AN ANALYSIS OF THE DEVELOPMENT OF SHOOT APICES IN EXCISED IMMATURE ZYGOTIC COTTON EMBRYOS *GOSSYPIUM HIRSUTUM* CV TEXAS MARKER-1

A Dissertation

by

MARIANNE KAY ARNOLD

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2011

Major Subject: Molecular and Environmental Plant Sciences

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Approved by:

Chair of Committee,	Jean H. Gould
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	Daniel Lineberger
	Carol Loopstra
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ABSTRACT

An Analysis of the Development of Shoot Apices in Excised Immature Zygotic Cotton Embryos (*Gossypium hirsutum* cv Texas Marker-1).

(December 2011)

Marianne Kay Arnold, B.A., San Francisco State University; B.S., New Mexico State University

Chair of Advisory Committee: Dr. Jean H. Gould

Although cottonseed is an important source of oil and fiber, the development of cotton embryos has not been investigated as well as development of cotton fiber. The development of cotton embryos in late heart-stage and early cotyledonary stage is less well investigated than the first 10-14 days after anthesis, or the late stages of embryo development during seed-fill and desiccation. This analysis focused on cotton embryos in the late heart-stage and early cotyledonary stage of development (1.5-4.0 mm or about 13-18 DPA).

In vitro analyses are important tools for studying embryos in isolation from the endosperm and fiber and when it is necessary to monitor the developing embryo continuously. The original goal of this work was to develop an in vitro culture method that would support continued development of excised zygotic embryos from the early cotyledonary stage into complete plants with true shoots, i.e. true leaves or visible buds and then to use this method to study aspects of developmental regulation during

cotyledonary stage and the transition to later stages. Not all embryos were competent to develop true shoots (an apical bud or a leaf plus a bud) in culture. A number of cultural variables were tested and eliminated. Embryo maturity at the time embryos were excised and the presence or absence of light during the first 14 days of culture affected the competence of immature embryos to developed true shoots. The effect of light was verified in several large replicated experiments. Morphological changes occurring during in vivo development were examined microscopically. The transition from heart-stage to early cotyledonary stage and the development of the first leaf from initials to a large structure were identified. Embryonic shoot apices continued to grow in cultured 1-3 mm embryos. The size and shape of light-treated and dark-treated embryonic apices was compared. A germination test of mature seeds identified seedlings with a similar phenotype occurring at similar rates in seedlings and light-cultured embryos and possible causes were discussed.

DEDICATION

This work is dedicated to my husband, Doug, who never let me quit.

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I would like to thank Dr. Jean Gould, my committee chair, and my committee members for their patience and support. I would like to thank John Hubstenberger at New Mexico State who encouraged me to go on for a graduate degree. I would also like to thank Dr. Greg Cobb, Dr. Alan Pepper, Dr. Dirk Hays, Dr. Scott Finlayson and Dr. Hisashi Koiwa, Dr. Stan Vitha and Dr. David Stelly for technical advice and the use of equipment and the Microscopy Center for microscopy training.

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Most of all, I would like to thank my husband, Doug Stocks, whose help, encouragement and support gave me the courage to undertake this project.

NOMENCLATURE

ABA	Abscisic acid
Abaxial	Away from the center of the meristem
Adaxial	Toward the center of the meristem
Anticlinal division	Cell divisions parallel to the surface
BT	Cotton ovule culture medium developed by Beasley and Ting (1973, 1974)
DPA	Days past anthesis
Fix	To kill and preserve tissue for microscopic examination
HIR	High irradiance response
LFR	Low fluence response
LED	Light emitting diode
MS	Cell culture medium developed by Murashige and Skoog (1962)
Periclinal division	Cell divisions perpendicular to the surface
PPF	Photosynthetic photon flux, a measure of light intensity expressed in μmol photons $m^{-2}s^{-1}$
SAM	Shoot apical meristem
SH	Cotton fertilized ovule culture medium developed by Stewart and Hsu (1977)
TM-1	*Gossypium hirsutum* cv Texas Marker-1 (Kohel et al., 1970)
True shoot	True shoot, for the purpose of this study a true shoot consists of an apical bud or a true leaf plus a bud
VLFR	Very low fluence response

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Although cottonseed is an important source of oil, protein and fiber, the regulation of fiber development on cotton embryos has received more investigative attention than development of the embryo itself. The early stages of cotton embryo development, within the first 10 to14 days post anthesis (DPA), have received significant attention focused on initiation and elongation of cotton fiber. Attention has also been focused on embryo development and physiology in later stages of embryo development, 20 to 55 DPA, during which cellulose is laid down in fibers, storage products accumulate and the embryo becomes dormant (Dure, 1975; Reeves and Beasley, 1935). Less attention has been focused on cotton embryo development in the critical 10 to 20 DPA period. In the 1970's and 1980's, cotton was the plant model used to study late embryo development in dicotyledonous crops and led to characterization of abscisic acid (ABA) in maturing embryos, and late embryo abundant (LEA) proteins in plants (Baker et al., 1988; Dure, 1975; Galau et al., 1986; Galau et al., 1987; Hughes and Galau, 1989; Hughes and Galau, 1991).

This dissertation follows the style of Plant Cell, Tissue and Organ Culture.

More recent research on dicot embryo development uses other models: *Arabidopsis* reviewed by Jenick (2007) and Chandler (2008), or legumes such as pea, fava bean and soybean, as reviewed in Weber et al. (2005). Though these plants serve as the current models for embryo development, there are differences between embryo development in legumes, *Arabidopsis*, and cotton.

The cotton embryo receives its nutrition from the same seed coat tissue that nourishes the endosperm and developing fiber (Ruan et al., 2003; Ruan et al., 1997), yet the complex relationship between these tissues which compete for the same nutrients has been little studied.

In very young embryos, 15 DPA or less, much of the gene expression data that is available refers to fertilized ovules. In cotton research, the term 'ovule' refers to the entire embryonic structure including embryo, endosperm and seed coat (Beasley, 1971; Brar and Sandhu, 1984; Eid et al., 1973; Joshi and Johri, 1972). In vitro studies are important tools for studying embryos in isolation from the endosperm and fiber and when it is necessary to monitor the developing embryo continuously during the developmental process.

In this study, I have focused on in vitro responses of excised cotton embryos in late heart-stage and early cotyledonary stage (1.5 mm to 4.0 mm; ~14 to 18 DPA) during which cell division was still taking place, but before the synthesis of large quantities of storage proteins and oils has commenced (Fig. 1).

Embryo development terminology

In many plants such as *Capsella* and *Arabidopsis* embryo development has traditionally been divided into ten stages: zygote, 2-cell, 4-cell, 8-cell, 16-cell, globular, heart-shaped, torpedo-shaped, walking-stick, maturation and desiccation (Goldberg et al., 1994). This staging system is difficult to apply to cotton. Cotton embryo development requires a long time, about 50 to 56 days from anthesis to desiccation during which time the cotyledons develop into very large convoluted leaf-like organs (Hughes and Galau, 1991; Ihle and Dure, 1972). Various terms, some of them predating Goldberg, have been used to describe cotton embryo development between heart-stage and maturation. Some researchers have used the term 'torpedo stage' (Pollock and Jensen, 1964; Reeves and Beasley, 1935) others have used 'dicotyledonous' (Pundir, 1972) and still others have used the terms 'early cotyledonary' and 'late cotyledonary' (Galau and Hughes, 1987; Galau et al., 1987; Hughes and Galau, 1991). Where possible in this work, I will use the terms heart-shaped to describe 1 to 2 mm embryos (~13-15 DPA), and early-cotyledonary to describe 2 mm to 5 mm (~14-20 DPA) development before storage products begin to accumulate and late-cotyledonary to describe development in 6 mm to 9+ mm embryos (~19-25 DPA) in which cell division is still taking place but storage products have also begun to accumulate.

Some researchers have used the term 'days after fertilization,' which correlates to about 36 hours after pollination, to describe embryo age (Reeves and Beasley, 1935), while others have used DPA, days post anthesis, when the flower opens and the pollen is

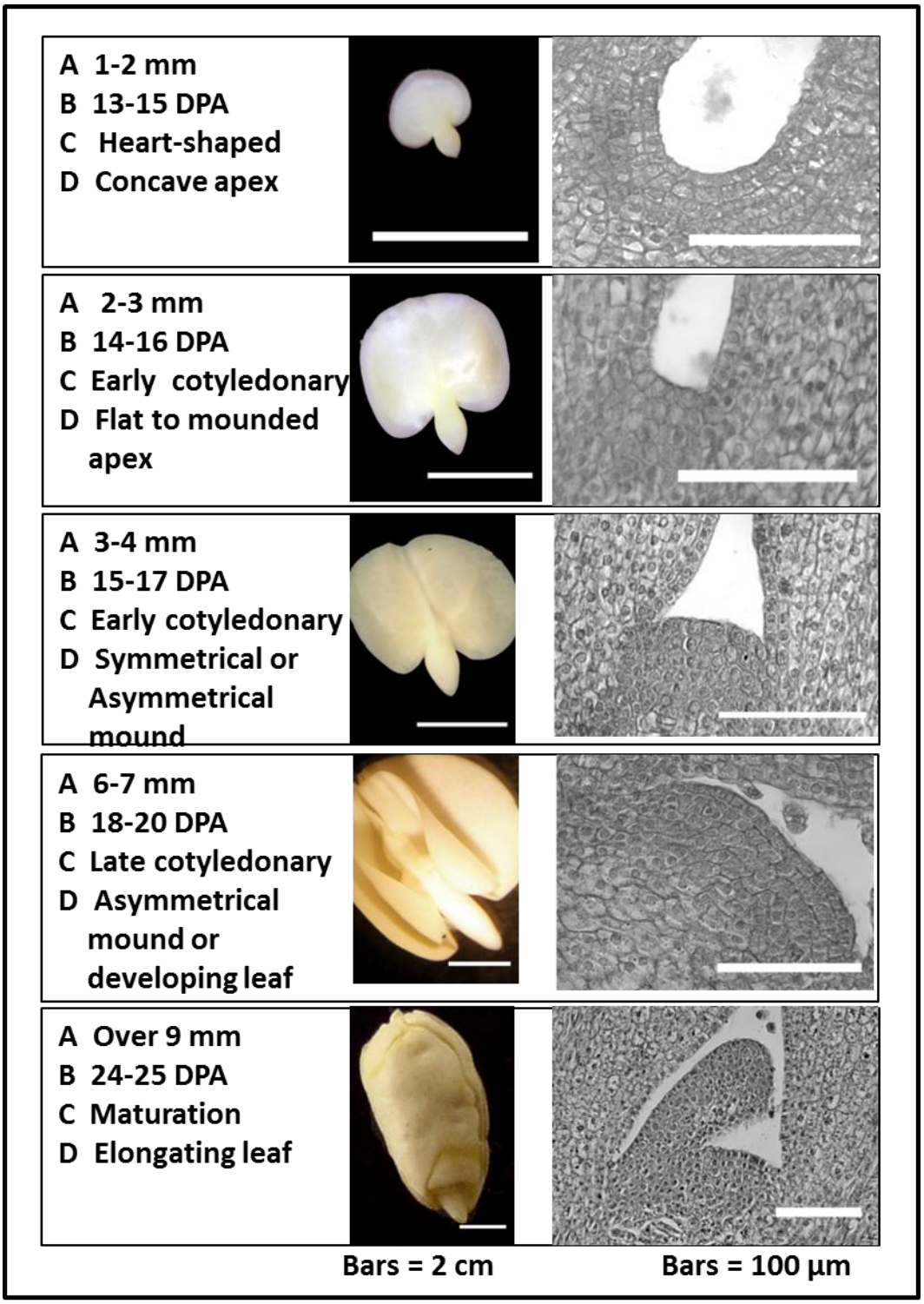

Fig. 1 Developmental staging of immature embryos from heart-shaped to maturation. (A) size-classes, (B) approximate DPA, (C) developmental stage, and (D) shoot apical morphology are shown. Bars (center) = 2 cm; bars (right) = 100µm.

released. Any mention of 'days after fertilization' will be followed by my estimate in DPA.

The criteria used for embryo developmental staging has also been inconsistent. Reeves and Beasley (1935) used three measures, days after fertilization, weight and length to characterize embryo development, whereas Galau et al. used DPA and weight (Galau et al., 1986; Galau and Hughes, 1987; Galau et al., 1987). Ihle and Dure used weight (Ihle and Dure, 1972). Pollock and Jensen (1964) estimated maturity by counting and mapping cells in the process of division while others only used DPA to estimate the maturity of the cotton embryo (Bi et al., 1999; Borole et al., 2000; Brar and Sandhu, 1984; Dhumale et al., 1996; Gill and Bajaj, 1984; Girhotra et al., 1999; Kalamani, 1996).

DPA is the easiest of the methods to use when staging development. Staging by DPA alone works fairly well during the summer months under field conditions. However, it does not identify the true developmental stage of an embryo. Embryos in differing stages of development occur within a single immature ovary on any given day of development. Summer field conditions are also difficult to replicate from one site to another and from one year to another.

Although weight is an extremely important growth parameter, the process of weighing tissue is extremely labor-intensive if performed on living in vitro tissue. Weighing must be done outside the laminar flow hood because the vibrations from the hood blower can affect the accuracy of the measurement. The sterile container must be weighed before and after the tissue has been transferred and multiple sterile transfers are required. On the other hand, embryo length can be measured from photographs and the

embryos need not be moved from container to container. Photography is nondestructive and subsequent development can be monitored. In this study I will use embryo length from top of cotyledon to tip of radicle as criteria for staging and also provide DPA (Fig.1).

Researchers have also used the terms 'provascular' (Reeves and Beasley, 1935) and 'procambium' (Pollock and Jensen, 1964) to describe the living, deeply staining cells in the embryo that will eventually give rise to cambium, xylem and phloem cells in the seedling. In this study I will use the more general term 'provascular.'

Two other development terms, 'leaf primordium,' and 'leaf initials' have also been used in a confusing manner. In this study I will use the terms 'initial' to denote the one or two cells that will divide to form a new tissue or organ, 'primordium' to designate the mound where a leaf or cotyledon is beginning to develop and 'elongating leaf' or 'elongating cotyledon' to designate a leaf or cotyledon that has grown taller than it is wide.

The American Heritage Dictionary (AHD, 2000) defines the term 'ovule' in botany as "a small body in seed-bearing plants that consists of the integument(s), nucellus, and embryo sac (containing the egg cell) and develops into the seed after fertilization." Cotton researchers use the term ovule in a very broad manner (Brar and Sandhu, 1984; Hendrix, 1990; Joshi and Pundir, 1966; Joshi and Johri, 1972; Sacks, 2008; Stewart and Hsu, 1977; Stewart, 1979) to encompass that developing embryo, integuments and endosperm as well as the unfertilized structure. In this study I will use the qualifiers 'unfertilized' and 'fertilized' with the word ovule to distinguish between

ovules before fertilization and the more mature stage after fertilization. I will use the term 'motes' to describe aborted ovules whether fertilized or unfertilized.

Embryo development in cotton

Although cotton follows the general dicotyledonous developmental pattern, to date the development in cotton has not yet been dissected into all the stages described for *Capsella* and *Arabidopsis* (Goldberg et al., 1994). In cotton embryo development, 1) zygote to early heart-stage (approximately 2 to 14 DPA), and 2) late cotyledonary, maturation and desiccation stages (20 through 56 DPA), are the most thoroughly investigated. Less is known of embryo development during the period from about 14 to 20 DPA which includes late heart-stage and young-cotyledonary stage of development. For a review of developmental stages see Turley and Chapman, (2010),

Hodnett, (2006), Mauney (1961), Pollock and Jensen (1964), Pundir (1972) and Reeves and Beasley (1935) have all documented aspects of cotton embryo development from zygote through globular stages. Researchers have variously reported the first cell division at two to five DPA (Hodnett, 2006; Pollock and Jensen, 1964; Pundir, 1972). After the first cell division, the cotton embryo, as measured by the area of a longitudinal section remained smaller than the zygote until it reached about 75 cells around 9 DPA (Pollock and Jensen, 1964). Between 9 DPA and 15 DPA the cell number increased to about 1000. Pollock and Jensen found that cell division occurred throughout the embryo in very young globular embryos but by the late globular stage cell division began to concentrate in the upper regions of the embryo, forming the primordia of the cotyledons (Pollock and Jensen, 1964).

Researchers have reported that the transition from globular to heart-shaped embryo and from heart-shaped embryo to cotyledonary embryo occurred at different times. These differences have been attributed to the use of different cotton varieties and/or different growing environments (Mauney, 1961; Mauney et al., 1967; Pollock and Jensen, 1964; Pundir, 1972; Reeves and Beasley, 1935; Turley and Chapman, 2010). Reeves and Beasley reported that embryos took on a heart-shaped appearance around six to nine days after fertilization (7-12 DPA); (Reeves and Beasley, 1935). Pundir reported that embryos were in late heart-stage at 14 DPA (Pundir, 1972). Two of Mauney's papers (Mauney, 1961; Mauney et al., 1967) reported apparently contradictory ages for heart-shaped embryos: in 1961 Mauney reported that the transition from 'proembryo' to heart-stage occurred about one week after fertilization (8-9 DPA) when the embryos were about 0.2 mm, but in 1967 he reported that heart-stage embryos were 0.2 to 0.3 mm (12 to 14 DPA). Pollock and Jensen observed embryos at young heart-stage at about 12 DPA (Pollock and Jensen, 1964).

Even less information is available about cotton embryo development in the transition from heart-shaped to cotyledonary stages. Pundir reported that *G. hirsutum* reached young cotyledonary stage around 17 to 18 DPA (Pundir, 1972). Reeves and Beasley (1935) reported the transition to cotyledonary stage 15 to 16 days after fertilization (16 to 18 DPA). Pollock and Jensen did not report an age or a size but did report that, the frequency of cell division in the apical region between the cotyledon primordia increased in torpedo (early cotyledonary) stage embryos compared to heart-stage embryos (Pollock and Jensen, 1964).

Development from 20 to 56 DPA has been well studied. Using RNA dot blots, the Galau laboratory (Dure et al., 1981; Galau et al., 1986; Galau et al., 1987; Hughes and Galau, 1989; Hughes and Galau, 1991; Hughes et al., 1993; Ritter et al., 1993) studied gene expression in whole embryos from 22 DPA which they defined as cotyledonary stage to 56 DPA which they defined as a mature embryo. Ihle and Dure (1969, 1972) studied RNA and DNA synthesis in embryonic 35 mg to 125 mg cotyledons (about 25 DPA to 50 DPA) using ^{32}P uptake. They found that DNA synthesis and cell division continued in developing embryos up to 32 DPA when the finiculus (funiculus) deteriorated (Ihle and Dure, 1969; Ihle and Dure, 1972).

Shoot apical organization in angiosperms

Schmidt (1924) reported that in angiosperms the SAM was organized into two regions: the tunica, comprised of one or more cell layers overlying the corpus. The number of tunica layers varies by species. In *Arabidopsis*, the tunica consists of two layers of cells while maize has a single sheet-like layer (Barton, 2010), and in *Coleus* the tunica consists of four layers of cells (Smith and Murashige, 1982).

Santina et al., reported that the SAMs of *Datura stramonium* consisted of three layers, the L1, the L2 and the L3, which contributed to different tissues and organs in the plant (Santina and Blakeslee, 1941; Santina et al., 1940).

Shoot apical development in *Arabidopsis*

Arabidopsis shoot apices have been extensively studied both during embryogenesis and afterward. The *Arabidopsis* SAM is derived from a group of cells in the upper two layers (epidermal and hypodermal) of the globular stage embryo. During the development from the late globular to the torpedo stage, the cells in the hypodermal layer divide again so that the upper region of the embryo becomes stratified into three layers. The upper two layers of cells, the L1 and L2 divide at right angles to the surface and will become the tunica in the germinated seed. The lowermost layer of cells, the L3, divides in all directions and will become the corpus (Barton and Poethig, 1993). The *Arabidopsis* SAM develops from these three layers of cells in the torpedo stage embryo (Barton and Poethig, 1993). In dicotyledonous embryos such as cotton and *Arabidopsis*, the SAM forms between the two cotyledons. Depending on the species, differing numbers of leaf primordia develop before the seed matures and dries (Barton, 2010). For example, *Arabidopsis* mature embryos have two very small leaf initials while peanut embryos have nine (Conway and Poethig, 1997; Yarbrough, 1949).

The first histological signs of leaf initiation occur when, instead of dividing at right angles (anticlinally) to the meristem surface, a small number of cells in the L2 layer divide parallel to the surface (periclinally), causing the formation of a "bump" or leaf primordium. Cells from the L1 and L3 layers surrounding this initiation point participate in the formation of the primordium; so the leaf consists of cells from all three meristem layers (Poethig, 1987).

Shoot apical development in cotton embryos

Compared to *Arabidopsis*, little has been studied in the shoot apex development in cotton embryos. Some early researchers have depicted developing apices in their *camera lucida* images. For example, Reeves and Beasley (1935) drew a plumule (shoot apex) of the cotton embryo 9 to 12 days after fertilization (10 to 14 DPA) and depicted a mound in the apical region 15 days after fertilization (16 to 17 DPA). Pundir's *camera lucida* images of 15 and 16 DPA embryos clearly lack apical mounds and his images of 35 DPA embryos have developed apical mounds (Pundir, 1972). Pollock and Jensen show two photographs of torpedo stage embryos that still have concave apices. Their study terminates at torpedo stage (Pollock and Jensen, 1964).

Christianson (1986) and Hodnett (2006) have reported that in *G. barbadense*, at least two leaves are initiated during embryogenesis. Both Christenson and Hodnett studied early embryo development in *G. barbadense* lines harbouring the of the *Semigamy* (*Se*) mutant (Christianson, 1986; Hodnett, 2006). This mutant was first reported by Turcotte and Feaster (1963) and mutant lines have been extensively used for sectorial analysis. However, as late as 2011 the sequence has not been published (Curtiss et al., 2011). Christianson proposed that initials of the first leaf developed from two cells and the second leaf developed from a single cell during the late globular stage (Christianson, 1986). Hodnett (2006) also relied on sectoral analysis of *Se* induced chimerism in his study of embryo development during the first 5 days after anthesis (Hodnett, 2006). Although his results differed somewhat from Christianson, Hodnett (2006) also found that the second leaf had a different pattern of chimeric sectors than

later leaves, presumably because the second leaf began to develop much earlier than the third and fourth leaves.

Mutations of the embryonic shoot apex in other systems

Research into shoot apical development has been conducted in *Arabidopsis* and a few model species, tomato (Keddie et al., 1998; Reinhardt et al., 2000; Schmitz et al., 2002), petunia (Stuurman et al., 2002), snapdragon (Waites et al., 1998), rice (Kurakawa et al., 2007), and maize (Brooks et al., 2009; Nogueira et al., 2009; Ohtsu et al., 2007). Tomato meristemetic mutations include *defective embryo and meristems (dem)* (Keddie et al., 1998) and the *blind (bl)* mutation which affects the development of lateral meristems (Schmitz et al., 2002).

Most research in apical meristem development has taken place in *Arabidopsis*. By use of mutants, laser micro dissection, microarrays and fluorescent cell sorting, apical development in *Arabidopsis* has been dissected almost to a cell-by-cell day-by-day level (Barton, 2010; Spencer et al., 2007; Yadav et al., 2009) Some of the better studied regulatory genes that affect *Arabidopsis* embryo apical development during heart-shaped stage through maturation are: *AINTEGUMENTA (ANT),* (Elliott et al., 1996); *CUP SHAPED COTYLEDON1* (*CUC),* (Takada et al., 2001); *WUSCHEL* (*WUS)*, (Laux et al., 1996; Mayer et al., 1998); *SHOOT MERISTEMLESS* (*STM)*, (Barton and Poethig, 1993); *ALTERED MERISTEM PROGRAM1* (*AMP1)*, (Helliwell et al., 2001); *CLAVATA (CLV3)*, (Fletcher et al., 1999); and *MONOPTEROS (MP),* (Hardtke and Berleth, 1998). For a more complete review of genes involved in meristem origination and maintenance see Barton (2010).

Apical meristem development in tissue culture systems

SAMS arise by other pathways besides zygotic embryogenesis. They can initialize in the axils of leaves as lateral meristems, they can develop adventitiously as when one cotyledon and the apical meristem of a seedling such as melon is removed (Amutha et al., 2009) or they can develop in somatic embryos. In somatic embryogenesis systems the embryos frequently fail to produce SAMs. This common problem has been reported in many species, including cotton (Hussain et al., 2009), sweet potato (Padmanabhan et al., 1998), *Tilia* (Kärkönen, 2000) and citrus (Tomaz et al., 2001).

Cotton embryo culture media

While it has been recognized that very young embryos have differing nutritional and/or hormonal requirements for growth and development than older embryos, data have not been available to correlate developmental processes such as apical meristem development with different nutritional, cultural or hormonal requirements. It has also been recognized that cotton embryos develop at different rates *in planta* under greenhouse and/or field conditions at different temperatures and at different times of the year (Beasley, 1974). Most embryo rescue studies have been made to allow excised immature embryos from wide crosses to develop into mature plants and most have utilized field-grown cotton developing under optimal light and temperature conditions. However DPA alone was used to classify development without reference to size or other developmental measure (Bi et al., 1999; Borole et al., 2000; Brar and Sandhu, 1984; Dhumale et al., 1996; Gill and Bajaj, 1984; Girhotra et al., 1999; Kalamani, 1996). I

have found that in greenhouse-grown plants, DPA and developmental stage of an embryo have often not been well correlated even in ideal summer conditions.

Early attempts to excise and culture 10 to 15 DPA embryos often failed because the nutrient culture media available were inadequate to sustain normal embryo growth and development (Beasley, 1942; Eid et al., 1973; Mauney, 1961). Liang found that increasing the pH of White's (White, 1934) culture medium to 7.0 improved the health of root systems and led to the recovery of more plants (Liang et al., 1978). Most later and more successful studies have used MS (Murashige and Skoog, 1962) which was developed for tobacco cultures or two modifications of MS, BT (Beasley and Ting, 1973; Beasley and Ting, 1974) or SH (Stewart and Hsu, 1977) for fertilized and unfertilized ovule and embryo culture. Beasley and Ting developed their media for fiber growth in fertilized (1 DPA) and unfertilized (0 DPA) ovules that had been removed from the boll. They modified their liquid media (BT) from the MS formulation by substituting 50 mM of KNO_3 for 41.2 mM NH_4NO_3 plus 18 mM of KNO_3, reducing the chelated iron concentration to 0.03 mM from 0.10 mM and substituting glucose for sucrose (Beasley and Ting, 1973; Beasley and Ting, 1974). Stewart and Hsu modified BT to develop a liquid medium, (SH) for the culture of embryos in fertilized ovules that had been removed from the boll. Stewart and Hsu found that when they substituted 15 mM NH_4NO_3 and 35 mM KNO_3 for the BT formulation (50 mM KNO_3) and when 40 gl^{-1} sucrose was used instead of glucose, DPA cotton embryos developed more rapidly than when cultured in BT (Stewart and Hsu, 1978). More recently, Sacks found that increasing KNO_3 concentration in MS (Murashige and Skoog, 1962) salts + B5 vitamins

(Gamborg et al., 1968) from 1.9 gl^{-1} to 3.8 gl^{-1} increased the frequency of germination in fertilized ovule cultures (Sacks, 2008).

None of these media were developed specifically for the in vitro culture of immature embryos that had been excised from the boll. For this reason I had to test each formulation for the one best suited to the recovery of plants from immature embryos. For a comparison of nutrient formulation of MS, BT and SH media, please see Appendix C.

The effect of light on cultured immature zygotic cotton embryos

While most researchers have cultured cotton embryos and fertilized ovules in the dark (Dhumale et al., 1996; Gill and Bajaj, 1987; Gill and Bajaj, 1984; Kalamani, 1996), others have cultured embryos and/or fertilized ovules in light (Mauney, 1961; Sacks, 2008; Umbeck and Stewart, 1985). The period of darkness has varied from three days for interspecific *Gossypium* hybrid fertilized ovules and embryos (Kalamani, 1996), 60-70 days for *G. hirsutum* fertilized ovule cultures (Eid et al., 1973) to 90 days of darkness for interspecific *Gossypium* hybrid embryos (Dhumale et al., 1996). On the other hand, Sacks cultured *G. hirsutum* X *G. arboreum* ovules under a 12 hr. fluorescent light regime (Sacks, 2008), and Mauney cultured *G. hirsutum* embryos in continuous light (Mauney, 1961; Mauney et al., 1967). Gill and Bajaj(1984) reported successful plant formation increased when excised immature *G. arboreum* embryos were cultured in dark for 15 days prior to transfer to light formation, and that *G. herbaceum* plant formation improved when excised immature embryos were cultured in the dark. Girhotra also found that culturing embryos in the dark for fifteen days before to transfer to light

improved plant formation (Girhotra et al., 1999). None of these studies addressed the development of shoot apices during culture.

Light in other tissue culture systems

Apical response to light quality and intensity appears to be species and even variety specific. Michler and Lineberger found that blue light at 27 µmol m-2s-1 reduced the number of somatic embryos and fresh weight in carrot suspension cultures relative to red light or darkness (Michler and Lineberger, 1987). A number of studies have associated far-red and blue light with lowered shoot production, while red and white light promoted shoot production. Burrit and Leung found that Begonia × erythrophylla petioles cultured under far-red, blue light or in the dark developed a reduced number of shoots per explant compared to petioles cultured under red or white light (Burritt and Leung, 2003). Hunter and Burrit found that in excised lettuce cotyledon explants, blue light inhibited shoot production while red light either promoted production or had no effect on the number of shoots, depending on variety. Treatment with blue plus red light also inhibited shoot development (Hunter and Burritt, 2003). Kadkade and Jopson tested growth and adventitious bud formation in Douglas-fir (Pseudotsuga menziesii) somatic embryos under narrow bandwidth lighting from 371 to 740 nm and found that red light increased adventitious bud formation compared to non-irradiated controls but blue and near UV had no significant effect (Kadkade and Jopson, 1978). Reuveni and Evanor (2007) tested the effects of varying periods of light or darkness on the ability of leaf explants to develop shoots in two petunia species, *P. hybrida and P. axillaris*. They found that in one species the ability to develop shoots

was completely extinguished after prolonged periods of darkness while in the other species it was not. By testing the progeny of an interspecific cross they found that two genetic loci controlled the ability of shoots to regenerate in the light while one locus controlled the ability of shoots to regenerate in the dark.

Recent advances in light emitting diode (LED) technology have led to a renewed interest in the effects of light in tissue culture systems. Li et al. tested the effect of red and blue LEDs on the in vitro development of cotton seedlings of Sumian 22 on biomass, chlorophyll content, root activity, leaf and stomatal anatomy but did not investigate the effect on shoot apical development (Li et al., 2010). Merkle et al. (2006) found that embryogenic cultures of loblolly pine (*Pinus taeda* L.), slash pine (*Pinus elliottii* Engelm.), longleaf pine (*Pinus palustris* Mill.) as well as a slash pine × longleaf pine hybrid went through a light-sensitive pre-germination stage at the point that the embryos had produced apical domes and cotyledon initials. During the pre-germination and radicle emergence stages they used either cool white or colored LEDS or both and found that generally embryos treated with red wavelengths resulted in higher frequencies of somatic embryo germination than the standard cool white fluorescent treatments or treatment with blue wavelengths. Germination and conversion to whole plants were further enhanced by sequential application of cool white fluorescent light and red light (Merkle et al., 2006).

Plant materials

Unless otherwise specified, all experiments were performed using *Gossypium hirsutum* cv Texas Marker-1, (TM-1) an inbred variety developed for use as a standard

reference cotton for genetic testing (Kohel et al., 1970). TM-1 has been used as the backcross parent for many aneuploid (lines with abnormal numbers of chromosomes) and recombinant inbred lines (lines produced from crosses of inbred parents by selfing and single seed decent), and extensive mapping and expression data have been published on it (Karaca et al., 2002; Kohel et al., 2002; Lee et al., 2006; Yang et al., 2006; Zhang et al., 2003). All experiments unless noted otherwise were from the same batch of seed.

CHAPTER II

EMBRYONIC SHOOT APEX DEVELOPMENT IN VIVO

Introduction

In this study, I focused on cotton embryos at the end of the heart-stage and during early cotyledonary stage (1.5 mm to 4.0 mm) while cell division is still taking place, but before embryos have begun to synthesize of large quantities of storage proteins and oils. However, in order to study in vitro development I needed to also observe the morphological changes that occurred in the embryo in vivo. Unlike *Arabidopsis*, there has been little recent study of cotton embryo morphology. The morphological studies that do exist often were made before the advent of digital photography (Joshi and Pundir, 1966; Mauney, 1961; Pollock and Jensen, 1964; Pundir, 1972; 1935). The early studies that have been made skipped over the early cotyledonary stage (Pundir, 1972) or concentrated on very early events in embryo development (Hodnett, 2006; Hodnett et al., 1997; Pollock and Jensen, 1964).

Terminology used to describe cotton embryo development is inconsistent and confusing (Chapter I). For the purpose of this study, I will describe embryo development by DPA and size-class based on embryo length from top of cotyledon to tip of radicle. Where possible, I will also indicate DPA and size-class in parentheses when reporting on the findings of other authors.

Reeves and Beasley (1935) reported the first appearance of plumules (embryonic shoot apices) of cotton embryos 9 to 12 days after fertilization (10 to 14 DPA) and depicted a mounded apex 15 days after fertilization (16 to 17 DPA). Pollock and Jensen

(1964) reported that during the heart-stage, cell division was infrequent in the apical region (Pollock and Jensen, 1964). During the torpedo stage, Pollock and Jensen observed that cell division resumed in the apical region (Pollock and Jensen, 1964). Although Pundir (1972) did not comment on the apical morphology, his images of 15 and 16 DPA embryos clearly lack mounding in the apical region and his images of 35 DPA embryos have developed apical mounds. Unfortunately, Pundir did not provide images of embryos between 16 and 35 DPA.

Materials and methods

G. hirsutum cv TM-1 cotton was grown in the greenhouse, allowed to self-pollinate and tagged on the day of anthesis. TM-1 self-pollinates efficiently in greenhouse conditions. Immature bolls from 1.3 to 9.6 mm (13 to 25 DPA) were harvested between 5/21/11 and 6/6/11 (Fig.2). Immediately after harvest the embryos were dissected from the bolls and fixed in modified FAA (5% formalin, 2.5% glacial acetic acid, 28.5% ethanol). In microscopy usage the word fix means to kill and preserve cells and their contents (Ruzin, 1999 pg. 33). Three embryos from the mid-region of each boll were set aside, photographed and measured.

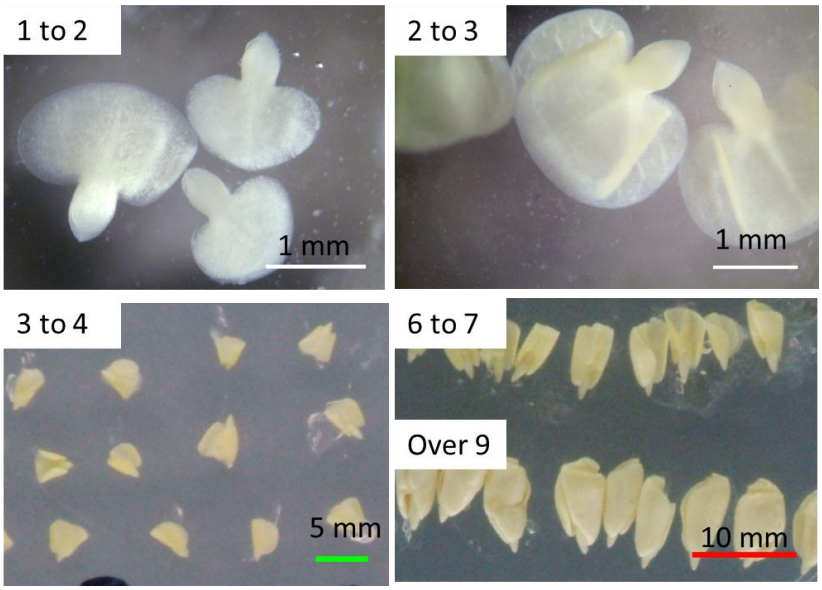

Fig. 2 Embryos by size-class. 1-2 mm (13 to14 DPA); 2-3 mm (14 to15 DPA); 3-4 mm (16 to17 DPA); 6-7 mm (19 to20 DPA); and over 9 mm 24 to 25 DPA.

At least 30 embryos (5 embryos per boll, 6 bolls) were sampled from each size-class (Fig. 2). The embryos were dehydrated in an ethanol tert-butanol series, (Stelly, personal communication), embedded in Paraplast Plus® and sectioned into 10 µm sections. After some experimentation it was found that the best sections resulted if embryos were embedded so that the knife blade was parallel to the long axis of the embryo. Longitudinal sections (Fig. 3) both perpendicular and parallel to the cotyledons were examined. The provascular strand had to be visible in both cotyledons for a section to be considered perpendicular. Both edges of the cotyledon had to be visible for a section to be considered parallel. Embryos that were not aligned correctly were discarded. Sections from 96 embryos correctly aligned embryos were selected for staining (Table 1). They were stained with Aniline Blue and Safranin O (Ruzin, 1999, pg. 64) and examined at 30X 100X and 400X magnifications.

Table 1 Number of shoot apices examined in each size-class.

Size-class	Number examined
Less than 2 mm	12
2-3 mm	18
3-4 mm	13
6-7 mm	30
Greater than 9 mm	23
Total	96

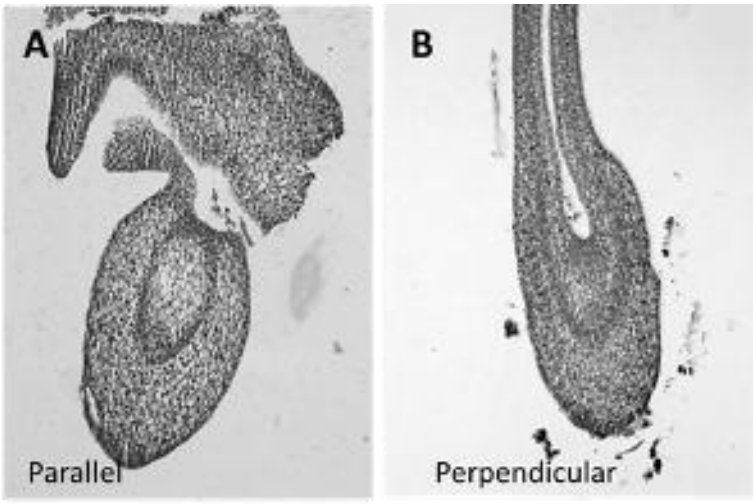

Fig. 3 Embryo sectioning. Embryos were sectioned longitudinally either (A) parallel or (B) perpendicular to the cotyledons.

Serial sections through the shoot apex were examined for general shape and for the number of visible apical layers. In order to determine shape it was necessary to examine the entire apical region of the embryo. The number of sections that were examined varied with the size of the embryo, from ~10 in 1-2 mm embryos to more than

30 in 9 mm embryos. A unique number was assigned to each embryo to avoid double counting when more than one slide was needed for the sections of one embryo.

Results

During embryo growth from 1.3 mm to 9.6 mm (13 to 25 DPA) the shoot apical region developed from a concave shape to a mounded shape (Fig. 5). The first true leaf developed from an asymmetrical bulge in 3-4 mm embryos to a tall curved structure (Fig. 4).

In the 1-2 mm size-class most of the embryo apical regions were concave or flat (Fig. 4A). Some embryos in the 2-3 mm size-class developed mounds, while others remained flat (Fig. 4B). All of the embryos in the 3-4 mm size-class had developed mounds (Fig. 4C), many asymmetrical. The asymmetrical shape was an indication that the first leaf had begun growing. Some embryos in the 6-7 mm size-class (Fig. 4D) and most embryos in the > 9 mm size-class had developed leaves (Fig. 4E), defined here as asymmetrical sickle shaped structures that were taller than they were wide. For percentages of embryos in each developmental stage please see Fig. 5.

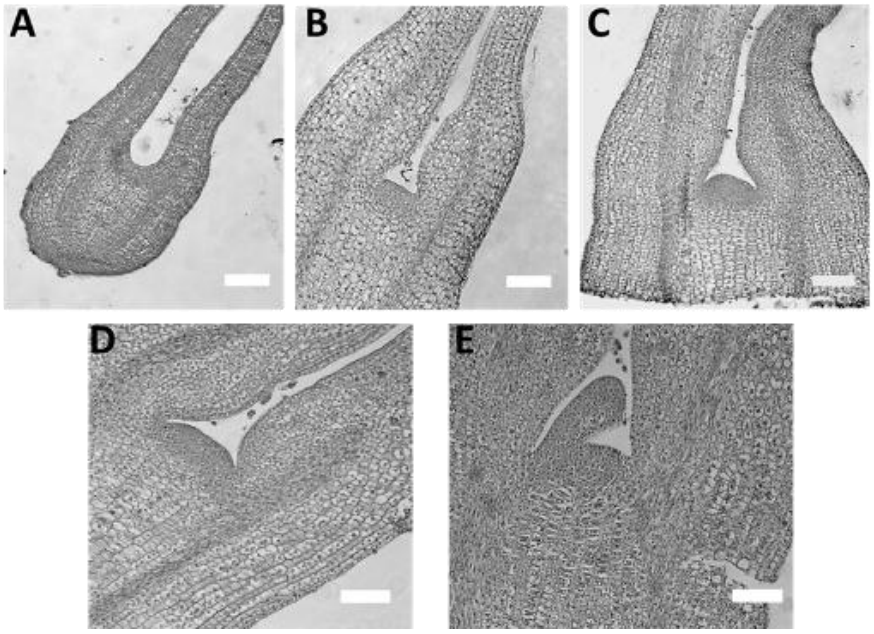

Fig. 4 Development of the embryo shoot apex during 14 to 25 DPA. (A) concave apex, 1-2 mm (14 DPA); (B) slightly mounded apex, 2-3 mm (16 DPA); (C) asymmetrical apex, 3-4 mm (17 DPA); (D) asymmetrical apex, 6-7 mm (20 DPA); (E) leaf, >9 mm, (24 DPA). Asymmetrical mounds had their highest point closer to one side of the embryo than the other; this asymmetry indicated the presence of a developing leaf primordium. Leaves were asymmetrical sickle-shaped, and were taller than they were wide. *bar =100 µM.*

Many times, longitudinal sections that first appeared to be symmetrically mounded were actually sections through the developing leaf and the actual meristem was displaced to one side (Fig. 6). By the time the embryos had reached 9 mm in length most embryos had developed a leaf. Some embryos 6 mm or larger developed a second "bump" caused by horizontal cell divisions in the L2 layer (Fig. 7C) that indicated the development of the second leaf initials (Barton, 2010). However, development of the second leaf beyond this rudimentary stage was not observed.

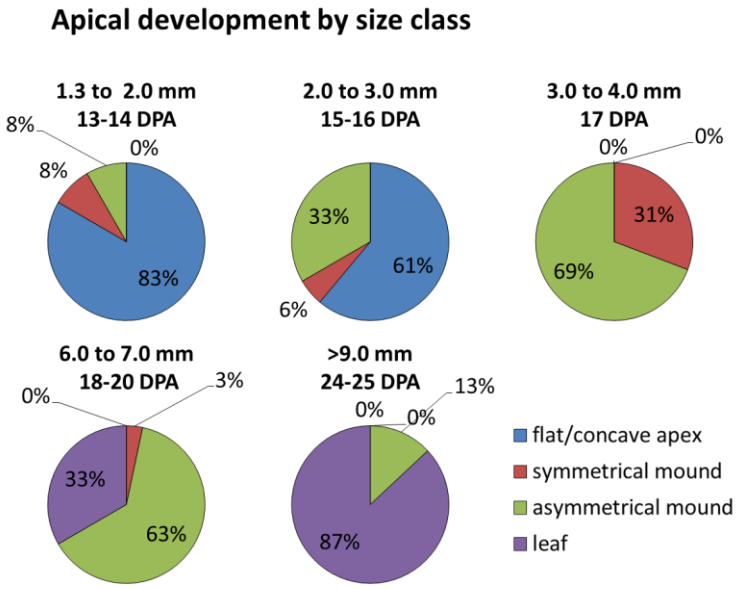

Fig. 5. Apical development by size-class. Leaves are defined here as asymmetric sickle shaped structures that are taller than they are wide.

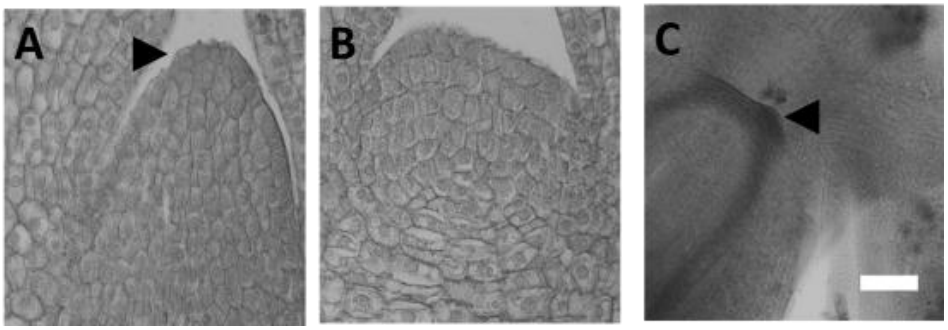

Fig. 6. Asymmetrical mounding in young embryos. (A) Apparently symmetrical mound lacks typical corpus tunica structure and is actually a developing leaf. (B) Another section of the same 6-7 mm (20 DPA) embryo showing the tunica and corpus morphology. Embryo has been sectioned perpendicular to the cotyledons. (C) 3-4 mm (18 DPA) embryo whole mount stained with Azure C and cleared with methyl salicylate. The plane of focus is parallel to the cotyledon. ◄or ► *first leaf, bar = 100 µM.*

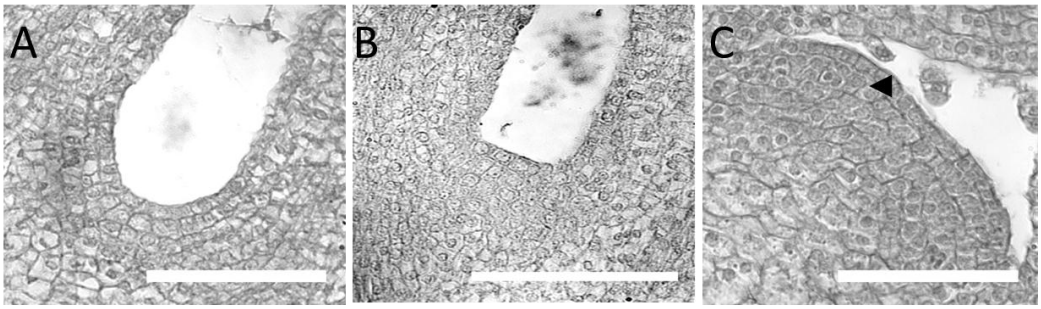

Fig. 7. Examples of layering patterns in developing apical regions. (A) 1-2 mm (14 DPA) embryo with concave apex; (B) 2-3 mm (17 DPA) embryo with flat apex; (C) 7 mm (20 DPA) embryo. All embryos show at least 3 distinct cell layers. *Bar* = 100 µM; ◄ indicates possible initiation site for second leaf.

Nearly all of the shoot apices that were examined had three distinct cell layers regardless of the size of the embryo (Fig. 7). A few appeared to have one or two or four layers (data not shown). In the embryos with concave apices the layers appeared as concentric rings, while in more mature embryos cells could be observed radiating from the corpus (Fig. 7).

Discussion

The cotton embryonic apex grows and changes shape from concave to a mounded structure during the embryo's growth from 1.5 to 4 mm. The first leaf primordium also begins to grow soon after the mounded apex becomes visible. During the embryo's development from 4 mm to 9 mm first leaf grows into a distinct structure that occupies much of the space between the cotyledons. It is not unusual for embryos of some species to develop one or more leaf primordia before the seed matures and dries down. For example, mature *Arabidopsis* embryos have two small leaf primordia and

mature peanut embryos have nine leaf primordia (Conway and Poethig, 1997; Yarbrough, 1949).

Previous research suggested that in cotton the first and second leaf initials were formed in the late globular stage of embryo development (Christianson, 1986). The data in this chapter show that the first leaf is actively growing during the early cotyledonary stage. The data suggest that the apices of 1-2 mm embryos correspond to Pollock and Jensen's (1964) description of heart-shaped embryos and the apices of 2-3 mm embryos correspond to their description of torpedo-shaped embryos.

CHAPTER III

TRUE SHOOT DEVELOPMENT IN EXCISED ZYGOTIC EMBRYOS 42 DAYS AFTER CULTURE

Introduction

The roles of light in seed germination and photomorphogenesis in seedlings have been thoroughly studied. For a review please see Chen et al. (2004) and Neff et al. (2000). Germination is the group of processes in seeds that begins with imbibition, water uptake by the seed and ends with the elongation of the axis (Bewley, 1997). Photomorphogenesis is the developmental process in seedlings that includes anthocyanin and chlorophyll synthesis, cotyledon expansion, cessation of hypocotyl elongation, activation of the SAM, and development of true leaves (Chory et al., 1991). Important roles for light in immature developing embryos of *Arabidopsis* (Cairns et al., 2006) and soybean (Ruuska et al., 2004) have been established.

However, the role, if any, of light in normal cotton embryo development has not been determined, nor has it been determined if the cotton embryo in the developing ovary (boll) is actually exposed to light. Embryos begin to turn green very soon after the initial cell divisions. This suggests that they are exposed to light. A single study (Kasperbauer, 2000) suggested that light, depleted in red and blue wavelengths, did penetrate immature cotton ovary walls. Kasperbauer did not report the size of the ovaries, but they must have been larger than the one cm diameter sensor window he used (Kasperbauer, 2000).

The roles that light may play in the development of excised embryos in culture have not been systematically studied. Some researchers have cultured fertilized cotton ovules and developing seed in the dark (Dhumale et al., 1996; Gill and Bajaj, 1987; Gill and Bajaj, 1984; Kalamani, 1996), others have cultured embryos and/or fertilized ovules in light (Mauney, 1961; Sacks, 2008; Umbeck and Stewart, 1985). The periods reported for the dark treatment varied from three days, for interspecific *Gossypium* hybrid fertilized ovules and embryos (Kalamani, 1996), 60-70 days for *G. hirsutum* fertilized ovule cultures (Eid et al., 1973), to 90 days of darkness for interspecific *Gossypium* hybrid embryos (Dhumale et al., 1996). Sacks (2008) cultured *G. hirsutum* X *G. arboreum* ovules under a 12 hour day length, and Mauney cultured *G. hirsutum* embryos in continuous light (Mauney, 1961; Mauney et al., 1967).

Because of the conflicting results described above it was necessary to test for the optimum period of darkness. I incubated embryos in the dark for 0, 3, 6, and 18 days but found that the optimum treatment was to incubate the embryos in the light. I verified the results in three large replicated experiments over three growing seasons.

Materials and methods

A single variety of cotton, *G. hirsutum* cv Texas Marker-1 (TM-1) was used for all of the experiments in this study. Cotton flowers were tagged on the day of anthesis and the young bolls were harvested at 13 to 29 DPA. Bolls were surface sterilized in 10% to 20% bleach for 20 minutes; the embryos were then excised from the fertilized ovules and placed on Medium I (Table 2) solidified with 7.5 to 15 gl^{-1} agar (Sigma A7921). Only two solidifying agents, Sigma A7921 agar and Gibco 10675-023

Phytagar were used for all of the experiments. At 14 (2006, 2007, 2008) or 18 (2005) days after culture (DAC) embryos were transferred to Medium II (Table 2) solidified with 7.5 to 8 gl^{-1} agar. Because the roots of young cultured cotton embryos frequently were frequently unable to penetrate into the agar, a groove was cut into the medium and the radicles were 'planted' root downward in the groove. At 28 DAC the embryos were transferred to Medium III (Table 2) solidified with 6 gl^{-1} Phytagar™ (Gibco 10675-023) or 8 gl^{-1} agar (Sigma A7921). At 42 DAC the embryos were removed from medium, photographed and the number of true shoots (shoot apex with developing leaves) counted.

Table 2 Embryo culture media employed in this study

Media	Purpose	Salts	Vitamins	Sucrose	Agar Phytagar gl^{-1}
I	Initiation	BT	BT	30 gl^{-1}	Agar (Sigma A7921) 7.5-15
II	Germination	BT	BT	30 gl^{-1}	Agar (Sigma A7921) 7.5-8
III	Rooting	½ MS	½ MS	15 gl^{-1}	Phytagar™ 6 Sigma A7921agar 8

Shoot development in response to increasing periods of darkness

In the first experiment, embryos (1.5 to 4 mm, 20 to 29 DPA) were cultured on Medium I in darkness for 3, 9 or 18 DAC or in light with a photoperiod of 8 hours dark and 16 hours light. Temperatures also fluctuated from about 19°C in the dark to 25°C in the light. Light-treated and dark-treated embryos were kept at the same temperature by placing the dark-treated embryos in a light-proof box beside the light-treated embryos Appendix A. Embryos cultured in the light and those first cultured in the dark for 3 or 9 days were transferred to low intensity light conditions (PPF ~14 μmol $m^{-2}s^{-1}$). At 18 DAC all embryos were transferred to a higher intensity light (PPF ~100 μmol $m^{-2}s^{-1}$) and recultured on Medium II (Table 2). At 28 DAC the embryos were transferred to Medium III (Table 2) and at 42 DAC they were photographed and the presence or absence of true shoots was recorded.

Table 3. Number of embryos per treatment in increasing periods of darkness

Size (mm)	Light-cultured 0 days	days of darkness 3 days	9 days	18 days
1.5 -2	22	20	19	23
2-3	42	55	49	55
3-4	24	23	21	29
Total	**88**	**98**	**89**	**107**

Three additional experiments were undertaken over successive years. Cotton flowers were tagged on the day of anthesis. At day 13 to 18 DPA the young bolls were harvested, surface sterilized, the developing embryos were excised from the ovules and placed on Medium I (Table 2) solidified with 8 to 15 gl^{-1} agar. Half the embryos were cultured in continuous light and half in darkness. At 14 DAC all of the embryos were transferred to Medium II (Table 2) in the light for germination. At 28 DAC the embryos were transferred to Medium III in the light and at 42 days they were photographed and the numbers of true shoots counted.

Because of the multiyear nature of these experiments, there were differences between the treatments from year to year. The first experiment outlined in this chaper (Table 3) was performed in the culture room under 16 hr. light 8 hr. dark photoperiod.

Table 4 Number of embryos light-treated or dark-treated by size-class and year

Size-class	light	dark
2006		
1.5 to 2	34	24
2-3	39	63
3-4	29	41
Total 2006	**102**	**128**
2007		
1.5 to 2.2	253	251
2. 2-3	277	277
3-4	191	213
Total 2007	**721**	**741**
2008		
1.5 to 2.2	148	136
2. 2-3	159	131
3-4	69	63
Total 2008	**376**	**330**

However, culture room temperatures also fluctuated from 26° C in the light to 19 C ° in the dark. Studies on the development of cotton fiber had established that nighttime temperatures of 22° C or less interfered with the development of cotton fiber (Haigler et. al. 1991). My own experience has since shown that in the greenhouse temperatures below 20° could delay embryo maturation for several days and lead to deformities in the flowers and ovaries (data not shown). All subsequent experiments (Table 4) in this chapter were conducted in in constant light or constant darkness at 28° to 32°C.

During the first two experiments, embryos were cultured on 2.3 mm filter papers placed over agar media. During subsequent years, filter paper was not used in order to

Table 5 Dates cultured, lighting, media changes and DPA 2005-2008

Year	Dates cultured	Fluorescent light source & photoperiod	PPF µmol m^{-2} sec^{-1}	Agar Media I (gl^{-1})	DPA
2005	11/21-12/16	Coolwhite 16 hr. light/8 hr dark	14/100	15	20 to 29
2006	7/29-8/27	Coolwhite 24 hr. light	100	15	13 to 17
2007	5/20-6/28 7/28-9/1 9/29-11/1	Coolwhite 24 hr. light	100	8-15	13 to 17
2008	3/14-4/13 4/16-5/5 5/6-6/27 8/10-9/15	Plant & aquarium 24 hr. light	60	12	14 to 18

speed up the culture process and to enable more embryos to be cultured on a single plate. The treatments were compared and no significant difference was found (data not shown). In the first year, the dark-treated embryos were placed together in a large cardboard box that was opened every two or three days in low light to add or remove plates. In subsequent years, dark-treated embryos were placed in Revco™ 5.5" x 5.5"x 2" (~14 x14 x 5 cm) paperboard freezer boxes and remained in total darkness for two weeks. In the third year the culture room facility that had been used previously was unavailable so the embryos were cultured at 28° to 32°C under GE Ecolux™ plant and aquarium fluorescent bulbs instead of Phillips Coolwhite™ fluorescent bulbs. Differences between the two illumination systems were tested and no significant difference was found (data not shown). For further details about the experimental size and conditions see Table 5.

Results

Shoot development in increasing periods of darkness (2005)

In the first experiment, embryos in the 2-3 mm and 3-4 mm size-classes developed significantly more true shoots when they were cultured in the light than when cultured in the dark for 18 days (Fig. 8). There was also a significant difference in response in all size-classes between nine days dark and 18 days dark. However, there seemed to be little difference in response between three days dark and nine days dark. Embryos in the 1.5 to 2 mm size-class responded erratically in no identifiable pattern (Fig. 8).

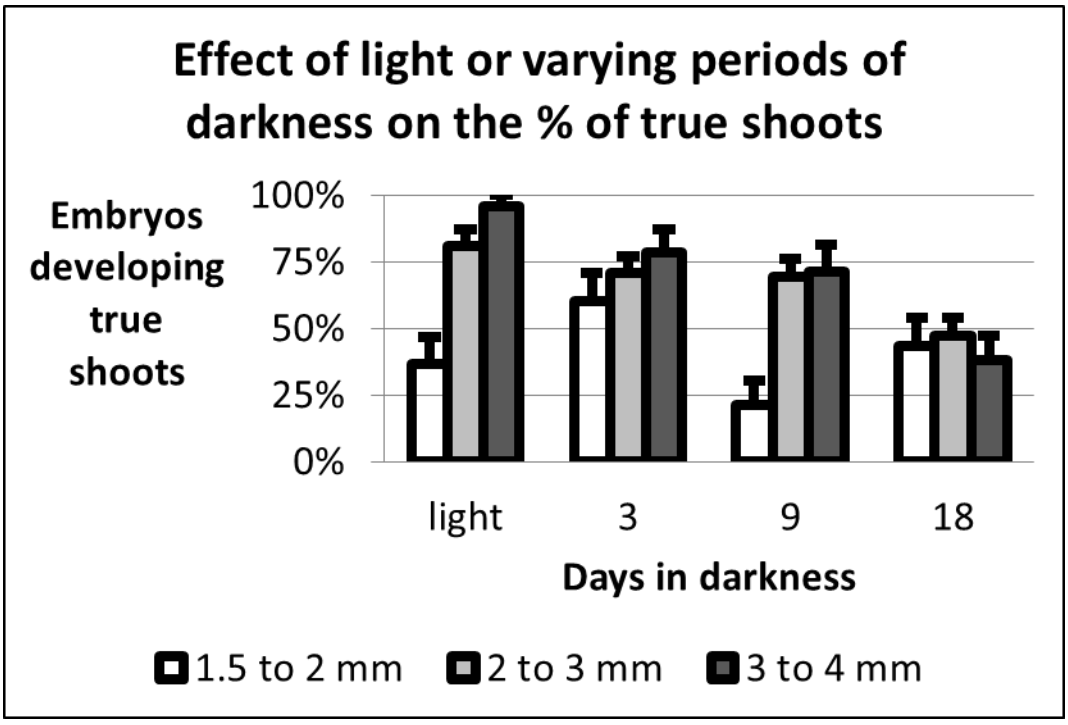

Fig. 8 Effect of light and varying periods of darkness on the % embryos developing true shoots. Embryos were cultured in low light (~14 µmol $m^{-2}s^{-1}$) or for 3, 9 or 18 days in the dark. Embryos cultured for 3 or 9 days in the dark were transferred to low light until 18 DAC. At 18 DAC all embryos were transferred to higher intensity light (100 µmol m^{-2s-1}). Error bars are standard error of the mean.

Light dark experiments (2006 to 2008)

Although there were year- to-year differences, possibly because the embryos were cultured in different times of the year (Table 5), the cumulative results of three years experimentation show that more embryos in all size-classes developed true shoots when incubated in the light for 14 days than when incubated in the dark. During all three years the percentage of embryos that developed true shoots was also significantly higher in the 3-4 mm size-class than those in the 1.5- 2.2 mm size-class (Fig. 9).

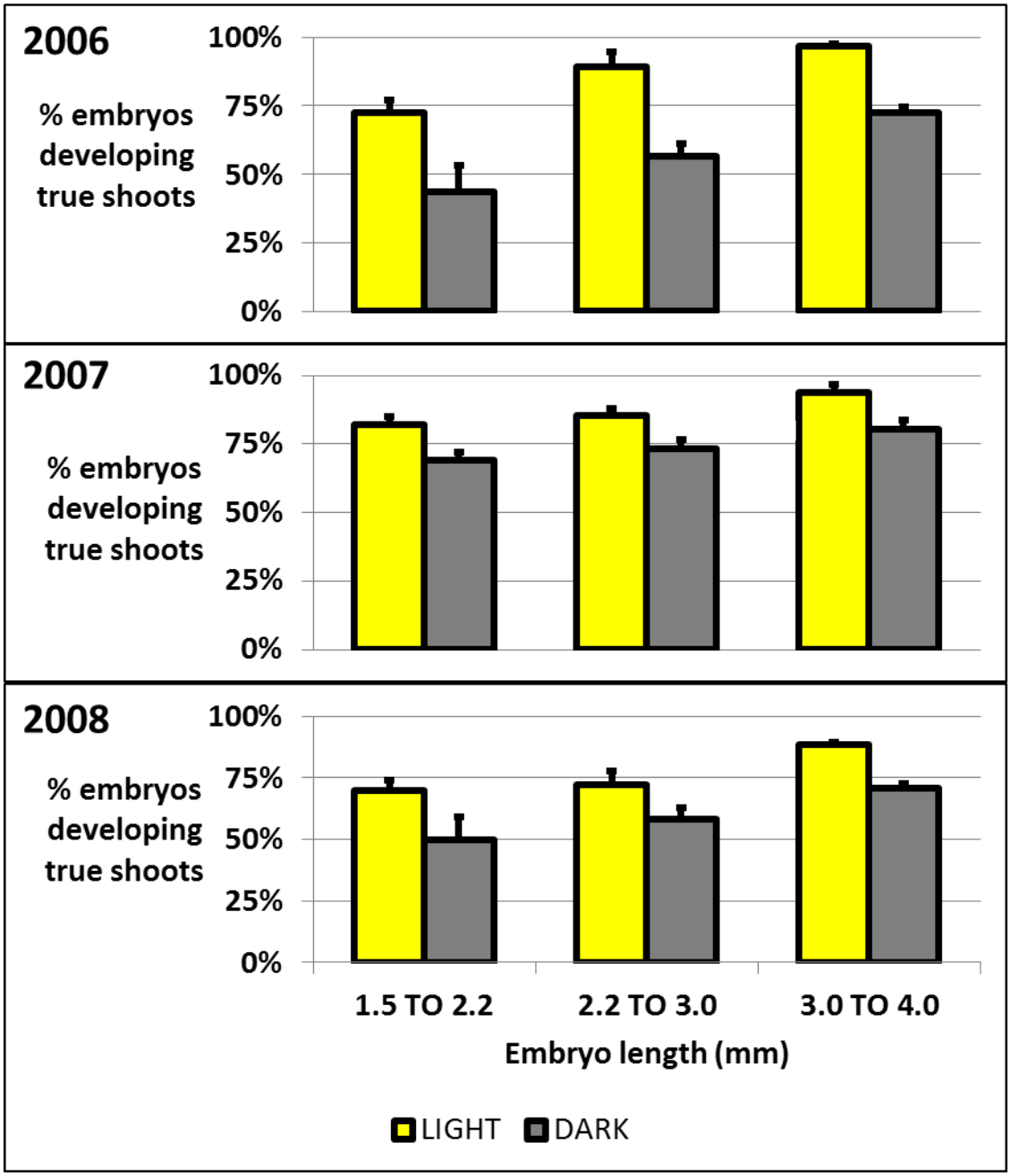

Fig. 9 The effect of light on development of true shoots in cultured immature embryos. Embryo length is the length of the embryos on the day they were excised and cultured. Error bars are the standard error of the mean.

Fig. 10 Plants from light and dark-cultured embryos 42 DAC. *indicates plant with true shoot; ■ indicates plant with normal hypocotyl but no visible shoot; ◄ indicates plant with short-hypocotyl phenotype.

Some of the embryos developed into plants with short thick hypocotyls but no shoots (Fig. 10) while others had not opened their cotyledons (data not shown) and still others seemed healthy except they lacked a true shoot (Fig. 11B). A whole range of plants with deformed or reduced shoot apices was found in addition to the plants which did not develop true shoots (Fig. 11 D-F). The leaves in some of the plants with deformed apices frequently lacked chlorophyll (Fig. 11D-E). Those plants with deformed or reduced shoot apices were still counted as true shoots.

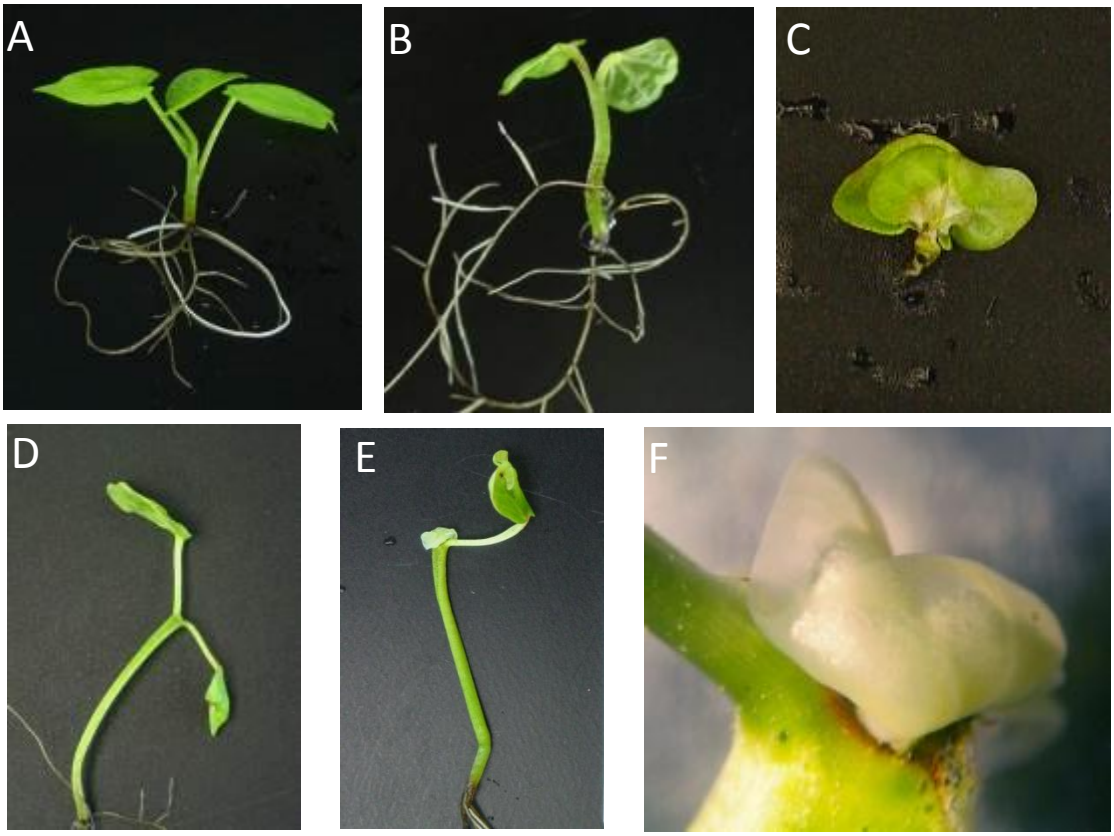

Fig. 11 Examples of 42 DAC plants developing from cultured embryos. (A) Normal plant with several true leaves; (B) healthy appearing 'plant' with no visible shoot apex; (C) 'plant' with extremely short hypocotyl and no apex; (D) plant with tiny vestigial apex; (E) plant with abnormal apex; (F) magnified image of plant with abnormal apex.

Discussion

The results in this chapter indicate that some very small 1.5 to 3 mm immature embryos can carry out part of the seedling photomorphogenetic program, i.e. activation of the SAM and the development of true leaves. As the embryos become larger, i.e. 3-4 mm, the capacity of cultured embryos to activate the SAM increases. These data also indicate that shoot development continues to be affected by prolonged darkness (14 days) for a long period (28 days or more) after the plants had been transferred to the light. If the difference between light-treated and dark-treated embryos was just 'normal'

photomorphogenesis versus ‘normal’ skotomorphogenesis, one would expect all of the dark-treated plants to resemble the dark-treated plants in the top row of Fig. 10, i.e. long hypocotyls with undersized shoots. If the deformities that occurred were simply tissue culture artifacts arising from an unnatural and somewhat stressful environment one would expect them to occur randomly.

CHAPTER IV

IN VITRO RESPONSE TO LIGHT INTENSITY AND WAVELENGTH

Introduction

Plants detect light in the red, far-red, blue UV A and UV B bandwidths, and regulate many of their physiological responses to these bandwidths through a complex system of light receptors and light-regulated signaling pathways. In *Arabidopsis*, for example, there are at least three types of molecules: phytochromes, cryptochromes and phototropins that act as light receptors (Chen et al., 2004; Neff et al., 2000). The complexity of the signaling system allows plants to respond to differences in light wavelength, light intensity, light duration and even light direction. Researchers have classified light responses as VLFR (very low fluence) LFR (low fluence) and HIR (high irradiance). In VLFR responses, plants respond to short pulses of light of 0.1 to 1.0 μmol m^{-2}, whereas the LFR response is to short pulses of light between 1.0 and 1000 μmol m^{-2}. Plant HIR responses require a relatively high intensity (>1000 μmol m^{-2}) for a relatively long period of time (Mancinelli, 1994).

Of special interest to this study is the role of light in germination and photomorphogenesis. Germination is defined here as the group of processes beginning with imbibition, water uptake by the seed and ending with the elongation of the axis, usually the radicle (Bewley, 1997). Different species can have very different light requirements for germination. Some require HIR while others respond to VLFR or both. In yet other species seed germination can be light insensitive (Takaki, 2001). Photomorphogenesis or de-etiolation is the developmental program that includes cotyledon expansion, cessation of hypocotyl elongation, activation of the SAM,

synthesis of chlorophyll and anthocyanins and development of true leaves (Chory et al., 1991).

The upper leaves and bolls of the field grown cotton plant can receive PPF up to 2000 µmol $m^{-2}s^{-1}$ (Taiz and Zeiger, 2010). It is unclear how much light cotton embryos receive during development in their natural state inside the boll. However, one researcher has reported that light, especially in the far-red wavelengths, can penetrate the walls of immature cotton bolls over 1 cm in diameter (Kasperbauer, 2000). The light available in a tissue culture environment, usually about 50 to 100 µmol $m^{-2}s^{-1}$, is much less intense than natural sunlight. The maximum light intensity used for the experiments in this chapter was a PPF of 60 µmol $m^{-2}s^{-1}$.

Two experiments were undertaken to test whether embryos in vitro responded to differences in light intensity and color. In the first experiment the effect of different intensities of light on the development of true shoots was examined. In the second experiment, the effect of different wavelengths of light on the development of true shoots was examined.

Materials and methods

For the purpose of this study, light intensity is stated in photosynthetic photon flux (PPF) measured in moles per square meter.

The effect of different light intensities on the development of true shoots was examined in the first experiment. A total of 915 embryos from 1.5 mm to 4.0 mm long (13 to 18 DPA) on the first day of culture were incubated under continuous full spectrum lighting for 14 days at light intensities of 0.0, 0.6, 6.0 or 60 µmol $m^{-2}s^{-1}$. Temperatures

were maintained at 28 to 32°C. Differences in light intensity were obtained by placing the embryos in a Revco™ freezer box covered by one to two layers of neutral wavelength Lee 211™ filters. Maximum light intensity was obtained by leaving the box uncovered and darkness was obtained by placing a lid on the box (Appendix A). Each treatment was replicated three times.

In the second experiment, the effect of different wavelengths (colors) of light on the development of true shoots was examined. A total of 1078 embryos of 1.5 to 3 mm (14 to 16 DPA) were tested. In order to avoid contamination with other wavelengths, all of the treatments of a single color were put into culture on the same day. The incubator was then closed for 14 days. This, however, limited the number of embryos that could be cultured at one time so 3-4 mm embryos were not used. During the first 14 days of culture the embryos were grown under continuous red, blue, far-red or full-spectrum GE Ecolux™ plant and aquarium bulbs or in the darkness at 28 to 32°C. In red, far-red and blue light treatments four light intensities (25, 2.5, 0.25 and 0.025 μmol m^{-2s-1}) were tested while only one light intensity, 60 μmol m^{-2s-1}, was used in the full spectrum control. Differences in light intensity were obtained by placing the embryos in a Revco™ freezer box covered by one to three layers of neutral wavelength Lee 211™ filters (http://www.leefilters.com). Maximum light intensity 25 μmol $m^{-2}s^{-1}$ was obtained by leaving the box uncovered and darkness was obtained by placing a lid on the box. Red light and far-red light were provided by banks of single wavelength LEDs, blue light was provided by Coralife™ actinic blue fluorescent bulbs. Light intensity (PPF) was measured using a Licor LI-185 quantum radiometer/photometer.

All of the embryos from both experiments were cultured on Medium I consisting of BT (Beasley, 1971; Beasley and Ting, 1973) salts and vitamins plus 30 gl^{-1} sucrose and 12 gl^{-1} agar at in sterile 20 X 100 mm Petri dishes. After 14 days the embryos were transferred to Media II consisting of BT salts and vitamins plus 30 gl^{-1} sucrose and 8 gl^{-1} agar and placed under full spectrum light at 60 μmol $m^{-2}s^{-1}$. At 28 days they were transferred to Medium III consisting of ½ MS salts ½ MS vitamins, plus 15 gl^{-1} sucrose and 8 gl^{-1} agar. At 42 days the number of true leaves on each plant was counted. A plant with at least one leaf or a visible apical bud (~ 0.5 mm) was considered to have a true shoot. Unopened buds and folded leaves were counted as ½ leaves. Each treatment consisted of 15 to 25 embryos and each treatment was repeated three to five times.

Analysis of variation was computed with the SAS v 9.1 General Linear Model. Under the Duncan Multiple Range model, means sharing the same letter were not significantly different at alpha < 0.05. Graphs were drawn using Microsoft Excel. All error bars represented the standard error of the mean of 3 to 5 replications.

Embryos were photographed on day 1, 14, 28 and 42 of culture. Images were measured using ImageJ™ software (Abramoff et al., 2004; Bearer, 2003). Length from the tip of the root to the top of the cotyledon was measured on day 1 and day 14.

Results

In the test of light intensity, overall results showed that more embryos developed true shoots in higher light intensities, PPF of 6 µmol $m^{-2}s^{-1}$ or 60 µmol $m^{-2}s^{-1}$ than at the lower intensity of 0.6 µmol $m^{-2}s^{-1}$ or in darkness (Table 6). However, when the data was analyzed by size-class it became apparent that only the embryos in the 3-4 mm size-class showed the most increase in the number of true shoots at PPF of 6 to 60 µmol $m^{-2}s^{-1}$ (Fig. 12). Embryos in the two smaller, 1-2 mm and 2.2-3 mm size-classes did show an increase in the rate of shoot development at 6 to 60 µmol $m^{-2}s^{-1}$ when compared to darkness. However, standard errors of the mean for embryos of the two smaller size-classes overlapped at 0.6 and 6 to 60 µmol $m^{-2}s^{-1}$.

Table 6 Effects of changing light intensity on the % of embryos developing true shoots

Light intensity µmol $m^{-2}s^{-1}$	**Embryos**	**% with true shoots**
60	220	75[a]
6.0	211	67[a]
0.6	264	56[b]
0.0	220	54[b]

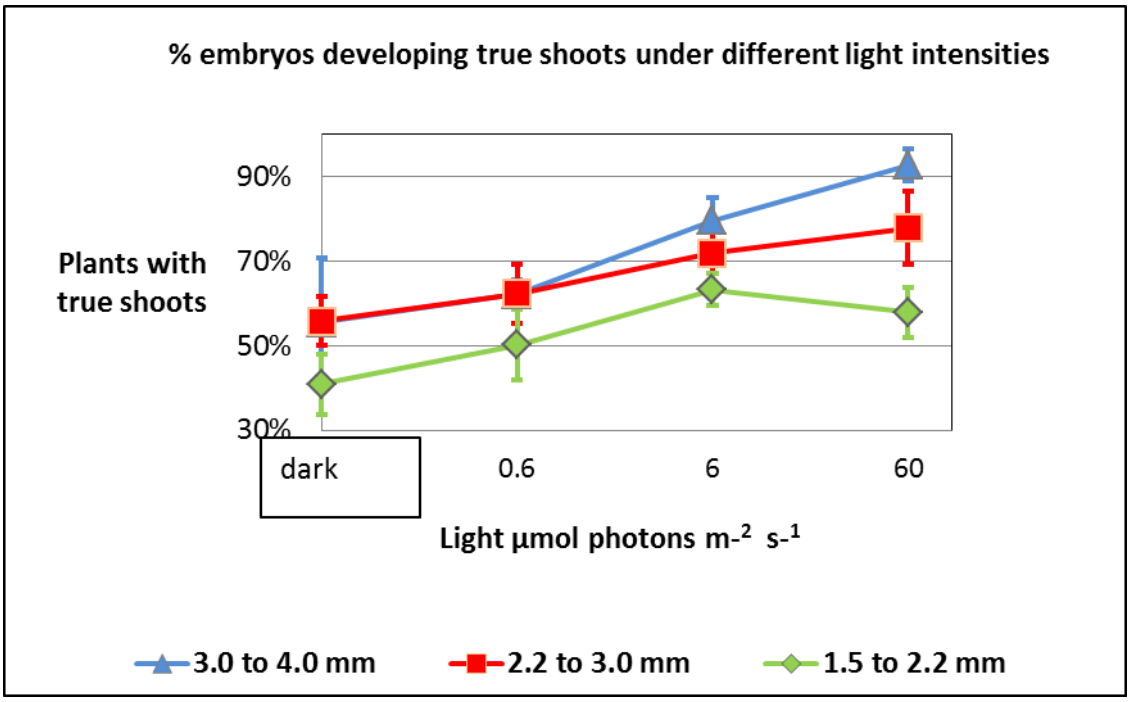

Fig. 12 The effect of light intensity and embryo size on the number of embryos developing true shoots. Error bars represent standard error of three replications.

In the test of the effects of light color, no single wavelength developed true shoots at the same rate as full spectrum light (Table 7). Embryos treated with blue light or far-red light produced true shoots at about the same rate as embryos left in the dark. However, embryos cultivated in red light produced significantly fewer true shoots than any of the other treatments. Light intensity did not have a significant effect on true shoot formation in the red or far-red light treatments but light intensity did affect embryos treated with blue light (Fig. 13). Embryos treated with blue light at lower intensities, PPF of 0.25 µmol $m^{-2}s^{-1}$ or 2.5 µmol $m^{-2}s^{-1}$, produced true shoots at about the same rate

Table 7 Effects of light wavelength on the % of embryos developing true shoots

Light wavelength	PPF (µmol $m^{-2}s^{-1}$)	Embryos	% with true shoots
Full spectrum	60	75	81[a]
Red	0.025 to 25	299	43[c]
Far-red	0.025 to 25	301	68[b]
Blue	0.025 to 25	341	66[b]
Dark	0	62	62[b]

as full spectrum light. When cultured in blue light at a full 25 µmol $m^{-2}s^{-1}$ the embryos frequently died (Fig. 13).

Discussion

Embryos in the 3-4 mm size-class developed significantly more true shoots at the higher intensity (PPF of 6 and 60 µmol $m^{-2}s^{-1}$) light than lower intensity (PPF of 0.6 µmol PPF $m^{-2}s^{-1}$) or in the dark (Fig.12). Embryos in the 1.5 to 2.2 mm size-class and the 22 to3 mm size-class did not develop significantly different numbers of true shoots as the light intensity increased. The 3-4 mm embryos seem not to be just bigger but have reached a different developmental stage than the 1.5 to 3 mm embryos.

At a PPF of 6 µmol $m^{-2}s^{-1}$ the HIR threshold would be reached in less than three minutes. The development of true shoots in 3-4 mm embryos appeared to be an HIR response. In Chapter II it was found that in most of the 3-4 mm embryos the primordia of the first leaf had already begun to grow. This raises the question of whether the buds

seen at 42 DAC were really from an activated meristem or whether what was seen was simply the first leaf.

None of the monochromatic light treatments produced more true shoots than full spectrum light (Table 7). Far-red light and darkness had the similar effects on the development of true shoots. Red light seemed to inhibit the development of true shoots while blue light response depended on the light intensity. Embryos cultured in blue light at lower intensities (0.025 µmol $m^{-2}s^{-1}$ to 2.5 µmol $m^{-2}s^{-1}$) produced as many shoots in culture as full spectrum lighting (Fig. 13). However, at a higher intensity (25 µmol $m^{-2}s^{-1}$) blue light sometimes killed the embryos.

The energy of a light source is inversely related to its wavelength so that 25 µmol of blue light at 420 nm produces much more energy than 25 µmol of red light at 660 nm. Much of the excess energy is dissipated in the form of heat (http://5e.plantphys.net). Instead of blue fluorescent bulbs, Li et al. used blue LEDs with an emission peak at 460 nm and a fluence of 50 µmol $m^{-2}s^{-1}$. They did not mention if there was tissue damage (Li et al., 2010). The one cm buds Li. et al. used were, however, much larger than the 1.5 to 3 mm embryos used in this experiment.

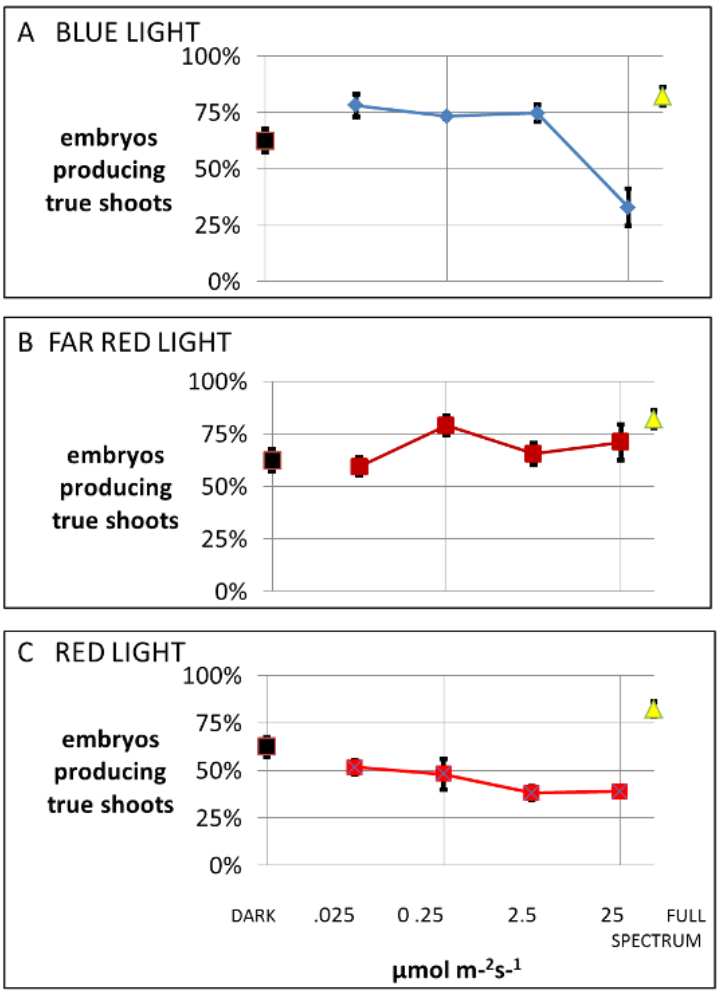

Fig. 13 The effect of different light colors and intensity on the % of embryos developing true shoots. A blue light; B far-red light; C red light. Error bars are the standard error of the mean. Dark and full spectrum light controls have been inserted for comparison. Black ■ indicates dark control; yellow ▲ indicates full spectrum light control.

Research into light wavelength in tissue culture applications has often produced conflicting results and results seem to be species specific. For a review of light in other tissue culture systems see Chapter I: light in other tissue culture systems. Li et al. (2010) found that in culture cotton seedlings developed more leaf area, leaf thickness,

chlorophyll and palisade tissue under blue LED lighting but a 1:1 proportion of red to blue LEDs was the most beneficial for overall development. He also found that the concentrations of sucrose, soluble sugar and starch were greatest in upland cotton plants under red LEDs compared to full spectrum lighting, blue LEDs or mixed red and blue. He suggested that the increased carbohydrates in red light were a result of reduced translocation of nutrients (Li et al., 2010).

Our results showed that significantly fewer embryos produced shoots in red light than in any other treatment and that more embryos treated with full spectrum light produced shoots than embryos treated with red or far-red light. These results appear to contradict Li's findings. We have repeatedly found that a difference in developmental stage was accompanied by a difference in response. Li worked with seedlings that had been germinated for one week (Li et al., 2010). When we germinated seeds for this study most seedlings had developed several leaf primordia by seven days (see Chapter VII).

CHAPTER V

EARLY APICAL DEVELOPMENT IN COTTON EMBRYOS DURING THE FIRST 14 DAYS OF CULTURE

Introduction

Previous experiments (Appendix B) have shown that embryos elongate during the first 6 to 14 DAC. This elongation could be from cell division or from expansion of existing cells. Ihle and Dure (1972) reported that cell division in the cotyledons of immature embryos (35 mg to 90 mg) stops when the embryos are germinated prematurely. By our estimate these embryos would have been about 25 to 35 DPA. The following two experiments attempt to answer three questions: 1) does cell division continue in immature embryonic SAMs after the embryos have been placed in culture; 2) does the meristem die in culture so that the shoots that develop are actually adventitious, and; 3) do light or dark treatments have any observable effect on the SAMS of cultured embryos during the first 6 to 14 days of culture?

Materials and methods

Two similar experiments were conducted over the growing season, the first for 6 days and the second for 14 days. Cotton (*G. hirsutum* cv TM-1) plants were grown in the greenhouse as described in Appendix A. Flowers were tagged on the day of anthesis, bolls harvested and embryos immediately excised and placed on media for 6 or 14 days. Embryos were cultured at 30°C on Medium I solidified by 12 gl^{-1} agar (Sigma A7921) under constant fluorescent light (PPF of 12µmol $m^{-2}s^{-1}$) or constant dark. After 6 or 14 days the embryos were photographed, fixed, sectioned and stained (See Appendix A).

Embryonic shoot apices were examined under the microscope and classified by general shape as described in Chapter II as flat or concave, symmetrically mounded, asymmetrically mounded and elongating leaf. An elongating leaf is defined here as an asymmetric, sickle shaped structure that is taller than it is wide.

In the first, 6-day experiment, fruit were harvested during the period 5/20/11 to 6/7/11. The embryos ranged in size 1.6 to 3.6 mm (14 to 17 DPA). Uncultured embryos from size-classes 1.3 to 2 mm, 2-3 mm, and 3-4 mm (13 to 17 DPA) harvested during the same time period (see Chapter II) were used as negative controls. Embryos sectioned both parallel and perpendicular to the plane of the cotyledons were examined (Fig. 3). The sections were examined for general shape (concave or flat, symmetrical mound, asymmetrical mound, elongating leaf) and for deeply staining cells.

In the second, 14-day experiment, fruit were harvested from 7/28/11 to 8/10/11. The embryos ranged in size from 1.9 to 3.0 mm (14 to 15 DPA). Embryos isolated from the same bolls as the cultured embryos were used as negative (uncultured) controls. In the 14-day experiment only embryos aligned perpendicularly to the plane of the cotyledons were used to facilitate comparison. The same general shape categories were used as described previously. In addition, the widths of the shoot apices were measured. Three longitudinal 10 μM sections from the center of each embryo were photographed, measured using ImageJ™ software and the number of cells counted.

Results

6-day experiment

In the 6-day experiment, no significant difference was found in apical development in light or dark-treated embryos (Fig. 14). Less than half of apices remained flat or convex in shape while most developed symmetrical or asymmetrical mounds. In the 6-day experiment apical response was different in each size-class (Table 8). On the day the embryos were excised and put into culture, 83% of the 1-2 mm size-class and 69% of the 2-3 mm size-class had flat or concave mounds (Chapter II). At six DAC only 44% of the 1-2 mm size-class and 2% of the 2-3 mm size-class had failed to develop a mound (Fig. 15).

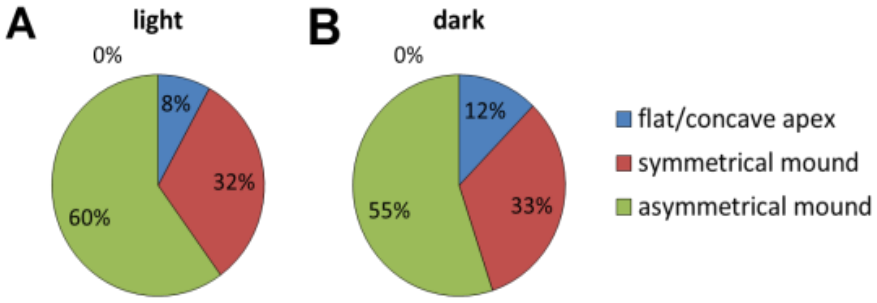

Fig. 14 Comparison of apical development between light and dark-treated embryos after six days of culture. Embryos from 1.6 to 4 mm were cultured six days in the light or the dark at 30° C. No significant difference was found in the degree of mounding between the two treatments. (A) cultured in light; (B) cultured in darkness.

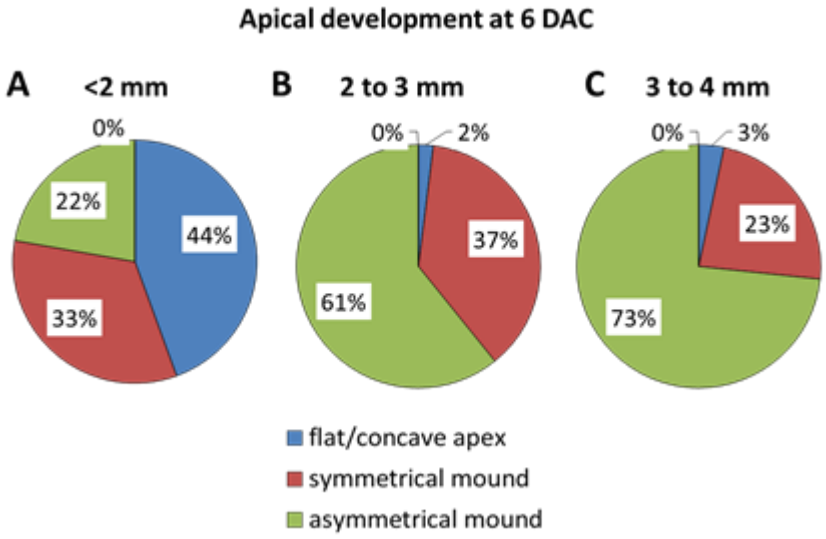

Fig. 15 Shoot apex morphology in six DAC cultured embryos. Embryos were cultured six days in the light or the dark at 30° C. (A) 1-2 mm on day of culture; (B) 2-3 mm on day of culture; (C) 3-4 mm on day of culture. Light and dark treatments are combined.

Table 8. Comparison of shoot apical morphology between uncultured embryos and embryos cultured six days

Treatment	Size-class (mm)	# embryos	Flat/concave shape	Symmetrical mound	Asymmetrical mound
Uncultured	1-2	13	85±10%	8±8%	8±8%
(Data from Chapter II)	2-3	18	67±11%	6±6%	28±11%
	3-4	13	0	15±10%	85±10%
6-day	1-2	18	44±12%	33±11%	22±10%
	2-3	51	2±2%	37±7%	61±7%
	3-4	30	3±3%	23±8%	73±8%

14-day experiment

Approximately ½ of the 2-3 mm embryos that were examined at 0 DAC had flat or concave-shaped apices. Nearly all of the embryos had developed mounds 14 DAC (Fig. 16; Fig. 17) Although more light-treated embryos developed asymmetrical mounds than dark-treated embryos the differences were not significant (Table 9). None of the cultured embryos developed leaves in the first 14 days.

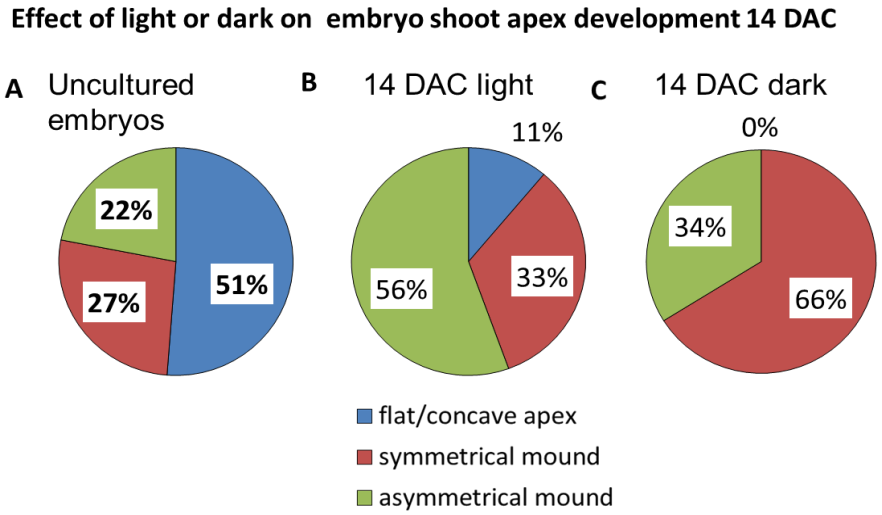

Fig. 16 Shoot apex morphology in 14 DAC cultured embryos. (A) uncultured control; (B) light-treated; (C) dark-treated. Embryos were 2-3 mm (14 to 15 DPA).

Table 9 Apical development in 2-3 mm embryos cultured 14 days

Size-class	#	Flat or concave apex %	Symmetrical mound %	Asymmetrical mound %
Uncultured embryos	14	51±14	27±12	22±11
Light-treated	18	11±7	33±11	56±12
Dark- treated	21	0	66±10	34±10

In order to compare development by a more objective measure, apical widths were also measured at 14 DAC and the measurements compared to embryos from the same bolls collected on the day of culture. The shoot apices of cultured embryos did continue to increase in width (mm) during the14 days of culture (Table 10). In order to determine whether the change in width was from cell expansion or cell division the number of cells along the line of measurement was also counted. The number of cells also increased during the14 days of culture. However, there was little or no difference in width between the light and dark treatments (Table 10).

Table 10 Apical width of 2-3 mm embryos cultured 14 days

Treatment	♯ embryos	Width (mm)	Number of cells wide
Uncultured	14	60±4	8.1±0.5
14 days dark	21	103±3	10.8±0.3
14 days light	18	97±3	10.1±0.3

Damaged or deeply staining tissue was observed in several embryos. Two out of 50 of the 6-day light-treated embryos developed dead or damaged cells in the apical region (Fig. 18). In the 14-day experiment one out of 14 of the dark-treated embryos also developed deeply staining cells in the apical region. The cells stained very dark red with Safranin O. Similar intensity of staining was seen in gossypol glands and lignifying xylem cells (data not shown).

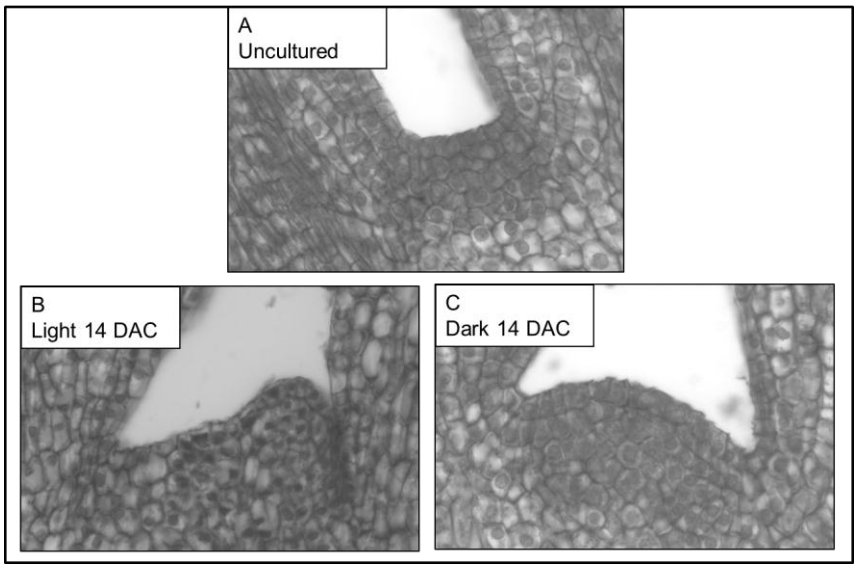

Fig. 17 Comparison of shoot apices between uncultured and 14 DAC embryos. Embryos were harvested the same day (2.1 mm 14 DPA). A flat apex uncultured embryo; B cultured in the light 14 days with asymmetrical mound; C cultured in the dark 14 days with symmetrical mound.

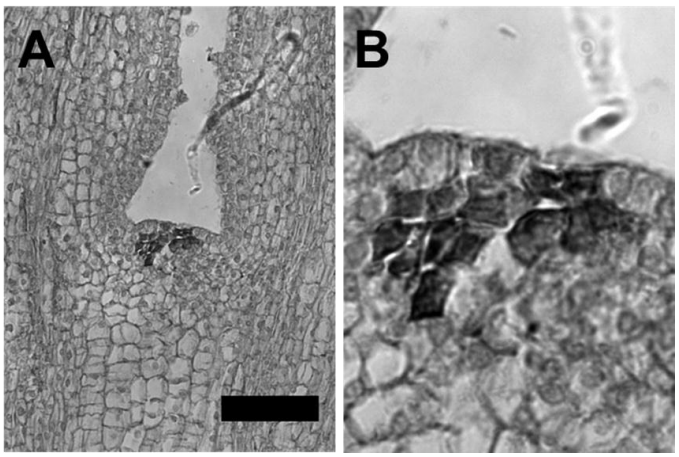

Fig. 18 Damage to shoot apex in embryo cultured 6 days in the light. (A) 1-2 mm embryo (14 DPA) after 6 days in light; (B) enlargement of the apical region. Bar = 100 µM

Discussion

These experiments were designed to determine whether: 1) cell division continued in immature embryonic SAMs after the embryos have been placed in culture; 2) the meristems died in culture so that the shoots that developed were actually adventitious, and; 2) whether light or dark treatments had any observable effect on the SAMS of cultured embryos during the first 6 to 14 days of culture.

Apical shape changed while the embryos were in culture both in the 6-day and 14-day experiments. Apical mounds developed when the cells in the apical region are dividing at a faster rate than the cells at the base of the cotyledons (Pollock and Jensen, 1964). This supports the hypothesis that cells in the shoot apices of the embryo continued to divide after the embryos were placed in culture. However, embryonic shoot apices developed at a slower rate than the shoot apices of embryos developing in vivo (see Chapter II). Furthermore, the shoot apices in 3-4 mm cultured embryos did not seem to develop any further after they were placed in culture (Table 8). In the 6-day experiment there was no visible difference in apical development between light and dark-treated embryos. Light-treated embryos in the 14-day experiment did develop more asymmetrical mounds than dark-treated embryos but did not show any difference in apical width. Six days may not have been sufficient time for morphological changes to occur.

The brief (1-2 hours) exposure to light while the embryo was excised from the ovary may have been sufficient to induce apical activation in embryos that were later cultured in the dark. Plants can respond to even brief light exposure. For example,

Powell and Morgan (1970) found that apical hook straightening in cotton hypocotyls could be induced by a two hour exposure to red light (Powell and Morgan, 1970) while Kojima and Oota (1980) used only a brief red or far-red pulse to control germination in lettuce seeds. Response appeared to have been delayed, i.e. there was a lag time between stimulus and change in morphology. In *Arabidopsis* SAMs, Barton (2010) found that transcriptional changes could be detected before cell divisions could be observed (Barton, 2010).

Only a few apices appeared to be dying in either the 6-day or the 14-day experiments. In the 6-day experiment two out of 50 of the shoot apices in light-treated embryos developed deeply staining cells (presumed dead) in the SAM. Previous experiments (data not shown) had shown that dark lesions were more common in light-treated embryos than dark-treated embryos and that the presence of dark lesions anywhere on the embryo at 14 DAC was negatively correlated with the ability of the embryo to develop a true shoot (data not shown). It is not known whether embryos with damage as extensive as shown in Fig. 18 could survive and develop a new shoot. Amutha et al. reported that after removal on the shoot apices and one cotyledon, about 46% of cotton seedlings had been able to regenerate a shoot. However, the leaves were extremely abnormal in appearance (Amutha et al., 2009). Agrawal was able to induce multiple shoots from the cotyledonary node in cultured seedlings that had their cotyledons and shoot apex removed but used growth regulators BAP and kinetin to aid in induction(Agrawal et al., 1997).

Even though the embryonic apices continued to grow in culture, growth was very slow compared to greenhouse grown embryos. Nearly all the greenhouse-grown embryos had developed a sickle-shaped leaf that was taller than it was wide by 25 DPA (Fig.4E). None of the apices developed beyond the asymmetrical mound stage in the 6-day or 14-day cultured embryos.

Apical development in 14-day embryos seemed no more advanced than in 6-day embryos. When embryos were grown in culture, elongation also slowed between 6 and 14 days in culture (Appendix B). The inability of the leaves to develop beyond a small mound in the first14 days of culture may have been due to the media drying out or the depletion of nutrients or the nutrients becoming bound up by the agar as pH decreased (Scholten and Pierik, 1998). In addition to the slowing of growth, dark and light-treated embryos often lost their green color (Appendix B). Embryos growing in the boll also lose their bright yellow-green color after about 25-30 DPA (data not shown). A developmental program change from embryonic growth and development to maturation and desiccation may have occurred.

CHAPTER VI

APICAL DEVELOPMENT IN GERMINATING SEEDLINGS

Introduction

In Chapter III the 42-day results showed that the presence or absence of light during the first 14 days of culture was one of the factors that affected the ability of the embryos to form a true shoot in vitro. The purpose of the following experiments was to investigate the natural occurrence in a fully mature seed population of the missing shoot apex phenotype seen in excised immature embryos in the absence of light and to establish a baseline for the number of germinating seeds that fail to produce shoots. Germination is the group of processes in seeds that begins with imbibition, water uptake by the seed and ends with the elongation of the axis (Bewley, 1997). A plant was considered to have germinated if the radicle had emerged from the seed coat or if plants lacked radicles, the cotyledons had expanded and ruptured the seed coat.

Materials and methods

Mature seeds were germinated both in vitro and in soil. In the first experiment the in vitro-germinated seeds were scored visually, without magnification in the same manner as cultured embryos. In the second experiment the seeds germinated in soil were screened first without magnification. The seedlings with missing or abnormal apices were examined with 16X magnification and were then fixed sectioned and stained and examined at 100X and 400 X magnifications.

Germination in vitro

A total of 189 *Gossypium hirsutum* cv TM-1 seeds (4 replications, 3 treatments, 14 -18 seeds) were sterilized. Seeds were sterilized after the method of Gould and Magallenes-Cedeno (1998) as follows: Seeds were sterilized for 30 minutes in 20% bleach to which one drop of detergent had been added. They were then rinsed five times with sterile deionized water and incubated at 30°C overnight in sterile deionized water. The next day they were again sterilized for 30 minutes in 20% bleach and rinsed five times sterile deionized water. The seed coats were removed and the seeds placed on media. They were germinated in 16 oz. (~500 ml) clear deli containers (ProKal 16SC) on ½ MS plus 8 gl^{-1} agar and 15 gl^{-1} sucrose at 28 to 32° C. The seeds were germinated in the dark, or under cool white fluorescent bulbs at a PPF of 0.06 μmol m^{-2} s^{-1} or 60 μmol m^{-2} s^{-1}. After 14 days the seedlings were photographed and the cotyledons removed. True shoots were scored visually after removing the cotyledons. Embryos with a visible (>0.5 mm) bud or a bud and a leaf were considered to have developed true shoots.

Germination in soil

On June 30, 2011 850 mature seeds were planted in three inch pots in the greenhouse. Five seeds were placed in each pot. Seven days after planting the seedlings were examined for abnormal or missing shoot apices. Seedlings with normal, abnormal and missing shoot apices were collected examined and fixed with modified FAA (5% formalin, 2.5% acetic acid, 28% ethanol) for microscopic analysis. The remaining seedlings were allowed to grow until 11 or 12 days after planting and once again

examined for abnormal or missing shoot apices. On day 12 the soil was dug up and seedlings that had not yet emerged from the soil were also collected and examined and fixed with modified FAA.

Results

In vitro germination of seeds showed a very direct relationship between the amount of light seedlings received and the appearance of visible true shoots (Fig. 19). Nearly all (90±3%) of the seedlings that were germinated in the light developed true shoots while only 19±6% of the seedlings germinated in the dark developed true shoots. About half of the seedlings germinated in low light (0.06 µmol m^{-2} s^{-1}), 50±7% developed true shoots (Fig. 19).

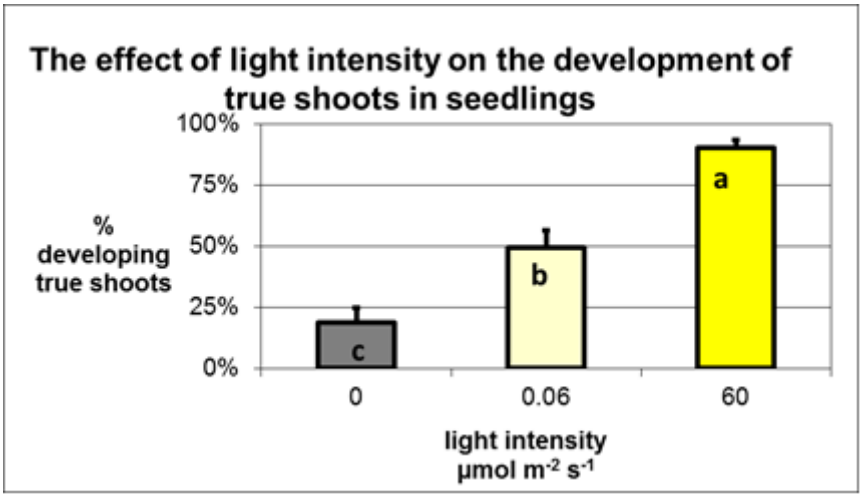

Fig. 19 Light intensity affects apical development in seeds germinated 14 days in vitro. True shoots were scored visually after removing the cotyledons. Embryos with a visible (>0.5 mm) bud or a bud and a leaf were considered to have a true shoot. Bars with different letters are significantly different under the Duncan Multiple Range test.

Out of the 850 seeds that were planted in soil, 85% germinated within 7 to12 days (Table 11), while 13 (1.9%) of the seeds that did germinate did not appear to have true shoots when examined with a 16X dissecting microscope (Fig. 20D). Most (9 out of 13) of the seeds that appeared to lack true shoots also failed to emerge above the surface of the soil, failed to open and had short thick hypocotyls and missing or dead radicles (Fig. 20B).

Five normal appearing seedlings and the seedlings without visible true shoots were fixed with modified FAA, sectioned and stained. Some of the seedlings with the short-hypocotyl phenotype had begun to decompose and simply fell apart when removed from the soil. The others were examined at 100X or 400X magnifications. Images at 100X or 400X magnifications showed that the seedlings actually did have apical meristems (Fig. 20) that were buried so deeply between the unopened cotyledons that they could not be observed even at 16X magnification.

Table 11 Normal and abnormal apical development in seeds germinated in soil

Number seeds planted	850	
Germinated	719	85% of total seeds planted
Not emerged from soil, no apex visible, short thick hypocotyl	9	1.25% germinated seeds
Emerged from soil, cotyledons not opened, no apex visible	3	0.42% germinated seeds
Emerged from soil, cotyledons opened, no apex visible	1	0.14% germinated seeds
Total seedlings with no visible apex	**13**	**1.9% germinated seeds**

A comparison of Fig. 21A to Fig. 21C suggested that seedlings with the short-hypocotyl phenotype also seemed to have more cells that stained darkly with Safranin O than normal seedlings. In some cases the entire layer of cells underlying the epidermis stained almost black (Fig. 21C).

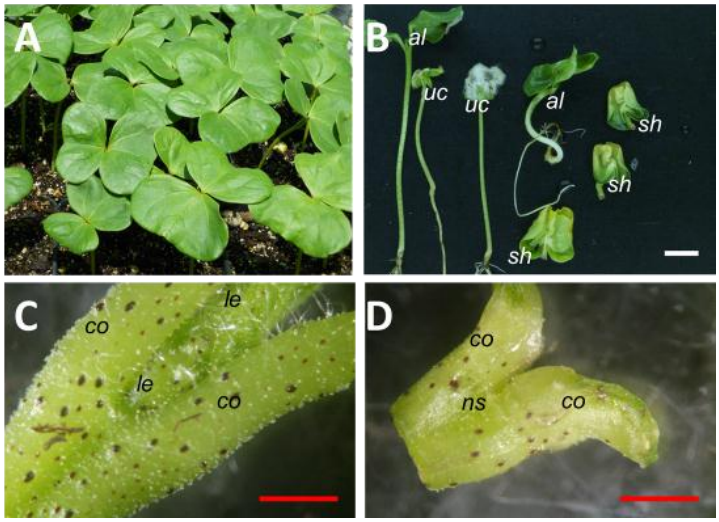

Fig. 20. Examples of normal and abnormal germinated seedlings. (A) Normal seedlings seven days after germination; (B) seedlings with abnormal leaves or missing shoot apices eleven days after germination; (C) magnified image of normal shoot apex seven days after germination; (D) Magnified image of the short-hypocotyl phenotype twelve days after germination. Part of the cotyledon has been cut away to show the apical region. White bars = 1cm; red bars = 1 mm; *al* abnormal leaf; *co* cotyledon; *le* normal unexpanded leaf; *ns* no visible shoot apex; *sh* short-hypocotyl plus missing radicle and missing shoot; *uc* unexpanded cotyledon.

Both the cotyledons and elongating leaves produced an abundance of glandular trichomes on both their adaxial (toward the center of the meristem) and abaxial (away from the center of the meristem) surfaces (Fig. 22). One seedling was observed to have an extremely disturbed leaf arrangement with three leaf primordia erupting adjacent to

one another (data not shown). Cotton leaf primordia normally develop in a spiral with a new primordium developing every 3/8 turn (McClelland and Neely, 1931).

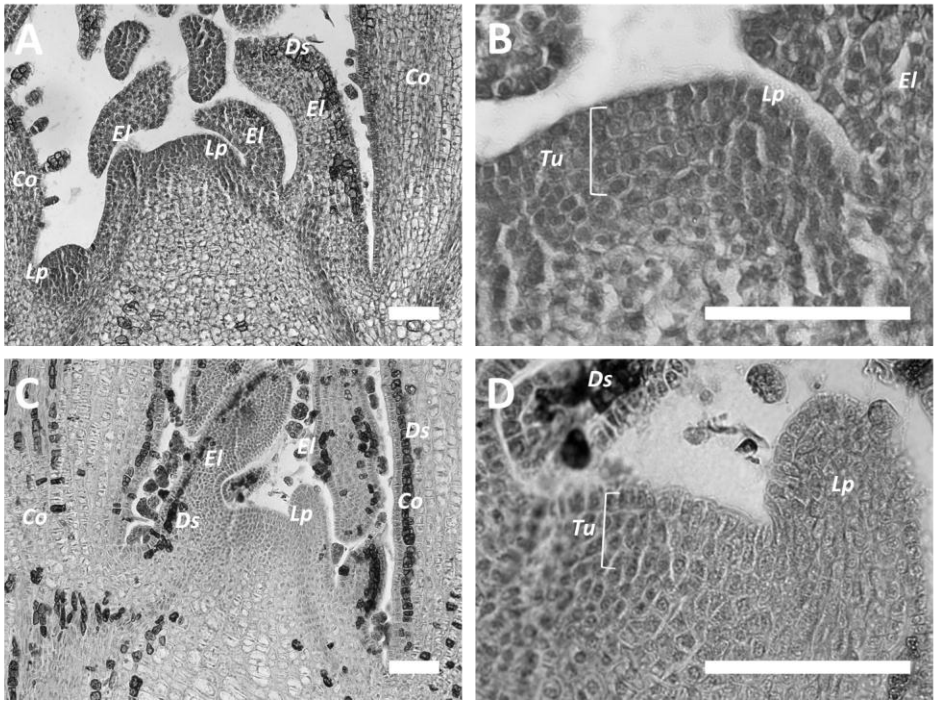

Fig. 21 Shoot apices of normal seedling and short-hypocotyl phenotype. (A) Apex of normal seedling seven days after germination; (B) enlarged image A; (C) apex of seedling with short-hypocotyl phenotype that has not yet emerged eleven days after germination; (D) enlarged image of C. *Tu* tunica; *Lp* leaf primordium; *El* elongating leaf; *Co* cotyledon; *Ds* deeply-staining cells; bar =100 µm.

Discussion

All of the seedlings, including the 'normal' seedlings were found to have patches of cells on the developing leaves and cotyledons that stained deeply with Safranin O (Fig. 21; Fig. 22). The reason Safranin O stained these cells so deeply is not known. However the seedlings with the short-hypocotyl phenotype appeared to have more dark patches. At times the entire layer of cells under the epidermis stained almost black.

Safranin O is a basic coal tar dye that has been used to stain chromosomes, cutins, and lignified cell walls (Sass, 1940 pg. 60). It stains most deeply at a pH > 7.0. It also stains gossypol glands (data not shown).

Seeds that were germinated in culture developed true shoots at a greater rate in the light than in the dark. There was a direct relationship between the intensity of light and the rate of true shoot formation (Fig.18). This is consistent with what is known about normal photomorphogenesis, development in light, and skotomorphogenesis or etiolation, development in the dark (Chory, 1997). However a small percentage of seeds failed to develop true shoots in the light and a small percentage of seeds did develop true shoots in the dark. A larger experiment was undertaken to see if there were one or more genetic mutations in our Texas Marker-1 seed population that interfered with shoot apical development. Out of 719 germinating seeds 13 (1.9%) failed to develop visible true shoots. However, a microscopic examination of the fixed and sectioned apices of four seedlings that failed to develop visible true shoots showed that there was a SAM with several small leaves developing in each of the seedlings. The two cotyledons were pressed together, hiding the apices. Two of the seedlings failed to shed their seed coats and their undersized SAMs could be explained by the failure of the cotyledons to receive light.

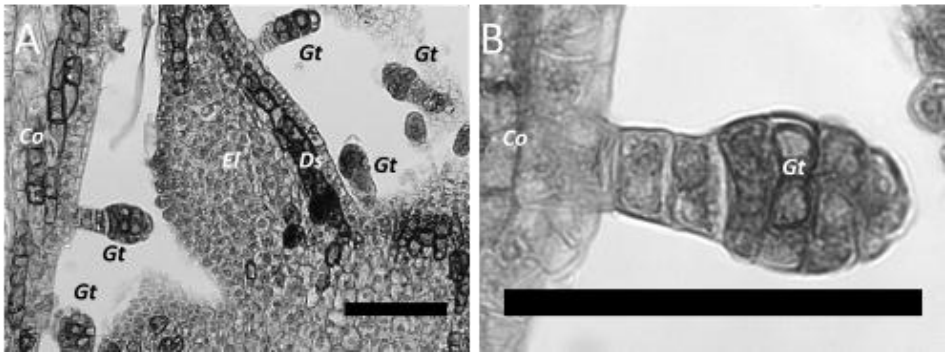

Fig. 22 Glandular trichomes on cotyledon and elongating leaf. (A) Apex of seven day seedling; (B) enlarged image of glandular trichome. *Gt* glandular trichome; *El* elongating leaf; *Ds* deeply staining layer of cells immediately under epidermis; *Co* cotyledon; bar =100 µm.

Fig. 23 Plants from 3-4 mm (21 DPA) dark-treated embryos. Circled plant has short thick hypocotyl phenotype and no true shoot. The other plants had true shoots. Embryos were put into culture 5/16/05.

Photomorphogenesis has been defined as the group of developmental processes in seedlings that includes anthocyanin and chlorophyll synthesis, cotyledon expansion, cessation of hypocotyl elongation, activation of the SAM, and development of true leaves. Skotomorphogenesis (etiolation) includes the group of developmental processes including formation of the apical hook, hypocotyl elongation and suppression of chloroplast and SAM development (Chory et al., 1994; Chory et al., 1996). The short-hypocotyl phenotype seen in mature seeds resembles neither photomorphogenesis nor skotomorphogenesis. The hypocotyl stops elongating even before the seed emerges from the surface of the soil. There is not an apical hook. The cotyledons remain closed. The radicle often deteriorates. It has been suggested that the short-hypocotyl phenotype in soil grown seedlings may have been due to disease. However, some of the seeds germinated under sterile conditions with their seed coats removed also developed the short-hypocotyl phenotype (data not shown).

It is beyond the scope of this study to prove that the cause of short-hypocotyl phenotype in rescued embryos is the same as the cause of the short-hypocotyl phenotype in germinated seeds. Tissue culture plants with a similar appearance to the short-hypocotyl phenotype had been observed many times in the course of this study (Fig. 23). Many of the roots of the short-hypocotyl seedlings grown in soil had deteriorated or died (Fig. 20). Plants in the 42-day experiments with the short-hypocotyl phenotype sometimes developed extensive adventitious roots arising from the base of the hypocotyl (Fig.22). Not all of the cultured embryos that failed to develop a visible true shoot had the short-hypocotyl phenotype. A reexamination of the images from the 2007 light-dark

experiment (see Chapter III) showed that 50 out of 741 of the dark-treated embryos but only 15 of the 721 light-treated embryos developed the short-hypocotyl phenotype. The similarity in frequency in the phenotype between the light-treated embryos and the germinated seedlings is striking. Very few plants in the 2007 experiment, twelve of the 741 dark-treated embryos and only two of the 721 light-treated embryos had cotyledons that failed to open (Table 12).

If the short-hypocotyl phenotype has the same cause in cultured immature embryos and mature seeds and that cause is genetic, it could be one or more recessive alleles. The phenotype is so severe that a dominant mutation would be lost in a single generation. However, heritability needs to be established. TM-1 is a highly inbred cotton line that had been self-pollinated for 22 generations (Kohel et al., 1970). Kohel

Table 12 Occurrence of short-hypocotyl phenotype in tissue culture population (2007)

Summary	Embryo size (mm)	Light treatment	%	Dark treatment	%
Number of embryos	1.5 to 2.2	253		251	
	2.2 to 3	277		277	
	3 to 4	191		213	
	Total	721		741	
Number of embryos without true shoots	1.5 to 2.2	46	18.0	77	31.0
	2.2 to 3	40	14.0	74	27.0
	3 to 4	12	6.0	42	20.0
	Total	98	14.0	193	26.0
Number with short-hypocotyl phenotype		15	2.0	50	6.7
Number with closed cotyledons		2	0.3	12	1.6

reported that TM-1 was free of any known mutations (Kohel et al., 1970). However, a recessive allele, especially an allele that interferes with germination could persist unnoticed in low numbers in a population for many generations. One common research practice is to multiply one's own seed from five or ten seeds provided by the USDA. If a recessive allele is present in one of the seeds, it would then occur at much higher frequencies in the research population than in the parental population.

Genetic contamination is possible. The parental plants could have accidentally been pollinated by another variety, and there could have been errors during harvest and ginning.

CHAPTER VII

CONCLUSIONS

The objective of this project was to develop an in vitro culture method that would allow growth and continued development of zygotic embryos from the early cotyledonary stage into complete plants as measured by the development of true shoots, i.e. true leaves or visible shoot apical bud. Once in place, this procedure would then be used to study aspects of developmental regulation during cotyledonary stage and the transition to later stages.

At the beginning of this study it was found that not all cultured embryos develop true shoots preventing development into viable plants. I developed two hypotheses: first, light promotes the development of SAM of *G. hirsutum* embryos in culture and darkness inhibits SAM development; and second, embryo maturity before the embryo is excised and placed into culture has a significant effect on SAM development in culture.

In order to test these hypotheses I conducted an investigation summarized in chapters II to VI. In Chapter II I described the development of shoot apices from ~ 1-2 mm (13 to 14 DPA) through late cotyledonary stage (~9 mm 25 DPA). The data led to the conclusion that the embryos in the 1-2 mm size-class corresponded to the description of heart-shaped embryos described by Pollock and Jensen (1964). My data established that the embryonic SAM continued to develop and grow after heart-stage and the first leaf also began to grow in the early cotyledonary stage (~ 3 mm 14 to 16 DPA). In Chapter III the results demonstrated that plants from embryos cultured 42 days

developed more true shoots if they were incubated in the light for the first 14 DAC than if they were incubated in the dark for the first 14 DAC. Furthermore, embryos that were three mm or larger responded more consistently than smaller embryos suggesting that the 3-4 mm embryos were not just larger but were developmentally distinct from embryos of 2 mm or less.

In Chapter IV embryos in the 3-4 mm size-class developed significantly more true shoots at the higher intensity (PPF of 6 and 60 μmol $m^{-2}s^{-1}$) light than lower intensity (PPF of 0.6 μmol $m^{-2}s^{-1}$) or in the dark. However, smaller embryos did not respond so consistently. Embryos in the 1.5 to 3 mm size-class developed no more shoots from any single wavelength light source than from full spectrum lighting. At PPF of 2.5 μmol $m^{-2}s^{-1}$ or less, embryos incubated under blue light produced shoots as frequently as under full spectrum light. Blue light at 25μmol PPF $m^{-2}s^{-1}$ sometimes killed the embryos.

Chapter V summarized the results from microscopic examination of apices of dark and light-treated embryos after 6 and 14 days of culture. The shoot apices of both dark and light-treated embryos that were less than 3 mm had continued to develop six days after they were excised and placed on media. Shoot apices of embryos that were over 3 mm did not appear to develop further. No difference between light and dark treatments was seen in the apices of embryos cultured six days. In embryos cultured 14 days there was evidence of growth in width and evidence of cell division. The leaf primordia of light-treated embryos seemed to be more developed than the dark-treated embryos but the apices were roughly the same width. The shoot apices were also

examined for unusual deeply-staining cells that might indicate that the apices were dying. Shoot apices of two out of fifty of the light-cultured 6-day embryos and one out of 14 of the dark-cultured 14-day embryos had unusually deeply-staining cells.

In Chapter VI the response of mature seeds was compared to immature embryos. Seeds germinated in culture developed true shoots in a manner that closely corresponded to the amount of light they received. Most seeds germinated in the light in the greenhouse developed true shoots. However, thirteen out of 719 of the seeds that germinated did not appear to have shoot apices upon visual inspection. Closer examination at a magnification of 100X and 400X showed that tiny apices surrounded by developing leaves had developed but were obscured by the tightly closed cotyledons.

The short-hypocotyl phenotype of greenhouse grown seeds with reduced apices resembled tissue culture plants with short hypocotyls and reduced apices that had been frequently observed in cultured. Photographs from the 2007 42-day light-dark experiments on cultured embryos were reexamined (see Chapter III) and plants with the short-hypocotyl phenotype were identified. 50 plants with the short-hypocotyl phenotype (and no apparent shoot) and 12 plants with closed cotyledons among the 741 dark-treated plants and only 15 plants with the short-hypocotyl phenotype and 2 plants with closed cotyledons among the 721 light-treated plants. The light-treated embryos showed a similar rate of short-hypocotyl plants as the soil germinated short-hypocotyl seeds. This is compelling evidence that one or more light-sensitive mutations affect cotton shoot apical development.

The data in this study suggests amendments to my hypotheses as follows:

1) Light promotes the development of the first leaf as well as the SAM of *G. hirsutum* embryos in culture. Darkness inhibits leaf development and the opening of the cotyledons and may be exacerbated by one or more light-sensitive alleles.

2) Embryo maturity before the embryo is excised and placed into culture also has a significant effect on apical development in culture. Larger embryos are not just more robust, apical morphology has changed, they respond to light differently, and the first leaf has begun to grow.

Using the in vitro system, I was able to identify a phenotype that could affect seed germination efficiency. A simple in vitro assay could be used to identify plants that are carriers of this phenotype and eliminate them from a breeding population.

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APPENDIX A

MATERIALS AND METHODS

Plant materials

Unless otherwise specified, all experiments were performed with *G. hirsutum* cv TM-1, an inbred line developed for use as a standard reference cotton for genetic and cytogenetic testing (Kohel et al., 1970). All experiments used the same batch of seed.

Embryo rescue

The complete process from culture of embryos to production of normal seedlings usually requires more than one culture step, and a medium has to be optimized for each culture step. All tissue culture experiments were performed using variations of the following procedure. Young bolls were harvested, disinfested in 10-20% bleach for 20 minutes and the embryos were dissected out of the fertilized ovules and placed on Medium I solidified with 7.5 to 15 gl^{-1} Sigma A7921 agar (Table 2). In early experiments (2005-2006) sterile filter paper was placed on the medium and the embryos were cultured on the filter paper while in later experiments filter paper was not used.

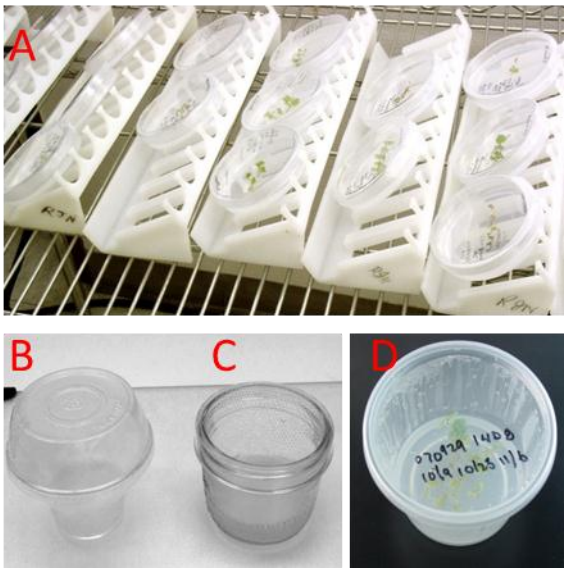

Fig. A1 Containers for growing 14-42 DAC embryos. (A) Embryos between 14-28 DAC growing on slants in Medium II; (B – D) Sterile containers used for rooting plants at 28 DAC: (B) 16 oz. Sundae Cup (C) 16 oz. autoclaved canning jar with 100 mm petri dish as lid, (D) 16 oz. clear polypropylene deli container (ProKal PKSC16).

At 14 to 21 DAC the embryos were transferred to Medium II solidified with 7.5 to 8 gl^{-1} Sigma A7921 agar for an additional 7 to 14 days. Because the young roots of cultured cotton embryos sometimes have difficulty penetrating into agar, a groove was cut in the agar and the radicles were 'planted' root downward in the groove. The plates were placed in the light on slants so that the embryo roots would grow downward and could be photographed (Fig.A1). At 28 DAC the embryos were transferred to Medium III solidified with 6 gl^{-1} Gibco 10675-023 Phytagar™ or 8gl^{-1} Sigma A7921 agar. Three different types of sterile containers were used for rooting: (1) 16 oz. plastic sundae cups (2) 16 oz. canning jars, or (3) 16 oz ~500 ml transparent polypropylene deli containers (ProKal PKS16C) ; (Fig. A1). At 42-49 DAC the embryos were removed from media, photographed and the number of true shoots (an apical bud or one or more true leaves)

counted. Culture temperatures varied from 19° to 26° C in early experiments (before 2006). In later experiments (after 2006) temperatures were maintained at 28° to 32 ° C.

Seed germination in vitro

Seeds were sterilized after the method of Gould and Magallenes-Cedeno (1998) modified by Gould and Raisor (unpublished) as follows: Seeds were sterilized for 30 minutes in 20% bleach to which one drop of detergent had been added. They were then rinsed five times with sterile deionized water and incubated at 30°C overnight in sterile deionized water. The next day they were again sterilized for 30 minutes in 20% bleach and rinsed five times sterile deionized water. The seed coats were then removed and the seeds placed on media.

Light-dark treatments

Except where otherwise noted, light was provided by coolwhite fluorescent or GE Ecolux™ plant and aquarium fluorescent bulbs. When darkness was required the petri dishes were placed into light-proof Revco™ 5.5" x 5.5"x 2" (~14 x14 x 5 cm) paperboard freezer boxes and incubated side by side on the shelf with the light-treated samples (Fig. A2). When filtered light was necessary the top of the Revco™ freezer box lid was cut out and one to three layers of neutral wavelength Lee 211 filters were taped over the edges of the box lid (http://www.leefilters.com). The lids of both the light-proof and filtered-light the boxes fit down over the edges of the boxes to exclude light but not air.

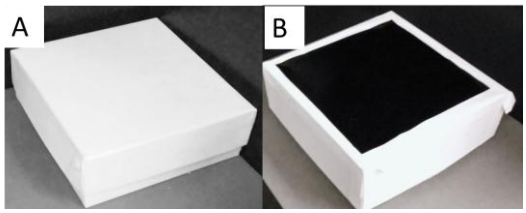

Fig. A2 Paperboard Revco™ boxes used for dark and filtered light experiments. (A) Light-proof and (B) filtered-light box with two layers of Lee 211 filters. Filtered light box lid was made by cutting top out of the box lid and taping filters over the edge of the lid.

Greenhouse conditions

Cotton plants (*G. hirsutum* cv TM-1) were grown under greenhouse conditions in five gallon plastic pots (Nursery Classic 2000) using Sunshine Metromix 700 or Metromix 900 potting mix. They were watered daily in the summer or every other day in the winter, fertilized weekly with ~8 ml Peters Professional 20-20-20 and with ~0.5 mL with Peters M77 trace minerals per plant. The plants were allowed to self-pollinate. Before each flush of blossoms the plants were also fertilized with ~15 ml triple super phosphate 0-46-0 per plant. Temperatures in the greenhouse were somewhat controlled and averaged 28° C but temperatures occasionally spiked as high as 42°C or as low as 18°C (Fig. A3). Depending on weather conditions, embryos healthy enough to culture could be harvested from March or April until the first frost in the autumn. After that time, greenhouse conditions did not support the reliable development of healthy cotton embryos. Cotton flowers were tagged on the day of anthesis and the fruit was harvested 13 to 30 DPA. Plants were kept for six to twelve months.

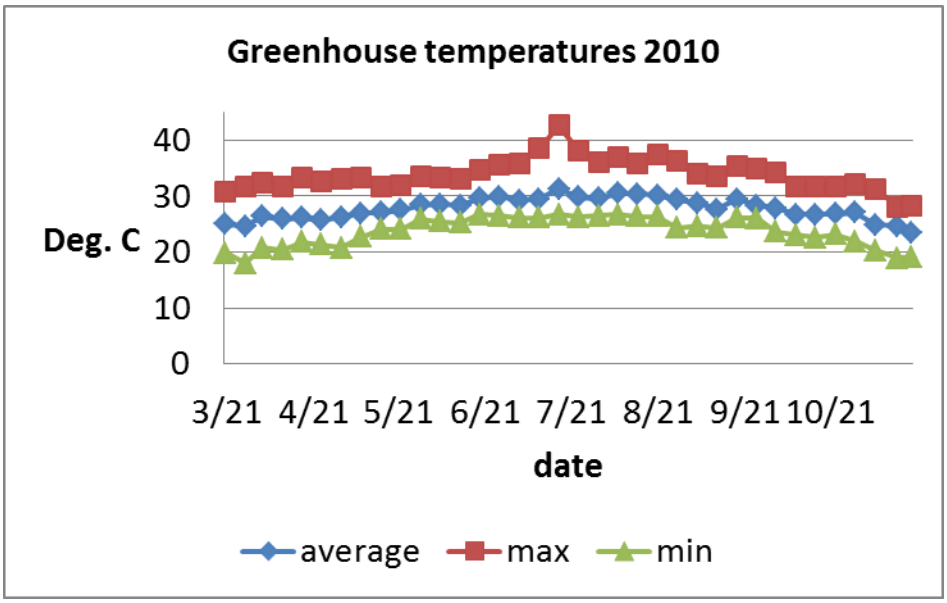

Fig. A3 Greenhouse temperatures for the 2010 growing season. Although weekly temperatures averaged between 24°C and 31°C, temperatures did spike as high as 42°C or as low as 18°C.

Microscopy

Cotton fruit were tagged on the day of anthesis, and harvested at 13 to 25 DPA. The embryos were immediately dissected out of the fruit and fixed with modified (Stelly, personal communication) FAA consisting of 5% formalin, 2.5% acetic acid, 28.5% ethanol for 24 to 48 hours. Embryos were dehydrated in an ethanol series with 5% increments starting at 35% to 70% (Cobb, personal communication). The embryos remained in each solution of the series for at least one hour. They were then stained overnight with 1% Safranin O dissolved in 70% ethanol. After staining dehydration was completed using a tert-butyl alcohol series (Lang, 1937) infiltrated and embedded in paraplast II™ (Stelly personal communication). They were sectioned in 10 µM sections, affixed to slides with Mayer's albumen (Ruzin, 1999 pg 85; Vitha personal

communication), deparafinized with Histochoice™ or Histoclear™ and stained sequentially with 1% Safranin O and 1 % Aniline Blue in 70% ethanol [118](Ruzin, 1999, pg. 111). The slides were cleared in an ethanol-methyl salicylate series (Hodnett et al., 1997). Glass ♯ 1 ½ cover slips were then mounted with Permount™.

Image analysis

For photography embryos were lit by two banks of parallel fluorescent lights to ensure consistent lighting and minimize shadows. Embryo length and area were measured using ImageJ™ software (Abramoff et al., 2004; Bearer, 2003).
Embryo color at 14 DAC varied greatly even among embryos from the same treatment (Fig. A4). Digital images have been used as a method of measuring foliage density (Stevens et al., 2008) and onion color (Kim et al., 2007). Here I used digital images to compare the percent of green surface between embryos. In order to compare color, I used ImageJ™ to separate color components. The digital cameras used in this study record color images in three channels red, green and blue. Within the red blue and green color channels, each pixel, the smallest unit of a picture that can be represented in a two dimensional grid, is assigned an intensity value of 0 (black) to 255 (brightest).

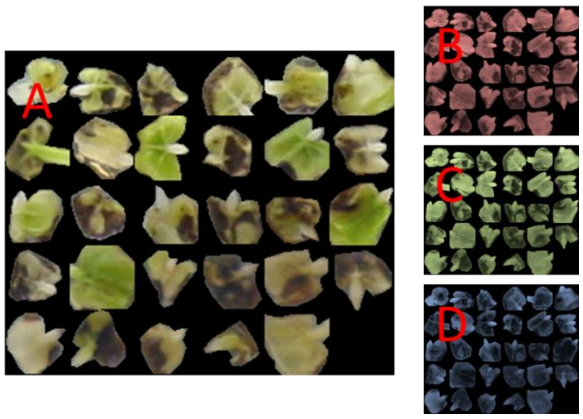

Fig. A4 Measurement of embryo color using ImageJ™. Digital image (A) was split into red (B) green (C) and blue (D) channels. Brightness on a scale of 0 (black) to 255 (brightest) was compared between red and green channels. Those pixels in which green had a higher value than red were counted. Color shown in B-D is for explanation purposes only.

After grey balancing and masking the background, color was measured by splitting the image of the embryo into the three channels, and using ImageJ™ to count pixels. The image math function of ImageJ™ was used to count pixels in which the intensity of the green channel exceeded the intensity of the red channel (Fig. A4). Dark lesions were measured by counting the pixels in which intensity values were 1 to 50.

Measurement of shoot apices

Three serial sections from the center of each shoot apex were measured using ImageJ™. The base of each shoot apex was measured from corner to corner (Fig. A5). All of the embryos measured were aligned perpendicularly to the plane of the cotyledons and cut in the same direction (Fig. A5).

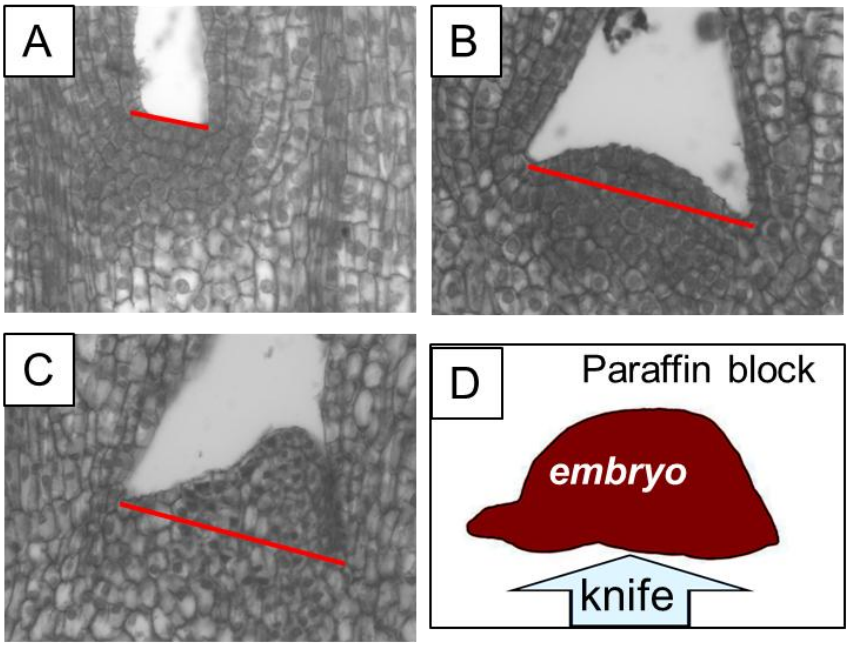

Fig. A5 Measurement of shoot apices. Embryonic shoot apices were measured across the base at the juncture with the cotyledons. Cells along the line of measurement were counted to determine cell size. (A) flat apex; (B) symmetrical apex; (C) asymmetrical apex (D) alignment of embryo in paraffin block. Embryo is aligned to be cut perpendicularly to the plane of the cotyledons. *Arrow* in D indicates the direction of cutting; *red bar* in (A-C) indicates line of measurement.

Statistics

Analysis of variation was computed with the SAS v 9.1 General Linear Model. Under the Duncan Multiple Range model, means sharing the same letter were not significantly different at alpha < 0.05. Coefficients of correlation were computed using the SAS v 9.1 CORR procedures. Correlations were considered significant if the probability was less than 5% that a value greater than $|r|$ (the coefficient of correlation) could occur randomly. Graphs were drawn using Microsoft Excel. All error bars represented the standard error of the mean of 3 to 5 replications.

APPENDIX B

EMBRYO GROWTH IN CULTURE

Introduction

Early during my experimentation, I noticed that embryos tended to stop growing after the first two weeks on Medium I. The cessation of growth might have been due to the gradual drying out of the media, the depletion of the nutrients or the embryos could be entering the maturation and desiccation phase of the embryo developmental program. More than one factor could be affecting growth. Not all embryos immediately resumed growth after transfer to fresh media (data not shown). If embryos in the light treatment began to elongate after transfer usually the embryos in the corresponding dark treatment dissected from the same boll(s) began to elongate (data not shown).

Experiments with phloroglucinol staining (Bell Arnold & Gould, not published) also indicated that gossypol in glands were most apparent during the first 10-14 DAC and declined thereafter (data not shown). Three experiments reported below were conducted to establish when growth occurred, when it slowed, and whether light, dark or agar concentration had an effect on growth during the first 14 DAC. The results of three experiments are included here.

Materials and methods

Embryos cultured in the dark at 25°C

1248 embryos from 1.3-4.2 mm (16 to 21 DPA) were cultured on Medium I, BT salts, BT vitamins with 30 gl^{-1} sucrose and 15 gl^{-1} Sigma agar (Sigma A7921) in the dark

at 25° C. Culture dates were 3/10/2006 to 4/8/2006. After 0, 4, 8, 12, 16, 20, and 28 DAC in the dark the embryos were photographed and measured.

Size comparison of embryos cultured in the light and dark at different agar concentrations

967 embryos from 1.5 to 4 mm (15 to 17 DPA) were put into culture between the dates 10/7/2007 and 11/1/2007. They were cultured on Medium I with 8 to 15 gl^{-1} agar (Sigma A7921) under constant light (PPF of 60 μmol $m^{-2}s\text{-}^{1}$) or darkness at 28° to 32° C for 14 days, then photographed and measured. Because the results of the other agar concentrations were similar, only the 12 gl^{-1} treatment is shown.

Size comparison of embryos cultured in the light and dark at a constant 30°C.

609 embryos from 1.5 to 4 mm (14 to 17 DPA) were put into culture between 9/6/09 and 10/11/09. They were cultured on Medium I solidified with 12 gl^{-1} agar for 0, 1, 3, 6, and 14 days in light (60 μmol PPF $m^{-2}s\text{-}^{1}$), then photographed and measured.

Results

Embryos cultured in the dark at 25°C

Embryos of all size-classes elongated rapidly the first four days of culture (Fig. B1). After four days, growth slowed, reaching a steady state by 8 or 12 days. Embryos appeared to reach a growth plateau. It is not known whether this was due to the embryos entering the normal desiccation process of embryo maturation or whether it might be caused by the media in the Petri dishes drying out, critical nutrients being depleted or

embryos depleting accumulated reserves of carbohydrates and proteins for metabolism. Germination was not observed.

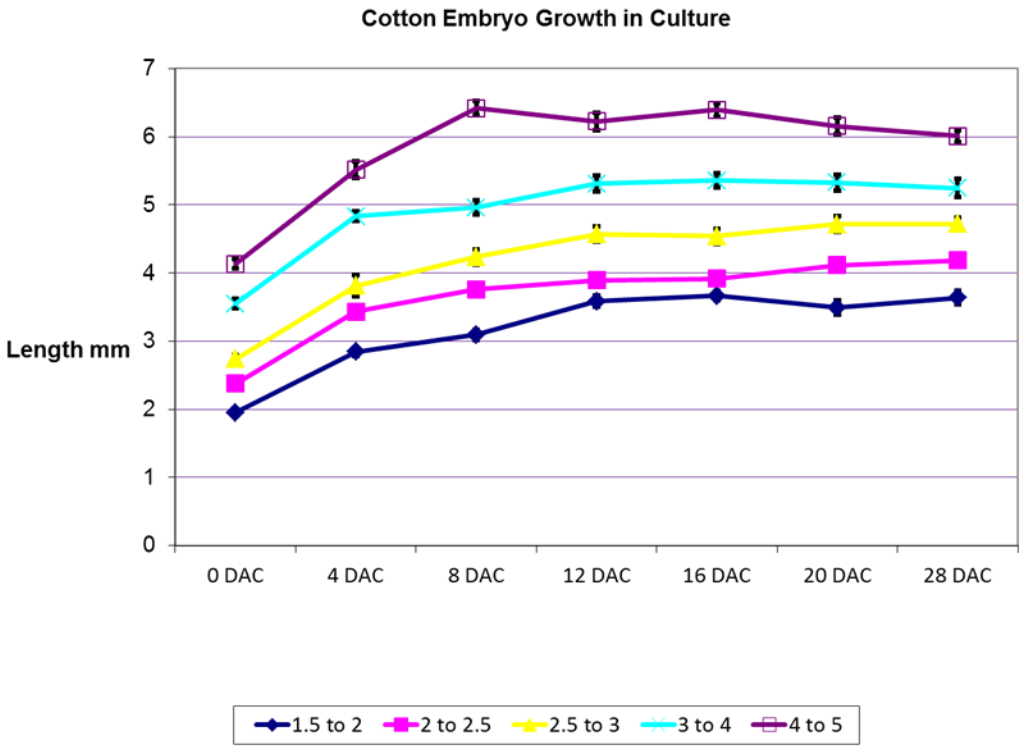

Fig. B1 Growth of excised immature embryos in culture. Embryos of different size-classes were grown in the darkness on Medium I at 25°C for periods from 0 to 28 DAC then photographed and measured. Error bars represent the standard error of the mean.

Excised immature embryos cultured in light or darkness at 28° to 32°C on different concentrations of agar

No significant difference was found between the lengths of embryos whether cultured 14 days in the light or darkness. Data from the 12 gl^{-1} agar (Sigma A7921) treatment are shown here (Fig. B2). Embryos cultured in media solidified with 8 gl^{-1} and 15 gl^{-1} agar also showed no significant difference in length between light and dark treatments. However, embryos cultured on 8 gl^{-1} agar were longer than those cultured on media with higher agar concentrations (Fig. B3).

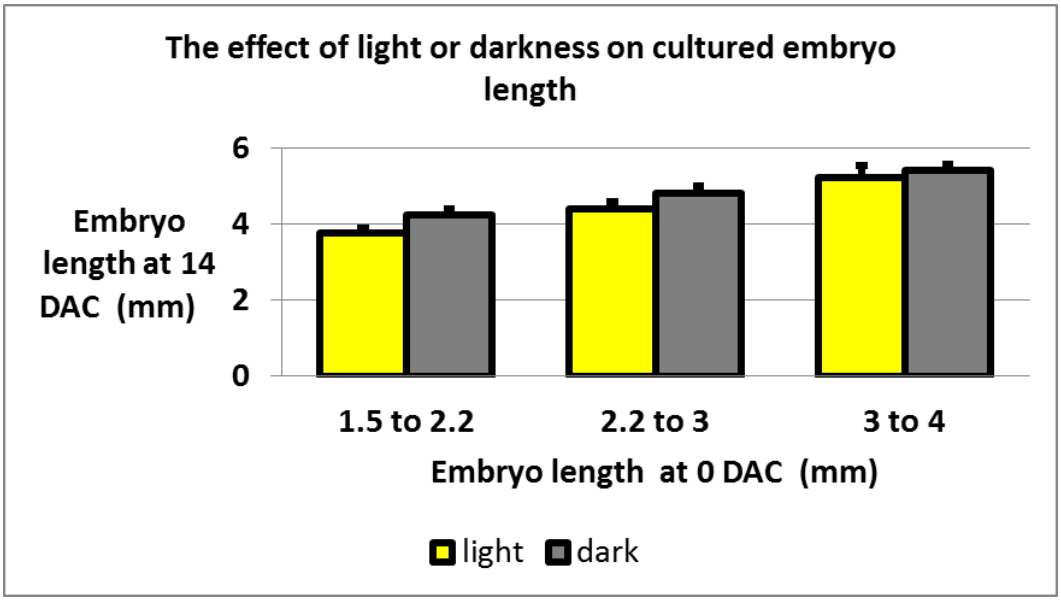

Fig. B2 Effect of light and darkness on length of cultured embryos. Embryos were cultured 14 days in light (PPF of 60 µmol $m^{-2}s^{-1}$) or darkness on Medium I solidified with 12 gl^{-1} agar (Sigma A7921).

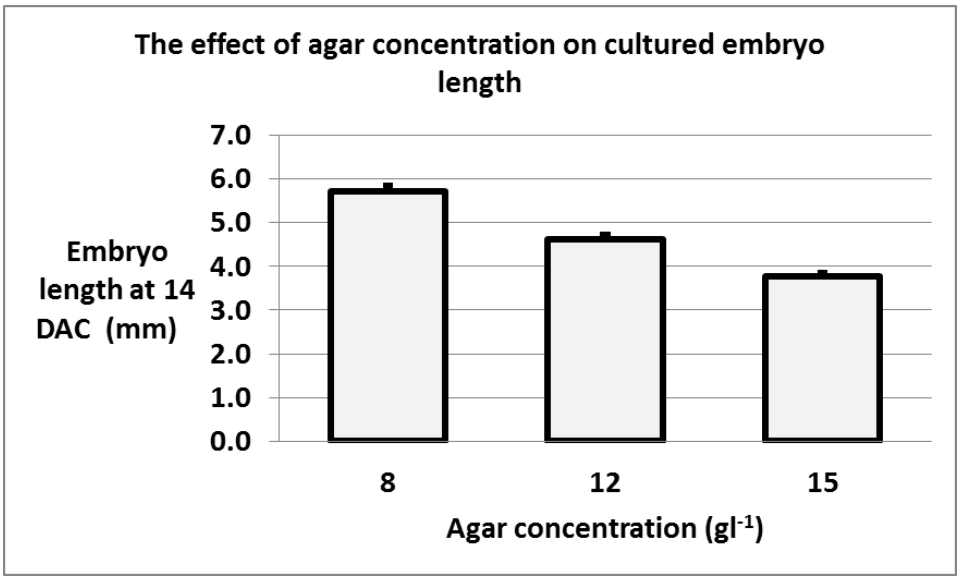

Fig. B3 Effect of increasing agar concentration on cultured embryo length. Light and dark treatments were combined here. Embryos were 1.5 to 4 mm, 14 to 17 DPA. They were incubated 14 days on 8, 12 and 15 gl-1 agar (Sigma A7921). Error bars are the standard error of the mean.

Growth of excised immature embryos cultured at a constant 30°C

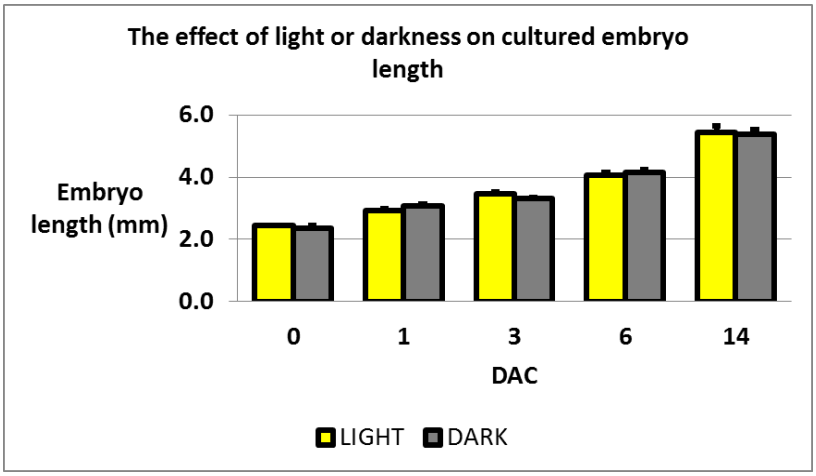

Fig. B4 The effect of light or darkness on embryo growth in culture. Embryos were cultured 1 to 14 days in light (60 µmol PPF $m^{-2}s^{-1}$) or darkness on Medium I solidified with 12 g^{l-1} agar. Error bars are the standard error of the mean.

No significant difference was found between growth of embryos in light or darkness (Fig. B4). Embryos almost doubled in length from 0 to 6 DAC. Growth slowed slightly from 6 DAC to 14 DAC (Fig. B4). Growth in the first 6 DAC appeared to be from cotyledon expansion while growth from 6 to 14 DAC appeared to be from hypocotyl elongation (Fig. B5). During 6 to 14 DAC embryos from both treatments often lost much of their green color. Hypocotyl shape differed somewhat between treatments at 14 DAC. Hypocotyls of 14 DAC light-treated embryos were often slightly curved down toward the media while the dark-treated embryos were usually straight (Fig. B5).

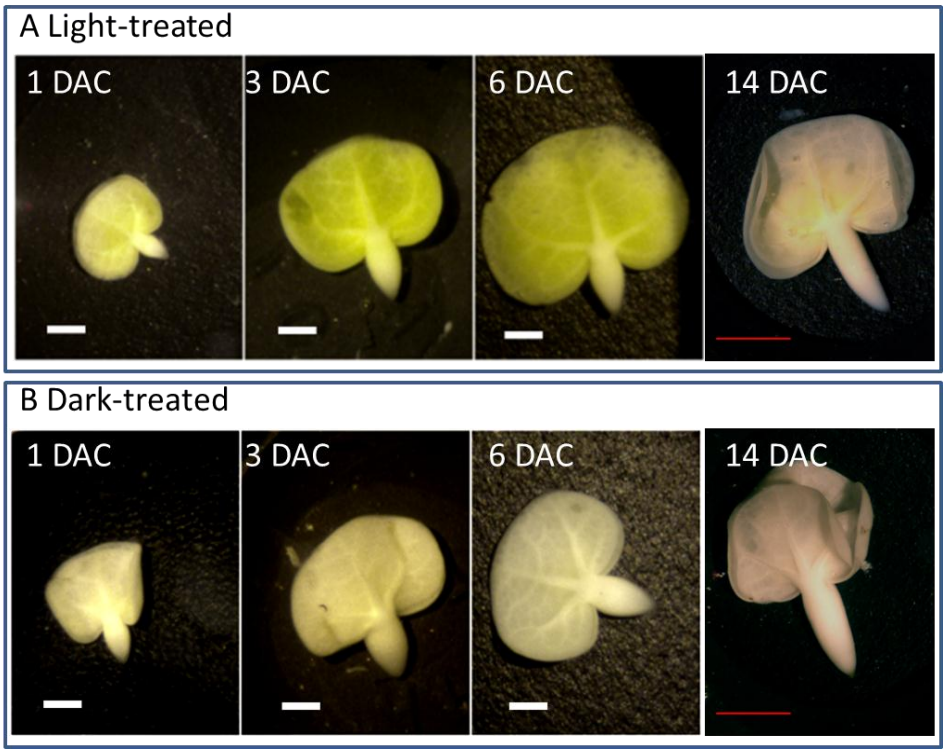

Fig. B5 Embryo length in culture. (A) Light-treated embryos; (B) Dark-treated embryos. Embryos (2-3 mm 15-16 DPA) were cultured between the dates 9/6/09 and 10/11/09. They were cultured 1 to14 days in light (60 µmol PPF $m^{-2}s^{-1}$) or darkness on Medium I solidified by 12 gl^{-1} agar. White bars = 1 cm; Red bars = 2 cm.

Light or darkness appeared to have no effect on the growth of immature embryos during the first 14 days. Constant temperatures of 25° and 30° C produced very similar growth rates (Compare Fig. B1 to Fig. B4). However, embryos grown on 8 gl-1 agar were significantly longer than those grown on 12 or 15 gl^{-1} agar.

APPENDIX C

TISSUE CULTURE MEDIA

Table C1 Comparison of salts and vitamins in MS, BT and SH media

Nutrient	MS		BT		SH	
	Murashige and Skoog (1962)		Beasley and Ting (1973; 1974)		Stewart and Hsu (1977)	
	mgl^{-1}	mM	mgl^{-1}	mM	mgl^{-1}	mM
Salts						
KH_2PO_4	170.0	1.25	272.2	2.0	272.2	2.0
H_3BO_3	6.2	0.10	6.18	0.10	6.18	0.10
$Na_2MoO_4.2H_2O$	0.250	0.001	0.242	0.001	0.242	0.001
$CaCl_2.2H_2O$	441.1	3.00	441.1	3.00	441.1	3.00
KI	0.83	0.005	0.83	0.005	0.83	0.005
$CoCl2.6H_20$	0.025	0.0001	0.024	0.0001	0.024	0.0001
$MgSO_4.7H_20$	370.0	1.5	493.0	2.0	493.0	2.0
$MnSO_4.H_20$	16.9	0.1	16.9	0.1	16.9	0.1
$ZnSO_4.7H_20$	8.63	0.03	8.63	0.03	8.63	0.03
$CuSO_4.5H_2O$	0.025	0.0001	0.025	0.0001	0.025	0.0001
NH_4NO_3	1650.0	41.2	0.0	0.0	1200.6	15.0
KNO_3	1900.0	18.8	5055.0	50.0	3538.5	35.0
$FeSO_4.7H_2O$	27.8	0.10	8.34	0.03	8.34	0.03
Na_2EDTA	37.3	0.10	11.17	0.03	11.17	0.03
Vitamins						
Nicotinic Acid	0.5	0.004	0.492	0.004	0.492	0.004
Pyridoxine-HCl	0.5	0.004	0.822	0.004	0.822	0.004
Thiamine-HCl	0.1	0.0003	1.349	0.004	1.349	0.004
Myo-Inositol	100.0	0.555	180.2	1.00	180.2	1.00

BT (Beasley and Ting, 1973; Beasley and Ting, 1974) was selected as the mineral and vitamin formulation for Medium I (initiation medium) and Medium II (germination medium). Previous testing (Fig. C1) showed that embryos initially cultured on the mineral plus vitamin formulations, BT, MS (Murashige and Skoog, 1962) and SH (Stewart and Hsu, 1977) developed similar numbers of true shoots. For the purpose of this report a true shoot was an apical bud or a true leaf plus an apical bud. Sucrose (30 gl^{-1} and 40 gl^{-1}) and glucose (20 gl^{-1}) were also tested in Medium I and

Medium II. While embryos smaller than 2 mm showed little difference between treatments, embryos 2 to 4 mm developed more true shoots when 30 gl^{-1} sucrose was used in Medium I and Medium II (Fig. C1). Medium III, (½ MS salts plus vitamins plus 15 gl^{-1} sucrose) was the standard rooting medium used in the laboratory.

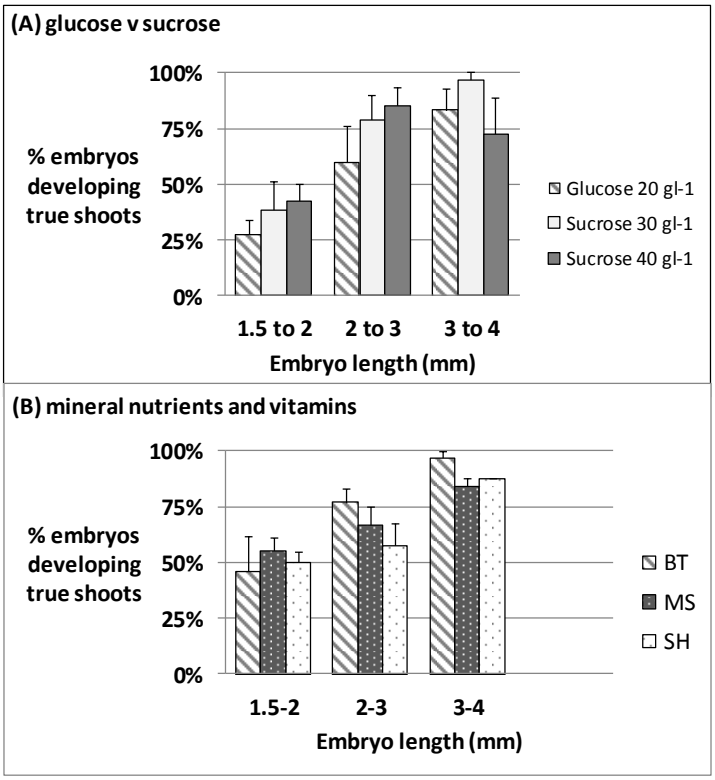

Fig. C2 Shoot development at 42 DAC in response to different formulations for Media I. Embryos were cultured on Media I in the dark at 25°C for 19-20 days before transfer to Media II in the light. (A) A total of 306 embryos from 16 to 19 DPA were cultured between 5/23/05 and 6/13/05 on BT with glucose 20 gl^{-1}, sucrose 30 gl^{-1} or sucrose 40 gl^{-1}. (B) A total of 380 embryos from 16 to 18 DPA embryos were cultured on BT, SH, or MS salts + vitamins with glucose 20 gl-1.

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