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Rhythmic Growth And Vascular Development In Brachypodium Distachyon

Dominick A. Matos

University of Massachusetts Amherst

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Rhythmic Growth and Vascular Development in *Brachypodium distachyon*

A Thesis Presented

By

Dominick A. Matos

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

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Rhythmic Growth and Vascular Development in *Brachypodium distachyon*

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By

Dominick A. Matos

Approved as to style and content by:

Samuel P. Hazen, Chair

Tobias I. Baskin, Member

Ana L. Caicedo, Member

Barbara A. Osborne, Department Head
Molecular and Cellular Biology
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Plants reduce inorganic carbon to synthesize biomass that is comprised of mostly polysaccharides and lignin. Growth is intricately regulated by external cues such as light, temperature, and water availability and internal cues including those generated by the circadian clock. While many aspects of polymer biosynthesis are known, their regulation and distribution within the stem are poorly understood. Plant biomass is perhaps the most abundant organic substance on Earth and can be used as feedstock for energy production. Various grass species are under development as energy crops yet several of their attributes make them challenging research subjects. *Brachypodium distachyon* has emerged as a grass model for food and energy crop research. I studied rhythmic growth, a phenomenon important to understanding how plant biomass accumulates through time, and vascular system development, which has biofuel feedstock conversion efficiency and yield. Growth rate changes within the course of a day in a sinusoidal fashion with a period of approximately 24 hours, a phenomenon known as rhythmic growth. Light and temperature cycles, and the circadian clock determine growth rate and the timing of rate changes. I examined the influences of these factors on growth patterns in *B. distachyon* using time-lapse photography. Temperature and, to a lesser extent, light influenced growth rate while the circadian clock had no noticeable effect. The vascular system transports important materials throughout the plant and consists of phloem, which conducts photosynthates, and xylem, which conducts water and nutrients. The cell walls of xylem elements and ground tissue sclerenchyma fibers are comprised of cellulose, hemicelluloses, and lignin. These components are important to alternative energy research since cellulose and hemicellulose can be converted to liquid fuel, but lignin is a significant inhibitor of this process. I investigated vascular development of *B. distachyon* by applying various histological stains to stems from three key developmental. My results described in detail internal stem anatomy and demonstrated that lignification continues after crystalline cellulose deposition ceases. A better understanding of growth cues and various anatomical and cell wall construction features of *B. distachyon* will further our understanding of plant biomass accumulation processes.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................ iii
ABSTRACT .......................................................................................................................... iv
LIST OF FIGURES ............................................................................................................. vii

CHAPTER
1. TEMPERATURE CYCLES DRIVE DAILY GROWTH RHYTHMS IN 
BRACHYPODIUM DISTACHYON ...................................................................................... 1
   1.1 Introduction ............................................................................................................. 1
   1.2 Material and Methods ........................................................................................ 5
       1.2.1 Plant material ............................................................................................... 5
       1.2.2 Imaging system ............................................................................................ 6
       1.2.3 Image analysis .............................................................................................. 7
   1.3 Results ................................................................................................................ 8
       1.3.1 Growth increases under warm light conditions ........................................... 8
       1.3.2 Growth is arrhythmic under constant light or constant dark conditions .... 9
       1.3.3 Growth is arrhythmic under light dark cycles and constant temperature 
               conditions ..................................................................................................... 10
       1.3.4 Temperature cycles induce rhythmic growth in constant light or constant 
               dark conditions ............................................................................................. 12
       1.3.5 Growth follows temperature cycles even in inverted conditions ............... 13
   1.4 Discussion ............................................................................................................ 14

2. VASCULAR TISSUE DEVELOPMENT AND DIFFERENTIAL DEPOSITION OF 
LIGNIN AND CELLULOSE IN BRACHYPODIUM DISTACHYON .................................. 17
   2.1 Introduction ........................................................................................................... 17
   2.2 Material and Methods ........................................................................................ 21
       2.2.1 Plant material ............................................................................................... 21
       2.2.2 Whole plant measurements ......................................................................... 21
2.2.3 Histochemistry.................................................................................................. 22
2.2.4 Image analysis and morphological measurements ........................................... 23
2.2.5 Statistical Analysis............................................................................................ 24
2.3 Results ...................................................................................................................... 24
  2.3.1 Developmental stage and vascular anatomy identification ......................... 24
  2.3.2 Cell wall biosynthesis in the stem occurs from elongation to senescence ....... 28
  2.3.3 Lignin deposition continues from stem elongation to senescence............... 30
  2.3.4 Crystalline cellulose deposition continues from stem elongation to inflorescence emergence............................................................................................ 34
2.4 Discussion ................................................................................................................ 36
BIBLIOGRAPHY............................................................................................................... 40
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Leaf growth imaging system and leaf length analysis</td>
<td>7</td>
</tr>
<tr>
<td>2.</td>
<td>Leaf growth in light dark and temperature cycle conditions</td>
<td>8</td>
</tr>
<tr>
<td>3.</td>
<td>Leaf growth in constant conditions</td>
<td>9</td>
</tr>
<tr>
<td>4.</td>
<td>Leaf growth in light dark and constant temperature conditions</td>
<td>11</td>
</tr>
<tr>
<td>5.</td>
<td>Leaf growth in constant light or dark with temperature cycles</td>
<td>13</td>
</tr>
<tr>
<td>6.</td>
<td>Leaf growth in inverted light dark and temperature cycles</td>
<td>14</td>
</tr>
<tr>
<td>7.</td>
<td>Three developmental stages for internode characterisation in <em>Brachypodium distachyon</em></td>
<td>25</td>
</tr>
<tr>
<td>8.</td>
<td>Whole plant characteristics of the three developmental stages in <em>Brachypodium distachyon</em></td>
<td>26</td>
</tr>
<tr>
<td>9.</td>
<td><em>Brachypodium distachyon</em> vascular tissue anatomy</td>
<td>27</td>
</tr>
<tr>
<td>10.</td>
<td>Internal characteristics of the three developmental stages in <em>Brachypodium distachyon</em></td>
<td>28</td>
</tr>
<tr>
<td>11.</td>
<td>Cell wall thickness increases throughout stem internode development</td>
<td>29</td>
</tr>
<tr>
<td>12.</td>
<td>Cell wall thickness increases throughout stem internode development</td>
<td>30</td>
</tr>
<tr>
<td>13.</td>
<td>Lignification increases throughout stem internode development</td>
<td>31</td>
</tr>
<tr>
<td>14.</td>
<td>Lignin deposition increases throughout stem internode development</td>
<td>32</td>
</tr>
<tr>
<td>15.</td>
<td>Lignin deposition increases throughout stem internode development</td>
<td>33</td>
</tr>
<tr>
<td>16.</td>
<td>Crystalline cellulose deposition in <em>Brachypodium distachyon</em> stem internode development</td>
<td>35</td>
</tr>
<tr>
<td>17.</td>
<td>Crystalline cellulose deposition of the three developmental stages</td>
<td>36</td>
</tr>
</tbody>
</table>
1.1 Introduction

Primary growth in plants is the product of cell division and elongation. Typically, cell division occurs in the meristems where pluripotent undifferentiated cells are located. Subsequently to cell division, some of the daughter cells replenish the meristem pool while others differentiate and elongate. The elongation process is largely caused by changes in turgor pressure via water intake and storage in the central vacuole accompanied by loosening of the cell wall. This process results in increases in leaf, stem, and root length. Interestingly, growth rate can vary throughout the day, manifested as a circadian growth rhythm. As with myriad behavioral and physiological traits in plants, growth rhythms are initiated by endogenous mechanisms and external cues (Harmer, 2009; Walter et al., 2009; Farré, 2012). Two external cues implicated in driving growth rhythms are daily light dark cycles and temperature cycles.

In Arabidopsis thaliana, light is perceived by phytochrome and cryptochrome photoreceptors that directly interact with growth promoting factors as well as entrain the circadian clock (Devlin & Kay, 2001; Nozue et al., 2007; Nusinow et al., 2011). In constant light and temperature conditions, A. thaliana hypocotyl growth exhibits a growth rhythm with a peak rate at subjective dusk (Dowson-Day & Millar, 1999). Several well-characterized clock mutants exhibit no such rhythm and thus this behavior is evidently the result of circadian clock function (Dowson-Day & Millar, 1999; Nozue et al., 2007). On
the other hand, in the presence of light dark cycles, growth rhythms peak at dawn rather than dusk (Nozue et al., 2007; Wiese et al., 2007). The concurrent effects of the circadian clock that are revealed in the absence of external cues and light regulated events dictate the timing of regulated growth processes (Nozue et al., 2007; Michael et al., 2008; Nusinow et al., 2011). The mechanism underlying this behavior is similar to that of the coincidence of external and internal cues that control photoperiodic flowering in *A. thaliana* (Imaizumi, 2010). Expression of the growth activating basic helix-loop-helix (bHLH) transcription factors *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*) and *PIF5* is repressed in the early evening by the circadian clock. Subsequent expression results in late night growth that is ultimately repressed by light regulated degradation of PIF4 and PIF5 proteins (Nozue et al., 2007). The components and behavior of the circadian clock in numerous plant species appears to be relatively conserved and similar to *A. thaliana*. This does not look to be the case for photoperiodic mechanisms and perhaps this should come as no surprise considering responses to seasonal day length vary greatly across species (Song et al., 2010). Daily growth rhythms are no exception (Walter & Schurr, 2005; Farré, 2012).

Light dark cycles are clearly an important external cue that determines specific time of day growth rhythms. Like *A. thaliana*, in the presence of light dark and temperature cycles, *Ricinus communis, Nicotiana tabacum, Flaveria bidentis*, and *Solanum lycopersicum* growth oscillations peak at dawn (Bertram & Lercari, 1997; Schmundt et al., 1998; Wiese et al., 2007; Poire et al., 2010). On the other hand, other eudicot species such as *Populus deltoides* and *Vitis vinifera* exhibit a maximum growth rate at dusk (Shackel et al., 1987; Walter et al., 2005). The growth rates of some eudicot species peak during the
day as seen for *Phaseolus vulgaris*, *Betula pendula*, and *Salix viminalis* or during the night as observed for *Glycine max* (Davies & Van Volkenburgh, 1983; Taylor & Davies, 1986; McDonald et al., 1992; Ainsworth et al., 2005). Timing of peak growth rate persists in *A. thaliana*, *N. tabacum*, *R. communis*, and *Dendrantherna grandiflorum* when temperature is held constant in the presence of light dark cycles (Tutty et al., 1994; Nozue et al., 2007; Poire et al., 2010). In constant light with temperature cycles, *D. grandiflorum* growth rhythms were similar to growth in light dark cycles (Tutty et al., 1994). When light and temperature are both held constant, *A. thaliana*, *R. communis*, and *D. grandiflorum* exhibit a circadian clock regulated growth rhythm (Tutty et al., 1994; Nozue et al., 2007; Poire et al., 2010). While the timing within a day may vary, light dark and temperature cycles as well as the circadian clock regulate daily growth rhythms in eudicot species.

The mechanisms that regulate rhythmic growth in grasses are likely distinct in some ways from eudicots. Rapid growth during the day has uniformly been observed in numerous grasses including *Zea mays*, *Oryza sativa*, *Sorghum bicolor*, *Sorghum vulgare*, *Triticum aestivum*, *Hordeum vulgare*, *Agropyron desertorum*, and *Pseudoroegneria spicata* (Johnson, 1967; Gallagher & Biscoe, 1978; Acevedo et al., 1979; Cutler et al., 1980; Kemp & Blacklow, 1980; Busso & Richards, 1992; Poire et al., 2010). While the number of species described is limited to two, *Z. mays* and *O. sativa*, above ground growth in monocots is arrhythmic in constant conditions (Poire et al., 2010). When temperature is held constant, growth oscillations can still be seen in light dark cycles in both *Z. mays* and *Fescue arundinacea*, but growth rate is greatest during the night and the rhythms are much less robust (Durand et al., 1995; Poire et al., 2010). On the other hand, growth behavior in constant light and temperature cycles mimics growth when both cues cycle (Poire et al.,
Cell division and elongation increase with temperature in grass leaves (Watts, 1971; Sadok et al., 2007; Parent et al., 2010; Poire et al., 2010). One possible mechanism is that temperature dependent enzyme activity coordinates the networks that influence growth. Another mechanism of coordination may be through a common regulator that senses temperature change and initiates a signal cascade. This latter possibility appears to be a more plausible model considering the variable activity of growth associated enzymes and photosynthetic rates measured within *A. thaliana*, *Z. mays*, and *O. sativa* (Parent et al., 2010). Regardless, temperature is the most important external cue influencing daily growth rhythms while the circadian clock appears to have no role in this respect.

As plants are sessile and some leaves and stems are rather large, growth can be measured with the simple use of a ruler. Another common method is to measure the displacement of a device attached to a leaf, referred to as a linear variable displacement transducer (Meijer, 1968). Today, time-lapse photography of leaf, root, and stem are common with high-resolution CCD cameras (Miller et al., 2007; Poire et al., 2010; Cole et al., 2011; Iijima & Matsushita, 2011). Plants lack the ability to sense wavelengths above far-red; therefore, infrared lighting can be used to illuminate plants in otherwise complete darkness (Nozue et al., 2007; Poire et al., 2010; Cole et al., 2011). Image sequence analysis can be performed using morphometric, particle tracking, or optical flow based approaches. Using these methods, total length or area can be calculated for each image within the time-lapse sequence. Indeed, numerous phenomic platforms have both automated image acquisition and analysis (Granier et al., 2006; Jansen et al., 2009; Walter et al., 2012).
It is essential to translate mechanistic understanding of growth regulation to energy crops where total biomass accumulation is the most vital attribute. Currently, several grasses are in various stages of development as energy crops including switchgrass, *Miscanthus sp.*, *Z. mays*, *S. bicolor*, and prairie grass mixtures (Tilman et al., 2006; Dhugga, 2007; Rooney et al., 2007; Schmer et al., 2008; Dohleman & Long, 2009). Several relevant differences between these species and the model system *A. thaliana* dictate that one or more grass species should serve as the focus of in depth study. Yet, energy crops have many features unfavorable to laboratory research, namely large and redundant genomes, large stature and long lifecycles, and inadequate genetic and genomic tools. On the contrary, *Brachypodium distachyon* exhibits many model system attributes including a sequenced genome, notable transformation efficiency, small stature, and a rapid life cycle (Initiative, 2010; Brkljacic et al., 2011). Here I investigated the effects of light, temperature, and the circadian clock on daily growth rhythms in *B. distachyon*.

1.2 Material and Methods

1.2.1 Plant material

Dry seed of *Brachypodium distachyon* accession Bd21-3 was imbibed and stratified in a wet paper towel at 6°C for sixteen days. Seeds were then sown towards one edge of 10 cm pots containing potting mix (#2; Conrad Fafard Inc., Agawa, MA, USA). Half of the soil was covered with 0.508 mm thick infrared absorbing paper followed by 0.254 mm infrared light absorbing paper (Edmund Optics; Barrington, NJ, USA). Plant were cultivated and imaged in a Percival model CU36L6 Growth Chamber (Percival
Scientific, Perry, IA, USA). Light conditions were 62 to 74 µmol of photons m$^{-2}$s$^{-1}$. The hot cold temperature parameters correspond to 28 ± 0.6°C and 12 ± 0.6°C, respectively. Percival conditions were monitored and recorded using a HOBO U12 Data Logger (Onset Computer Corporation, Bourne, MA, USA).

### 1.2.2 Imaging system

Time-lapse photography was conducted using modified Canon SD870 IS cameras (Canon, Lake Success, NY, USA). This 8 megapixel digital camera has a 3.8x wide-angle optical image stabilized zoom and a 28 mm lens. The camera was modified with a deep black white infrared filter by Life Pixel (Mukilteo, WA, USA) in order to capture only the infrared spectrum ranging from 750 to 1000 nm. The Canon Hack Development Kit (CHDK) advanced operating system file on an 8 GB standard SHDC memory card along with a DiskBoot BIN file allowed the Canon SD870 IS camera to boot into the CHDK operating system via the firmware enhancement option found in the camera operating system menu. A script was adapted from the freely available lapse.lua script written by Fraser McCrossan to provide the camera with time-lapse capability featuring an internal tick clock, fixed focus, camera flash disablement, and infinite capturing ability (File 1). This new script was used to capture an image every 30 minutes with a -2 exposure and macro and black and white settings enabled. The cameras were placed 50 mm away from the pots (Fig. 1A). A 100 by 100 mm LED backlight emitting 880 nm infrared light (Edmund Optics, Barrington, NJ, USA) was placed 90 mm away from the pots to the right of the camera to illuminate the growing plants (Fig. 1A-B). The cameras and LED backlight were placed on a horizontal plane as the soil surface (Fig. 1B).
1.2.3 Image analysis

The images were compiled into movies using Quicktime Pro (Apple, Cupertino, CA, USA) at a rate of 24 frames per second and captions were added to the movies using Windows Live Moviemaker (Microsoft, Redmond, WA, USA). ImageJ was used to analyze the image sequences (Abramoff et al., 2004). The first leaf image was converted to a binary image (Fig 1C-E). Any gaps present in the foreground were colored with white, and 2 to 3 morphological erosions were implemented (Fig 1E). Afterwards, plant images were skeletonized and measured to determine leaf length (Fig 1F). This process was done for every frame in which the leaf was visible (Fig 1G-H). Growth rate was calculated as the leaf length measurement minus the leaf length from the previous measurement and plotted as the average of every two hours.
1.3 Results

1.3.1 Growth increases under warm light conditions

I first characterized growth under diurnal conditions of 12 hr light, 28°C and 12 hr dark, 12°C (LDHC) by measuring leaf length (Fig. 2A-B). Growth rhythms were observed with a 24 hr period. Growth rate dramatically increased within the first hour of day and peaked at an average of 8.5 hr after lights whereupon it decreased rapidly following the transition from light to dark (Fig. 2B, Movie 1-2). The length of the first leaf increased by an average of 5.2 mm during hot light conditions and 1.4 mm during cold dark conditions. Accordingly, the average growth rate was considerably greater during simulated day conditions, 432 µm/hr, than nighttime condition, 120 µm/hr.

Figure 2. Leaf growth in light dark and temperature cycle conditions. (A) Temperature (black) and light intensity (gray) of a 12 hr light:12 hr dark temperature cycle time course. (B) Leaf length (gray) and growth rate (black) of the first leaves. Leaf length data are means ± SEM, n = 6 - 7.
1.3.2 Growth is arrhythmic under constant light or constant dark conditions

To elucidate the effect of the circadian clock on growth rhythms, I measured leaf length under constant light and temperature conditions. After entrainment in 5 days of LDHC, growth condition were changed to constant light and 28°C, LLHH (Fig. 3A). Growth remained constant, similar to what was observed during daytime condition in the diurnal time course (Fig. 3B, Movie 3-4). Leaf length increased by an average of 7.2 mm during subjective day and 7.3 mm during subjective night. Likewise, growth rate was continuous with a subjective day average of 589 µm/hr and subjective night average of

![Figure 3](image-url)

**Figure 3. Leaf growth in constant conditions.** (A) Temperature (black) and light intensity (gray) of a 12 h light:12 h dark temperature cycle time course transferred to constant (A) 28°C light or (C) 12°C dark conditions. (B, D) Leaf length (gray) and growth rate (black) of the first leaf. Leaf length data are means ± SEM, n = 4 - 7.
603 µm/hr (Fig. 3B).

To test if constant light drives growth and masks a circadian clock regulated growth oscillation, I measured leaf length under constant dark and 28°C conditions, DDHH, following LDHC entrainment (Fig. 3C). Initially, growth persisted similar to daytime conditions (Fig 3D, Movie 5-6). Leaf length increased by an average of 4.4 mm during subjective day and 4.7 mm during subjective night. Within the course of the first day of constant darkness, growth rate began to diminish to a rate of 250 µm/hr likely the effect of an absence of photosynthetic activity. The average growth rate in DDHH was 364 µm/hr during the subjective day and 389 µm/hr during the subjective night. As might be expected, growth was slower in DDHH than in LLHH. Nonetheless, the similar and constant growth rate observed under 28°C regardless of constant light or constant dark conditions suggests that the circadian clock does not influence growth rate in *B. distachyon*.

**1.3.3 Growth is arrhythmic under light dark cycles and constant temperature conditions**

I next investigated the effects of light dark cycles on growth rhythms. Following LDHC entrainment, growth conditions were changed to a constant temperature of 28°C with 12 hr light and 12 hr dark, LDHH (Fig. 4A). Growth was arrhythmic and remained somewhat constant, similar to what was observed during daytime condition in the diurnal time course (Fig. 4B, Movie 7-8). Leaf length increased by an average of 4.6 mm during the subjective day and 4 mm during the subjective night. Accordingly, the average growth
rate was greater during subjective day, 391 µm/hr, than in subjective night, 351 µm/hr (Fig. 4B).

To further elucidate the effects of light on growth, I measured leaf length under a constant temperature of 12°C with 12 hr light and 12 hr dark, LDCC (Fig. 4C). Growth rate was similar to the rate observed in the nighttime conditions of LDHC conditions with no discernible rhythm to growth (Fig. 4D, Movie 9-10). Leaf length increased by 0.8 mm with an average rate of 65 µm/hr at subjective day by 1.1 mm with an average of 95 µm/hr
during subjective day (Fig 4D). Total growth was much slower during LDCC, 80 µm/hr, than LDHH, 378 µm/hr (Fig 4B, D). The arrhythmic growth observed in these growth conditions indicates that light dark cycles do not influence growth rhythms in a noticeable manner.

1.3.4 Temperature cycles induce rhythmic growth in constant light or constant dark conditions

Considering the waveform properties observed in LDHC were not emulated in the four constant temperature conditions, LLHH, DDHH, LDHH, or LDCC, I measured leaf growth in constant light with a 12 hr 28°C and 12 hr 12°C temperature cycle, LLHC (Fig. 5A). Growth was similar to that observed in LDHC (Fig. 5B, Movie 11-12). Leaf length increased by an average of 5.4 mm during the day and 1.5 m during the subjective night. Accordingly, the growth rate was considerably greater during the day, 453 µm/hr, than nighttime condition, 124 µm/hr. Similar to LDHC, daytime growth rate increased gradually and peaked 3 hr prior to subjective night (Fig. 5B).

To test if temperature could drive growth rhythms in the absence of light, I measured leaf length under constant darkness with a 12 hr 28°C and 12 hr 12°C temperature cycle, DDHC (Fig. 5C). As in LLHC, growth patterns were similar to LDHC (Fig. 5D, Movie13-14). The length of the first leaf increased by an average of 6.5 mm during the subjective day and 1.8 mm during subjective night. The average subjective day and night growth rate was 542 and 152 µm/hr, respectively. While growth rate was faster during DDHC than LLHC, the peak rate occurred at three hours before subjective
dusk in both conditions (Fig. 5B, D). Taken together, these results strongly suggest that temperature cycles can drive rhythmic growth.

**Figure 5.** Leaf growth in constant light or dark with temperature cycles. (A) Temperature (black) and light intensity (gray) of a 12 hr light:12 hr dark temperature cycle conditions transferred to constant (A) light or (C) dark conditions with continues temperature cycles. (B, D) Leaf length (gray) and growth rate (black) of the first leaf. Leaf length data are means ± SEM, n = 4 - 6.

### 1.3.5 Growth follows temperature cycles even in inverted conditions

Since growth rhythms were observed in conditions with either temperature or light dark cycles, I tested the effect of inverting the treatments. Following growth in LDHC for three days, temperature cycles were inverted to 12°C during the light period and 28°C
during the dark period, thus LDCH (Fig. 6A). Leaf length increased by 5.3 mm in response to 28°C dark conditions and only by 1.1 mm in 12°C light conditions (Fig. 6B, Movie 15-16). Appropriately, growth rate was greater at the daytime temperature in the dark, 431 µm/hr, than the nighttime temperature in the light, 90 µm/hr.

Interestingly, peak growth rate occurred 3 hr into the 28°C dark period similarly to what was seen in LDHH and LDCC (Fig 4B, 4D, 6B). Also, amplitude changes when conditions changed from nighttime to daytime and vice versa were sharp and occurred within 5 hr evoking what is typically seen when conditions are changed from 28°C to 12°C in LDHC, LLHC, and DDHC (Fig 5B, 5D, 6B).

1.4 Discussion

Growth rhythms of *B. distachyon* under diurnal growth conditions were typical of other grass species grown both in the field and controlled environments with a peak growth rate observed during the day (Acevedo et al., 1979; Busso & Richards, 1992; Poire
et al., 2010). In the absence of external cues, oscillations were lost, demonstrating that the circadian clock had no influence on daily growth rhythms. This observation has also been noted in both Z. mays and O. sativa (Poire et al., 2010). Interestingly, the lack of growth rhythms in constant conditions is not due to an absence of a circadian clock in monocots (McClung, 2010; Song et al., 2010). Growth was arrhythmic in light dark cycles and constant temperature conditions, a result unlike what was observed in Z. mays and F. arundinacea (Durand et al., 1995; Poire et al., 2010). It was suggested that plant water relations may have played a role as evaporative demand increases over the course of a day and decreases at night; growth responds inversely in Z. mays (Sadok et al., 2007; Poire et al., 2010). In our study, growth was fastest during the subjective day.

Temperature is clearly a major driver of growth rate in B. distachyon, a result also observed in other monocots such as Z. mays O. sativa, and F. arundinacea (Watts, 1971; Durand et al., 1995; Parent et al., 2010; Poire et al., 2010). Growth oscillations during temperature cycles in constant light and darkness were very similar to the ones seen during the diurnal time course suggesting that temperature was an important cue driving growth oscillations. Also, in constant 28°C and light, dark, or light dark conditions, average growth rate during the first and second 12 hr period of a day followed the faster pace seen during daytime conditions of the diurnal time course. Plants grown in constant 12°C and light dark cycles had a slow growth rate similar to the diurnal nighttime conditions. Taken together, growth rate appears to be influenced by temperature. The importance of the effect of temperature was more evident when light dark and temperature cycles were inverted and growth was always greatest at 28°C regardless of light conditions. The stems of two eudicot species, D. grandiflorum and L. esculentum, have
been shown to exhibit similar behavior in inverted light dark and temperature cycles (Bertram & Karlsen, 1994; Tutty et al., 1994). To our knowledge, this observation has not been made previously in monocots.

The effects of light, temperature, and the circadian clock on the daily growth rhythms of *B. distachyon* and other monocots are distinct from eudicots. This may be the result of differences in the exposure of the apical meristem to temperature (Walter et al., 2009). The eudicot growth zone is typically located apically and relatively exposed to the environment. In monocots, the growth zone is often surround by leaf sheathes. As it is not exposed to light, the grass growth zone is not photosynthetically active and thus the regulation of this process typically regulated by the circadian clock is unnecessary (Harmer, 2009). Perhaps with buffered temperature exposure and restricted light exposure, there is a limited or nonexistent role for growth signaling from the circadian clock in grasses. An intriguing question is whether growth rate in monocots is a passive reaction sensitive to temperature either through changes in enzyme activity or biomechanics of stress physiology, or actively regulated similar to the action of photoreceptors by a temperature sensing mechanism.
CHAPTER 2

VASCULAR TISSUE DEVELOPMENT AND DIFFERENTIAL DEPOSITION OF LIGNIN AND CELLULOSE IN BRACHYPODIUM DISTACHYON

2.1 Introduction

Vascular tissue first emerged in land plants in the mid-Silurian period. This development allowed vascular plants to grow large and colonize areas further away from bodies of water. The vascular systems of early tracheophytes were organized in a prostele pattern of a single column. Subsequent diversification included the appearance of numerous types of arrangements of vasculature and among the most advanced and diverse are the angiosperms (Worsdell, 1902; Ye, 2002). Angiosperms first appeared in the fossil record in the early Cretaceous period and subsequently diversified 140-150 million years ago into an array of plant life including eudicots and grass (Chaw et al., 2004). Along with numerous other distinctions, eudicots and monocots developed unique vascular patterning and internal anatomy (Ye, 2002).

The vascular system transports water and nutrients throughout the plant and is comprised of xylem and phloem found conjointly in bundles. The phloem consists of bilateral pathways that conduct photosynthates produced mostly in leaves to the non-photosynthetic tissues such as roots and seeds. Hormones, proteins, and nucleic acids, for example, are also ferried by these cells. Living companion cells are interspersed throughout interconnected conductive yet dead sieve elements to perform cellular functions. Xylem stores and transports water, mineral nutrients and hormones from the roots shootward. These cells are either conductive tracheids and vessel elements or non-
conducting xylary parenchyma cells. Both the tracheids and vessel elements have thick secondary cell walls, a characteristic that provides the mechanical strength necessary to withstand the negative pressure generated during water transport. After completing secondary cell wall biosynthesis, vessel elements are generally thought to undergo cell death and are perforated at both ends to form the final vessel, a continuous hollow column where water transport occurs. On the other hand, tracheids do not form a vessel (Carlquist & Schneider, 2002).

In the eudicot stem, vascular bundles are fused in a ring to create a pattern known as eustele. In the grass stem, the vascular tissue is organized in an atactostele pattern described as vascular bundles scattered throughout the ground tissue. Within the vascular bundle, the phloem is outside of the xylem. The sieve elements and their companion cells are generally smaller than their xylem counterparts. Within the xylem, the vessel elements typically are larger and have thicker cell walls than tracheids (Ye et al., 2002). The area between the vascular bundles, the interfascicular region, may be comprised of two different cell types in both monocots and eudicots: parenchyma and sclerenchyma (Zhong et al., 2001). Parenchyma cells typically have a large central vacuole to facilitate storage of polysaccharides, fats, and even proteins. These very large cells are predominantly found in the pith although in some species they can be found in vascular bundles and in the interfascicular region. They remain alive throughout the life cycle of the living plant and have only a primary cell wall. On the other hand, sclerenchyma cells undergo cell death following secondary cell wall biosynthesis and provide mechanical support. In monocots, sclerenchyma fibers form the bundle sheath, a layer of protective fibers that surround the vascular bundle and separate phloem from xylem. In dicots, the
cortex, which is the layers of cells found between the epidermis and vascular tissue, is made up of collenchyma and parenchyma cells. Collenchyma cells have thick primary cell walls and provide support to the stem. In monocots, these cells tend to be absent although the cortex may still refer to layers of ground tissue found just below the epidermis and above the outermost vascular bundle. Chlorenchyma cells can also be found in both dicots and monocots, and are characterized as having chloroplasts and thin primary cell walls. These photosynthetic cells tend to be located near the epidermis when present.

A defining aspect of plant cell function is the wall. Following cell division, primary cell walls are formed and thicken during cell elongation. Once a cell has taken final shape, some specialized cell types, which include tracheary elements and sclerenchyma cells, undergo further wall thickening by secondary cell wall biosynthesis (Cosgrove, 2005). Cell walls are mostly comprised of three different components: cellulose, hemicellulose, and lignin. The most abundant polysaccharide in the majority of tissues is the glucan cellulose, which exists as an unbranched chain containing up to 15,000 β-1,4-linked glucose molecules (Somerville, 2006). The glucan chains are cross-linked to each other via hydrogen bonds and in turn are thought to spontaneously assemble to form cellulose microfibrils that provide tremendous tensile strength to plant cell walls and can exist in crystalline, para-crystalline, and non-crystalline states (Tomme et al., 1995; Genet et al., 2005; Harris & DeBolt, 2008). In contrast, the shorter hemicelluloses are chemically and physically more complex and their monomer compositions vary among species, tissues and cell types within an individual plant (Scheller & Ulvskov, 2010). For example, eudicots contain large amounts of pectin and
xyloglucan, while commelinoid monocotyledons, including the grasses and cereals, have little pectin, large amounts of glucuronoarabinoxylan and a unique hemicellulose, mixed linkage (1,3;1,4)-β-glucan (Vogel, 2008; Scheller & Ulvskov, 2010). Lignin is built from three monolignols: p-coumaryl, coniferyl, and sinapyl alcohols that polymerize to form p-hydroxyphenyl, guaiacyl, and syringyl phenylpropanoid units (Bonawitz & Chapple, 2010). Crosslinking lignin with hemicellulose in secondary cell walls of vascular tissue increases hydrophobicity and thus gives these functional tissues the capacity to efficiently conduct water (Donaldson, 2001). Lignin also provides the structural rigidity needed to keep the plant continuously erect as it grows.

In addition to a long history of serving as sources of fiber for paper making and other applications, plant cell wall polysaccharides can be saccharified and fermented by some microorganisms that make byproducts capable of functioning as fuel (Carroll & Somerville, 2009). Therefore, the biosynthesis of plant cell walls and the relative efficiencies with which they can be converted to sources of energy is of keen interest. Directly working with cultivated species or emerging bioenergy crops would be ideal, but several attributes make them challenging subjects. In general, food and energy crops are large, requiring considerable space for cultivation, and have relatively long life cycles. Crops also tend to have large and redundant genomes (Bennett & Leitch, 1995). Recently, Brachypodium distachyon has emerged as a model species for various food and bioenergy crops (Draper et al., 2001). It exhibits most of the model system properties of Arabidopsis thaliana, but as a grass B. distachyon serves as a model for potential energy crops such as Panicum vergatum, Sorghum bicolor, and Miscanthus sp., as well as for the cereal crops that constitute a large part of the world’s diet (Catalán & Olmstead, 2000;
Brkljacic et al., 2011). Here I investigate the deposition of lignin and cellulose and describe the anatomy of the *B. distachyon* stem.

2.2 Material and Methods

2.2.1 Plant material

Dry seed of *B. distachyon* accession Bd21-3 was imbibed and stratified in a wet paper towel at 6°C for seven days. Seeds were then sown in 10 cm pots containing potting mix (#2; Conrad Fafard Inc., Agawa, MA, USA). Growth chamber temperature was maintained at 20 °C with 20 h light:4 h dark cycles at a fluence rate of 220 µmol of photons m\(^{-2}\) s\(^{-1}\) and relative humidity of 67 to 69. For further histochemical analysis, the first internode of the tallest stem was removed from plants of three different stages. The first stage, stem elongation, corresponded to when the first internode above the crown of the tallest stem was elongating. The second stage, inflorescence emergence, was when the first internode was completely elongated and the inflorescence began to emerge from the flag leaf. The third stage, senescence, was when the first internode has senesced and the stem reached its maximum height with all of the leaves showing signs of senescence.

2.2.2 Whole plant measurements

Tiller count was recorded as the number of stems per plant and height as the tallest tiller per plant to the tip of the inflorescence of fully senesced plants. Biomass accumulation was quantified as the weight of the total above ground biomass. Internode length was determined by removing the leaf sheath and imaging the tissue using a stereo
dissecting Leica MZ16 F microscope (Leica Microsystems, Buffalo Grove, IL, USA) and the line measurement feature of ImageJ (Abramoff et al., 2004).

2.2.3 Histochemistry

Cross-sections of the first internode were made manually using a No. 11 scalpel blade and a Nikon SMZ445 dissecting microscope (Nikon, Melville, NY, USA). Sections were transferred to 1.5 mL Eppendorf microcentrifuge tubes containing distilled water. Cross-sections were subjected to two different histological stains: toluidine blue or the Wiesner reagent. Following a 30 second treatment with 7.4 µM toluidine blue, sections were rinsed twice with distilled water. To stain and visualize lignin, sections were treated with the Wiesner reagent (79 µM phloroglucinol-ethanol in 13.7 mM HCl) for 2 minutes. Stained cross-sections were mounted on microscope slides and visualized using a Nikon Eclipse E200MV R microscope (Nikon, Melville, NY, USA) attached to a PixeLINK 3 MP camera (PixeLINK, Ottawa, Canada). Images were captured using the associated PixeLINK uSCOPE software (PixeLINK, Ottawa, Canada) and further processed with Adobe Photoshop CS5.5 (Adobe, Waltham, MA, USA). The cellulose-binding module CBM3a (PlantProbes, Leeds, England) was used to detect crystalline cellulose content (Blake et al., 2006). Stem cross-sections were rinsed twice with phosphate-buffered saline (PBS; 33 mM Na₂HPO₄, 1.8 mM NaH₂PO₄ and 140 mM NaCl, pH 7.2) prior to incubation with 100 µL of 10 µg/mL CBM3a diluted in PBS for 1 hour. The solution was then removed, and two 5 minute long washes were followed by two rapid changes of PBS. The cross-sections were then treated with 100 mL of anti-His antibody produced in mouse (Sigma-Aldrich, St. Louis, MO, USA) and diluted in PBS at a 1:1000 ratio for 1
hour. The solution was then removed and two five minute long washes followed by two rapid changes of PBS. The cross-sections were then treated with 100 µL of rabbit anti-mouse antibody conjugated to Texas Red fluorophore (Invitrogen, Grand Island, NY, USA) and diluted in PBS at a 1:100 ratio for 1 hour. Afterwards, the solution was removed and two 5 minute washes were followed by two changes of PBS. Fluorescence microscopy was performed using a Leica MZ16 F microscope equipped with a mercury bulb attached to a Leica DFC300FX 1.4 MP camera (Leica Microsystems, Buffalo Grove, IL, USA). The violet filter (425/40 nm) was used to visualize lignin autofluorescence, and the Texas Red filter (560/40 nm) was used to visualize Texas Red fluorescence. Images were captured using the Image-Pro Plus imaging software (Media Cybernetics, Bethesda, MD, USA) and further processed using Adobe Photoshop CS5.5.

2.2.4 Image analysis and morphological measurements

ImageJ was used to analyze images by automatically measuring selected areas of interest after scale calibration (Abramoff et al., 2004). Stained whole stem images were used to observe total vascular bundle count. To measure stem cross-section and vascular bundle area, the ImageJ freehand selection tool was used to trace the appropriate anatomy. For interfascicular region measurements, the area in between vascular bundles was traced using the polygon tool. For vascular bundle cell wall thickness, lines was drawn across the cell walls of vessels and four adjacent bundle fibers and then averaged. For interfascicular region cell wall thickness, lines were drawn across the adjacent cell walls of the first and second row of cells outside the bundle sheath of a vascular bundle and then averaged. The adjacent cell walls of the second and third row of cells were also
measured using the previous technique. For the various fluorescence quantification measurements, the corrected total fluorescence formula was used (Burgess et al., 2010). The freehand selection tool was used to trace vascular bundles, interfascicular regions, and the whole stem cross-section with the square select tool being used to select background regions to quantify the background fluorescence of each image.

2.2.5 Statistical Analysis

For each measurement, 10 to 25 independent plants were sampled. Analysis of variance and Dunnett’s contrasts were performed in R v2.15.0.

2.3 Results

2.3.1 Developmental stage and vascular anatomy identification

I selected three developmental stages to characterize internode and vascular development in *B. distachyon*: stem elongation, inflorescence emergence, and senescence. For all three stages, the first stem internode above the crown was sampled. During the first developmental stage sampled, the first and only internode was elongating at the base of the tallest tiller (Fig. 7A-B). This was the first appearance of stem tissue, which occurred 18 to 22 days after germination. At the second stage, the inflorescence first emerged from the flag leaf sheath (Fig. 7C-D). This developmental stage occurred 25 to 30 days after germination. These first two stages are roughly equivalent to stages 30 and 51 of the BBCH-scale for cereals (Lancashire et al., 1991). The third stage sampled was when the plants had reached their maximum height and the first internode and basal
leaves were completely senesced (Fig. 7E-F). This stage, which occurred 39 to 45 days post germination, is more difficult to equate with the BBCH crop phenology system as it describes grain development characteristics at senescence with developmental stages. The number of stems per plant from the elongation stage to senesce did not change (Fig. 8A). Total stem height significantly increased at each stage by nearly 10 cm, and fresh weight significantly increased from 144 mg to 394 mg (Fig. 8B). The length and width of the
first internode increased significantly before inflorescence emergence, but not after (Fig. 8C). These three stages encompass nearly the complete development of a stem internode.

I characterized numerous anatomical features of the stem at all three developmental stages.

*Brachypodium distachyon* possesses an atactostele arrangement of vascular bundles with two circles at the periphery of the stem (Fig. 9A). The innermost vascular bundles are considerably larger than the outer ring of bundles. The cell types within the vascular bundles exhibit a pattern typical of other monocots with the phloem at the outside, and the xylem oriented towards the center of the stem (Fig. 9C). Each vascular bundle is surrounded by bundle sheath cells that also separate the xylem and phloem. The xylem is comprised of large vessels located at the interior end and both sides of the bundle with tracheids located in between the vessels. While not very

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**Figure 8. Whole plant characteristics of the three developmental stages in *Brachypodium distachyon*.** (A) Stem count. (B) Stem height (black) and stem weight (gray). (C) Internode length (black) and stem area (gray). Growth stages correspond to stem elongation (E), inflorescence emergence (F), and senescence (S). Data are means ± standard deviation, n = 13 - 25. Points annotated with the same letter are not significantly different at P < 0.05.
pronounced, some bundles have a lacuna, which is a region of variable size and shape where protoxylem may have existed. The areas in between the vascular bundles, also known as the interfascicular region, are mostly comprised of sclerenchyma fibers (Fig. 9B). These fibers along with chlorenchyma are also located in the layer of cells between the epidermis and outer vascular bundle, a region referred to as the cortex. The center of the stem, sometimes referred to as the pith, is populated with parenchyma cells that are larger than surrounding cell types (Fig. 9B). A few parenchyma cells are located in the interfascicular region since the outermost exterior of the pith can be located in between inner vascular bundles (Fig. 9B). Interestingly, all of the vascular bundles were formed at or before the point of stem elongation, as the number did not significantly change (Fig. 10A). Similarly, the size of the vascular bundles did not change over time with an average size of 3100 µm² for outer bundles and 7800 µm² for inner bundles (Fig. 10B). On the other hand, the area between the vascular bundles significantly increased by 5300 µm² from stem elongation to inflorescence emergence (Fig. 10C). Therefore, the increase

**Figure 9. Brachypodium distachyon vascular tissue anatomy.** (A) Cross section of whole stem and (B) higher magnification of the first stem internode. Red, inner vascular bundles; pink, outer vascular bundles; cyan, interfascicular region compromised mostly of sclerenchyma fibers; gray, pith; lime green, chlorenchyma and sclerenchyma cells comprise the cortex; brown, epidermis. (C) High magnification of a vascular bundle. Green, bundle sheath (B); dark purple, phloem (P); blue, xylem vessels (XV); orange, xylem tracheids (XT); magenta, lacuna (Lc); yellow, xylem parenchyma cells (XP). (A-B) Bar = 0.1 mm, (C) bar = 0.01 mm.
2.3.2 Cell wall biosynthesis in the stem occurs from elongation to senescence

First internode cross-sections were treated with a polychromatic dye, toluidine-blue, that stains polysaccharides violet and lignin turquoise allowing for the visualization of plant cell walls. At stem elongation, the cells of the vascular bundle appear darker and have thicker cell walls than other cell types (Fig. 11A-B). At inflorescence emergence, the cells of both the vascular bundle and interfascicular region appear slightly darker and have noticeably thickened cell walls (Fig. 11C-D). At senescence, these same cell types appear darker, and their cell walls are substantially thicker (Fig. 11E-F).

For both inner and outer vascular bundles, the total cell wall size of adjacent xylem in interfascicular region size accounts for the increase in stem area between elongation and flowering and not a change in vascular bundle size.
Figure 11. Cell wall thickness increases throughout stem internode development. Whole stem (A, C, E) and higher magnification (B, D, F) of *Brachypodium distachyon* cross-sections stained with toluidine-blue. (A-B) Elongating, (C-D) inflorescence emergence, and (E-F) senesced stem internode transverse cross-sections. Images were taken using brightfield microscopy. Bars = 0.1 mm.
vessels and bundle sheath significantly increased from stem elongation to senescence (Fig. 12A). This growth was quite dramatic with cell wall thickness increasing from 2.7 to 4.6 µm for inner vascular bundles and from 2.0 to 3.3 µm for outer vascular bundles. For the interfascicular region, wall thickness of neighboring sclerenchyma also increased significantly from stem elongation to senescence (Fig. 12B). The sclerenchyma closest to the bundle sheath thickened from 1.7 to 5.0 µm while the sclerenchyma second nearest to the bundle thickened from 1.7 to 4.1 µm. Thus, cell wall biosynthesis appears to continue throughout development in both the vascular bundle and interfascicular region.

2.3.3 Lignin deposition continues from stem elongation to senescence

Stem cross-sections were treated with the Wiesner reagent to observe changes in lignin deposition across development. The Wiesner reagent stains lignin yellow at low concentrations and becomes increasingly red at higher

![Figure 12. Cell wall thickness increases throughout stem internode development. (A) Cell wall thickness of adjacent vessel and bundle fibers of inner (black) and outer (gray) vascular bundles. (B) Cell wall thickness of adjacent sclerenchyma nearest (black) and second nearest (gray) to the bundle sheath. Data are means ± standard deviation, n = 96 - 100. Points annotated with the same letter are not significantly different at P < 0.05.](image-url)
Figure 13. Lignification increases throughout stem internode development. Whole stem (A, C, E) and higher magnification (B, D, F) of Brachypodium ditachyon cross sections of all three developmental stages stained with the Wiesner reagent. (A-B) Elongating, (C-D) inflorescence emergence, and (E-F) senescenced stem internode transverse cross-sections. Images were taken using brightfield microscopy. Bars = 0.1 mm.
Figure 14. Lignin deposition increases throughout stem internode development. Whole stem (A, C, E) and higher magnification (B, D, F) of Brachypodium distachyon cross sections of all three developmental stages observing the autofluorescence of lignin. (A-B) Elongating, (C-D) inflorescence emergence, and (E-F) senescence internode transverse cross-sections. Images were taken using wide field epifluorescence microscopy. Bars = 0.1 mm.
concentrations. At stem elongation, the vascular bundles stained a pale red while the interfascicular region appeared bright yellow (Fig. 13A-B). At inflorescence emergence, the vascular bundles stained a more distinctive red and the interfascicular region a darker yellow (Fig. 13C-D). At senescence, the vascular bundles and interfascicular region both stained a dark red (Fig. 13E-F). Since lignin is autofluorescent, fluorescence microscopy was used to further observe and quantify lignin content within stems. When elongating, the vascular bundles and interfascicular region faintly appeared and was more apparent at higher magnifications (Fig. 14A-B). At inflorescence emergence, both the vascular bundles and interfascicular region were noticeably more fluorescent (Fig. 14C-D). At senescence, the vascular bundles were especially bright, and the interfascicular region became strikingly more fluorescent (Fig. 14F).

Figure 15. Lignin deposition increases throughout stem internode development. (A) Corrected total autofluorescence of whole stem, (B) inner (black) and outer (gray) vascular bundles, and (C) interfascicular region. Data are means ± standard deviation, n = 16 - 27. Points annotated with the same letter are not significantly different at $P < 0.05$. 
14E-F). I then quantified the total corrected lignin autofluorescence of the whole stem, vascular bundles, and interfascicular region. The whole stem significantly increased in total autofluorescence from stem elongation to senescence (Fig. 15A). Accordingly, both inner and outer vascular bundles increased significantly in total autofluorescence over time (Fig. 15B). The interfascicular region had the most substantial change in total autofluorescence where it nearly increased by three-fold from stem elongation to senescence (Fig. 15C). These observations demonstrated that lignification occurred throughout development in both the vascular bundle and interfascicular region.

2.3.4 Crystalline cellulose deposition continues from stem elongation to inflorescence emergence

Stem cross-sections were immunostained with the CBM3a recombinant protein probe and Texas Red fluorophore in order to observe crystalline cellulose content throughout development. At stem elongation, the xylem was more fluorescent than the interfascicular region (Fig. 16A-B). The interfascicular region appeared slightly more luminous at inflorescence emergence but the vascular bundles did not increase (Fig. 16C-D). Interestingly, neither the interfascicular region nor the vascular bundles seemed more fluorescent at senescence (Fig. 16E-F). I then quantified the total corrected crystalline cellulose fluorescence of the whole stem, vascular bundles, and interfascicular region. The total crystalline cellulose fluorescence of the whole stem significantly increased from stem elongation to inflorescence emergence where it appeared to plateau (Fig. 17A). This same trend was observed for inner vascular bundles and the interfascicular region (Fig. 17B-C). For outer vascular bundles, there was no change in fluorescence throughout
Figure 16. Crystalline cellulose deposition in *Brachypodium distachyon* stem internode development. Whole stem (A, C, E) and higher magnification (B, D, F) images of the Texas red fluorescence of *Brachypodium distachyon* cross sections immunostained with the CBM3a probe. (A-B) Elongating, (C-D) inflorescence emergence, (E-F) and seneseced stem internode transverse cross-section. Images where taken using wide field epifluorescence microscopy. Bars = 0.1 mm.
crystalline cellulose appears to be deposited in vascular bundles and the interfascicular region from stem elongation to inflorescence emergence and then seemingly ceases afterwards.

2.4 Discussion

Here I have described and quantified growth and cell wall deposition of three stages of *B. distachyon* stem internode development. Sampling was conducted based on important developmental milestones rather than time after germination, thus allowing for simple application by different investigators to diverse accessions grown under a variety of conditions.

Interestingly, the size and number of vascular bundles did not change after the point of stem elongation. Therefore, any increase in stem area was due to changes in the interfascicular region. On the other hand, changes in fresh weight were the
result of secondary cell wall thickening, which again, did not result in a change in
vascular bundle size. While I cannot rule out changes at earlier stages of growth, vascular
bundle structure appears to be established at the point of meristem differentiation.

The term for grass vascular patterning is atactostele; atacto meaning disarranged. However, the arrangement is clearly not random and scattered. For example, the two
circles of vascular bundles, the inner bundles being considerably larger than the outer,
observed in B. distachyon are regularly spaced and of fairly predictable number. This
design is similar to closely related C₃ crop species such as Oryza sativa and Triticum
aestivum with some subtle differences (Patrick, 1972; Li et al., 2003). In O. sativa and T.
aestivum, the inner and outer vascular bundles are located directly across from each other
and the inner bundles are surrounded by parenchyma cells while outer bundles are
surrounded by sclerenchyma fibers. In B. distachyon, inner and outer vascular bundles
are diagonal to each other, and both types of bundles are surrounded by sclerenchyma
fibers. For this reason, the interfascicular region, a term commonly used to describe
dicots yet applicable to monocots, is mostly comprised of sclerenchyma fibers in B.
distachyon. Larger grass species such as Zea mays and S. bicolor have many more
vascular bundles with a spiral pattern from the center of the stem (Kiesselbach, 1949;
Wilson et al., 1993; Sindhu et al., 2007). The rind is the region near the epidermis with
small vascular bundles surrounded by highly thickened sclerenchyma fiber. Interestingly,
this area has all the hallmarks of the interfascicular region of B. distachyon. In C₄ grasses,
bundle sheath cells contain chloroplast and thin walls (Sage et al., 2012). I use the term
bundle sheath in C₃ grass B. distachyon as an anatomical term, rather than a functional
label. These cells appear to be thick walled fibers without chloroplasts in B. distachyon.
The cell types and organization of the ground tissue outside of vascular bundles is typical of other monocots. Chlorenchyma cells also are found within the stem adjacent to the epidermis and collenchyma cells are not observed in the cortex, an observation typical of monocots.

The cell walls of the vascular bundle and interfascicular region continued to thicken throughout development indicating that cell wall biosynthesis continued up until plant senescence. Previous transcriptomic analysis at the resolution of whole tissue types revealed a strong correlation among lignin and cellulose structural gene expression (Brown et al., 2005; Persson et al., 2005; Ruprecht et al., 2011). Histological observation of different cell types revealed differences in both the temporal and special deposition of lignin and crystalline cellulose in *B. distachyon* stem. Lignification continued from stem internode elongation to senescence in sclerenchyma fibers and tracheary elements, cells known to undergo exclusive secondary cell wall biosynthesis, a process typified by high lignin deposition (Ye, 2002). Continued lignification was also observed in parenchyma cells, which do not undergo secondary cell wall biosynthesis. While primary cell walls are often not considered lignified, this observation is not unusual (Chesson et al., 1997; Lin et al., 2002). Unlike lignin, crystalline cellulose stained content did not increase in the first internode following inflorescence emergence and was most abundant in xylem vessels and tracheids. While relatively little crystalline cellulose was observed in the bundle sheath cells, they were heavily lignified. Thus, differences in timing of deposition and cell type were apparent through the analysis of three stages of *B. distachyon* internode development.
Xylem cells undergo apoptosis before becoming functional for water transport (Roberts & McCann, 2000; Bollhoner et al., 2012). As the cell matures, secondary wall biosynthesis occurs while the autolytic factors associated with cell death such as hydrolytic enzymes are suppressed by inhibitors and by storage in the central vacuole (Obara et al., 2001). After a critical level of serine protease is reached in the extracellular space, an influx of calcium ions into the cytoplasm triggers a rupture of the central vacuole releasing the autolytic factors that lead to rapid cell death (Groover & Jones, 1999; Obara et al., 2001). Remarkably, even after death, the cell walls of tracheary elements continue to lignify (Stewart, 1966; Pesquet et al., 2010). Although this process is not well understood, xylem parenchyma cells may deposit lignin monomers into the walls of dead cells and provide the hydrogen peroxide needed for polymerization (Hosokawa et al., 2001; Ros Barceló, 2005; Tokunaga et al., 2005). In the developing *B. distachyon* stem internode, cell death likely occurs following the biosynthesis of cellulose, sometime between elongation and inflorescence emergence. After this point, lignification of xylem and fiber cells continues. These mechanisms could explain the differential deposition of crystalline cellulose and lignin throughout development since lignification would continue even after cellulose and lignin deposition during secondary cell wall biosynthesis has concluded.


