes (CHI, FLS, FHT, and ANS) demonstrated conservation of sequence integrity for low copy nuclear genes. At varying annealing temperatures these primer sets were indicative of some of the diversity within *Syringa* spp. Consistency within the original assessments required refinement for downstream analysis of somatic fusion hybrids. Degenerate primers from DFR regularly amplified greater information content and were subsequently targeted for refinement through primer redesign and expansion of the original assessment panel.

Changing the pairing affinity for the DNA and the primer sets by lowering annealing temperature yielded expected changes in the number of amplified bands. Multiple bands from *S.* x*prestoniae* 'Miss Canada' along with the predominant bands from the other cultivars were sequenced to assess the amplification of intron 2 of DFR. Early attempts had a variety of sequences that would align to nonspecific regions of the genome. The first redesigned primer set, as described within the materials and methods section, was designed within the exon to provide an increase in the amount of conserved exon sequence present for alignment purposes.

Sequence diversity for the tested varieties demonstrated indel polymorphisms unique to each taxonomic series (Fig. 4-1.). Common indels grouped taxonomic series together without exception in the panel assessed. The *Villosae* series was the only group to have a SNP at the sixth base pair downstream of the forward primer (Fig. 4-2.). This SNP uniquely distinguished *S.* x*prestoniae* 'Miss Canada' from the remainder of the group. The conservation of sequence data within the tested population bolsters the utility of low copy nuclear molecular markers for the screening of a somatic fusion hybrid population.

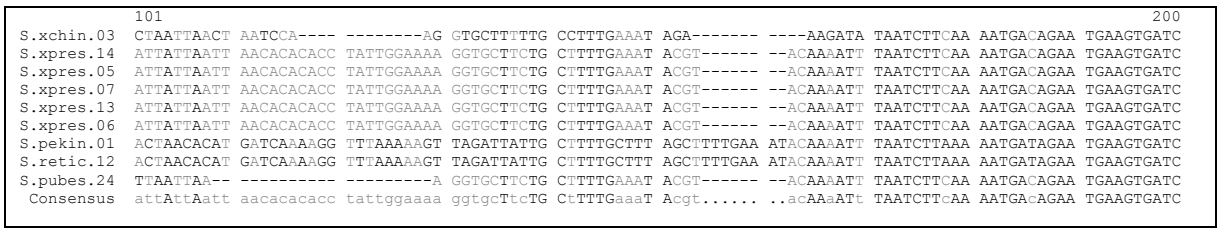

Fig. 4-1. Nucleotide polymorphisms among tested *Syringa* spp. intron 2 of dihydroflavonol 4-reductase.

Black letters indicate consensus sequence. Gray letters indicate low consensus sequence. Dashes (-) indicate indels. (01) *Syringa pekinensis* 'Zhang Zhaming', (03) *Syringa* x*chinensis*, (05) *Syringa* x*prestoniae* 'James Mcfarlane', (06) S. x*prestoniae* 'Miss Canada', (07) S. x*prestoniae* 'Donald Wynman', (12) *Syringa reticultata*, (13) S. x*prestoniae* 'Minuet', (14) S. x*prestoniae* 'Dancing Druid', (24) *S.* 'Penda'.

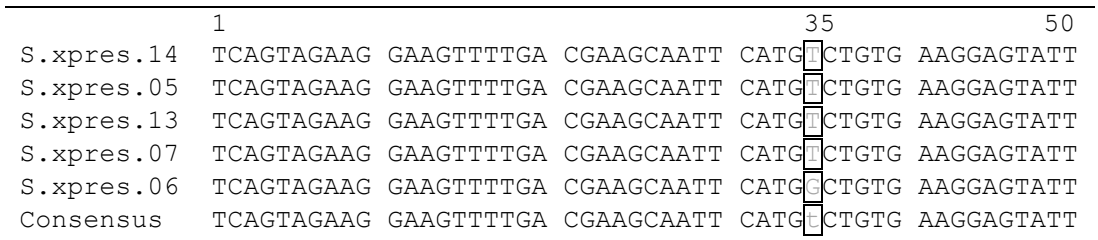

Fig. 4-2. Single nucleotide polymorphism (SNP) located 5 bases downstream of the forward primer (FiDFR-i2) within the *Villosae* taxonomic series of the genus *Syringa.*

(05) Syringa x*prestoniae* 'James Mcfarlane', (06) S. x*prestoniae* 'Miss Canada', (07) S. x*prestoniae* 'Donald Wynman', (13) S. x*prestoniae* 'Minuet', (14) S. x*prestoniae* 'Dancing Druid'

References

Brady, L., M.J. Bassett, and P.E. McClean. 1998. Molecular markers associated with T and Z, two genes controlling partly colored seed coat patterns in common bean. Crop Sci. 38:1073-1075.

Corpet, F. 1988. Multiple sequence alignment with hierarchical clustering. Nucl. Acids Res., 16(22):10881-10890.

Hall, B.E. 2013. Building phylogenetic trees from molecular data with MEGA. Mol. Biol. Evol. doi:10.1093/molbev/mst012.

Kim and Jansen, 1998 Kim, K.J. and R.K. Jansen. 1998. A chloroplast DNA phylogeny of lilacs (Syringa, Oleaceae): Plastome groups show a strong correlation with crossing groups. Am. J. Bot. 85(9):1338-1351.

Kochieva, E.Z., N.N. Ryzhova, O.I. Molkanova, A.M. Kudryavtsev, V.P. Upelniek, and I.B. Okuneva. 2004. The genus *Syringa*: Molecular markers of species and cultivars. Russian J. Genetics 40(1):37-40.

Lendvay, B., A. Pedryc, and M. Höhn. 2013. Characterization of nuclear microsatellite markers for the narrow endemic *Syringa josikaea* Jacq. fil. ex Rchb. Not. Bot. Hort. Agrobo. 41(1):301-305.

Li, J., B. Goldman-Huertas, J. DeYoung, and J. Alexander III. 2012. Phylogenetics and diversification of Syringa inferred from nuclear and plastid DNA sequences. Castanea 77(1):82-88.

McClean, P.E., R.K. Lee, and P.N. Miklas. 2004. Sequence diversity analysis of dihydroflavonol 4-reductase intron 1 in common bean. Genome 47:266-280.

McClean P.E., R.K. Lee. 2007. Genetic architecture of chalcone isomerase non-coding regions in common bean (*Phaseolus vulgaris* L.) Genome 50:203-214.

Sang, T. 2002. Utility of low-copy nuclear gene sequences in plant phylogenetics. Crit. Rev. Biochem. Molec. Biol. 37(3):121-127.

APPENDIX

Table A-1. Composition of *Syringa* micropropagation media

	mass ($mg \cdot L^{-1}$)	μM
Organics		
Myo-Inositol	150	832.5933
Glycine	2	26.6418
Nicotinic Acid	0.5	4.061738
Pyridoxine HCl	0.5	2.431907
Thiamine HCL	1	2.964984
Phosphates & Oxides		
Potassium Phosphate Monobasic	174.26	1280.514
Boric Acid	6.2	100.2701
Sodium Molybdate	0.25	1.033485
Nitrates		
Ammonium Nitrate	1231.4	15384.81
Potassium Nitrate	1242	12284.87
Calcium Nitrate	556	2354.436
Calcium		
Calcium Chloride	96	653.0612
Calcium Gluconate	650	214.1439
Sulfates		
Magnesium Sulfate Heptahydrate	374.52	1519.474
Manganese Sulfate Monohydrate	22.3	131.937
Zinc Sulfate Heptahydrate	8.6	29.91304
Cupric Sulfate Pentahydrate	0.25	1.001201
Potassium Sulfate	990	5681.166
Iron		
Disodium Ethylenediaminetetraacetic Acid	37.25	100.0698
Ferrous Sulfate Heptahydrate	27.85	100.1799
Carbohydrates		
Sucrose	10000	
Maltose	10000	
Gelling Agents		
Agar	4000	
Gelrite® (Gellan Gum)	1400	
pH (post autoclavation target)	5.4	

Table A-2. Analysis of variance for the effects of hybrid counts and hybrid portion for both experimental runs of the *Syringa* x*chinensis* X *S. reticulata* 'Ivory Silk' tests of the Epppendorf Multiporator® electroporation settings (1000, 2000, and 4000 V•cm^{-1}) and 40 or 80 μs of voltage application.

Effect	Num DF	Den DF	F Value	Pr > F
volts	2	11	0.17	0.8429
durat	1	11	2.28	0.1595
volts*durat	2	11	1.67	0.2332

Run 1 Hybrid counts

Effect	Num DF	Den DF	F Value	Pr > F
volts	2	10	0.02	0.9831
durat	1	10	1.87	0.201
volts*durat	2	10	1.18	0.3455

Run 2 Hybrid counts

Effect	Num DF	Den DF	F Value	Pr > F
volts	2	11	0.32	0.7328
durat	1	11	3.24	0.0992
volts*durat	2	11	2.38	0.1388

Run 1 Hybrid portion

Effect	Num DF	Den DF	F Value	Pr > F
volts	2	10	0.02	0.9809
durat	1	10	1.74	0.2167
volts*durat	2	10	1.17	0.3491

Run 2 Hybrid portion

Table A-3. Analysis of variance for the effects of hybrid counts and hybrid portion for both experimental runs of the *Syringa* x*prestoniae* 'Dancing Druid' X *S. reticulata* 'Ivory Silk' tests of the Epppendorf Multiporator® electroporation settings (1000, 2000, and 4000 V•cm^{-1}) and 40 or 80 μs of voltage application.

Effect	Num DF	Den DF	F Value	Pr > F
volts	2	10	5.43	0.0253
durat	1	10	2.82	0.1242
volts*durat	2	10	0.07	0.9337

Run 1 Hybrid counts

Effect	Num DF	Den DF	F Value	Pr > F
volts	2	10	6.62	0.0148
durat	1	10	3.12	0.108
volts*durat	2	10	1.98	0.1892

Run 2 Hybrid counts

Effect	Num DF	Den DF	F Value	Pr > F
volts	2	10	5.43	0.0254
durat	1	10	2.82	0.1238
volts*durat	2	10	0.07	0.9334

Run 1 Hybrid portion

Effect	Num DF	Den DF	F Value	Pr > F
volts	2	10	3.75	0.0608
durat	1	10	0.23	0.6392
volts*durat	2	10	3.88	0.0566

Run 2 Hybrid portion

Table A-4. Analysis of variance for the effects of enzyme treatment and duration of exposure on microscope collected data of protoplast counts and viable protoplast counts as well as flow cytometric collected data on total protoplast counts for *Syringa* x*chinensis*.

Effect	Num DF	Den DF	F Value	Pr > F
trt	5	5	7.08	0.0255
Duration	2	2	7.16	0.1226
trt*Duration	10	10	2.16	0.1205

Flow cytometric collected data on protoplast counts

Effect	Num DF	Den DF	F Value	Pr > F
trt	5	5	7.41	0.0232
Duration	2	2	7.21	0.1218
trt*Duration	10	10	3.43	0.0325

Fluorescence microscopy collected data on viable protoplast counts

Effect	Num DF	Den DF	F Value	Pr > F
trt	5	5	6.14	0.0341
Duration	2	2	84.39	0.0117
trt*Duration	10	10	9.56	0.0007

Bright field microscopy collected data on total protoplast counts

Table A-5. Analysis of variance for the effects of enzyme treatment and duration of exposure on microscope collected data of protoplast counts and viable protoplast counts as well as flow cytometric collected data on total protoplast counts for *Syringa* x*prestoniae* 'Dancing Druid'.

Effect	Num DF	Den DF	F Value	Pr > F
trt	5	5	21.47	0.0022
Duration	2	2	2.11	0.322
trt*Duration	10	10	1.72	0.2036

Flow cytometric collected data on protoplast counts

Effect	Num DF	Den DF	F Value	Pr > F
trt	5	5	6.35	0.0318
Duration	2	2	3.18	0.2393
trt*Duration	10	10	3.48	0.0309

Fluorescence microscopy collected data on viable protoplast counts

Effect	Num DF	Den DF	F Value	Pr > F
trt	5	5	21.44	0.0022
Duration	2	2	8.34	0.107
trt*Duration	10	10	4.72	0.011

Bright field microscopy collected data on total protoplast counts