New Approaches in Engineering Somatic Embryogenesis in Loblolly Pine Suspension Cultures

Elizabeth Morgan Cummings Bende

University of Massachusetts Amherst

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NEW APPROACHES IN ENGINEERING SOMATIC EMBRYOGENESIS IN LOBLOLLY PINE SUSPENSION CULTURES

A Dissertation Presented
By
ELIZABETH MORGAN CUMMINGS BENDE

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY
May 2018

Chemical Engineering
NEW APPROACHES IN ENGINEERING SOMATIC EMBRYOGENESIS IN LOBLOLLY PINE SUSPENSION CULTURES

A Dissertation Presented
By
ELIZABETH MORGAN CUMMINGS BENDE

Approved as to style and content by:

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Susan C. Roberts, Chair

________________________________________
Sarah L. Perry, Member

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Samuel P. Hazen, Outside Member

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John Klier, Department Head
Chemical Engineering
DEDICATION

To my Mom, who is the reason I knew I wanted to pursue science at the age of 10, and to my Dad, who always knew I was meant to be an engineer, even when I didn’t.

Thank you both for your encouragement, support, and love.
ACKNOWLEDGEMENTS

This dissertation work would not have been possible without the support of my advisor, Professor Susan Roberts. Sue has been a positive influence on my career since the beginning, providing perspective from beyond the PhD and constantly challenging me. In her group, I’ve learned the confidence to give a good talk and ask questions. I’ve also learned the benefits and challenges to working with plant cell culture and am excited to apply this perspective in my future endeavors. I’m forever thankful for all the opportunities Sue has afforded me throughout my PhD and I look forward to applying what I’ve learned in my career.

I would also like to thank my committee members. Sarah Perry has given me very helpful advice throughout the PhD journey and provided a unique perspective on my project. We somehow always ended up on the same flights to AIChE, even when I was flying from Cleveland! Sam Hazen also provided invaluable help, and I enjoyed working with his post-docs in recent years to discuss loblolly pine cultures. In such a small academic niche, I feel so fortunate to have Sam on my committee since he works with the same system. Numerous other faculty members have supported me and encouraged me throughout my work, including Danny Schnell (Michigan State) who briefly served on my committee in my second year and Luis Vidali (WPI) who shared his microscopy expertise and was always a smiling face in the lab. Lou Roberts was invaluably helpful and never said no when I needed guidance (or supplies!) in my protein work and provided encouragement no matter what the situation.

The entire team at Weyerhaeuser made this research a reality. When I joined Sue’s group in my first year, I was surprised to be the first to focus my research entirely on a plant system that wasn’t Taxis. The project started with samples shipped to UMass, and eventually evolved into overnight shipments of cultures and the transfer of protocols for embryo development. I learned so much in this process, and I could not have done so without...
the confidence and support of the team in Seattle. A very special thank-you to Tony Swanda and Pat Brownell, who were in it from the start and made the journey across the country to visit the lab and talk about the project. Their passion and support truly unmatched. Also thanks to Robin Horst, who took over the collaboration about halfway through and provided excellent procedural and writing advice. So many others worked on this project, Pramod Gupta, Steve Lund, Jim Grob, and Eric Hanczyc. Thank you for your support, advice, and all of the knowledge you’ve shared with me. And thanks Pat, for the lab care packages!

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ABSTRACT

NEW APPROACHES IN ENGINEERING SOMATIC EMBRYOGENESIS IN LOBLOLLY PINE SUSPENSION CULTURES

MAY 2018

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Directed by Professor Susan C. Roberts

Many industries including agriculture and healthcare require efficient methods for replication of plants with optimal traits. The loblolly pine (Pinus taeda) is a valuable crop in the timber industry, occupying 30 million acres of U.S. land, and breeding efforts aim to produce a crop with ideal phenotypic traits, including superior growth and wood quality.

One method to large-scale clonal crop propagation is somatic embryogenesis (SE), the process through which asexual (somatic) plant cells undergo differentiation in vitro, resulting in germination-competent embryos. There are three main stages of growth and development that lead to the production of embryos: 1) aggregated cells that form embryonic suspensor masses (ESMs) are grown and scaled up in maintenance cultures; 2) ESMs are plated on solid media to initiate SE; and 3) embryos are separated from the ESM and germinated to generate plants. However, this process is not fundamentally understood, leading to large, unpredictable variability in embryo yield, a number only determined 8-12 weeks downstream of SE initiation. This work aims to glean fundamental insight into culture dynamics and correlations with outcomes while simultaneously providing engineering strategies to
improve embryo yield.

Here, a variety of process manipulations and their effects on SE process outcomes and performance are presented. Induction of stress-related pathways through exogenous addition of plant stress hormones prior to moving a culture into development was shown to improve the rate of SE, and this knowledge was used to explore a means for more efficient embryo production. A clear link between the total volume of small cell aggregates and embryo yield was demonstrated, enabling yield prediction at a timepoint 12 weeks before previously possible. Finally, methods for culture protein modification without genetic transformation were developed through inhibition or supplementation of endogenous extracellular arabinogalactan proteins (AGPs), which were found to significantly influence SE in loblolly pine. Techniques were developed to characterize the influence of these treatments on embryo yield as well as the molecular response of the cultures, including culture stress (determined by phenolic content), growth (using a Coulter counter), and total and specific protein biomarkers. The work presented here is among the first studies that consider process engineering as a simple and cost-effective means to improve the overall feasibility of SE on both an academic and industrial scale.
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CHAPTER 1
INTRODUCTION

1.1 A critical need for streamlined pine crop production

There is a critical world-wide need for efficient production and reproduction of high-value crops. The global impact of suboptimal crops is manifold, resulting in global problems including hunger due to crop death, insufficient supply of pharmaceutical natural products due to natural or seasonal variation in production, and resource waste in the production of subpar crops. Natural fertilization in plants leads to genetic variation from crossover of genes during meiosis [1, 2] as well as fusion of gametes from two parents; as such, crops traditionally bred from two elite parents can still vary greatly. The resultant unpredictability hinders commercial feasibility of plant-scale processes, including biofuels feedstock production and production of valuable plant specialized compounds.

One major industry that will benefit from an idealized pipeline for crop production is the timber industry. Timber milling and logging in the United States alone is a $100 billion annual industry [3], and there are 521 million acres of land devoted to commercial wood production in the United States [4]. This land is designated as the timberlands, and since the time-frame for lumber crops to reach maturity ranges from 30-40 years, there is a large investment of land and time tied to the development of wood crops. One important crop in the timberlands is Pinus taeda, or the loblolly pine tree, shown in Figure 1.1. The loblolly pine comprises one half of the standing pine volume in the United States [5] and is an important construction crop used for pulpwood and sawlogs. Loblolly pine is also the principal species used in paper making [6], dictating its importance across numerous industries. At least 1.5 billion loblolly seedlings are planted annually to meet demand [7, 8], underscoring the need for an efficient production scheme.
Due to the long reproductive timespan of pine tree crops, unpredictable variability that arises from natural fertilization in pine cones can be particularly damaging. Reproductive diversity can lead to large variability in crop quality, and the resulting crops can display poor wood quality, slow growth rates, or blight susceptibility. Determination of phenotypic properties (and therefore, prediction of downstream success) of a tree based on genetic information is a possible solution but remains an ambitious goal since in loblolly pine trees characteristics are often determined by several genes [9, 10]. Regardless, this method has been attempted through assembly of expressed sequence tags resulting in development of complex gene linkage maps [9]. More recently the loblolly pine genome was published [7] and at 22 billion basepairs the genome was the largest genome sequenced.
at the time of publication (in 2016 the California Sugar Pine genome was published at 33 billion basepairs [11]). The sequenced genome is useful for tree breeding since more insight into genetic links to success is possible with the availability of a high quality reference genome. Improvements remain underway for the genome assembly; recently, the genome was reassembled with longer reads resulting in a less fragmented and more useful genome assembly for improved annotation and ultimately improved phenotypic linkage maps [12].

One way to avoid the detrimental genetic and therefore phenotypic variation that arises in traditionally bred pine crops is through bypassing this random genetic diversity through clonal propagation of elite trees, made possible through somatic embryogenesis (SE) [13]. SE enables cultivation of superior genotypes with known properties and eliminates wasted resources such as crop land, time, and money spent on unsuccessful crops. SE not only provides a method to limit crop variation with the potential to implement a genetically identical crop [14] but also provides a means to genetically engineer a species [15]. Since growth and overall livelihood of the loblolly pine crop across different regions has been linked to tree genotype, there is an established basis for efficient selection of elite genotypes to propagate for particular climate and soil conditions [16].

1.2 Somatic embryogenesis

This work was carried out in collaboration with Weyerhaeuser NR Co (Seattle, WA), where loblolly pine seedlings are propagated and sold. Since loblolly pine is an important timber crop, efficient reproduction is imperative to timberland productivity. SE is a relatively new technology in loblolly pine, with first reports in the literature beginning in 1987 [17]. The technology has been implemented at an industrial scale at Weyerhaeuser, with patent literature focusing on both improvement and scale-up of the process [18–20].

In SE, asexual (somatic) plant cells form embryos capable of germination in vitro
[21, 22], in contrast to typical seed formation through which plant sexual (germ line) cells fuse to produce a seed. SE bypasses random variation inherent to traditionally bred crop generations rather than relying on the fusion of gametes and any resulting genetic variation due to recombination. SE cultures can be scaled up as needed to the bioreactor level [23], enabling large-scale propagation of a superior crop and providing an invaluable tool especially in plants with long reproductive cycles or in plants that take many years to reach maturity [24]. SE allows selection for traits including superior biomass quality, fast growth rate, disease resistance, drought tolerance, or production of high value metabolites. Therefore, SE has applications in biofuels feedstocks, pharmaceutical crops, timber, and agriculture. Since the entire process of SE occurs in vitro, crops can be produced throughout the year without the need to wait for the next reproductive cycle [24].

To initiate an in vitro loblolly pine embryogenic culture, immature seed is sterilized and a dissection is placed onto initiation medium with growth hormones, including auxins (e.g., 2,4-dichlorophenoxyacetic acid) and cytokines (e.g., benzylaminopurine) [25]. Embryogenic suspensor masses (ESMs) propagate from the dissected tissue and can be scaled up in liquid suspension culture for further scale-up and subsequent embryo development (Figure 1.2). Next, the ESMs are transferred onto solid development medium with the hormone abscisic acid (ABA) and an increase in osmolality, initiating the transition from pre-embryogenic material into somatic embryos. The transfer from maintenance cultures to development media initiates a change in culture metabolism, leading to a differentiation process [21, 23]. In loblolly pine, somatic embryo development takes place over 10-12 weeks on solid media, at which point a final embryo yield value is assessed, defined as the number of embryos produced from a particular culture per plate or tray [26]. After development, embryos undergo a maturation stage (4-7 weeks) that increases germination potential.

It is important to note that not all aggregates in vitro become embryos and not all embryos are capable of germination, leading to limitations in commercial feasibility [28].
Figure 1.2 Overview of the stages of SE in loblolly pine from suspension culture to embling. Total time from maintenance culture to seedling is 6-8 months [25]. Images rearranged and reproduced with permission from SA Wilson [27].

The scope of this work is concerned with improving or predicting embryo yield, since this value alone provides enough variability to significantly impact commercialization. In fact, Weyerhaeuser experiences extremely variable loblolly SE yields, with yields ranging from 30 to 2000 embryos per plate in a period of just two months (see Chapter 3). Since the value for embryo yield can only be measured 10-20 weeks after the establishment of a maintenance culture, and germination success takes several additional months [25], this variability severely limits commercialization. Currently, there are no quantitative metrics to predict culture success, so all cultures are transitioned through all three phases of development, resulting in high experimental cost and time requirements and reducing productivity and profitability. By identifying a biomarker that can predict culture success after culture initiation (i.e., high embryo yield), experimental and commercial cost would decrease. Yield prediction would enable screening of cultures throughout maintenance, allowing poorly performing cultures to be eliminated from plating endeavors as early as the first phase of development.
1.3 Cell types and the importance of extracellular signaling processes in somatic embryogenesis

ESM tissue in maintenance cultures is comprised of various cell types spanning a large size range. Plant cell cultures typically grow in aggregates ranging from 100-500 \( \mu m \) in diameter [29]. SE cultures also aggregate, but can be much larger, with diameters up to 1000 \( \mu m \) in banana [30], greater than 1000 \( \mu m \) in date palm [31], and up to 1500 \( \mu m \) in loblolly pine (see Chapter 2). Embryogenic suspension cultures contain aggregates called proembryonic masses (PEMs), designated PEM I, II, or III depending on developmental state (Figure 1.3) [32]. PEM I’s are the least complex cell type, and are generally comprised of a single vacuolated suspensor cell attached to a dense mass that comprises the pre-embryonic head. The PEM I cell types grow and divide and form a larger embryonic head (made of embryogenic cells, as opposed to the long vacuolated cell types) attached to more than one suspensor as they transition into PEM II cell types [33]. As they become more aggregated and organized, the aggregates transition into PEM III’s. Based on isolation of individual Norway Spruce PEM types in individual agar gels, it is hypothesized that PEM III’s are the aggregates capable of transitioning into somatic embryos [32]. The cycling of these cell types has also been studied in *Araucaria angustifolia* (Brazilian pine) by microscopically examining aggregate types removed from an actively proliferating culture.

While it is interesting to consider that these more organized cells are themselves capable of SE, this hypothesis was developed through isolation of PEMs [34] or observed via microscopy after removal from a culture without dynamic insight into development [33]. Since isolating cellular structures can compromise many important cell-cell interactions and signaling pathways involved in SE, the results may not be representative of what happens during typical SE.

Numerous studies across several systems have demonstrated that soluble signaling
molecules play a critical role in SE fate [23, 35, 36], and these extracellular signals can be generated by other cells in vitro. Many specific extracellular proteins have been implicated in SE signaling, such as arabinogalactan proteins (AGPs) in cotton [37] and carrot [38], lipid transfer proteins in carrot [39], and numerous other classes of extracellular proteins, as reviewed by Smertenko and Bozhkov [24]. Therefore, by isolating individual aggregates, critical extracellular interactions in SE cultures are ignored and critical information regarding somatic embryo development may be overlooked. Motivated by these limitations, previous work in our laboratory has been done to develop methods to characterize loblolly tissue using the Coulter counter [27]. The work presented here aims to gain more comprehensive insight into factors that affect SE progression by studying the entire culture and its
response rather than disturbing the integrity of a culture to glean insight into molecular and cellular processes.
1.4 Dissertation outline: a multi-scale approach to analyze somatic embryogenesis

In this dissertation work, I discuss methods to both improve the feasibility of SE on an industrial scale as well as provide fundamental insight into the complex culture dynamics in loblolly SE cultures. This project entails a unique approach to study and optimize loblolly pine SE by considering the system from a holistic point of view, including extracellular, intracellular, and intercellular factors (Figure 1.4). Cellular interactions are critical to the success of a culture. Plant cells *in vitro* exist in an aggregated state, where cell-cell contact and signaling (*intercellular* events) can influence culture performance. To study these interactions in loblolly SE in supplementation to hypotheses based on existing literature [32, 33], culture size and morphology and its influence on embryo yield were considered in Chapter 2.

Molecular characteristics in the extracellular space may be indicative of the state and potential of a particular culture to generate embryos and can be engineered to influence embryo yield. In Chapter 3, a screening platform for embryo yield within a culture using media samples to study extracellular signals is presented. Following our results, proteins identified in Chapter 3 are considered to develop methods to engineer and manipulate these extracellular proteins in Chapter 4. Moving away from protein work
and into the *intracellular* space, the influence of intracellular stress levels are considered in terms of impact on embryo yield. Plants rely on an intricate chemical defense system (i.e., a stress response) to adapt to adverse conditions, characterized by the synthesis of specialized products that can enhance survivability. In Chapter 5, a shift in metabolism is implemented by manipulating culture conditions using a plant stress hormone, and is demonstrated to improve embryo yield, suggesting a link between plant stress-related pathways and the SE process. Our work provides a basis for future studies that had not been established in loblolly pine, as well as cost effective engineering and screening strategies to improve or predict embryo yield at an industrial scale.

### 1.5 Research in an understudied system and broader impacts

Research concerning loblolly pine is particularly important because, despite its commercial importance, there is very limited work on this SE system in published literature. The research described herein was developed through close analysis of SE literature in related plant culture systems. There are several proteins that have been linked to SE in a variety of plant species, most notably AGPs in Norway spruce [41] and peach palm [42] and chitinases in *Lotus* spp. [43] and Norway spruce [44]. As a result, the findings of this project are not exclusive to loblolly pine and can be applied and tested in any plant species capable of SE, thereby impacting a broad range of industries, including other lumber crops, pharmaceuticals, and biofuels. Other coniferous species can be propagated through SE, including *Picea abies, Picea mariana,* and *Pinus radiata* [21]. Many medicinal plants can also be produced through SE, including *Panax ginseng* (herbal stimulant) [45], *Petiveria alliacea* (anti-cancer, antibiotic medication) [46], and *Corydalis yanhusuo* (neuropathic pain treatment) [47]. Efficient production of these crops is critical to ensure a constant supply of important medications to a growing population, and crop production via SE eliminates seasonal variability as well as low specialized metabolite yield within the crop.
SE also has applications in biofuels feedstocks based on a loblolly pine crop [48], and provides a valuable tool for genetic modification of crops with a high biomass yield. For example, embryogenic tissue can be transformed using *Agrobacterium* and then used in high-throughput reproduction of genetically modified crops including *Panicum virgatum* (switchgrass) [49]. Other crops that produce biofuel precursors that can be propagated via SE include *Jatropha curcas* Linn. [50], cited by Goldman Sachs in 2007 as one of the best crops for biofuel production for its ability to grow in unfavorable conditions and for its high oil yield [51]. Collectively, the work presented here describes novel engineering and screening strategies for loblolly SE systems but can be easily tested and adapted to other culture systems that use liquid phase culturing for propagation of ESM.
A RAPID METHOD TO SCREEN CULTURES FOR EMBRYO YIELD OUTCOMES

Loblolly SE cultures consist of unique cellular morphologies, ranging from PEM I to PEM III cell types that span a wide size range. It is hypothesized that the largest, most developed PEM III cell types transition into somatic embryos, but this hypothesis is built upon studies that identified the transition by isolating individual aggregates, destroying any inherent intercellular interactions. Here, aggregate size distributions were collected from cultures on the day they were transitioned to development media and retroactively screened for links between these distributions and the downstream embryo yield. By sampling directly from the tissue that was plated, a snapshot of the in vitro morphology and insight into the developmental state of the culture is determined. In control cultures, the total volume of aggregates with a mean diameter below 273 μm negatively correlates with embryo yield with p<0.01, implicating accumulation of these smaller aggregates with a culture state that leads to lower embryo conversion frequency. Application of the same screen to all treated cultures spanning a wide range of culture manipulations allows identification of a similar trend, but it is also noted that catastrophic treatments (those that result in severe inhibition of SE) deviate from the expected relationship. Collectively, these results indicate that a simple and rapid assay using the Coulter counter and only a small culture sample can result in yield prediction results during routine plating of cultures to development media, or in control cultures as part of an experiment to determine the baseline yield results. This method is more rapid and insightful than microscopy alone, since insight is gleaned into not only size distributions but

Parts of this chapter are reproduced from EM Cummings Bende†, SA Wilson†, and SC Roberts. “Aggregate size distributions reveal predictive markers for embryo yield in loblolly pine somatic embryogenesis suspension cultures.” In preparation. †Contributed equally
also the total amount of cellular volume within a liquid-phase culture.

### 2.1 Introduction

The Coulter principle is a commonly used technology with applications in blood counting [52], bacterial cell culture analysis [53], and can even be scaled down to microfluidic chips capable of accurately analyzing nanoscale colloidal particles [54]. The method is simple and robust: as particles are pulled through an aperture, the Coulter counter measures a change in the resistance in the fluid across the aperture [55]. This pulse in resistance is proportional to the volume of the particle, which can then be used to calculate the mean diameter of each particle in the sample. The volume is equivalent to the volume of fluid displaced, and for plant cells (which are not spherical) this enables insight into aggregate size. The diverse morphology in plant cell culture (especially SE cultures, see Figure 1.3) and any morphological response to treatments is traditionally analyzed only through microscopy, and has been extensively studied in SE in this manner [32, 33, 56, 57]. Recently, the Roberts lab has applied the Coulter counter technology to determine aggregation states of *Taxus* suspension cultures [58]. This method was extended to loblolly SE cultures [27] and since, application of the Coulter counter has become a routine part of SE experiments in loblolly pine (see Chapters 4 and 5).

A previous student in the Roberts lab, Sarah Wilson, developed a method to apply the Coulter counter to loblolly pine suspension cultures which was thereafter applied to the industrial bioreactors at Weyerhaeuser NR Co [27]. Using cultures fractioned with filters of known size, the Coulter counter was applied to accurately measure the size of loblolly PEMs, verified through microscopic analysis. Further, the measured size was shown to approximately link the determined volume to developmental state (PEM I, II, III) since aggregates that have progressed to PEM III are typically large [27]. While microscopy can provide the most accurate determination of extent of development, the Coulter counter
provides a higher throughput method to screen multiple cultures on a routine basis while providing total biomass levels as well as size information [58]. Total runtime for accurate determination of biomass size and quantity is two minutes, requiring only two 2-mL culture samples per analyzed flask.

This method was further extended in a data mining endeavor, collecting cell volume and particle size distribution data from culture flasks as a part of independent experiments. The data were then mined for a correlation between embryo yield and volume parameters, size distributions, and contributions of specific populations to the total volume. A link between the aggregate size distribution in loblolly pine SE suspension cultures and the downstream embryo yield was determined, for the first time introducing a rapidly screenable marker for SE outcomes 12 weeks earlier than previously possible. This was made possible through a retroactive screen of particle size distributions measured using a Coulter counter and correlation with outcomes, and has implications on SE in not only loblolly pine but potentially other coniferous SE systems. Implementation of this method would allow continuous yield prediction, thereby enabling predictable outcomes in the industrial SE system.

2.2 Materials and methods

2.2.1 Loblolly pine embryogenic culture and sampling

Media formulations are described in detail in US Patent #7,598,073 [59] (for formulation and protocol, see Appendix A). Maintenance cultures were shipped overnight with ice packs from Weyerhaeuser NR Co (Seattle, WA) and immediately moved to Erlenmeyer flasks incubated at 23°C in the dark at 100 RPM in a New Brunswick™ Innova 44R incubator (Eppendorf, Hamburg, Germany). Maintenance cultures were subcultured weekly into fresh maintenance media by settling cells for 20 minutes, then transferring one part settled cell volume (SCV) into four parts fresh media. For the experiments described herein,
well-mixed culture was split into 250 mL flasks with 10 mL SCV and 40 mL fresh media. Treatments were applied according to each experimental protocol. For weeks that cultures were merely transferred and not used for experimental analysis, cultures were maintained in 500 mL flasks with 72 mL fresh media and 18 mL SCV. Summary of experiment start dates, genotype and cryopreservation accession number, as well as experimental IDs referenced in Figure 2.7 are outlined in Table 2.1.

Table 2.1 Summary of experiments included in screen of Coulter counter data for relationships with yield.

<table>
<thead>
<tr>
<th>Experiment ID</th>
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<th>Date counted</th>
<th>Genotype</th>
<th>Cryopreservation ID</th>
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<td>6/2/2016</td>
<td>A</td>
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<td>1</td>
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</tr>
</tbody>
</table>

2.2.2 Embryo development and yield assessment

Cultures were plated to development media on day seven of culture according to the methods in US Patent #7,785,884 [26] (outlined in Appendix A). On day 7 of culture for each experiment, 10 mL of well-mixed culture were placed into a sterile conical tube and allowed to settle for 20 minutes. The conditioned media was removed, and a volume of rinse media (prepared according to US Patent #7,598,073 [59]) equal to the volume of settled cells was added to the culture and allowed to incubate for 10 minutes, at which point half of the added rinse media was removed with a serological pipette. To plate, a well mixed
750 μL sample of this culture was pipetted onto a nylon mesh (2”x2” with a 100 μm weave as described in US Patent #7,785,884 [26]) on a Buchner funnel. The excess media was pulled off by pulling a vacuum and the mesh was transferred into a petri dish containing the solid development media [59] (for formulation and protocol, see Appendix A). Three petri dishes were used per treatment flask for a total of 9 development plates per treatment within each experiment.

The petri dishes were placed in a well-ventilated box with foil-covered holes to preserve darkness at room temperature for a 12-week period. After 12 weeks, embryo yield was assessed by hand-counting the embryos that transitioned into cotyledonary embryos using forceps [25, 28, 59]. Fresh weight was determined by weighing the dishes before counting and after removing all biomass from the plate. Photos were taken of all plates prior to counting using a Panasonic Lumix FZ200 camera with manual focus.

2.2.3 Coulter counter

The biomass levels of the liquid maintenance cultures were determined using a Beckman Coulter Multisizer™ 3. Cultures were analyzed in duplicate using a 2 mL well mixed culture sample placed into an electrolyte solution containing of 6.5 g L⁻¹ sodium chloride and 0.325 g L⁻¹ sodium azide in 35% v/v glycerol. The Coulter counter was equipped with a 2,000 μm aperture for all analyses. During routine plating experiments (for example, Section 4.2.1.2), the Coulter counter was run on days 0, 4, and 7 (the day of plating). Each flask was sampled and run twice, and the data averaged to provide more accurate volume distributions. As a result, a library of size distribution data within loblolly suspension cultures was generated for the day of plating as well as 3 and 7 days prior to plating to development media.

2.2.4 Meta-analysis of experimental controls

Aggregate size distributions determined using the Coulter counter throughout 13 independent routing plating experiments were compiled to search for relationships amongst
particle size, quantity, and embryo yield. Aggregate size data obtained from the Coulter counter were separated into size bins based on mean diameters selected to represent the distinct morphological stages in suspension cultures (150, 273, 495, and 691 μm). Data were plotted and screened for significance using MATLAB or R using the corrplot [60], ggplot2 [61], and GGally [62] packages. Individual relationships were plotted and further analyzed using GraphPad Prism version 7.03 for Windows (GraphPad Software, La Jolla, CA, www.graphpad.com) to determine Pearson correlation coefficients and p-values for each relationship.

2.3 Results and discussion

The compilation of the data from completely independent plating experiments was a large endeavor and resulted in a significant amount of data: three days of analysis run in duplicate on the Coulter counter for each flask, each comprised of particle size data distributed among 50 size bins. In such a large dataset, trends were identified by separating out populations of aggregates into size bins chosen to represent approximate PEM size cut-offs. However, numerous additional parameters were analyzed before determining the most prominent and directly applicable relationships that enable screening aggregate size distributions for the ultimate embryo yield. Here, an easy to screen population for correlation with outcomes is discussed, but other interesting relationships and/or properties identified in the data can be found in Appendix B.

2.3.1 A relationship between volume of small particles and embryo yield

During routine experiments in the laboratory, the Coulter counter was run as an integral part of culture analysis to determine any change in growth in response to treatments. Coulter counter size distributions measured on the day the experiments were plated for 13 independent experiments were combined. In each experiment, three replicate flasks were
run for each treatment group alongside three replicate control (untreated) flasks for a total of 39 control measurements and 156 treated-flask measurements. Treatments were diverse and included application of compounds including the heavily glycosylated class of proteins known as arabinogalactan proteins (AGPs) or application of the plant stress hormone methyl jasmonate.

Volume distributions over a range of particle sizes were determined from the Coulter counter volume output for each particle. The distributions were separated into size bins based on diameter and plotted against embryo yield in a correlation matrix including each control flask from each of 13 independent experiments (Figure 2.1). The size bins chosen for analysis were for diameters < 150, 150-273, 273-495, 495-691, and > 691 \( \mu \text{m} \), selected based on Coulter counter output (e.g., 495 \( \mu \text{m} \) instead of 500 \( \mu \text{m} \)) as well as rough cutoffs that map approximately to cell type as determined previously (See Figure 7.3 in [27]). Here, the term diameter is used to represent a mean diameter calculated from the volume of displaced fluid as aggregates pass through the Coulter counter aperture. This calculated value provides a representation of aggregate size as a function of the diameter of a sphere with the same volume as each aggregate. Since loblolly pine PEMs are not spherical and can have highly variable morphologies, this number does not represent the actual diameter but estimates the extent of aggregation for each particle. To ensure that any identified relationships were not due to an extenuating factor, all analyses were plotted in a correlation matrix to test for collinearity of all variables (Figure 2.1).
Figure 2.1 Correlation matrix between the total volume of aggregate size populations determined by the Coulter counter and embryo yield. The diagonal shows distribution of the data across each domain. The upper half of the matrix shows Pearson correlation coefficients for each relationship and the lower half shows corresponding scatter plots for data visualization. Each point is one control flask within each of 13 experiments (3 control flasks per experiment for n=39 flasks). Volumes represent an average of two runs on the Coulter counter. Embryo yield was determined 12 weeks after the volume distributions were obtained and represent an average embryo yield among n=3 replicate plates. Plots were drawn and data analyzed using the GGPlot package in R [61]. The relationship between total volume of aggregates with diameters in the range of 150-273 μm provides the strongest predictive relationship with embryo yield, R=-0.69.
A negative correlation appears when embryo yield is plotted against the total volume of particles between 150 and 273 μm (R=-0.69, Figure 2.1). This cellular population is correlative with the population of aggregates below 150 μm (R=0.89), so subsequent analyses were performed with both populations separate as well as combined. In *Cyclamen persicum* SE cultures, small (<150 μm) PEM aggregates had low embryo conversion frequency compared with the larger counterparts [63], supporting the results here that a large total volume of small cell types results in lower embryo yield. Unexpectedly, the total volume of larger aggregates expected to be PEM II or III cell types was not correlative with embryo yield, a result expected based on previous determination that the larger cell types transition into embryos [32]. The volume of particles between 273 and 495 μm appears to be predictive of the total volume measured in each culture (R = 0.91), which is surprising since this population makes up only 26.5 +/- 3.4% of the total volume measured in these experiments. This is particularly interesting because the volume of the population of aggregates in the 273-495 μm range does not correlate with any other analyzed cellular population.

### 2.3.2 A relationship between total number of small particles and embryo yield

The distribution of the number of aggregates that fell into each size bin was also explored (Figure 2.2). The number of aggregates measured in each sample with diameters greater than 495 μm ranged from only 22 particles to 158 particles, accounting for only 1 +/- 0.5% of the total number of measured aggregates (Figure 2.3). For this reason, the >495 μm number population was not further fragmented as it was in the volume population analysis. On the lower end of the size spectrum, aggregates with diameters that fell below 150 μm accounted for a much larger number of particles that encompassed a much larger range of numbers across all control flasks analyzed. The range in the number of aggregates that fall within each bin size is illustrated in Figure 2.3a. To allow better visualization of the two larger size bins, the 273-495 and >495 μm size bins are also plotted on a separate plot, Figure 2.3b. The majority of aggregates had diameters below 150 μm and were not
further fractioned due to the lower size cutoff for particles measured by the Coulter counter (72.3 μm). Unsurprisingly, since an average of 77% of aggregates fell into the <150 μm bin, the number of aggregates below 150 μm correlated strongly with the total number of aggregates (R=1, Figure 2.2). Similar to the results from the volume analysis, Figure 2.2 shows a correlation between the number of aggregates in the 150-273 μm range and embryo yield with a Pearson correlation coefficient of -0.68, and there was no relationship between the number of cellular aggregates >495 μm and embryo yield (R=0.01).
Figure 2.2 Correlation matrix between the total number of aggregates in each size population determined by the Coulter counter and embryo yield. The diagonal shows distribution of the data. The upper half of the matrix shows Pearson correlation coefficients for each relationship and the lower half shows corresponding scatter plots for data visualization. Each point is one control flask within each of 13 experiments (3 control flasks per experiment for n=39 flasks). Numbers represent an average of two runs on the Coulter counter. Embryo yield was determined 12 weeks after the volume distributions were analyzed and represent an average embryo yield among n=3 replicate plates. Plots were drawn and data analyzed using the GGPlot package in R [61]. The relationship between number of aggregates with diameters in the range of 150-273 μm provides the strongest predictive relationship with embryo yield, R=-0.68.
Figure 2.3 Distribution of the number of aggregates in each size bin among 13 independent experiments. The vast majority of aggregates fall in the <150 μm bin, with increasingly small spread as the size of the bin increases. (a) All size bins plotted on the same axes. (b) Enlarged look at the two largest size bins, 273-495 and >495 μm, showing a small spread of number of aggregates in the >495 μm size bin. Whiskers outline the entire range of the data except for outliers, which are shown as empty circles. The middle 50% of the data is outlined by the box, with the median shown as the horizontal line within the box.

2.3.3 Further exploration of interesting populations

The measured populations in each control flask were averaged within each experiment for further statistical analyses. Relationships between embryo yield and the volume of populations measured were considered. The total volume of aggregates measured did not correlate with embryo yield, p=0.095 (Figure 2.4a). Further, the relationship between the volume of cellular aggregates measured with a diameter below 150 μm and the downstream embryo yield was not significant (Figure 2.4b). As determined previously, the population of aggregates between 150 and 273 μm correlates negatively with embryo yield (p=0.0063), indicating that a higher volume of these smaller aggregates is indicative of a lower embryo yield and providing confidence that this population is an easily observable marker for embryo yield outcomes (Figure 2.4c). Due to the previously identified relationship between particles below 150 μm and particles between 150 and 273 μm (Figure 2.1), the total population of aggregates below the 273 μm cutoff was further explored. Unsurprisingly, the vol-
Volume of aggregates with diameters below 273 $\mu$m correlates negatively with embryo yield with a Pearson correlation coefficient of -0.657 ($p=0.015$), providing an easier to screen population (Figure 2.4d) requiring less precision in measurement.

Figure 2.4 Closer look at relationships between volume of cellular populations with embryo yield reveals useful relationships. All three replicate experimental control flasks were averaged within an experiment such that each point represents one experiment. Vertical error bars are +/- standard deviation with $n=9$ and horizontal error bars are +/- standard deviation with $n=6$. Lines are the lines of best fit and the dotted lines illustrate the 95% confidence interval for the line of best fit. Displayed $R$ values represent the Pearson correlation coefficient, and * denotes significance of the correlation with * $p<0.05$ and ** $p<0.01$ and ns = not significant. (a) Total volume of aggregates determined by the Coulter counter does not correlate with embryo yield. (b) Volume of aggregates with mean diameter less than 150 $\mu$m does not correlate with embryo yield. (c) Volume of aggregates with mean diameters in the range of 150-273 $\mu$m correlates with embryo yield ($p<0.01$). (d) Volume of aggregates less than 273 $\mu$m correlates negatively with embryo yield ($p<0.05$).
This predictive relationship is promising and provides a rapid method to determine the viability of a culture with respect to embryo yield using only a small sample of culture on the desired day of plating. Since the development process in loblolly pine lasts 8-12 weeks, this relationship has the potential to streamline SE in loblolly pine. Only cultures that have potential for high embryo yield would be moved into development, saving time and resources. While the strongest correlation between volume of aggregates within each bin was identified in the 150-273 \( \mu \text{m} \) bin, the \(<273 \mu\text{m}\) bin may be more useful as it is easier to screen and requires less precision in measurement. The correlation between volume of culture below 273 \( \mu \text{m} \) is strong (\(R=-0.657\)) and provides confidence that this low-diameter population is predictive of SE outcomes. Interestingly, the quantity of larger cell types was not predictive of embryo yield (Figure 2.5 a and b), a finding in opposition to the hypothesis identified in related systems that larger cellular aggregates (PEM III types) progress to form embryos [32, 33]. Though neither the volume or the number of larger cell types is correlative with embryo yield, this does not disprove the working hypothesis; rather, it underscores the importance of cell-to-cell interactions in the SE process and verifies that isolation of cell types may not reveal the intricacies of the developmental progression of SE cell types. The results suggest that cell types in addition to PEM III aggregates \textit{in vitro} influence embryo yield outcomes, and that the measure of the larger cellular aggregates does not give a complete picture of a culture’s developmental state or potential. Therefore, future analyses of culture morphology and its influence on embryo yield should study an intact culture to develop theories concerning SE developmental progression.
Figure 2.5 Volume populations of large cell types are not predictive of embryo yield outcomes. All three replicate experimental control flasks were averaged within an experiment such that each point represents one experiment. Vertical error bars are +/- standard deviation with n=9 and horizontal error bars are +/- standard deviation with n=6. (a) Volume of aggregates with mean diameter greater than 495 $\mu$m does not correlate with embryo yield, $p=0.859$. (c) Volume of aggregates with mean diameter greater than 691 $\mu$m does not correlate with embryo yield, $p=0.799$.

The total volume of aggregates measured in the cultures was not predictive of embryo yield, underscoring the importance of the small cell types in SE process outcomes. The small aggregates ($d<273$ $\mu$m) accounted for only 34 +/- 14% of the total volume but 94.5 +/- 1.9% of the total number of aggregates counted, so while the majority of individual SE aggregates fall into the $<273$ $\mu$m category, the bulk of the culture volume is larger. Upon elucidation of the relationship within control flasks, individual experiments were analyzed to determine if the trend between these small aggregates with mean diameter less than 273 $\mu$m and embryo yield was upheld within an experiment and after application of numerous experimental treatments. The trend is not maintained within individual experiments (Figure 2.6) but when all data are combined they follow a trend similar to that of the controls (Figure 2.7). Figure 2.7 shows the embryo yield plotted against the total volume of aggregates with diameters less than 273 $\mu$m and is color coded by each experiment. Here, each point represents a flask within an experiment. The data fall into two regimes: a steep drop-off in embryo yield values at volumes less than 10 mm$^3$ and then a plateau for volumes above
10 mm³. When looking within an experiment in this analysis, several entire experiments deviate from the general trends, and these deviant experiments are always experiments with overall low embryo yield (Figure 2.7). When considering the data in Figure 2.7 with the deviant points excluded, the relationship appears to revert to a single negative correlation.

![Graphs showing embryo yield vs. volume of aggregates for individual experiments and pooled analysis.](image)

Figure 2.6 Individual experiments do not follow the trend identified from combined data from all experiments. Embryo yield plotted against the volume of aggregates below 273 μm for a representative set of 4 independent experiments. The negative correlation identified among the controls from 13 experiments does not hold within an experiment.

The pooled analysis of all treated experimental and control flasks (Figure 2.7) elucidates a similar relationship between small diameter cell populations and embryo yield that the control flask analysis revealed, but also provides additional insight into the effect of experimental treatment on morphological development. In general, points that break the linear trend are the treatments that were ultimately catastrophic with respect to embryo yield. For example, the three points on the graph that show the lowest yield as well as the lowest volume of aggregates below the 273 μm cutoff (solid circled points in Figure 2.7) were cultures subjected to total inhibition of extracellular AGPs, an essential class of proteins in SE systems [36, 41, 64]. The next most extreme points fall only slightly higher on the <273 μm volume range but even lower in embryo yield outcomes (big dashed circled points in Figure 2.7) and were treated with high levels of a stress-inducing compound (methyl jas-
Figure 2.7 Volume of small aggregates from all treated flasks follow a two-phase trend with respect to embryo yield. Compiled data from all control and all treated flasks among 13 independent experiments (n=195 flask observations) showing relationship between embryo yield and total volume of aggregates with diameter <273 μm. Data follow a two-phase trend: at volumes less than 10 mm$^3$ data follow a steep, negative trend and at volumes greater than 10 mm$^3$ data form a flat plateau. Deviant data (circled) are from treatments that either inhibited an entire extracellular protein class (solid circle) or subjected flasks to catastrophic stress treatment via application of methyl jasmonate (dashed circle). Data in the small-dotted circle were from experiments performed outside the typical useful age range of cultures and also stand out as different than the norm.

Collectively, it is anticipated that the trend depicted in Figure 2.7 will be conserved among experimental treatment groups, except in cases where catastrophic treatments are applied. Since the goal of SE experimental treatments is typically to find a method to improve embryo yield, this is not anticipated to be a problem; additionally, the negative trend does not hold within an experiment and as such as not useful to predict results within a short-term experiment. Therefore, SE yield predictions via Coulter counter size analysis is recommended for either experimental controls or for routinely subcultured SE culture.
Using this methodology, cultures can be screened for effective estimation of downstream yield results and subpar cultures would not be plated, eliminating the resources wasted on nonideal SE cultures.

Limitations of this analysis include the lower cutoff of the Coulter counter, which was set to 72.3 μm based on our method development and to accommodate all large aggregates. It has been previously determined that there are few aggregates that fall into this population [27]; however, future work should include further analysis of all aggregates that fall into the <273 μm bin using a smaller aperture on the Coulter counter as well as further elucidation of morphological state via microscopy. Likely, the population below 273 μm is comprised of not only PEM I cell types but also cellular debris or even small PEM II cell types, an analysis that requires further investigation since the morphology of measured aggregates cannot be determined through Coulter counter analysis alone. Based on these results it is hypothesized that an abundance of PEM I cell types in vitro negatively influences embryo yield. SE cultures are typically not immortal [65, 66], and loblolly SE cultures are no exception and thus have a finite useful lifespan (in our case, approximately four months after retrieval from cryopreservation). Future work should include analysis of a culture with the Coulter counter and simultaneous plating throughout its useful lifetime, potentially providing a link between aggregate size distributions and the acquired recalcitrance in embryogenic potential due to aging.

Regardless, the correlation remains predictive of embryo yield, and its utility should be verified with size fractionation experiments. Preliminary work in our lab suggests that separation and immediate plating of the larger cell types using a >710 μm stainless steel mesh filter results in an improvement in embryo yield over unfiltered culture and the <710 μm filtered population, a finding which should be explored in detail in this and other coniferous SE systems. Other attempts evaluate culture morphology or ultimate culture success via morphology analysis have included image analysis techniques in carrot [67] and in Dou-
glas fir [68] to classify the extent and normality of embryo development in the development stage. Image analysis has also been used to quantify growth of early somatic embryo tissue during development-stage culturing in Norway Spruce [69]. These techniques are successful at either embryo quality classification or development-stage growth quantification, but do not attempt to analyze multiplication-stage suspension cultures. Furthermore, our analysis of total volume of aggregates provides more detail about the true size of a PEM than just the two-dimensional area measurement of each aggregate.

2.4 Outlook

Meta-analysis of aggregate size distributions determined by the Coulter counter allowed for a detailed analysis of size populations and their relationships to embryogenesis outcomes. This screening method could allow for the propagation of only cultures with ideal properties and therefore enable predictable embryo yield outcomes. On each potential plating day, cultures can be quickly analyzed using the Coulter counter. If the morphology falls into an acceptable predicted embryo yield range on the correlation, the culture should be plated. Further, instead of maintaining the culture in suspension and plating a little each week hoping for best outcomes, more culture could be plated on plating days during which high yield is projected. This would eliminate excessive propagation of unsuccessful cultures by determining optimal plating times. The analysis here was carried out on loblolly pine ESM tissue, but the methodology can be extended to other coniferous SE systems. While the exact correlation identified here may not be directly applicable, a library of size distributions and embryo yield outcomes can be collected to search for and identify an equivalent trend in other systems. Since analogous morphology is expected in related gymnosperm SE systems, similar results are expected among closely related species.
2.5 Acknowledgements

Funding and all loblolly cultures for the work was provided by Weyerhaeuser NR Co. I thank Sarah A. Wilson, PhD, for the Coulter counter method development in loblolly pine, without which I would not have had these data to analyze. Undergraduates Michael Bodanza and Nathan Hague performed preliminary size fractionation plating experiments referenced in this text.
There is a critical need for efficient reproduction of high-value crops including timber crops, biofuels feedstocks and medicinal plants. Somatic embryogenesis (SE), an in vitro cellular process that leads to the formation of a viable embryo, provides a method to create a clonal crop and enables selection for plants with superior characteristics. Our work focuses on the use of SE for the loblolly pine, a crop essential to the US timber industry. The primary limitation to large-scale implementation of SE is that the process is not fundamentally understood, leading to large variability in embryo yield—a number not determined until 3-4 months after SE initiation. Arabinogalactan proteins (AGPs) are a promising biomarker implicated in other SE systems. In this work, AGPs are demonstrated to be influential on loblolly pine somatic embryo yield using novel characterization techniques to elucidate the role of AGPs in culture, including a new colorimetric assay for extracellular AGP and colorimetric ELISAs using antibodies with AGP subclass specificity. While the total extracellular AGP concentration did not correlate with embryo yield, a negative correlation was identified between extracellular JIM8- and JIM13- AGP concentrations and embryo yield, indicating that specific AGPs play a critical role in SE. It is also demonstrated that the JIM8 and JIM13 antibodies bind proportionally in the loblolly pine culture system, a system-specific phenomenon. Understanding the complex properties of AGPs and their influence on SE will enable a better fundamental understanding of SE and identification of strategies to promote high embryo yield.

Parts of this chapter are reproduced from EM Cummings Bende and SC Roberts. “Extracellular arabinogalactan proteins are a screenable and manipulatable marker for somatic embryo yield in loblolly pine.” In preparation.
3.1 Introduction

Loblolly maintenance culture AGPs were characterized to identify molecular markers associated with high embryo yield. The quantification of relevant proteins provided a glimpse into in vitro molecular processes in maintenance cultures and led to the potential to link a molecular condition to embryo yield. There are several proteins involved in complex interactions throughout the process of SE, and this work focuses on AGPs. AGPs are a diverse class of proteins consisting of a core polypeptide backbone, accounting for 2-10% of the protein by mass, with many branched glycan chains branching from the core polypeptide [70]. The structure of AGPs is not fully characterized, as they are heavily glycosylated with branched carbohydrate side chains of various sizes [70]. The proteins can be linked to the cell surface via a glycophosphatidylinositol linkage or free within the culture media [71]. The most widely held hypothesis about AGP structure is the "wattle blossom model" [70], in which (like the wattle blossom flower) sugar moieties branch out from the central peptide, forming a disperse spheroidal glycoprotein [72].

Some traditional molecular biology approaches have been implemented to study AGP functionality and analyze structural differences in different proteins, as reviewed by Tan et al [73]. Due to the high degree of glycosylation inherent to AGP diversity, application of molecular cloning strategies to introduce and overexpress specific AGPs is limited since post-translational modifications can result in different structures in the final AGP products. Despite these challenges, AGPs have been successfully manufactured and overexpressed to probe functionality in plant systems [74–76]. Further, AGP peptides have been sequenced to identify differences between AGPs identified to have different functionalities. For example, embryogenic AGPs in cotton are different than nonembryogenic AGPs, and the embryogenic AGP fraction was sequenced and subsequently manufactured in Nicotiana tabacum [37]. These studies indicate that while glycosylation does provide a unique challenge to AGP analysis, molecular cloning techniques can be applied to begin to analyze
AGP structure-based differences in functionality. Additionally, carbohydrate analysis techniques can be applied to determine structural differences in AGPs. The molecular makeup of the sugars present in the glycoproteins can be discerned using gas chromatography mass spectrometry [37, 77], providing insight into carbohydrate structures in AGPs (e.g. 50% galactose, 35% D-arabinose, etc).

AGPs are highly diverse and are involved in a variety of cellular processes across the plant kingdom. Recently AGPs were determined to be the key component of ivy vine secretions that enable the plant to 'climb' the sides of buildings [78]. A specific type of AGP, the fasciclin-like AGP (FLA), contains an adhesive fasciclin domain involved in intercellular interactions as well as adhesion to the extracellular matrix [79]. The fasciclin domain was originally identified in *Drosophila* [80] and is also found in bacteria, fungi, algae, insects, and animals [81] as well as plants. In plants, the presence of FLAs has been linked to flour yields during the milling of wheat; in fact, human selection for high-yielding wheat genotypes has resulted in crop cultivars with reduced levels of FLA [82], suggesting a role for FLAs in toughness of plant tissue, and again implicating AGPs in the plant extracellular space.

AGPs are also highly involved in plant morphogenic processes [71], including root tip and shoot morphogenesis in *Arabidopsis* [83] and xylogenesis in loblolly pine [84] and maize [85]. Inhibition of these important proteins in *Arabidopsis* induced programmed cell death (PCD) [86], indicating a role of AGPs in PCD. In maize, it has been suggested that JIM13, an antibody specific to an AGP subclass, marks cells destined for PCD [85], implicating these extracellular proteins in the regulation of PCD. This apparent role of AGPs in PCD in plants is interesting given the link between PCD and SE processes; in Norway Spruce, there are two waves of plant PCD throughout the SE morphogenic process: 1) when the PEM surrounding the newly formed somatic embryo degrades and 2) when terminally differentiated cells that previously supported the newly formed embryos die off [34].
AGPs have been linked to SE success or failure in other systems [71]. For example, when exogenous AGPs are applied to *Daucus carota* (carrot) protoplast cultures, the previously lost embryogenic potential was restored [36]. Antibodies (JIM8 and JIM13) that bind subsets of AGPs implicated in SE provide an additional tool to study the role of specific AGPs [87]. For example, cells carrying the JIM8 epitope need to be present for SE to occur in carrot cultures, but these JIM8-labelling cells are themselves unable to form embryos [35]. In Norway spruce (*Picea abies*), the ratio of JIM13-epitope AGP levels to total AGP levels negatively correlates with embryo yield [41]. In addition, JIM13 has been shown to label pre-embryogenic masses but not embryos, providing a potential marker to be explored [32]. The β-glucosyl Yariv (βgluY, Figure 3.1a) reagent also provides a useful tool in AGP research; part of the definition of AGPs is their ability to bind βgluY [85, 88]. This bright red colored reagent selectively binds to AGPs and precipitates AGPs out of salt solution [89, 90]. βgluY has been used to precipitate AGPs out of chicory cell culture, resulting in decreased embryogenicity in a concentration-dependent manner [91], further implicating AGPs as critical to the SE process.
In this work, molecular assays were created in order to screen cultures for different AGP states and correlation with embryo yield outcomes. Study of AGPs in the loblolly pine system began with the generation of a library of samples from industrial bioreactors. Assays were then developed to study the proteins within media samples associated with cultures that had various embryo yields. An assay to quantify total extracellular AGP was streamlined, enabling rapid quantification of soluble AGPs at lower levels than previously possible. Through the development and application of two new ELISA techniques using
JIM8 and JIM13 antibodies, we determined that both of these AGP subclasses correlate negatively with embryo yield, enabling estimation of embryo yield 12 weeks before previously possible. We also identified that these two AGP subclasses exist proportionally in this loblolly SE system, a finding that is species specific. Together, these results provide methods to quantify extracellular AGPs as well as the ability to screen for highly successful cultures using a benchtop assay.

3.2 Materials and methods

3.2.1 Cell culture and sampling

Loblolly pine SE cultures were maintained as described (Section 2.2.1) and according to published methods [59]. For the biomarker screen in this Chapter, these cultures were maintained at Weyerhaeuser NR Co (Seattle, WA) at the bioreactor level. Embryo yield is reported here as a normalized value, such that a reported normalized yield value of 1 will be equal to the average embryo yield within a dataset, a value greater than 1 will be above average, and a value less than 1 will be below average embryo yield.

A library of media and well mixed culture samples (cells plus media) from industrial bioreactors was generated at Weyerhaeuser NR Co. (Seattle, WA). Media samples were taken from bioreactors during the maintenance stage, filtered to remove cell debris, flash frozen in liquid nitrogen, and preserved at -80°C for future analyses (Table 3.1). Cultures are identified as unique by their genotype (defined by the initial source of the culture: a single fertilized seed from a pine cone) and a number corresponding to when the culture was established; thus, the sample library was developed from several unique cultures representing commercially important lines (Table 3.1).
Table 3.1 Summary of all media samples analyzed in the extracellular AGP analysis

<table>
<thead>
<tr>
<th>Sample</th>
<th>Genotype</th>
<th>Cryo-preservation ID</th>
<th>Days out of cryopreservation</th>
<th>Days in liquid culture</th>
<th>Days in Wave reactor</th>
<th>Normalized embryo yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>1</td>
<td>197</td>
<td>156</td>
<td>111</td>
<td>0.03</td>
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<tr>
<td>2</td>
<td>A</td>
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<td>85</td>
<td>41</td>
<td>1.55</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
<td>2</td>
<td>155</td>
<td>99</td>
<td>55</td>
<td>1.10</td>
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<td>B</td>
<td>1</td>
<td>204</td>
<td>184</td>
<td>140</td>
<td>1.27</td>
</tr>
<tr>
<td>5</td>
<td>B</td>
<td>2</td>
<td>134</td>
<td>107</td>
<td>76</td>
<td>1.43</td>
</tr>
<tr>
<td>6</td>
<td>B</td>
<td>3</td>
<td>105</td>
<td>78</td>
<td>41</td>
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<tr>
<td>7</td>
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<td>126</td>
<td>99</td>
<td>62</td>
<td>1.54</td>
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<tr>
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<td>170</td>
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<td>225</td>
<td>191</td>
<td>153</td>
<td>0.74</td>
</tr>
<tr>
<td>16</td>
<td>E</td>
<td>2</td>
<td>113</td>
<td>72</td>
<td>41</td>
<td>0.69</td>
</tr>
<tr>
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<td>E</td>
<td>2</td>
<td>148</td>
<td>107</td>
<td>76</td>
<td>0.56</td>
</tr>
</tbody>
</table>

### 3.2.2 Total extracellular protein

The total extracellular protein was determined using the Pierce™ Coomassie (Bradford) Protein Assay Kit (Pierce Biotechnology, Waltham, MA). Concentrations were determined to fall in the range of [0, 30] μg mL⁻¹, so the micro preparation was used (with samples diluted 10-fold as needed to fall within the range of the standard curve). 150 μL media samples were added to the wells of a 96-well plate along with a bovine serum albumin (BSA) standard curve mixed by serial dilution with water of a 2000 μg mL⁻¹ stock (Pierce Biotechnology, Waltham, MA). 150 μL of the Bradford reagent was added and pipetted to mix. After 10 minutes, the absorbance of each well was read at 600 nm using the ELx800 absorbance reader (BioTek, Winooski, VT).
3.2.3 Total extracellular arabinogalactan protein

Media samples were concentrated using 10 kDa cutoff Nanosep (Pall, Port Washington, NY) ultrafiltration columns. The columns were passivated with 500 μL filter-sterilized (0.22 μm) 1% bovine serum albumin (BSA) in 1x PBS (Sigma Aldrich, St. Louis, MO) incubated at room temperature for one hour. The BSA was discarded and the reservoir rinsed twice with nanopure water. Next, the columns were filled with 500 μL nanopure water and centrifuged for 5-10 minutes, and the flow-through was discarded. 500 μL media samples were thawed over ice and were transferred to passivated columns. The tubes were centrifuged at 3.75 g at 4°C for 30-90 minutes, checking every ten minutes to see how much volume remained above the filter (dependent on the protein concentration in each sample). When roughly a 4x concentration was achieved (between 100 and 150 μL sample remaining above the filter), the concentrated samples were transferred into a clean tube, weighed, and stored on ice and assayed immediately or stored at -80°C for later use. The concentration factor for each sample was determined by comparing the mass of the concentrated sample to the mass of the original sample.

100 μL of concentrated cultured media was mixed with an equal volume of 2% w/v NaCl and 12 μL of 3.33 mg mL⁻¹ (0.04 mg) βgluY (GlycoSyn, Gracefield, New Zealand) and vortexed. To create a standard curve, dilutions of gum arabic (Acros Organics, New Jersey, USA) in the range of 0-150 mg mL⁻¹ were prepared and treated identically to media samples. The tubes were incubated at room temperature overnight to precipitate the AGPs. The precipitate was pelleted by centrifuging at 3.75 g for 10 minutes. The supernatant was removed, and the pellet was washed three times by vortexing with 200 μL 1% NaCl (Sigma, St. Louis, MO), centrifuging for 10 minutes at 3.75 g, and removing the supernatant. After the final wash step, 200 μL 100 mM NaOH (Fisher Scientific, Waltham, MA) was added and the deep red AGP-Yariv complexes were resolubilized by vortexing. The absorbance of the resulting solution at 450 nm was proportional to the concentration of AGP, and when
compared to a gum arabic (GA) standard and adjusted for individual concentration factors, the concentration of AGP in the original media sample was determined.

### 3.2.4 ELISA

An ELISA was developed to measure the concentration of extracellular JIM13 AGP. Using GA in 1x PBS as a standard solution, a uniformly binding 96-well ELISA plate (Nunc-Immuno, Sigma Aldrich, St. Louis, MO) was coated with media samples diluted 1:10 000 in 1x PBS (Sigma Aldrich, St. Louis, MO) and incubated for 90 minutes at room temperature. The wells were washed four times by adding 300 μL 0.05% TWEEN-20 in PBS to each well and then gently shaking the plate by tapping the side of the plate for 30 seconds before disposing of the rinse buffer by pouring out the solution and lightly tapping against paper towels to remove excess buffer. The plates were blocked with 200 μL 5% bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, MO) in PBS for 30 minutes. The plates were washed three times, as previously described. Next, 100 μL of 1:10 JIM13 (CarboSource, Athens, GA) in PBS solution were added to the wells and incubated for one hour, followed by washing the wells five times, as previously described. The secondary antibody, Goat anti-rat IgG (Fisher Scientific, Waltham, MA) was diluted 1:15 000 and 100 μL were added to each well. The plates were incubated for 45 minutes, followed by washing five times as described above. Ultra-streptavidin conjugated to horseradish peroxidase (Life Technologies, Waltham, MA) was diluted 1:15 000, 100 μL were added to the wells, and the plates were incubated for 30 minutes. The wells were washed seven times, as previously described to thoroughly remove any unbound components. 100 μL of TMB substrate (TMB substrate kit, Pierce Biotechnology, Waltham, MA) were added and allowed to react at room temperature until the desired color had developed (approximately 15 minutes), at which point the reaction was stopped using 50 μL 2 M sulfuric acid (Sigma Aldrich, St. Louis, MO). The absorbance of the plates was immediately read at 450 nm using the ELx800 absorbance reader (BioTek, Winooski, VT). GA was used as a standard, and the absorbance was found to be linear with concentration. Concentrations can be expressed in terms of GA equiva-
lent concentration. Each sample and standard was run in duplicate on each ELISA plate, and runs of the ELISA wherein the two values were not in agreement were excluded from analysis. All results from the ELISAs are presented as the average and standard deviation determined from three independent ELISAs, each with two technical replicates.

The ELISA used to quantify the extracellular JIM13 epitope was adapted for use with the JIM8 antibody (CarboSource, Athens, GA), and the protocol is identical except for the following changes: 1) media samples were diluted 1:2000, and 2) JIM8 was the primary antibody.

### 3.3 Results and discussion

The samples included in this study represent a range of commercially important genotypes. Each genotype represents one clone, or a culture initiated from the same fertilized pine cone. Since genotypes behave differently and can have drastically different morphologies and cultures can even vary from one to the next, it is important to consider culture-to-culture variability and strive to identify a universal marker for high yield. Therefore, a diverse library of samples was curated representing cultures at different ages in maintenance cultures, different genotypes, and different cryopreservation accession numbers. Fortunately, the cultures sampled had a large range of embryo yields, allowing comparison of potential biomarkers within different yield ranges.

#### 3.3.1 Total extracellular protein

Before studying specific proteins as biomarkers for embryo yield, methods were established to quantify total extracellular protein. Extracellular proteins were studied since little to no processing is required prior to analysis: no extraction steps are needed and secreted proteins are inherently soluble. In an industrial-scale bioprocessing system, procurement of media samples is the simplest process to automate and ultimately decreases time spent at the bench, enabling rapid screening of cultures on a regular basis. Extracellular
proteins can be signaling molecules and are known to influence SE [41, 42, 94], providing confidence that the extracellular space is a suitable target for screening.

Before analysis of AGPs, total extracellular protein levels were analyzed to determine what level of protein is available for quantification in the secreted, extracellular space. The choice of an appropriate assay for total extracellular protein is not trivial; compounds included in media formulations can result in interference that makes accurate determination of protein concentration impossible (see Table 3.2). In this case, the use of the BCA assay provided the false result that there is no inherent variation in the concentration of protein among the media samples in Table 3.1 due to interference of components in the liquid media. This interference could arise due to the inclusion of casamino acids since the BCA assay measures peptides with lengths of three amino acids or longer [95]. The maintenance media formulation includes 1 g L^{-1} casamino acids, a partially hydrolyzed protein mixture that contains both amino acids and small peptides. Uncultured (fresh) media assayed with the BCA assay provides a baseline protein concentration of 914 μg mL^{-1}: an unsuitable baseline for determination of the concentration of secreted protein.

Table 3.2 Summary of relevant interfering substances in assays for total protein

<table>
<thead>
<tr>
<th>Assay</th>
<th>Operating Concentration Range</th>
<th>Examples of Interfering Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCA</td>
<td>25-2000 μg mL^{-1}</td>
<td>Detergents, ascorbic acid, small chain amino acids</td>
</tr>
<tr>
<td>Bradford</td>
<td>25-2000 μg mL^{-1}</td>
<td>Detergents</td>
</tr>
<tr>
<td>Micro Bradford</td>
<td>2.5-25 μg mL^{-1}</td>
<td>Detergents</td>
</tr>
</tbody>
</table>

Next, the Bradford assay was tested and ultimately chosen for application; the Bradford assay (Pierce Biotechnology, Waltham, MA) provided a baseline protein concentration in uncultured media of 0, enabling detection of the low-levels of secreted proteins present in cultured media. Protein concentrations in the media were determined to fall in the range of [0, 30] μg L^{-1}, but total extracellular protein did not correlate with embryo yield (data not
shown). These results may be useful for normalization of other important protein concentrations and are important to identify baseline secreted protein levels in loblolly suspension cultures.

### 3.3.2 Total extracellular AGP

Characterizing AGPs in loblolly pine began with development of an assay for quantifying total extracellular AGP, which is notably simpler than measuring cell-associated values since no extraction steps are required. The currently accepted method for total AGP quantification is single radial diffusion [93], in which a thin gel is poured with 10 μg mL\(^{-1}\) of \(β\text{gluY}\) and 0.15 M sodium chloride, small wells are punched out with a Pasteur pipette, and samples are added to each well. The gels incubate overnight at room temperature, and the samples diffuse from the well and through the gel (Figure 3.1b). If there is AGP in the sample, the AGP will form precipitation complexes within the gel, resulting in a reddish ring that has an area proportional to the concentration of total AGP in the original sample. However, this single radial diffusion method is difficult to control, time-consuming (requiring an overnight incubation), and costly as the gel leaves much of the expensive reagent unused.

Therefore, a colorimetric assay that provides a more rapid method for AGP quantification was developed, resulting in an end product that is still useful for AGP characterization. This assay was developed based on a cell-associated AGP assay, as previously described [96]. The assay results in a strongly linear (\(R^2 = 0.995\), Figure 3.1c) standard curve with GA in the range of 10-150 μg mL\(^{-1}\), which is approximately five times more sensitive than the single radial diffusion method [93]. The assay was made more sensitive by the addition of an ultrafiltration step to concentrate AGPs in the media samples, enabling determination of concentrations as low as 1 μg mL\(^{-1}\). The colorimetric total AGP assay was run on samples taken the day of plating, and values for total extracellular AGP were plotted against final yield values. The results showed no correlation between the two values (Figure
3.2). Figure 3.2 Total extracellular AGP does not correlate with embryo yield. Graph of embryo yield plotted against total extracellular AGP in μg mL⁻¹. Data show no correlation, with a Pearson correlation coefficient of 0.22.

The lack of correlation between total extracellular AGP and embryo yield is not surprising since AGPs are comprised of numerous structurally diverse compounds. Polypeptide backbones in AGPs vary greatly both within a species and between different plant species [97]. This variety is amplified by the diversity of the remaining 90%+ of the AGP: the many branched sugar moieties which are largely comprised of arabinose and galactose [98]. These branching polysaccharides can be linked and branched in numerous ways, with lengths spanning anywhere from 30-150 residues, adding to the diversity of AGPs [97]. In fact, previous research on a variety of SE systems has demonstrated it is either the type [36] or fraction [41] of AGPs that is linked to SE success, rather than total extracellular AGP concentration. The size and structure of these proteins are variables which provide opportunity to further characterize the AGPs. For example, antibodies against subclasses of AGPs (including JIM8 and JIM13) have been linked to SE success in other systems.
3.3.3 JIM13 and JIM8 AGPs correlate negatively with embryo yield

To begin the analysis of AGP subpopulations, ELISAs were developed using JIM13 and JIM8 antibodies to quantify secreted subsets of AGPs. JIM13 was chosen for analysis due to prior work observed in related systems. For example, in Norway spruce, a higher proportion of extracellular JIM13 epitope relative to total AGP was correlated to lower embryogenicity [41]. Another study visualized the JIM13 epitope in Norway spruce using a tagged secondary antibody and fluorescence microscopy, and found that the JIM13 antibody recognizes only non-embryogenic tissue (PEMs but not somatic embryos) [32]. These studies both suggest that the presence or the amount of the JIM13 epitope in culture might be indicative of a nonembryogenic culture, and led to the hypothesis that a higher extracellular concentration of JIM13 will correlate to a low embryo yield.

The GA equivalent concentration of JIM13 or JIM8 epitopes in each media sample was determined by comparing the absorbance of a sample to that of a standard curve created from serial dilutions of GA. Each sample was measured from at least three independently run ELISAs with duplicate wells in each assay and averaged, with standard deviations less than 20% considered acceptable. Yield values were normalized against the average yield in the data presented, such that the average yield will have a normalized yield value of one, normalized yield values less than one have a mean yield below average, and normalized yield values greater than one have above average yield. Figure 3.3 graphs the normalized embryo yield against the determined JIM13 values. The data show a negative linear correlation with a Pearson correlation coefficient of -0.63, indicating a strong linear correlation. As hypothesized, a higher quantity of the JIM13 epitope in the media was associated with lower-yielding cultures. Based on these results, extracellular JIM13 AGPs may be a biomarker of low embryo yield, indicating that an early prediction of SE success may be possible (Figure 3.3).

The JIM8 antibody was raised against protoplasts isolated from beet suspension cul-
Figure 3.3 JIM13 AGP concentration correlates negatively with embryo yield. Graph of normalized embryo yield plotted against the concentration of JIM13 AGPs in μg mL\(^{-1}\). Data show a negative correlation with a Pearson correlation coefficient of -0.64. Embryo yield is normalized to the average yield within the dataset, and JIM13 is shown as the GA equivalent concentration and the average of at least 3 independent ELISAs +/- the standard deviation.

Figure 3.4 JIM8 ELISA results are shown in Figure 3.5, showing a negative trend and indicating that JIM8 AGPs may also be indicative of embryo yield outcomes. The trend also appears to be reminiscent of the JIM13 results (Figure 3.3).
Figure 3.4 Hypothesized cell cycle for JIM8-labeling cell types. Cells undergo an asymmetric division to yield a JIM8 positive and a JIM8 negative cell. Only the JIM8 positive cells can become embryos and only in the presence of JIM8 negative cells [35].

The similarities between the graphs are further explored in Figure 3.6a. Figure 3.6a plots the JIM8 data against the JIM13 data for each sample, and shows a positive linear correlation, indicating a relationship between the binding epitopes of the antibodies. It is important to note that the JIM13 epitope was present in a higher amount than the JIM8 epitope, since dilutions of 1:10000 and 1:2000 were required, respectively, for the absorbance to fall within the range of the standard curve for the ELISA. Figure 3.6b shows the JIM8 and JIM13 concentration divided by the total AGP for that sample, resulting in a very strong linear trend with an Pearson correlation coefficient of 0.99.

3.3.4 JIM antibodies bind proportionally in loblolly pine somatic embryogenesis

Conflicting accounts of the similarities in binding epitopes of JIM8 and JIM13 antibodies were found in other systems. For example, the antibodies bound identically in carrot suspension cultures [87] and Arabidopsis thaliana [100], and proportionally in maize roots [64] and sugar beet [101]. However, other reports found the epitopes to be entirely different, such as in a P. abies embryogenic system [41] and as a stress response in tomato plants [102]. The negative correlation between JIM8 and JIM13 and embryo yield AGPs in loblolly pine, however, is supported by prior experimental results. Results in carrot suggest
Figure 3.5 JIM8 AGP concentration correlates negatively with embryo yield. Graph of normalized embryo yield plotted against the concentration of JIM8 AGPs in μg mL\(^{-1}\). Data show a negative correlation with a Pearson correlation coefficient of -0.29. Embryo yield is normalized to the average yield within the dataset, and JIM8 is shown as the GA equivalent concentration and the average of at least 3 independent ELISAs +/- the standard deviation.

that JIM8-labelling tissue is nonembryogenic [35] in the same way that JIM13-labeling tissue is nonembryogenic in Norway spruce [32]. Both AGP types having a similar negative correlation with embryo yield in loblolly pine further suggests that these specific proteins are associated with nonembryogenic tissue types.

Recently, the binding epitopes of 130 antibodies against plant cell wall glycans were classified, and JIM8 and JIM13 bound different classes of carbohydrates [103]. The collection of antibodies was screened against a library of carbohydrates, and from the recognition patterns the antibodies were grouped into clades with other antibodies with similar binding patterns. JIM8 and JIM13 were placed into different clades, with the clade including JIM13 binding a larger number of carbohydrate epitopes than JIM8. This is consistent with the data in Figures 3.3 and 3.5 for the loblolly pine SE system, as more JIM13 epitope than JIM8 epitope was observed for every sample. The study also showed that JIM8 and JIM13
Figure 3.6 JIM8 and JIM13 AGPs are related in loblolly pine SE cultures. (a) Extracellular JIM8 AGP concentration is related to the JIM13 AGP concentration in loblolly pine SE cultures with a Pearson correlation coefficient of 0.44. Concentrations are shown as GA equivalent concentrations and as the average of at least three independent ELISAs. For clarity, no error bars are shown. (b) Extracellular JIM8 AGP concentration normalized to total extracellular AGP correlates to the JIM13 AGP concentration normalized to the total extracellular AGP in loblolly pine SE cultures with a Pearson correlation coefficient of 0.99.

also react to pectin residues, indicating that these two antibodies are not specific to only AGPs and the carbohydrate region recognized by the antibodies may be present in other carbohydrates [103]. Since pectin is localized within the cell wall rather than secreted as an extracellular signal [104], we do not anticipate that quantification of JIM8- or JIM13-epitopes in media samples will be complicated by this cross-reactivity. All accounts in the literature along with new data presented here lead to the hypothesis that JIM8 and JIM13 antibody binding similarity is system-specific and must be validated within each culture system.

3.4 Conclusions

Here, JIM8 and JIM13 AGPs are shown to correlate negatively with embryo yield in loblolly pine. The concentration of JIM8 and JIM13 AGPs were shown to be related in this system, and when normalized to total extracellular AGP demonstrate a linear relationship. It is therefore concluded that the antibodies bind related epitopes in loblolly pine, and that
either JIM8 or JIM13 could be used to screen cultures for embryo yield outcomes. To further validate these results, industrial-scale SE cultures can be sampled within and across genotypes throughout the duration they are maintained in culture. These assays can be used to first screen for embryo yield outcomes and then to further validate the identified trend by providing results across several cultures throughout their useful lifetime. These results will supplement the trends identified here. After trend validation, samples can be used to identify other proteins or genes influential to culture success using next generation sequencing technologies (i.e. RNA-seq) to identify differentially expressed pathways that are positively or negatively correlated with high embryo yield, providing potential targets for metabolic engineering or potential end products (e.g., proteins) to focus additional work.

To further study AGP subclasses and their influence on SE, analytical chemistry techniques including reversed-phase high performance liquid chromatography (RP-HPLC) or high pressure size exclusion chromatography (HPSEC) can be applied to characterize AGPs in high and low yielding SE cultures. In subsequent experiments, a protein extraction was optimized for loblolly cultures to enable this future work (Appendix C). The assay for total and specific AGPs can be run on cellular protein extracts to determine levels of cell-associated AGPs. By comparing extracellular AGP levels with cell-associated AGPs, insight can be gleaned into AGP signaling mechanisms and the relationship to embryo yield. The results in this chapter indicating a role of specific AGPs on SE success in loblolly pine is further explored in Chapter 4, in which JIM13 and JIM8 positive AGPs are introduced to suspension cultures prior to moving to development.

### 3.5 Acknowledgements

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AGPs have been implicated in somatic embryogenesis (SE) across many plant species, including extracellular AGPs in loblolly pine as demonstrated in Chapter 3. Culture engineering strategies were implemented to manipulate extracellular AGPs in vitro and further characterize the relationships between extracellular AGPs and embryo yield. Elimination of AGP functionality through binding with βgluY in vitro completely inhibited SE and development stage growth at 50 μM, confirming the critical importance of AGPs in the SE process. Next, culture AGPs were supplemented to probe the effect of AGP manipulation via addition of exogenously derived AGPs. Gum arabic (GA) and gum ghatti (GG), two widely used commercial plant gums comprised largely of AGPs, were both supplemented in cultures and unexpectedly had opposing effects. GA reduced SE success by inhibiting embryo yield while low concentrations of GG were able to improve outcomes. Since both plant gums are bound by the JIM13 antibody, the results presented here complicate the hypothesis of JIM13 AGPs negatively impacting embryo yield. The effect of the plant gums on maintenance phase growth determined using the Coulter counter is also discussed; GG at high concentrations promoted the growth of aggregates with mean diameter below 273 μm. The work described here can be used as a platform to further elucidate the role of AGPs on loblolly pine SE.

Parts of this chapter are reproduced from EM Cummings Bende* and SC Roberts. “Extracellular arabinogalactan proteins are a screenable and manipulatable marker for somatic embryo yield in loblolly pine.” In preparation.
4.1 Introduction

Extracellular AGPs have been manipulated in other SE systems to probe the role of these heavily glycosylated proteins in SE. To further elucidate the function of AGPs in the loblolly SE system and introduce engineering strategies based on this functionality, AGPs were manipulated in the extracellular space. First, complete inhibition of extracellular AGPs was achieved through application of the $\beta$-glucosyl Yariv reagent ($\beta$gluY) \textit{in vitro}. $\beta$gluY has been applied to other plant cell culture systems; for example, in \textit{Rosa} suspension cultures the addition of $\beta$gluY inhibited culture growth in a concentration-dependent manner without affecting cellular viability or size, thereby implicating AGPs in proliferation [105]. Through addition of $\beta$gluY to Brazilian pine suspension cultures, it was demonstrated that $\beta$gluY reduces growth by decreasing cellular viability through initiation of programmed cell death (PCD) [106]. The compound also induced PCD in \textit{Arabidopsis} suspension cultures, implementing AGPs as critical molecules involved in PCD [86] (See Section 3.1 for further discussion of a link between SE, PCD, and AGPs).

The compound has also been introduced in embryogenic systems to probe the effect of a lack of AGP functionality in these processes. In a \textit{Brassica napus} L. cv. Topas microspore embryogenic system (in microspore embryogenesis, an embryo is formed from either a male or a female gamete as opposed to the vegetative tissue of SE [107]), $\beta$gluY inhibits embryo formation thereby implicating AGPs as critical to this analogous morphogenic process [108]. In a \textit{Chicorum} SE culture, the addition of $\beta$gluY inhibited SE in a dose-dependent manner, further supporting the critical nature of AGPs in the SE process [91]. Based on this prior work, we studied the essential nature of AGPs on SE in loblolly pine by completely and partially (50 and 10 $\mu$M $\beta$gluY, respectively) inhibiting AGP functionality \textit{in vitro} within maintenance stage cultures before moving to development media. The results presented here supplement the relationship between extracellular JIM13-binding AGPs and the downstream embryo yield by further underscoring the importance of AGPs.
The opposite approach to extracellular AGP manipulation is then presented by way of supplementation of AGPs in the extracellular domain. This has been attempted in other SE systems using different AGPs with various outcomes. In cotton, the supplementation of embryogenic AGPs improved SE rates while nonembryogenic AGPs decreased SE rates [37], suggesting an influence of specific AGPs in the SE process. In Norway spruce, the addition of concentrated extracellular AGPs stimulated SE [41], providing further evidence that SE success can be influenced through manipulation of extracellular AGPs in vitro. When JIM8 AGPs were applied to carrot SE cultures, embryo yield was reduced, further supporting this hypothesis [109]. Based on these results as well as the hypotheses developed in Chapter 3, it is anticipated that the introduction of more JIM13- or JIM8-binding AGPs (such as GA) will inhibit embryo yield.

Here, the commercially available gum arabic (GA) and gum ghatti (GG) were chosen for their ready availability, affordability, and ability to bind JIM8 [103], and JIM13 [103, 110]. Both gums are harvested in southeast Asia by making an incision on a tree trunk and allowing the gum (sap) to exude from the wound. The resulting product can be processed to various purity grades, ranging from large amber-colored crystals to an almost white, homogeneous powder (Figure 4.1). Both GA and GG are widely utilized plant gums with large AGP components. GA is a natural biopolymer derived from *Acacia senegal* and is a common food and pharmaceutical additive used for its beneficial emulsifying properties [111], as a flavor protectant through microencapsulation [112], and use in combination with sodium alginate to form beads for controlled drug delivery [113]. GA is commonly used across numerous industries, including solubilization of flavor compounds and sugars in soda as well as color stabilization in wines [114]. Other useful properties include its high solubility and stability across a wide pH range, as well as its nontoxic nature and generally recognized as safe (GRAS) status [115–117]. GG is a plant gum derived from the sap of
Anogeissus latifoliae and is sometimes used as a replacement for GA [118]. GG has also achieved GRAS status and is very useful for its emulsification and thickening properties, but has not been thoroughly studied and thus remains limited in its application [119].

Figure 4.1 Commercially available plant gums. a) GA ready to be used to prepare sweets in Jabalpur, India. b) GA is available in a variety of purity levels, ranging from unprocessed crystals (left) and a sieved powder (right). "Acacia gum, pieces and powder" by Simon A. Eugster is reproduced here under CC-BY-SA 3.0.

Here, embryo yield was manipulated through supplementation or elimination of AGPs, thereby verifying the importance of these glycosylated proteins in the extracellular space and confirming they can be influenced using simple engineering strategies. A single addition of a compound to SE cultures prior to induction of development is a simple process manipulation that is easily implementable across any scale and is advantageous over genetic modification due to its fast nature and preservation of the clonal integrity of the resultant crop. The response of a culture to the exogenous plant gums was analyzed with respect to growth, aggregate size distributions, as well as the accumulation of plant stress-related phenolic compounds. Here, it is demonstrated that embryo yield can be manipulated through the exogenous application of plant gums, providing an experimental basis for culture AGP engineering.
4.2 Materials and methods

4.2.1 AGP engineering experiments

Cultures were maintained as described in Section 2.2.1. Plating experiments were designed to allow direct comparison of results across time (Figure 4.2). The cultures were mixed together before dividing well-mixed aliquots of SCV into experimental flasks in triplicate, with 10 mL of SCV added to 40 mL media in a 250 mL flask. On day 7, each flask was plated to three replicate Petri dishes with development media for a total of nine plates per treatment. During the maintenance culture phase, well-mixed culture samples (cells plus media) were taken on days 0, 4, and 7, and stored at -80°C for future analysis. The Coulter counter was run to quantify biomass and aggregate size distributions (see Section 2.2.3 for methods) on days 0, 4, and 7. On the day of plating, additional samples were taken: media samples to allow analysis of extracellular proteins and cell samples for future studies of cell-associated proteins. These samples were stored at -80°C. Development occurred over a 12-week period as described in Section 2.2.1, at which point embryo yield was determined by hand-counting embryos with forceps. Total biomass was weighed to quantify growth in the development stage.

The experiments were designed such that data could be studied over time both within individual cultures and amongst unique cultures. This became particularly useful when the library of Coulter counter data was mined for relationships with embryo yield in Chapter 2. The biomass levels as determined by the Coulter counter were also useful for normalizing other data determined from culture, media, and cell samples. To visualize aggregate size distributions determined by the Coulter counter, a 7-point weighted moving average (WMA) (Equation 4.1) was used:

\[
WMA(x_n) = \frac{x_{n-3} + 2x_{n-2} + 3x_{n-1} + 4x_n + 3x_{n+1} + 2x_{n+2} + x_{n+3}}{16}
\]  
(4.1)
Figure 4.2 Schematic of general experimental design for laboratory scale loblolly pine SE plating experiments. The experiments lasted the duration of the 7-day culture period with sampling on days 0, 4, and 7 prior to plating cultures to development media on day 7. Treatments were usually applied on day 0, but could be shifted to tune the effect (see Chapter 5).

where \( n \) is the index of the each bin output by the Coulter counter, and the WMA is calculated for each bin within the distribution to yield a smooth particle size distribution curve. This method of averaging based on adjacent bin measurements helps to give a better estimate of the size distributions since a finite number of particles are analyzed using the Coulter counter.

To quantify biomass levels in maintenance flasks, a dry weight (DW) correlation was used to approximate the dry weight in the cultures as a function of volume measured by the Coulter counter, developed by measuring the contribution of different sized cellular populations separated using filtration to the dry weight (See [27]). The function calculates DW (g L\(^{-1}\)) as a function of the volume of particles with \( d < 233 \mu m \) (\( V_{<233} \)) and the volume
of particles with d>233 μm ($V_{233}$):

$$DW = A \times V_{<233} + B \times V_{>233}$$

(4.2)

where $A$ and $B$ have units of g L$^{-1}$ μm$^{-3}$, and $A$ is equal to $4.2 \times 10^{-10}$ and $B$ is equal to $2.6 \times 10^{-10}$. Volumes determined from the Coulter counter are input in μm$^3$. The dry weight was used to assess growth rates in liquid cultures as well as to normalize phenolic content determined by the Folin Ciocalteu assay (see Section 5.2.6 for methodology).

4.2.1.1 AGP elimination experiments

To inhibit AGP functionality in vitro, β-gluY (GlycoSyn, Gracefield, New Zealand) was added on day 0 of culture at 10 or 50 μM. β-gluY was dissolved in nanopure water at 3.3 mg mL$^{-1}$ and filter sterilized (0.22 μm PVDF syringe filter unit). The resulting solutions were added to maintenance flasks to reach the desired concentration. These experiments were performed at a smaller scale (15 mL total culture volume in a 50 mL Erlenmeyer flask) to minimize cost, and thus only culture, media, and cell samples were taken on day 7 prior to plating.

4.2.1.2 Gum arabic and gum ghatti addition experiments

For both of the AGP supplementation experiments discussed here (GA and GG experiments), cultures were the same genotype and cryopreservation accession number and the experiments were performed within three weeks of eachother to enable the elimination of culture age as a factor for analysis.

GA (Acros Organics, New Jersey, USA) was dissolved in nanopure water at 40 mg mL$^{-1}$ and filter sterilized (0.22 μm PES syringe filter unit, EMD Millipore). The resulting solution was added to 32 mL maintenance media in 250 mL flasks before adding 8 mL SCV to achieve the desired GA concentration (volumes of 50, 100, and 200 μL of prepared GA
solution for 50, 100, and 200 mg L$^{-1}$, respectively).

GG (HiMedia, West Chester, PA) was dissolved in nanopure water by stirring overnight to a final concentration of 6% w/v. Insoluble matter was filtered using vacuum filtration through filter paper. The insoluble mass was dried on the benchtop overnight and weighed to determine the soluble fraction and concentration of the resulting amber-colored, viscous solution. The GG solution was filtered at 0.22 μm (Nalgene™ Rapid-Flow™ bottle top PES filter) before adding to the maintenance media in 250 mL flasks to achieve the desired concentration.

4.2.1.3 Statistical analyses

All statistical analyses were performed using GraphPad Prism version 7.03 for Windows (GraphPad Software, La Jolla, CA, www.graphpad.com). For comparing two data-points, the Student’s T-test was used, and for greater than two datapoints a one-way ANOVA with Dunnett’s test for multiple comparisons was used to determine significance among the treatment levels. All barplots were made using GraphPad Prism software.

4.3 Results and discussion

4.3.1 Arabinogalactan protein starvation inhibits embryogenesis in loblolly pine

To probe the important nature of AGPs in this coniferous loblolly pine SE system, experiments were designed to effectively starve the maintenance stage cultures of AGPs one week prior to moving cultures to development conditions. On day 0, the culture media treated with βgluY appeared red in color due to the brightly colored red βgluY (Figure 4.3a). Throughout the culture period, βgluY associated with the cells and made the cell surface appear red in color (Figure 4.3b), indicating that the reagent has associated with cell-wall
AGPs.

Figure 4.3 Cultures with the $\beta$gluY added show that the reagent associates with the cell surface after seven days in culture. a) On day 0, just after $\beta$gluY is added, treated culture media appears a deep red (left) compared to the control flasks (right). b) After seven days in culture with $\beta$gluY, the red-colored $\beta$gluY has associated with AGPs on cell surface, pulling the red from the surrounding media (shown: 50 $\mu$M $\beta$gluY culture).

Due to the reagent’s high cost, these experiments are unique in their scale: the total culture volume was capped at 15 mL as opposed to the typical 50 mL. Thus, the effect of $\beta$gluY on culture biomass accumulation was determined by visual quantification of the settled cell volume (SCV) in a graduated 15 mL centrifuge tube, a common method to measure growth in plant cell culture. In the 50 $\mu$M treated cultures, growth was inhibited in maintenance cultures (p<0.001 by the Student’s T-test, Figure 4.4a), while 10 $\mu$M $\beta$gluY had no effect on maintenance phase growth (Figures 4.4b). The growth inhibition at 50 $\mu$M $\beta$gluY suggests that AGPs play a role in ESM proliferation in the maintenance phase, as the ESM was unable to grow to the same level as the untreated cultures. Similar observations were made in carrot SE cultures in which application of high levels of $\beta$gluY largely reduced growth [120].

Figure 4.5 shows the early influence of $\beta$gluY on development at a timepoint of five weeks (out of the full 12 weeks allowed for development) for the control and 50 $\mu$M treated cultures. The control plate (Figure 4.5a) shows ESM proliferation as well as early signs of embryo development, apparent from the opaque, white, and torpedo-like structures. Compared to the control plates, the 50 $\mu$M plates have very sparse growth and no signs of
Figure 4.4 βgluY inhibits growth in maintenance phase cultures as determined by fold-change in settled cell volume. a) 50 μM βgluY inhibits growth in the maintenance phase and b) 10 μM βgluY has no effect on growth within the maintenance phase. Error bars for both graphs are +/- SEM with n=3. *** denotes statistically different than the control within an experiment using the Student’s t-test with p<0.001.

embryo formation (Figure 4.5b). Throughout the seven day maintenance culture period, βgluY in the media associated with the cell surface, staining the cells bright red and pulling the red pigment from the media (Figure 4.3) and as a result, the βgluY treated ESM on development media was also red (Figure 4.5b). Similarly in Rosa suspension cultures treated with βgluY, 95% of the reagent was found to be bound to the cell wall [105].
Figure 4.5 βgluY applied during maintenance phase inhibits proliferation in development stage cultures. The control plate (a) shows proliferation of the ESM and early signs of embryo development, while the 50 μM plate (b) shows little to no proliferation. Photos were taken five weeks after plating.

Twelve weeks after the initiation of development, embryos were counted to quantify the ultimate response of loblolly SE cultures to treatment with βgluY. Figures 4.6a and 4.6b show the effect of 50 μM βgluY on the final embryo yield and development stage biomass levels. SE was completely inhibited at 50 μM βgluY, as evidenced by a complete lack of embryos across the 18 replicate plates, compared with the moderate embryo yield levels (an average of approximately 150 embryos) in the control plates (Figure 4.6a). Growth in development was also inhibited by 50 μM βgluY with p<0.0001 (Figure 4.6b). Similar though less dramatic results were observed with the addition of a lower concentration of 10 μM βgluY. Figure 4.6c shows inhibition of embryo yield and Figure 4.6d shows inhibition of growth in the development stage, both with p<0.05. Though inhibited, the 10 μM βgluY cultures were still able to proliferate and form embryos.
Figure 4.6 \( \beta_{\text{glu}}Y \) inhibits embryo formation. Final development results for \( \beta_{\text{glu}}Y \)-treated SE cultures. a) and b) show embryo yield and growth results, respectively, for 50 \( \mu M \) \( \beta_{\text{glu}}Y \) treated cultures. c) and d) show embryo yield and growth results, respectively, for 10 \( \mu M \) \( \beta_{\text{glu}}Y \) treated cultures. Error bars are +/- SEM with \( n=18 \). * denotes statistically different than the control within an experiment using Student’s t-test with * \( p<0.05 \) and **** \( p<0.0001 \).

This dose-dependent response of the cultures to various levels of the AGP-binding \( \beta_{\text{glu}}Y \) suggests that AGPs are essential to both growth and development in loblolly SE cultures throughout both the maintenance and development stages. When \( \beta_{\text{glu}}Y \) was added at 50 \( \mu M \) to maintenance stage cultures, SE was completely inhibited as AGPs were bound by the bright-red \( \beta_{\text{glu}}Y \). The reduction in the magnitude of response at 10 \( \mu M \) indicates that not all AGPs were bound by \( \beta_{\text{glu}}Y \) at that concentration, and therefore some of the unaffected PEMs were able to overcome AGP inhibition and yield embryos. Growth inhi-
bition and SE inhibition in loblolly pine SE (Figures 4.4 and 4.6) were likely, since \( \beta \text{gluY} \) had similar effects in other systems. \( \beta \text{gluY} \) has been previously introduced to other culture systems to probe AGP functionality in the SE process; for example, \( \beta \text{gluY} \) inhibits SE in a dose-dependent manner in Chicorum SE cultures, and the compounds bound by the reagent were confirmed to be AGPs using JIM antibody immunostaining [91]. In peach palm SE, addition of \( \beta \text{gluY} \) to the culture medium decreased embryo formation and increased callus proliferation with increasing concentration of \( \beta \text{gluY} \) [42]. These results further support the dose-dependent inhibition of embryo yield in response to \( \beta \text{gluY} \) presented here as well as the working hypothesis that AGPs are imperative to proliferation and development within embryogenic cultures.

Despite removing the cultures from \( \beta \text{gluY} \)-dosed media by plating the cultures to development media after seven days, the 50 \( \mu \text{M} \beta \text{gluY} \) cultures were unable to recover their initial potential, resulting in a halt in the developmental process. These results indicate a fundamental change within cultures after application of \( \beta \text{gluY} \), which should be further explored. Yariv treated cultures should be tested for viability (through fluorescein diacetate and propidium iodide staining) and markers of PCD (such as DNA laddering and cyttoplasmic shrinkage [34]) to identify the mechanism through which AGP inhibition prevents growth and SE. Regardless, the results presented here support the hypotheses developed in Chapter 3, further implicating AGPs as influential on loblolly SE.

4.3.2 Arabinogalactan protein supplementation reveals differences between commercial AGPs

After confirmation of the critical importance of AGPs in loblolly pine SE through in vitro inhibition, culture AGP supplementation was performed to determine if the addition of commercially available plant gums affects embryo yield. Our first studies with exogenous AGP application to loblolly SE focused on the easily obtainable GA and GG to gather results to support the hypothesis that the presence of higher extracellular levels of JIM8 and JIM13
AGPs in vitro results in lower embryo yields.

Here, GA was introduced to the liquid culture medium seven days prior to plating to development media to allow sufficient interaction time. Concentrations were selected based on previously measured extracellular AGP concentrations (Figure 3.2) to determine if SE success can be influenced with an exogenously applied foreign plant gum. No change in growth was observed within maintenance-phase cultures, with consistent growth rates among all experimental flasks (Figure 4.7a). Further, no change in the accumulation of the stress-related phenolic compounds was observed (Figure 4.7b), indicating that the tested GA concentrations did not induce stress in the maintenance stage cultures (see Chapter 5 for further discussion of phenolic compounds and culture stress in SE systems).

Figure 4.7 Gum arabic has no effect on maintenance culture growth or accumulation of stress-related compounds (phenolics). (a) No change in growth as determined by the Coulter counter is observed in response to any level of GA. (b) No difference in phenolic compound content normalized to dry weight is observed in GA treated cultures. Results are statistically identical by One-way ANOVA with Dunnett’s test.

Dose dependent inhibition of embryo yield with increasing concentration of GA was observed (Figure 4.8a), with embryo yield decreasing with increasing concentrations of GA. Interestingly, this inhibition was identified in combination with a lack of change in development-stage growth (Figure 4.8b). Therefore, throughout the twelve weeks on devel-
development media, the cultures continued to grow and proliferate but were less likely to undergo SE with increasing GA doses. These results in combination with the lack of influence of GA on maintenance-stage growth and phenolic compound accumulation suggest that the JIM13-binding GA applied extracellularly acts as a signaling molecule in loblolly pine SE that prevents the conversion of PEM to embryo. Since in Norway Spruce, JIM13 AGPs were found on only PEM tissue and not embryos [32], it is possible that the supplementation of soluble JIM13 AGPs signalled the cells to halt differentiation into embryos.

Figure 4.8 Gum arabic inhibits embryo yield in a dose-dependent manner. Final development results for GA treated cultures. (a) As concentration of GA increases, embryo yield decreases. (b) GA does not affect culture growth in the development stage. Error bars are +/- SEM with n=9. * denotes statistically different than the control by One-way ANOVA with * p<0.05, ** p<0.01, and *** p<0.0001.

GG has not been used as extensively as GA in SE studies, potentially due to its less widespread availability in its pure form or because such little work has been done in the SE community with the substance. Regardless, extra purification was required to work with GG in the loblolly pine cultures. The gum came in large, crystalline chunks with apparent woody contaminants (Figure 4.9). The gum was allowed to swell and dissolve overnight on a stir plate. The next day, the solution was filtered over a vacuum flask before sterile filtration and subsequent addition to the culture flasks.

Differences in growth within maintenance flasks in response to GG were apparent
Figure 4.9 Gum ghatti dissolution and filtration process is complicated by viscosity and insoluble pieces. (a) Unprocessed piece of GG weighing 4.2 g. (b) GG in 50 mL nanopure water left to dissolve overnight. (c) After sitting overnight, liquid is amber colored with undissolved, wood-like chunks throughout. Solution is thick and viscous. (d) After filtration, flow-through is frothy. (e) Insoluble matter, which accounted for 2.23% of the original mass of GG.

from the aggregate size distributions determined by Coulter counter analysis. The day 7 aggregate size distributions were overlayed (Figure 4.10a), revealing an increase in accumulation of particles with a low diameter for the two highest GG concentrations. This peak in particle volume has a mean diameter of approximately 200 μm. Smaller aggregates in SE cultures are typically PEM I aggregate types, indicating an earlier developmental state in cultures with a larger volume of this cell type. Growth curves of the cultures in response to GG are presented in Figure 4.10b, with biomass levels presented as the total volume within a culture determined through analysis with the Coulter counter. Though there is no significant difference in biomass accumulation between the control and any GG concentration, the growth was further explored since there was such an apparent difference in the aggregate size distributions as revealed in Figure 4.10a.
Figure 4.10 Maintenance-stage growth effects from gum ghatti application. Higher concentrations of GG promoted accumulation of small aggregates in the maintenance stage. (a) Weighted volume distributions from day 7 Coulter counter measurements from GG experiment. An accumulation of particles with a mean diameter around 230 μm is observed at 10 and 50 mg L⁻¹ GG. (b) Growth curves from GG experiment showing the total volume of aggregates measured using the Coulter counter on days 0, 4 and 7 of culture. Error bars are +/- standard deviation with n=3. Measured volumes on each day are statistically identical with p>0.06.

As suggested by the aggregate size distributions (Figure 4.10), a significant increase in accumulation of aggregates with diameter below 273 μm was observed in both the 10 and 50 mg L⁻¹ flasks, (p<0.05, Figure 4.11). While the developmental state was not determined microscopically, prior work in our lab has confirmed that size fractionation of loblolly ESM cell types using 300 μm mesh filters leaves a population largely comprised of PEM I cell types and single vacuolated suspensors in the <300 μm fraction [27], and therefore the accumulation of these smaller aggregates in vitro is indicative of a shift to an earlier developmental state. Further, the population of aggregates with mean diameter below 273 μm were found to negatively correlate with embryo yield, (see Chapter 2), suggesting a developmental change in response to GG. However, despite the accumulation of a significantly larger population of aggregates with mean diameter below 273 μm at the two highest GG levels, there was no significant difference in overall growth rates determined by the fold-change in dry weight for any treatment groups compared to the control (p≥0.06, Figure 4.12a). Further, there was no significant difference between levels of phenolic content among treatment levels (Figure 4.12), indicating no shift toward stress-induced metabolic...
Figure 4.11 Two highest gum ghatti levels promote accumulation of aggregates with mean diameter below 273 μm. Error bars are +/- SEM with n=3. * denotes statistically different than the control by One-way ANOVA with * p<0.05.

Figure 4.12 Gum arabic has no effect on maintenance culture growth or stress-related compound (phenolics) accumulation. (a) No change in growth as determined by the Coulter counter is observed in response to any level of GA. (b) No difference in phenolic compound content normalized to dry weight is observed in GA treated cultures. Results are statistically identical by One-Way ANOVA and error bars represent +/- SEM with n=3.

Despite the significant effect that 10 and 50 mg L⁻¹ GG had on morphological dynamics within the maintenance phase, these levels did not have a significant effect on embryo yield or growth within development, as shown in Figure 4.13. Based on results discussed in Chapter 2, a lower embryo yield is expected in cultures with significantly more pathways in GG treated cultures.
aggregates in the <273 μm size bin (for example, the 10 and 50 mg L\(^{-1}\) GG flasks, see Figure 4.11), an effect that was not observed in these studies. In Chapter 2, the relationship between the total volume of aggregates <273 μm in development and the ultimate embryo yield is shown to not hold within an experiment, but rather serves as a marker for screening routinely maintained cultures. The embryo yield results (Figure 4.13) in combination with the different total volumes of aggregates with diameters less than 273 μm (Figure 4.11) confirms the finding that the correlation (Figure 2.4d) does not hold within an experiment and is only applicable for screening for baseline yield potential.

![Figure 4.13 Gum ghatti promotes SE at lowest tested concentration. Final development results for GG treated cultures. (a) GG promotes embryo yield at lowest dose and has no effect at higher doses. (b) GG does not affect culture growth in the development stage. Error bars are +/- SEM with n=9. * denotes statistically different than the control by One-way ANOVA with * p<0.05.](image)

However, the lowest concentration of GG (1 mg L\(^{-1}\)) in fact promoted embryo yield without affecting growth (Figure 4.13, p=0.03) indicating that aggregates are more likely to undergo SE at this treatment level. Since, like GA, GG is recognized by both the JIM13 and JIM8 antibodies, the lack of inhibition of embryo yield across these treatment levels is surprising and complicates the overarching hypothesis that higher extracellular levels of the JIM13-binding AGPs is predictive of lower embryo yield. Since both GA and GG are natural products, it is likely that there are components present in the gums in addition to AGPs, so it is possible that these confounding compounds complicate the results. Particularly, the
impure nature of the GG source was discussed here (Figure 4.9) and therefore further work is necessary to elucidate the role of any other components.

AGPs have been supplemented extracellularly in closely related systems with varying degrees of success. Notably, in Norway spruce, concentrated extracellular proteins applied to embryogenic cultures improved embryo yield outcomes, and AGPs were determined to be the most influential component of the extract [41]. These results simultaneously implicate endogenous AGPs as critical to SE and open doors for potential supplementation in vitro to improve outcomes in recalcitrant or low-yielding lines. Further, AGP activity does not seem to depend on species of origin; in Cyclamen persicum, the addition of AGPs isolated from carrot seeds improved proliferation of PEM tissue in the liquid phase cultures [63].

Subsequent work in the literature focuses on identifying differences in AGPs that improve SE outcomes. In cotton, AGPs extracted from embryogenic culture promoted SE when supplemented in media [37]. Similarly, GA was introduced into the cotton cultures with no significant effect on the embryo yield [37], indicating that while GA is AGP-rich, these AGPs did not influence SE in the same way endogenous AGPs were able to. It is possible that the epitope was present at too low a concentration to have a significant effect, as we identified extracellular AGP concentrations in loblolly pine to be on the order of 10 mg L\(^{-1}\) (see Chapter 3). Plant gums have been used to benefit SE in other circumstances. For example, cashew exudate gum, a less common gum than both GA and GG, was determined to largely consist of AGPs and was subsequently supplemented to carrot SE culture media resulting in an improvement of both embryo yield and embryo conversion to plant [121]. This gum was included in media throughout several weeks of culture throughout the development stage rather than the seven day exposure tested within the experiments here [121], so it is possible that varying the length of treatment will provide a different result.

Further work should involve fractioning the GA using ultrafiltration filters with a
3.5 kDa molecular weight cutoff such that any small molecule contaminants inherent to GG and GA natural products can be removed. These fractions of GA and GG can be introduced to cultures to probe the effect on SE in loblolly pine and determine if the AGP component is the influential factor. Future work should also include the extraction of cell-associated AGPs from loblolly SE cultures and supplementation with these extracted (native) AGPs. Methods have been developed for extraction of AGPs from cell samples taken from loblolly pine suspension cultures as part of routine experiments, and this newly developed methodology and the analysis methods are presented in Appendix C. AGP properties are consistently different between embryogenic and non-embryogenic samples [37, 41, 63] and supplementation of embryogenic AGPs is expected to improve embryo yield outcomes. It is possible that supplementation of low-yielding or recalcitrant cultures with AGPs extracted from a successful culture can restore embryo yielding potential to these lines.

4.4 Outlook

Complete AGP inhibition prevents embryo production, confirming that AGPs play an essential role in SE. The subsequent AGP supplementation experiments seemingly contradict these results, as the addition of some commercial AGPs (GA) actually inhibit embryo yield. Further confounding the results is the improvement in embryo yield by the addition of yet another AGP (GG). These results suggest that although some AGPs are detrimental to embryo yield, it is possible that certain AGPs may be beneficial when introduced to a culture. Future work should pursue two major routes: 1) characterization of the two plant gums and endogenous extracellular loblolly pine AGPs to determine the structural differences leading to the contradicting responses, and 2) supplementation of loblolly SE cultures with AGPs isolated from other (highly successful) loblolly pine cultures.
4.5 Acknowledgements

This work was carried out throughout my PhD career, and I was therefore assisted by a large number of undergraduates: Marcus Lundgren (WPI), Kara Upton (WPI), Rachael Messier (Worcester State University), Elizabeth Humble (UMass Amherst), Julie Boshar (UMass Amherst), and Laura Ornes (UMass Amherst) all played a role in this work. Cultures and funding for this work were provided by Weyerhaeuser NR Company. I would like to thank the R&D team at Weyerhaeuser for their invaluable intellectual contributions to the countless iterations of assay optimization.
CHAPTER 5

EARLY STRESS ACTIVATION VIA METHYL
JASMONATE IMPROVES LOBLOLLY PINE SOMATIC
EMBRYO YIELD

Somatic embryogenesis (SE) provides a means for large scale production of a clonal crop, enabling propagation of crops with ideal phenotypes. The loblolly pine is an important timber crop that can be propagated through SE, but variable yields of viable embryos are often reported. SE involves a change in the culturing conditions of cells from maintenance to conditions associated with embryo development, and some of these changes are related to stress in plants. In this chapter, embryo yield was improved through early activation of stress in the cultures prior to moving to SE-inducing media using methyl jasmonate (MeJA), a largely conserved activator of plant specialized metabolism. Using a three-way ANOVA to identify consistent outcomes in a group of experiments, addition of 0.1 μM MeJA one day prior to plating was shown to significantly improve embryo yield (p<0.01) despite other variability-inducing factors (e.g., culture age and genotypic differences). Contrastingly, higher levels (10 μM-100 μM) of MeJA added one or three days prior to plating decreases embryo yield and simultaneously upregulates phenolic compound accumulation, indicating that these levels shift metabolism to the point of detriment. Finally, image analysis was used to quantify 2D proliferation in development. The area of the plate occupied by biomass is shown to strongly correlate with embryo yield (R≥0.5), allowing yield assessment without sacrificing culture integrity. Together, these results provide a simple and robust method to improve

Parts of this chapter are reproduced from EM Cummings Bende, Y Feng, RJ Messier, NP Bende, TB Peiris, SC Roberts. “Early stress activation via methyl jasmonate improves loblolly pine somatic embryo yield.” In preparation.
embryo yield across a number of process variables and a platform for experimental and statistical determination of beneficial treatments in this cell culture process.

5.1 Introduction

SE is a method to produce a clonal crop and eliminate detrimental variability that can arise due to the random nature of fertilization in plants. Crops can be established from an ideal lineage selected for traits including fast growth rate, disease or drought tolerance, or superior wood quality. SE is particularly useful in crops with long timespans to reach maturity, such as pine tree crops in the timber industry. Here loblolly pine is discussed, a crop that occupies half of the standing pine volume in the United States. At 1.5 billion seedlings planted annually, more loblolly pine trees are planted each year in the US than any other species by the US timber industry [7, 8], underscoring the critical importance of efficiently producing a superior crop. SE provides a means to large scale clonal propagation of commercial crops, but yields are often variable hindering experimental and commercial feasibility [65, 122, 123].

Plants have unique metabolic pathways, largely as a response to external stressors including drought, temperature changes, and herbivorous pests. While there are many metabolic pathways involved in SE, several pathways are regulated by genes also involved in plant stress responses [124, 125]. Application of known stress treatments can elicit positive responses in SE cultures [126]. For example, abscisic acid (ABA) plays a key regulatory role in the stress response [43] and is also a critical component of development media in many plant systems including loblolly pine [8, 25, 127], Brazilian pine [128], and carrot [129]. Drought stress plays a role in the SE process; a key change between maintenance media types and embryo development media for conifers is a sharp increase in osmolality, typically achieved by the addition of polyethylene glycol (e.g., PEG-8000 in loblolly pine [25] or PEG-4000 in Norway spruce [130]) or gellan gum in maritime pine [57]. The increased
osmolality results in reduced water availability for the cultures plated to the development media, stimulating somatic embryo maturation [131]. In maritime pine, the addition of gellan gum enables SE through this reduction in water availability, and when the concentration was decreased the cultures yielded fewer embryos and a reduced capacity to produce endogenous ABA [57].

Methyl jasmonate (MeJA), or the closely related jasmonic acid (JA), are widely conserved activators of specialized metabolism in plants [132–135] and have also been linked to SE and other plant developmental processes [136]. In cotton, treatment of developing embryos with JA improved not only somatic embryo yield but also improved embryo morphology [124]. Further, increased expression of JA-responsive genes was found in cotton somatic embryos when compared to zygotic embryos, implicating SE as a stress response in cultures and as a means to reproduce under stressful conditions [125]. Contrasting results were observed in *Medicago sativa* (alfalfa), in which MeJA added to development stage SE media inhibited both growth and embryo yield [137], and any supplementation of the endogenous JA levels was detrimental to the ultimate embryo yield [138]. Despite these promising findings, little work has been done to determine the effect of MeJA or JA on the SE process [139, 140].

Phenolic compounds are comprised of an aromatic ring with at least one hydroxyl group and are a major class of plant specialized metabolites. The presence and/or amount of phenolic compounds has been linked to embryogenicity in several systems. For example, high levels of phenolic compounds (including caffeic, ferulic, and salicylic acids) and the presence of other phenolic compounds (including p-coumaric acid, benzoic acid, trans-resveratrol, catechin, and niringenin) in cotton were linked to success in embryogenic induction [141]. In *Feijoa sellowiana* (pineapple guava) embryogenic cultures, the addition of exogenous caffeic acid during development increased the rate of SE [142], implicating phenolic compounds as signaling molecules in SE. An embryogenic profile of cell wall
phenolic compounds was restored in maize with application of polyethylene glycol (PEG), transforming a non-embryogenic callus into embryogenic tissue [143], suggesting the critical nature of phenolic compounds in SE. These data support other works showing that phenolic compounds play an important role in plant morphogenic processes such as shoot proliferation [144], rooting [145], and rhizogenesis [146]. Because of the importance of phenolic compounds in embryogenic processes, quantification is important to characterize cultures prior to induction of embryo development. Here, we quantify the total phenolic content as a measure of culture stress and as a screen for a potential link between the total phenolic content and SE success or failure.

Here, we explore early activation of stress-induced pathways via application of MeJA to loblolly pine maintenance phase cultures just days before transfer to development media, at an earlier time than previously studied in other SE systems. Simple process manipulations that improve embryo yield are advantageous as a cost-effective and easy-to-implement means to improve SE, and these results can be extended and tested on other commercial crops capable of SE. Insights into the SE process are gleaned through quantification of phenolic compound accumulation during maintenance-stage culturing as a screenable marker to determine stress levels in vitro. Finally, a strong relationship is identified between two-dimensional culture growth on solid media and embryo yield, potentially simplifying embryo yield quantification and enabling more high-throughput studies. Together, these results provide a simple method to improve embryo yield as well as time-saving screening steps for this valuable industrial process.

5.2 Materials and Methods

5.2.1 Cell culture and application of treatments

Cell cultures were established at an industrial partner facility as described previously [25] and shipped overnight on ice. Once received, they were cultured weekly by settling the
cultures for 20 minutes, removing excess conditioned media, and transferring one part of the
remaining settled cells into four parts fresh media (for typical maintenance, this amounted
to 72 mL fresh media plus 18 mL settled cells in a 500 mL flask); media formulations were
as previously described [59]. Cultures were incubated at 23°C at 110 RPM in the dark. For
experiments, cultures were divided into 250 mL Erlenmeyer flasks with foam stoppers at a
total culture volume of 50 mL. On day 4 or 6 of the seven-day culture period, treatments
were applied to the cultures as follows.

Methyl jasmonate stock was prepared by mixing 435 μL 100% ethanol (Sigma Aldrich, St.
Louis, MO), 523 μL nanopure water, and 42 μL methyl jasmonate solution (Sigma
Aldrich, St. Louis, MO). The stock solution was filter sterilized using a 0.22 μm PVDF
syringe filter unit (MilliporeSigma, Billerica, MA) and dilutions were created using filter
sterilized ethanol (50% v/v). For controls, a mock solution of 50% v/v ethanol was applied
to the cultures. Treatments were applied on day 4 or day 6 by adding a total volume of
23.7 μL solution to each flask to attain the desired concentration. Since flasks had been
sampled for analysis, total culture volume at the time of MeJA application was 44 mL for
all experiments. All treatments were assessed in biological triplicate flasks.

5.2.2 Embryo development

SE cultures were plated as described in Section 2.2.2. Three Petri dishes were used
per flask for a total of nine development plates per treatment within each experiment. The
petri dishes were stored in the dark at room temperature for a 12-week period. After 12
weeks, the embryos were counted by hand using forceps. Fresh weight was determined by
weighing the dishes before counting and after removing all biomass from the plate.

5.2.3 Sampling

Samples were taken throughout the seven-day culture period. On days 0, 4, and 7
well-mixed culture samples were taken using a 5 mL pipette with a cut tip. On day 7, media
samples were taken after allowing the cells to settle for 20 minutes. Cell samples were taken
by filtering 15 mL of well mixed culture on a sterile coffee filter and pulling a vacuum to remove any excess media. Cells were scooped into microcentrifuge tubes. All samples were frozen at -80°C for future analysis.

5.2.4 Image analysis

Photos were taken of all plates prior to counting using a Panasonic Lumix FZ200 camera with manual focus. To determine the area of the Petri dish occupied by the biomass on each individual plate, MATLAB was used for image analysis. Since a camera mount was used such that the distance between the plate and the camera was constant for all photographs, image calibration of each individual photo was unnecessary. Color channels were thresholded to allow identification of ESM and embryos using machine vision, and the area occupied by the biomass was quantified in pixels (code reproduced in Appendix D). Areas of the image containing other elements in the development plates such as the mesh and media were discarded from this measurement.

5.2.5 Coulter counter

The Coulter counter was run as described in Section 2.2.3 for quantification of dry weight for normalization of phenolic content with respect to biomass levels. A dry weight (DW) correlation was used to approximate the dry weight in liquid cultures as a function of volume measured by the Coulter counter [27]. DW (g L⁻¹) was calculated as a function of the volume of particles with d<233 μm ($V_{<233}$) and the volume of particles with d>233 μm ($V_{>233}$):

$$DW = A \times V_{<233} + B \times V_{>233}$$

(5.1)

where $A$ and $B$ have units of g L⁻¹ μm⁻³, and $A$ is equal to $4.2 \times 10^{-10}$ and $B$ is equal to $2.6 \times 10^{-10}$. Volumes determined from the Coulter counter are input in μm³. Here, the dry weight was used to normalize phenolic content to biomass within maintenance cultures.
5.2.6 Determination of phenolic content

Culture samples were dried in an Eppendorf Vacufuge Plus (Eppendorf, Hamburg, Germany) overnight at 30°C. 500 μL methanol (Sigma Aldrich, St. Louis, MO) were added and samples were incubated at room temperature for one week. Samples were then placed into an Aquasonic Model 75HT sonicator bath (VWR, Radnor, PA) with ice water for 20 minutes to effectively disturb the biomass. The samples were then centrifuged at 20,000 x g for 10 minutes to clear the extract of insoluble particles.

Total phenolic content was determined, as previously described [147]. Briefly, 20 μL of sample were added to a microcentrifuge tube. For a standard curve, dilutions of a gallic acid solution were made in the range of 0-0.2 mg mL⁻¹. Then 40 μL of 0.2 N Folin-Ciocalteu reagent (Sigma Aldrich, St. Louis, MO) and 160 μL of 700 mM sodium carbonate were added. Tubes were incubated at room temperature for 10 minutes and then centrifuged for 60 seconds at 20,000 x g to clear any precipitate. The absorbance of each sample was read at 750 nM using the Multiskan GO Microplate Spectrophotometer (Fisher Scientific, Waltham, MA) and the concentration was determined using the gallic acid standard curve. Phenolic concentrations were normalized to the biomass levels determined by the Coulter counter for the same day the samples were taken.

5.2.7 Experimental summary

Experiments were performed as outlined in Table 5.1. The first four experiments aimed to screen the effect of MeJA across a wide range of culture conditions, including two distinct genotypes and two days of MeJA addition to cultures. Here, a genotype is defined as a culture established from the same parent trees, such that a genotype in cultures produces genetically identical (clonal) embryos [14, 59]. Experiments were then streamlined toward the most successful conditions to test the robustness of the approach across culture age and genotype.
Table 5.1 Summary of experiments included in MeJA analysis, including experiment ID (titles) and the combinations of treatments therein

<table>
<thead>
<tr>
<th>Exp. ID</th>
<th>Cell line</th>
<th>Age when plated (days)</th>
<th>Day of MeJA addition</th>
<th>Treatments (MeJA Concentration, μM)</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1a</td>
<td>A-1</td>
<td>104</td>
<td>6</td>
<td>x</td>
</tr>
<tr>
<td>1b</td>
<td>A-1</td>
<td>111</td>
<td>4</td>
<td>x</td>
</tr>
<tr>
<td>2a</td>
<td>B-1</td>
<td>118</td>
<td>6</td>
<td>x</td>
</tr>
<tr>
<td>2b</td>
<td>B-1</td>
<td>125</td>
<td>4</td>
<td>x</td>
</tr>
<tr>
<td>3</td>
<td>B-1</td>
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<td>6</td>
<td>x</td>
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<td>4</td>
<td>A-1</td>
<td>251</td>
<td>6</td>
<td>x</td>
</tr>
</tbody>
</table>

5.2.8 Statistical methods and analysis

To determine significance of the responses to MeJA treatment levels within a single experiment, GraphPad Prism version 7.03 for Windows (GraphPad Software, La Jolla, CA) was used to perform a One-way ANOVA with Dunnett’s test for multiple comparisons. All corresponding figures were made using R.

To determine robust responses conserved across multiple experiments and treatment parameters, a three-way ANOVA was used with Tukey’s post-hoc test to determine differences among means using SAS Studio software version 3.06 for Windows. The effects of treatment level, genotype, and day of MeJA addition were determined on output variables (e.g., embryo yield, fresh weight after development, phenolic compound accumulation in maintenance, and biomass levels in maintenance determined by the Coulter counter). These methods were first applied to experiments 1a-2b. The same methods were subsequently applied to experiments 1a, 2a, 3, and 4, but with culture age introduced as the third factor instead of day of addition. After determination of statistical groupings and significance levels in SAS software, the final boxplots were created in R for data presentation. The boxplots presented in this work provide an effective data visualization tool, since many parameters are included in their construction. The whiskers encompass all of the data except for outliers, which are illustrated as empty circles. The grey boxes outline the middle 50% of the
data, and the horizontal line within the box denotes the median. Finally, the mean is plotted as the solid circle, providing a full visualization of the influence of parameters on the results.

5.2.9 Estimation of missing parameters

Because of the long timespan of SE experiments in loblolly pine, the feedback loop for successful treatment levels is lengthy and imperfect. For example, it was determined that the most successful treatments were in the lower concentration range, and so for experiments 3 and 4 an additional low-concentration treatment was added and the two highest concentrations were not included, as discussed below. Therefore, the response of cultures to 0.5 μM MeJA for experiments 1a-2b was estimated to enable analysis across all experiments. To do so, the response variables (e.g., embryo yield, fresh weight, phenolic compound accumulation) of cultures to 0.5 μM MeJA were assumed to follow a normal distribution. This is anticipated to be a valid assumption based on the normality of the response variables to 0.1 and 1 μM MeJA.

The results from 0.1 and 1 μM MeJA treated cultures were used to estimate the mean and the variance of the predicted normal distribution at 0.5 μM. Observations were culled from this estimated response population to represent the response at 0.5 μM MeJA. These estimated results are used in the data analysis only and should not be used to recommend treatment levels.

5.3 Results and discussion

SE in loblolly pine is a three-phase process, in which: 1) embryonic suspensor masses (ESMs) are grown in a liquid maintenance culture for propagation, 2) ESMs are moved to solid development media to initiate differentiation into an embryo, and 3) embryos are isolated and placed on a third media type to germinate [25]. Here, the goal was to improve embryo yield outcomes in the development stage by manipulating the maintenance
phase cultures. To ensure robust results, an engineering solution is sought for SE cultures that is beneficial across numerous parameters that can influence yield. The treatments were tested on both fresh cultures and old cultures, since loblolly SE cultures are recalcitrant with age; embryo yield typically declines throughout routine culture (data not shown), a finding consistent with other systems [65, 148]. Multiple genotypes were tested, as significant yield variation is possible from one genotype to the next.

The first four experiments described herein (Table 5.1, Experiments 1a, b and 2a, b) were performed to screen loblolly SE cultures for the best treatment combination, and so a large range of MeJA concentrations (0-100 μM) and two different addition days (day 4 and day 6 of a 7-day culture period) were tested across two distinct cell lines. The upper limit for MeJA application (100 μM) was selected based on concentrations that elicit a defense response in other systems for production of specialized metabolites, including \textit{Taxus} for paclitaxel production [149]. 100 μM MeJA was expected to act as a negative control in this study by shifting metabolism away from growth and development and toward specialized metabolic pathways, and the lower concentration limit was selected based on the benefit of MeJA in the development stage in cotton SE [124]. Since treatment of loblolly SE in the maintenance phase only exposed cultures to MeJA for 1-3 days instead of the entire development stage, the mid-range tested concentrations were expected to be most beneficial.

The experiments also include two loblolly pine genotypes, which perform differently from batch to batch [150], and by introducing these additional cultures the resultant hypotheses are strengthened and benefits are expected to be translatable to additional genotypes.

The first four experiments were run within a four-week period to better enable comparison of results across experiments and to eliminate culture age as a factor in the analysis. Experiments were designed to determine which treatment level(s) should be further investigated and which should be excluded from future analyses. Since in the subsequent experiments an additional concentration of MeJA (0.5 μM) was included, the results from this treatment
were estimated for experiments 1a,b and 2a,b, as described for the data analysis.

5.3.1 Morphological changes in response to methyl jasmonate

In loblolly pine SE cultures, ESMs exist across three types of proembryonic masses (PEMs I, II, and III) spanning various developmental stages (see Figure 1.3). PEM I’s consist of vacuolated suspensors attached at a small proembryonic head and proliferate to form the larger PEM II’s. These aggregates have a longer and denser mass of suspensors attached to the proembryonic head, and these aggregates grow and divide to form PEM III’s, the most developed aggregate types present in maintenance phase culturing and the types hypothesized to form embryos [32]. Morphological responses at early stages of development have been linked to downstream success or failure in other embryogenic systems including pecan [151] and coffee [152]. Since MeJA induces stress in plants, culture morphology was characterized in response to hormone addition as an early determination of culture health and potential.

Figure 5.1a-d compares the culture morphology at the PEM level in the maintenance stage on the day of plating across a range of MeJA concentrations for genotype B during the day 6 addition experiment. Here, the morphology for a single genotype is shown, but results were comparable across both genotypes (data not shown). In the control cultures, a typical morphology was observed (Figure 5.1a) where cell types range from single vacuolated suspensor cells to the most organized and developed PEM III’s. Comparable morphologies were observed in cultures exposed to the lower ranges of MeJA concentrations (0.1 μM and 10 μM: Figures 5.1b and 5.1c, respectively). However, at the highest concentration of MeJA, the culture showed signs of stress, including asymmetric PEMs, an atypical observation in the loblolly SE developmental pathway and an early indication that 50 and 100 μM MeJA is detrimental to embryo yield, Figure 5.1d. Asymmetric, or fused, early embryos have been found to be less likely to successfully convert into plantlets in other systems [153, 154], so based on the culture morphology observed here (Figure 5.1) we anticipated lower
embryo yields at 100 μM MeJA.

Twelve weeks after initiation of development, an observable difference was observed in the culture growth and development progression. Figure 5.1 e-j shows final development images for genotype A with day 6 addition of MeJA, and Figure 5.1 k-p shows the same genotype with day 4 addition of MeJA. In both cases, the two highest MeJA concentrations (Figure 5.1i, j and Figure 5.1o, p) showed less outward growth and more gaps in the biomass than the control plates (Figure 5.1e and k). Further, the day 4 addition development plates (Figure 5.1 k-p) displayed these effects more strongly; less growth was observed on the day 4, 50 μM MeJA plate (Figure 5.1o) than on the comparable day 6 addition plate (Figure 5.1i). The morphology of the plates with 10 μM MeJA appeared consistent with the controls for the day 6 addition experiment (Figure 5.1e-h). Contrastingly, the 10 μM
MeJA day 4 addition experiment exhibited sparse growth at lower concentrations than its
day 6 counterpart (Figure 5.1k versus Figure 5.1n), indicating that day 4 addition may be
more harmful to cultures than the day 6 MeJA addition. To quantify the effect of MeJA on
culture proliferation in the development stage, the total mass of ESM and embryos after 12
weeks on development media was determined and the results compared using a three-way
ANOVA and Tukey’s post hoc test. The data for experiments 1a,b and 2a,b were combined
for analysis so that only robust responses would be identified. Figure 5.2 shows inhibition
of growth at 10, 50 and 100 μM MeJA for both day 4 and day 6 addition with p<0.001,
consistent with growth inhibition at high MeJA levels in other SE culture systems [155,
156].

Figure 5.2 Effect of MeJA on development-stage growth among two different genotypes
and two different days of addition. The highest concentrations of MeJA inhibited growth.
Different letters represent different statistical grouping (p<0.05) by three-way ANOVA and
Tukey’s post-hoc test. For each concentration, n=36 and the box outlines the middle 50%
of the data, the line inside the box represents the median and the filled in circle the mean,
whiskers encompass all of the data except for outliers which are shown as empty circles.
Data with different letters are statistically different with p<0.05 by three-way ANOVA with
Tukey’s post-hoc test.
5.3.2 Low doses of methyl jasmonate improve embryo yield outcomes

A three-way ANOVA with Tukey’s post-hoc analysis was used to determine the effect of MeJA concentration, day of MeJA addition, and genotype on the embryo yield. Figure 5.3 shows the embryo yield response for both genotypes across all concentrations of MeJA. Here, data were pooled to determine the overall effect that each of the treatment levels had on both genotypes and both MeJA addition days. While the absolute value of the average embryo yield was highest in the 0.1 μM MeJA cultures, this effect was not statistically significant (p=0.507). After identifying positive trends in the day 6 addition experiments, the day 6 addition experiments from both genotypes (experiments 1a and 2a) were plotted separately (Figure 5.4). Individual experiments were analyzed using a one-way ANOVA with Dunnett’s test for multiple comparisons. Experiment 1a (Figure 5.4a) showed no significant yield increase at 0.1 μM MeJA but yield values trend toward beneficial. Experiment 2a demonstrated a significant increase in embryo yield at 0.1 μM (p<0.01, Figure 5.4b), which encouraged further investigation of the effect of MeJA on loblolly pine SE.

The three highest concentrations of MeJA (10, 50, and 100 μM) significantly inhibited embryo yield (Figure 5.3, p<0.0001). Similar results were demonstrated at high levels of MeJA in *M. sativa* L. [156] and cotton [124], though these results were demonstrated after constant exposure of MeJA throughout culture rather than the short term exposure explored here. Our results also show a significantly increased accumulation of phenolic compounds in the maintenance phase at 10, 50, and 100 μM MeJA (Figure 5.5, p<0.0001). As plant cell cultures are subjected to external sources of stress, the metabolism changes and carbon flux shifts away from growth and toward defensive compounds [133]. Phenolic compounds comprise a large class of plant specialized metabolites [157] and their measurement provides a metric to identify such a shift in metabolism. Here, the upregulation of phenolic compounds at the 10, 50 and 100 μM MeJA treatment levels (Figure 5.5) indicates that at
Figure 5.3 Embryo yield in response to MeJA treatments in two different genotypes and two different days of addition follows a similar pattern as growth. Embryo yield is inhibited at the three highest concentrations of MeJA. For each concentration, n=36 and the box outlines the middle 50% of the data, the line inside the box represents the median and the filled in circle the mean, whiskers encompass all of the data except for outliers which are shown as empty circles. Data with different letters are statistically different with \( p<0.05 \) by three-way ANOVA with Tukey’s post hoc test.

these levels, MeJA induces a detrimental stress \textit{in vitro}. Combined with the significantly inhibited embryo yield at these levels (Figure 5.3) and visible and quantifiable growth inhibition in development (Figure 5.1 i, j, o, and p; Figure 5.2), high concentrations of MeJA are surmised to push the cultures too far down jasmonate-responsive stress pathways to benefit embryo development and yield. Since the goal of this research is to improve embryo yield, the concentrations of 50 and 100 \( \mu \text{M} \) were dropped for all subsequent experiments. 10 \( \mu \text{M} \) MeJA was included as a negative control for embryo yield improvement at the lower MeJA concentrations.

Genotypes A and B had significantly different embryo yields with \( p<0.0001 \); cultures from genotype A on average yielded more embryos than genotype B (Figure 5.6).
Figure 5.4 Embryo yield in response to day 6 addition of MeJA in individual experiments shows improvement in yield at low concentration. (a) Experiment 1a shows no significant yield benefit at any concentration but yield inhibition at higher concentrations. (b) Experiment 2a shows significantly improved embryo yield at 0.1 μM MeJA (p<0.01) and similar yield inhibition at higher concentrations. For each concentration, n=9 and the box outlines the middle 50% of the data, the line inside the box represents the median and the filled in circle the mean, whiskers encompass all of the data except for outliers which are shown as empty circles. * denotes statistically different than the control culture using One-way ANOVA with Dunnett’s test for multiple comparisons, with ** p<0.01 and **** p<0.0001.

Since unique cultures often do not behave identically in SE systems [65, 150] this is an expected result. The difference in yield between genotype and the conserved effect of MeJA across genotypes provides confidence in our hypothesis that low-level MeJA concentrations improve embryo yield and both genotypes were used in subsequent analyses. Day 4 and day 6 MeJA addition resulted in statistically identical embryo yield responses with p=0.5225, and thus one addition day was dropped for all subsequent experiments. Based on the morphology of the embryos after 12 weeks on development (Figure 5.1 i,j versus Figure 5.1 o,p), the day 6 treated plates had more acceptable growth in the development stage. Further, improved embryo yields were observed in the day 6 experiments when analyzed alone (Figure 5.4 a and b), and therefore day 6 was chosen as the day of addition for additional experiments.
Figure 5.5 Phenolic compound accumulation in two different genotypes and two different days of addition in the maintenance phase cultures in response to MeJA. The increase in phenolic content at 10, 50, and 100 μM MeJA indicates a shift in culture metabolism toward stress-induced pathways. For each concentration, n=12 and the box outlines the middle 50% of the data, the line inside the box represents the median and the filled in circle the mean, whiskers encompass all of the data except for outliers which are shown as empty circles. Different letters represent different statistical grouping by three-way ANOVA with Tukey’s post-hoc test (p<0.05).

5.3.3 Benefit of methyl jasmonate treatment does not depend on culture age

The same cultures used in the time of addition experiments were aged and the experiments were repeated. For these experiments, MeJA treatments were used on the lower end of the treatment range with an additional low-range concentration (0.5 μM MeJA). As a culture ages, lower embryo yields are expected since SE culture embryo yields decrease over time [65]. Therefore, this approach guaranteed insight into the response of low-yielding cultures to MeJA treatments. Culture age has a significant effect on embryo yield for both genotypes A and B (p<0.0001, Figure 5.7a). Cultures that were over 200 days old at the
Figure 5.6 Genotypes A and B had significantly different mean embryo yields across all MeJA concentrations and both days of addition, providing confidence that experimental results can be translated to other genotypes. For each genotype, n=126 and the box outlines the middle 50% of the data, the line inside the box represents the median and the filled in circle the mean, whiskers encompass all of the data except for outliers which are shown as empty circles. **** denotes statistically different by three-way ANOVA with Tukey’s post-hoc test with p<0.0001.

time of plating yielded, on average, approximately one third the embryos that cultures 104-125 days old at the time of plating yielded. Additionally, both genotypes had a statistically different embryo yield (p<0.0001, Figure 5.7b), which remains consistent with the results in Figure 5.6 despite the negative influence that advanced culture age had on embryo yield (Figure 5.7a).

Embryo yield was significantly improved at 0.1 μM MeJA with p<0.01 (Figure 5.8). This result is consistent across both cell lines at both culture ages, despite the inherent variation caused by these two variables (see Figure 5.7). Since this is a robust effect independent of culture age or cell line, we propose the addition of a low concentration of MeJA one day prior to moving cultures to development as a cost-effective means to improve SE yield. MeJA has been identified as beneficial within other embryogenic systems (albeit no other coniferous systems), but has not been analyzed at this early point in the process with a robust test across variable factors. For example, low levels of MeJA applied to a cotton SE system
Figure 5.7 Effect of culture age and genotype on embryo yield from day 6 MeJA addition experiments. a) Age has a significant effect on embryo yield in day 6 MeJA addition experiments. Cultures around 200 days old yielded fewer embryos than cultures around 100 days old (p<0.0001). b) Embryo yield is significantly different in both genotypes tested (p<0.0001) in day 6 MeJA addition experiments. For each culture age or genotype, n=90 and the box outlines the middle 50% of the data, the line inside the box represents the median and the filled in circle the mean, whiskers encompass all of the data except for outliers which are shown as empty circles. **** denotes statistically different by three-way ANOVA with Tukey’s post-hoc test with p<0.0001.

during the development stage improved not only embryo yield but also embryo morphology [124], but this was applied later in the process and as a long-term exposure throughout the entire development stage.

Other analogous embryogenic systems have proposed the manipulation of MeJA levels through exogenous addition to influence yield outcomes. Propagation of orchid is achieved through the formation of protoderm-like bodies (PLBs, which are analogous to somatic embryos [158]). The inclusion of 1 mg L\(^{-1}\) MeJA (approximately 5 μM) in orchid micropropagation culture medium improved the rate of formation of PLBs [159], suggesting a beneficial strategy to improve PLB yield. In a *Brassica napus* microspore embryogenesis system, short-term exposure of cultures to JA improved embryo yield but resulted in lower germination success downstream of embryo development [140]. These studies support our
findings that low levels of MeJA can benefit embryogenesis but also underscore the need to continue future experiments through until the end of the germination stage to ensure long-term culture success.

Figure 5.8 Low concentrations of MeJA added on day 6 improve embryo yield despite variation that arises from genotype and culture age. 0.1 μM MeJA significantly improves embryo yield among pooled data (p<0.01) despite variability introduced by age and genotype effects (see Figure 5.7). For each concentration, n=36 and the box outlines the middle 50% of the data, the line inside the box represents the median and the filled in circle the mean, whiskers encompass all of the data except for outliers which are shown as empty circles. Different letters represent different statistical grouping (p<0.05) determined with a three-way ANOVA with Tukey’s post-hoc test.

5.3.4 Phenolic compound accumulation depends on culture age

In this work, total phenolic compound accumulation appears to be a stress response rather than a marker for embryo yield success. Figure 5.5 shows an upregulation of phenolic compound accumulation in cultures after treatment with 10-100 μM MeJA. Contrastingly, there was no change in phenolic compound accumulation at any MeJA concentration in day 6 treated cultures (Figure 5.9a). Since Figure 5.5 includes data from experiments where MeJA was added on either day 6 or day 4, the response of some cultures exposed to the
stress hormone for three days prior to moving to development were included in this analysis. These cultures had a longer contact time with MeJA prior to moving to development media and thus a longer time to react and shift metabolism, thereby accumulating higher levels of phenolic compounds. Figure 5.9a suggests that 1 day of exposure to low-levels of MeJA does not shift culture metabolism significantly in the direction of the specialized metabolic pathways involved in phenolic production. No change in phenolic accumulation compared to the control was seen in any cultures treated with 1 μM MeJA or less (Figure 5.5 and Figure 5.9a). These results suggest that while there was a shift in culture metabolism at low concentrations evidenced by the increased downstream embryo yield, the cultures were not pushed down stress-related pathways to the point of detriment.

Figure 5.9 Effect of MeJA concentration and culture age on phenolic compound accumulation among day 6 MeJA treated cultures and both genotypes. a) Day 6 MeJA treatments cultures do not affect phenolic compound accumulation. b) Older cultures produce significantly less phenolic compounds than fresh (p<0.0001). For each concentration, n=12, and for each culture age n=30. The box outlines the middle 50% of the data, the line inside the box represents the median and the filled in circle the mean, whiskers encompass all of the data except for outliers which are shown as empty circles. **** denotes statistically different with p<0.0001, ns denotes not statistically different results.

Another interesting result from these analyses was the effect of aging on culture metabolism. As expected, older cultures yielded significantly fewer embryos than newer cultures (Figure 5.7a). Among all MeJA concentrations and genotypes within the day 6 ad-
dition experiments, older cultures accumulated significantly less phenolic compounds than their fresher counterparts (p<0.0001, Figure 5.9b). Based on these results, loblolly SE cultures are hypothesized to be less metabolically active and therefore less capable of responding to treatments. This is supported by lower embryo yield with age; the cultures did not respond to the development conditions in the same way as the fresher cultures (Figure 5.7). This recalcitrance is largely typical of coniferous species, which become non-responsive to treatments as they age (as reviewed by Bonga et al [66]).

5.3.5 Outward growth correlates with embryo yield

This set of experiments is unique in that it is comprised of six distinct experiments that represent two commercially important genotypes. Thus, a large dataset was created providing the unique opportunity to search for trends. When developing the experimental protocol, the goal was to identify a simpler and more efficient method to determine the final embryo yield since counting nine plate replicates per treatment that can have over 300 embryos each is a time-consuming endeavor. To automate embryo yield assessment, a code was developed for image analysis to quantify the area of the plate occupied by biomass after twelve weeks of development. A digital camera (Panasonic LUMIX FZ200) was used to photograph all development plates through a clear lid at a constant distance from the plate. A MATLAB code (reproduced in Appendix D) was used to discern what area of the plate was covered in culture, using the color for detection (Figure 5.10). By thresholding color channels within each entire photograph, the code is able to discern embryos and ESM from everything else including the media, mesh, and any glare in the photograph. Areas were not normalized within individual plates since photos were taken from a constant distance. To verify this, ImageJ software [160] was used to measure the length of the mesh (known to be 2”x2”) within 9 randomly selected photos. The coefficient of variation was determined to be only 1.6%, confirming that normalization of areas determined from each individual plate image is not necessary.
When plating loblolly ESM culture to development media, the initial spread of the culture across the plate is imperative to plating success. This diameter of the ESM tissue placed on development media is influenced by pipetting speed, distance from plate, and angle of pipetting. Throughout these experiments, every effort was made to generate uniform spread among the plates to eliminate sources of variation. Therefore, uniform distribution of spread at the time of plating is assumed, and those effects should be negligible in this analysis. The two-dimensional area occupied by culture on development plates correlated strongly with the mass of the cultures. Figure 5.11 demonstrates that these two parameters are correlative among four independent experiments, with Pearson correlation coefficients ranging from 0.48 to 0.87, indicating that the two dimensional growth area can be used to approximate the culture growth even though total biomass accumulation depends on
the third dimension of upward growth. Based on observations throughout this dissertation work, cultures initially grow simultaneously upward and outward, but never grow beyond approximately 5 mm from the media. This could be due to transport limitations; as cultures grow upward on the Petri dish the aggregates at the top would not have immediate access to nutrients from the media source, and therefore it is more advantageous for the culture to proliferate outward.

Figure 5.11 Area of plate occupied at the end of development correlates with development stage growth. Correlations between the area of the plate occupied (pixels) against development-stage growth for four of the stress experiments. All plots show data points for individual development plates. For each plot, the linear line of best fit is shown with a 95% confidence band and R values were calculated using Prism. a) Genotype A with day 6 application of MJ, R = 0.87; b) Genotype A with day 4 application of MJ, R = 0.76; c) Genotype B with day 6 application of MJ, R = 0.48; and d) Genotype B with day 4 application of MJ, R = 0.81.

After twelve weeks of development, a strong relationship between area occupied by the cultures (in pixels) and the counted embryo yield was also identified (Figure 5.12). Experiments 1a, 1b, 2a, and 2b provided a large range in embryo yield, and so these experiments were used to determine applicability of any identified trends. Here, each experiment
was plotted individually resulting in strong linear correlations as indicated by Pearson correlation coefficients ranging from 0.5 to 0.88. Using this method, embryo yield can be predicted based on a photograph of a development plate and subsequent computer analysis rather than counting by hand. Typically growth and SE progression is assessed visually or through determination of fresh weight [161]. Here it is demonstrated that a photograph of culture can be used as a predictor of total biomass and embryo accumulation without sacrificing the plate. Therefore, cultures can be photographed and then immediately processed for the next stage in SE (germination).

Figure 5.12 Area of plate occupied at the end of development correlates with embryo yield. Correlations between the area of the plate occupied (pixels) against embryo yield for four of the stress experiments. All plots show data points for individual development plates. For each plot, the linear line of best fit is shown with a 95% confidence band and R values were calculated using Prism. a) Genotype A with day 6 application of MJ, R = 0.88; b) Genotype A with day 4 application of MJ, R = 0.70; c) Genotype B with day 6 application of MJ, R = 0.50; and d) Genotype B with day 4 application of MJ, R = 0.78.
5.4 Conclusions

A simple and robust method to improve embryo yield in loblolly pine somatic embryogenic cultures is presented and analyzed here. The method is inexpensive and easy to implement across both the laboratory and the industrial scale, as it only requires a single addition of MeJA solution 24 hours prior to moving to development. Other studies have attempted to analyze the influence of MeJA on SE success, but this is the first work to implement such short-term exposure prior to moving a culture development-inducing media and is the first study using application of MeJA to coniferous SE systems to influence yield.

Further work must be done to confirm the scalability of the work and that the method can be applied to industrial bioreactors as it can be to culture flasks. Since this work aimed at improving embryo yield and did not study germination, future experiments should be extended through the end of the germination stage to determine the germination rate of the resultant embryos as well as the long-term viability of the resultant crop. Future studies should also include further optimization of concentration of this inexpensive compound, as it is possible that there is another concentration less than 0.1 μM that improves SE at an even greater rate. Since the largest benefit was observed at 0.1 μM and no concentrations were tested below this threshold, this is the logical next step. Additional work could involve introducing other plant stress hormones in the maintenance stage to validate the working hypothesis that application of low concentrations of stressors prior to moving cultures to development is beneficial to embryo yield.

5.5 Acknowledgements

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culture samples for phenolics. I thank Professor Thelge Buddhika Peiris and his student Yutong Feng for their contribution to the data analyses.
CHAPTER 6

SUMMARY AND OUTLOOK

SE is a plant cell culture process useful for large scale propagation of crops with predictable characteristics due to the clonal nature of the resultant offspring. The process is currently limited for widespread, large-scale application due to unpredictable or low yields of germination-competent embryos. This dissertation aimed to glean fundamental insight into the earliest stage of the SE process and develop engineering strategies based on this acquired insight. First steps in this process included adaptation of the industrial scale cell culture process to the laboratory scale, an endeavor that required consideration of patent literature as well as work with experienced professionals. Since the timeline for each experiment spanned 13 weeks from experimental start to final embryo yield assessment, the development of experimental protocols that would provide as much information and potential for future analyses was critical. This experimental outline is discussed in Section 4.2.1 and can be used as a framework for implementation of future SE studies in this coniferous system. Considering the ultimate goal of improvements or screening platforms for an industrial scale system, only easy-to-implement engineering strategies were considered and hypotheses were developed based on SE in similar systems. The findings presented here were developed through analysis of the loblolly pine SE system, but these strategies are easily tested across other SE workflows. The results are expected to be most directly applicable to other coniferous systems but are also likely to be beneficial to any SE system that is maintained in liquid suspension cultures.
6.1 Aggregate size in somatic embryogenesis influences embryo yield outcomes

In Chapter 2, a rapid and robust screening method was identified that links aggregate size distributions *in vitro* to loblolly pine SE outcomes. Through a first-of-its-kind meta-analysis of cellular size distribution data culled from 13 independent experiments, a relationship between small cell types and the downstream embryo yield was identified. This strong, negative correlation between the total volume of aggregates with mean diameter below 273 μm in maintenance cultures and the ultimate embryo yield has the potential to streamline the SE process at any scale by implementing a two-minute assay prior to determine whether to move a culture to development. Further, if a culture is identified to be at a cellular state anticipated to yield a high number of embryos, more of that culture can be plated at that particular timepoint to guarantee sufficient embryo yield.

To further support the identified relationship between accumulation of a large volume of small cell types and low embryo yields, the relationship can be artificially verified in a loblolly SE system. First, a population of aggregates below 273 μm can be isolated by filtration of cultures through a sterile mesh. These small cells can be introduced to intact cultures to mimic different total volumes of these small cell types and plate to development media, effectively creating an artificially produced standard curve. The expectation is a confirmation of the correlation identified in Chapter 2, and the result would provide confidence in direct application of the correlation for embryo yield prediction.

To gain insight into both the progression of cellular morphologies within maintenance cultures as well as its influence on embryo yield, cultures can be fractioned using sterile mesh filters to create distinct populations (<250 μm, 250-500 μm, 500-750 μm, and >750 μm) corresponding to single suspensors, PEM I, PEM II, and PEM III cell types. These filter sizes are recommended based on cellular morphology and size as determined
via microscopy [27]. To enable comparison of data over time and across experiments, the same filter sizes should be applied to all genotypes. These filtered populations can be used in two distinct experimental platforms: 1) fractionation and immediate plating to determine the impact of the artificially induced morphology on embryo yield, and 2) maintenance of these isolated populations for plating 1, 2, and 3 weeks after fractionation to determine growth dynamics across the varying isolated morphologies. The Coulter counter can be used to monitor bulk size distributions, and when combined with microscopy the precise morphological state can be verified. Finally, a culture can be analyzed weekly with the Coulter counter in combination with weekly plating to embryo-producing media. This procedure would enable insight into how a culture morphology changes throughout its useful lifetime and provide insights into developmental links to loblolly pine’s acquired recalcitrance with age. These methods will provide fundamental information about cell cycle progression in loblolly SE cultures as well as strengthen hypotheses developed through the meta-analysis of Coulter counter size distributions in this dissertation.

6.2 Arabinogalactan proteins play a critical role in somatic embryogenesis

In Chapter 3, new assays were developed to enable analysis of AGPs in samples taken from industrial bioreactors. The development of a frozen library of media samples enabled a screen of samples with a wide range of embryo yield values. While the total concentration of secreted AGPs did not correlate with embryo yield outcomes, a subset of AGPs (those recognized by JIM8 and JIM13 antibodies) was identified as correlative with embryo yield using a colorimetric ELISA. Using this newly developed ELISA method to screen for these JIM13 AGPs, media samples can be obtained from a culture of interest and analyzed for a prediction of embryo yield.

With the knowledge that an extracellular subset of AGPs is related to the ultimate
embryo yield, the essential nature of AGPs on this process in loblolly pine was probed by implementing culture engineering strategies in Chapter 4. Here, the necessity of AGPs in loblolly SE was confirmed by starving cultures of AGP through application of the AGP-binding βgluY. High levels of the compound resulted in complete inhibition of embryo yield and growth in the development stage, indicating that AGPs are necessary to both proliferation and differentiation in loblolly SE. Next, extracellular AGP levels were supplemented with two JIM13-binding plant gums. As initially hypothesized, supplementation of GA in vitro inhibits embryo yield in a dose-dependent manner in loblolly pine, supporting the negative correlation between JIM13 AGPs and embryo yield identified in Chapter 3.

Surprisingly, GG improved embryo yield outcomes at low doses despite its binding affinity for JIM13. These results complicate the AGP hypothesis developed in Chapter 3. Further work should be done to investigate fundamental differences between the gums, and analytical techniques including the U-HPLC method presented in Appendix C and the SDS-PAGE and Western Blot techniques developed in Appendix C can be employed to glean insight into these complex plant gums. Further, since both gums exist as natural products and we simply seek the AGP component, the gums can be fractioned using a column with a molecular weight cutoff of 3.5 kDa to remove any small, non-AGP contaminants therein. These fractioned gums can be introduced to loblolly SE culture as an additional culture engineering experiment. After completion of extracellular AGP analysis, the next step would be analysis of cell-associated AGPs. Using the protein extraction method detailed in Appendix C, a larger quantity of AGP can be isolated from loblolly SE cell samples. These extracts can be supplemented into other loblolly SE cultures to determine the influence of the supplementation of these AGPs on embryo yield outcomes.

Another interesting consideration in the study of AGPs in loblolly pine SE would be the analysis of chitinases, the proteins that catalyze the degradation of chitin, an N-acetyl-β-D-glucosamine (GlcNAc) polymer [162]. Plant chitinases are involved in the both
the defense against pathogens and developmental processes [163], and studies have shown that chitinases play an important role in SE [36, 38, 44]. In fact, a search for a chitinase substrate in plant culture was critical in the initial discovery of the AGP [71]. GlcNAc is a component of plant glycosylation chains, making chitinases prime candidates for the mediation of AGP signaling via modification of AGP glycosylation [36]. Treatment of embryogenic carrot cultures with AGPs pre-incubated with chitinases increase SE yield [36], suggesting that chitinases may modulate embryogenicity through modification of AGPs. In other studies, a chitinase was able to restore embryogenic potential to a mutant carrot line [38], and a chitinase-related protein improved early embryo development in Norway spruce [56]. Future work should characterize the effect of application of chitinases and/or AGPs pre-incubated with chitinases to maintenance phase culture media and the influence on downstream embryo yield. The chitinases can be produced using a Baculovirus insect cell expression system, as previously described [36, 164]. Purified chitinases can be incubated with AGPs isolated from cultured medium (using βgluY) and characterized using the same AGP analyses techniques described in this work, and the modified AGPs can be reintroduced to maintenance cultures to test the influence on yield.

The ultimate objective of follow-up experiments to this dissertation work should be a proteomics analysis within differentially yielding cultures. Differential proteomic analyses have been performed in other systems, usually between embryogenic and nonembryogenic tissue within the same species [165] or analyses at different stages of development [166]. An analysis of samples from high, middle, and low yielding maintenance cultures in loblolly pine would be the first analysis of this kind, providing insight into protein states that lead to different yield levels. Throughout the work detailed here, hundreds of cell, total culture, and media samples were taken and stored at -80°C. These samples were taken so that samples from high-, medium-, and low- yielding cultures spanning several distinct genotypes could be processed for proteomic analysis. This sample library is in place as is a protein extraction protocol that can be tested for applicability with the proteomics workflow. The results of this
analysis would be a library of differentially expressed proteins among cultures that resulted in different quantities of embryos. Using this information, fundamental insight into the molecular mechanisms underlying the SE process would be uncovered while simultaneously providing screenable protein biomarkers for varying SE success rates.

6.3 Culture stress plays a role in somatic embryogenesis success

In Chapter 5, low levels of the plant stress hormone methyl jasmonate (MeJA) were demonstrated to robustly benefit embryo yield outcomes across a number of parameters that typically influence yield including culture age and genotype. The influence of this hormone is best when applied just one day prior to moving the culture to development conditions. Insight into fundamental differences between fresh and aged cultures is also gleaned; as a culture ages the response of the culture to a change in culture conditions is reduced, demonstrated both by the overall lower embryo yield as well as the lack of phenolic compound accumulation in the older cultures in response to MeJA. Finally, a method to streamline embryo yield assessment at the culmination of development is achieved through image analysis of a photograph.

Since the lowest tested concentration of MeJA was determined to be the most beneficial to embryo yield, concentrations below this nanomolar level of the hormone should be tested to determine the optimal level for application. Embryos that were treated with MeJA during the maintenance phase should be tested for germination competence by extending the experiments beyond the twelve-week development mark and transitioning the embryos to germination conditions. Additional stress hormones can be tested to determine the influence of these compounds on SE. Using the same experimental platform, hormones such as ABA can be introduced to cultures one day prior to plating to compare outcomes with different hormone supplementation. ABA is a promising compound for subsequent stress-engineering experiments since it is present in the development media, and as such
is already known to benefit the SE workflow at this later timepoint. By introducing the compound shortly prior to moving the culture to development, the culture metabolism may shift toward its embryo-producing state prior the transition to solid media and potentially improve outcomes. Since very little literature exists with short term exposure of SE cultures to treatments like this, it is possible that this strategy could benefit the SE workflow.

6.4 Final remarks

These are the first studies of their kind to enable early predictive screening for embryo yield, a big feat considering that the final data point arrives 12 weeks after plating to development media. For commercial feasibility of such a lengthy process with outcomes that influence the next 30 years of timberland productivity, it is imperative to develop methods that result in predictable yields of germination-competent embryos. As such, this dissertation aimed to provide methods to screen an existing culture for outcomes as well as strategies to improve yield in any given culture. In this vein, results are presented that span several commercially important genotypes of loblolly pine over a period of several years to provide robust strategies for process improvement. These milestones are achieved without genetic modification, thereby preserving the ideal lineage and offering quicker and cheaper engineering strategies. The methods and analyses presented here along with the future recommendations could simultaneously provide simple strategies for early stage SE process optimization as well as fundamental insight into the dynamics of the SE process.
APPENDIX A

MEDIA FORMULATIONS

Here, the protocols and formulations for making both maintenance and development media are outlined. These protocols are based on US Patent #7,785,884 [26] and US Patent #7,598,073 [59]. The protocol for rinse media is not outlined, but the formulation can be found in US Patent #7,598,073 [59] and it can be prepared using similar procedures as detailed in the maintenance media protocol (Section A.3).

A.1 Salt stock protocol and formulation

To expedite media formulations, 4 L of a 10x salt stock was first mixed with amounts detailed in Table A.1. The following protocol was used, and all chemicals were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted:

1. Measure 3.5 L of nanopure water into a large (at least 4 L in volume) beaker and place with a stir bar onto a stir plate in a chemical fume hood.

2. Working one salt at a time, measure out the specified mass of salt in the hood (according to Table A.1) and add to the beaker of water.

3. Allow each salt to dissolve completely before adding the next component. Repeat steps (2) and (3) until all salts are dissolved.

4. Bring volume to 4 L and store at 4°C for up to 6 months.
Table A.1 Summary of salts and quantities included in a 4 L batch of salt stock for maintenance and development media according to US Patent # 7,785,884 [26].

<table>
<thead>
<tr>
<th>Salt</th>
<th>Mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium nitrate</td>
<td>6.000</td>
</tr>
<tr>
<td>Potassium nitrate</td>
<td>36.400</td>
</tr>
<tr>
<td>Potassium phosphate monobasic</td>
<td>5.440</td>
</tr>
<tr>
<td>Calcium nitrate tetrahydrate</td>
<td>9.448</td>
</tr>
<tr>
<td>Magnesium sulfate heptahydrate</td>
<td>9.860</td>
</tr>
<tr>
<td>Magnesium nitrate hexahydrate</td>
<td>10.260</td>
</tr>
<tr>
<td>Calcium chloride dihydrate</td>
<td>2.000</td>
</tr>
<tr>
<td>Magnesium chloride hexahydrate</td>
<td>2.000</td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>0.166</td>
</tr>
<tr>
<td>Boric acid</td>
<td>0.620</td>
</tr>
<tr>
<td>Manganese sulfate monohydrate</td>
<td>0.420</td>
</tr>
<tr>
<td>Zinc sulfate heptahydrate</td>
<td>0.576</td>
</tr>
<tr>
<td>Sodium molybdate dihydrate</td>
<td>0.005</td>
</tr>
<tr>
<td>Copper sulfate pentahydrate</td>
<td>0.005</td>
</tr>
<tr>
<td>Cobalt chloride hexahydrate</td>
<td>0.005</td>
</tr>
</tbody>
</table>

A.2 Additional stock solution formulations

Stock solutions were prepared with the following final concentrations:

1. Iron chelate solution (light sensitive, stored at 4°C for up to 1 year)
   
   (a) 7.450 g L⁻¹ ethylenediaminetetraacetic acid disodium salt (Na₂EDTA)
   
   (b) 5.570 g L⁻¹ iron (II) sulfate heptahydrate

2. Vitamins stock (aliquot into 1 mL tubes and store at -20°C until use)
   
   (a) 1 g L⁻¹ nicotinic acid
   
   (b) 1 g L⁻¹ pyridoxine hydrochloride
   
   (c) 2 g L⁻¹ thiamine hydrochloride
   
   (d) 4 g L⁻¹ glycine
3. 2,4-D stock (store at -20°C until use)
   (a) 10 mg mL$^{-1}$ 2,4-Dichlorophenoxyacetic acid (2,4-D)

4. 6-Benzylaminopurine stock (store at -20°C until use)
   (a) 10 mg mL$^{-1}$ 6-Benzylaminopurine (BAP)

5. Kinetin stock (store at -20°C until use)
   (a) 10 mg mL$^{-1}$ kinetin

6. Abscisic acid (ABA) stock (light sensitive) (aliquot into 25 mL samples and store at -20°C until use)
   (a) 2 g L$^{-1}$ ABA

7. Amino acids stock (aliquot into 1 mL tubes and store at -20°C until use)
   (a) 100 g L$^{-1}$ L-proline
   (b) 50 g L$^{-1}$ L-arginine
   (c) 20 g L$^{-1}$ L-alanine
   (d) 20 g L$^{-1}$ L-serine

**A.3 Maintenance media formulation and protocol**

Maintenance media was used for routine subculturing of liquid-phase culture in this work. The media was prepared as follows, with all chemicals purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted:

1. Add 400 mL prepared salt stock (see Section A.1) to 2 L nanopure water in a 4 L beaker. Add a stir bar and place onto a stir plate.
2. Add the following components, one at a time, waiting for each to dissolve before adding the next component:

   (a) 2.0 g casamino acids (BD Bacto, BD Biosciences, Franklin Lakes, NJ)
   (b) 0.8 g myo-inositol
   (c) 4.0 g L-glutamine
   (d) 120 g maltose

3. Add the following liquid stocks, allowing sufficient time for mixing before adding the next:

   (a) 20 mL iron chelate solution
   (b) 2.0 mL Vitamins stock
   (c) 440 μL 2,4-D stock
   (d) 40 μL 6-Benzylaminopurine stock
   (e) 40 μL kinetin stock

4. Adjust pH to 5.7 using 1M potassium hydroxide solution and 1M hydrochloric acid as needed.

5. Bring volume to 4 L.

6. Filter sterilize at 0.22 μm (Nalgene™ Rapid-Flow™ bottle top PES filter) and store at 4°C for up to 6 months.

**A.4 Development media formulation and protocol**

Development media was prepared as follows, with all chemicals purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted. This protocol yields 4 L development media, sufficient for 120 petri dishes (100mm x 25 mm).
1. Preheat a large water bath (sufficient to hold eight 500 mL bottles) to 68°C.

2. Add 400 mL salt stock and 1.6 L nanopure water to a 4 L flask with a stir bar. Place flask on a stir plate.

3. Add 20 mL iron chelate solution and mix.

4. Add the following components, one at a time, waiting for each to dissolve before adding the next component:
   (a) 0.4 g L-asparagine
   (b) 2.0 g casamino acids (BD Bacto, BD Biosciences, Franklin Lakes, NJ)
   (c) 4.0 g L-glutamine
   (d) 0.4 g myo-inositol
   (e) 400 g PEG-8000 (Acros Organics, Fisher Scientific, Hampton, NJ)

5. Add the following liquid stocks, allowing sufficient time for mixing before adding the next:
   (a) 4 mL amino acid stock
   (b) 2 mL vitamins stock

6. Bring volume of solution to 3.6 L.

7. Divide solution between eight 500 mL bottles, with 450 mL solution into each bottle. Add 0.5 g (acid washed) activated charcoal and a stir bar to each bottle.

8. Shake on an orbital shaker at low speed (approximately 25 rpm) at room temperature for 30 minutes.

9. Working one bottle at a time, adjust the pH of each bottle to 5.7 using 1M potassium hydroxide solution and 1M hydrochloric acid. Add 1.25 g Gelrite® to each bottle and return to shaker.
10. Autoclave at 121°C and 15 psi above atmospheric pressure for 20 minutes. Immediately remove media from autoclave, stir briefly on a stir plate, and place into preheated water bath.

11. Prepare the sugar/ABA solution:

   (a) Add 40 g glucose and 100 g maltose to 240 mL nanopure water. Stir until completely dissolved.

   (b) Adjust volume to 400 mL.

   (c) Add 50 mL ABA stock and immediately filter sterilize the solution at 0.22 μm (Nalgene™ Rapid-Flow™ bottle top PES filter). Store in the dark until use.

12. After approximately one hour, or when media bottles reach 68°C, move one bottle to a stir plate in a laminar flow hood. While stirring, add 56.5 mL sugar/ABA solution and mix well.

13. Divide prepared media bottle between 15 petri dishes (100 mm x 25 mm, Kord-Valmark, Bristol, PA), aiming for 34 mL media in each dish.

14. Allow to cool in the hood for 1 hour. Store in the dark at room temperature for up to one month.
APPENDIX B

FURTHER ANALYSIS OF COULTER COUNTER
SIZE DISTRIBUTIONS

The output from the Coulter counter provides a large dataset in which relationships or trends in aggregate size distributions can be discerned in loblolly ESM cultures. Such an analysis is the first of its kind: a bulk size analysis \textit{in vitro} has not been performed in another SE system. In the process of identifying the strong link between aggregates with mean diameters less than 273 \( \mu \text{m} \) and embryo yield discussed in Chapter 2, many additional analyses were performed. Here I discuss other analyses performed with less direct implications on SE process optimization. The analyses presented in this appendix provide a more complete picture of how analyzed variables are related (or not) within the loblolly SE cell culture system.

B.1 Materials and methods

Data included in this appendix are the same dataset analyzed and discussed in Chapter 2 in the search for a link between size distributions and embryo yield. Correlation matrices are plotted using the corrplot feature in R [60] (Figure B1) or corrplot in MATLAB (Figure B2). 3-dimensional scatterplots were made using MATLAB. Statistical analyses were performed using GraphPad Prism version 7.03 for Windows (GraphPad Software, La Jolla, CA) to determine Pearson correlation coefficients (R values) and p-values for each relationship.
B.2 Results and discussion

B.2.1 Bulk screen of compiled data

Using the corrplot function in R [60] with hierarchical clustering and significance analysis, the curve parameters were arranged in a correlation matrix, Figure B1. All relationships are significant with $p<0.05$ unless marked by an ‘x,’ indicating that many variables are interrelated. Since many of these values are calculated from others, this is largely expected, and any interesting relationships identified from this plot are further discussed in Chapter 2 or in this appendix. Largely these analyses were focused on embryo yield, since this is ultimately the most useful parameter to predict. However, other unique populations that arose from our plots were further explored.
Figure B1 Correlation matrix between all variables analyzed while searching for a trend between loblolly embryo yield and size markers in the maintenance phase. Red ovals are positive trends while blue are negative, and the more narrow the ovals the higher the Pearson correlation coefficient and therefore the tighter the trend. All relationships are significant correlations (p<0.05) unless marked by an ‘x,’ indicating codependence of several variables analyzed. Color is scaled with the R value and the legend is presented at the right indicating the value.

When all treatments were plotted in addition to the controls in a correlation matrix with each flask individually plotted for better data visualization, unique features of the data were identified. Figure B2 shows total volume of aggregates in different size bins and em-
bryo yield. This plot includes the data from Figure 2.7 (embryo yield versus volume of aggregates with diameter less than 273 \( \mu \)m for all individual experimental flasks) as well as the other discussed size bins (e.g., diameters in the range of 273-495 \( \mu \)m). No other size bin had a strong correlation with embryo yield.

### B.2.2 Curve parameters are related to particle size

Aggregate size distributions from each experiment were characterized in a number of ways. One of the ways they were considered was a Peak Broadness calculation, or a modified standard deviation using Bessel’s correction for finite sample sizes, Equation B.1.

The peak broadness used here is a method of describing the distribution of the data in terms of the standard deviation, or a quantitative description of the width of the aggregate size distributions determined from the weighted moving average (Equation 4.1). The peak broadness is calculated as follows:

\[
\text{Peak Broadness} = \left[ \frac{N}{N-1} \sum_{i=1}^{N} \frac{V_i}{V_{tot}} (d_i - \bar{d})^2 \right]^{1/2}
\]  

(B.1)

where \( V_{tot} \) is the total volume measured by the Coulter counter:

\[
V_{tot} = \sum_{i=1}^{N} V_i
\]

(B.2)

\( \bar{d} \) is the mean diameter calculated from the weighted moving average in each flask:

\[
\bar{d} = \frac{1}{N} \sum_{i=1}^{N} d_i
\]

(B.3)

and \( N \) is the number of size bins data are allocated into (within the data output from the Coulter counter) and \( i \) is the bin index. In Figure B3, the volume of aggregates in several size bins are plotted against the peak broadness for all experimental flasks. Here, an increasingly
Figure B2 Correlation matrix between volume populations and embryo yield for each experimental flask analyzed. Each point represents one flask in an experiment. Diagonal shows the distribution of the datasets along each axis.
correlative relationship is seen as the lower diameter cutoff increases. Logically, this makes sense: the more 'large' particles exist in a culture (given that small aggregates are also observed, as they are in cell culture) the higher the mean diameter is anticipated to be (Figure B4) as the aggregate size distribution curves simultaneously widen.

Figure B3 Peak broadness is related to volume of large aggregates. Volume of increasingly large size bins plotted against the calculated peak broadness (μm) for each experimental flask. (a) Total volume of aggregates with d>273 μm against peak broadness, R=0.72, (b) Total volume of aggregates with d>495 μm against peak broadness, R=0.80, and (c) Total volume of aggregates with d>691 μm against peak broadness, R=0.83. All correlations are significant with p<0.0001.

Figure B4 shows the volume of large aggregates in cultures plotted against the mean diameter for each flask. Here an interesting phenomenon arises: apparent populations in the data. There is a cluster of datapoints that fall on the low-volume, low $\bar{d}$ portion of the data, as well as two populations that deviate in the mid-range of the data. This was further explored for Figure B4b by adding a third dimension to the plot. Embryo yield, the focus of our investigation, was plotted as the third dimension in Figure B5. All high-yielding flasks (colored orange or yellow, with embryo yield greater than 300) fell within the same population on the plot.
Figure B4 Mean diameter is related to volume of large aggregates. Volume of increasingly large size bins plotted against the calculated mean diameter (μm) for each experimental flask. (a) Total volume of aggregates with $d>273$ μm against $\bar{d}$, $R=0.77$, (b) Total volume of aggregates with $d>495$ μm against $\bar{d}$, $R=0.89$ and (c) Total volume of aggregates with $d>691$ μm against $\bar{d}$, $R=0.90$. All correlations are significant with $p<0.0001$.

These relationships alone are not conclusive but open the possibility of further analysis. It is possible that there is an underlying relationship that yet to be uncovered that could be even more predictive of embryo yield outcomes, even within an experiment. Since the current predictive relationship is only effective 1) after accumulation of a baseline range in yields and Coulter counter size distributions and 2) for control or routinely plated flasks, it would be beneficial to predict yield outcomes even after application of treatment. To do so, it is recommended to work from a larger dataset than is currently available.

B.2.3 Populations that were not significant

Here, I provide a brief summary of some of the additional analyses performed to characterize the cultures in terms of size distributions.

- The fold change in measured parameters between days 4 and 7 were calculated to determine if the growth rate of any analyzed population was correlative with embryo
Figure B5 Plotting third dimension provides some insight into apparent populations identified in mean particle diameter analysis. Volume of particles with \(d>495\, \mu\text{m}\) plotted against \(d\) color coded by the ultimate embryo yield. All high embryo yield (yield > 300) flasks fell in the same region on the plot.
yield. This division process, when applied to the data, yielded very large error bars that obscured any trends in the data and thus no useful relationships with growth rates were determined.

- The size distributions on day 4 of culture (three days prior to plating to development media) were analyzed. While the trend between aggregates with mean diameter below 273 \( \mu \text{m} \) and embryo yield was still significant, the trend was less strong and therefore less useful for application. All other relationships were similarly diminished at day 4, indicating that the optimal time to measure the culture’s size parameters for yield prediction is as close to plating as possible (preferably the day of plating).

- Using size distribution equations inspired by molecular weight distribution characterization equations from polymer science, the dispersity in the diameters of the aggregates was calculated. The dispersity function quantified the heterogeneity diameter within the aggregates measured:

\[
\text{Dispersity} = \frac{\sum_{i=1}^{N} N_i d_i^2}{\sum_{i=1}^{N} N_i d_i} \times \frac{\sum_{i=1}^{N} N_i d_i}{\sum_{i=1}^{N} N_i} \quad (B.4)
\]

where \( N \) is the number of particles in each bin output by the Coulter counter, \( i \) is the bin index, and \( d_i \) is the diameter in each bin. There was no significant relationship between the culture heterogeneity and the embryo yield.
APPENDIX C
EXTRACTION AND ANALYSIS OF
CELL-ASSOCIATED AGPS

After identification of extracellular JIM13 as a potential biomarker for embryo yield, analysis of cell-associated JIM13 AGP epitope is the next step. Most work with the JIM13 antibody tests for the presence and location of the JIM13 epitopes, rather than quantity [42, 167, 168]. One recent study used a protein extraction and identified JIM AGPs within a protein gel and Western blot [102], indicating that traditional protein extraction methods can work to isolate these heavily glycosylated proteins. A robust method to extract plant proteins from loblolly suspension cultures was developed, which overcame challenges unique to plant culture systems including pH and interfering compounds in the media. Once a clean protein extract in the desired buffer was created, an extraction process was developed for AGPs for quantification and potential characterization work.

To begin to understand the molecular differences in AGPs, methods were developed for analysis given a protein or AGP extract using SDS-PAGE, JIM13 Western blots, and U-HPLC to characterize purified AGPs. HPLC has been performed on cotton [37], cashew [121], and Arabidopsis [169] AGPs. U-HPLC is a preferred method due to the more rapid runtimes which enable more high-throughput analysis of many samples, lower mobile phase consumption leading to lower operating cost, and improved peak resolution. The adaptation to the smaller-scale, higher pressure U-HPLC system included 1) column selection based on AGP characteristics, 2) scale-down to the smaller U-HPLC column volume (flow rate, sample volume, etc) and 3) mobile phase selection, including a solvent gradient throughout each run. The baseline setup was a reversed-phase system using a Waters ACQUITY U-HPLC system.
C.1 Materials and methods

C.1.1 Protein extractions

To prepare for a protein extraction, one scoop (approximately 100 μL) 0.5 mm zirconium oxide beads were placed into locking microcentrifuge tubes and weighed. The protein extraction buffer was prepared using 100 mM NaCl (Fisher Scientific, Waltham, MA), 20 mM sodium acetate pH 5.5 (Sigma Aldrich, St. Louis, MO), 10% glycerol (Alfa Aesar, Ward Hill, MA), and 1% Triton X-100 (Sigma Aldrich, St. Louis, MO) and stored at 4°C until use. Just prior to use, Halt™ Protease Inhibitor Cocktail and EDTA were added to a concentration of 1x (Pierce Biotechnology, Waltham, MA) and the buffer was placed on ice to chill before use.

Next, cell samples were taken from suspension cultures and poured over sterile coffee filters in a buchner funnel. A vacuum was pulled over the funnel to remove the media until the cells appeared dry, white, and fluffy. Cells were immediately scooped into the prepared tubes, weighed, and placed on ice. 800 μL prepared extraction buffer was added to each tube. Cells were lysed using the Bullet Blender Gold (Next Advance, Averill Park, NY) run at speed 6 for 3 minutes using the 4°C cooling feature with dry ice. The tubes were immediately removed and placed onto ice and allowed to rest for 30 minutes. Next the tubes were centrifuged at 3.75 g for 30 minutes and the supernatant was transferred into clean tubes. The blending process was repeated once more with 400 μL buffer, and the supernatants were pooled.

C.1.2 Downstream processing of protein extract

To remove debris, the extract was filtered using a low-binding 0.22 μm PES syringe filter unit (EMD Millipore, Billerica, MA). The extracts were then dialyzed against 100 mM NaCl in 5 mM acetate buffer pH 5.5 using Snakeskin Dialysis Tubing with a 3.5 kDa molecular weight cutoff (Fisher Scientific, Waltham, MA). The tube of extract was floated
in a beaker of dialysis buffer at 4 °C on a slow moving stir plate. A total volume of dialysis buffer 3000 times the sample volume was used, changing to fresh buffer every 4-8 hours a total of 3 times. After dialysis, the purified extract was recovered, the volume recorded, and was frozen until later use.

C.1.3 AGP extraction

To extract AGPs from the dialyzed extract, the colorimetric AGP assay was performed as described (section 3.2.3) with several changes. First, 400 μL dialyzed extract was mixed with 400 μL 1.4156% w/v sodium chloride and 48 μL 3.3 mg mL⁻¹ βgluY. Standard were prepared using 100 μL gum arabic solutions (0-150 mg mL⁻¹), 100 μL 2% w/v sodium chloride and 12 μL 3.3 mg mL⁻¹ βgluY. Samples were incubated overnight before washing the pellet as described in Section 3.2.3. For quantification, samples and standards were both resolubilized in 100 μL 20 mM NaOH, resulting in an effective concentration of the dialyzed AGPs and enabling quantification. NaOH was found to denature the AGPs, so for downstream analyses water was used to resolubilize the AGP-Yariv complexes.

C.1.4 SDS-PAGE

SDS-PAGE and Western Blots using the JIM13 antibody were used to validate extraction methods and to determine the size range of JIM13-binding AGPs. Analyzed samples included gum arabic (GA), gum ghatti (GG), dialyzed and non-dialyzed protein extracts (prepared as described above), and bovine serum albumen as a negative control. For preparation for gel analysis, 60 μL sample was mixed with 15 μL Pierce™ Lane Marker Reducing Sample Buffer (Thermo Fisher Scientific, Waltham, MA) in a microcentrifuge tube. The samples were placed with caps open into a 100°C heating block for 5 minutes and then placed immediately on ice. A 4-20% Mini-PROTEAN® TGX™ Precast Protein Gel gel (Bio-Rad Laboratories, Hercules, CA) with 30 μL well capacity was loaded into a Bio-Rad gel assembly. Tris-glycine running buffer was prepared with final concentrations of 3.02 g L⁻¹ Tris base (Thermo Fisher Scientific), 18.8 g L⁻¹ glycine (Thermo Fisher Scientific), 1% w/v SDS (prepared using 10% w/v SDS stock solution, Thermo Fisher Scientific). The
running buffer was added to the gel assembly.

Samples were loaded into the gel (25 μL), and 10 μL Broad Range Color Prestained Protein Standard (New England Biolabs, Ipswich, MA) was used as a ladder and loaded into the edge wells. 2 identical gels were run in parallel so that one could be used for total protein staining and the other could be used for Western Blot analysis. The gel was run for 5 min at 100 V to resolve proteins within the gel. The gel was then run at 150 V until the pink dye reached the bottom of the cassette, keeping in mind that AGPs are in the range of 70-200 kDa [37] and watching the corresponding bands in the ladder progress on the gel to ensure sufficient separation of AGPs.

C.1.5 Staining for total protein

The gel was removed from the running buffer well and the gel carefully removed from its casing according to the manufacturer’s instructions (BioRad). The gel was washed by submerging in deionized water in a shallow dish and incubating for 5 minutes before pouring the water off. The gel was then submerged in Imperial™ Protein Stain (Fisher) for 15 minutes. The stain was poured off, washed twice with deionized water as described, and submerged in deionized water overnight to destain. The gel was imaged by photographing with a Panasonic Lumix DMC-FZ200 camera.

C.1.6 JIM13 Western Blot

The remaining gel was carefully removed from its casing as described. The gel was layered (from bottom to top, using forceps to handle membrane and filter papers): two layers of filter paper cut to the size of the gel soaked in running buffer, the gel, nitrocellulose membrane soaked in running buffer, two more layers of cut and soaked filter paper. The proteins were transferred using a semi-dry electrotransfer method with 50 mA constant current for 1.5 hours. Using forceps, the nitrocellulose membrane was transferred into a shallow dish containing 100 mL block buffer (5% dry milk, 0.1% TWEEN-20 in 1x PBS). The dish was rocked at room temperature for 1 hour before pouring off block buffer.
To stain with the primary antibody, 3 mL JIM13 (Carbosource, Athens, GA) was diluted using 12 mL block buffer. The JIM13 dilution was added to the dish with the nitrocellulose membrane and gently rocked for 1 hour at room temperature before moving the assembly to 4°C overnight. Then the liquid was poured off and washed 3 times with PBST (0.1% TWEEN-20 in 1x PBS) by adding 50 mL PBST to the dish, rocking for 5 minutes, and pouring off the liquid. To stain with secondary antibody, 5 μL Goat Anti-Rat IgG/HRP conjugate (Fisher) was diluted using 25 mL PBST, added to the dish, and incubated for 45 minutes on the plate rocker. The membrane was washed 3 times as described. The membrane was then rinsed one time with 50 mL PBS. Approximately 15 mL, or enough solution to coat, 1-Step TMB Blotting solution (ThermoFisher) was added to the dish using a transfer pipet to gently coat the membrane. The color was allowed to develop until clear bands were apparent, about 5 minutes. At this point, the remaining solution was gently poured off and the reaction was stopped using 1x PBS, poured over the membrane gently from the edge of the dish. The membrane was imaged using the Panasonic Lumix DMC-FZ200 camera.

C.1.7 U-HPLC Method Development

A 1.7 μm 2.1 mm x 50 mm ACQUITY UPLC Protein BEH C4 300Å column was selected for application with these heavily glycosylated proteins. The UPLC setup was a Waters ACQUITY U-HPLC H-class system (Waters Corporation, Milford, MA). Mobile phases consisted of A) aqueous: 10% acetonitrile and 0.1% phosphoric acid in HPLC grade water and B) organic: 80% acetonitrile and 0.08% phosphoric acid in HPLC grade water. A gradient was run for 2.5 minutes from 100% solvent A to 100% solvent B, at which point the flow was continued for an additional 3.5 minutes. For analysis, 2 mg mL\(^{-1}\) GA was dissolved in 90:10 water:acetonitrile and filtered using a 0.22 μm PVDF syringe filter unit (MilliporeSigma, Billerica, MA) into a crimp-top U-HPLC vial. 5 μL sample was injected for each run and the absorbance was monitored at 215 nm (for peptide bond analysis) and 280 nm (for tryptophan, tyrosine, and cysteine). The max plot was also monitored over the entire spectrum measured by the U-HPLC.
C.2 Results and discussion

C.2.1 SDS-PAGE and JIM13 western blots

SDS-PAGE and Western blots were used to verify that protein extraction methods successfully extracted AGPs from cell samples. Further, quantification of cell-associated AGP provided information regarding the levels of these proteins that can be found associated to the ESM. In colorimetric AGP assay in which both pre-dialysis and post-dialysis extracts were run, it was shown that 50% of AGP is lost during dialysis. This is not ideal, but still leaves enough remaining AGP for downstream processing in a more pure form with no contaminating compounds in the buffer.

Figure C1 SDS-PAGE and JIM13 Western blots confirm success of protein extraction method for AGP extraction. (a) SDS-PAGE stained for protein using Imperial™ Protein Stain. GA has a protein band around 70 kDa, and GG has a similar band as well as a larger MW band. Protein extract shows a range of extracted proteins. Yariv supernatant and Yariv-isolated AGPs suggest further optimization would be needed for AGP isolation. (b) Same gel as (a) but after transfer to nitrocellulose membrane and run in a JIM13 Western Blot protocol. BSA shows no reaction to the JIM13 antibody, verifying that bands represent JIM13-reactivity. GA demonstrates no JIM13 binding, indicating a potential problem. GG and the protein extract show a wide band of JIM13-positive proteins in the correct range for AGPs. AGPs are still present in the supernatant after precipitation with βgluY, but appear all but vanished in the resolubilized AGP precipitate band.

SDS-PAGE analysis with stain for total protein in combination with the JIM13 stain-
ing after Western Blot analysis verified that the protein extraction worked to extract the heavily glycosylated AGPs. GA and GG both stained positive for protein, but after Western Blot analysis the signal for GA disappeared. This disappearance could be due to insufficient loading.

The staining for the supernatant and resolubilized AGP-Yariv complexes was the first indication that further optimization would be necessary in the AGP isolation scheme. The supernatant after precipitation of AGPs using $\beta$gluY stained positive for both total protein (expected) and JIM13 AGPs (unexpected, Figure C1), while the signal all but disappeared in the resolubilized AGP-Yariv complex band whereas it should have stained positive for the JIM13 epitope. This could be simply because there was more protein in the supernatant band (as unfortunately the same amount of protein couldn’t included in each band since AGPs are largely carbohydrate based), but also indicated that insufficient time was included for the AGP precipitation. The time of incubation with samples, NaCl, and $\beta$gluY was then optimized, with sufficient and complete precipitation occurring after 24 hours at 4°C. Another possible cause is that the NaOH used to resolubilize AGP-Yariv complexes breaks down the JIM13-binding region on the isolated AGPs, and as such we recommend using water to resolubilize AGPs for any downstream analysis [170]. Preliminary work using water to resolubilize the AGP-Yariv complexes suggested difficulty, as even after heavy vortexing, shaking for 24-48 hours at 125 rpm, and sonicating a red pellet remained in samples and GA standards. The literature suggest that sufficient AGP may have come into solution [170, 171], a concept which still requires validation in our system in U-HPLC.

C.2.2 U-HPLC Analysis of AGPs

Several rounds of optimization culminated in the method presented here. The spectra from the GA samples are shown in figure C2 which show the 215 (a) and 280 (b) nm spectra. Key peaks are highlighted and area underneath calculated using Waters software, with the area under the peaks displayed in Figure C2 below the associated spectrum. The
Figure C2 U-HPLC spectra of gum arabic at 215 and 280 nm demonstrates U-HPLC method for AGP analysis. (a) Spectrum at 215 nm. (b) Spectrum at 280 nm.

shapes of the spectra is consistent with reports in the literature, where both peaks were identified to contain AGPs based on subsequent analysis of the components using SDS-PAGE and βgluY to stain for AGP [37].

The results presented here are preliminary but provide an excellent basis for U-HPLC on AGPs based on methods for HPLC analysis in the literature [37, 171, 172]. For application with extracted loblolly AGPs, protein extracts can be prepared from plant cell culture samples as described in Section C.1.1 for analysis. For the simplest, initial runs, an appropriate volume of acetonitrile should be added to dialyzed protein extracts to match the polarity of mobile phase A. The samples should then be filtered and run as described here for direct comparison among samples and to commercial plant gums. For cleaner analysis of AGPs, βgluY can be used to isolate AGPs from dialyzed protein extracts (Section
C.1.3). After resolubilization in water, the more pure AGP sample should be analyzed. It is possible that the remaining $\beta$gluY might interfere with analyses, as the compound exists in an aggregated state in aqueous solution [173]. In this case, sodium dithionite can be used to dissociate the red-colored $\beta$gluY until the solution clears [37, 171].

C.3 Outlook

Here, a method was developed to extract AGPs from a loblolly pine SE tissue sample and the extracts were analyzed using conventional protein analysis techniques including SDS-PAGE and Western blotting. Next, I show that supplementation of culture AGPs is influential on embryo yield. These results can be combined: extracted loblolly AGPs should be supplemented into SE cultures to probe for improved embryo yield outcome. This proposed methodology has the potential to enable 1) extension of the useful lifetime of maintenance-stage cultures and 2) improvement of embryo yield outcome in less responsive genotypes.

In addition to analysis of extracted AGPs via the elution time and peak size using U-HPLC, further analysis should be performed to determine the nature of the fractionated AGPs. The method can be adapted to the preparative scale following protocols outlined in the literature [37, 172], collecting the elute at the appropriate times. Samples can be analyzed using SDS-PAGE and staining with Imperial™ Protein Stain (Thermo Scientific, Waltham, MA) to identify protein bands or $\beta$gluY to identify AGP bands [37]. These isolated AGP populations can also be reintroduced into cultures as a follow-up to culture engineering strategies discussed in Chapter 4, with the hypothesis that AGPs isolated from high-yielding SE cultures will improve embryo yield outcomes when introduced during maintenance phase culturing.
C.4 Acknowledgements

I would like to thank Lou Roberts with his invaluable help with method development for SDS-PAGE and Western Blots. I would also like to thank Greg Andrews for his help with the U-HPLC operation.
APPENDIX D

IMAGE ANALYSIS TO QUANTIFY BIOMASS

In this appendix, I present a MATLAB script that takes a picture of loblolly ESM and embryos on development media as input and quantifies the area within the picture occupied in pixels. If all images are taken from the same distance (which can be verified using ImageJ’s measure function to measure the 2”x2” nylon mesh on each image), then the area of the plates can be directly compared to one another. This code was written by Nakul Bende.

%% Areal analysis of cultured plates %%
% This code analyzes photographs of petri dishes containing somatic embryos and ESM after 12 weeks of development. We first load all the images in memory, followed by some operations for adjusting color channels. This allows detection of areas covered with embryos or ESM based on color detection algorithms. Necessary assumption: the photographs must be taken at the same height and lighting conditions. Though not included, an internal calibration for scale can be calculated based on a scale if included while capturing images.
% Written by Nakul Bende in 2016

%% Finding details on images from working directory into matlab
files = dir('*.JPG');
files = sort(files, 'descend')
nfiles = size(files, 1);
im = imread(files(1).name); % im will be a multidimensional image stack
[m n p] = size(im); % all the 'p' images will have resolution mxn,

% Defining variables, preallocating matrices for speed
im_gray = zeros(m, n, p);
im_cropped = zeros(m, n, p);
im_lab = zeros(m, n, p);
im_thresholded = zeros(m, n, p);
im_thresholded_dilated = zeros(m, n, p);
im_thresholded_dilated_filled = zeros(m, n, p);

%% In a loop, loading each individual image, processing them using
% colorspace, and calculating areal fraction of embryos. For the first
% image, this should be run with line 22 as 'for i = 1'. While running the
% first time, un-comment lines 29-31 and 43-56 for calculating appropriate
% masks and thresholds for a successful analysis

for i = 1:nfiles

% Loading an image file from folder
fname = files(i).name;
im = imread(fname);

% Cropping image. Line 29-31 open a GUI for selecting a freehand polygon
to be used as a mask. Once the polygon is drawn, right click for
copying coordinates for polygon vertices. To close, double click on the
polygon to generate mask. This should be pasted into line 33 as
variable mask_freehand, and line 29-31 should be commented out for
automating image analysis of further images

% imshow(im)
% mask_freehand = imfreehand();
% im_mask = mask_freehand.createMask(); % Creates a binary mask

mask_freehand = [REPLACE THIS WITH VERTICES FOR MASK]; % Please paste the
vertices of polygon

im_mask = poly2mask(mask_freehand(:, 1), mask_freehand(:, 2), m, n);

% Making a image (im_masked) with only regions inside the polygon showing
im_masked = im;

im_masked(im_mask==0) = 0; % Deleting all pixels outside the masked area

% Convert RGB image from camera into L*a*b colorspace
im_lab = rgb2lab(im_masked);

% The following code when un-commented (line 43-56) can be used to find
threshold from GUI, choose appropriate values which include the embryos
, but exclude the background%%%%%\\

% % Define thresholds for channel 1 based on histogram settings
% ref_channel1Min = 6.849;
% ref_channel1Max = 64.555;
% % Define thresholds for channel 2 based on histogram settings
% ref_channel2Min = -17.739;
% ref_channel2Max = 2.330;
% % Define thresholds for channel 3 based on histogram settings
% ref_channel3Min = -9.952;
% ref_channel3Max = 7.704;
%
% % ref_im_thresholded = (im_lab(:, :, 1) >= ref_channel1Min ) & (im_lab
% ( :, :, 1) <= ref_channel1Max) & ... % Generates a reference image with colorspace
% (im_lab(:, :, 2) >= ref_channel2Min ) & (im_lab(:, :, 2) <=
% ref_channel2Max) & ... % thresholding applied
% (im_lab(:, :, 3) >= ref_channel3Min ) & (im_lab(:, :, 3) <=
% ref_channel3Max); % Generates a reference image with colorspace

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% imshow(ref_im_thresholded); % Displays the reference image for visual analysis

% Find the area covered by cells
% Define thresholds for channel 1 (L) based on histogram settings, clipping the desired regions of in this color channel
channel1Min = 15.000;
channel1Max = 100.000;

% Define thresholds for channel 2 (A) based on histogram settings, clipping the desired regions of in this color channel
channel2Min = -16;
channel2Max = 38;

% Define thresholds for channel 3 (B) based on histogram settings, clipping the desired regions of in this color channel
channel3Min = 11;
channel3Max = 60;

im_thresholded = (im_lab(:,:,1) >= channel1Min ) & (im_lab(:,:,1) <= channel1Max) & ...
(im_lab(:,:,2) >= channel2Min ) & (im_lab(:,:,2) <= channel2Max) & ...
(im_lab(:,:,3) >= channel3Min ) & (im_lab(:,:,3) <= channel3Max); % Calculate a thresholded image

% Dilating helps in reducing error and smoothening the edges
se90 = strel('line', 3, 90);
se0 = strel('line', 3, 0);
im_thresholded_dilated = imdilate(im_thresholded, [se90 se0]);

% Filling areas between the pixel for an effective measurement of
contiguous area. This introduces a small error, but greatly improves
region detection

```matlab
im_thresholded_dilated_filled = imfill(im_thresholded_dilated, 'holes');
```

% This section analyzes the regions of interest (embryo coverage)
```
im_analyzed = regionprops(im_thresholded_dilated, 'Centroid', 'Orientation', 'BoundingBox', 'Area', 'ConvexArea');
```
```
files(i).Analyzed_area = sum([im_analyzed(1:size(im_analyzed, 1)).Area]);
```
```matlab
% Storing the area in a variable

% Removing thresholded data from masked image, giving a final image (im_thresholded) with only embryos selected
```
im_masked = im;
im_masked(im_thresholded==0) = 0;
```

% If image output is desired, uncomment lines 86-102 Write image for analysis later. Note that this is computationally expensive
```
figure('Color', [1 1 1], 'Visible', 'off'); % Remove visible off handle to actually see images - CAUTION: further slows down the algorithm drastically, is quite useless unness a visual inspection is needed for plots
```
```
% subplot(2, 2, 1) % Original image
```
```
% imshow(im);
```
```
% title('Original image');
```
```
% subplot(2, 2, 2) % Brightness channel
```
```
% imshow(im_lab(:,:,1),[0 100]);
```
```
% title('L channel');
```
```
% subplot(2, 2, 3) % Raw thresholded image
```
```
% imshow(im_thresholded);
```
```
% str = sprintf(': Area = %0.0f', files(i).Analyzed_area); str = strcat('str = strcat('}
Threshold', str);

% title(str);

% subplot(2, 2, 4) % Detected areas
% imshow(im_masked)
% title('Detected')
% im_name = strcat(fname, '_analyzed'); Saving the image with same
% filename as original image
% export_fig(im_name, '-png', '-q90', '-r300')
% clf % Clearing this figure for next loop

end

%% Making a cell array for organizing data
files_image_analysis = struct2cell(files)';
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