CELL WALL PLASTICITY REGULATION IN POLLEN TUBES RELATED TO MECHANICAL STRESS

CHRISTINE CAMERON

DEPARTMENT OF PLANT SCIENCE
MCGILL UNIVERSITY MACDONALD CAMPUS, MONTREAL

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ABSTRACT

The pollen tube is a rapidly growing plant cell that features particular traits that can only be understood when integrating cell biological with cell mechanical concepts. Firstly, regular temporal variations in the growth rate are governed by a feedback mechanism involving the regulation of the mechanical properties of the apical cell wall and mechanosensitive ion channels. Recent developments in image analysis have allowed making significant progress in the quantification of this behavior. Secondly, a pollen tube uses invasive growth to penetrate the flower tissues with the aim to transport the male sperm cells to their target, an ovule. Turgor was recently found to be the driving force of this invasive behavior and the mechanical underpinnings have been characterized using Lab-on-Chip (LOC) technology. Thirdly, the pollen tube is able to reorient its growth direction upon exposure to a guidance cue and the steering mechanism involves the sophisticated choreography of intracellular transport processes serving to fuel the polar growth process. This morphogenetic process relies on the precise spatial control of a gradient in the mechanical properties of the cell wall. Sophisticated imaging and micromanipulation techniques have been instrumental for the advancement in characterizing the biomechanical features of this crucial cell in the plant reproductive cycle. It has been recently shown that the pollen tube diameter changes in response to mechanical hindrance; this change in diameter suggests there is an adaptive mechanism within the cell wall formation pathway. In order to investigate this, pollen tubes from four plant species, *Camellia sinensis, Nicotiana tabacum, Solanum chacoense* and *Arabidopsis thaliana,* were germinated in media of varying stiffness to expose them to different degrees of mechanical stress during cell wall formation. Diameter has been correlated with the characteristic spatial profile of the distribution of cell wall polysaccharides. The pollen tubes were stained and immunolabeled to show beta-glycan contents as well as that of methyl esterified and de-esterified homogalacturonan (HG) to determine if the biochemical composition of the cell wall was affected by exposure to different types of mechanical environment.

Though they grow in different pistil environments in vivo, both *C. sinensis* and *A. thaliana* had higher de-esterified HG content at the tip during growth in the stiff media suggesting the formation of a stiffer tip in response to stress. Some *N. tabacum* and *S. chacoense* pollen tubes displayed swollen bulbous tips with abnormal tip cell wall biochemistry suggesting this is an adaptive mechanism in response to stress. The abnormal swollen tips contained low levels of de-esterified HG in the bulbous tip cell wall suggesting this polysaccharide is necessary in maintaining pollen tube diameter during growth.

RÉSUMÉ

Le tube pollinique est une cellule végétale dont la croissance rapide présente des caractéristiques particulières que l'on ne peut assimiler qu'en associant biologie cellulaire et concepts de mécanique cellulaire. Premièrement, les variations temporelles, à intervalles réguliers, du taux de croissance sont régies par un mécanisme de rétroaction associé à la régulation des propriétés mécaniques de la paroi cellulaire apicale et des canaux ioniques mécano-sensibles. Les récents développements en analyse d'images ont permis de faire d'importants progrès dans la quantification de ce mécanisme. Deuxièmement, la croissance invasive du tube pollinique lui permet de pénétrer les tissus du pistil afin de transporter les cellules spermatiques vers leur cible, un ovule. La force exercée par la turgescence est le principal moteur de ce comportement invasif dont les fondements mécaniques ont été caractérisés à l'aide de la technologie Lab-on-Chip (LOC). Troisièmement, le tube pollinique est capable de réorienter sa direction de croissance en réponse à un signal de guidage. Cette réorientation nécessite une régulation complexe des processus de transport intracellulaire contrôlant la croissance polaire. Ce processus morphogénétique repose sur le contrôle spatial précis d'un gradient de propriétés mécaniques de la paroi cellulaire. Les techniques de pointe en imagerie et en micromanipulation ont été déterminantes pour la caractérisation des propriétés biomécaniques de cette cellule, dont le rôle dans le cycle reproductif des plantes est crucial. Il a été

récemment démontré que le diamètre du tube pollinique change en réponse à un obstacle mécanique. Ce changement de diamètre suggère l'existence d'un mécanisme adaptatif dans la formation de la paroi cellulaire. Afin de comprendre ce mécanisme, j'ai induit la germination de tubes polliniques de quatre espèces végétales, *C. sinensis*, *N. tabacum*, *S. chacoense* et *A. thaliana*, dans des milieux de différentes rigidités. Le but étant de les exposer à différents degrés de stress mécanique lors de la formation des parois cellulaires. Le diamètre des tubes a été comparé au profil de distribution spatial des beta-glycanes pariétaux. Les tubes polliniques ont été spécifiquement marqués par des anticorps afin de montrer la présence de beta-glycanes spécifiques ainsi que les pectines méthyl-estérifiées et dé-estérifiées. Le but de ces marquages est de déterminer si la composition biochimique de la paroi cellulaire serait affectée par les différentes propriétés mécaniques des milieux de croissance des tubes polliniques. Bien que les tubes polliniques de *C. sinensis* et d'*A. thaliana* germent in vivo dans différents environnements pistillaires, leurs apex sont caractérisés par une forte concentration en pectines de-estérifiées lorsqu'ils poussent, in vitro, dans des milieux rigides, suggérant ainsi la formation d'un apex plus rigide en réponse au stress. Certains tubes polliniques de *N. tabacum* et de *S. chacoense* sont caractérisés par des apex de forme bulbeuse et par une composition biochimie pariétale atypique au niveau apical, suggérant la présence d'un mécanisme adaptatif en réponse au stress. Les apex de forme atypique bulbeuse ont contenu des bas niveaux de pectines de-estérifiées dans la paroi cellulaire en soulignant que les pectines de-estérifiées sont nécessaires pour maintenir le diamètre du tube pollinique pendant la croissance.

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TABLE OF CONTENTS

CONTRIBUTIONS

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CHAPTER 1: CELL MECHANICS OF POLLEN TUBE GROWTH

1.1 INTRODUCTION

Male gametes in plants are delivered to the female partner by way of a mobile intermediate generation, the male gametophyte or pollen grain [2]. The final step of this delivery entails a transport of the gametes from the top of the flower pistil where the pollen grain lands, to an ovule containing a female gametophyte, nestled deeply within the ovary [3]. Delivery is executed through elongation of a conduit: the pollen tube, that penetrates the transmitting tract of the pistil, growing at unparalleled rates to precisely hone in on its target [4]. Formation of the pollen tube is governed by the general principles of plant cell growth, but optimized for the purpose of invasive and directional growth by confining the growth activity to the very tip of the cell [5]. It is here that cell wall gets assembled through both exocytosis and membrane-located polysaccharide synthesis [6]. Cell wall assembly is fueled by precisely choreographed cytoplasmic streaming of vesicles which deposit cell wall material and synthesizing enzymes at the tip [7,8] (Figure 1). The rapid polar elongation of the pollen tube depends on the superb coordination of multiple cellular processes that have been investigated over the past few decades including cytoskeletal dynamics, cell polarity and intracellular transport [9-12]. The physical interplay between the cell wall and internal turgor pressure plays an important role in invasive growth which has motivated researchers to consider the fundamental cell mechanical underpinnings of the growth process [13]. Several remarkable features characterizing pollen tube growth have garnered particular attention in recent years, due to technological developments that have finally allowed studying them in more detail. Some of the most recent trends are briefly summarized in the following.

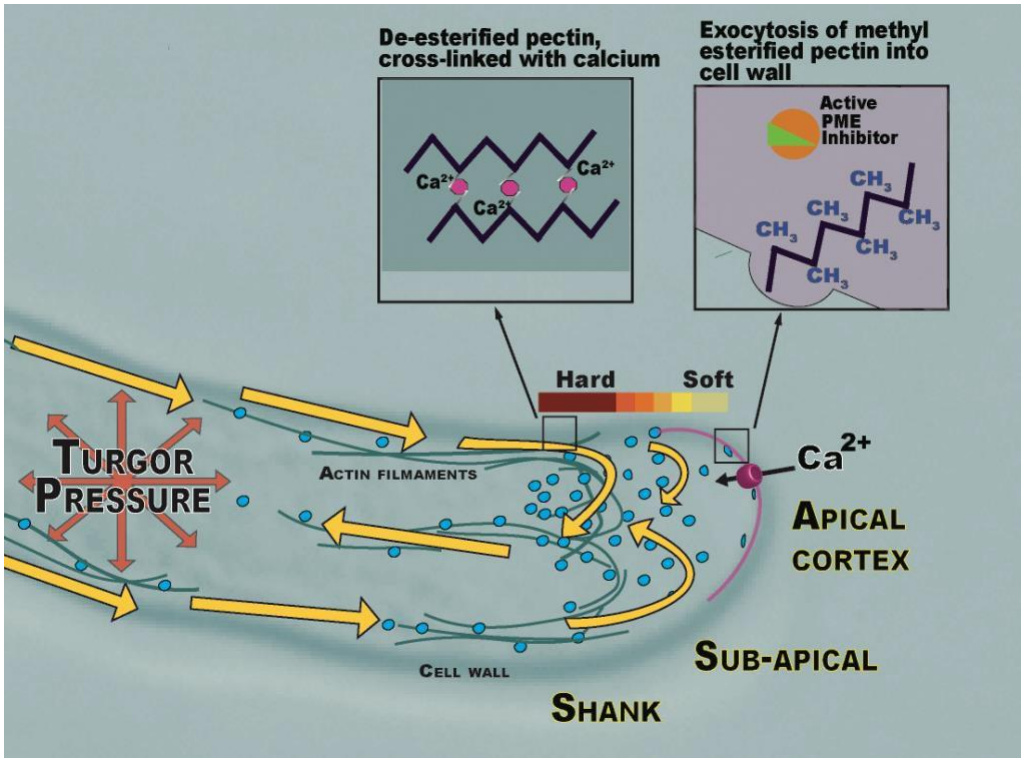


Figure 1. Pollen tube elongation is driven by turgor. At the apex, where the wall yields the cylindrical cell elongates. Only where the wall yields is the force created by the pressure effective against external obstacles. Wall assembly at the tip is fueled by the delivery of intracellular vesicles, transported in a reverse fountain stream (indicated by the yellow arrows). The vesicles contain cell wall material as well as cell wall synthesizing enzymes which are released through exocytosis at the tip. The cell wall at the tip is made up of methyl esterified pectin that is pliable allowing for deformation and continued elongation. Maturation of the pectin involves de-methyl esterification by pectin methyl esterases (PME), secreted at the tip. Ca^{2+} influx occurs at the tip through calcium channels.

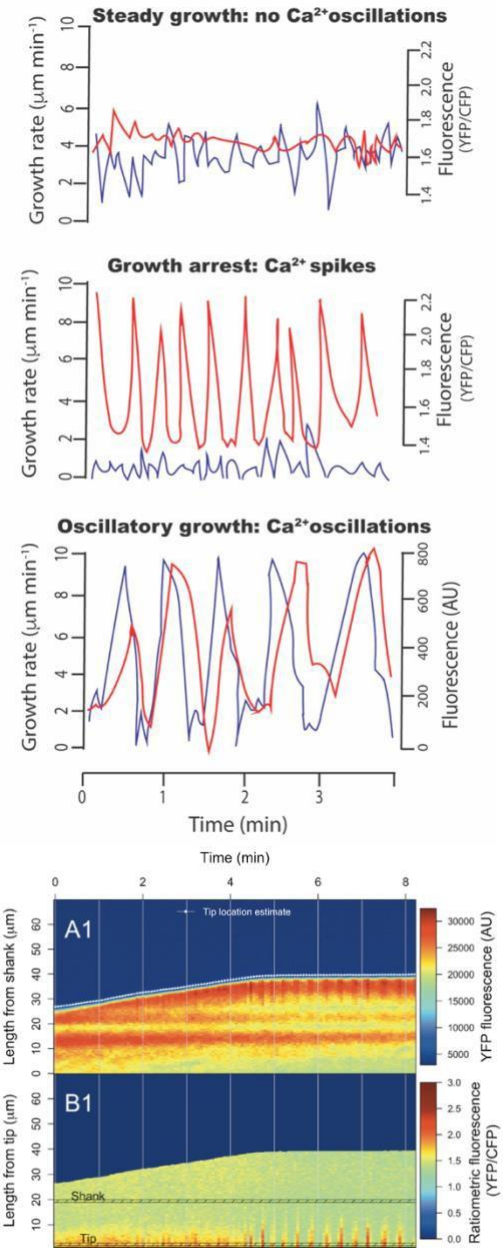
1.2 OSCILLATORY POLLEN TUBE GROWTH

Pollen tube growth kinetics exhibits oscillatory changes, suggesting that the temporal regulation of the growth process underlies a feedback mechanism [14,15]. In parallel with the growth rate, many cellular parameters in the pollen tube fluctuate including the cytoplasmic Ca^{2+} concentration, ion fluxes across the plasma membrane, the thickness of the apical cell wall and local enzyme concentrations suggesting some of these processes may be linked [7,16,17].

One pivotal parameter is the exocytosis rate which has been documented to oscillate along with cell growth and is suspected to be a key factor involved in mediating spatio-temporal growth regulation [18]. Correlating with exocytosis, the amount of cell wall material at the tip shows temporal variations and it has been observed that the status of the cell wall predicts the magnitude and length of pollen tube growth [18]. The deformability of the cell wall at the apex is determined by both its biochemical composition and its thickness, and only quantitative, real-time monitoring of both will allow determining any true causal relationship governing temporal control, similar to the question of spatial control of growth [18,19]. Whether or not the oscillatory phenomenon has a biological purpose is up for debate, but it has inspired many to investigate the regulatory network controlling the rapid growth process [20,21]. Oscillatory phase delays of individual processes have been used to deduce relationships between these different phenomena [14]. However, this approach is not without fallacies, as elegantly pointed out by Damineli et al. (2017), who provide an excellent overview of the current state of research on oscillatory growth and propose two distinct oscillatory systems: one at the tip and the other at the shank [21].

Prominent among the oscillating features, and therefore incorporated into many of the modeling approaches, are the cytoplasmic concentration and the transmembrane flux of Ca^{2+} [16,22]. Calcium has a particularly complex role in plant cell growth both outside of the plasma membrane, where it influences the biochemical properties of the cell wall by cross-linking pectin, as well as inside the cytoplasm, where it influences the dynamics of the actin cytoskeleton, for example mediated by actin-binding proteins or Rho GTPase interactions [23]. The concentration of cytoplasmic free Ca^{2+} has been observed to oscillate [16,24], but the concentration peak was found to be delayed with respect to growth [25]. The result was initially puzzling, but a feedback mechanism relating exocytosis, cell wall mechanics and mechanosensitive calcium channels provided a possible explanation [15,26]. The reality is likely to be more complex, however [14,21,27,28]. As the modeling attempts progress, it becomes increasingly obvious that the quality and quantity of experimental data are limiting

factors when investigating the regulation of pollen tube growth. One particular challenge is posed by the resolution of the optical microscope which limits the spatial and temporal resolution at which oscillatory phenomena can be detected.



This is a challenge in particular for *A. thaliana*, the primary model organism for plant cell biology, whose pollen tubes are small in diameter and grow slower than those of many other species. Progress was recently made through the development of a novel computational method named CHUKNORRIS which was used to investigate the correlation between oscillatory tip growth and calcium dynamics in *Arabidopsis thaliana* pollen tubes [29]. Using this image processing technique, frequent high amplitude Ca^{2+} oscillations in non-growing pollen tubes were detected suggesting that calcium oscillations can occur independently of growth oscillations [29] (Figure 2). In addition, Ca^{2+} spikes were recorded during the slowed growth of the pollen tube towards the embryo sac, suggesting a possible role for calcium signaling in ovular perception [29] (Figure 2). These observations confirm that the role of calcium in regulating pollen tube growth is more complex than previously thought.

Figure 2. Characterization of the oscillatory features of *Arabidopsis* pollen tubes growing in vitro by CHUKNORRIS. Representative time series of the three growth regimes and underlying oscillatory signatures obtained from kymographs (example at the bottom). Growth rate is shown in blue while fluorescence indicating Ca^{2+} concentration is shown in red. Redrawn after Damineli et al. (2017) JXB with permission.

1.3 MECHANICS OF INVASIVE GROWTH

In order for successful fertilization to occur, the pollen tube must push through the pistil matrix to overcome the mechanical resistance of pistillar and ovular tissues [13,30]. Until recently, it was unknown how the pollen tube generates the requisite invasive growth force. In animal cells with invasive capacity such as cancer cells or neuronal growth cones, invasive forces are generated through cytoskeletal mechanisms [13,31,32]. Cytoskeletal forces are considered to be too small to effect substantial mechanical action against the pollen tube cell wall, but evidence for a different mechanism was lacking [13]. It is known that the mechanics of polar elongation in pollen tubes and the generation of a cylindrical tube geometry rely on the interplay between the internal turgor, a hydrostatic pressure, to force tip cell wall deformation and the biochemical cell wall properties at the tip. The deformation, or stretching, of the cell wall is accompanied by the incorporation of new cell wall material [33,34] involving the breaking and formation of cross-links between existing and newly added polymers [18,28,35,36] (Figure 1).

Although the interplay between turgor and cell wall was known to drive cell growth, it was unknown whether the turgor is also the source of invasive forces, in part due to a lack of precision in micro-measurement techniques. In recent years, technological advances have allowed for the measurement of the invasive force generated by individually growing pollen tubes. Measurements were done using a microfluidic device, termed the TipChip. This device was constructed using Lab-on-Chip (LOC) technology [37,38] (Figure 3) and designed to present the growing pollen tube with a calibrated mechanical obstacle [38]. The measured force was found to correspond in magnitude to the typical turgor found in pollen tubes corroborating the hypothesis that the invasive force is generated through this pressure. However, can turgor be the driving force of invasive growth, given that it is a scalar parameter without directionality? Rather than through turgor, growth directionality is provided by the cell wall resisting this

driving force; it is related to the spatial gradient of mechanical properties generated by polar release of new cell wall material [39]. The high deformability or yielding behavior of the cell wall at the tip allows for the turgor to act on the outside of the cell deforming the invaded tissue.

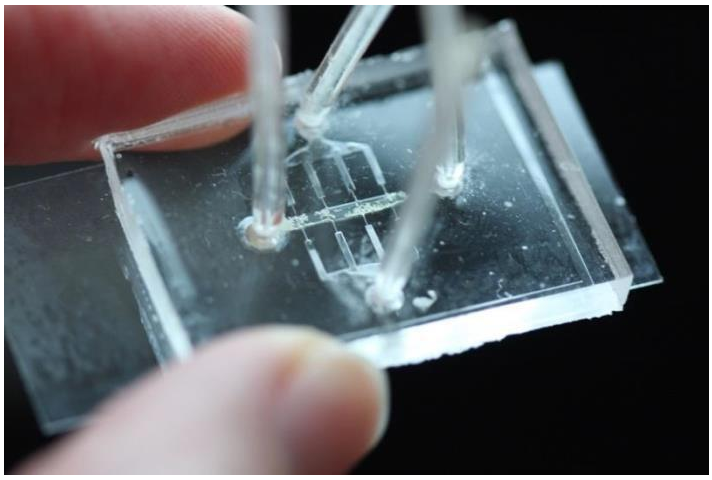


Figure 3. TipChip, a microfluidic device designed to manipulate individual pollen tubes for the purpose of micromechanical testing, assessment of chemotropic behavior, and other cell biological assays. The TipChip consists of a patterned PDMS layer forming a microfluidic network sealed by a cover slip. The microfluidic network features a larger chamber into which the pollen grains are injected, traps accommodating individual pollen grains, and microchannels guiding elongating pollen tubes towards experimental features such as mechanical obstacles, levers, actuators and electrical fields [38].

Measuring the mechanical gradient in the cell wall is not straightforward [19,40,41], but the strain pattern in the cell wall clearly proves its differential deformation behavior [36]. The cell wall at the tip is composed of highly deformable methyl-esterified pectin allowing for pressure induced cell expansion, whereas the shank is reinforced by de-esterified pectin cross-linked with Ca^{2+}, crystalline cellulose, and a layer of callose [9,40] (Figure 1). The gelation transition of pectin from stiff shank to flexible tip is particularly important for the polar morphogenetic process as it correlates with a drastic change in deformation behavior, which has been observed experimentally and predicted by modeling [9,42-44]. The use of cellular force microscopy on Lilium *longliforum* and

Arabidopsis thaliana pollen tubes cultured in a microfluidic set-up yielded spatial correlation between stiffness and cell wall biochemistry [45], confirming earlier data obtained through micro-indentation [40,41]. Performing indentation experiments on a cylindrical cell with a hemisphere shaped end necessitates complex geometrical considerations when interpreting force-deformation data, however. These arise because of the changing contact angle between the indenter and the cell surface. Even if these technical challenges will be circumvented in the future, for example by using multi-degree of freedom force sensors or by applying soft indentation over larger surfaces [46], it remains to be seen how meaningful microindentation data are for assessing the deformability of the cell wall under turgor induced tensile stress. Given the different types and orientation of the experimentally induced and the naturally occurring stresses, experiments performed with micro-indentation [9,40,41] or cellular force microscopy [19,45] may be limited in their meaning, although a comparative approach has successfully correlated temporal changes in apical stiffness associated with growth oscillations [41]. The identification of turgor as the pushing force in invasive behavior was a key finding and opens new avenues of research to address tube pathfinding within the maze of pistillar tissues related to species specific biology [47].

1.4 STEERING MECHANISMS

The direction of pollen tube growth is susceptible to external and internal chemical, electrical and mechanical guidance cues [7]. Steering mechanisms are crucial to enable the pollen tube to find its way towards an unfertilized ovule [10]. Several of the molecular players involved in guiding pollen tube growth have recently been identified; they involve various signals emitted by both the male and female gametophytes [43,48].

A directional cue has to be translated into a change in growth direction and the secretory growth machinery controlled by the actin cytoskeleton is

instrumental in mediating the cellular response. The actin cytoskeleton does so by orchestrating the delivery of the cell wall material to the side of the tube that needs to expand [49]. However, many steps in the mechanism translating external cues into directional growth responses remain to be identified. Recently, it has been observed that a disruption in the cytoplasmic pH promotes actin fragmentation and disrupts the reverse fountain vesicle streaming, allowing for large organelles to move into the clear zone and arrest cell growth [50] (Figure 1). This suggests that the mechanical relationship between the cytoskeleton and cell wall development is dependent on the pH gradient as well as other ion gradients in order to successfully maintain polar growth [50-52]. The observation that calcium and proton gradients fluctuate in different regions of the pollen tube are consistent with the notion that these ions have essential roles in tip growth polarity [7,52]. Most recently, LOC has been exploited to measure the effect of the proton gradient on directional growth by exposing the pollen tube to a microelectrode within the channel. The change in localized pH led to growth arrest or changes in growth direction depending on where proton influx occurs in relation to the pollen tube [50]. While the cellular response was obvious, the cellular events mediating the effect remain to be identified. On the one hand, a local decrease in cytoplasmic pH might interfere with actin functioning thus affecting delivery of cell wall material, but on the other hand, protons might also act on the outside of the cell by changing cell wall polymer linkages through acidification [50]. The high reproducibility of experimental set-ups based on microfluidics and LOC technology will make significant progress in addressing how chemical signaling and biochemical composition influence directional growth [37,45,50,53,54]. Through LOC-based single cell analysis, it was also revealed how pollen tubes respond to highly localized electrical fields [55] and it was shown that while they respond quickly to a chemical guidance cue, they do not have an internal directional memory [42,56]. This illustrates two key features of pollen tube growth: the ability to self-organize tip growth in absence of external signals and the capacity to respond to guidance cues through re-orientation of

growth [42,57]. These growth characteristics offer exciting opportunities for future research.

1.5 CONCLUSION

Recent technical advances in imaging and micromechanics technology have enabled researchers to observe and measure both cell biological processes and cell mechanical features simultaneously. Future studies are likely to further develop LOC-based experimental strategies to address the many questions about pollen tube biology that remain unanswered [37]. The ability to measure parameters that were previously impossible to quantify on a single cell basis will be combined with improved strategies to circumvent the limitations in resolution of live cell imaging. Computational analysis software such as that developed by Damineli et al. [29] illustrates that subpixel detection can be accomplished for more accurate and reproducible results. Single cell manipulation and analysis have clearly transformed the field of cell biology in general and has opened exciting new avenues for pollen tube research in particular.

TABLES

Table 1. Glossary of scientific terms used in this paper

Cellular Force Microscopy (CFM)	Micro-indentation technique operating with a capacitive force sensor probe mounted on a three-axis microrobotics actuator. The device is designed to measure the apparent stiffness of cells at whole-cell or subcellular scale. The force resolution ranges from submicronewton to the millinewton range.
CHUKNORRIS	Computational method developed to analyze time-lapse series of micrographs acquired to monitor the oscillatory behavior of growing cells. The name stands for **C**omputational **H**euristics for **U**nderstanding **K**ymographs and a**N**alysis of **O**scillations **R**elying on **R**egression and **I**mproved **S**tatistics
Invasive growth	Behavior exhibited by specialized cell types able to penetrate tissues or matrices surrounding them. Examples include fungal hyphae, root

	hairs, pollen tubes and neuronal growth cones.
Lab-on-Chip	Device that integrates one or several experimental set-ups on a single integrated platform with the size of only millimeters to a few square centimeters.
Micro-indentation	Technique to measure stiffness of a (microscopic) sample by applying a calibrated local force with a stylus or tip and measuring the resulting deformation of the sample. The concept is identical to that of an atomic force microscope (AFM), but the spatial resolution is usually in the micrometer range, whereas the AFM operates in nanometer range. Micro-indentation has been performed with tools such as the 'cell poker' and the CFM.
Microfluidics	Technology used in micro-devices containing a network of microscopic conduits that can be designed to control fluid flow with the aim to create a microscopic test assay
Pistil	Flower organ housing the female reproductive tissues. The structure consists of the stigma (the landing platform for the pollen), the style and the ovary housing the ovules which in turn harbour the female gametophytes
Polar growth	Cell growth occurring in a specific direction, typically yielding a cylindrical cell or cell protrusion.
TipChip	Lab-on-Chip device developed for the investigation of tip growing cells such as pollen tubes
Turgor	Hydrostatic pressure in a cell established through water uptake driven by differences in osmotic potential across the semi-permeable plasma membrane.

CHAPTER 2: CELL WALL PLASTICITY REGULATION RELATED TO MECHANICAL STRESS

2.1 INTRODUCTION

For successful plant reproduction to occur, the pollen tube needs to penetrate specific pistillar tissues, also referred to as the transmitting tract, in order to fertilize the ovules located in the ovary at the bottom of the pistil [58]. Pollen tube-pistil interactions vary between species due to the different structure of the transmitting tract tissues in the style which can be either solid tissue or a hollow space lined with transmitting tract cells [59]. The pistil-pollen tube interaction is species-specific suggesting that pollen tube cell wall formation is also species-specific depending on whether the pistil has enclosed tissue or a hollow space surrounding the pollen tube as it elongates [60]. Further, the specificity of the transmitting tract acts as barrier for the flowers to exclude pollen tubes from inappropriate species [60]. *Camellia sinensis* is an example for a species in which the pollen tubes grow through a hollow canal suggesting the pistillar tissue is not needed as a structural support to stabilize the growing pollen tube tip [59,60]. *Arabidopsis thaliana, Solanum chacoense* and *Nicotiana tabacum* are three species whose pollen tubes grow through solid transmitting tract tissue which suggests their pollen tubes may share similar physical characteristics while piercing and invading the solid pistillar tissue [58]. Whether they share similar cell wall biochemistry and stiffness differentials remains to be assessed. Further differentiation between species was discovered when comparing wet stigma, *N. tabacum*, and dry stigma, *A. thaliana*, suggesting the hydration roles of the pollen coat are different between these two species and that there is genetic variation in pollen tube formation, and therefore variation in pollen tube adaptation to its environment [60]. Although all 4 species are angiosperms, they differ in the size of their pollen grain, an evolutionary adaptation related to the stylar environment, either hollow or solid [61]. Pollen grain size suggests the heterotrophic growth rate of the pollen tube through the

style is different due to the difference in nutrient availability between the solid and hollow environment [61]. Growth rate and nutrient availability are two factors that seem to have a major effect on cell wall organization.

It has been recently shown that the pollen tube diameter expands in response to a narrow mechanical hindrance suggesting there is endogenous control of cell shape [38]. Most interestingly, the diameter returned to its original size after expansion through the narrow gap suggesting this diameter is hardwired within the pollen tube system and also that the expansion of the diameter after the obstacle may be a result of the effort of the pollen tube to release more of the internal pressure onto the mechanical impedance by making the cell wall more flexible [38]. The mechanism behind diameter control has been shown through modeling and experimentation to rely on a change in cell wall biochemistry (from methyl esterified HG to de-esterified HG) that occurs during maturation and that locks the diameter into place in a transition region between apical growth region and distal part of the tube [9]. Given the variation in pollen tube diameter among plant species, this suggests there is species-specific genetic control over the diameter transition point pathway and that in response to mechanical hindrance different species would have different adaptive mechanisms within the cell wall formation pathway [38]. Further, this suggests that a change in cell wall diameter correlates with a change in cell wall stiffness due to a change in cell wall biochemistry.

In order to study the mechanism that regulates cell wall diameter in pollen tubes and the morphological and biochemical response of the cells to mechanical stress, four species of pollen tubes were germinated in media of various stiffness and the composition of their cell walls was monitored using fluorescence microscopy. The pollen tubes were also analyzed for average diameter, average maximum length attained under in vitro conditions, average diameter:length ratio. The fluorescence intensity of labeled pollen tube cell wall materials was plotted to identify the spatial patterns in distribution of beta-glycans (referring to cellulose content), methyl esterified HG (referring to pectin with low degree of calcium

crosslinks) and de-esterified HG (referring to pectin with high degree of calcium crosslinks).

2.2 MATERIALS AND METHODS

OBJECTIVE 1: POLLEN TUBE CHARACTERISTICS ANALYSIS IN STIFF MEDIA

2.2.1 PLANT MATERIAL

Fresh pollen was collected from *A. thaliana* eco-type Col-0 the day of the experiment from fully bloomed flowers. Around 30 flowers were collected and added to a 1.5 ml eppendorf tube with 0.5 ml liquid *Arabidopsis* growth medium pH 7.0 containing 0.1 mg/ml H_3BO_3, 1 mM $Ca(NO_3)_2 \cdot 4H_2O$, 1 mM $CaCl_2$, 10 mM KCl, 2 mM $MgSO_4 \cdot 7H_2O$, pH 7, and 18% (w/v) sucrose in ddH_20 [62]. The mixture was vortexed for 40 seconds twice and then placed in the centrifuge at 13000 rpm for 3 minutes. The residual plant material at the top and 0.4 ml supernatant was removed and the pellet containing pollen was resuspended in the 100 µl of medium to be used for germination. *S. chacoense* pollen was collected from the Matton lab at the *Institut de recherche en biologie végétale* and either used fresh or frozen. *N. tabacum* pollen was harvested from plants grown by our own lab, and *C. sinensis* was collected from a plant in the Montreal Botanical Garden. Pollen from these three species was left on silica gel for 24 hours to promote desiccation and then stored at -20°C in gel capsules on silica within falcon tubes.

2.2.2 POLLEN GERMINATION

Pollen grains of multiple species (*C. sinensis*, *N. tabacum* and *S. chacoense*) were removed from -20°C freezer stocks and hydrated for 1 hour prior to germination. Rehydrated or fresh pollen grains were added to Brewbaker and Kwack (BK) medium of pH 7.4 containing 100 µg ml^{-1} H_3BO_3, 300 µg ml^{-1} $Ca(NO_3)_2 \cdot H_2O$, 100 µg ml^{-1} KNO_3, 200 µg ml^{-1} $MgSO_4 \cdot 7H_2O$ in liquid or in presence of 1% (0.05 g in 5 ml) or 4% NuSieve® GTG® low melting temperature agarose (0.20 g in 5 ml) that was liquefied in solution using a microwave [63].

The grains were added to the liquefied agarose solution that was cooled to 35°C and then pipetted into a 1cm x 1cm 30 µm thick silica square attached to a glass slide to solidify. The slides were then put in a hydration chamber with a cover slip and left to germinate for the allotted time: 3 hours (*C. sinensis* and *N. tabacum*), 4.5 hours (*S. chacoense*) and 6+ hours (*A. thaliana*). Timing was determined through preliminary experiments to identify the HAG (hours after germination) before the pollen tubes burst.

2.2.3 MICROSCOPY

For epifluorescence microscopy, all samples were imaged on a Zeiss Axio Imager Z1 with Apotome at 20X under brightfield optics. For confocal laser scanning microscopy, all pollen tubes were imaged on a Zeiss LSM 710 Airyscan confocal microscope. Pollen tubes stained with calcofluor white were imaged under a 20X objective (numerical aperture = 0.8) at maximum excitation 514 nm wavelength, and maximum emission wavelength of 440 nm and immunolabeled pollen tubes were imaged under a 20X objective lens at maximum excitation 594 nm wavelength, and maximum emission wavelength 618 nm. Statistics for objective 1 was calculated using excel standard error function (referred to as SE in figure headings). FIJI (FIJI Is Just ImageJ) was used to quantify pollen tube diameter/length and to measure the fluorescence intensity of all pollen tube micrographs. Diameter was measured at 15 µm from the pollen tube tip and the tube length was measured from the pollen tube tip to the growth initiation point at the grain. The diameter:length ratio was calculated by dividing the average diameter by the average length; the larger the diameter:length ratio the smaller the length is compared to the diameter on average. Relative fluorescence intensity was normalized to the highest pixel intensity present in the image. The fluorescence profile was measured over a distance of 20.5 µm along the pollen tube cell wall (from the very tip to the shank on each side) and then both sides were averaged. Statistics for the relative fluorescence intensity of label of beta-glycan, de-esterified HG and methyl esterified HG were calculated by taking the average variance and taking the square root to obtain the average standard

deviation for the replicas within each sample. 10 pollen tubes were measured per fluorescence profile.

Statistical testing was performed through a 2 factor ANOVA test using R. Species and treatment type were each independent variables. 3 ANOVA tests were performed on diameter, length and diameter:length ratio. Post hoc testing was performed on significant ANOVA results using a Tukey test to determine the significant difference between two means of the independent variable influencing the dependent variable affecting cell wall formation in the pollen tube. The species' population size were all different due to the species-specific germination rate and flowering time. The *n* for each species and treatment is reported as follows: for *C. sinensis* there were 93 individuals in 0% agarose, 96 individuals in 1% agarose, 114 individuals in 4% agarose; for *A. thaliana* there were 10 individuals in 0% agarose, 25 individuals in 1% agarose, 21 individuals in 4% agarose; for *N. tabacum* there were 77 individuals in 0% agarose, 115 individuals in 1% agarose, 86 individuals in 4% agarose; and for *S. chacoense* there were 36 individuals in 0% agarose, 51 individuals in 1% agarose, 63 individuals in 4% agarose.

OBJECTIVE 2 AND 3: SPATIAL PROFILE OF THE POLLEN TUBE CELLWALL BIOCHEMISTRY THROUGH STAINING AND IMMUNOLABELLING

2.2.4 FLUORESCENCE LABEL OF CELL WALL COMPONENTS (BETA-GLYCANS)

Pollen was germinated as described and then fixed after 3 hours for *C. sinensis* and *N. tabacum,* after 4 hours for *S. chacoense* and after 6 hours for *A. thaliana*. The fixing solution was a 3.5% formaldehyde solution in PIPES buffer containing 50 mM PIPES, 1 mM EGTA and 0.5 mM $MgCl_2$ with ddH_20 added to 10 ml. The solution was prepared by adding 0.15g of solid paraformaldehyde pellets to 5 ml of the buffer and dissolving them using heat. Before use, the solution was cooled to room temperature. The fixing solution was pipetted onto the solidified media + pollen square within the silica mat and then put in the microwave oven (Pelco BioWave 34700 equipped with a Pelco Cold Spot)

operating at 150 W at a controlled temperature of 32°C ± 1°C for 40 seconds. This was followed by a 3-minute pause out of the microwave and then a second 150 W for 40 seconds. The fixing solution was then washed away by PIPES buffer five times at 150 W for 40 seconds each time. 0.4% calcofluor white mixed with ddH_20 was added on top of the solid media + pollen square in the silica mat and then vacuumed under 21 in of Hg using the microwave at 150 W for 10 minutes. Calcofluor white binds with a high affinity to cellulose microfibrils but it also stains variety of beta-glycans such as callose and chitin. Therefore, structures stained by calcofluor white will be referred to as beta-glycans from here on [9]. This was followed by five washes of ddH_20 at 150 W for 40 seconds each. Following the labeling procedure and final washes, a drop of Citifluor (Electron Microscopy Sciences) for microscopical observations was put on top of the solid media and a glass cover slip was mounted onto the silica using quick drying clear nail polish. Fluorescence methods adapted from [9].

2.2.5 IMMUNOFLUORESCENCE LABEL

For immunolabeling of pectin, after the fixation steps the pollen tubes were washed three times with phosphate-buffered saline (PBS) containing 135 mM NaCl, 6.5 mM Na_2HPO_4, 2.7 mM KCl and 1.5 mM KH_2PO_4 at pH 7.3 with 3.5% (w/v) bovine serine albumin (BSA) at 150 W for 40 seconds in the microwave oven (Pelco BioWave 34700 equipped with a Pelco Cold Spot). For *C. sinensis*, 5% (w/v) BSA was used to penetrate the pollen tube cell wall. LM19 was used as the primary antibody to label de-esterified HGs and LM20 was used as the primary antibody to label methyl esterified HGs and 3.5 µl (or 5 µl for *C. sinensis*) of the primary antibody was added to 170 µl of 3.5 % (or 5%) BSA/PBS solution. LM19 binds to de-esterified or weakly methyl esterified HG epitopes of pectin and LM20 binds to highly methyl esterified HG epitopes of pectin which will be referred to as de-esterified HG and methyl esterified HG, respectively [64]. The primary antibody was pipetted over the solid media + pollen square within the silica mat and put under 21 in of Hg vacuum in the microwave for 15 minutes at 150 W. The primary antibody was washed three times using 3.5% (or 5%)

BSA/PBS solution at 150 W for 40 seconds. Then 1.2 µl (or 1.7 µl) of the secondary antibody Alexa anti rat 594 was added to 150 µl of 3.5% (or 5%) BSA/PBS solution and pipetted onto the solid media + pollen square within the silica mat. This was put under 21 in of Hg vacuum in the microwave for 15 minutes at 150 W. This was then followed by three washes of 3.5% (or 5%) BSA/PBS solution at 150 W for 40 seconds each. Controls were performed by omitting incubation with primary or secondary antibody. Immunolabeling methods adapted from [9].

2.3 RESULTS

Of the four species of pollen tubes germinated in this study, *C. sinensis* had the highest germination rate and grew the longest pollen tubes with the largest diameters at maximum pollen tube length (Figure 1, Table 1). The maximum length was determined to be the length of the pollen tubes before the majority of the cells started bursting. The time necessary to achieve this length differed between species.

ANOVA tests were performed on diameter, length and diameter:length ratio results (Figure 1, Supplementary Table 1). It was found that the effect of species on diameter changes depending on the growing conditions ($F = 3.8$, $P < 0.001$). Post hoc Tukey comparison revealed that there was a significant difference between growth in 1% agarose and 4% agarose on the pollen tube diameter ($P < 0.001$) as well as growth liquid media and 1% agarose ($P < 0.001$) (Supplementary data Figure 1). There was no significant difference between 0% agarose and 4% agarose in all four species (Supplementary Figure 1). However, the interaction effect between species and growing condition was not found to be significant on length ($F = 1.1$, $P = 0.4$). Further the interaction effect between species and growing condition was not found to be significant on the diameter:length ratio ($F = 0.9$, $P = 0.5$).

Table 1. Experimental results of *C. sinensis, A. thaliana, N. tabacum* and *S. chacoense* in mechanical stress growth experiments resulting in differing diameters, lengths and tip cell wall biochemistry in 4% agarose media.

	In vivo pistillar environment	Average Diameter (µm) in 0%, 1%, 4% agarose respectively	Average Length (µm) in 0%, 1%, 4% agarose respectively	Resulting tip biochemistry in 4% agarose media (comparing tip to shank)
C. sinensis	Hollow	16.9, 18.9, 15.2	464, 351, 387	Higher de-esterified HG content
A. thaliana	Solid	8.7, 9.1, 10.2	51.5, 91.7, 99.9	Higher beta-glycan, de-esterified HG and methyl esterified HG content
N. tabacum	Wet solid	9.7, 10.8, 9.5	332, 234, 255	Higher beta-glycan, de-esterified HG and methyl esterified HG content
S. chacoense	Solid	8.3, 9.5, 8.6	152, 110, 147	Lower de-esterified and methyl esterified HG content

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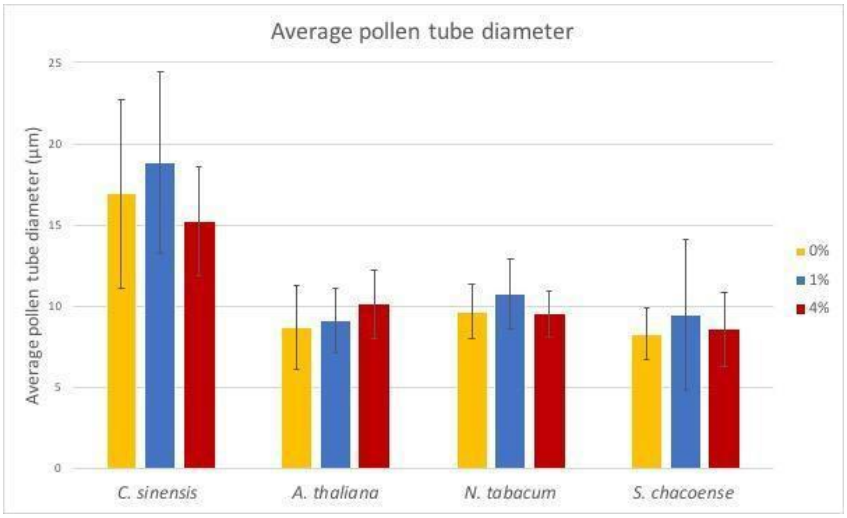

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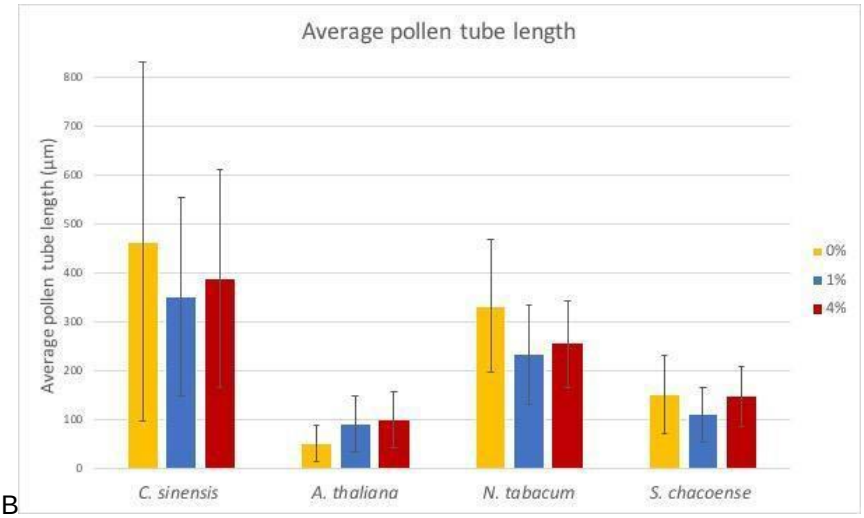

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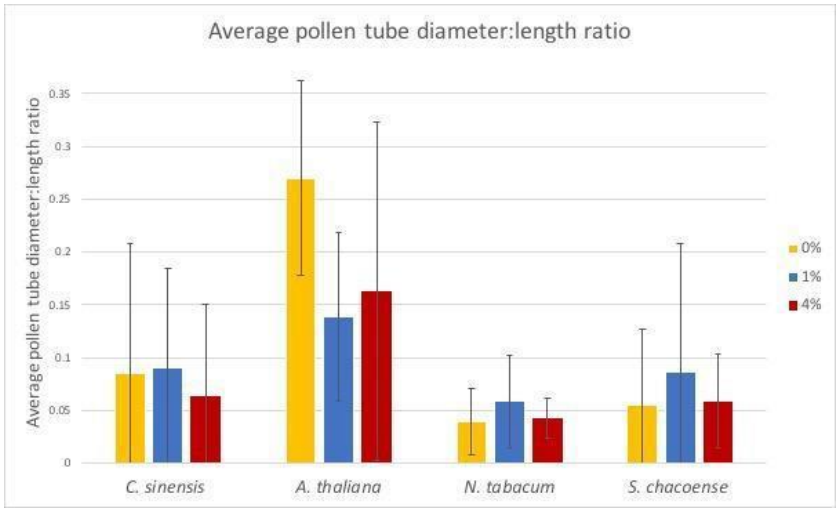


Figure 1. Pollen tube dimensions of different species in different growth media. A, Average pollen tube diameter (µm), B. average maximum pollen tube length under in vitro conditions (µm), C. average diameter:length ratio of *C. sinensis, S. chacoense, A. thaliana and N. tabacum* in liquid

media (yellow), 1% agarose media (blue) and 4% agarose media (red). Standard deviation bars included.

2.3.1 CAMELLIA SINENSIS POLLEN TUBES EXHIBIT SMALLER DIAMETERS AND LENGTHS IN STIFF GROWTH ENVIRONMENT – A FEATURE THAT CORRESPONDS WITH HIGH LEVELS OF DE-ESTERIFIED HG AT THE TIP

The *C. sinensis* pollen tubes had the highest diameters on average compared to the other three species (Figure 1A). The average diameter of *C. sinensis* pollen tubes in 0% agarose media was 16.9 µm (Figure 1A). Increasing the agarose stiffness to 1% agarose media also increased the average pollen tube diameter in this species to 18.9 µm (Figure 1A). Further increasing the agarose content of the media from 1% agarose media to 4% agarose media had an opposite effect on the average diameter and it decreased to 15.2 µm as the media became stiffer (Figure 1A). The increase in diameter in the presence of 0% and 1% agarose followed by the dramatic decrease in diameter of the pollen tubes in the presence of 4% agarose suggest the degree of media stiffness affected the diameter formation of the pollen tubes (Figure 1A). This observation together with the fact that the average lengths of *C. sinensis* pollen tubes differs with media stiffness suggests that the mechanical properties of the media caused a change in growth behavior (Figure 1). The longest average pollen tube length was recorded at 464 µm for *C. sinensis* pollen tubes in liquid media suggesting the liquid environment may be the most favorable growth environment for this species (Figure 1B). The average maximum pollen tube length in 1% agarose was calculated at 351 µm, observationally these *C. sinensis* pollen tubes were the shortest in length compared to the other *Camellia* agarose conditions (Figure 1B). Increasing the agarose content of the media from 1% agarose to 4% did not result in a significant change in maximum pollen tube length; the average maximum length of pollen tubes in 4% agarose was 387 µm (Figure 1B). The length results show no significant interaction effect which suggests the change in media did not affect the maximum length (Supplementary Table 1).

The *C. sinensis* pollen tubes in the presence of 1% agarose had a higher diameter:length ratio than the pollen tubes in the presence of 4% agarose providing evidence that the growth media affected the pollen tube formation. The "long length and narrow diameter" pollen tube phenotype was also seen in the pollen tubes in the presence of 4% agarose of *S. chacoense* and *N. tabacum* suggesting these species responded similarly to the stress of stiff media in their growth environment (Figures 1 A, B, C). Since the diameter of pollen tubes is known to depend on the biochemical profile of the pollen tube cell wall, I investigated the spatial distribution of cell wall components using fluorescence staining and immunolabeling.

Using calcofluor white staining, the relative fluorescence intensity of beta-glycan content in *C. sinensis* was found to be uniform throughout the cell wall from tip to shank for pollen tubes grown in 1% and 4% agarose (Figure 2A). There was an increase of beta-glycan content in the cell wall at the tip of the *C. sinensis* pollen tubes in the absence of agarose compared to tubes grown in 1% and 4% agarose and compared to the shank of the pollen tube (Figure 2A). The pectin content of the cell wall was assessed through immunolabeling of de-esterified HG using LM19 and methyl esterified HG using LM20. The de-esterified HG content of the pollen tubes in the presence of 4% agarose was much greater at the tip of the pollen tube and decreased dramatically towards the shank (Figure 2B).

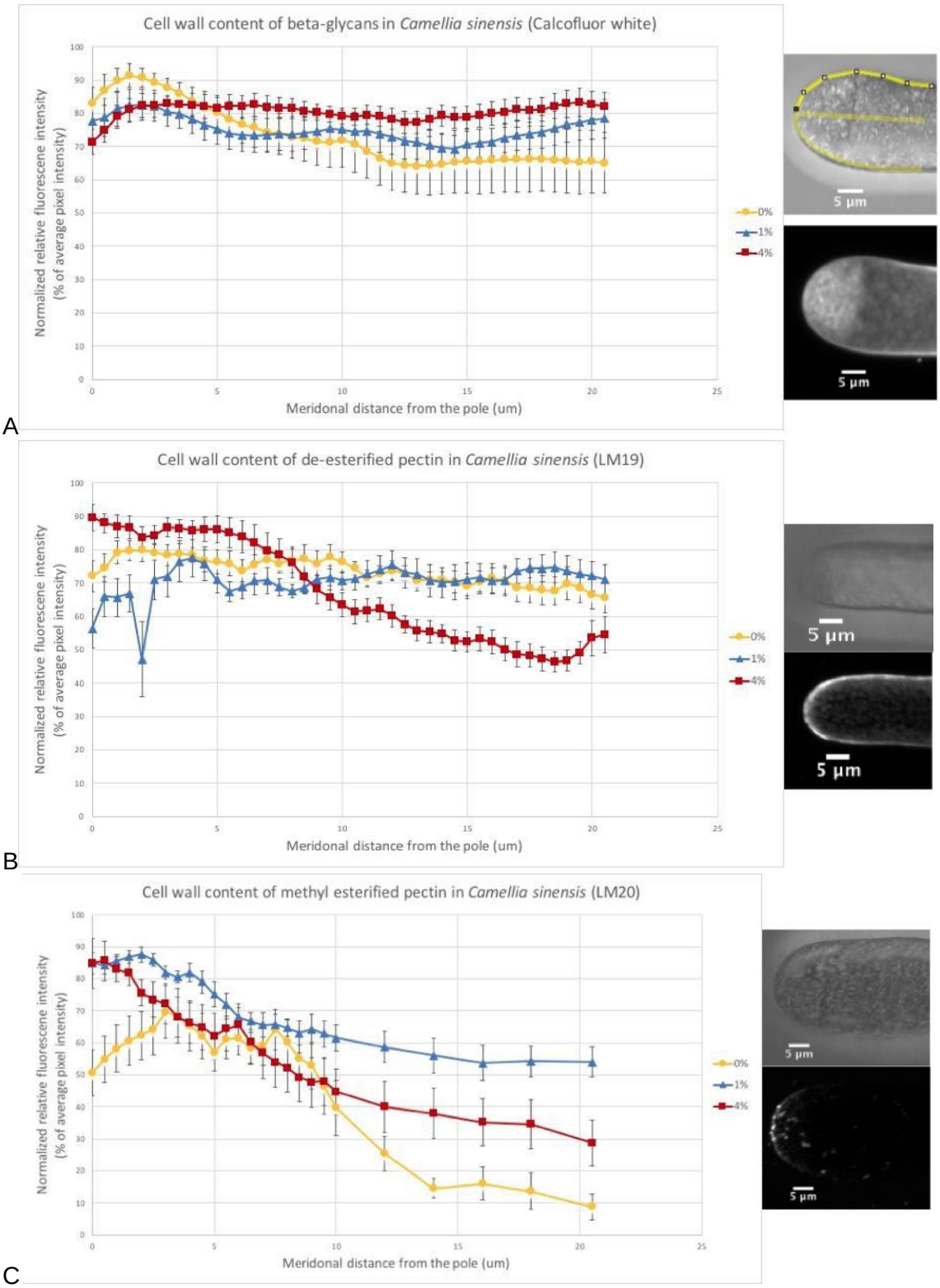


Figure 2 A. Left: Relative fluorescent intensity profile of beta-glycans in *C. sinensis* cell wall, normalized to the highest relative pixel intensity Right: Maximum projection of confocal optical sections of *C. sinensis* stained with calcofluor white growing in 1% media. Yellow lines indicate where the fluorescence was measured along the cell wall and averaged. B. Left: Relative fluorescence intensity profile of de-esterified HG in *C. sinensis* cell wall, normalized to the

highest pixel intensity. Right: Maximum projection of confocal optical sections of *C. sinensis* immunolabeled with LM-19 growing in 4% media C. Left: Relative fluorescence intensity profile of methyl esterified HG in *C. sinensis* cell wall, normalized to the highest pixel intensity. Right: Maximum projection of confocal optical sections of *C. sinensis* growing in 4% media immunolabeled with LM-20. Standard error bars included.

Inversely, the de-esterified HG content pollen tubes in 1% agarose was much lower at the tip and increased slightly towards the shank (Figure 2B). The methyl esterified cell wall content at the tip was dramatically higher than the shank cell wall content in pollen tubes germinated in both 1% agarose and 4% agarose pollen tubes (Figure 2C).

The similarity of these results suggests the growing conditions had no effect on the methyl esterified HG organization of the tip. The methyl esterified HG cell wall content decreased substantially from tip to shank in *C. sinensis* pollen tubes grown in the absence of agarose (Figure 2C). The drastic spatial gradient in the methyl esterified HG fluorescence intensity suggests there is a difference in the biochemical content of the cell wall between tip and shank influenced by the absence of agarose in their growth medium.

2.3.2 A STIFF GROWTH ENVIRONMENT INFLUENCES CELL WALL BIOCHEMISTRY AT THE TIP OF *ARABIDOPSIS THALIANA*

The average diameter profile of *A. thaliana* showed a higher average diameter for the pollen tubes in the presence of 4% agarose at 10.2 µm compared to 1% agarose at 9.1 µm suggesting that the average diameter increased as the media stiffness increased (Figure 1A). The *A. thaliana* pollen tubes grown in the absence of agarose had an average diameter of 8.7 µm and achieved an average maximum length of 51.5 µm which was the smallest recorded length of the three growth conditions (Figures 1A, 1B). The average maximum pollen tube length in 1% agarose was 91.7 µm and in 4% agarose was 99.9 µm (Figure 1B). The average diameter:length ratio for the pollen tubes in the presence of 4% agarose was larger than for the pollen tubes in the presence of 1% agarose (Figure 1C). *A. thaliana*'s diameter:length ratio was different compared to the other three species (Figure 1C). Both the length and the

diameter of *A. thaliana* pollen tubes in the presence of 4% agarose are larger than that in the presence of 1% agarose suggesting these tubes grew more efficiently in the 4% media.

Since the diameter of *A. thaliana* pollen tubes increased as the media stiffness increased it was necessary to assess if the cell wall biochemistry changed with increasing diameter (Figure 3). The cell wall content of beta-glycan and de-esterified HG of *A. thaliana* pollen tubes grown in 1% agarose was constant from tip to shank (Figure 3A, 3B). Comparatively, the 4% agarose tubes show increased cell wall content of both beta-glycan and de-esterified HG at the tip (Figure 3A, 3B). During the beta-glycan stain experiment performed for Objective 2, the pollen tubes grown in the absence of agarose did not germinate successfully and there is no data to present for these replicates. Therefore, there is no comparison between beta-glycan staining and de-esterified HG immunolabeling of *A. thaliana* for the pollen tubes in the absence of agarose (Figure 3A). Figure 3A and 3B suggest a pattern in the pollen tube growth of *A. thaliana*. There is a peak increase of polysaccharides, beta-glycans and de-esterified HG at the tip of pollen tubes grown in 4% agarose (Figures 3A, 3B). The methyl esterified HG content of the tip cell wall at the shank was similar for all replicates indicating that the growth condition has no measurable effect on the methyl esterified HG content in the tubes of *Arabidopsis thaliana* (Figure 3C).

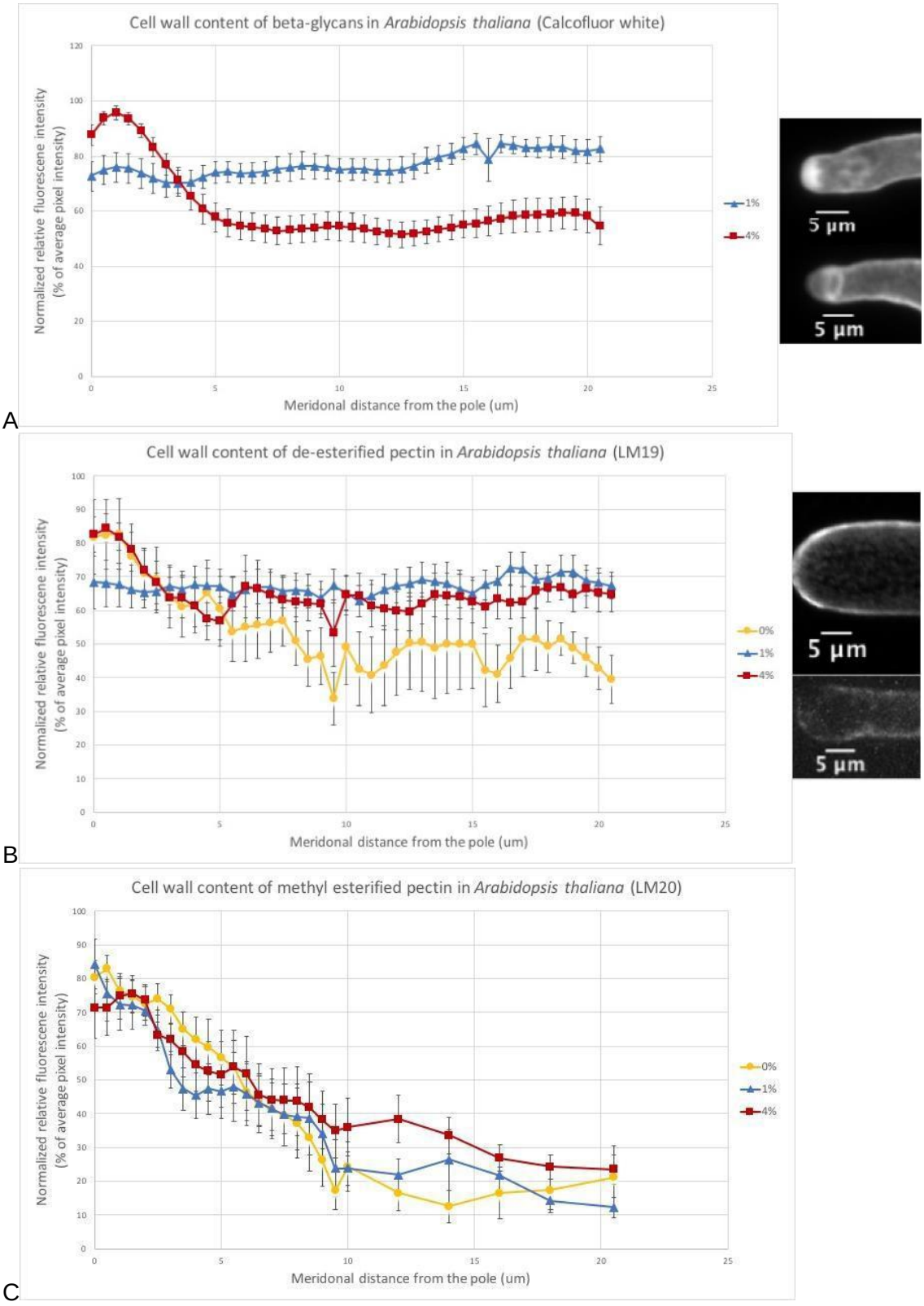
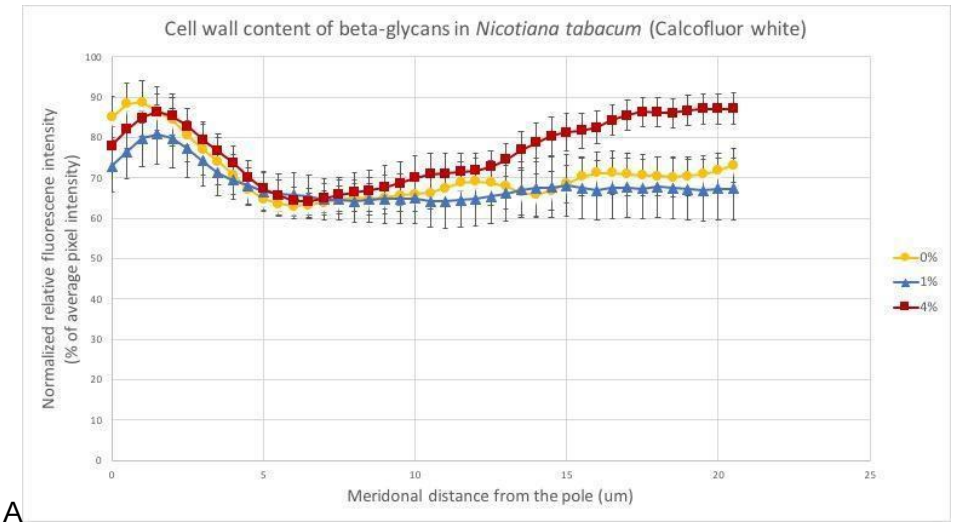


Figure 3. A. Left: Relative fluorescence intensity profile of beta-glycans in *A. thaliana* cell wall germinated in 1% and 4% media, normalized to the highest relative pixel intensity Right: Maximum projection of confocal optical sections of *A. thaliana* pollen tube stained with calcofluor white growing in 4% agarose media (top) and 1% agarose media (bottom) B. Left: Relative

fluorescence intensity profile of de-esterified HG in *A. thaliana pollen tube* cell wall germinated in liquid, 1% and 4% media, normalized to the highest pixel intensity. Right: Maximum projection of confocal optical sections of *A. thaliana* pollen tube immunolabeled with LM-19 growing in 4% agarose media (top) and 1% agarose media (bottom) C. Relative fluorescence intensity profile of methyl esterified HG in *A. thaliana* cell wall germinated in liquid, 1% and 4% media, normalized to the highest pixel intensity. Standard error bars included.

2.3.3 IN *NICOTIANA TABACUM* POLLEN TUBES STIFFER GROWTH ENVIRONMENT CAUSES BIOCHEMICAL CHANGE AT THE TIP

Similar to *S. chacoense* and *C. sinensis*, the *N. tabacum* pollen tubes had an average diameter:length profile that decreased with increasing agarose concentration (Figure 1C). The average diameter of pollen tubes grown in 1% agarose was 10.8 µm, an increase compared to liquid medium at 9.7 µm (Figure 1A). The pollen tubes grown in 4% agarose had a diameter similar to the pollen tubes in the absence of agarose, at 9.5 µm (Figure 1A). *N. tabacum* maximum pollen tube lengths slightly increased as the agarose stiffness increased from 1% media to 4% media (Figure 1B). The pollen tubes in the absence of agarose had average maximum lengths of 332 µm, those in 1% agarose measured 234 µm, and those in 4% agarose 255 µm (Figure 1B). Analysis into the biochemistry of the cell wall revealed that the beta-glycan content of the *N. tabacum* pollen tube cell wall had all similar peak shapes at the tip which decreased gradually towards the shank (Figure 4A). The de-esterified HG content at tip, on the other hand, was different between the 1% and 4% agarose concentrations indicating that the change in diameter may correlate with and possibly be caused by the change in biochemistry at the tip (Figure 4B).



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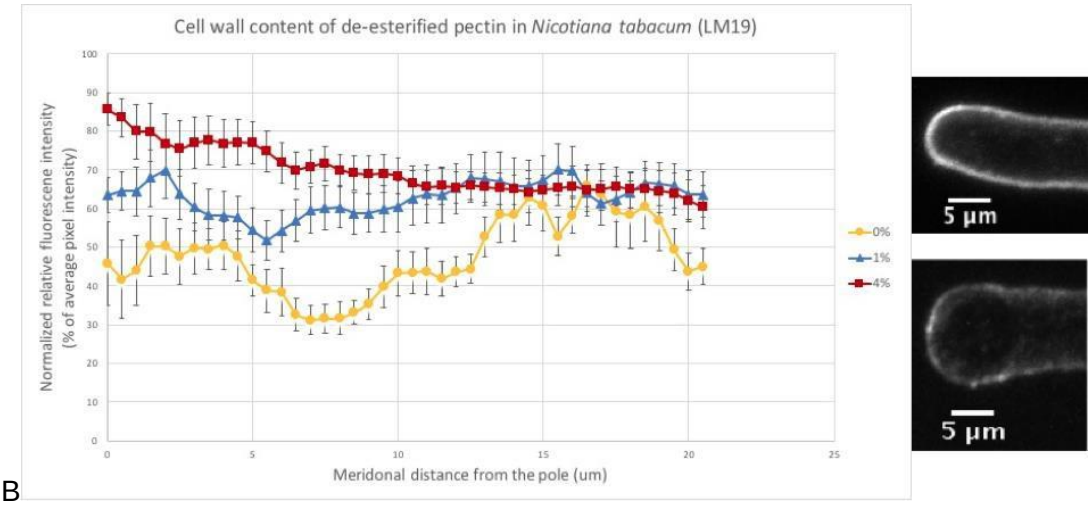

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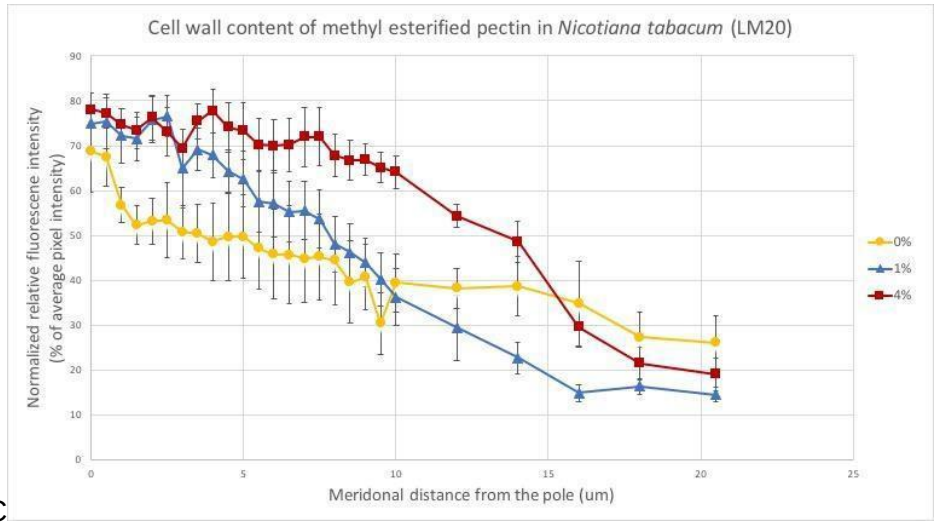

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Figure 4 A. Relative fluorescence intensity profile of beta-glycans in *N. tabacum* cell wall germinated in liquid, 1% and 4% media, normalized to the highest relative pixel intensity. B. Left: Relative fluorescence intensity profile of de-esterified HG germinated in liquid, 1% and 4% media, normalized to the highest pixel intensity. Right: Maximum projection of confocal optical sections of *N. tabacum* pollen tube immunolabeled with LM-19 in 4% agarose media (top) and 1% agarose

media (bottom) C. Relative fluorescent intensity profile of methyl esterified HG content of the *N. tabacum* pollen tube cell wall germinated in liquid, 1% and 4% media, normalized to the highest relative pixel intensity. Standard error bars included.

Further, the pollen tubes grown in the absence of agarose had similar amounts of de-esterified HG in their cell wall from tip to shank suggesting the liquid media did not influence polysaccharide accumulation in one section of the cell wall over another (Figure 4B). The methyl esterified HG content decreased from the tip to shank in all three agarose growth conditions suggesting the tip of *N. tabacum* pollen tubes have more methyl esterified HG in the cell wall compared to the shank (Figure 4C).

2.3.4 HIGH BETA-GLYCAN CONTENT CHARACTERIZES THE CELL WALL OF SWOLLEN *NICOTIANA TABACUM* POLLEN TUBES

N. tabacum pollen tubes showed abnormal growth strategies in the 0% media and 1% media growth environments resulting in swelling during tube elongation (Figures 5, 6). Abnormal growth was seen in 20-30% of the *N. tabacum* pollen tubes per experiment depending on agarose media. The bulbous tips were characterized based on having diameters that were 1.5 times larger than the straight growing tubes. After identifying the abnormal tubes, it was found that the cell walls of the *N. tabacum* swollen tips had increased beta-glycan content compared to the average straight growing pollen tubes in the same agarose environment (Figure 5). Further, the beta-glycan fluorescence profiles showed peak increases that corresponded to areas on the pollen tube were the diameter transitioned from the average diameter to the swollen diameter (Figures 5, 6).

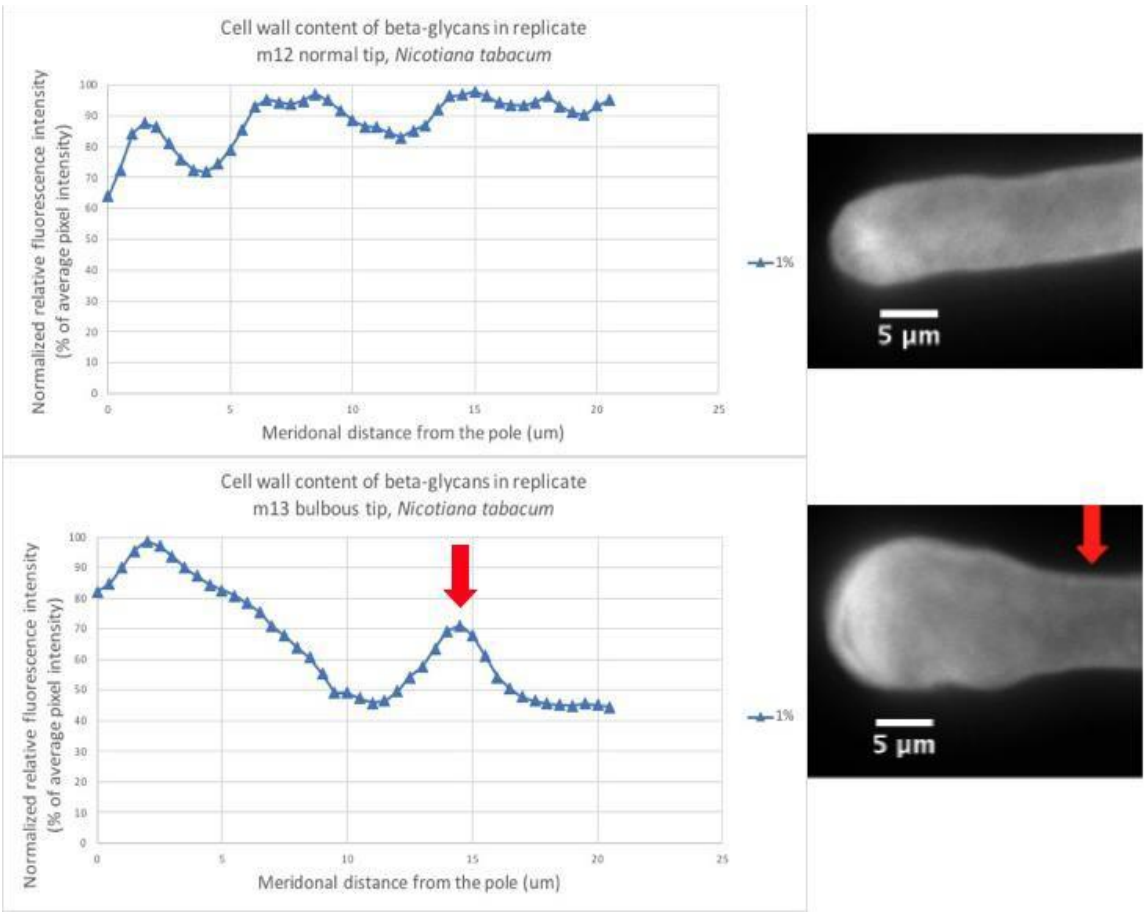

Figure 5. Comparison of the relative fluorescence intensity profile of beta-glycan content normalized to the highest pixel intensity present in normal tip *S. chacoense* pollen tube in 1% agarose media (top) and bulbous tip *S. chacoense* pollen tube growing in 1% agarose media (bottom). Red arrows indicate the peak abundance of beta-glycans corresponding to the point on the pollen tube cell wall indicating the probable diameter transition point.

The abnormal growth pattern was also identified in *N. tabacum* pollen tubes growing in the absence of agarose through swelling of the shank but was not observed in pollen tubes in the presence of 4% agarose (Figure 6). The peak abundance in beta-glycans is located distal of the swollen tip, at a point on the cell wall of average pollen tube diameter and seems to identify the diameter transition point (Figure 6).

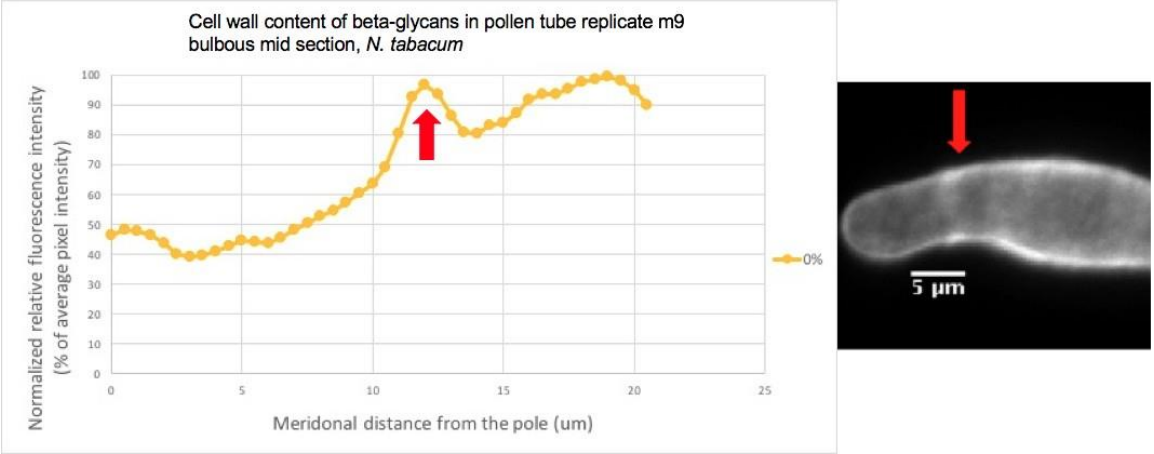

Figure 6. Relative fluorescence intensity profile of beta-glycan content normalized to the highest pixel intensity present in bulbous shank *N. tabacum* pollen tube in 0% agarose media. Red arrows indicate the peak abundance of beta-glycans corresponding to the point on the pollen tube cell wall indicating the probable diameter transition point.

This small peak of beta-glycan abundance is found in Figure 5 as well and alludes to the pollen tube diameter change and consequently a cell wall biochemistry change from high beta-glycan content at the tip to low beta-glycan content in the shank (Figure 5). This singular peak is not observed in the fluorescence profile of a normal straight growing pollen tube suggesting it's a characteristic relating to swelling of the cell wall (Figure 5).

2.3.5 A STIFFER GROWTH ENVIRONMENT CAUSES A DECREASE IN HG CONTENT AT THE TIP OF *S. CHACOENSE* POLLEN TUBES

S. chacoense pollen tubes had the smallest diameters compared to the other three species (Figure 1A). Adding 1% agarose to the liquid medium increased the average diameter of the pollen tube from 8.3 µm to 9.5 µm (Figure 1A). Further increasing the agarose content from 1% to 4% resulted in a decreased pollen tube diameter to 8.6 µm. This diameter profile is similar to the one seen in *C. sinensis* and *N. tabacum* treatment results (Figure 1A). With 147 µm, the pollen tubes in the presence of 4% agarose attained a longer maximum length compared to those in the presence of 1% agarose at 110 µm suggesting that the 4% growth medium had an effect on the pollen tube growth strategy (Figure 1B). Therefore, the cell wall composition was investigated to determine if

the observed change in pollen tube appearance reflected a difference in the cell wall biochemistry. The beta-glycan content in the cell wall of the *S. chacoense* pollen tubes was different depending on the growth condition (Figure 7A). In the absence of agarose, the beta-glycan content was highest at the tip and decreased towards the shank (Figure 7A). The pollen tubes grown in 1% agarose media on average had lower beta-glycan content in their tips which drastically increased towards the shank (Figure 7A). The pollen tubes grown in 4% agarose showed a slight increase in beta-glycan cell wall content 5 µm from the tip towards the shank, suggesting the distal end's cell wall had higher beta-glycan content (Figure 7A). Further investigation into the biochemistry of the pollen tubes showed striking differences in the tip biochemistry between the agarose concentrations (Figures 7B, 7C). The de-esterified HG content was highest at the tip of *S. chacoense* pollen tubes grown in the absence of agarose and decreased towards the shank (Figure 7B). In pollen tubes grown in 1% agarose, the de-esterified HG content was constant between tip and shank (Figure 7B). Most interestingly, the normalized fluorescence profile of pollen tubes grown in 4% agarose showed three large peaks between the tip and shank, suggesting the pollen tubes that were averaged had very different individual fluorescence profiles (Figure 7B). The error bar of the first noticeable peak had the largest difference indicating the individual pollen tubes had different fluorescent profiles whereas the third peak had a small error bar indicating this peak increase of de-esterified pectin was present in most of the pollen tubes grown in 4% agarose (Figure 7B).

Regarding the growth observations, the *S. chacoense* pollen tubes had difficulties growing in 4% agarose and roughly 30% showed abnormal shapes through swollen pollen tube tips. All the growth conditions in Figure 7C had higher methyl esterified HG content in their tips compared to their shank portions which is consistent with the other 3 species methyl esterified HG results (Figure 7C).

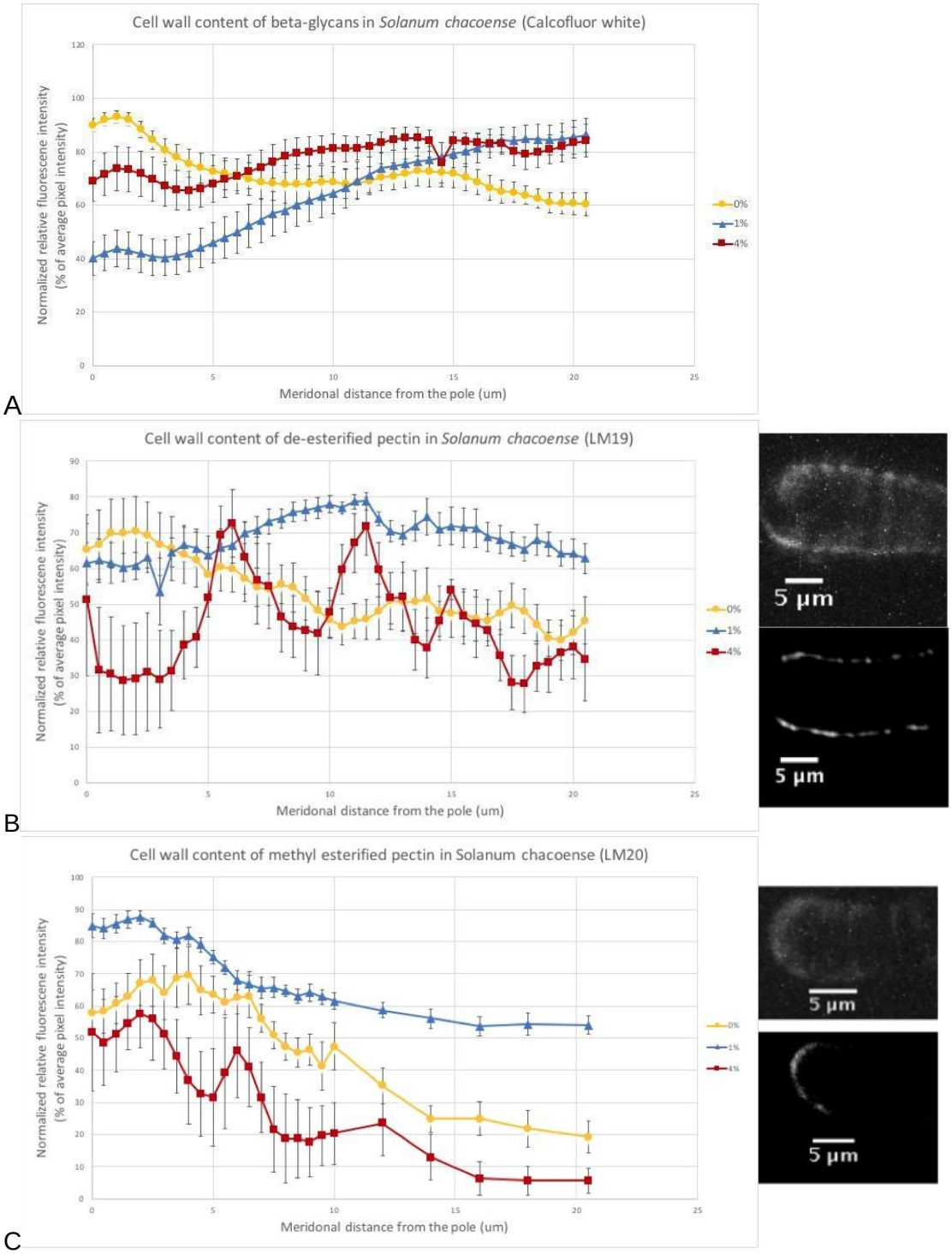

Figure 7. A. Relative fluorescence intensity profile of beta-glycans in *S. chacoense* cell wall germinated in liquid, 1% and 4% media, normalized to the highest relative pixel intensity. B. Left: Relative fluorescence intensity profile of de-esterified HG in *S. chacoense* cell wall germinated in liquid, 1% and 4% media, normalized to the highest pixel intensity present in the image. Right: Maximum projection of confocal optical sections of *S. chacoense* pollen tube immunolabeled with LM-19 in 1% agarose media (top) and 4% agarose media (bottom). C. Left: Relative fluorescence intensity profile of methyl esterified HG in *S. chacoense* cell wall germinated in liquid, 1% and 4%

media, normalized to the highest pixel intensity. Right: Maximum projection of confocal optical sections of *S. chacoense* pollen tube immunolabeled with LM-20 in 1% agarose media (top) and 4% agarose media (bottom). Standard error bars included.

2.3.6 THE BULBOUS TIP OF *S. CHACOENSE* HAS HIGHER METHYL ESTERIFIED HG CONTENT THAN NORMAL TUBES GROWN IN 4% AGAROSE MEDIA

S. chacoense pollen tubes exhibited low amounts of methyl esterified HG and de-esterified HG content in the tip cell wall when growing in 4% agarose media (Figure 7C). Interestingly, some *S. chacoense* pollen tubes displayed swollen tips during germination in 4% growth media, suggesting the stress of the stiff environment affected the cell wall formation and turgor may have forced a diameter expansion at the tip (Figure 8).

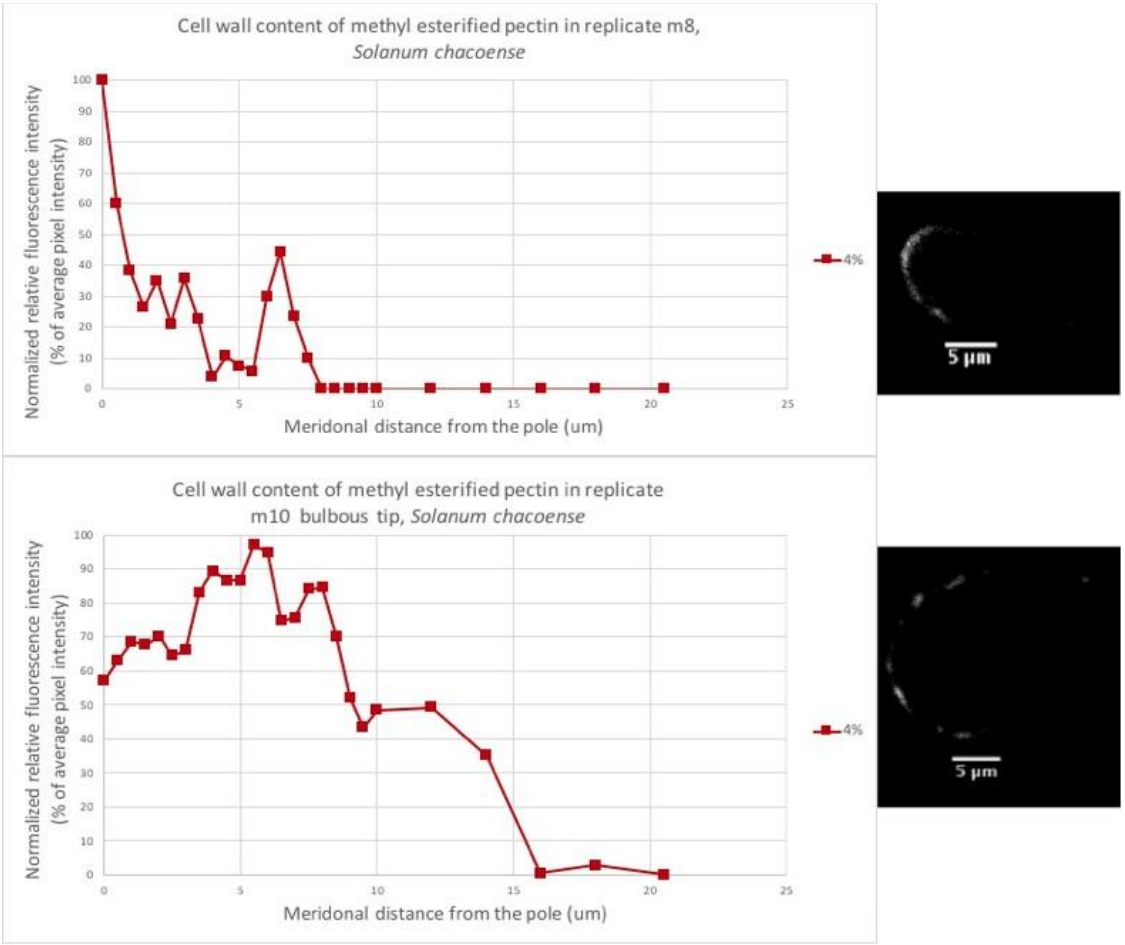

Figure 8. Comparison of the relative fluorescence intensity profiles of methyl esterified HG (pectin) in cell wall of two *S. chacoense* pollen tubes in 4% agarose media. Top pollen tube is normally growing and bottom has swollen (bulbous) tip. Methyl esterified HG was immunolabeled with LM-20 antibodies and pixel intensity percentage was averaged.

Comparing a straight growing pollen tube with a swollen tipped pollen tube there was an obvious increase in methyl esterified HG content at the swollen tip (Figure 8). Further, bulbous tipped pollen tubes were found among the replicates grown in the absence of agarose. These pollen tubes showed a decrease in de-esterified HG at the distal portion of the bulbous tip whereas it increased at the portion of the tube where the transition diameter was maintained (Figure 9).

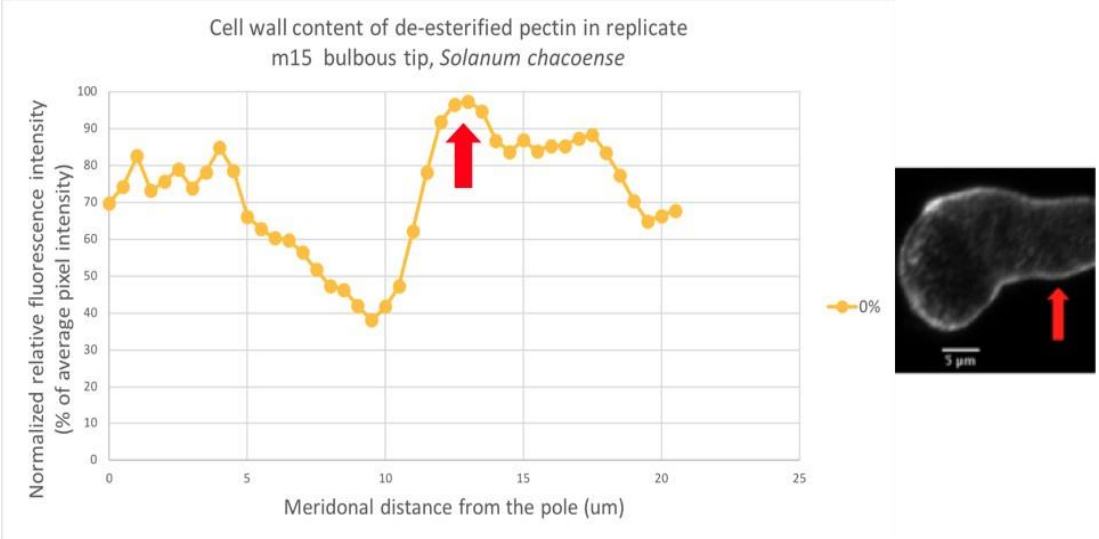

Figure 9. Relative fluorescence intensity profile of de-esterified HG content normalized to the highest pixel intensity present in bulbous shank *S. chacoense* pollen tube growing in 0% agarose media (right). Red arrows indicate the peak increase of de-esterified HG on the relative fluorescence intensity profile at the meridonal distance of the arrow that's measured along the pollen tube cell wall indicating the probable diameter transition point.

2.4 DISCUSSION

In summary, by growing different species of pollen tubes in different stiffness of agarose media and measuring their size and cell wall polysaccharide content, it was found that growth media stiffness affected the biochemistry of the pollen tube cell wall. A model, suggested by Chebli et al. (2012), dictates that the mechanism behind diameter control relies on a change in cell wall biochemistry (from methyl esterified HG to de- esterified HG) which correlates the diameter transition point with the stiffening of the cell wall [9]. We found these changes in

cell wall biochemistry occurred parallel with changes in pollen tube diameter and confirmed that the effect of species on diameter changes depending on the growing conditions. Further by using several species in the model, it allowed for investigation into species-specific genetic control of the diameter transition point associated with the differences of the species-specific in vivo pistillar environment. This was observed in *N. tabacum* and *S. chacoense* pollen tubes which adapted to mechanical stress of unnatural media stiffness through abnormal growth strategies such as the swelling of the cell wall at the tip.

In order to consider how the different stiffness of media would influence how each species would grow in vitro, it was necessary to include species in the experiment that grow in solid and others that grow in hollow in vivo pistillar environments. Four plant species were chosen based on the different in vivo pistil environments of their pollen tubes: *C. sinensis* and its hollow pistil environment, *S. chacoense* and *A. thaliana*'s solid pistil environments and *N. thaliana*'s wet solid pistil environment. The four species all had different average diameter and maximum lengths ensuring their diameter:length ratios would be on different scales. This would suggest that the cell wall biochemistry of each species would be different in order to form the unique biological structuring required for that species to function in its specific pistillar environment.

By changing the agarose growth condition, there is an observed change in pollen tube cell wall biochemistry suggesting that the addition of mechanical stress using different media stiffness has a correlative effect on growth strategy. Further, the biochemical change in the cell wall was specific for each species when growing in a particular agarose growth condition. These included stiffer agarose media influencing: increased de-esterified HG content at the tip of *C. sinensis* pollen tubes, increased polysaccharide levels at the tip of *A. thaliana* and *N. tabacum* pollen tubes and decreased de-esterified and methyl esterified HG content at the tip of *S. chacoense* pollen tubes. In addition, both *N. tabacum* and *S. chacoense* pollen tubes exhibited abnormal growth

strategies in different agarose conditions resulting in swollen tips and swollen shank portions during pollen tube elongation.

In *C. sinensis, A. thaliana,* and *N. tabacum*, the pollen tubes grown in 1% agarose media, on average, had larger diameters and smaller maximum lengths in comparison to the other agarose growth conditions establishing the “small length and wide diameter” observation for the 1% agarose grown pollen tubes. The exception was observed in *A. thaliana,* which exhibited small diameters and small lengths in 1% agarose and large diameters and large lengths in 4% agarose comparatively. The structural difference of the *A. thaliana* tube compared to the other species is unexpected since it would seem that small organisms invade stiff matrices more easily due to their shape [7]. The pollen tubes with high diameter:length ratios were often categorized as the “small length and wide diameter” pollen tubes. Repeated results showed the pollen tube growth strategy changed in different agarose conditions suggesting that the biochemistry of the cell wall plays a role in establishing the overall diameter of the pollen tube [1]. Chebli et al. (2012) suggest that the diameter transition point of pollen tubes is the region where the concentration of de-esterified HG becomes higher than the concentration of methyl esterified HG due to the activity of pectin methyl esterase (PME) [9]. The pectin transition point is also associated with a stiffness differential to stabilize the shank while it expands at the tip [7].

The main component of the pollen tube cell wall are polysaccharides, such as cellulose and pectin, whose spatial distribution and percentage of cell wall content contribute to forming the diameter of the pollen tube [9]. Therefore, any change in polysaccharide content would subsequently affect the cell wall formation and its maintenance of the pollen tube diameter. ANOVA testing on diameter showed a significant interaction effect between diameter, species and treatment suggesting that the effect of species on diameter changes depending on the treatment. The post hoc test results suggest that the 1% and 4% treatment groups affect a change in diameter in each species. Further, there was a visible difference in all polysaccharide fluorescence intensities at the tip

between the 1% and 4% treatments of all species. Coupling these results suggests the change in tip diameter was correlated with a change in the biochemistry of the tip cell wall (in this experiment only the first 20 µm of cell wall measured from the tip was observed as it is in this region that the growth activity and the determination of the diameter occur) as an effect of the change in media stiffness. The measured maximum lengths of the pollen tubes had no significant interaction effect from all media stiffness treatments on the species. The average maximum length results of the in vitro grown pollen tubes were shorter in comparison to the in vivo pollen tube lengths on record for the 4 species, which are recorded to be around several centimeters long depending on the species [65]. Further research is warranted to establish whether pollen tube length has a significant interaction effect on species in vivo.

2.4.1 INCREASED HG CONTENT AND A SMALLER AVERAGE DIAMETER IN *C. SINENSIS* POLLEN TUBE TIPS ARE EFFECTS RELATED TO A STIFFER GROWTH MATRIX

Since the recorded in vivo length of the *Camellia* pistil is ~3 cm, and since the maximum pollen tube length measured in vitro was only 1216 µm, it seems the pollen tubes require a pistillar environment in order to successfully grow to a maximum length. Clearly, the artificial in vitro medium cannot fully mimic the in vivo pistillar environment. The *C. sinensis* pollen tubes had the highest average diameter of all four species. The beta-glycan fluorescence staining in the *C. sinensis* pollen tube infers that the cell wall's cellulose content is a constant level along the whole tube in 1% and 4% agarose grown pollen tubes. The cell wall content of cellulose was slightly increased in the tip compared to the shank in the pollen tubes grown in the absence of agarose but it seemed the difference in beta-glycan content between the tip and shank was minor when comparing all three growth conditions. Interestingly, another species from the *Camellia* family, *C. japonica,* has been reported to have low amounts of cellulose at the pollen tube tip compared to the shank suggesting cellulose is not a major contributor to diameter formation in the pollen tube [58].

Regarding HG content, there was increased de-esterified HG content in the *C. sinensis* apical cell wall compared to the shank for 4% agarose grown pollen tubes suggesting that *C. sinensis* pollen tubes growing in stiff media formed stiffer pollen tube tips. Parre and Geitmann (2005) used pectinase coupled with micro-indentation experiments to correlate high local cell wall stiffness with lowered degree of pectin methyl-esterification [66]. Interestingly, the methyl esterified HG content is also higher at the tip portion in the pollen tubes in the presence of 4% agarose indicating many cell wall materials were concentrated into the *C. sinensis* apical cell wall of the 4% agarose grown pollen tubes. The *C. sinensis* pollen tubes grown in the absence of agarose had lower methyl esterified HG content at the tip comparatively, suggesting the liquid environment caused the pollen tube to modulate the mechanical properties of the apical cell wall.

There are multiple reasons why the pollen tube may need to modulate the mechanical properties of its apical cell wall. Firstly, when growing through a stiff medium, the tube must produce the force necessary to displace and/or deform the matrix in its way. Secondly, the expanding apical wall likely experiences friction against the external matrix. Friction in a growth medium is necessary to prevent push back in the distal regions of the pollen tube but too much friction at the pollen tube tip impedes growth and subsequent biochemistry [67]. *N. tabacum* pollen tubes grown in the absence of agarose also exhibited decreased HG content, specifically de-esterified HG, at the tip compared to pollen tubes grown in agarose. These results all suggest that liquid media influenced a change in the tip cell wall biochemistry and subsequently influenced a change in cell wall mechanical properties. In contrast, the reduced tip diameter of 4% agarose grown pollen tubes suggests pollen tube elongation may have occurred faster compared to the other agarose treatments. According to fracture theory, a pollen tube with a smaller diameter would invade the stiffer matrix more efficiently, ultimately allowing for a longer length to be established in a shorter time [7]. As *C. sinensis* grow in hollow pistils in vivo, increasing the stiffness of their surrounding matrix may have affected the formation of their cell wall through

an adaptation to reinforce the tip by allocating higher amounts of de-esterified HG to the apical cell wall.

2.4.2 LARGER AVERAGE DIAMETER CORRELATES WITH INCREASED AMOUNTS OF POLYSACCHARIDE IN THE APICAL CELL WALL OF *A. THALIANA* POLLEN TUBES GROWN IN 4% AGAROSE MEDIA

Calcofluor white (CW) is used to identify the cellulose content in the pollen tube cell wall due to its specific affinity to beta-glycan microfibrils [68]. There is no evidence that supports the interaction of CW with pectin, which may be due to the presence of galacturonic acid in pectins. Therefore, it is assumed that the CW signal reflects the total beta-glycan content present in that section of the cell wall [69].

Interestingly, the beta-glycan and de-esterified HG micrographs of *A. thaliana* pollen tubes showed a similar pattern of increased polysaccharide content at the tip of 4% agarose grown pollen tubes and a constant level of polysaccharide content from tip to shank in 1% agarose grown pollen tubes. This patterning suggests the change in agarose concentration (from 1% agarose to 4%) had a direct effect on the cell wall biochemistry of *A. thaliana* pollen tubes. In addition, the increase in fluorescence intensity of polysaccharides at the tip of 4% agarose grown pollen tubes suggests the recorded large average diameter of *A. thaliana* tubes grown in 4% agarose relates to an increase in cell wall material at the tip. The growth strategy of the Arabidopsis pollen tubes grown in 4% agarose and the related increased diameter may be indicative of their behavior in the in vivo biological environment, which is a transmitting tract made of solid pistillar tissue [70].

The stiffness of solid tissue surrounding the pollen tube is important in order to maintain stability during tip deformation while the pollen tube is incorporating new cell wall building materials into the tip during exocytosis [1]. While internal turgor pressure is the driving force of tip expansion, it interacts with

the pressure outside of the pollen tube and is regulated to stabilize the cell during penetration of different tissue barriers [71]. Thus, fluctuations in turgor depending on the environment suggest a pollen tube's size will depend on the surrounding environmental pressure. Considering that *A. thaliana* pollen tubes had a larger average diameter in the presence of stiff media, it is interesting to see that other species with solid styles such as *S. chacoense* and *N. tabacum* do not display a larger diameter in stiffened medium. An increase in diameter does therefore not seem to be a global strategy to cope with the mechanical impedance of a stiffer growth matrix.

Although the spatial profile of the label for beta-glycan and de-esterified HG in *A. thaliana* pollen tubes had similar peak fluorescence levels in the 4% agarose grown pollen tubes and similar consistent fluorescence levels in the 1% agarose grown pollen tubes, the pollen tubes grown in the absence of agarose for the beta-glycan experiment were not included due to experimental failure. This experiment was repeated several times but the pollen grains did not germinate in liquid media and at that point the *A. thaliana* plants in the greenhouse were no longer flowering. In general, *A. thaliana* pollen tubes had a low germination rate for all performed experiments which is not unusual for the species [62]. Compared to other angiosperm species, *A. thaliana* has been reported to have higher beta-glycan content at their tip but it is unknown if liquid media would result in a similar apical peak increase as seen in the de-esterified HG fluorescence profile [58].

Further exploration into the in vivo biochemistry of the pollen tube cell wall would provide further evidence of its biological adaptations in particular through investigation of semi in vivo pollen tube growth. Semi in vivo pollen tube germination involves pollinating a stigma, cutting it from the pistil and placing it in media to allow for pollen tube growth [72]. I conducted this experiment with *A. thaliana* pollen tubes in 1% and 4% agarose media and successfully stained the tubes with calcofluor white but did not acquire sufficient data for them to be included in this thesis. Repeating this experiment would allow to quantitatively

compare the cell wall biochemistry differences between semi in vivo pollen tube germination and in vitro germination.

The topic of in vitro germination is highly debated since agarose media cannot truly mimic the extracellular matrix of the transmitting tract within the pistil. The transmitting tract, the plant tissue within the style connecting the stigma and the ovaries, contains biological nutrients that affect the morphology and behavior of pollen tubes [65]. The typical *Arabidopsis* pistil length is ~ 2-3 mm; the fact that under in vitro conditions pollen tubes do not reach this length suggests that germination is not sufficiently supported by the in vitro growth conditions [73].

2.4.3 *N. TABACUM* POLLEN TUBES EXHIBIT ABNORMALLY SWOLLEN TIPS IN AGAROSE MEDIA DISSIMILAR FROM THEIR IN VIVO SOLID PISTILLAR ENVIRONMENT

Another species that grows in solid styles in vivo is *N. tabacum*; these pollen tubes had the longest measured maximum length in 4% agarose media compared to the other three pollen tube species. Maximum length was measured before pollen tube bursting, which was around 3 hours after germination for *N. tabacum*. Since the *N. tabacum* style has been reported to be ~4 cm long in vivo, it seemed plausible that the pollen tubes did not reach their maximum length in vitro in comparison to their in vivo requirements [74]. However, the relatively small diameter of the *N. tabacum* pollen tubes correlates with the anatomy of their in vivo transmitting tract, which is made up of solid epithelial cells, creating a minimal space (~2 mm) through which multiple pollen tubes need to grow [74]. The growth success of the small pollen tubes in stiff media reiterates the theory that the smaller the width of a penetrating structure, the easier the structure invades a matrix [7]. Interestingly, the measured diameter of *N. tabacum* pollen tubes grown in 4% agarose was 10.5 µm, which is similar to the recorded diameter of 10 µm in vivo. This similarity in diameters suggests that the 4% stiff media was most similar to this species' in vivo environment [74]. Although the diameters may be similar, in this case, it has to be acknowledged that the in vitro

agarose media lacks biological nutrients such as arabinogalactan-proteins (AGPs) that are found in pistillar tissue [74]. The difference in media nutrients is an indication of why the in vivo pollen tube lengths are vastly different from the in vitro pollen tube lengths.

Although both *A. thaliana* and *N. tabacum* grow in solid pistil environments in vivo and exhibited increased de-esterified and methyl esterified HG tip content in the 4% agarose grown pollen tubes, their diameter profiles were different. The difference in the results suggests the optimal diameter for pollen tubes is species dependent and not dependent solely on the environmental type. In addition, the increase in de-esterified HG content at the tip cell wall of *A. thaliana* and *N. tabacum* suggests tip stiffness plays a role in maintaining cell wall stability during tube elongation.

A major observation occurred in experiments with less stiff media such as 1% agarose media or media lacking agarose in which some *N. tabacum* pollen tubes displayed tubes with swollen tips and swollen shanks. When comparing these swollen tipped tubes to a straight growing *N. tabacum* pollen tube growing under the same conditions, it was found that the cell wall of the swollen tips had increased beta-glycan content compared to the “normal” straight growing pollen tube. Since this increase in beta-glycan content was specific to the swollen portion of the cell wall, there may be a correlation between the increase in diameter and the increase of beta-glycan content in the bulbous tip cell wall. This phenomenon is curious for the *N. tabacum* species because of their characteristic low cellulose content [75]. In contrast, *S. chacoense’s* abnormal tip swelling is correlated with a decrease in beta-glycan content related to the expanded tip diameter supporting the conclusion that cell wall biochemistry is species specific [76]. Therefore, any changes in cell wall biochemistry will affect species phenotypes differently and consequently affect the diameter transition point. It was found that the peak increase on the fluorescence profile corresponded to a point on the pollen tube cell wall where the diameter visibly changes. The peak is visible in *N. tabacum’s* bulbous tip and swollen shank and

in the de-esterified HG fluorescence profile of *S. chacoense*'s bulbous tip. The reoccurrence of the peak in these abnormal growth profiles contributes evidence to the mechanical model of cell wall coordination by Chebli et al. (2012) that identified de-esterified HG as the determinant in cell wall diameter formation [9] (Figure 9). It appears the diameter transition point peak phenomenon is a valuable indicator of the cell wall's attempt to maintain diameter homeostasis while stabilizing the incorporation of new cell wall material.

2.4.4 *S. CHACOENSE* POLLEN TUBES ADAPT TO MECHANICAL STRESS THROUGH ABNORMAL SWOLLEN TIPS SUGGESTING THE GROWTH RATE SLOWS DOWN

Comparing the maximum length results achieved by *S. chacoense* in all agarose growth conditions and the recorded *Solanum* pistil length of 10 mm in vivo, it seems, like with *A. thaliana,* that even the stiffest in vitro agarose media conditions are insufficient for the tubes to attain in vivo length [77]. *S. chacoense* pollen tubes had the most difficulty growing in vitro and would exhibit abnormal growth phenotypes such as bulbous tips that would arrest tube growth, displaying an inefficient growth strategy for reaching the ovary. This suggests that pollen tubes that lack necessary biochemical components adapt to maintain stability of the cell wall. By changing their growth strategy while continuing sustain tip elongation, these pollen tubes produce bulbous tips or tube swelling [66]. Since the function of the pollen tube is to elongate as quickly as possible towards the ovary, it seems counterintuitive to preserve cell wall stability before tip elongation. In lily pollen tubes it was found that exocytosis of cell wall materials at the tip continued to occur during slow growth, which resulted in a thickening of the tip cell wall without elongation [78]. Therefore, it seems possible that pollen tube tip swelling seen in the abnormal pollen tubes of *N. thaliana* and *S. chacoense* is a product of the growth rate slowing down and a subsequent thickening of the cell wall [78].

The presence of the abnormal growth strategies in pollen tubes suggests that one of the ways the pollen tube adapts to mechanical stress is by slowing down its growth rate. In particular, this phenomenon was evident in 4% agarose grown *S. chacoense* pollen tubes due to the observed high number of bulbous tipped pollen tubes in these experiments. The stress of the 4% agarose growth medium may have triggered pollen tubes to execute some abnormal variations of growth patterns in order to pierce through the stiff gel and maintain the internal turgor pressure in a new environment. Since turgor is the driving force of invasion, this environmental change may have influenced an imbalance in the species' ability to regulate its apical HG content. Both methyl esterified HG and de-esterified HG content in the 4% agarose grown *S. chacoense* pollen tubes were dramatically lower at the tip.

Similarly, Parre and Geitmann (2005) observed a dramatic decrease in apical de-esterified HG content using JIM5/JIM7 immunolabeling of pectins in *S. chacoense* [66]. Parre and Geitmann (2005) suggest that externally added pectinase digested most of the pectin in the apical cell wall and thus turgor forced diameter expansion [66]. In contrast to their results, it was found that the bulbous tipped pollen tubes in the presence of 4% agarose had high contents of methyl esterified HG at the tip in comparison to the shank (Figure 8) [66]. Therefore, it may be that a swollen tip shows no correlation with a change in cell wall biochemistry and in fact may be an indication of unregulated turgor pressure within the pollen tube.

The lack of both cell wall esterified and de-esterified HG at the *S. chacoense* tip suggests that the pollen tubes in the presence of 4% agarose had other cell wall components, such as callose or crystalline cellulose, that were maintaining the form of the pollen tube tip. Aouar et al. (2010) found that *Solanum* pollen tubes treated with cellulase enzyme achieved larger than normal diameters when growing, suggesting cellulose has a role in maintenance of cell wall diameter transition point [76]. Although results obtained with different labelling techniques cannot be compared directly, it is interesting to compare the

treatment conditions that were labelled with the same technique and identify if the patterning between 1% and 4% agarose grown pollen tubes was similar with different labels. In this case, it seemed that 4% agarose grown pollen tubes had higher beta-glycan fluorescence intensity at the tip compared to the 1% agarose grown pollen tubes. When comparing the de-esterified HG fluorescence intensity of the pollen tubes grown in 4% agarose to 1% agarose, the average de-esterified HG content was found to be much less at the tip in 4% agarose grown pollen tubes. This may suggest that the apical cell wall of *S.* chacoense pollen tubes was more reactive to the beta-glycan label or that there was a higher proportion of cellulose in this region. However, there was also an increase in methyl esterified HG in the apical cell wall of bulbous tipped *S. chacoense* pollen tubes, also grown in 4% agarose. This increase in methyl esterified HG may be related to the expanded apical diameter. Further, when silencing the pectin methylesterase gene in *N. tabacum* pollen tubes, the resultant pollen tubes retarded their growth prematurely suggesting the esterification process is essential for tube elongation [79].

2.5 FUTURE

Recent innovative research has engineered new tools to investigate pollen tube invasive growth and pollen tube cell wall formation through adaptations to microscopy like cellular force microscopy (CFM) coupled with a microindentator tip and most recently a lab-on-a-chip device with a MEMS-based capacitive force sensor [19,80]. CFM microindentation may one day be able to determine the difference in tip stiffness of *A. thaliana* and *S. chacoense* pollen tubes grown in 4% media; where *A. thaliana* pollen tubes have higher de-esterified HG content and *S. chacoense* pollen tubes have lower de-esterified HG content at their respective tips (Figures 3B, 7B). Investigation into the correlation of high de-esterified HG tip content and tip stiffness could lend evidence to form a finite conclusion on an observation that is highly debated in pollen tube research. That being said, focusing on microindentation on pollen tube grown semi-in vivo, for

example with *A.* thaliana, would provide a more realistic observation into the in vivo tip stiffness of pollen tubes as they expand through the style.

Further, using microindentation to investigate the stiffness of swollen bulbous tips could determine if diameter expansion correlates with a flexible cell wall or if the stiffness is maintained through homeostatic internal turgor pressure (Figures 8, 9). Technical advances in pollen tube research on the biological side regarding microscopy adaptations are paralleled with advancements on the biomechanical side regarding MEMS-based devices that allow the investigation of invasive force and adaptive behavior of pollen tubes. The MEMS-based force sensors developed by Ghanbari et al. (2018) and Burri et al. (2018) will allow quantitative calculation of the force of pollen tube invasive growth which coupled with the addition of mechanical stress or mechanical obstacles could determine if mechanical stress affects invasive force [80]. Since A. thaliana and S. chacoense have different amounts of pectin content in the pollen tube tips in cells grown in 4% media the comparison of these pollen tubes could clarify if tip stiffness or tip biochemistry affects invasive force.

Finally, connecting genotype with phenotype will indicate how the cell wall synthesis pathway adapts to mechanical stress. Using forward genetics similar to the phenotype sequencing developed by Harper et al. (2011) that screens multiple mutants from sequencing pools could compare the genome of straight growing pollen tubes to the genome of bulbous tipped or abnormally growing pollen tubes [81,82]. Since *S. chacoense* and *N. tabacum* both showed phenotypic abnormalities in their pollen tube growth, investigating the genes behind their biological growth strategies will provide insight into adaptation during cell wall formation under mechanical stress (Figures 5, 6, 8, 9).

SUPPLEMENTARY DATA

Table 1. A. Two factor ANOVA test on diameter as the independent variable B. Two factor ANOVA test on length as the independent variable C. Two factor ANOVA test on diameter:length ratio as the independent variable. * denotes level of significance

A.

	Degrees of freedom	Sum of Squares	Mean Square	F value	Pr (>F)
Species	3	10114	3371	263.601	<2e-16 ***
TR	2	543	271	21.225	1.06e-9 ***
Species:TR	6	292	49	3.801	0.000968 ***
Residuals	775	9912	13		

B.

	Degrees of freedom	Sum of Squares	Mean Square	F value	Pr (>F)
Species	3	9552507	3184169	95.577	< 2e-16 ***
TR	2	940067	470033	14.109	9.58e-7 ***
Species:TR	6	215008	35835	1.076	0.375
Residuals	775	25819209	33315		

C.

	Degrees of freedom	Sum of Squares	Mean Square	F value	Pr (>F)
Species	3	0.562	0.18727	27.332	<2e-16 ***
TR	1	0.040	0.04022	5.870	0.0156 *
Species:TR	3	0.018	0.00587	0.875	0.4633

Residuals	779	5.338	0.00685		

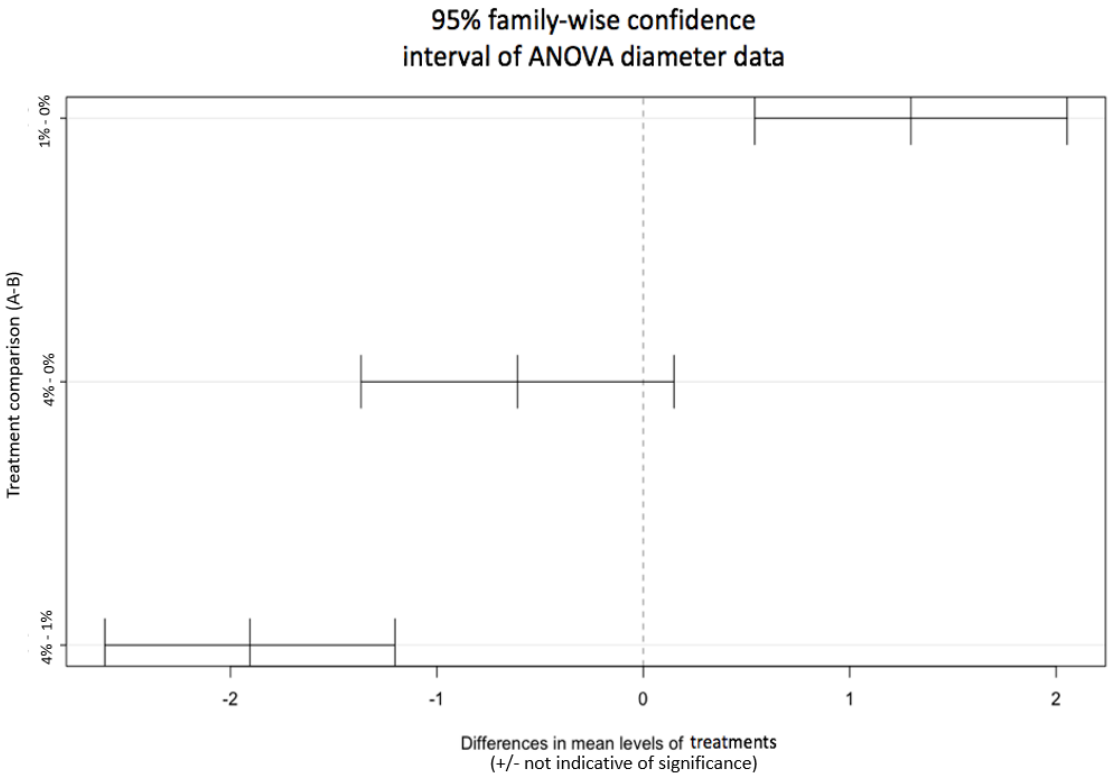

Figure 1. Post hoc Tukey test plot displaying the differences in treatment means between 0%, 1% and 4% agarose experiments from Table 1A's ANOVA data. Means that fall across the 0 line are deemed insignificant.

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